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# Effects of soybean variety and *Bradyrhizobium* strains on yield, protein content and biological nitrogen fixation under cool growing conditions in Germany

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ABSTRACT

Soybean (*Glycine max* (L.) Merr.) is able to fix atmospheric nitrogen in symbiosis with the bacteria *Bradyrhizobium japonicum*. Because these bacteria are not native in European soils, soybean seeds must be inoculated with *Bradyrhizobium* strains before sowing to fix nitrogen and meet their yield potential. In Central Europe soybean cultivation is still quite new and breeding of early maturing soybean varieties adapted to cool growing conditions has just started.

Under these low temperature conditions in Central Europe the inoculation with different, commercially available *Bradyrhizobium* inoculants has resulted in unsatisfactory nodulation. The aim of this study was: (i) to test the ability of commercially available inoculants to maximize soybean grain yield, protein content and protein yield, (ii) to study the interaction of different inoculants with different soybean varieties for two different sites in Germany under cool growing conditions over three years and (iii) to determine the variability of biological nitrogen fixation. Field trials were set up on an organically managed site at the Hessische Staatsdomäne Frankenhausen (DFH) and on a conventionally managed site in Quedlinburg (QLB) for three consecutive seasons from 2011 to 2013. Three early maturing soybean varieties—Merlin, Bohemians, Protina—were tested in combination with four different *Bradyrhizobium* inoculants—Radicin No.7, NPPL-Hi Stick, Force 48, Biodoz Rhizofilm—and compared with a non-inoculated control. Effective inoculation with *Bradyrhizobium* strains increased grain yield, protein content and protein yield by up to 57%, 26% and 99%, respectively. Grain yield, protein content and protein yield were generally higher in DFH. Average grain yield was 1634 kg ha$^{-1}$ in QLB (2012–2013) and 2455 kg ha$^{-1}$ in DFH (2011–2013), average protein content was 386 g kg$^{-1}$ in QLB and 389 g kg$^{-1}$ in DFH and average protein yield was 650 kg ha$^{-1}$ in QLB and 965 kg ha$^{-1}$ in DFH. The percentage of nitrogen derived from air (Ndfa) ranged between 40% and 57%. Soybeans inoculated with Radicin No. 7 failed to form nodules, and crop performance was identical to the non-inoculated control. Biodoz Rhizofilm, NPPL Hi-Stick and Force 48 are suitable for soybean cultivation under cool growing conditions in Germany. Interactions between soybean variety and inoculant were significant for protein content and protein yield at both sites, but not for nodulation, grain yield, thousand kernel weight and Ndfa. The variety Protina in combination with the inoculant Biodoz Rhizofilm can be recommended for tofu for both tested sites, while Merlin and Protina in combination with Biodoz Rhizofilm are recommended for animal fodder production in DFH. Animal fodder production was not profitable in QLB due to low protein yields.



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## 1. Introduction

Soybean (*Glycine max* (L.) Merr.) is an important high-quality protein source for human and animal nutrition. With the soil bacteria *Bradyrhizobium japonicum* symbiotically colonizing the plant's roots, soybeans are able to fix atmospheric nitrogen ($N_2$). Because this bacterium is not native to European soils, soybean seeds are generally inoculated with *Bradyrhizobium* strains before sowing. Salvagiotti et al. (2008) reported in a review article that soybean biological $N_2$ fixation (BNF) ranged between 0 and 337 kg N $ha^{-1}$ and on average 50-60% of soybean N demand originates from BNF.

Efficient symbiosis depends on environmental factors such as soil temperature, water and aeration, pH, salinity, amount of N in soil, as well as on the *Bradyrhizobium* strain, inoculation formulation, and soybean genotype (Keyser and Li, 1992; Stephens and Rask, 2000; Zhang et al., 1996). Thus, among other factors, nodulation is affected by low soil temperature and by inoculation procedure (Zhang and Smith, 1996; Zhang et al., 1996). A root temperature in the range of 25–30 °C is reported as optimal for BNF (Subramanian and Smith, 2013). In Central Europe soil temperature regimes is categorised as mesic, with mean annual soil temperature ranges between 8° and 15 °C (USDA-NRCS Soil Science Division, 2003). It is possible to select *Bradyrhizobium* strains and soybean genotypes to fix biological nitrogen efficiently for the environmental conditions in a given production area (Alves et al., 2003; Zhang et al., 2003). The combination of soybean variety and *Bradyrhizobium* strain can also be important, which was shown by Luna and Planchon (1995) and Solomon et al. (2012).

In Central Europe soybean cultivation is still quite new and breeding of early maturing soybean varieties adapted to cool growing conditions has just started. There are several different *Bradyrhizobium* inoculants available in Europe. However, they were mainly developed for the environmental conditions in the USA, and nodulation results after inoculation with these *Bradyrhizobium* inoculants have been unsatisfactory under low temperature conditions in Germany (Kadiata et al., 2012). Our hypothesis is that certain inoculants might be better suited for Central European growing conditions than others. To our knowledge, France is the only European country where the effectiveness of *Bradyrhizobium* inoculants has been tested before commercialization (Herridge et al., 2002), while no systematic field studies on the efficacy of commercial inoculation products with early maturing soybean varieties under cooler German farming conditions are available.

The aim of this study was: (i) to test the ability of commercially available inoculants to maximize soybean grain yield, protein content and protein yield, (ii) to study how different inoculants interact with different early maturing soybean varieties for two different sites in Germany under cool growing conditions over three years, and (iii) to determine BNF variability.

## 2. Material and methods

### 2.1. Site description

The experiments were conducted on two different sites in Germany: Hessische Staatsdomäne Frankenhausen (DFH) in Grebenstein, Hesse and Quedlinburg (QLB) in Saxony-Anhalt.

The field trials at DFH were operated under organic farming conditions. DFH is the research farm of the University of Kassel (51.4N; 9.4E) and is located 230 m above sea level. The farm was converted to organic farming in 1998 and is certified as organic by two organic farming associations, Bioland and Naturland. Soil type is a Haplic Luvisol and soil texture is a silty loam. Mean annual precipitation is 650 mm and the 30-year mean annual temperature is 8.5 °C. The average crop heat units calculated from sowing to harvest over the three experimental years 2011–2013 was 2953 °C (Brown and Bootsma, 1993).

The field experiments at QLB were operated under conventional farming conditions on the research station (51.4N; 11.8E) of the Julius Kühn-Institute. The research station is located 140 m above sea level and its soil type is a Chernozem with a loamy soil texture. Mean annual precipitation is 497 mm and the 30-year mean annual temperature is 8.9 °C. The average crop heat units calculated from sowing to harvest over the three experimental years 2011–2013 was 2525 °C (Brown and Bootsma, 1993).

### 2.2. Trial description

Field trials were conducted on both experimental sites in three consecutive seasons (2011–2013). Each year the soybeans were planted on different fields where soybeans had never been cultivated before. Three very early maturing soybean varieties (Merlin, maturity group (MG) 000; Bohemians, MG0000/000; Protina, MG000/00) were tested in combination with four different *Bradyrhizobium* inoculants (Radicin No. 7, NPPL-Hi Stick, Force 48, Biodoz Rhizofilm) and a non-inoculated control (Table 1). All three soybean varieties are cold tolerant varieties commonly cultivated under cool growing conditions in Central Europe. Merlin was chosen because this variety is the standard for stable grain yields under cool growing conditions. Bohemians variety is characterized as earlier maturing than Merlin and Protina is the earliest cold tolerant soybean suitable for tofu production.

