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Title: Accumulation of biologically fixed nitrogen by legumes cultivated as cover crops in Switzerland

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Abstract

Aims Biological nitrogen fixation by legumes is expected to play a greater role in future cropping systems. Our study evaluated 19 legume species grown as cover crops in Swiss agroecosystems.

*Method*s Two field experiments were set up to monitor the biomass production and nitrogen content of 19 legumes and two non-legumes. The proportion of nitrogen derived from atmospheric N_2 (%Ndfa) was assessed using the ^{15}N natural abundance method. In parallel, a pot experiment was set up to determine the species-specific B values necessary to apply this method.

Results Some species produced an important amount of biomass in three months, up to 6.86 t DM/ha for *Vicia faba*. Five species, *Lathyrus sativus, Pisum sativum*, *Vicia sativa*, *V. villosa* and *V. faba*, acquired more than 100 kg N/ha through biological fixation. Important amounts of nitrogen were also derived from the soil. %Ndfa values showed high variability between and within species, ranging from 0% to almost 100%.

Conclusions Some legumes showed high N accumulation even in a short growing period, and could play an important role in fixing renewable nitrogen in crop rotation.

Keywords

^{15}N natural abundance method, B value, legumes, nitrogen accumulation, symbiotic N_2 fixation

Introduction

With the ongoing decrease of fossil fuel reserves, the reliance of agricultural systems on industrial fertilizers, especially nitrogen fertilizers, must be challenged. In this respect, the revival of legumes in crop rotation plays an important role (Bohlool et al. 1992). About 20-22 million tons of nitrogen (N) are fixed each year globally by crop legumes through biological fixation, compared to 85 million tons of N applied as fertilizer (Peoples et al. 2009). Thus, a potential for an increased utilization of biologically fixed N exists. Legumes are used as seed or forage crops, as cover crops between cash crops, or associated with other crops. In Europe, the area sown with grain legumes has decreased from 4.6% to 1.8% of arable land in fifty years (Bues et al. 2013) and varied regionally between 0.5% and 6.5% of the arable land (GL-Pro 2005). The environmental benefits of introducing grain legumes in the crop rotation are largely brought by N fixation (Nemecek et al. 2008). Legumes modify the N cycle in different ways and proportions, depending on their utilization. As grain crops, the input of legumes to the N pool is limited as a great amount of N is exported with the grain (Peoples et al. 2009). In contrast, cultivated in intercropping, legumes are known to transfer N to the associated crop or plants in arable and grassland systems (Høgh-Jensen and Schjoerring 1994; Dahlin and Stenberg 2010; Pirhofer-Walzl et al. 2012; Frankow-Lindberg and Dahlin 2013) and in arable systems (Jensen 1996ab; Moyer-Henri et al. 2006; Hauggaard-Nielsen et al. 2009). As cover crops, legumes are expected to offer additional agro-system services, along with N fixation (Thorup-Kristensen et al. 2003; Justes et al. 2012). Similarly to non-legume cover crops, legumes can increase N availability to the succeeding crop as well as reduce N leaching, sustain soil organic matter formation, improve soil structure and reduce weed pressure (Thorup-Kristensen et al. 2003; Justes et al. 2012). However, the additional N inputs brought by legumes to the cycle can promote weed growth or increase N leaching and denitrification (Thorup-Kristensen et al. 2003; Hofstra and Bouwman 2005; Justes et al. 2012). Therefore, the balance between positive and negative effects of legumes strongly depends on species-intrinsic properties such as growth rate, N uptake ability and symbiotic association rate, and on extrinsic factors such as timing of seeding, destruction modalities, pedo-climatic conditions, and soil type. Thus, legumes have to be properly managed in order to increase their usefulness in agricultural systems (Unkovich et al. 1997).

Consequently, the amount of N accumulated by the crops during the growing period is a key factor for the optimal integration of legumes in a rotation. In addition, the separate evaluation of the different N origins is important in order to estimate the amount of N derived from fixation and the amount accumulated from the soil solution. Biological N fixation is highly

77 variable depending on the species and on environmental conditions. The proportion of total
78 plant N resulting from biological fixation (%Ndfa) can vary from 5% to 97% (Unkovich and
79 Pate 2000). Many parameters determine the intensity of N fixation, particularly legume
80 species, soil fertility, fertilization rate, soil tillage and climatic conditions (Jensen 1996c;
81 Carranca et al. 1999; van Kessel and Hartley 2000).
82 Several methods aim at evaluating the rate of biological N fixation (Unkovich et al. 2008).
83 Among these, the ^{15}N natural abundance method has been rather successfully applied in
84 agricultural systems (Amarger et al. 1979; Shearer and Kohl 1986; Unkovich et al. 2008). The
85 use of this method requires an accurate evaluation of the so-called "B value", which
86 corresponds to the "$\delta^{15}N$ of shoots of legumes that are solely dependent upon N_2 fixation and
87 sampled at the same growth stage as the field plants" (Unkovich et al. 2008). B values can be
88 found in the literature for several legume species. However, they are known to be influenced
89 by several factors such as cultivar, growth stage, rhizobial strain, and soil conditions. So,
90 ideally, B values have to be assessed in the same conditions as the field experiment, with
91 plants to be sampled at the same growth stage as the plants sampled in the field, and with the
92 same rhizobial strains.
93 In this study, 19 legume species were cultivated in two field experiments in Switzerland, in
94 order to assess their potential use as cover crops in the context of a short three-months
95 growing period. In particular, we aimed to evaluate the performance of each species in terms
96 of biomass production, N accumulation, and the amount of N derived from biological fixation
97 by the ^{15}N natural abundance method. At the same time, specific B values were determined in
98 a pot experiment for the 19 legumes.
99
100
101 **Materials & Methods**
102 Nineteen legume species were chosen according to their actual or potential use as cover crops
103 in Swiss agroecosystems, including some Mediterranean species (Table 1). Phacelia (*Phacelia*
104 *tanacetifolia*) and oat (*Avena sativa*) were used as non-legume reference cover crops.
105
106 *Field experiments*
107 The field experiments were conducted in 2011 at two different sites in Switzerland (Changins:
108 46° 24’ N - 06° 14’ E, 426 m asl; Zollikofen: 46° 59’ N - 07° 28’ E, 544 m asl). The two sites
109 had similar soil clay content and pH, showed comparable cumulative rainfall from seeding to
110 harvest, but differed markedly in the soil organic matter and mineral N content (0-90 cm,

