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Does a Soybean Intercrop Increase Nodule Number, N Uptake and Grain Yield of the Followed Main Crop Soybean?

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Abstract: It is not known whether seed inoculated soybean intercropping can increase the number of nodules, nitrogen uptake and yield of the subsequent main crop, soybean. For this reason, the soybean intercropping approach, sole or mixed cropping with buckwheat, was adopted to examine the influence of inoculation and intercropping of soybean and buckwheat on the subsequent main crop, soybean. Field trials were conducted from 2016 to 2019 in Germany and Poland. For this purpose, soils on which soybeans had not been grown in the past were selected as experimental plots and laid out in a split-plot design. It was surprising that even without inoculation a nodule growth could be documented. However, intercrop inoculation resulted in an average of 12 times more nodules per plant at four out of five sites. In addition, a 43% higher number of nodules was found on the lateral roots of the main soybean crop when intercropping with inoculated soybean occurred. The influence of the intercrop on the main crop soybean also depended on their growth in late summer and autumn. Further, there was a medium relationship (R = 0.7) between the number of nodules in the intercrop soybean and the nitrogen content of the soybean grain in the main crop. In terms of soybean grain yield, a single inoculation of the intermediate soybean crop contributed an average of 5% higher yield and inoculation of both the intercrops, and the main crop improved yield by 15%.

Keywords: *Glycine max* (L.) *Merr.*; intercropping; inoculation; soybean/buckwheat; nodules; nitrogen content; sulfur content

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1. Introduction

Li et al., (2001), Bedoussac et al., (2015) and Maitra et al., (2021) defined intercropping as a system in which two or more crops are planted simultaneously in the same field during the same growing season [1–3]. Intercropping is an important agri-environmental measure, which has been reported to have positive effects on nutrient retention, water and soil protection, weed suppression and soil nitrogen enrichment. In order to benefit from the aforementioned effects, good establishment of the intercrop is mandatory. This depends, amongst other factors, on the type of catch crop selected, the sowing date, soil moisture content, precipitation and temperature. In Central Europe an optimal period for sowing the catch crop is the end of July to the beginning of August, as there is a wide margin for the selection of a suitable intercrop [4].

Intercropping with soybeans has not been established in central Europe so far, which could be due to the high cost of seeds. Fast-growing and frost sensitive crops such as buckwheat or mustard are typically observed as intercrops in Central Europe. Various international field trials have been carried out on intercropping with soybeans, for example, in combination with corn [5], sorghum [6,7] or sugarcane [8]. Previous experiments conducted in Poland [9] and France [10] studied intercropping with buckwheat and soybean. However, these studies have mainly focused on weed suppression and an associated increase in yield through better resource utilization of the soybean main crop. There exists a research gap regarding influence of intercropping with soybeans on nodule growth and yield of the main soybean crop cultivation. Soybeans are legumes that form a symbiotic relationship with bacteria from the genus *Bradyrhizobium* sp. and are thus able to fix atmospheric nitrogen (N_2). Due to their origin, these specific soybean bacteria do not occur naturally in the soils of Central Europe. Therefore, the seeds usually have to be inoculated before sowing, with the appropriate bacteria. In this context, various preparations are available on the market, in liquid or powder form, as well as the already fully inoculated seed. Various studies have already demonstrated the presence of soybean-specific bacteria after a long time in Central European soils where seed inoculated soybean had been cultivated many years before [11–13]. Previous reports have indicated a strong to moderate correlation between intensity of nodulation and the associated nitrogen fixation and, thus, a higher grain yield or grain N content [14,15]. In practice, the seeds are usually mixed with the inoculant and then sown directly. As an alternative to seed inoculation, the inoculant can also be applied directly to the soil. Previous reports have applied different inoculation methods at varying time points. For example, Bai et al., (2003) applied the inoculant directly into the seed furrow [16]. Wichern (2018), on the other hand, describes a one-year seed or soil inoculation trial in which the inoculant was placed in a furrow and was subsequently worked in about 5 cm by a rotary tiller [17]. No significant difference in grain yield or number of nodules was found between the seed or soil inoculation variants [17]. Further to inoculation, agricultural management also strongly influences rhizobial diversity. It has been observed that rhizobia diversity increases with organic farming, as well as direct seeding methods. Moreover, there is higher rhizobial quantity when soybeans are included in the crop rotation compared to monoculture with non-legumes [18,19]. This effect could be due to the incorporation of crop residues.

