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Abstract

Host-plants may rarely leave their ancestral niche and in which case they tend to be surrounded by phylogenetically distant neighbors. Phylogenetically isolated host-plants might share few mutualists with their neighbours and might suffer from a decrease in mutualist support. In addition host plants leaving their ancestral niche might face a deterioration of their abiotic and biotic environment and might hence need to invest more into mutualist partners.We tested whether phylogenetic isolation of hosts from neighbours decreases or increases abundance and activity of their mutualists and whether mutualist activity may help to compensate deterioration of the environment. We study oak-hosts and their ectomycorrhizal fungi mutualists established in the litter layer formed by the phylogenetically closely or distantly related neighbourhood. We find that oaks surrounded by phylogenetically distant neighbors show increased abundance and enzymatic activity of ectomycorrhizal fungi in the litter. Moreover, oaks surrounded by phylogenetically distant neighbors also show delayed budburst but ectomycorrhizal fungi activity partly compensates this negative effect of phylogenetic isolation. This suggests decreased nutrient availability in a phylogenetically distant litter partly compensated by increased litter-degradation by ectomycorrhizal fungi activity.Most observed effects of phylogenetic isolation cannot be explained by a change in baseline soil fertility (as reflected by nutritional status of fresh oak litter, or soil microbial biomass and activity) nor by simple reduction of percentages of oak neighbours, nor by the presence of gymnosperms.Our results show that colonizing new niche represented by the presence of distantly related neighbours may delay plant phenology but may be supported by mycorrhizal mutualists. Studies on other host-plant species are required to generalize our findings.

Key words: community phylogeny, mycorrhiza, forest trees, enzymatic activity, breaking with niche conservatism, mutualism strength, budburst phenology

55

1. Introduction

Mutualists provide essential services to numerous species. For instance, more than 80% of terrestrial plant species interact with mutualistic mycorrhizal fungi (Parnischke, 2008) that play a vital role for the plants. Mycorrhizal fungi take up nutrients from the soil via their extraradical mycelium and translocate these nutrients to the plant partner, receiving carbohydrates in return (Smith and Read, 2008). However, mutualistic relationships are exposed to threats. In particular spatial isolation between individual hosts of the same plant species may lead to a loss of mutualist species and a decrease in their ecological functions, as already observed with pollinators (Ghazoul and Shaanker, 2004), predators of plant enemies (Magrach et al., 2011) and mycorrhizal fungi (Dickie and Reich, 2005; Peay et al., 2010; Frank et al., 2009).

Mutualists may be host-plant-specific and may conserve hosts throughout evolutionary history (e.g. Powell et al., 2009; Shefferson et al., 2010 but see van der Heijden and Horton 2009 and below). Thus phylogenetically close host-species may be expected to share mutualists and their services (Peay et al. 2010 and above references). It has been shown that host plant species surrounded by distantly related plant neighbors (i.e. phylogenetically isolated hosts) can have lower herbivore load than host plant species surrounded by closely related species (Goßner et al., 2009; Yguel et al., 2011). Similar conclusions may apply to mutualists: phylogenetic isolation of host plants from neighboring species could lead to a decrease in abundance of host mutualists and associated services. However, some mutualists are not or only partly host-specific (Molina et al., 1992; Fontaine et al., 2009; Morris et al., 2009), and phylogenetically distant host species may hence harbor the same generalist mutualist species (Selosse et al., 2006). In such case, host plants surrounded by phylogenetically distant plant species may not experience any loss in mutualist species.

Individual host plants that are surrounded by phylogenetically distant species might also face a deterioration of their living conditions. Phylogenetically distantly related species may have different functional traits resulting in different levels of litter decomposition (Pan et al. in press). Therefore colonizing an environment composed of distantly related species may correspond to different and often deteriorated abiotic living conditions like nutrient availability in the litter.Moreover, as a general tendency, phylogenetically distant species often have different environmental preferences (Wiens et al. 2010 for multiple examples and references), and thus, an environment dominated by phylogenetically distant neighbours is likely suboptimal for a given focal species, independent of the impact of these neighbours on the environment. As an extreme example, fish have conserved an aquatic niche. Thus, a distantly related species like an ape for instance surrounded by fish will be in extreme abiotic living conditions.In addition, within the environment colonized, and possibly created by phylogenetically distant species, these species are likely the superior competitors.Mutualistic interactions may then beparticularly important to compensate for such deteriorated and suboptimal living conditions (Hacker and Gaines, 1997; Dickie and Reich, 2005). In that case, host plants surrounded by phylogenetically distant species would need to invest more into mutualistic interactions. As a consequence, the abundance and activity of mutualists are expected to increase with increasing phylogenetic isolation of a host plant.

Three contradictory hypotheses are hence plausible: for a given host plant species, interactions with mutualists (i) decrease, (ii) remain unchanged or (iii) increase with increasing phylogenetic distance of host plants to their plant neighbors. In this study, we tested these hypotheses with plant-mycorrhizae interactions, focusing on deciduous oak trees, their ectomycorrhizal fungi (EMf) mutualists and their enzymatic activity related to budburst, a vital function. Deciduous oaks (*Quervus petraea/rubra*) are particularly suitable to test such hypotheses: these oaks occur in a wide range forest compositions and neighbourhoods, and are important for forestry.

In spring, oaks and other deciduous trees need to quickly produce their leaves within a narrow time window between winter frosts and the annual peak of solar irradiation in order to maximize annual carbon uptake (e.g. Morecroft et al., 2003). Oak budburst starts in a period without support from photosynthetic apparatus resulting in an unbalancedplant carbon-budget during this period. Budburst could be delayed by deteriorated soil or microclimates as they may occur in phylogenetically distant neighborhoods (Courty et al., 2007). But budburst may be supported by mycorrhiza: Breda et al. (2013) have shown that carbon is derived from litter during spring reactivation and is channelled from the soil to oak roots via EMf, supporting the hypothesis that oaks are partially mixotrophic plants (Courty et al., 2007).EMf may do so by producing extracellular and cell wall-bound hydrolytic and oxidative enzymes to degrade C- and N-compounds contained in soil organic matter (Courty et al., 2010;Rineau et al., 2012; Tedersoo et al., 2012). In fact, in deciduous oaks, spring reactivation modifies the activity of EMf resulting in greater mobilization of carbon and nitrogen from soil organic matter (Courty et al., 2007). Without the contribution of EMf to nutrient uptake, budburst would be significantly delayed (Dickie et al., 2007; Breda et al., 2013). Such and other functions of EMF can now be portrayed by measures of enzymatic activitiesof excised ECM root tips. Such a functional approach may be more powerful than looking at taxonomic identity of ECMf, given that enzymatic functions can vary within ECMf taxa or remain constant betweenECMf taxa (Courty et al., 2005, Buée et al., 2007). This functional approach permits testing whether phylogenetic distant neighborhoods change ECMf support and whether this support may compensate for a possible delay in budburst. We studied the effect of phylogenetic isolation of oak trees (*Quercus robur* L. and *Quercus petraea* Mattuschka Liebl) from their tree neighbours on budburst phenology and on the abundance and activity of associated EMf in root tips within the litter layer formed by this neighborhood. We focused on enzymes involved in the mobilization of carbon, but also nitrogen during budburst. In particular, we addressed the following questions:

- Does the phylogenetic isolation of oak trees change the abundance and activity of litter EMf?