bare soil) in mid November (Table 2). The cover crops were sown in plots (10 m^2) with a plot seeder at the beginning of August, after a winter wheat crop (straw exported, ploughed). Seeding rates were adapted from recommended rates for cash crops to fit to a utilization as cover crops (Table 1). Target plant density was homogenised among species as a function of plant architecture and to offer a consistent high soil cover. The experimental design followed a randomized complete block design with three replicates. No fertilization and no inoculation with specific rhizobia were done, in order to correspond to basic management of cover crops. At the beginning of November, the whole plot surfaces were machine harvested at 5 cm and total aboveground dry matter of each crop was determined (drying at 65°C for 48h). The biomass N content (%N) was determined according to the Swiss standard methods (FAL et al. 2004). Shoot N yields (Ntot in kg/ha) were calculated from the product of biomass and N concentration (%N).

The measurement of $\delta^{15}N$ values and additionally the %C and/or %N of the samples was performed using a Flash EA 1112 Series elemental analyzer (Thermo Italy, former CE Instruments, Rhodano, Italy) coupled to a Finnigan MAT Delta^{plus}XP isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) via a 6-port valve (Brooks et al. 2003) and a ConFlo III (Werner et al. 1999). The measurements of the δ values and used laboratory standards followed the scheme described by Werner and Brand (2001). The $\delta^{15}N$ values are expressed in ‰ and represent the deviation from atmospheric ^{15}N concentration assumed to be constant at 0.3663 atom% ^{15}N. For each legume, the part of N derived from atmospheric N_2 (%Ndfa) was computed using the ^{15}N natural abundance method (Amarger et al. 1979; Unkovich et al. 2008). This method takes advantage of the naturally higher ^{15}N concentration of the N available in the soil compared to atmospheric N_2.

$$\%Ndfa = 100\ \frac{\delta^{15}N\text{ref} - \delta^{15}N\text{leg}}{\delta^{15}N\text{ref} - \text{B}}$$

where $\delta^{15}N_{leg}$ is the isotopic composition of the N-fixing legume, $\delta^{15}N_{ref}$ is the isotopic composition of a non-N_2-fixing species (reference species), and B is the $\delta^{15}N$ of shoots of the respective legume when grown in total dependence upon N_2 fixation. B values were assessed specifically for this study in a separate pot experiment (see below).

For each cover crop species, %Ndfa was computed separately for each site and each replicate (to reduce the influence of spatial variation in ^{15}N abundance on the %Ndfa estimates),. To obtain accurate estimations of %Ndfa, the use of several reference plants is recommended (Unkovich et al. 2008). Ideally, the reference plants should have a root system and N uptake ability similar to the legumes studied, in order to correctly reflect the N pool available for the

144 legumes in the soil. It is assumed that dicot species are closer to legumes than grasses in terms
145 of root system (Unkovich and Pate 2000), but both types of species have been used in the
146 literature. Here phacelia was chosen as a dicot reference plant, and oat as an alternative
147 monocot reference, known to grow well as cover crop on the studied sites. Alternatively with
148 oat and phacelia as reference plants, twelve %Ndfa values were provided in combination with
149 the two sites and three replicates.
150 When necessary, the %Ndfa values <0% (δ^{15}N of the legume species higher than the δ^{15}N of
151 the reference plant) were corrected to 0% (11 cases from 228). The %Ndfa values >100%
152 (δ^{15}N of the legume species smaller than the corresponding B value) were rounded to 100%
153 (20 cases from 228), following the recommendations of Unkovich et al. (2008) for use when
154 less than 10% of legume samples are concerned. For these cases, an alternative correction
155 method was also applied for comparison. This method consisted in using the lowest δ^{15}N
156 values observed in the field as the full N_2 fixing reference instead of the measured B value,
157 which is sometimes proposed when B values cannot be determined experimentally (Peoples et
158 al. 1997, 2002; Unkovich et al. 2008; Roscher et al. 2011).
159 The total quantity of N symbiotically fixed in the aboveground biomass Ndfa (in kg/ha) was
160 computed from the product of %Ndfa and Ntot. For each legume in each replicate, the mean
161 %Ndfa between the values obtained from the two reference plants was used. The quantity of
162 N accumulated from the soil was derived by subtraction of Ndfa from the total N content of
163 dry biomass.
164 Overall and within site analyses of variance were performed to test for the effect of sites and
165 species on the main variables presented here. All data and statistical analyses were done using
166 R 3.1.1 (R Core Team 2014).
167
168 *Determination of the B values*
169 B values are known to be influenced by several important factors. The rhizobial strains
170 responsible for N fixation may exhibit differential isotopic fractionation properties (Ledgard
171 1989; Unkovich et al. 1994, 2008). Different host cultivars or genotypes can show divergent
172 isotopic fractionation (Unkovich et al. 1994), which is also influenced by soil moisture and
173 nutrition levels (Ledgard 1989). Just after emergence, shoot δ^{15}N reflects the δ^{15}N of the seed
174 reserves and then decreases gradually with plant growth as symbiotic activity increases and
175 shoots accumulate fixed N (Unkovich et al. 1994, 2008; Boddey et al. 2000). Thus plant
176 growth stage also influences B values. The harvested plant organs have to be identical for B
177 value determination and δ^{15}N field measures, because roots and shoots exhibit contrasted

$\delta^{15}N$, with accumulation of ^{15}N in nodules (Turner and Bergersen 1983; Yoneyama et al. 1986). Thus, the best way to obtain reliable B values is to experimentally determine these values under the same conditions as the studied setting. For these reasons, an experiment where all 19 legume species were grown in 10 L pots (3 replicates) was set up in 2011 in the same period, in Zollikofen, close to field conditions. The pots were placed outside, protected from the rain by a plastic cover, with ambient light and temperature conditions.

