Nodulation of lucerne (*Medicago sativa* L.) roots: depth distribution and temporal variation

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ABSTRACT

This study was undertaken in order to test whether the development of nodule density over the vegetation period is different in lucerne stands grown for 1, 2 or 3 years continuously. For rapidly assessing nodule density in the field, a modified profile wall method was applied. Nodules were counted on a vertical profile wall, after spraying away a 2 cm layer of soil. For validating this method nodule density was determined on roots washed from monolith samples. Field data indicate that there is a shift of nodulation towards deeper soil layers with increasing maturity of lucerne stands. In 1-year lucerne nodulation was limited virtually to the top 15 cm of soil. In the 15–30 cm soil layer and in the subsoil (30–80 cm), nodule density increased with the cropping duration (1 year < 2 years < 3 years). Temporal decreases in nodule density during the vegetation period associated with dry spells were more pronounced for 2-years as compared with 3-years lucerne.

Keywords: alfalfa; perennial cultivation; profile wall; soil monolith; subsoil

Lucerne (*Medicago sativa* L.) is recognized as a beneficial crop for soil fertility building, especially by forming deep penetrating continuous biopores and by providing shoot and root residues rich in N (Pietsch et al. 2007, Yang et al. 2011). Thus, cultivation of lucerne has the potential to result in increased earthworm population, microbial activity as well as increased soil organic matter and N contents (e.g. Kautz et al. 2010). Furthermore, plant growth promoting microorganisms may be supported by H_2 which is released into soil as a consequence of nitrogenase activity in nodules (Ruiz-Argüeso et al. 1979). Release of H_2 could be of particular relevance for microorganisms in the subsoil, where readily available energy sources are scarce and microbial activity is generally lower as compared with the topsoil (Fierer et al. 2003, Salome et al. 2010).

Generally, nodulation of *Medicago* roots seems to vary largely with site conditions and is influenced by various biotic and abiotic factors such as presence of rhizobia and arbuscular mycorhizal fungi (e.g. Guo et al. 2010), Ca contents and soil acidity (Munns 1970), soil nitrate content (Streeter 1988) as well as soil moisture (Athar and Johnson 1996). Carter and Sheaffer (1983) reported the nodule density of non-irrigated lucerne to decrease continuously with soil depth to almost zero in the 45–60 cm deep soil layer. In a soil influenced by a groundwater table, lucerne nodules were reported down to soil depths of 2.50 m (Fox and Lipps 1955).

It is well established that N_2 fixation varies considerably between 1-year, 2-years and 3-years stands of lucerne (Kelner et al. 1997). However, little is known about how the distribution of nodules changes over time and space in perennial lucerne stands.

Our paper presents a rapid assay for determining nodules under field conditions. The assay was used to test (i) whether the depth distribution of nodules is different between lucerne stands in the 1st, 2nd and 3rd year of cultivation, and (ii) whether the nodulation changes over the vegetation period.

MATERIAL AND METHODS

A field trial (randomized block design, three field replications) was established on a silty loam soil (WRB: Haplic Luvisol) at the Klein Altendorf

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experimental research station (50°37'N, 6°59'E) near Bonn, Germany. Mean annual temperature is 9.6°C with average annual precipitation of 625 mm. At the beginning of the experiment pH in the Ap horizon was 6.5 ($CaCl_2$), soil organic C content was 1.02 g/kg and $CaCO_3$ content was < 1 g/kg. Prior to this experiment, lucerne (*Medicago sativa* L.) was not grown on the site for at least 2 decades.

In order to allow simultaneous investigation of lucerne cv. Planet in the 1st, 2nd and 3rd year of cultivation, the crop was sown consecutively in the spring of 2009, 2010 and 2011 with a row width of 12 cm and a plot size of 6 × 10 m. In 2009, spring rye was sown in the plots designated for 1-year or 2-years cultivation of lucerne. In 2010, oats was grown in the plots nominated for 1-year cultivation of lucerne. Before sowing of lucerne, spring rye or oats, the soil was ploughed to a depth of 30 cm. No mineral fertilizer or organic manure was applied. Quantification of nodules took place from May 3rd (calendar week 18) to September 22nd (calendar week 38) 2011. During the period of investigation, dry spells occurred between calendar weeks 18 and 21 as well as between weeks 27 and 29 (Figure 1).

For estimating nodule densities in the field we used an adapted form of the profile wall method that was described for estimating root length densities by Böhm and Köpke (1977). In 9 field plots (3 cropping durations × 3 field replicates) 1.50 m deep trenches were arranged. The profile wall was smoothened with a sharp edged spade in a way that lucerne taproots were visible close to the wall's surface. A counting frame with a grid size of 12 × 5 cm was placed on the wall in order to estimate nodule densities in a total area of 36 × 80 cm. Lucerne taproots were located in the center of the 12 cm wide grids. The top 2 cm soil layer was washed away from the profile wall with tap water using a pressure spray bottle and visible nodules were counted within each grid.