A factorial treatment was arranged in a split-plot design with inoculant (I) as main plots and soybean variety (SV) as subplots. The main plot factor was laid out according to a randomized complete block design with four replications (REP). The subplot size was 15 $m^2$ (1.5 m × 10 m). Soybeans were sown with 65 kernels capable of germination per $m^2$ and a space between rows of 37.5 cm.

Seeds were inoculated according to the manufacturer recommendations (Table 1). For Radicin No. 7, concentration of bacteria per seed recommended by the producer was 100–400 folds lower than for the other inoculation products. Due to the unsuccessful inoculation in 2011, Radicin No. 7 was applied undiluted in 2012 (20 fold higher concentration than recommended). Inoculation was done just prior to sowing. For each plot the respective amount of inocula was added to the plastic bag containing the soybean seeds and thoroughly mixed. To avoid cross contamination, a thorough cleaning of the plot seeder was done after each inoculant, first by running 5 kg of barley seed though the plot seeder, followed by a thorough cleaning by air pressure.

Detailed descriptions of the trial management, soil characteristics, fertilizer and plant protection and time of sampling and harvesting are given in Table 2.

### 2.3. Measurements

To assess nodulation parameters, three (DFH) and four plants (QLB) per subplot were sampled from the second and the third row of each plot. Sampling was done twice, six weeks after sowing (nodulation1) and at flowering (nodulation2). The plants were carefully uprooted using a spade to obtain unharmed roots and nodules. The whole root system was exposed and the adhering soil was gently removed by hand over a metal sieve. The two subsamples from each subplot were used to assess nodulation (number of nodules per plant), record the inside color of nodules and to assess percent damage of nodules caused by pea and bean weevil (*Sitona lineatus*). There was no negative effect of *Sitona lineatus* damage, since *Sitona lineatus* was only observed for three plants in site DFH and year 2012.

At physiological maturity, soybean plants were harvested by a plot combine harvester. Grain yield measured in kilograms per

**Table 1**
Product name and producer of *Bradyrhizobium* inoculant tested in the field trials.

| Product name of inoculant | Concentration (viable *Bradyrhizobium* cells per g or ml) | Recommended dose per ha | Formulation | Manufacturer |
|---|---|---|---|---|
| Control (non-inoculated) | – | – | – | – |
| Radicin No. 7 | $1 \times 10^7 - 10^8$ | 75 ml | Liquid | Jost, Germany |
| Force 48 | $1 \times 10^9$ | 400 g | Peat based plus liquid adhesive | Becker Underwood, USA |
| NPPL Hi-Stick | $4 \times 10^9$ | 400 g | Peat based | Becker Underwood, USA |
| Biodoz Rhizofilm | $1 \times 10^9$ | 400 g | Peat based plus liquid adhesive | De Sangosse, France |

**Table 2**
Description of the field trials conducted at the two experimental sites: Hessische Staatsdomäne Frankenhausen (DFH) and Quedlinburg (QLB) over three experimental years 2011, 2012 and 2013 with respect to fertility, soil management, plant protection, sampling time, harvesting time and soil parameters.

| | DFH organic farming | | | QLB conventional farming | | |
|---|---|---|---|---|---|---|
| | 2011 | 2012 | 2013 | 2011 | 2012 | 2013 |
| Previous crop | Beetroot | Carrots | Carrots | Green oat | Green oat | Winter wheat |
| Fertilizer | | | | | | |
| Carbokalk (99.1 kg ha$^{-1}$ MgO, 1660 kg ha$^{-1}$ CaO) | – | – | – | 29-10-07 | – | – |
| Carbokalk (101 kg ha$^{-1}$ MgO, 1700 kg ha$^{-1}$ CaO) | – | – | – | – | 13-10-08 | 29-10-09 |
| Tillage | 25-10-10 | 26-10-11 | 29-10-12 | 28-10-10 | 25-10-11 | 26-10-12 |
| Sowing date soybean | 28-04-11 | 08-05-12 | 07-05-13 | 20-04-11 | 25-04-12 | 25-04-13 |
| Plant protection including herbicides | | | | | | |
| Bow hoe | 25-05-11 | 25-05-12 | 06-06-13 | – | – | – |
| Bow hoe | – | 30-05-12– | – | – | – | – |
| Manual weeding | 06-06-11 | 14-06-12 | 01-07-13 | – | – | – |
| Manual weeding | 04-10-11 | 18-10-12 | – | – | – | – |
| Stomp Aqua (2.5 l) | – | – | – | 21-04-11 | 26-04-12 | 25-04-13 |
| Karate zeon (0.075 l) | – | – | – | 19-05-11 | 21-05-12 | 17-05-13 and 02-07-13 |
| First nodule sampling | 15-06-11 | 19-06-12 | 24-06-13 | 01-06-11 | 06-06-12 | 06-06-13 |
| Second nodule sampling | 11-07-11 | 24-07-12 | 22-07-13 | 30-06-11 | 02-07-12 | 02-07-13 |
| Soybean harvest | 05-10-11 | 23-10-12 | 04-10-13 | 02-09-11 | 21-09-12 | 05-09-13 |
| Precipitation (mm from sowing to harvesting) | 196 | 308 | 280 | 187 | 330 | 134 |
| Crop heat unit (from sowing to harvesting) | 3528 | 2795 | 2536 | 2576 | 2494 | 2506 |
| Soil parameters and nutrient status before sowing | | | | | | |
| Soil type | Haplic Luvisol | | | Chernozem | | |
| Soil texture | Silty loam | | | Loam | | |
| pH | 6.9 | 7.3 | n.a. | 6.8 | 6.9 | 6.9 |
| $K_2O$ (mg per 100 g soil) | 12.9 | 11 | n.a. | 18 | 14 | 30 |
| $P_2O$ (mg per 100 g soil) | 14.4 | 12 | n.a. | 13.2 | 11.7 | 21 |
| Mg (mg per 100 g soil) | 15.1 | 9 | n.a. | 11.6 | 11.2 | 10.6 |
| Nmin (0–30 cm) (kg ha$^{-1}$) | | 87 | n.a. | 56 | 76 | 29 |
| Nmin (0–90 cm) (kg ha$^{-1}$) | 138 | 159 | n.a. | 160 | 233 | 74 |

n.a. Data not available.

hectare (kg ha$^{-1}$) at 100% dry matter (DM) and thousand kernel weight (TKW) was determined at final harvest. NIR reflectance spectra of all soybean samples in the 1100–2100 nm regions were measured with the polychromator PSS-2120 (Polytec GmbH, Waldbronn, Germany). For the measurement in reflection the combined illumination and sensor unit PSS H-A01 (Polytec GmbH, Waldbronn, Germany) was used. Absorbance data were stored as log (1/R) ($R$ = reflectance) at 2 nm intervals. All spectra data were processed using the Senso*Logic* package (Senso*Logic* GmbH, Norderstedt, Germany). NIRS statistics for estimated parameters are shown in Table S1. The protein content was derived from the estimated N content by following formula:

$$\text{Protein content (g kg}^{-1}) = \text{N content (g kg}^{-1}) \times 6.25 \quad (1)$$

Grain yield and protein yield could not be measured at QLB in 2011 because of burst pods, lost seeds and other damage caused by a severe hail storm that occurred on the 24th of August.