- Does phylogenetic isolation delay budburst phenology?

- Can enzymatic activity of litter EMf compensate for the negative effect of phylogenetic isolation on budburst phenology?

We also explored multiple environmental variables by which the neighborhood of a tree or the corresponding baseline conditions may operate on a focal tree. We finally explored whether the effect of phylogenetic isolation can be explained by the percentage of oaks in the neighborhood or the presence of gymnosperms.

2. Materials & methods

2.1. Site description and experimental design

Given that this study is on adult trees, experimentation is virtually impossible, so we followed a correlative approach, profiting from a natural experiment of variation in phylogenetic canopy composition around a focal species across a forest canopy. Such an approach cannot entirely control for sampling effects, such as a high abundance of oaks being related to a low phylogenetic distance, but this can be accounted for in later analyses (see below) Specifically, our study was conducted in the Forest of Rennes (surface area: 2000 ha), Brittany (France; Supporting information S1). A total of 17 different tree species were in contact with the focal oaks canopy. Twenty two c. 60-year-old oaks were sampled, with age estimated from tree circumference at breast height [total mean equal to 62.1 cm (SD = 16.7)] and from information from forest managers. Oak trees were chosen by pair, with one surrounded mainly by other oaks and beech trees, and the other surrounded mainly by pine and holly trees, plus some other angiosperm trees. Because oaks in pine stands are generally in the lower part of the canopy, we restricted ourselves to such low-canopy trees everywhere. Oak trees within a pair were close to each other (30–150 m apart), and belonged to the same species, *Quercus petraea* or *Q. robur* (note that these oak species are closely related and tend to hybridize). Pairs were spread across the entire forest. Such an approach of pairing or blocking has been recommended to partial out spatially varying environmental factors such as soil composition (Legendre et al., 2004).

2.2. Phylogenetic isolation of host trees within the surrounding canopy

For each focal oak, we quantified its mean phylogenetic distance to all neighboring trees with which its crown was in contact. Phylogenetic distances were extracted from published phylogenies (Table S1) following procedures applied previously (Vialatte et al., 2009; Yguel et

al., 2011) and using phylogenetic classification (APG, 2009). In order to quantify phylogenetic distance, we used the younger of the crown ages of the two lineages involved, i.e. of the two ages of earliest diversification within the two lineages. For instance, we ranked the comparison between oak and pine species as a comparison between two classes, Gymnosperms and Angiosperms, between which the younger is approximately 140 million years old (the crown age of angiosperms), and the phylogenetic distance is hence 140 million years. Thus, the younger of the two crown ages represents biologically the time when the oak lineage and the other lineage started to be physically and physiologically distinct from a point of viewof mycorrhizal fungi or of other species tightly interacting with the tree. Moreover, this age also avoids giving overly weight to gymnosperms, in contrast tostem-age distance which would in many cases simply be a descriptor of % of gymnosperms in the neighborhood. Overall, mean phylogenetic isolation ranged from 10 to 125.66 million years, and varied continuously between these extremes.

2.3. Abundance and activities of EMf

For each focal oak tree, we took four soil samples at a distance of 0.5-1.5m from the trunk in the four cardinal directions to take into account possible within-tree variation in EMf colonization or activity (note that neighbouring trees were at a distance mostly superior to 2 m, often much more).One sample corresponded to a soil core of 4 cm in diameter by 10 cm depth (125 cm^3). This depth exceeds the litter layer, but we found that 100% of the root tips were restricted to the litter layer, notably the litter corresponding to the previous falls. Sampling was repeated twice, before and after budburst, the 21stof March and the 21stof April 2011, respectively. Litter thickness was measured for each sample. Oak roots from soil cores were soaked in tap water for 15 min before being gently washed. Shape and colour were used to separate oak roots from those of other tree species. Moreover, genetic analyses on two root tips per sample confirmed this determination (See below, verification of root tree species)

188 Oak root tips were observed in water with a stereomicroscope (x40) and the total number of root
189 tips with EMf was recorded in each sample. Abundance of root tips with EMf per tree was
190 calculated as mean of abundances in the four soil samples. Then, ten root tips with EMfwere
191 analyzed for activity using the high-throughput microplate assays described by Courty et al.
192 (2005) and recently optimized by Pritsch et al. (2011).The enzymatic activities were measured
193 successively on each root tip with EMf: decomposition of cellulose and hemi-cellulose by β-
194 glucosidase (EC 3.2.1.3) and β-glucuronidase (EC 3.2.1.31), and decomposition of phenolic
195 substrates by laccase (EC 1.10.3.2) activities (Courty et al., 2007; Breda et al., 2013). As a
196 control we also considered β-glucosidase, which expression is independent to oak budburst
197 (Courty et al., 2007; Breda et al., 2013). Enzyme activities were expressed per unit of time and
198 per EMf root tips projected area as described in Pritsch et al. (2011). The mean activity of the 10
199 sampled root tips with EMf was then calculated per tree.

200 As explained in the Introduction we chose a functional approach to characterize EMf based on
201 enzymatic activity, rather than a taxonomic approach identity given that enzymatic functions can
202 vary within taxa or be constant between(Courty et al., 2005;Buée et al., 2007). Thus, EMf
203 species were not identified.

204

205 **2.4. Verification of species identity of roots**

206 Two root-tips in each soil sample were snap frozen and kept individually at -20°C in Eppendorf
207 tubes. DNA was extracted using the REDExtract-N-Amp Plant PCR Kit (XNAP, Sigma,
208 FRANCE). The oak ITS was amplified according to the procedure detailed in Courty *et al.*
209 (2008) and with oak specific primers (Oak-ITSF, 5'-CGAAACCTGCACAGCAGAACGACCC-
210 3'; Oak-ITSR, 5'-CGCGGGATTCGTGCAATTCACACC -3'). PCRs were run on a 0.8%
211 agarose gel (Bioprobe, QBiogene) in 1% Tris-Acetate-EDTA buffer and stained with Midori

212 Green (LabGene, Switzerland). The expected size of the band (320 bp) was verified with a 1-kb
213 ladder (Gibco BRL, France), confirming the species identity of the root.All 352root tips tested
214 were identified as oak trees (*Quercus robur* or *petraea)*. We note that the correct species identity
215 does not guarantee that the roots belong to the adjacent oak, but the probability that they do is
216 very high.