Additionally, a total of 4 soil monolith samples (24 × 12 × 80 cm each) were excavated from the same field trial. Starting from the previously described trenches, the monoliths were formed out of the profile wall by using a motor hammer. A wooden box (24 × 12 × 100 cm) accurately fitting for the monolith size was prepared. The open back and bottom sides of the box allowed framing of the monolith. Two steel blades (24 × 15 cm, 24 × 120 cm) fitting into angle irons fixed to the back and bottom sides of the box were used to cut the undisturbed monolith from the profile wall. After re-opening the boxes' back walls in the lab, lucerne nodules were first counted after spraying away the top 2 cm layer using a grid as described for the *in situ* determination above. In a next step the soil was soaked in tap water for at least 1 h and then successively washed away with a showerhead in 5 cm steps starting from the bottom. Nodules accidentally washed away were carefully recovered from the washing water by sieves. After washing, roots were cut in 5 cm layer steps from the bottom and nodule numbers counted by visual inspection.

Data were tested for normality by means of Shapiro-Wilk tests. Normal distribution was hardly given and could not be achieved by standard transformation procedures throughout the dataset. For this reason, significant differences between the cropping durations were estimated by pairwise

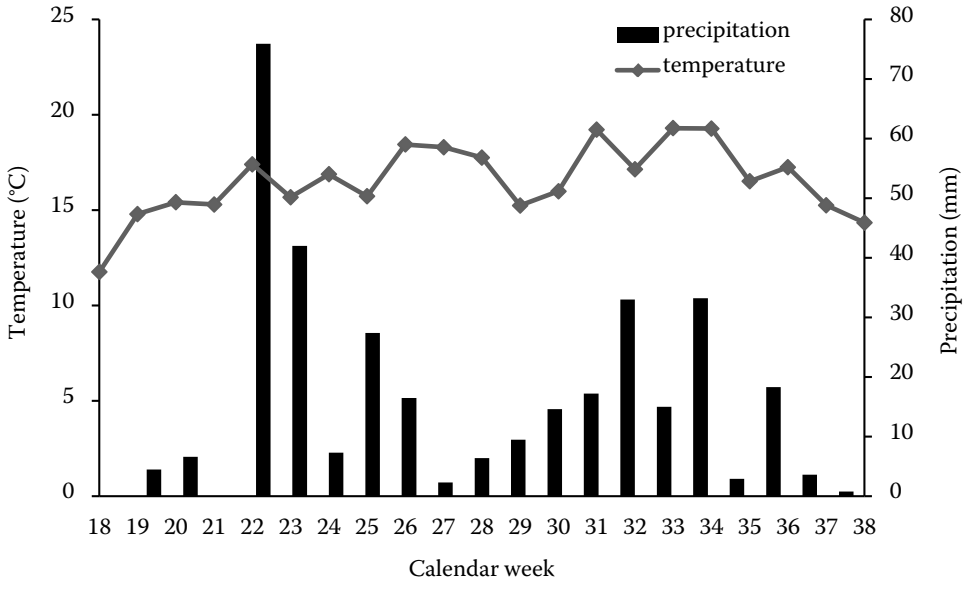

Figure 1. Mean temperature and sum of precipitation during the study period in 2011 on a weekly basis

comparisons with non-parametric Mann-Whitney-U-tests using the software package IBM SPSS 20, (IBM, Armonk, USA).

RESULTS AND DISCUSSION

For validation of the profile wall method, we calculated the correlation with the nodule densities determined after removing the soil from a monolith sample. Figure 2 shows that field counting of nodules on a profile wall is obviously less accurate and tends to underestimate nodule densities compared with the extraction by soaking and washing. Thus, the latter method should be preferred, whenever possible. However, in field experiments, when simultaneous investigations of different treatments and soil depths in many field replicates are necessary, the profile wall nodule counting can be a reasonable alternative, especially since more possible samples in limited time may result in higher precision. In any case, data obtained from the profile wall should be calibrated. For estimating root-length densities from the profile wall, conversion factors between 2 and 3 are commonly applied (Köpke 1981). For the nodules counted on the profile wall in our study, we determined conversion factors of 1.67 for the topsoil (0–30 cm soil depth) and 4.00 for the subsoil (30–80 cm soil depth) from the functions in Figure 2. These conversion factors were used for calculating the nodule densities shown in Figure 3.