In order to determine the efficiency of BNF, it was necessary to measure how much nitrogen the plant derived from the air, based on the different ratios of the stable nitrogen isotopes $^{15}N$:$^{14}N$ in air and soil, respectively. In DFH 2012 and 2013 and in QLB 2013, $^{15}N$ air of soybean grains was determined by the $^{15}N$ natural abundance method (Kohl et al., 1980; Schweiger et al., 2012). The percentage of N derived from air (Ndfa) was calculated by the standard equation according to Unkovich et al. (2008):

$$\text{Ndfa (\%)} = \frac{(\delta^{15}\text{N of reference plant} - \delta^{15}\text{N of } N_2 \text{ fixing legume})}{\delta^{15}\text{N of reference plant} - \text{B}} \times 100, \quad (2)$$

where $\delta^{15}N$ is the sample natural abundance expressed as parts per thousand relative to atmospheric $N_2$ of air (Unkovich et al., 2008). As reference plant we used the data of the non-inoculated control of each variety in each replication and as $N_2$ fixing legume we used data of the inoculated soybean plots (soybean variety × inoculum × replication). As 'B' value we used −0.85 based on 'B' values for soybean grain determined by Oberson et al. (2007) in Switzerland.

The absolute amount of nitrogen uptake in soybean grains derived from BNF in kg ha $^{-1}$ (Nfix) was computed for DFH 2012 and 2013 and in QLB 2013 by the following equation:

$$\text{N fix (kg ha}^{-1}) = \frac{\text{N uptake in grain} \times \text{Ndfa}}{100} \quad (3)$$

### 2.4. Statistical analysis

Analysis of variance (ANOVA), adjusted means and standard error estimates were computed using the MIXED procedure of

the software package SAS 9.2 (SAS Institute 2002–2008). Pairwise comparison was done using the Tukey-test ($p \leq 0.05$). Normal distribution and homogeneity of variance of residuals were tested by diagnostic plots generated by PROC UNIVARIATE and PROC GPLOT in SAS. Nodulation at second nodule sampling (nodulation2), grain yield, thousand kernel weight (TKW), protein content, protein yield, Ndfa and Nfix were analysed according to the basic model for a split-plot experiment as defined by Piepho et al. (2003):

$$\text{Response} = \text{I} + \text{SV} + \text{I} \times \text{SV} + \text{REP} : \text{REP} \times \text{I} + \underline{\text{REP} \times \text{I} \times \text{SV}} \quad (4)$$

The model used the following factors: REP, complete replicate; I, inoculant and SV, soybean variety. The effects I, SV, I × SV and REP were considered fixed and the main plot error REP × I and the sub-plot error $\underline{\text{REP} \times \text{I} \times \text{SV}}$ were modelled as random. The sub-plot error is underlined to indicate that it corresponds to the residual error term. Fixed and random effects in the model are separated with a colon.

In this study the field trials were conducted in three consecutive seasons, but on different fields. The model used for statistical analysis including year (Y) as a random factor was as follows:

$$\text{Response} = \text{I} + \text{SV} + \text{I} \times \text{SV} : \text{Y} \times \text{REP} + \text{Y} + \text{Y} \times \text{I} + \text{Y} \times \text{SV} + \text{Y} \times \text{I} \times \text{SV} + \text{Y} \times \text{REP} \times \text{I} + \underline{\text{Y} \times \text{REP} \times \text{I} \times \text{SV}} \quad (5)$$

where Y × REP × I is the main-plot error and $\underline{\text{Y} \times \text{REP} \times \text{I} \times \text{SV}}$ is the sub-plot error.

Since seasonal weather is not predictable Y was treated as a random factor, which allows making a general statement about the treatments. This model was applied for all traits except Ndfa and Nfix, where we had only single year data.

Due to different pedoclimatic conditions and different farming management the two sites were analyzed separately. In the non-inoculated control we have detected no nodules in all the replications, resulting in zero nodules and zero variance. Therefore the nodulation data of the non-inoculated control was excluded from ANOVA, because with no nodules and no variance, the data do not conform to the model's assumptions. For the protein yield in DFH data, an arcsin transformation was used to conform to the ANOVA model's assumption of a normal distribution. Adjusted treatment means were transformed back for presentation of the data (Piepho, 2009).

For presentation of data, means followed by a common letter are not significantly at the 5% level of probability using the Tukey-test. If the *F* test of source of variance for an interaction of the two factors was significant, the Tukey-test was only applied for the interaction and not for the two factors. For description of stochastic variability the average of the Standard Error of a Difference (mean SED) is shown.

## 3. Results

For the site DFH, results are shown for the three experimental years (2011–2013). For the site QLB, results of nodulation, TKW and protein content are shown over three years, whereas grain yield and protein yield are only shown for 2012 and 2013 due to severe crop damage by hail in 2011 (Table 3).

### 3.1. Nodulation

At both sites inoculation of soybean seeds with Radicin No. 7 at recommended concentration in 2011 and undiluted in 2012 did not result in an effective development of nodules (viability of *Bradyrhizobium* inoculant was tested in a pot trial (Hertenstein, 2013; Messmer et al., 2012)). For both years the Radicin No. 7 treatment and the non-inoculated controls showed similar results for all traits. Thus, Radicin No. 7 was not tested in 2013. Inoculation with Force 48, NPPL Hi-Stick and Biodoz Rhizofilm all consistently resulted in nodulation at both sites for all years. These three are collectively referred to as the 'effective inoculants'.

Samples taken of the non-inoculated control did not show any nodules at QLB and less than 0.5 at DFH at both sampling dates. Hence, major effects of cross contamination can be excluded. Except for the ineffective inoculum Radicin No. 7, which did not result in any nodules, the *Bradyrhizobium* inoculation with Force 48, NPPL Hi-Stick and Biodoz Rhizofilm produced nodules in each site and year. The inside of nodules appeared whitish-pink, which showed that bacteria were well developed and active in the synthesis of the protein leghemoglobin (Vance et al., 1988). There was no negative effect of *Sitona lineatus* damage, since *Sitona lineatus* was only observed for three plants in site DFH and year 2012. Plants at the second sampling had a significantly higher number of nodules during anthesis—33.8 or 225% more in DFH and 74.4 or 116% in QLB—when compared to the first nodule sampling six weeks after sowing. Both assessments were correlated – DFH 2011–2013: $r = 0.36$, $p = 0.001$; QLB 2011–2013: $r = 0.82$, $p < 0.001$ – but second nodule sampling had greater differentiation. Therefore, only nodulation at flowering (nodulation 2) is reported.

In DFH, nodulation 2 was neither significantly affected by soybean variety, inoculation, nor by the interaction inoculation × soybean variety (Table 3). In 2011 the number of nodules was generally lower than in other years (Table S3). The highest number of nodules was observed in 2012 for the combination Merlin/Biodoz Rhizofilm (33) and the lowest number in 2011 for the combination Protina/NPPL Hi-Stick (7).

