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Biogeosciences Discussions

Interactive comment on "Diversity pattern of nitrogen fixing microbes in nodules of Trifolium arvense (L.) at different initial stages of ecosystem development" *by* S. Schulz et al.

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Introduction section: p. 4, l. 25-27: "we assumed that the level of diversity of rhizobia nodulating clover will change with ongoing succession, being more diverse at the beginning of ecosystem development." Why? Please write any justification for this assumption. Rhizobium-legume symbioses are very specific, and, for example, R. leguminosarum bv. trifolii will not nodulate alfalfa, regardless of presence or absence of S. meliloti in soil (no compatible microsymbiont = no symbiosis). Of course some not-rhizobial bacteria could be sometimes isolated from nodules, but they are rather additions, "contaminations" which are present in nodules together with rhizobia, but

not real nitrogen-fixing microsymbionts which are able to induce nodulation.

Thankx for this comment. Indeed we were not clear enough in this part on our goals. The aim of our study was not to characterize the presence of different Rhizobia species in nodules (the reviewer is of course right that there is a strict specificity between the different partners which form the symbiosis) but to investigate intraspecies diversity of R. leguminosarum bv. trifolii and to characterize different strains or ecotypes of this species. We assume that the diversity pattern of strains and ecotypes might indeed differ during ecosystem development and that over time there might be a selection of the optimal ecotypes for the particular plant cultivar and the given environmental conditions. Accordingly we changed this part of the introduction as follows in the revised version: "Moreover, annual legumes like T. arvense face the problem that the symbiosis must be established each year again (Sessitsch et al., 2002). However, with each passing year the rhizobial population might increase, as they are released from the nodules at the end of each growing season (Sadowsky and Graham, 2006) dominated by those ecotypes which form the most efficient symbiosis related to the particular plant cultivar and the given environmental conditions.. . .. Due to the higher environmental stress level at the very beginning of ecosystem development and the enrichment of effective R. leguminosarum ecotypes over time because of the successive presence of T. arvense, we assumed that the level of intraspecies diversity of R. leguminosarum nodulating clover will change with ongoing succession, being more diverse at the beginning of ecosystem development." Related to the "contaminations" mentioned, we discuss this also in the Discussion section, as we think that those microbes co-colonizing the nodules may play an important role in the establishment of the symbiosis which has been overseen in the past and thus might not be only simple "contaminations"

Results section: p. 10 l.3-18 It should be clearly stated which differences are statistically significant, and which are not. This was written for differences in C/N ratio (p.10, l. 16-17) but I think this should be done also for other values reported in this part of the manuscript.

Whereas differences in soil derived carbon and nitrogen were not significantly different, we could observe significant differences in plant derived C and N from plants grown at the different sites. We changed these parts accordingly in the revised version: “Analyses of both soils revealed only little differences in carbon and nitrogen contents, which were not statistically significant. However, by tendency total carbon (TC) and nitrogen (TN) concentrations were higher in soil samples from the 5a site compared to the 2a site.”

Results section, p. 11, l.13-14: “The maximum number of OTUs per nodule was 3”. Is it true? Yes, it is true. The number of 3 OTUs is related to the medium sized nodules at a similarity level of 97%. We changed the sentence accordingly in the revised version: “The analysis of rarefaction curves on a 97% DNA homology level revealed no differences in the diversity of nifH harboring microbes in medium sized nodules from plants grown on the two different sites. At that similarity level the maximum number of OTUs per group of nodules was 3 (Figure 3).”

In Material and Methods section (p.6, l. 23-24) Authors wrote: “Plants from three different plots were treated as true replicates” and (p. 7 l. 22): “In total 12 clone libraries were prepared (2 sites, 2 nodule sizes, and 3 plots)”. Therefore there were no analyses of single, individual nodules – in one clone library DNA of microsymbionts from three nodules were present. So, I think that it should be written for example: “: : :the maximum number of OTUs per group of nodules: : :” (or per one experimental group or something like this) In the revised version of the manuscript the sentence in the result section has been changed: “At that similarity level the maximum number of OTUs per group of nodules was 3 (Figure 3). If the rarefaction curves were analyzed on a 99 % similarity level for the medium sized group of nodules however, up to 6 OTUs for nodules from plants grown on the 2a site were detected, whereas the number of OTUs from the group of nodules grown on the 5a site were significantly lower (3 OTUs), indicating a higher diversity in nodules of this size class in plants grown on the younger site.”