Field counting started in early May 2011. Since 1-year lucerne was sown just 3 weeks before, this treatment was neglected at the first sampling date (Figure 3). In the following, a rapid increase of nodule density was recorded under 1-year lucerne in the upper topsoil (0–15 cm soil depth). In early August, nodule densities under 1-year lucerne increased up to 49 nodules/cm^3 and even outnumbered nodule densities under perennially grown lucerne. The rapid nodulation was probably favoured by the soil's pH of 6.5. Cheng et al. (2002) reported that nodules on lucerne roots appeared earlier and in higher numbers when plants were grown in a soil of pH 7.0 as compared with plants grown in a soil of pH 4.3. Furthermore, the absence of mineral N-fertilizer was shown to be beneficial for rapid increase of nodule numbers on roots of newly sown lucerne (Vasileva et al. 2011). Taking into account that 2-years and 3-years lucerne shoots were cut in weeks 19, 26, 32 and 37 whereas 1-year lucerne was only cut in week 28 and 37, the nodulation might also have been affected by different cutting regimes. Carter and Sheaffer (1983) showed that cutting may reduce nodulation in lucerne. However, nodules on 1-year lucerne roots were almost exclusively found in the upper topsoil, despite roots being present at least down to 80 cm soil depth from late May. In the lower topsoil (15–30 cm) nodule densities were generally increasing in the order of 1 year < 2 years < 3 years (Figure 3). Nodules on roots of perennial lucerne are considered to decay over winter (Thornton 1930), thus it can be virtually excluded that this effect is based on relics.

Temporal variations in nodule density were more pronounced for 2-years as compared with 3-years lucerne in both topsoil levels. Changes in nodule density over time can reflect the plant's current supply with nutrients and water. Drought was reported to have a negative impact on nodulation (reviewed by Serraj et al. 1999). In our field trial, comparatively dry periods can be related to the comparatively low nodulation densities recorded during calendar weeks 21 and 31, especially in the 2-year lucerne.

Under 1-year lucerne, nodules were almost exclusively recorded in the upper topsoil. No differences between nodule density under 2-years and 3-years lucerne were found in the upper topsoil, whereas nodule density was higher under 3-years lucerne as compared with 2-years lucerne in the subsoil (i.e. the soil beneath the ploughed horizon,

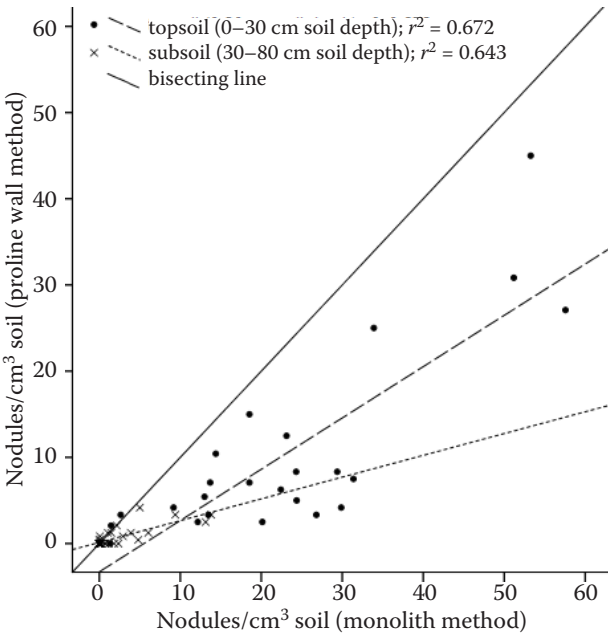

Figure 2. Linear regression of nodule density assessed by monolith method and nodule density estimated by the profile wall method. Topsoil (0–30 cm soil depth): $y = 0.60x - 3.28$; $P < 0.001$. Subsoil (30–80 cm soil depth): $y = 0.25x + 0.13$; $P < 0.001$