In QLB, nodulation 2 was significantly influenced by inoculation but not by soybean variety (Table 3). No significant interaction was detected. Across the three soybean varieties inoculation with Biodoz Rhizofilm resulted in a significantly higher number of nodules (12.3) compared to Force 48 (9.0) and NPPL Hi-Stick (7.3) (Table 5). The highest number of nodules was observed with 14.2 nodules per plant in 2013 followed by 8.2 and 5.6 nodules per plant in 2011 and 2012, respectively (Table S4).

### 3.2. Grain yield

Grain yield of the effectively inoculated soybeans were consistently higher than for the non-inoculated soybean at both sites. The average grain yield of the effectively inoculated soybeans over three years increased by 57% in DFH and by 16% in QLB compared to the non-inoculated control. The average grain yield in DFH was 2455 kg ha$^{-1}$ and 1634 kg ha$^{-1}$ in QLB. Soybean variety, inoculant, and the interaction inoculation × soybean variety all were significant factors in the DFH experiments (Table 3).

For the variety Merlin and Bohemains, the combination with Force 48, NPPL Hi-Stick or Biodoz Rhizofilm resulted in similar grain yields that were not significantly different from each other (Table 4). For the variety Protina, inoculation with Biodoz Rhizofilm and Force 48 produced the highest grain yields. Inoculation of Protina with Biodoz Rhizofilm increased grain yield by 71% above the non-inoculated control. Merlin had the highest grain yield each year; Protina's grain yield was approximately the same as Merlin's in 2013. Grain yield levels were overall highest in 2013 (Table S3).

In QLB grain yield was with 1634 kg ha$^{-1}$ 55% lower than in DFH (2524 kg ha$^{-1}$) across the two years (2012–2013; Table S4) and neither significantly affected by soybean variety, inoculant, nor by the interaction inoculation × soybean variety (Table 3). Just like in DFH, Merlin was the variety that achieved the highest grain yield in both years in QLB (Table S4). The three inoculants and the non-inoculated control were at the same grain yield level in 2012 at QLB. In this year, soil mineral nitrogen was very high at sowing (233 kg ha$^{-1}$ in 0–90 cm, Table 2). Plants grew relatively tall and

**Table 3**
*P*-values for *F* tests of sources of variation (ANOVA) for nodulation at flowering (nodulation 2) and yield parameters under organic management in DFH and under conventional management in QLB for three soybean varieties (Merlin, Bohemians, Protina) and four *Bradyrhizobium* treatments (Force 48, Biodoz Rhizofilm, NPPL Hi-Stick and non-inoculated control) from 2011 to 2013.

| Treatment | Nodulation 2 (nodules per plant) | | Grain yield (kg ha$^{-1}$ at 100% DM) | | Thousand kernel weight (g) | | Protein content (g kg$^{-1}$) | | Protein yield (kg ha$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | DFH | QLB | DFH | QLB[a] | DFH | QLB | DFH | QLB | DFH | QLB[a] |
| Soybean variety (SV) | 0.296 | 0.072 | **0.034** | 0.251 | **<0.001** | **0.005** | **0.003** | **<0.001** | **0.046** | 0.443 |
| Inoculant (I) | 0.070 | **0.002** | **<0.001** | 0.589 | **<0.001** | 0.133 | **<0.001** | **0.009** | **<0.001** | 0.166 |
| I x SV | 0.952 | 0.285 | **0.006** | 0.291 | 0.361 | **0.026** | **<0.001** | **0.007** | **0.043** | **0.019** |

*P*-values in bold represent significant effects at the 5% level.
Estimates of variance components for random effects are shown in Table S2.
[a] Data of 2011 not considered because of hail damage.

**Table 4**
Average of soybean variety (SV), inoculant (I), year (Y), and the interaction soybean variety and inoculant (SV × I) of nodulation at flowering (nodulation2), grain yield, thousand kernel weight, protein content and protein yield in DFH (2011–2013) and average of the standard error of differences between two means (mean SED).

| | | Nodulation2[a] (nodules per plant) | Grain yield (kg ha$^{-1}$ at 100% TM) | Thousand kernel weight (g) | Protein content (g kg$^{-1}$) | Protein yield[b] (kg ha$^{-1}$) | |
|---|---|---|---|---|---|---|---|
| Soybean variety (SV) | | | | | | | |
| Merlin | | | 17.7 | 2705 | 165 b | 367 | 967 |
| Bohemians | | | 18.1 | 2218 | 207 a | 371 | 804 |
| Protina | | | 14.8 | 2443 | 162 b | 432 | 1024 |
| mean SED | | | – | – | 3.11 | – | – |
| Inoculant (I) | | | | | | | |
| Control | | | – | 1750 | 156 b | 333 | 573 |
| Force 48 | | | 15.4 | 2661 | 185 a | 405 | 1069 |
| NPPL Hi-Stick | | | 12.8 | 2651 | 185 a | 403 | 1058 |
| Biodoz Rhizofilm | | | 22.4 | 2752 | 187 a | 418 | 1138 |
| mean SED | | | | – | 3.14 | – | – |
| SV | I | | | | | | |
| Merlin | Control | | – | 1987 cd | 144 | 299 g | 589 c |
| | Force 48 | | 16.1 | 2861 ab | 171 | 384 df | 1093 ab |
| | NPPL Hi-Stick | | 13.6 | 2930 ab | 172 | 385 df | 1125 ab |
| | Biodoz Rhizofilm | | 23.4 | 3039 b | 173 | 398 ce | 1204 ab |
| Bohemians | Control | | – | 1600 d | 184 | 325 g | 514 c |
| | Force 48 | | 17.5 | 2463 bc | 214 | 382 ef | 940 ab |
| | NPPL Hi-tick NPPL Hi-Stick | | 13.6 | 2432 bc | 215 | 380 ef | 922 b |
| | Biodoz Rhizofilm | | 23.2 | 2377 ac | 214 | 395 cd | 938 b |
| Protina | Control | | – | 1663 d | 139 | 376d e | 622 c |
| | Force 48 | | 12.6 | 2675 ab | 169 | 448 b | 1188 ab |
| | NPPL Hi-Stick | | 11.3 | 2593bc | 168 | 443 b | 1141 ab |
| | Biodoz Rhizofilm | | 20.5 | 2841 ab | 174 | 462 a | 1304 a |
| mean SED | | | – | 143.69 | – | 7.47 | |

If variance of the interaction (SV × I) was significant, Tukey-test was only applied for the interaction and not for the two factors.
Values followed by the same letter(s), within each column and treatment effect, are not significantly different at $p \leq 0.05$.
[a] For number of nodules at second sampling (nodulation 2), the inoculant treatment Control was excluded as requirements for ANOVA were not met due to lack of variation in nodules (<1 nodule per plant).
[b] Arcsin transformed data of protein yield with SED are shown in Table S5.

the variety Bohemians lodged. In contrast, QLB's 2013 grain yield of the non-inoculated control was 14–16% lower than grain yield of the three effectively inoculated crops.

### 3.3. Thousand kernel weight

Thousand kernel weights were significantly influenced by soybean variety and by inoculant in DFH (Table 3). TKW was highest for the variety Bohemians, with 207 g (Table 4). The non-inoculated control had the lowest TKW. Inoculation of soybeans increased TKW by up to 20% over the non-inoculated control.