217

218 **2.5. Measurement of soil humidity, temperature and pH**

219 Soil humidity, temperature and pH were measured within a few cm from the root samples.
220 Humidity and temperature were measured with a wet sensor (WET-2 - WET Sensor, AT delta-T
221 devices) in March and April 2011 while pH (pH-H_2O) was measured only once between March
222 and April. Two statistical outliers of abiotic conditions data were identified graphically and
223 excluded from the analyses presented in the results section. But including or not these outliers
224 did not change the effect of phylogenetic isolation on activity of EMf (TablesS2). The mean, the
225 standard deviation and the range of the soil temperatures are respectively: 11.09, 1.79 and 10.20
226 in March and 16.47, 2.84 and 16.60 in April. The mean, the standard deviation and the range of
227 the soil humidity are respectively: 33.07, 11.47 and 60.80 in March, and 22.97, 12.29 and 57.80
228 in April. The mean, the standard deviation and the range of the soil pH are respectively: 4.03,
229 0.24 and 1.14.

230

231 **2.6. Measurement of air temperature and humidity**

232 Temperature and humidity were measured from the 25th March to the 30thApril 2011. We useda
233 sensor placed in the middle of the crown and in a mesh bag under a branch, protected from
234 precipitation as well as sun (DS1923 Hygrochron Temperature/Humidity Logger iButton, 8KB
235 Data-Log Memory), with hourly records, averaged per day and summed per month. Four sensors

did not work at all and three were lost, thus 7 trees were excluded from all analysis with temperature and humidity of air (n=15 for all measurements).The mean, the standard deviation and the range of the air temperatures are respectively: 85.61, 1.76 and 6.61 in March and 412.55, 9.19 and 31.09 in April. The mean, the standard deviation and the range of the air humidity are respectively: 83.53, 21.23 and 94.60 in March and 72.75, 10.70 and 48.72 in April.

2.7. Inferring variation in soil fertility for trees and microbes

We used an integrative approach to infer variation of the nutritional soil status in which the oaks and the mycorrhizal fungi were growing. A site dominated by phylogenetically distant neighbours may be characterized by abiotic basesline conditions that are suboptimal, regardless of the impact of the neighbourhood on the litter. First, we used the chemical composition of oak leaves; A low C/N ratio of tree leaves indicates a high nutritional status of the leaves and hence of the soil in which the tree is growing. We studied C,N and C/N ratios both in the fresh leaves in spring and in the fresh litter of the oaks. Both types of samples led to the same results and we only present the latter as it better integrates across the entire year. Fresh litter of the oak trees was sampled during autumn 2010 by gently shaking branches, i.e. the sampled leaves were in turn of abscission and leaves had not touched the ground and its decomposers at the ground. Carbon and nitrogen concentrations and carbon isotope ratios of litters were measured by dry combustion on a NA 1500 elemental analyser (Carlo Erba, Rodana, Italy).

Second, a high microbial biomass of the soil may be related to high soil nutritional status, and high microbial activity or biomass of the soil is directly related to high carbon mineralization (Doran and Parkin, 1994). Soils were sampled in August 2011 and February 2012 under each tree. Microbial biomass of the soil was analyzed using the substrate-induced respiration (SIR) method (Anderson and Domsch, 1978). The microbial respiratory response was measured in an

electrolytic O_2 microcompensation apparatus at hourly intervals for 24 h at 22°C (Scheu, 1992). Microbial activity (basal respiration; mg O_2 $h^{-1}g^{-1}$ dw) was determined without substrate.The mean, the standard deviation and the range are respectively 1.17, 0.45 and 2.22 for nitrogen concentration, 49.50, 3.11 and 15.35 for carbon concentration, 45.53, 10.69 and 4.40 for the ratio C/N, 23.50, 15.16, 59.61 for microbial activity in February, 2453.82, 1782.61, 7479.78 for microbial biomass in February and 15.01, 5.10, 21.24 for microbial activity in August and 1781.76, 655.39, 2487for microbial biomass in August.

2.8. Budburst phenology

Budburst phenology was monitored from the 15th March to the end of budburst in 2011, by scoring the phenological state of 10 random apical buds from the upper layer of the crown of each sampled oak, every 3.5 days. Phenological state corresponds to a three-rank scale described in Wesolowski and Rowinski (2008). Then, we calculated the budburst phenology as the number of days required to reach the score indicating full budburst for all buds. The sampling procedure is detailed in Yguel et al. (2011). The effect of tree pair and the effect of the focal oak species on budburst phenology were non-significant (respectively d.f. = 11; F=1.10; p=0.43 and d.f. = 20; F=2.66; p=0.11). Hence, these variables were not included in further analyses.

2.9. Statistical analysis

We used simple regression analyses to test the effect of phylogenetic isolation of the host, the effect of the abiotic soil conditions (i.e. soil temperature, soil humidity, soil pH, litter thickness and more generally all factors related to tree pair) and the effect of focal oak species (i.e. *Q. petraea* or *Q. robur*) on abundance and activity of EMf. In this and all further analyses we confirmed that residuals approached normality and homoscedasticity We also performed

multiple linear regressions in order to explore the effect of phylogenetic isolation on abundance and enzymatic activity of EMf while accounting for the effect of other co-variables. Only co-variables that scored at least marginally significant in simple regression analyses were included in the latter multiple regression analyses. In multiple regression analyses, standardized regression coefficients are presented instead of unstandardized regression coefficient, because the former allow a better comparison between the effects of independent variables (Schielzeth, 2010).As month had an effect on most of the variables, data from March and April were analyzed separately.

We tested the effect of phylogenetic isolation,EMf abundance and enzymatic activity on budburst using simple regression analyses. We tested the interaction effects between phylogenetic isolation and activity of EMf enzymes on budburst to investigate the hypothesis of compensation of a negative effect of phylogenetic isolation by increased EMf activity. Contrary to the other hypotheses this hypothesis is one tailed and so were the tests of the interaction terms (Tab. 1). It was not possible to incorporate in the same model all possible explanatory variables, i.e. phylogenetic isolation, all enzymatic activities, abiotic factors and their respective interactions due to strong multicollinearity and limited sample size. We hence made an effort to reduce the number of covarying independent variables in the model explaining budburst phenology. We discarded the abiotic factors as in simple regression analyses they had a lower explanatory power than either phylogenetic isolation or enzymatic activities. Due to strong correlations among activities of different enzymes, we calculated a separate model for each enzyme separately. We only retained enzymes for which the activity was significantly correlated with budburst phenology in simple regression analyses. Even in these models multicolinearity among independent variables was still strong. In order to decrease the remaining collinearity, we transformed data by centering the enzymatic activities, phylogenetic isolation and the interaction

term by their own means. Using these transformed data increased the tolerance values (Table S3) and hence decreased collinearity but did not change the significance of the interactions term.