Thus, in addition to soil properties (e.g., pH or soil texture), crop management also plays an important role in soybean cultivation. This information raises the question as to whether indirect inoculation of the soil via a soybean intercrop using seed inoculated soybean leads to a better distribution of bacteria in the soil and thus to a higher nodule number and yield performance of the consecutive soybean main crop. Indirect soil inoculation by soybean intercropping is expected to result in better distribution of rhizobia in the soil and thus increased nodule population, especially on the lateral roots. This could have yield- or quality-enhancing effects on the main soybean crop. Studies by Hardarson et al., (1989) and McDermott and Graham (1989) have already demonstrated the positive effect of soil inoculation at the time of soybean seeding [20,21]. Moreover, they reported improved N_2 fixation through better nodulation at the lateral roots. However, other additives, such as the bacterium *Azospirillum*, can also have a positive effect on plant growth and lead to higher yields [21].

In the present work, virgin soils, where to the best of our knowledge, soybean had never been grown, were used to test whether inoculated seeds of soybean sole crop and soybean mixed with buckwheat could increase nodulation, plant nitrogen and sulfur content, and yield performance of the subsequent soybean main crop. Due to lack of data on this issue, the main objective of this study is to investigate the effect of inoculation by soybean intercropping on the subsequent soybean main crop.

2. Materials and Methods

2.1. Experimental Design and Site Description

From 2016 to 2019 field trials were carried out at several sites in Germany (Pillnitz & Ebersbach) and Poland (Pawlowice near Wroclaw) (Table 1) to test the effect of inoculation and intercrop cultivation with soybean and buckwheat on the subsequent soybean main crop. For the field experiment, virgin areas were selected on which soybean had not been cultivated before.

Field trials were conducted in experimental fields of the Dresden University of Applied Sciences at Pillnitz (Pi) and the Wrocław University of Environmental and Life Sciences at Pawlowice (Pa). In 2018/2019 a field trial was carried out on an arable field in Ebersbach near Görlitz (Eb). The arable field at the Pi site had been farmed organically since 2004, while those in Pa and Eb were managed conventionally. The temperatures and precipitation in the individual experimental years, as well as before sowing, are listed in Table 2. The field trials were performed in a split-plot design (main plots: cover crop; subplots: without or with inoculation of the intercrop or main crop) with four replications and a single subplot size of 10.0 $\times$ 1.50 m.

A trial year always consisted of an intercrop followed by soybean as the main crop. In the period of intercrop cultivation two factorial treatments were conducted: seed inoculation (i) or uninoculated seeds (u) and pure soybean (S) or buckwheat (B), as well as soybean/buckwheat (BS) mixtures. At the Pawlowice and Ebersbach sites, buckwheat (B) was also sown as sole crop, in uninoculated or inoculated form. In 2018/2019, the trial was expanded to the Eb site, as no corresponding area was available in Pi. For this purpose, in the main crop soybean the variants of uninoculated intercrop and inoculated main crop were sown. An overview of the individual treatments for the different field experiments is shown in Table 3. Inoculation was performed with two teaspoons of peat preparation (HiStick, BASF) per plot (equivalent to about 5.7 kg/ha). The inoculant HiStick was mixed with the seed before sowing and then applied together. The sole crop seeding densities were 80 germinating seeds m^{-2} for soybean (cv. Merlin) and 150 germinating seeds m^{-2} for buckwheat (cv. Kora). The mixed system was designed following the replacement design, with 50% of the sole soybean crop density replaced with 50% of the sole associated crop density of buckwheat (distance between rows equal in each plot: 0.30 m).