In QLB, average TKW was 148 g compared to 178 g in DFH. TKW was influenced by soybean variety and by the interaction inoculation × soybean variety (Table 3). When looking at the interaction, TKW of the three effective inoculants did not differ significantly within the respective variety (Table 5). The highest TKW was reached in combination with the variety Bohemians independently of the inoculants. The comparison to DFH, the increase in TKW by effective inoculation was minor and not significant.

### 3.4. Protein content

Nitrogen fixation plays a role in protein formation in legumes. A minimum protein content of >450 g kg$^{-1}$ is needed for soybeans to meet the grade required for tofu (Miersch, 2013). At DFH and QLB, protein content significantly differed by variety, inoculant and the interaction inoculation × soybean variety (Table 3). As expected the protein content of the variety Protina was higher than that of the other two varieties. Protein content of non-inoculated soybeans was significantly lower than protein content of the inoculated soybeans for all varieties with the exception of Bohemians in QLB. Inoculation of soybeans increased protein content up to 26% at DFH and 24% at QLB compared to the non-inoculated control.

At DFH, for each of the three soybean varieties protein content was highest in combination with Biodoz Rhizofilm (Table 4). The highest protein content was observed for the combination Protina/Biodoz Rhizofilm in 2012 (478 g kg$^{-1}$; Table S3). In 2011 the needed protein threshold was not reached for any of the soybean variety inoculation combinations. In 2012 and 2013 the protein

**Table 5**
Average of variety of soybean variety (SV), inoculant (I), year (Y), and interaction soybean variety and inoculant (SV × I) of nodulation at flowering (nodulation 2), grain yield, thousand kernel weight, protein content and protein yield in QLB (2011–2013) and the average of the standard error of differences between two means (mean SED).

| | | Nodulation2[a] (nodules per plant) | Grain yield[b] (kg ha$^{-1}$ at 100% DM) | Thousand kernel weight (g) | Protein content (g kg$^{-1}$) | Protein yield[b] (kg ha$^{-1}$) |
|---|---|---|---|---|---|---|
| Variety of soybean (SV) | | | | | | |
| Merlin | | 9.1 | 1929 | 129 | 368 | 685 |
| Bohemians | | 7.9 | 1375 | 175 | 358 | 535 |
| Protina | | 11.6 | 1598 | 140 | 429 | 731 |
| mean SED | | – | – | – | – | – |
| Inoculant (I) | | | | | | |
| Control | | – | 1473 | 138 | 332 | 534 |
| Force 48 | | 9.0 b | 1703 | 151 | 401 | 706 |
| NPPL Hi-Stick | | 7.3 b | 1689 | 149 | 395 | 682 |
| Biodoz Rhizofilm | | 12.3 a | 1670 | 153 | 413 | 678 |
| mean SED | | 2.86 | – | – | – | – |
| SV | I | | | | | |
| Merlin | Control | – | 1681 | 122 d | 315 e | 576 a |
| | Force 48 | 9.8 | 2011 | 133 d | 385 cd | 757 a |
| | NPPL Hi-Stick | 6.2 | 1953 | 129 d | 372 ce | 671 a |
| | Biodoz Rhizofilm | 11.4 | 2070 | 132 d | 400 bc | 734 a |
| Bohemians | Control | – | 1198 | 169 abc | 327 de | 475 a |
| | Force 48 | 6.6 | 1480 | 176 ab | 365 ce | 583 a |
| | NPPL Hi-Stick | 7.4 | 1428 | 173 abc | 364 ce | 536 a |
| | Biodoz Rhizofilm | 9.8 | 1394 | 182 a | 376 ce | 547 a |
| Protina | Control | – | 1539 | 124 d | 352 ce | 552 a |
| | Force 48 | 10.8 | 1619 | 143 cd | 454 ab | 778 a |
| | NPPL Hi-Stick | 8.3 | 1686 | 146 bcd | 450 ab | 839 a |
| | Biodoz Rhizofilm | 15.8 | 1547 | 146 bcd | 462 a | 753 a |
| mean SED | | – | – | 7.75 | 12.70 | 127.93 |

If variance of the interaction (SV × I) was significant, Tukey-test was only applied for the interaction and not for the two factors.
Values followed by the same letter(s), within each column and treatment effect, are not significantly different at $p \leq 0.05$.

[a] For number of nodules at second sampling (nodulation2), the inoculant treatment control was excluded as requirements for ANOVA were not met due to lack of nodules (no nodule per plant).

[b] Data of 2011 not considered because of hail damage.

content needed was attained for Protina inoculated with any of the effective inoculants Force 48, NPPL Hi-Stick, and Biodoz Rhizofilm.

A significant linear regression of protein content on nodulation 2 was found for individual soybean varieties, i.e., increased nodulation resulted in increased protein content. However, the number of nodules can only explain a small proportion of the variation observed in protein content. Merlin had the strongest relation with $R^2 = 0.49$ ($p < 0.001$), followed by Protina with $R^2 = 0.24$ ($p = 0.002$) and Bohemians with $R^2 = 0.18$ ($p = 0.01$).

Protein contents were very similar between DFH (389 g kg$^{-1}$) and QLB (386 g kg$^{-1}$). Also in QLB highest protein content was reached for Protina in combination with Biodoz Rhizofilm (Table 5). In contrast to DFH the minimum protein content of 450 g kg$^{-1}$ was reached with Protina in 2011 and 2012 with any of the effective inoculant as well as in 2013 with the inoculant Biodoz Rhizofilm only (Table S4). In 2012, the year with relatively high mineral N content in QLB's soil, the protein content was on average higher (407 g kg$^{-1}$) compared to 2011 (383 g kg$^{-1}$) and 2013 (366 g kg$^{-1}$) (Table S4). No significant regression was found for the individual soybean varieties.

### 3.5. Protein yield

Protein yield was significantly influenced by variety, inoculants and the interaction inoculation*soybean variety at DFH and by the interaction inoculation × soybean variety in QLB (Table 3).

Protein yield of effectively inoculated soybeans was increased by up to 114%, 92%, and 124% compared with the non-inoculated control grown at DFH, in 2011, 2012, and 2013, respectively. The protein yield was higher on average in 2012 and 2013 compared to 2011 (Table S3). In each year, protein yield was highest for Protina up to 1480 kg ha$^{-1}$ in 2013 (Table S3). Across the three years Protina inoculated with Biodoz Rhizofilm had the highest protein yield (1304 kg ha$^{-1}$; Table 4), an increase of 110% compared to the non-inoculated control. Protina inoculated with Biodoz Rhizofilm had significantly higher protein yield than Bohemians inoculated with NPPL Hi-Stick or Biodoz Rhizofilm (Table 4).

Protein yield was considerably lower at QLB (650 kg ha$^{-1}$) than at DFH (1025 kg ha$^{-1}$) in 2012–2013. The increase in protein yield of effectively inoculated soybean compared to non-inoculated control was also smaller in QLB (+29%) than in DFH (+48%). In QLB protein yield was significantly influenced by the interaction inoculants × soybean variety but pairwise comparisons of the Tukey-test were not significant (Tables 3 and 5). The highest protein yield over two years was measured for Protina in combination with NPPL Hi-Stick, with an increase of 52% compared to the non-inoculated control. In 2013 protein yield of the three effectively inoculated crops was up to 70% higher compared to the non-inoculated control (Table S4), whereas in 2012, the year with the high soil mineral N content, the increase was less pronounced (9–18%).