We used the method described in Aiken & West (1991) to illustrate graphically the interaction effect between two quantitative predictors. This method uses the unstandardized coefficients of the regression model including the interaction, i.e. $Y = a + b_1X + b_2Z + b_3X*Z$, with a the intercept, $b_1$the regression coefficient of the phylogenetic isolation, b_2, the regression coefficient of the enzymatic activity, b_3 the regression coefficient of the interaction term, Y the budburst phenology, Z the enzymatic activity and X the phylogenetic isolation. The equation was then rearranged in order to obtain a linear equation to be used for graphical illustration: $Y = (b_1 + b_3Z) X + (a + b_2Z)$. Three values of Z were used to represent medium, low and high enzymatic activity: the mean of observed enzymatic activity, plus and minus the standard deviation respectively. Phylogenetic isolation is given as X axis, covering the observed values from 10 to 125.5 million years. We predicted Y (budburst) for each of 50 levels of phylogenetic isolation separated by an increment of 2.31 million years (i.e. (125.5-10)/50=2.31).

All statistical analyses were performed using Statistica Version 9.0 (Statsoft, Maisons-Alfort, France).

3. Results

3.1. Abundance of EMf increased with phylogenetic isolation

Abundance of EMf (recorded in the litter layer only) increased with increasing phylogenetic isolation of host trees in simple regression analyses.This relationship was significant in April and marginally significant in March (Fig. 1; Table S4a). Phylogenetic isolation, however, explained only 16 and 18% of the variation of the EMf abundance.Focal oak species (*Q. robur* vs. *Q.*

petraea) had also a significant effect on EMf abundance in March but not in April (Table S4).There was no significant effect of nutritional status of fresh oak litter, or of soil microbial biomass or activity on ectomycorhizal abundance (Table S4).In the multiple regression analyses including environmental condition as covariables, phylogenetic isolation had a significant effect on EMf abundance in April in all models. In March, the effect of phylogenetic isolation on EMf abundance was marginally significant with focal oak species as co-variable and non-significant with focal oak species and soil humidity as co-variables (Table S4b, S4c).Phylogenetic isolation was always among the two most important explanatory variables of EMf abundance (see standardized regression coefficients in Table S4b, S4c).

3.2. Enzymatic activities of EMf increased with phylogenetic isolation

In simple regression analyses, the laccase activity in March and April, and the β-glucuronidase activity in April only significantly increased withthe phylogenetic isolation of host trees (Fig. 2a, 2b, tablesS5a, S5d). Phylogenetic isolation had no significant effect on β-glucosidase activity,which is unrelated to budburst(Figure 2c and Table S5g).None of the enzymatic activities were significantly related to nutritional status of fresh oak litter, microbial biomass or activity with one exception: strangely soil microbial activity in February has a significant effect on β-glucuronidase activity in March (See table S5a, c, d, g). After removing two outliers, soil humidity had a significant effect on β-glucuronidase activity in April and laccase activity in March and April (TablesS2a). Soil pH had also a significant effect on β-glucuronidase in April and on laccase activity in March (Table S2b). Air temperature of had a significant effect on β-glucuronidase activity in April, and marginally significant on laccase activity in March (TablesS5a, S5d). Humidity of air had a marginally significant effect on β-glucuronidase and β-glucosidase activity in March and a significant effect on laccasein April (TablesS5a, S5d, S5g).

However, in multiple regression analyses accounting for abiotic variables and phylogenetic isolation of the host tree as independent variables, phylogenetic isolation was the only predictor which had a significant effect on β-glucuronidase activity in April, and on laccase activity in March and April (TablesS5b, S5c, S5e, S5f). Removing the two outliers did not change the results; in multiple regression analyses, phylogenetic isolation was still the only predictive variable which had a significant effect on β-glucuronidase activity in April and laccase activity in March and April (TablesS2c S2d, S2e, S2f). The effect of phylogenetic isolation on β-glucosidase activity remained non-significant in March and in April (TablesS5h, S5i). Phylogenetic isolation was always the variable with the strongest effect on laccase and glucuronidase activities (see standardized regression coefficients in tables S5b, S5c, S5e, S5f).

3.3. Budburst phenology was delayed with phylogenetic isolation

In simple regression analysis, budburst phenology was significantly delayed with increasing phylogenetic isolation of host trees(Fig. 3. Table S6). The low r^2 may be explained by the lack of experimental control and unknown environmental variation and the fact that budburst was a semi-quantitative variable, with three categories, recorded every 3.5 days, reducing inevitably the explicable variance. Budburst wasalso significantly delayedwithincreasing laccase activity in March and April, and also with increasing β-glucuronidase activity in April (Table S6). Inversely, budburst phenology was accelerated marginally significantly with increasing air temperature in March and April (Table S6). We note that in a prior study in 2010 we observed the same effect of phylogenetic isolation but not of air temperature on budburst (Yguel et al., 2014 in press).Thus the effect of phylogenetic isolation on budburst, though of limited R^2, is consistent across year and could not be due to year to year fluctuation (See Yguel et al., 2014 in

379 press).Besides, there was no significant effect of nutritional status of fresh oak litter, soil
380 microbial biomass or activity on budburst phenology (Table S6).

381

382 **3.4. Enzymatic activity of EMf partly compensated for the delay of budburst in**
383 **phylogenetically isolated trees**

384 We performed multiple regression analyses including an interaction term between phylogenetic
385 isolation of host trees and the enzymatic activities of EMfthat were significant in simple
386 regression analyses (i.e. laccase activity in March and April; β-glucuronidase in April). The
387 interaction between laccase activity in April and phylogenetic isolationhad a significant effect on
388 budburst phenology (Table 1). Figure 4 illustrates the direction of the interaction. Budburst was
389 far less delayed in phylogenetically isolated oaks when they showed high laccase activity. No
390 other tested interactions were significant.