The pots were filled with quartz sand (washed and sterilized at 105°C for 24 hours), and then inoculated with a soil suspension obtained from a fresh soil sample from Changins. This soil was chosen because, in preceding years, it supported the cultivation of various legume crops with the appropriate rhizobium inoculation. The seeds were sterilized using the method described in Hellsten and Huss-Danell (2000). The pre-germinated seedlings were then transplanted into the pots (8 plants/pot). Pots were watered as needed with an N-free nutrient solution adapted from Unkovich et al. (1994). The nutrient solution contained distilled water and macronutrients (in mM): $MgSO_4$ (2), $CaCl_2$ (2) K2SO4 (2), KH_2PO_4 (0.5), K_2HPO_4 (0.5), Fe-EDTA (1) and trace elements (in µM) H_3BO_3 (4.5), $MnCl_2$ (1.8), $ZnCl_2$ (1), $CuSO_4$ (0.3), Na_2MoO_4 (0.1), $CoCl_2$ (0.2).

After harvest, aboveground biomass was dried at 50°C for 72 hours and then analyzed to determine shoot $^{15}N/^{14}N$ sample ratios as described above, and to compute the corresponding B values. For each legume, the mean B value was used in order to assess %Ndfa.

Results

B values

B values differed significantly between species (Table 3), ranging from -2.6‰ for *Cicer arietinum* to -0.08‰ for *Vicia faba* (Table 4). Most of them (14 out of 19) lay between 0‰ and -1‰. Five species, *Vicia sativa*, *Trifolium alexandrinum*, *Lens culinaris*, *Glycine max* and *Cicer arietinum,* showed B values lower than -1‰. Intraspecific variation in B value was low, except in *Trigonella foenum-graecum* (Figure 1).

Biologically fixed and total nitrogen concentration

The mean isotopic composition $\delta^{15}N$ measured in the two field experiments was lower in the legumes than in the two reference species (Table 4). The $\delta^{15}N$ of phacelia was consistently higher than that of oat. Some legumes also showed high $\delta^{15}N$ values in the field: *Cicer arietinum, Glycine max, Lupinus albus, Trifolium subterraneum*, and *Trigonella foenum-*

graecum. The δ^{15}N values were generally higher in Zollikofen than in Changins (respectively 1.83 and 0.09, $p<0.001$, Table 3). The values of the two reference plants, oat and phacelia, were also markedly lower in Changins (Table 4).

Consequently, this site showed higher mean %Ndfa than Zollikofen (respectively 68% and 59%), though this difference was not significant ($p<0.05$, Table 3), due to the high variability between species, ranging from 7% to 100% (Figure 2). Species differed significantly in their %Ndfa ($p<0.001$, Table 3), and the interaction with the experimental site was also significant ($p=0.010$, Table 3). Four species, *Trifolium repens*, *Lathyrus sativus*, *Vicia villosa* and *Vicia pannonica*, showed a mean %Ndfa higher than 80%, and two species, *Cicer arietinum* and *Glycine max*, a mean %Ndfa lower than 20%.

The use of the alternative method to assess %Ndfa in the cases where it exceeded 100% led to the following changes in the mean estimated values of the concerned species: for *Lathyrus sativus* from 84.5% to 82.7%, for *Trifolium incarnatum* from 75.7% to 68.0%, for *Trifolium repens* from 84.0% to 83.5%, for *Trifolium resupinatum* from 74.4% to 59.7%, for *Vicia faba*, from 78.6% to 74.5%, for *Vicia pannonica* from 87.6% to 79.2% and for *Vicia villosa* from 87.2% to 82.4%.

The %Ndfa obtained with phacelia as reference plant was systematically higher than that estimated with oat as reference plant (mean difference of 7%, paired t-test: $p<0.001$), due to their difference in δ^{15}N. However, the correlation between the mean %Ndfa estimated with oat and phacelia was very high (Kendall's rank correlation tau: 0.89, $p<0.001$).

Nitrogen concentration in shoots also varied between species ($p<0.001$, Table 3), with mean values lower than 2% for *Lupinus albus* and mean values higher than 4% for *Lathyrus sativus*, *Vicia pannonica*, *Vicia sativa* and *Vicia villosa* (Table 4). Nitrogen concentration %N correlated positively with %Ndfa at the two sites (Kendall's rank correlation tau: 0.65, $p<0.001$ in Changins, and 0.56, $p<0.001$ in Zollikofen). In Zollikofen, %Ndfa apparently reached a limit around 80%, regardless of the %N value achieved, whereas in Changins, %Ndfa continued to increase with increasing %N values (Table 4). The mean carbon/nitrogen ratio (C/N) varied between species ($p<0.001$, Table 3), from 9.8 in *Vicia pannonica* to 32.5 in *Lupinus albus* (Table 4). As expected, oat and phacelia had high C/N ratios, of 36.7 and 35.7 respectively. The average N concentration and C/N ratio were similar between sites (Table 3).