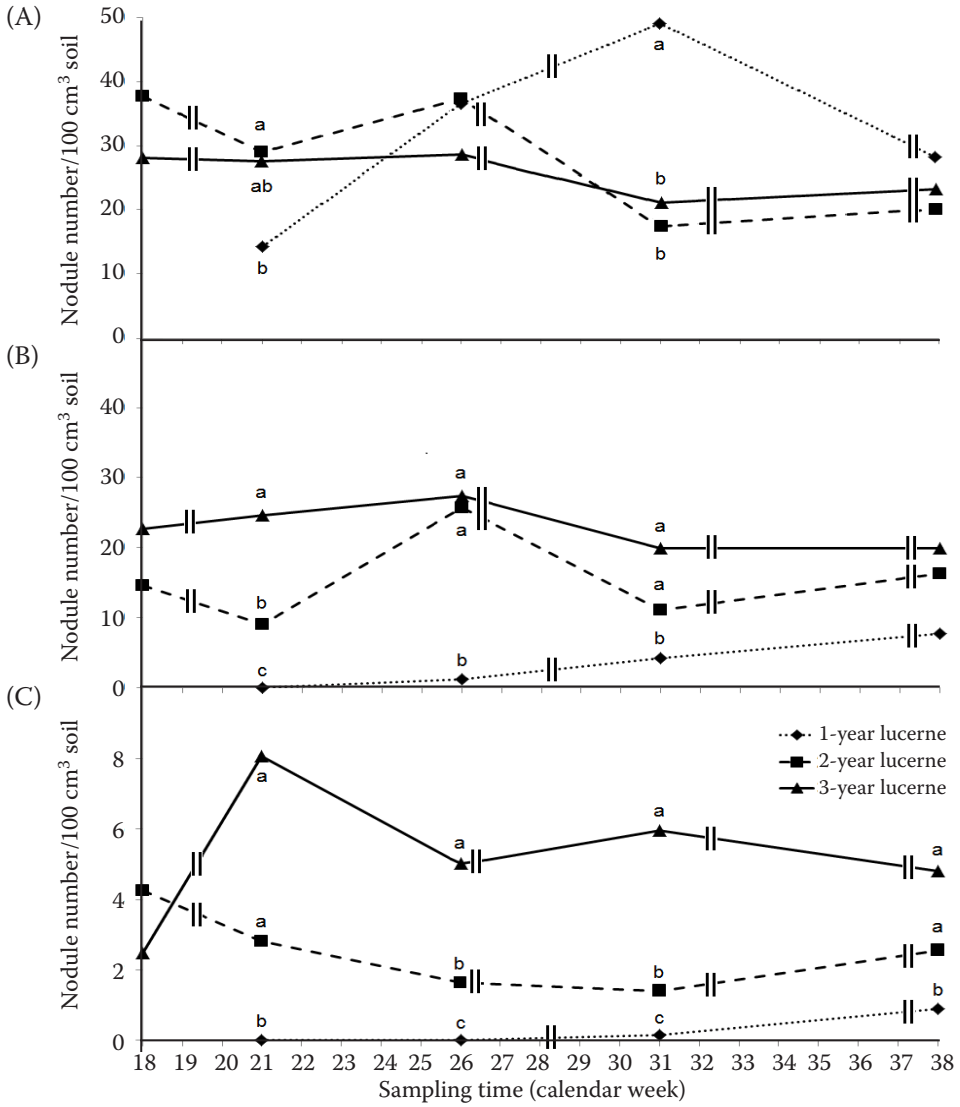

Figure 3. Temporal variation of lucerne root nodulation in three soil depths. (A) upper topsoil (0–15 cm depth); (B) lower topsoil (15–30 cm depth); (C) subsoil (30–80 cm depth). Double vertical lines indicate cutting dates of shoots. Different letters indicate significant differences within each sampling date (Mann-Whitney-U-tests, $P < 0.05$)

> 30 cm soil depth). Thus, it can be concluded that there is a shift of nodulation towards deeper soil layers with increasing maturity of lucerne stands. However, for 3-years lucerne nodule density was still generally about 5-fold lower in the subsoil than in the topsoil layers.

Brockwell et al. (1995) reported that during the 5th growing season of lucerne grown on a red podsolic soil very few nodules were found in 0–10 cm soil depth, although the contribution of N derived from the atmosphere to the plants' N supply ranged from 79–92%. Even though nodulation in deeper soil layers was not investigated in their study, this result indicates that the relevance of shallow soil layers for the nodulation of mature lucerne stands decreases whereas the relevance of deep soil layers potentially increases.

Nodules under 1-year lucerne appeared to be more reddish in color, whereas nodules under 2-years and 3-years lucerne tended to be more yellowish or brownish which possibly indicates differences in nodule activity. Thus, higher total nodule numbers must not necessarily reflect higher N_2-fixation rates.

Apparently, most of the nodules recorded in the subsoil were attached to roots growing in large biopores which we recognized as earthworm burrows. Earthworm activity can increase root colonization of lucerne by rhizobia as well (Stephens et al. 1994). In our field experiment, deep-burrowing earthworms were more abundant under 2-years and 3-years stands as compared with 1-year stands (Kautz et al. 2011). Thus, it is plausible that biopore densities in the subsoil or

activity of earthworms influenced depth distribution of nodules on lucerne roots.

Our results show that the cropping duration of lucerne had a pronounced effect on the depth distribution of nodules. While in the 1st year nodulation was limited almost exclusively to the upper 15 cm of soil, increased nodule densities were recorded in deeper soil layers as a function of cropping duration. The cropping duration of lucerne also influenced the temporal variation of nodule densities: After periods of drought, nodule density of 2-years lucerne apparently decreased in the topsoil, whereas nodulation of 3-years lucerne remained rather constant.

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