Discussion section, p.14, l. 6-10: “Therefore, higher nitrogen contents of the plants from the 5a site might be attributed to more efficient strains which comprise higher nitrogen fixing activities compared to strains that are associated with nodules of plants derived from the 2a site. To address that question, nifH clone libraries and the influence of soil age on the nodule community were compared.” The deliberation about different nitrogen fixing abilities of strains is well-founded, but studying of nifH sequences will not provide an answer for this question – the best method will be plant test. Of course the Authors did not isolate strains but only their DNA, so they are not able to perform plant tests – so the only way to correct it will be not do write about relationships between symbiotic efficiency and diversity of nifH sequences.

This objection is absolutely valid; therefore we excluded the question about the nitrogen fixing ability from that part of the discussion and added a sentence on this for future research (see below; reviewer 2): “As the evaluation of the amount and distribution of small, medium and large nodules resulted in no significant difference between plants from the two different sites. The question arose if the symbiotic microbial community of the differently sized nodules differs. . . . This study was based on one sampling time point during the vegetation period. Although we assume that during flowering a maximum of nitrogen is fixed in the nodules, nodule dynamics over time might be indeed a topic of interest for future research, mainly when the size of the nodules can be linked to their particular contribution to nitrogen fixation.”

Discussion section, p. 14, l. 16-19: “Hence it is very likely that the medium sized nodules represent the most active nodules, which is further underlined by the tendency of nifH copy numbers in nodules of the medium size class mainly from 2a site: : :”. I do not think so – there were more nifH copies in these nodules (fig. 2), but plants from 2a site had more medium nodules than plants from 5a site, and in spite of this - nitrogen content of plants from 2a site was lower comparing to plants from 5a site (Tab. 1) – therefore those nodules couldn,t be “the most active”

With respect to the comment above about the nitrogen fixation activity, we skipped this

part of the discussion and focused on differences of the microbial diversity only.

Discussion section, p.15 l. 8-12: “However, one might ask why these nodules did not develop to a medium nodule. Due to low nutrient contents in the soil and the fact that the plants does not spend more energy than needed in nodule production, it is obvious that there might be not enough energy available to promote the maturation of more medium or large nodules.” In my opinion this is not supported by presented data – plants grown on “better” soil – 5a (Table 1) had more small and less medium or large nodules (Fig. 1). I think the answer is simpler: not all nodules emerge at the same time; clover produces indeterminate nodules, therefore young, small spherical nodules evolve into mature, larger, rod-like nodules – if there is enough time: Of course spherical nodules might be old and ineffective (and therefore small, not supported by plant with nutrients), but (more likely) they may be effective but young (and therefore small), and only nitrogenase (reduction of acetylene) assay could answer this question.

As indeed the question on the dynamics of nodules overtime in their size and their contribution to N fixation is a topic of high interest and has been also raised by reviewer 2, we added some points to that issue and added this as part of future needs for research (see below) “This study was based on one sampling time point during the vegetation period. Although we assume that during flowering a maximum of nitrogen is fixed in the nodules, nodule dynamics over time might be indeed a topic of interest for future research, mainly when the size of the nodules can be linked to their particular contribution to nitrogen fixation.”

Table 1 – The abbreviations used in the table (i.e. “DON”, “TN” etc.) should be explained in table legend

The explanation of the abbreviations has been implemented: “Table 1. Chemical parameters of bulk soil and plants. Dissolved organic carbon (DOC), dissolved organic nitrogen (DON) and total carbon (TC) and nitrogen (TN) contents were measured in bulk soil samples from the 2a and the 5a site (n = 3, standard deviations in parenthe-

sis). Plant carbon and nitrogen contents were measured in Trifolium arvense (L.) from the 2a and the 5a site (n = 9, standard deviations in parenthesis). Nitrate concentration was below the detection limit for all samples (< 0.3 μg g-1). A significant impact of soil age on the measured parameters was tested with one-factor ANOVA ($p < 0.05$) and indicated by an asterisk.“

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