The intercrop was sown at the end of July/beginning of August after the cereal grain harvest and a basic soil preparation with the cultivator (at a depth of 25 cm). The main crop, soybean, was sown in late April/early May of the following year (Table 1). Before sowing, the frozen intercrop was mulched and incorporated using a cultivator. During the sowing process, first the plots with non-inoculated soybean were sown to prevent contamination. This was followed by sowing of the inoculated variants. The crops were managed without fertilizers, fungicides or insecticides. In Pa and Eb, there was a pre-emergence herbicide application in the main soybean crop. At the Pi site, weed control in the summer of 2018 was carried out by hand at the time of the two-leaf stage and at the beginning of pod formation.

Table 1. Characterization of trial sites.

Year	Site	Acronym Environment	Coordinates	m. a.s.l. (m)	Soil Texture	Soil Index	Mean Annual Temperature [a] (°C)	Annual Precipitation [a] (mm)	Date of Sowing		Date of Harvesting		pH	P K Mg ($mg \cdot 100\ g^{-1}$ Soil)			N_t (%)	Humus (%)
									Inter-Crop	Main Crop	Inter-Crop	Main Crop						
2016/2017	Pillnitz	Pi 16_17	51°00′04.7″ N 13°53′23.3″ E	118	sL	65	10.5	542	26 July 2016	1 May 2017	25 October 2016	12 October 2017	5.9	5.2	17.8	12.1	0.10	1.7
	Pawlowice	Pa 16_17	51°17′70.6″ N 17°11′60.2″ E	240	sL	70 *	9.8	557	24 August 2016	25 April 2017	31 October 2016	29 September 2017	5.8	8.0	13.6	11.5	0.10	1.6
2017/2018	Pillnitz	Pi 17_18	51°00′03.5″ N 13°53′26.2″ E	118	sL	65	11.6	298	24 July 2017	2 May 2018	30 October 2017	29 August 2018	6.9	6.7	12.3	13.9	0.12	2,6
	Pawlowice	Pa 17_18	51°17′72.6″ N 17°11′40.2″ E	240	sL	70 *	10.5	399	16 August 2017	27 April 2018	29 October 2017	3 September 2018	6.4	8.2	14.5	8.2	0.10	1.7
2018/2019	Ebersbach	Eb 18_19	51°09′40.1″ N 14°55′37.2″ E	208	sL	63	10.7	406	15 August 2018	25 April 2019	27 October 2018	12 September 2019	6.3	3.5	12.0	12.8	0.13	2.1
	Pawlowice	Pa 18_19	51°17′76.1″ N 17°10′79.6″ E	240	sL	70 *	10.8	448	7 August 2018	6 May 2019	31 October 2018	20 September 2019	6.3	5.8	16.0	9.0	0.10	1.7

m.a.s.l. = meters above sea level; * soil index converted as per Link et al. [22], since the scale for the soil index in Poland ranges from 18 to 100, 100 = "very fertile"; sL = sandy loam; a = weather data according to State Office for Environment, Agriculture and Geology [23] and Deutscher Wetterdienst [24].

Table 2. Precipitation during growing season over the three experimental years.

		Precipitation per Month (mm)																
Year	Environment	Precipitation Four Weeks before Sowing	July [I]	August [I]	September [I]	October [I]	November [I]	December [I]	January	February	March	April	May [M]	June [M]	July [M]	August [M]	September [M]	October [M]	Σ
16_17	Pi	91.4	93.2	43.1	51.5	71.5	29.7	26.8	10.6	22.0	41.0	39.9	26.4	56.3	92.0	82.8	32.2	69.4	788.4
	Pa	33.2	–	27.1	44.7	83.8	36.3	36.1	16.9	24.2	31.1	57.0	24.1	52.5	112.2	43.6	65.7	–	655.3
17_18	Pi	73.1	92.0	82.8	32.2	69.4	41.5	27.8	21.9	2.0	20.3	36.5	18.5	30.2	18.5	44.6	–	–	538.2
	Pa	110.2	–	43.6	65.7	71.4	28.4	29.6	22.6	3.1	27.6	19.0	54.3	36.6	79.1	20.3	38.4	–	539.7
18_19	Eb	22.6	36.9	16.0	52.2	35.7	7.7	67.4	40.6	24.0	47.6	14.5	74.2	31.4	42.3	53.2	22.4	–	566.1
	Pa	16.5	–	20.3	38.4	45.3	14.7	38.0	56.2	27.7	22.5	24.2	76.8	27.0	44.5	59.8	42.0	–	537.4