Biomass production and nitrogen accumulation

The mean dry biomass accumulated by legumes during the three months period was highly variable ($p<0.001$, Table 3), ranging from a minimum of 0.75 t/ha in *Cicer arietinum* to a

maximum of 6.86 t/ha in *Vicia faba* (Table 4). For comparison, the two non-legume reference plants reached a mean biomass of 4.21 t/ha in oat and 5.37 t/ha in phacelia. The corresponding total amounts of N accumulated above ground were 16 kg/ha for *Cicer arietinum* and 186 kg/ha for *Vicia faba* (Table 4). Oat and phacelia accumulated respectively 53 kg/ha and 64 kg/ha, exclusively from the soil solution. Concerning the quantity of N acquired through biological N_2 fixation, five species, *Pisum sativum*, *Vicia sativa, Lathyrus sativus*, *Vicia villosa* and *Vicia faba*, exceeded 100 kg/ha (Table 4). The quantity of N derived from the soil by the legumes could reach high values, similar to those observed for the two non-legume reference plants. In particular, *Glycine max* removed on average 70 kg N/ha from the soil in three months, which was more than the uptake of oat and phacelia.

The quantities of N accumulated were significantly linked to biomass production and the rates varied as a function of the sources. Modeled by linear regression, this led to a total accumulation rate of about 25 kg of N / t of DM (Changins: Ntot=16+25*DM, R^2=0.73, $p<0.001$; Zollikofen: Ntot=18+25*DM, R^2=0.58, $p<0.001$), with 18 kg N / t DM coming from the atmosphere (Changins: Ndfa=12+18*DM, R^2=0.43, p=0.002; Zollikofen: Ndfa=9+17*DM, R^2=0.36, p=0.006) and 7 kg N / t DM from the soil (Changins: Nsoil=4+7*DM, R^2=0.33, p=0.010; Zollikofen: Nsoil=9+7*DM, R^2=0.62, $p<0.001$).

The amount of aboveground biomass was not correlated with either %N (Kendall's rank correlation tau: -0.18, p=0.30 in Changins, -0.16, p=0.37 in Zollikofen) or %Ndfa (Kendall's rank correlation tau: -0.02, p=0.95 in Changins, 0.03, p=0.89 in Zollikofen).

Discussion

B values

In general, the B values measured in this study were in the range of the values found in the literature, except for *Cicer arietinum* (lower values) and *Vicia faba* (higher values) (Figure 1). The species for which no literature values were found tended to have B values similar to those of closely related species. For example, *Trifolium incarnatum* had values similar to the other *Trifolium* species, *Lupinus albus* similar to *Lupinus angustifolius* (Figure 1), and *Vicia pannonica* similar to *Vicia faba*.

The values found in the literature show that B values vary significantly between species but above all within species (Figure 1 and Online Resource 1). Notably, literature B values ranged from -3.61 (Okito et al. 2004) to +1 (Turner and Bergersen 1983) for *Glycine max*, from -0.95 (Yoneyama et al. 1986) to +3.14 (Turner and Bergersen 1983) for *Medicago sativa*, from -

1.65 (Shipanski and Drinkwater 2012) to +1.88 (Kohl and Shearer 1980) for *Trifolium pratense*, from -2.48 (Riffkin et al. 1999) to +0.78 (Unkovich et al. 2008) for *Trifolium repens* and from -0.78 (Unkovich et al. 2008) to +2.68 (Bergersen and Turner 1983) for *Trifolium subterraneum*. These variations within species can be explained by several factors, such as crop variety, developmental stage, rhizobial strain, pedoclimatic conditions, which are known to influence B values (Unkovich et al. 1994, 2008). These could thus explain the variations observed between literature data and our own results on one hand and among literature data on the other hand. This indicates the necessity to identify specific B values when simultaneously studying different species. When specific B values cannot be determined, using the median value of literature values could be a good alternative. However the comparison of the median literature values with the B values determined in this study showed important discrepancies for some species such as *Cicer arietinum*, *Vicia faba*, *Lens culinaris* and *Trifolium subterraneum* (Figure 1). Mean %Ndfa based on the median literature B values differed from %Ndfa evaluated in this study by more than 10% for *Lens culinaris*, *Trifolium subterraneum* and *Vicia faba*. However, this apparently reasonable difference would have led to a significant misestimation of the quantity of N acquired through biological fixation by those species.

Biologically fixed nitrogen

For the species that had no growth problem on our experimental sites, the %Ndfa values observed were similar to those reported in literature (Unkovich and Pate 2000; Peoples et al. 2009). The %Ndfa depends not only on the species but also on the quantity of N available from the soil (Høgh-Jensen and Schjoerring 1994; Unkovich and Pate 2000). Here, lower %Ndfa were observed in Zollikofen compared to Changins, which can be explained by the difference in the soil mineral N values measured at the end of the growing period (124 kg N/ha in Zollikofen compared to 78 kg N/ha in Changins) and the higher soil organic matter content in Zollikofen. In addition, %Ndfa attained an upper limit around 80% in Zollikofen, while higher values were observed in Changins for the same resulting %N in the aboveground biomass. Some species also showed differential response to the two sites. In particular, *Glycine max, Lupinus albus* and *Trigonella foenum-graecum* achieved very low %Ndfa in Zollikofen, although they produced rather reasonable amounts of dry matter. This could be due to the absence of the appropriate symbiotic rhizobia in the Zollikofen soil, in contrast to Changins where *Lupinus albus* and *Glycine max* are regularly cultivated. It also suggests that the capacity to establish successful symbiosis may depend on complex plant-rhizobia-soil