391

392 **3.5. Most effects significant phylogenetic isolation cannot be fully explained by a lower**
393 **percentages of oaks in the neighbourhood of the focal tree.**

394 We explored whether the significant effects ($p \leq 0.05$) of phylogenetic isolation described above
395 could be explained simply by a reduction in the percentage of oaks in phylogenetically isolated
396 neighbourhoods. For this purpose we included percentage oaks as a covariable in the
397 corresponding analyses (plus percentage of oaks x laccase activity in the analysis of possible
398 compensatory effects). We found that in two cases phylogenetic isolation became non-significant
399 and less important than percentage of oaks after inclusion of percentage of oaks: on laccase
400 activity in March and tree budburst (Tables S7 c, e). For these two processes phylogenetically
401 isolation hence operates primarily via the absence of very closely related individuals (i.e. other
402 *Quercus*). In one case phylogenetic isolation become non significant but more important than

403 percentage oaks: EMf abundances in April (Table S7. a). In three cases the effect of phylogenetic
404 isolation remained significant: onβ-glucuronidase activity in April and on laccase activity in
405 April, and, most interestingly, the interaction term laccase activity x phylogenetic isolation on
406 budburst (Tables S7. b, d, f, g). Overall, the effect of phylogenetic isolation on abundance and
407 enzymatic activity in April represent more than just a dilution of oaks. While budburst delay is
408 more due to a dilution of oaks, the compensatory effect of enzymatic activity on budburst is
409 triggered by phylogenetic isolation.

410

411

412

413 **4. Discussion**

414 In our study, EMf abundance increased significantly (although only moderately) with the
415 phylogenetic isolation of a host tree. The effect of phylogenetic isolation was much stronger on
416 enzymatic activity involved in C and N mobilization during budburst while an enzyme not
417 related to budburst was not affected.These EMf were entirely in the litter layer, formed by the
418 oak and its respective neighbours. Phylogenetic isolation also delayed budburst but this effect
419 was due to the dilution of oaks.Interestingly, the increase in EMf enzymatic activity appeared to
420 partly compensate for the negative effect of phylogenetic isolation on budburst phenology. Most
421 of these effects of phylogenetic isolation could not be entirely explained by an effect of
422 percentage of oak neighbors, notably the compensatory effect of laccase activity on delayed
423 budburst in phylogenetically distant neighborhoods.

424

425 **4.1. Why should EMf abundance and enzymatic activities increasewith phylogenetic**
426 **isolation ?**

Phylogenetic isolation might have affected EMf via changes in abiotic or biotic conditions, which indeed varied strongly among trees (Methods).Abiotic conditions might in part reflect the baseline environments dominated by phylogenetically distant species rather than the impact of the neighbours themselves. First, we indeed found that with increasing phylogenetic isolation,soil humidity, air temperature and pH decreased (Table S8). These abiotic factors may indeed affect abundance and activity of EMf (Bago, 1998; Courty et al., 2008). Nevertheless, these effects of abiotic factors were probably negligible compared to biotic factors since effects of abiotic factors were less significant than those of phylogenetic isolation and not significant anymore when accounting for phylogenetic isolation. Second, a dominance of phylogenetically distant neighbours might, theoretically, reflect a low soil fertility for our focal oaks and low soil fertility in turn might trigger an increase in EMf abundance and activity. However we found neither a change of soil-fertility indicators (i.e. nutritional status of oak leaves, soil microbial biomass/activity) with phylogenetic isolation nor a clear affect of these variables on mycorrhizal abundance or activity(see Table S8). Thus, a difference in fertility is unlikely in order to explain the increasing activity and abundance of EMf in phylogenetically isolated trees.

In our study, biotic factors appear hence to be the most important factors affecting abundance and enzymatic activities of EMf. Increasing phylogenetic isolation of host trees corresponds to a change in neighboring tree species composition. This change affects litter composition which could in turn modify the composition and activity of EMf communities (Conn and Dighton, 2000; Tedersoo et al., 2003; Buee et al., 2007). Increasing phylogenetic isolation from neighboring species may correspond to an increasing difference in chemical composition between oak litter and that of tree neighbors. This is for instance the case with gymnosperm neighbours compared to angiosperm neighbours, both occurring in our study system. Litter from angiosperm species is known to be more easily decomposed than that of gymnosperm species (Weedon et al., 2009; Osono, 2011). In particular, lignin (Osono, 2011) and hemicellulose

(Weedon et al., 2009) decompose more slowly in gymnosperms than in angiosperms. Also, lignin concentration is often higher in gymnosperms compared to angiosperms (Weedon et al., 2009) whereas the opposite is true for phosphorus or nitrogen (Weedon et al., 2009). We therefore suggest that both the quality and the decomposition rate of the litter decreased with increasing phylogenetic isolation of oak trees. This was also supported in our study by the fact that litter thickness also increased with increasing phylogenetic isolation of host trees ($p =< 0.03$, results not shown). Phylogenetic isolation might hence decrease the mobility of carbon in cellulose, hemicellulose and lignin, which should necessitate increasing enzymatic activity related to cellulose, hemicellulose and lignin degradation (Colpaert and van Tichelen, 1996; Conn and Dighton, 2000). This is actually what we observed: enzymatic activity strongly increased with phylogenetic isolation.We note that the relationship between phylogenetic isolation and enzymatic activity was not solely due to an increase in abundance of gymnosperm neighbours. In fact, studying only the oaks exclusively surrounded by angiosperm neighbours gave the same results in April (Table S9).

Overall the effect of phylogenetic isolation on enzymatic activity appears to be more related to functional differences reflected by phylogenetic distance in general, including among Angiosperms. It is not only the difference between gymnosperms and angiosperms that countsNevertheless, we acknowledge that our results might be specific to our focal species, oaks,which may require more and invest more into support by EMf than other tree species. Studies on other host-plant species are therefore required to verify the generality of our findings. Moreover, future research will need to identify the precise functional traits conveying the effect of a phylogenetically distant neighbourhood, including little known physiological and root traits that may influence interactions of trees with mycorrhizal fungi. Finally, further soil parameters may help to definitively conclude about the role of abiotic baseline conditions on which distantly

related neighbours dominate vs. the deterioration in conditions (e.g. litter composition) caused by theseneighborhood.

Besides litter decomposabilitythere might be biotic factor that could explain the positive relationship between phylogenetic isolation and EMf abundance. Natural enemies of EMf, i.e. fungivores, might have been less abundant around phylogenetically isolated host trees. Actually, fungivores of EMf associated with neighbouring phylogenetically distant tree to might not accept EMf associated with oak or, if they do, might suffer reduced fitness and population growth (Bertheau et al., 2010). On the other hand, fungivores specialized on oak EMf might not penetrate phylogenetically distant neighborhoods (see also Prinzing, 2003). Such reduced abundance of consumers and associated "consumption" in phylogenetically isolated trees may be equivalent to the reduced insect herbivore abundance and herbivory (Vialatte et al., 2009; Yguel et al., 2011).