### 3.6. Percentage of nitrogen derived from air in the harvested seeds (Ndfa)

Table 7 shows the percentage of nitrogen derived from air (Ndfa). At DFH in 2012 and at QLB in 2013 significant effects of soybean variety were recorded. Differences between the three effective inoculants were significant only at QLB in 2013. No significant soybean variety × inoculant interaction was detected in the three trials (Table 6). Protina and Merlin had significantly higher Ndfa than Bohemians at DFH in 2012, whereas at QLB in 2013 Merlin was more efficient in Ndfa than Protina and Bohemians (Table 7). The highest percentage of Ndfa across varieties was achieved in all three trials with the inoculant Biodoz Rhizofilm (up to 56% in QLB 2013) and lowest percentage of Ndfa with NPPL Hi-Stick (40.1% in DFH 2013). However, Ndfa was significantly higher for soybeans inocu-

**Table 6**
*P*-values for *F* tests of sources of variation (ANOVA) for percentage of nitrogen derived from air (Ndfa) and absolute amount of N derived from air of soybean seeds (Nfix) under organic management in DFH in 2012 and 2013 and under conventional managmeent in QLB in 2013 for three soybean varieties (Merlin, Bohemians, Protina) and three *Bradyrhizobium* treatments (Force 48, Biodoz Rhizofilm, NPPL Hi-Stick).

| | DFH | | | | QLB | |
|---|---|---|---|---|---|---|
| | 2012 | | 2013 | | 2013 | |
| | Ndfa (%) | Nfix (kg ha$^{-1}$) | Ndfa (%) | Nfix (kg ha$^{-1}$) | Ndfa (%) | Nfix (kg ha$^{-1}$) |
| Soybean variety (SV) | **0.009** | **<0.001** | 0.437 | **0.008** | **<0.001** | **<0.001** |
| Inoculant (I) | 0.129 | **0.013** | 0.706 | 0.221 | **0.006** | **0.003** |
| I x SV | 0.237 | 0.158 | 0.608 | 0.726 | 0.141 | 0.816 |

*P*-values in bold represent significant effects at the 5% level.

**Table 7**
Average of variety of soybean variety, inoculant and interaction soybean variety and inoculant (SV × I) of nitrogen derived from air (Ndfa) and fixed nitrogen (Nfix) in DFH (2012 and 2013) and in QLB (2013) and the average of the standard error of differences between two means (mean SED).

| | DFH | | | | QLB | |
|---|---|---|---|---|---|---|
| | 2012 | | 2013 | | 2013 | |
| | Ndfa (%) | Nfix (kg ha$^{-1}$) | Ndfa (%) | Nfix (kg ha$^{-1}$) | Ndfa (%) | Nfix (kg ha$^{-1}$) |
| Soybean variety (SV) | | | | | | |
| Merlin | 49.3 a | 98 a | 43.4 | 84 ab | 54.7 a | 65 a |
| Bohemians | 43.3 b | 67 b | 46.4 | 69 b | 48.8 b | 48 b |
| Protina | 49.7 a | 100 a | 43.1 | 100 a | 47.0 b | 49 b |
| mean SED | 2.0 | 0.05 | – | 0.08 | 2.25 | 0.04 |
| Inoculant (I) | | | | | | |
| Control[a] | – | – | – | – | – | – |
| Force 48 | 48.0 | 91 ab | 46.5 | 85 | 49.6 b | 54 ab |
| NPPL Hi-Stick | 44.6 | 79 b | 40.1 | 74 | 44.9 b | 46 b |
| Biodoz Rhizofilm | 49.9 | 95 a | 46.3 | 90 | 56.0 a | 62 a |
| mean SED | – | 0.05 | – | – | 2.25 | 0.4 |

Values followed by the same letter(s), within each column and effect, are not significantly different at $p \leq 0.05$.
[a] The non-inoculated control was used as reference plant for calculating Ndfa. For each soybean variety the same non-inoculated variety was used as reference plant. Non-inoculated soybeans did not form any nodules.

lated with Biodoz Rhizofilm compared to soybeans inoculated with Force 48 or NPPL Hi-Stick only at QLB during the 2013 crop season (Table 7).

At DFH in 2012 and at QLB in 2013 a significant linear regression of protein content on Ndfa was found for Merlin ($R^2_{DFH} = 0.39$, $p_{DFH} = 0.039$, $R^2_{QLB} = 0.71$, $p_{QLB} < 0.001$) and for Bohemians ($R^2_{DFH} = 0.32$, $p_{DFH} = 0.040$, $R^2_{QLB} = 0.48$, $p_{QLB} = 0.007$) but not for Protina ($R^2_{DFH} = 0.06$, $p_{DFH} = 0.428$, $R^2_{QLB} = 0.25$, $p_{QLB} = 0.099$). Thus, the prediction power of Ndfa for protein content is very limited.

### *3.7. Total amount of nitrogen derived from BNF in the harvested seeds (Nfix)*

Soybean varieties differed significantly in the total amount of nitrogen derived from BNF in the seed (Nfix) at DFH in 2012 and 2013, and at QLB in 2013. The inoculant also had significant effects on Nfix in DFH 2012 and QLB 2013. No significant soybean variety × inoculant interaction was detected for Nfix (Table 7). Protina and Merlin fixed significantly more nitrogen than Bohemians at DFH during the 2012 crop season. At DFH in 2013 most nitrogen was fixed by Protina, followed by Merlin and then Bohemians. Merlin fixed significantly more nitrogen than Bohemians or Protina at QLB in 2013. At all sites Biodoz Rhizofilm had the highest Nfix values measured, followed by Force 48 and NPPL Hi-stick (Table 8). These differences were significant in DFH in 2012 and in QLB in 2013 (Table 7).

## 4. Discussion

Successful soybean cultivation in Europe depends on effective soil inoculation with non-native *Bradyrhizobium* bacteria. In our study, non-inoculated soybeans did not form any nodules, and grain yield, thousand kernel weight, protein content and protein yield were significantly increased by up to 57%, 20%, 26%, and 99%, respectively, after successful inoculation with *Bradyrhizobium* strains. Inoculation of soybean with Radicin No. 7 did not result in a formation of nodules, and soybean performance was identical to the non-inoculated control. Insufficient nodulation in combination with Radicin No. 7 was also reported in pot trials conducted in Austria and Germany (Bonell, 2010; Wächter et al., 2013). However, effective nodulation of soybeans inoculated with Radicin No. 7 was reported in recent pot trials (Hertenstein, 2013). Nevertheless, they used a much higher concentration of inoculant – $10^6$ bacteria per seed – than our field trial and in their experiment the soil was sterilized before inoculation. Product formulation or insufficient colony forming units might be possible reasons for the failure of Radicin No. 7 under field conditions. Stephens and Rask (2000) indicated that liquid inoculants have limited shelf life and require cool temperature storage. While both conditions were followed in our study, Radicin No. 7 failed at both sites in two years, as well as in on-farm trials conducted at two additional sites in Germany (data not shown). Therefore, Radicin No. 7 cannot be recommended for inoculation of soybeans.