interactions or on local conditions such as soil pH or moisture at the onset of the field experiment. The high soil pH measured at both sites in this study could have affected a sensitive species like *Lupinus albus*, but the lime activity of soil is known to be low at Changins. Although the lime activity at Zollikofen was unknown, *Lupinus albus* grew well there, presumably making use of soil N. Here %Ndfa values were not correlated with dry matter yield, showing that species producing low biomass were not necessarily limited by a poor functioning of the N_2 fixation processes but rather by soil or climatic limiting factors. The present study showed that some species were able to fix more than 100 kg/ha of N during a period of just three months. In particular, *Vicia faba* fixed a lot of N (150 kg/ha), confirming the observations by Lopez-Bellido et al. (2006) for this crop in wheat rotation in Spain. Some species with low %Ndfa exhibited very high accumulation from the soil N, similar to what was observed for the two non-N_2-fixing reference species. Similarly, better fixing legumes absorbed also a significant amount of N from the soil solution. These results show that legume cover crops could also play an important role as catch crops. Due to the very large amounts of nitrogen accumulated by legumes, proper management is required to reduce N leaching potential. Besides climatic conditions, the C/N ratio of the residues is the main factor influencing the dynamics of mineralization of the nitrogen accumulated by the cover crops and consequently its availability for the succeeding crop and the risk of leaching (Thorup-Kristensen and Nielsen 1998; Justes et al. 2009). An optimal management will take into account the timing and modality of the breakdown of biomass, as well as the identity of the succeeding crop (Justes et al. 2012).

In Switzerland, cover crops are mainly used in the following systems: short term cover after rapeseed, before winter wheat; long term cover before spring crops (e.g. summer cereals, sugar beet, sunflower, maize). To avoid too much N accumulation and to optimise C/N ratio, legumes are generally seeded in mixture with other species (e.g. oat, phacelia). In addition, the destruction of the cover crops is performed at minimum one month before the seeding of spring crops. Our results will thus help to identify the best performing legumes in terms of biomass and amount of fixed nitrogen and to design new cover crop mixtures.

This study evaluated the nitrogen accumulated in the aboveground biomass (shoots), but it is necessary to consider the N contained in the roots to accurately estimate the total N brought by the legumes into the system. Although shoot:root N ratios are expected to vary with growth conditions, the total N accumulated (shoot + root) could be estimated from published values of such ratios. Applying the specific values of Unkovich and Pate (2000) to our data, we obtained total plant N amount of 27 kg/ha for *Cicer arietinum* (shoot:root N ratio: 1.4),

137 kg/ha for *Lens culinaris* (1.8), 126 kg/ha for *Lupinus albus* (1.4), 143 kg/ha for *Medicago sativa* (1.0), 310 kg/ha for *Vicia faba* (1.5) and 235 kg/ha for *Vicia sativa* (2.1). Rhizodeposition is also an important process responsible for N input in the soil, although its contribution to soil N is difficult to quantify (Fustec et al. 2010). N deposition values ranging from 4% to 71% of total plant N were reported by Fustec et al. (2010). These cumulative quantities of N are so substantial, showing that legumes could be successfully used as short period cover crops if the aim is to increase N inputs in the rotation.

Limits of the work

The applicability and precision of the ^{15}N natural abundance method depends strongly on the accurate determination of the B values and on the choice of the reference plants (Pate et al. 1994; Unkovich et al. 1994). The precision of the used B values is particularly important at high %Ndfa estimates (Unkovich et al. 2008). Thus, the used B values should be determined under conditions most similar to the field conditions including rhizobial strain and developmental stage of plants.

Nevertheless, some species exhibited lower $\delta^{15}N$ values than their respective B values, leading to unrealistic values of %Ndfa greater than 100% at Changins. This could be due to differences in the growth conditions or in effective rhizobial-plant association between the field and pot situation (Unkovich et al. 2008). Further, it shows that the use of a field inoculum for the B value determination does not guarantee identical rhizobium symbiosis, although in our experiment, the source of soil suspension was obtained from one of the experimental sites, historically cultivated with different legume species (including *Glycine max* and *Lupinus albus*, but not *Cicer arietinum*). These deviating values were rounded to 100%, but the use of the alternative correction method slightly decreased the estimates. The use of several soil solutions to experimentally determine B values should thus be recommended, but would considerably increase the effort and time required and probably goes beyond the aim of most of the studies using the ^{15}N natural abundance method.

The effect of the choice of the reference plant was observed in our experiments by systematically lower values of $\delta^{15}N$ in oat compared to phacelia. These low $\delta^{15}N$ values for oat lead to several estimations of %Ndfa < 0, occurring when the $\delta^{15}N$ of the legumes in the field were higher than that of the reference plant (9 cases with oat, only 2 cases with phacelia as reference plant). In these cases, the symbiosis with rhizobia was probably absent or not effective, or large amounts of N were available from the soil. This suggests that oat was indeed not the best choice for a reference plant in our experiment, although it grew well as

cover crop. This confirms that dicot species are probably better reference plants for legumes due to their more similar root systems (Unkovich and Pate 2000). Gehring and Vlek (2004) showed that the use of non-N_2-fixing legume references allowed to overcome the problem of negative %Ndfa estimates.

The accuracy of %Ndfa estimations is also influenced by the level of the soil ^{15}N abundance. Values of reference $\delta^{15}N$ higher than 2% (or 4%) are generally recommended for use in %Ndfa computation (Unkovich et al. 2008). Here the soil ^{15}N available for plants (estimated from the $\delta^{15}N$ of the non-fixing plants oat and phacelia) in the Changins site was lower than in Zollikofen, and was lower than 2% in oat. Similarly, it is recommended that the difference between the reference $\delta^{15}N$ and B values should be at least 5‰ (Högberg 1997). With the low $\delta^{15}N$ values in Changins, this minimal difference could not be attained. This can lead to poor accuracy in estimating %Ndfa (Unkovich et al. 1994), as found at this site.