Finally, the increased EMf and activity on roots of phylogenetically isolated trees might simply reflect an increased microbial activity of these litters fertility. However, delayed budburst in a more active litter is little plausible. Moreover, we found no relationship between phylogenetic isolation and microbial biomass or respirational activity in the litter (unsigned $t < 1.34$, $p > 0.19$, results not shown), or between either of the latter and EMf abundance or enzymatic activity (unsigned $t < 1.57$, $p > 0.13$, except for a negative relationship microbial activity vs. EMf glucoronidase activity (April at $t = -2.2$, $p = 0.041$, results not shown). This, tentatively, suggests that denser and more active mycorrhiza colonizers of roots are more than a random sample from the ambient microbial litter community.

499 **4.2. Why budburst phenology was delayed with phylogenetic isolation, and how this might**
500 **be compensated by higher enzymatic activity of EMf?**

501 Carbon sources are required to achieve budburst but carbon reserves in oak are largely depleted
502 before budburst (Courty et al., 2007). Hence, oaks need additional source of carbon to achieve
503 budburst and associated EMf could partly mobilize this missing carbon (Courty et al., 2007;
504 Bréda et al., 2013). As previously mentioned, phylogenetic isolation of trees was probably
505 associated with a change in litter composition that resulted in a lower decomposition rate of the
506 litter, which in turn delayed budburst. In addition, phylogenetic isolation may be associated with
507 a deterioration of microclimatic conditions during budburst such as stronger shading under
508 gymnosperm crowns. Again this would render budburst more difficult, limit the photosynthetic
509 activity of budding leaves and increase the need for soil-derived carbon. Air temperatures are
510 indeed related to phylogenetic isolation of crowns in April (Table S8) and we found that
511 budburst can be related to air temperature (Table S6). However, as we state in the results, the
512 effect of air temperatures was not consistent across years.Regardless of the exact mechanism by
513 which phylogenetic isolation affects budburst, increasing enzymatic activities of EMf might
514 partly compensate for the delaying effect of phylogenetic isolation on budburst time. This is
515 what we found for laccase: increased laccase activity decreased the negative effect of
516 phylogenetic isolation on budburst. Such partial compensation of the effect of phylogenetic
517 isolation on the physiological performance of trees during budburst may dampen any farther-
518 reaching effects of phylogenetic isolation on tree growth (and, consistently, Yguel et al., unpubl.,
519 find such effects).However, the observed delay of 3-4 days in budburst between phylogeneticaly
520 isolated and non-isolated oaks could nevertheless have other major consequences by altering
521 competitive balance between plant species and thereby geographic distribution of tree species
522 (Vitasse et al., 2013).

523

4.3. Why budburst was still delayed?

Budburst was still delayed in phylogenetically isolated trees. Several reasons might explain this result. On the one hand, EMf might be unable to entirely compensate for the deterioration of abiotic and biotic conditions triggered by phylogenetic isolation. On the other hand, trees generally have to reward EMf for their services (for arbuscular mycorrhizal plants: see Kiers and Van der Heijden, 2006; Kiers et al., 2011). Hence, trees might have to "pay back" more for the high activity of their EMf under high phylogenetic isolation than for the low activity of EMf under low phylogenetic isolation. High costs for sustaining interactions with mutualists may limit tree performance and delay budburst.

4.4. What are the possible evolutionary implications and future directions?

At least for mixotrophic or heterotrophic plants, our study suggest that phylogenetic isolation of host plants may enhance the recruitment and activity of mutualists in response to the deterioration of environmental conditions. Phylogenetic isolation from neighbours can be interpreted in terms of niche evolution. Many lineages show phylogenetic conservatism in species niches (Wiens et al., 2010 or "signal" sensu Losos 2008), including the flora considered in our study (Prinzing et al., 2001). In case of such phylogenetic conservatism we expect that the ancestral niche is dominated by closely related species and a non-ancestral one is dominated by distantly related species. A plant colonizing such a non-ancestral environment would hence find itself phylogenetically isolated from its neighours.If leaving the ancestral niche exposes an individual to a deterioration of its environmental conditions, we might hypothesize that a stronger support from mutualists is then required for plants to colonize a new niche (Brundett et al., 2002). Such support from mutualists may be a case of niche construction i.e. a process that improves the response to a niche attribute affecting the fitness of individuals (Kylafis and

548 Loreau, 2011). In our study, niche construction was likely achieved by increasing investment
549 into interactions with EMf that decompose litter and hence facilitate nutrient uptake. Inversely,
550 EMf may benefit from host plants breaking with niche conservatism due to increased investment
551 of energy by host-plants into the support by mutualists. This is consistent with the idea that
552 mutualistic interactions between fungi and plants have evolved particularly during colonization
553 of new niches (Prinzing, 1999; Brundrett et al., 2002).

554

555 **5. Conclusion**

556 Here, we study a situation in which oak individuals converge with individuals of distantly related
557 species onto the same local environment. Success of such oaks would, theoretically, contribute to
558 an evolutionary expansion of niches of oaks towards niches of distantly related species. Our
559 study shows that EMf contribute to this success. The evidence remains correlative and
560 experiments manipulating mycorrhiza across the full life span of trees are needed to identify the
561 outcome of mutualist support for tree fitness. Moreover, future studies have to investigate
562 whether more intense interactions between EMf species and trees in the new niche involve the
563 same EMf species as in the ancestral niche, or whether new associations between trees and EMf
564 species are being formed. In other words to answer the following question: Do ancestral partners
565 help their hosts to colonize a new niche - or does the new niche provides colonists with the
566 partners they need to succeed in their colonization?

567

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SUPPORTING INFORMATION

Appendix S1. Details on the Rennes Forest and the species composition of trees surrounding the focal oaks studied.

Table S1. Phylogenetic distance between oak and neighboring species

Table S2. Relationship between soil humidity or pH and enzymatic activity excluding two outliers.

Table S3. Effect of phylogenetic isolation, enzymatic activities and the interaction term on the budburst phenology analysed without any transformation of the data.

Table S4. Effect of phylogenetic isolation, abiotic conditions and focal oak species on EMf abundance in simple and multiple regression analyses

Table S5. Effect of phylogenetic isolation, abiotic conditions and focal oak species on enzymatic activity of EMf in simple and multiple regression analyses

Table S6. Effect of phylogenetic isolation, abiotic conditions abundance and enzymatic activity of EMf on budburst phenology in simple regression analyses

Table S7. Variables significantly related to phylogenetic isolation: the effect of accounting for percentage of oaks as co-variable.

Table S8. Effect of phylogenetic isolation on abiotic conditions

Table S9. Effect of phylogenetic isolation on enzymatic activity of EMf in March and April, in simple regression analyses, considering only trees without gymnosperm neighbors.