In general, the number of nodules was low for all inoculants when applied according to manufacturer instructions with an average of 10 nodules per plant for QLB and 17 nodules per plant for DFH. The latter is in the range reported for soybean cultivation without soybean history (20–35 nodules per plant) and is much lower than the number of nodules reported after repeated soybean cultivation with 87–125 nodules per plant (Grossman et al., 2011).

QLB had lower production potential with an average grain yield of 1634 kg ha$^{-1}$. The advantage of successful inoculation was less pronounced than in DFH, where the average grain yield was 2455 kg ha$^{-1}$. The missing impact of inoculation in 2012 can be

explained according to findings of Keyser and Li (1992) by the high mineral N content (233 kg ha$^{-1}$) in the soil in that year. Also the number of nodules per plant was lower in 2012 than in 2013, where mineral N content in the rhizosphere was not at a high level. Also Albareda et al. (2009) showed that N fertilization had a negative effect on soybean-*Bradyrhizobium* symbiosis and resulted in a reduction of nodulation of soybeans and no improvement of grain yield compared to unfertilized plots.

Protina is cultivated for tofu production in Southern Germany with a minimum protein content target of 450 g kg$^{-1}$ in the seeds (Miersch, 2013). Such high protein contents could also be achieved at the less favorable growing conditions in DFH in 2 of 3 years and QLB in all three years with variety Protina preferably inoculated with Biodoz Rhizofilm, whereas Bohemians and Merlin never reached more than 420 g kg$^{-1}$, protein content.

Highest protein yield for soybeans grown at DFH was achieved by the combination Protina—Biodoz Rhizofilm at 1304 kg ha$^{-1}$ followed by Merlin—Biodoz Rhizofilm at 1204 kg ha$^{-1}$, at which the former profited from increased protein content and the latter from the increased grain yield. Similarly, the highest protein contents in QLB were achieved by Protina followed by Merlin but the difference between both was not significant as protein yield was much lower <840 kg ha$^{-1}$. The low protein yield observed at the conventional manage site QLB is not very competitive of animal feed production.

The percentage of Ndfa ranged between 40% and 53% in DFH and between 40% and 57% in QLB, which is consistent with other studies in Switzerland (Oberson et al., 2007) and Austria (Schweiger et al., 2012). Considering that soybeans were grown for the first time in these fields under unfavorable growing conditions, we can assume that the inoculation of the three products resulted in successful symbiosis and BNF.

Inoculants tested in this study contained different *Bradyrhizobium* strains as well as different concentrations and formulations. Radicin No. 7 is liquid, while Force 48, NPPL Hi-Stick and Biodoz Rhizofilm use peat-based dry carriers. Biodoz Rhizofilm and Force 48 are both supplied with a sticking agent, and NPPL Hi-Stick had a concentration four times greater than these two products. As mentioned before, liquid inoculants have a few disadvantages compared to peat inoculants. While peat-based inoculants might be detached from the soybean seed when sown, the incorporation of a sticking agent improves attachment and uniformity of seed coverage (Stephens and Rask, 2000). The higher number of nodules of Biodoz Rhizofilm and Force 48 compared to NPPL Hi-Stick in this study could be a result of better adapted strains or due to the formulation, which might be even more important if seeds are planted with pneumatic seeder. New inoculants are formulating seed coatings with long shelf lives so the seed company can sell pre-inoculated seed (e.g., FixFertig). Successful inoculation with coated seed has been questionable in the past, as reported in Switzerland (Agroscope, 2010), Austria (Hein et al., 2013) and Germany (LfL, 2014). Co-inoculation with other plant growth promoting bacteria (PGPR) (Bai et al., 2003; Cassán et al., 2009) and beneficial fungi, such as Mycorrhiza (Antunes et al., 2006) have been developed and are already marketed in North America, but not yet in Central Europe.

Under cool growing conditions at the DFH and QLB sites, soybeans inoculated with Biodoz Rhizofilm fixed the highest amount of nitrogen and obtained the highest grain yield, protein content and protein yield, whereas NPPL Hi-Stick was less effective. These results were consistent with the results of a pot trial at different temperature regimes (Messmer et al., 2012), where Force 48 and Biodoz Rhizofilm performed well under cool temperatures of 12–16 °C, whereas NPPL Hi-Stick was cold sensitive and performed best under higher temperatures of 20–22 °C. We observed a significant interaction between soybean variety and inoculant for protein content and protein yield at both sites. However, the differences between the inoculants were minor for other agronomic traits. An examination of the effectiveness of *Bradyrhizobium* inoculants before commercialisation, as practiced in France, should become standard in all countries, as insufficient inoculation resulted in up to 45% losses of protein yield.

## 5. Conclusions

The effectiveness of commercially available inoculants is very important for successful soybean cultivation in new cultivation regions. Radicin No. 7 failed completely while soybeans inoculated with Biodoz Rhizofilm obtained the highest yield, protein content and protein yield. Biodoz Rhizofilm, NPPL Hi-Stick and Force 48 are suitable for soybean cultivation under cool growing conditions in Germany. Biodoz Rhizofilm and Force 48 are especially recommended due to their formulation (with liquid adhesive) for pneumatic sowing machines and potentially higher cold tolerance. Protina in combination with Biodoz Rhizofilm reached sufficient protein content for tofu production at both tested sites. Merlin and Protina in combination with Biodoz Rhizofilm can be recommended for animal fodder production at DFH. Animal fodder production was unprofitable at QLB due to very low protein grain yield level.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eja.2015.09.008.

## References

Agroscope, 2010. Vorsicht bei Vor-Inokulation von Soja-Saatgut. Medienmitteilung.

Albareda, M., Rodríguez-Navarro, D.N., Temprano, F.J., 2009. Soybean inoculation: dose, N fertilizer supplementation and rhizobia persistence in soil. Field Crop Res. 113, 352–356, http://dx.doi.org/10.1016/j.fcr.2009.05.013.

Alves, B.J.R., Boddey, R.M., Urquiaga, S., 2003. The success of BNF in soybean in Brazil. Plant Soil 252, 1–9, http://dx.doi.org/10.1023/A:1024191913296.

Antunes, P.M., de Varennes, A., Rajcan, I., Goss, M.J., 2006. Accumulation of specific flavonoids in soybean (*Glycine max* (L.) Merr.) as a function of the early tripartite symbiosis with arbuscular mycorrhizal fungi and *Bradyrhizobium japonicum* (Kirchner) Jordan. Soil Biol. Biochem. 38, 1234–1242, http://dx.doi.org/10.1016/j.soilbio.2005.09.016.

Bai, Y., Zhou, X., Smith, D.L., 2003. Enhanced soybean plant growth resulting from coinoculation of strains with. Crop Sci. 43, 1774, http://dx.doi.org/10.2135/cropsci2003.1774.

Bonell, M., 2010. Bestimmung der Stickstofffixierleistung von biologisch angebauter Soja im ostösterreichischen Raum anhand isotopenbasierender Methoden.

Brown, D.M., Bootsma, A., 1993. Crop heat units for corn and other warm—season crops in Ontario. www.sojaförderring.de. URL https://www.sojafoerderring.de/wp-content/uploads/2014/02/Berechnung-CHU-Uni-Guelph-Ontario.pdf (accessed 5.19.14.).