Conclusions

Our study on the performance of 19 legume species, potentially useful as cover crops, showed important differences in terms of biomass production and N accumulation. In addition, this study provided specific B values to compare the legume species in terms of symbiotic activity and N accumulation. Substantial amounts of N were accumulated by *Vicia faba, Vicia villosa* and *Lathyrus sativus*. However, taking into account the seed cost per hectare of the different legumes, the cultivation of *Vicia villosa* in Changins and *Vicia sativa* in Zollikofen would be recommended based on the aims and results of this study. The 150 kg/ha of N biologically fixed by *Vicia faba* in only three months corresponds to about 230 kg/ha oil equivalent in the form of mineral N fertilizer (ecoinvent Centre 2014), making a major contribution to the overall favorable results of legumes in environmental life cycle impact assessments (Nemecek et al., 2008). Legumes support the development of the succeeding crop through N inputs to the system, contribute to the constitution of soil organic matter, and modify the availability of phosphorus (Gallet et al. 2003; Peoples et al. 2009). The best performing legumes are also good candidates for use in crop mixtures, as they promote the performance of associated species. The association of complementary species potentially enlarges the services provided by cover crops and increases their profitability. In addition, legumes can play an important role in mitigating climate change (Jensen et al. 2012). Thus the important and multiple services provided by legume cover crops bring new and interesting perspectives for the management of the non-productive intercrop period, and the revival of legumes in agroecosystems must be a priority in the near future.

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695 **Table and figure legends**

696

697 **Table 1** Targeted plant density of the studied legumes species (english name, latin name and
698 cultivar when available)

699

700 **Table 2** Characteristics of the two sites used for the field experiments. The temperature
701 corresponds to the mean value from sowing (beginning of August) to harvest (beginning of
702 November), and the rainfall to the cumulated rainfall values during the same period. SOM:
703 soil organic matter

704

705 **Table 3** Analyses of variance for the variables measured. Factors: site and species. df: degree
706 of freedom, MS: mean square, p: p-value. Significant p-values at 0.05 are indicated in bold

707

708 **Table 4** Mean values of the variables measured for each species, separately for the two sites.
709 The p-value from the analysis of variance within each site is indicated below, along with the
710 corresponding 5% LSD. Ndfa: nitrogen derived from atmospheric N_2, Ntot: total aboveground
711 nitrogen, C/N: carbon/nitrogen ratio

712

713 **Fig. 1** B values from the pot experiment (boxplots) and from the literature (crosses, the
714 median literature value is represented by the circle). The literature value points for *Lupinus*
715 *albus* correspond to data for *Lupinus angustifolius.* For data sources see Online Resource 1

716

717 **Fig. 2** %Ndfa for each legume species, for the two different sites. White: Changins, black:
718 Zollikofen. Each box represents the distribution of the n=6 estimations (3 replicates x 2
719 reference plants) of %Ndfa in each site. Different letters (within each site) indicate significant
720 differences in mean %Ndfa according to a Tukey HSD post hoc test performed independently
721 for each site (within site analyses of variance for differences among species are significant at
722 each site)

723

724 **Table 1**

Species	Latin name	Cultivar	Targeted plant density [pl/m2]
Chickpea	*Cicer arietinum*	unknown, from Canada	150
Soybean	*Glycine max*	Gallec	150
Grasspea	*Lathyrus sativus*	Merkur	150
Lentil	*Lens culinaris*	unknown, from Canada	150
White lupin	*Lupinus albus*	Amiga	150
Alfalfa	*Medicago sativa*	Sanditi	1000
White sweet clover	*Melilotus albus*	unknown	1500
Field pea	*Pisum sativum*	Hardy	100
Berseem clover	*Trifolium alexandrinum*	Tigri	600
Crimson clover	*Trifolium incarnatum*	Carmina	800
Red clover	*Trifolium pratense*	Suez	1000
White clover	*Trifolium repens*	Pepsi	2500
Persian clover	*Trifolium resupinatum*	Lightning	1500
Subterraneum clover	*Trifolium subterraneum*	Daliak	500
Fenugreek	*Trigonella foenum-graecum*	unknown	400
Faba bean	*Vicia faba*	Fuego	150
Hungarian vetch	*Vicia pannonica*	Beta	250
Common vetch	*Vicia sativa*	Ebena	250
Hairy vetch	*Vicia villosa*	unknown	250
Oat	*Avena sativa*	Expander	400
Phacelia	*Phacelia tanacetifolia*	Boratus	400

725

726

727 **Table 2**

	Climate		Soil			
	Temperature [°C]	**Rainfall [mm]**	**Clay [%]**	**pH**	**SOM [%]**	**mineral N [kg/ha]**
Changins	15.8	233	23	7.4	2.0	78
Zollikofen	13.9	199	20	7.7	4.0	124

728 **Table 3**

		B value			$\delta^{15}N$		%Ndfa		%N		Biomass		Ndfa		Ntot		C/N	
	df	MS	p		df	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
site	-	-			1	86.87	**<0.001**	1888	0.121	0.02	0.772	2.72	0.098	3534	0.058	1936	**0.037**	16.1	0.203
error	-	-			4	0.82		488		0.23		0.59		512		203		7.0	
species	18	0.92	**<0.001**		18	6.02	**<0.001**	2989	**<0.001**	4.17	**<0.001**	14.24	**<0.001**	10742	**<0.001**	12662	**<0.001**	179.7	**<0.001**
site:species	-	-			18	2.31	**<0.001**	542	**0.010**	0.31	**<0.001**	0.99	**<0.001**	1471	**0.007**	1545	**<0.001**	15.1	**<0.001**
error	36	0.04			72	0.41		248		0.10		0.28		646		529		3.4	