TABLES AND FIGURES

Table 1. Multiple regression analyses explaining budburst phenology by the effect of phylogenetic isolation and enzymatic activity of EMf, and the interaction term between both. Enzymes considered show significant relationship to budburst in simple regression analysis. Phylogenetic isolation is expressed in million of year before present (MYBP) and enzymatic activity is expressed per unit of time and per EMf root tips projected area ($\mu M\ mm^{-2}\ min^{-1}$). Data are centered by their means (see Appendix S10 for analysis of uncentered data). Tolerances characterize the mutual independence among independent variables (i.e. 1 if an independent variable is entirely uncorrelated to the other independent covariables). P-values are for one tailed hypotheses.

		Effect on budburst phenology				
		Df	T	P	Standardized regression coeffcient	Tolerance
Model 1	Phylogenetic isolation[V1]		0.08	0.46	0.07	0.04
P=0.06	EMf β-glucuronidase (April)[V2]	18	0.55	0.29	0.46	0.05
r^2=0.31	V1* V2		-0.69	0.24	-0.19	0.45
Model 2	Phylogenetic isolation[V1]		0.44	0.33	0.13	0.39
P=0.03	EMf Laccase (March))[V2]	18	1.41	0.08	0.44	0.35
R^2=0.37	V1*V2		-0.72	0.24	-0.15	0.77
Model 3	Phylogenetic isolation[V1]		1.67	0.05	0.98	0.10
P=0.04	EMf Laccase (April) [V2]	18	-0.79	0.26	-0.45	0.10
R^2=0.35	V1*V2		-1.85	0.04	-0.50	0.48

Figure 1. Relationship between phylogenetic isolation of host trees and abundance of ectomycorrhizal fungi in March and April per soil core. The statistics for these relationships are respectively: d.f. = 20, $r^2 = 0.16$, t = 1.99, P = 0.06 (March); d.f. = 20, $r^2 = 0.18$, t = 2.16, P = 0.04 (April).

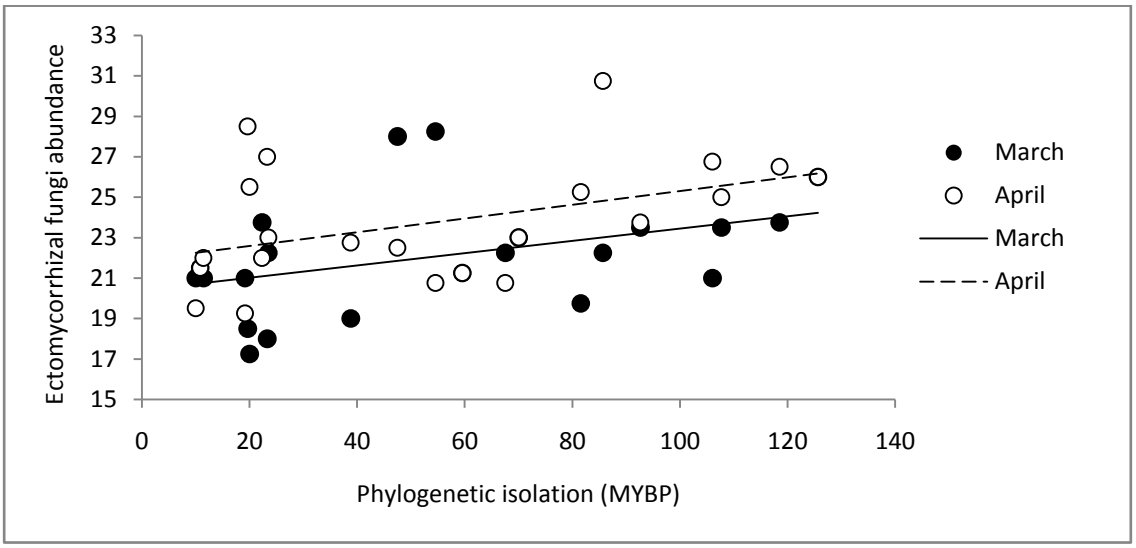
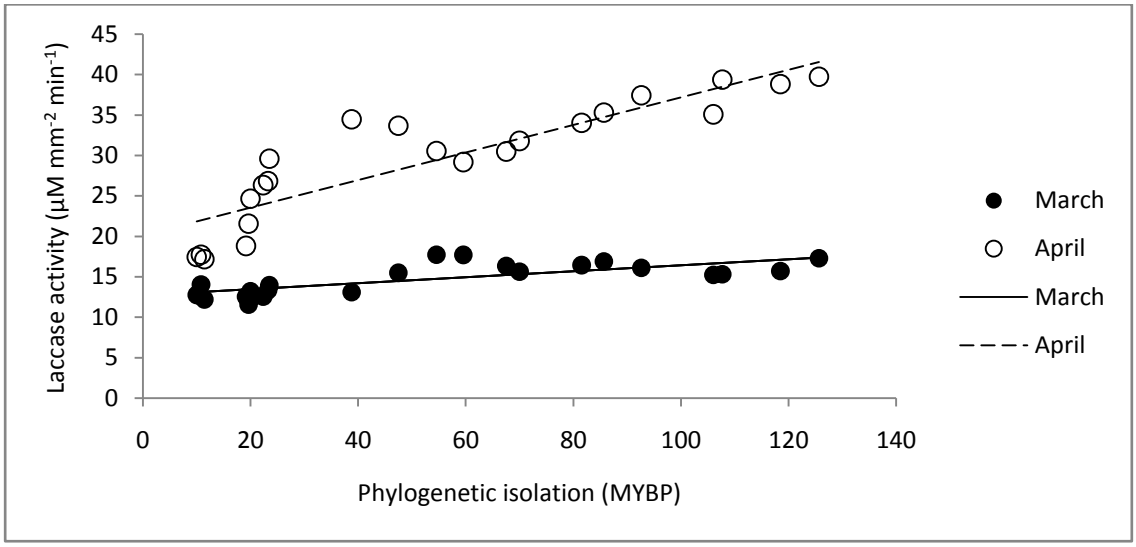
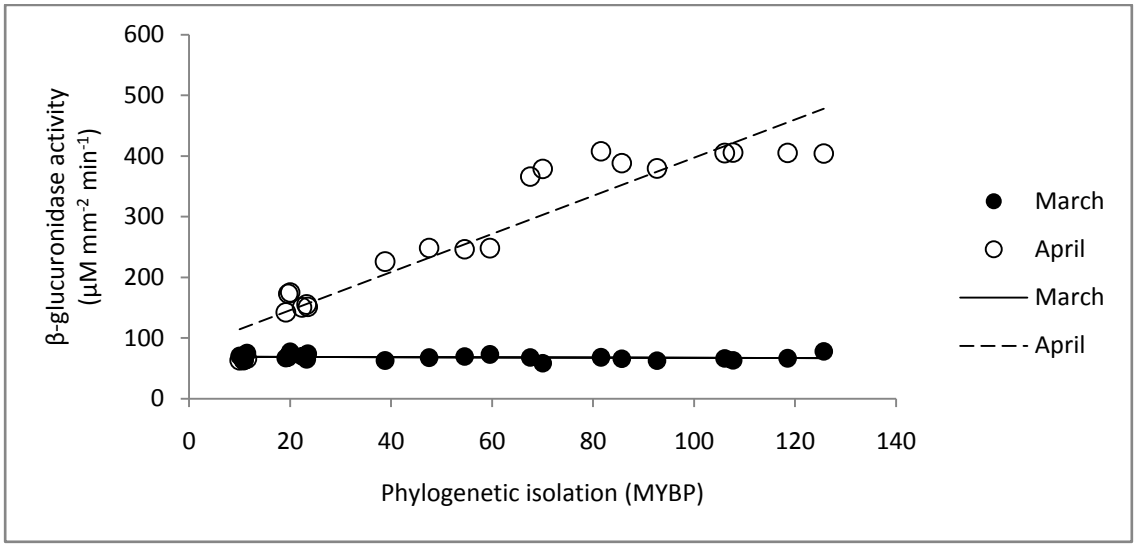