Cassán, F., Perrig, D., Sgroy, V., Masciarelli, O., Penna, C., Luna, V., 2009. Azospirillum brasilense Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). Eur. J. Soil Biol. 45, 28–35, http://dx.doi.org/10.1016/j.ejsobi.2008.08.005.

Grossman, J.M., Schipanski, M.E., Sooksanguan, T., Seehaver, S., Drinkwater, L.E., 2011. Diversity of rhizobia in soybean [*Glycine max* (Vinton)] nodules varies under organic and conventional management. Appl. Soil Ecol. 50, 14–20, http://dx.doi.org/10.1016/j.apsoil.2011.08.003.

Hein, W., Fischl, M., Traudtner, F., Surböck, A., Gadermaier, F., Heinzinger, M., Kober, W., 2013. Biofrühjahrsanbau 2013. Ländliches Fortbildungsinstitut Österr. 40.
Herridge, D., Gemell, G., Hartley, E., 2002. Legume Inoculants and Quality Control, in: Inoculants and Nitrogen Fixation of Legumes in Vietnam. Australian Centre for International Agricultural Research, Hanoi, Vietnam.
Hertenstein, F., 2013. Early growth performance of soybean cultivars and bradyrhizobia strains under cool growing conditions. In: Master Thesis. Univ. Münst.
Kadiata, B.D., Schubert, S., Yan, F., 2012. Assessment of different inoculants of *Bradyrhizobium japonicum* on nodulation, potential N2 fixation and yield performance of soybean (*Glycine max* L.). J. Anim. Plant Sci. 13, 1704–1713.
Keyser, H.H., Li, F., 1992. Potential for increasing biological nitrogen fixation in soybean. Plant Soil 141, 119–135, http://dx.doi.org/10.1007/bf00011313.
Kohl, D.H., Shearer, G., Harper, J.E., 1980. Estimates of N2 fixation based on differences in the natural abundance of 15N in nodulating and nonnodulating isolines of soybeans. Plant Physiol., 61–65.
Luna, R., Planchon, C., 1995. Genotype × *Bradyrhizobium japonicum* strain interactions in dinitrogen fixation and agronomic traits of soybean (*Glycine max* L. Merr.). Euphytica 86, 127–134, http://dx.doi.org/10.1007/bf00022018.
Messmer, M., Berset, E., Zimmer, S., Haase, T., Habekuss, A., Palaez, S., Hertenstein, F., Thonar, C., Salomé, C., Ordon, F., Heß, J., Wilbois, K.-P., 2012. Breeding for improved soybean-bradyrhizobia symbiosis for cool growing conditions in Central Europe. In: Presented at the 10th European Nitrogen Fixation Conference, Munich.
Miersch, M., 2013. Sojaverarbeitung zu Tofu. Soja-Tag 2013, Frankfurt [WWW Document]. sojainfo.de. URL http://www.sojainfo.de/fileadmin/soja/documents/Sojatag_2012/soja_sojatag2012_miersch.pdf (accessed 7.30.14.).
Oberson, A., Nanzer, S., Bosshard, C., Dubois, D., Mäder, P., Frossard, E., 2007. Symbiotic N2 fixation by soybean in organic and conventional cropping systems estimated by 15N dilution and 15N natural abundance. Plant Soil 290, 69–83, http://dx.doi.org/10.1007/s11104-006-9122-3.
Piepho, H.-P., 2009. Data transformation in statistical analysis of field trials with changing treatment variance. Agron. J. 101, 865, http://dx.doi.org/10.2134/agronj2008.0226x.
Piepho, H.P., Buchse, A., Emrich, K., 2003. A Hitchhiker's guide to mixed models for randomized experiments. J. Agron. Crop Sci. 189, 310–322, http://dx.doi.org/10.1046/j.1439-037X.2003.00049.x.
Salvagiotti, F., Cassman, K.G., Specht, J.E., Walters, D.T., Weiss, A., Dobermann, A., 2008. Nitrogen uptake, fixation and response to fertilizer N in soybeans: a review. Field Crops Res. 108, 1–13, http://dx.doi.org/10.1016/j.fcr.2008.03.001.
Schweiger, P., Hofer, M., Hartl, W., Wanek, W., Vollmann, J., 2012. N2 fixation by organically grown soybean in Central Europe: method of quantification and agronomic effects. Eur. J. Agron. 41, 11–17, http://dx.doi.org/10.1016/j.eja.2012.01.011.
Solomon, T., Pant, L.M., Angaw, T., 2012. Effects of inoculation by *Bradyrhizobium japonicum* strains on nodulation, nitrogen fixation, and yield of soybean (*Glycine max* L. Merill) varieties on nitisols of Bako, Western Ethiopia. ISRN Agron. 2012, 1–8, http://dx.doi.org/10.5402/2012/261475.
Stephens, J.H., Rask, H., 2000. Inoculant production and formulation. Field Crop Res. 65, 249–258, http://dx.doi.org/10.1016/S0378-4290(99)00090-8.
Subramanian, S., Smith, D.L., 2013. A proteomics approach to study soybean and its symbiont *Bradyrhizobium japonicum*—a review. In: Board, J. (Ed.), A Comprehensive Survey of International Soybean Research—Genetics, Physiology, Agronomy and Nitrogen Relationships. InTech.
Unkovich, M., Herridge, D., Peoples, M., Cadisch, G., Boddey, R., Giller, K., Alves, B., Chalk, P., 2008. Measuring plant-associated nitrogen fixation in agricultural systems. Australian Centre for International Agricultural Research, Canberra, ACT.
USDA-NRCS Soil Science Division, 2003. Soil Temperature Regimes Map [WWW Document]. URL http://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/use/worldsoils/?cid=nrcs142p2_054019 (accessed 2.3.14.).
Vance, C.P., Egli, M.A., Griffith, S.M., Miller, S.S., 1988. Plant regulated aspects of nodulation and N2 fixation. Plant Cell Environ. 11, 413–427, http://dx.doi.org/10.1111/j.1365-3040.1988.tb01365.x.
Wächter, K., Gruber, S., Claupein, W., 2013. Unterscheidet sich das Inokulationsergebnis verschiedener Impfmittel bei Soja? J. Für Kult., 401–410.
Zhang, F., Charles, T.C., Pan, B., Smith, D.L., 1996. Inhibition of the expression of *Bradyrhizobium japonicum* nod genes at low temperatures. Soil Biol. Biochem. 28, 1579–1583, http://dx.doi.org/10.1016/S0038-0717(96)00261-1.
Zhang, F., Smith, D.L., 1996. Inoculation of soybean (*Glycine max* (L.) Merr.) with genistein-preincubated *Bradyrhizobium japonicum* or genistein directly applied into soil increases soybean protein and dry matter yield under short season conditions. Plant Soil 179, 233–241, http://dx.doi.org/10.1007/bf00009333.
Zhang, H., Prithiviraj, B., Charles, T.C., Driscoll, B.T., Smith, D.L., 2003. Low temperature tolerant *Bradyrhizobium japonicum* strains allowing improved nodulation and nitrogen fixation of soybean in a short season (cool spring) area. Eur. J. Agron. 19, 205–213, http://dx.doi.org/10.1016/S1161-0301(02)00038-2.