729

730 **Table 4**

	B value	$\delta^{15}N$		N [%]		Biomass [tDM/ha]		Ndfa [kg/ha]		Ntot [kg/ha]		C/N	
Species	**Pots**	**C**[a]	**Z**[a]	**C**	**Z**	**C**	**Z**	**C**	**Z**	**C**	**Z**	**C**	**Z**
Cicer arietinum	-2.60	1.32	3.98	2.03	2.25	1.11	0.40	3	2	22	9	21.5	20.4
Glycine max	-1.46	0.81	5.36	2.88	1.75	4.83	3.41	55	4	143	60	16.2	24.3
Lathyrus sativus	-0.54	-0.39	0.87	4.05	4.44	3.99	2.95	149	101	161	129	10.7	9.6
Lens culinaris	-1.18	-0.37	0.49	3.03	3.39	2.23	3.20	50	81	67	108	14.1	13.0
Lupinus albus	-0.74	0.70	4.47	1.60	1.24	5.60	4.68	40	9	88	59	27.8	37.2
Medicago sativa	-0.79	0.64	0.81	2.95	3.68	2.03	2.27	27	62	60	83	14.9	12.5
Melilotus albus	-0.91	0.15	1.39	3.03	3.50	1.72	1.57	32	35	52	55	14.8	12.7
Pisum sativum	-0.56	0.13	1.09	3.01	3.14	5.52	4.46	115	102	166	139	14.4	12.9
Trifolium alexandrinum	-1.11	0.43	0.71	2.33	2.54	3.24	3.19	35	59	76	81	18.5	17.2
Trifolium incarnatum	-0.67	-0.58	1.57	3.22	2.77	3.27	3.25	90	57	105	90	13.6	15.3
Trifolium pratense	-0.83	-0.19	1.14	3.42	3.38	1.40	1.61	36	37	47	54	13.1	13.3
Trifolium repens	-0.53	-0.28	0.66	3.77	3.95	0.97	1.47	32	47	36	58	11.4	11.2
Trifolium resupinatum	-0.81	-0.51	0.72	2.88	3.28	2.17	2.61	46	65	62	85	14.2	14.2
Trifolium subterraneum	-0.81	-0.08	3.20	3.44	2.83	1.70	1.49	43	19	59	43	12.7	15.2
Trigonella foenum-graecum	-0.42	0.84	4.37	2.28	1.93	3.84	1.76	62	7	90	33	19.6	22.9
Vicia faba	-0.08	0.29	1.17	2.71	2.68	7.45	6.27	172	129	204	169	16.5	17.1
Vicia pannonica	-0.14	-0.34	1.12	4.23	4.80	1.90	2.00	78	74	80	96	10.2	9.5
Vicia sativa	-1.09	-0.36	0.58	4.04	4.00	3.54	4.39	107	131	143	176	10.4	10.2
Vicia villosa	-0.35	-0.50	1.15	4.44	4.32	3.57	3.23	159	100	159	135	10.0	10.3
Avena sativa		1.48	5.07	1.04	1.40	3.60	4.82			38	68	40.5	32.9
Phacelia tanacetifolia		2.24	6.13	1.05	1.34	5.23	5.50			55	74	37.5	33.8
site mean[b]	-0.82	0.09	1.83	3.12	3.15	3.16	2.85	70	59	96	88	15.0	15.7
p-value[c]	<0.001	0.040	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
LSD 5%	0.32	1.14	0.96	0.54	0.50	1.04	0.72	55	23	47	26	3.2	2.9

731 [a]C: Changins; Z: Zollikofen

732 [b]The site means and analyses of variance are computed without the two reference plants oat and phacelia

733 [c]Within site analyses of variance for differences among species

line figure 1
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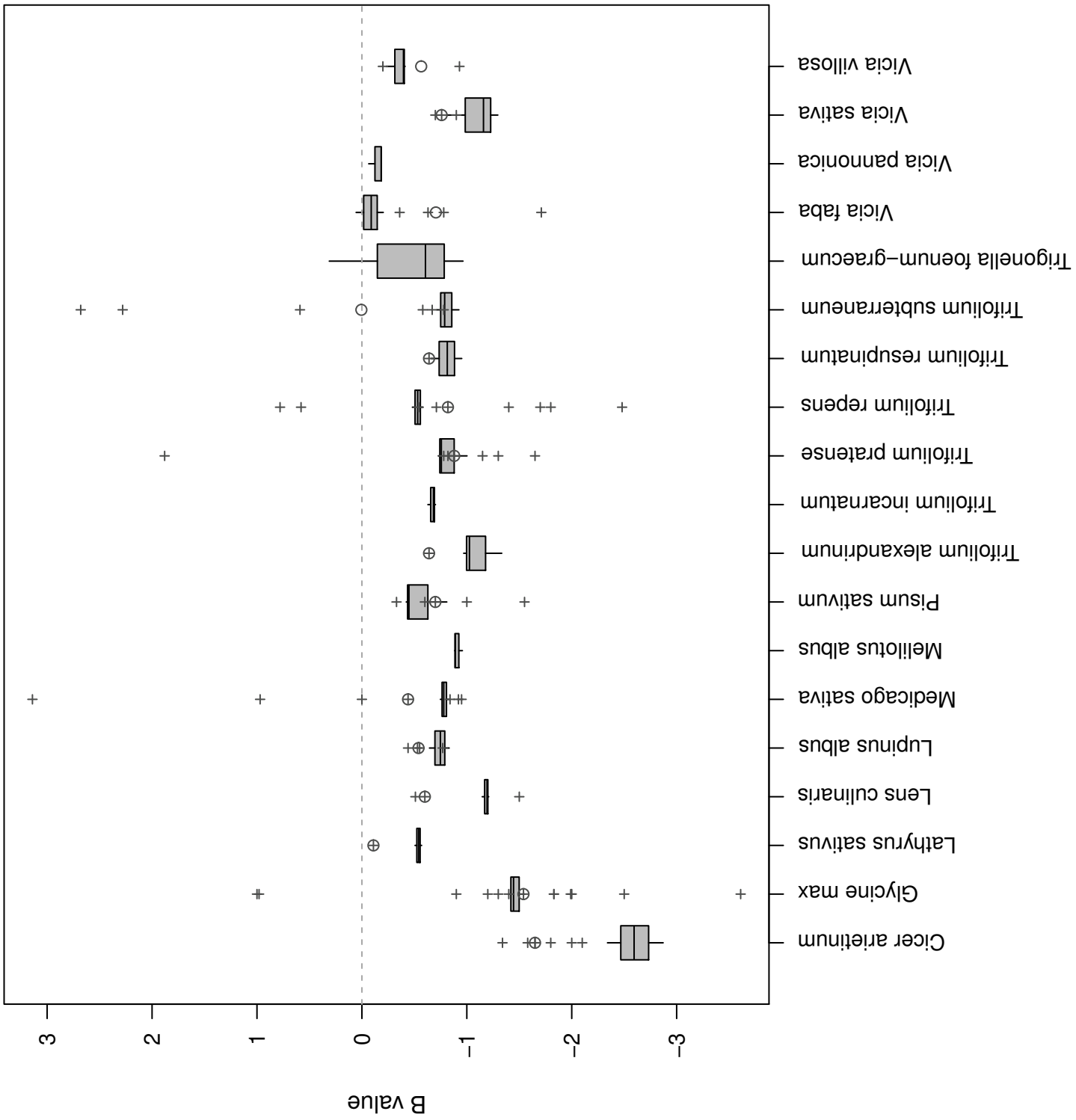