Figure 2. Relationship between phylogenetic isolation of host tree and EMf laccase activity in March and April (a), EMf β-glucuronidase activity in March and April (b), and EMf β-glucosidase activity in March and April (c). The statistics for these relationships are respectively: (a) March d.f. = 20, $r^2 = 0.54$, t = 4.92, $P = 8*10^{-5}$; April d.f. = 20, $r^2 = 0.78$, t = 8.61, $P = 3*10^{-8}$; (b) March d.f. = 20, $r^2 = 0.02$, t = -0.73, P = 0.47; April d.f. = 20, $r^2 = 0.89$, t = 12.96, $P = 3*10^{-11}$; (c) March d.f. = 20, $r^2 = 0.04$, t = -0.98, P = 0.33; April d.f. = 20, $r^2 = 0.09$, t = -1.43, P = 0.16.

(a)



(b)



779 **(c)**

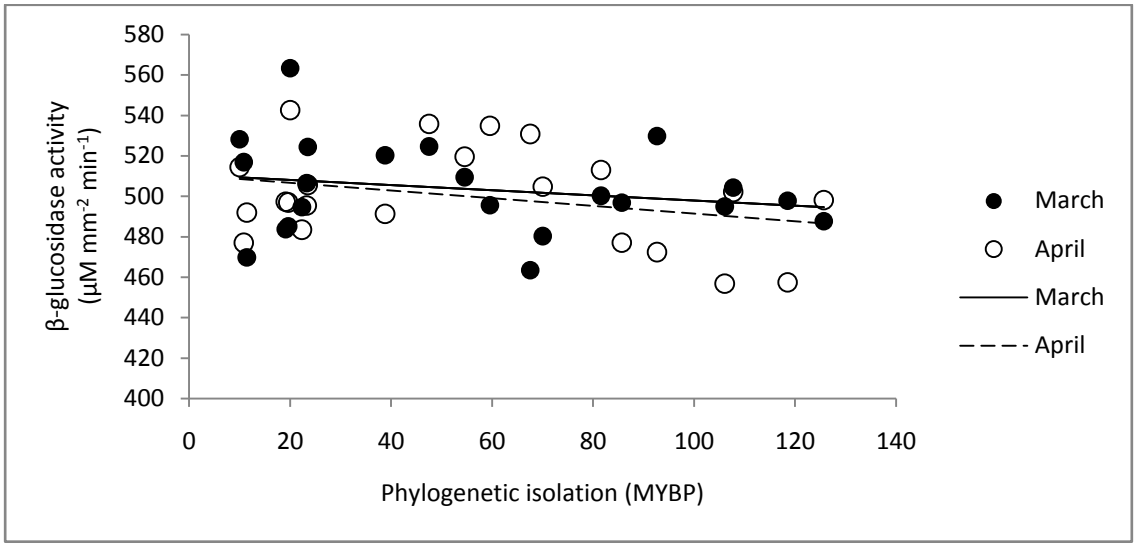


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Figure 3. Relationship between phylogenetic isolation and budburst phenology in oak trees. The statistics of the relationship are : df=20; t=2.34; p=0.02; r^2=0.21.

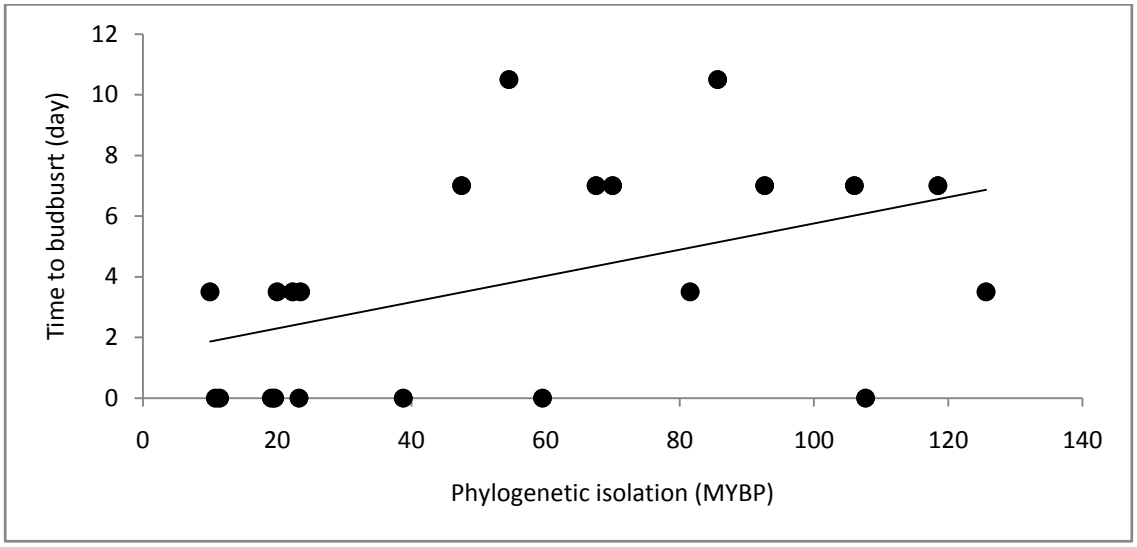

Figure 4. Representation of the statistical interaction effect between phylogenetic isolation and laccase activity of ectomycorrhizal fungi in April on budburst phenology based on the non-standardized regression coefficients of the variables in the interactive model (See Methods). Phylogenetic isolation strongly delayed budburst in oak trees with low laccase activity See table 1 for tests statistics.