line figure 2

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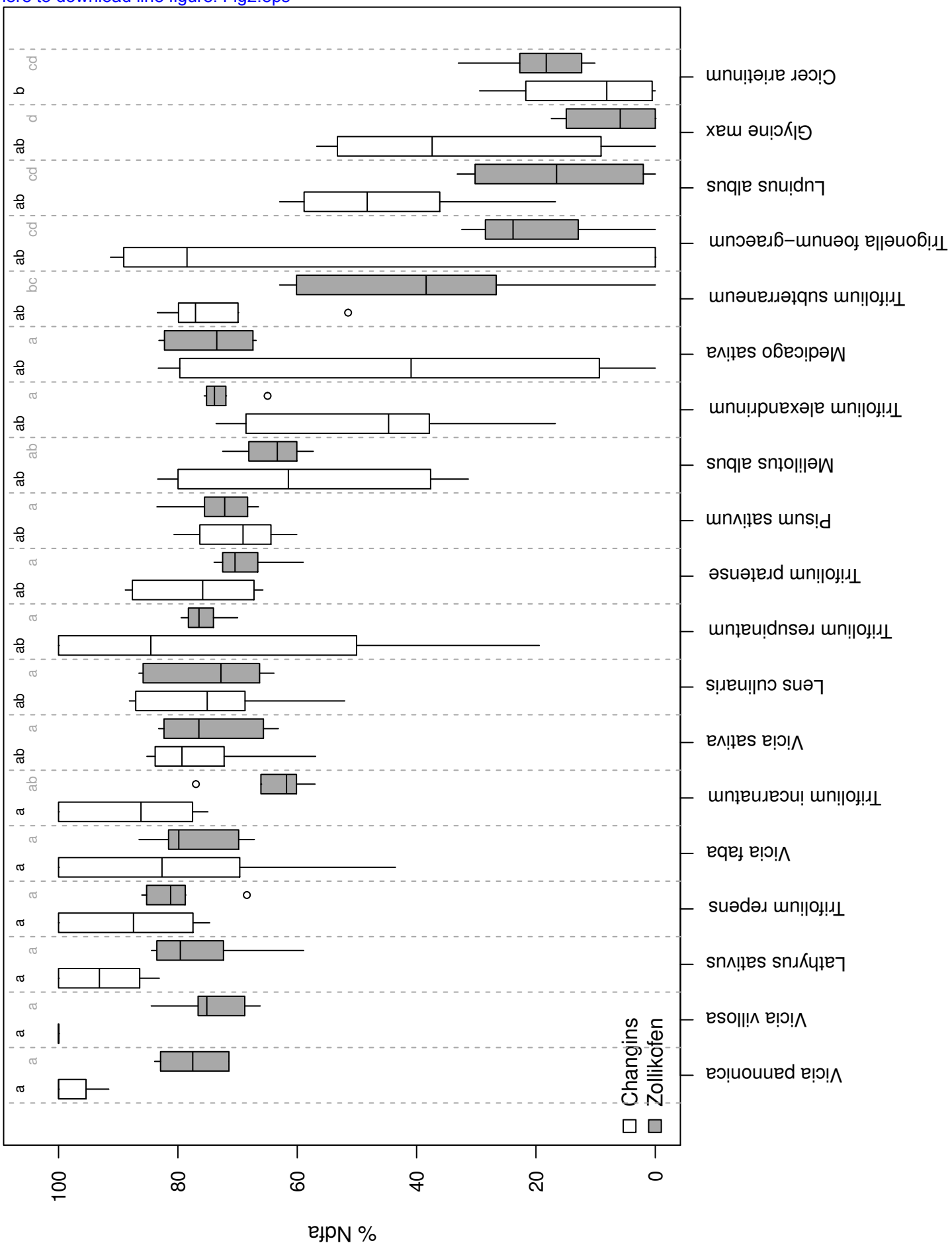

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