I = intercrop; M = main crop; Pi = Pillnitz; Pa = Pawlowice; Eb = Ebersbach.

Table 3. Overview of the individual test variants.

	Intercrop		**Soybean Main Crop**			
	i	**u**	**i**	**i**	**u**	**u**
Cultivated Crop	soybean (i)	soybean (u)	(ii)	(ui) **	(iu)	(uu)
	buckwheat (i) *	buckwheat (u) *	(ii)	(ui) **	(iu)	(uu)
	soybean/buckwheat (i)	soybean/buckwheat (u)	(ii)	(ui) **	(iu)	(uu)
	winter fallow (u) **		(ui) **		(uu) **	

i = inoculated; u = uninoculated; (x/x) = the first letter in the parenthesis stands for the inoculation of the intercrop and the second letter for the inoculation of the main crop; * these variants were not established in Pillnitz; ** these variants existed only in Eb 18_19.

2.2. Site Conditions

In order to classify the soil conditions and the supply levels, a basic nutrient analysis was carried out on the test areas before soybean main crop (mixed sample at 30 cm depth). A basic chemical analysis was carried out on each soil sample in accordance with the guidelines for soil analysis of the Association of German Agricultural Analytic and Research Institutes e.V. (VDLUFA). The pH value [25] and the phosphorus (P), potassium (K) [26], magnesium (Mg) [27], humus [28], and total nitrogen (Nt) contents [29,30] of the soil were determined accordingly (Table 1).

At the Pawlowice (Poland) site, collection of all chemical data was not possible. For this reason, some data on soil conditions are not available.

2.3. Determination of Yield and Nutrient Analysis

During the intercrop, an intermediate harvest was carried out towards the end of October, consisting of two parts. First, an area of 2.25 m^2 per plot was harvested by hand to determine the shoot mass of intercrops. The total fresh mass was sorted by hand into soybean, buckwheat and weeds, weighed individually and dried at 55 °C for 48 h. Specific weed identification was not performed. Second, ten individual soybean plants were dug out from the soybean plots, using a spade. The soybean roots were carefully rinsed with tap water. Then the root was separated from the stem and the root nodules were isolated and counted to quantify the number of nodules per plant. The other parts of the plant were also dried at 55 °C to determine the dry matter.

In total, two harvests of the main crop, soybean, were conducted. Nevertheless, complete harvesting of the main crop was only possible at the sites in Pi and Eb. For this reason, the collected data for Pa are incomplete. The first harvest was conducted at the end of flowering/beginning of pod forming. Sample processing was performed in the same way as that described for the intermediate crop. Additionally, the individual plants were differentiated in summer 2019 in Eb according to whether the nodules were located at main root or at the secondary roots. The stems and roots were also dried at 55 °C and the dry matter was determined. Furthermore, the chlorophyll content was measured in soybean leaves with a chlorophyll meter (SPAD-502Plus) in summer 2018 and 2019 in Pi and Eb. The youngest fully developed leaf was measured on ten individual plants per plot and a mean value was calculated for the respective plot. At BBCH 89 [31], the soybeans were harvested with a plot combine (13.5 m^2). Harvested seeds were cleaned, weighed, and dried at 55 °C; dry weight was then determined.

Elemental analysis (EuroEA3000-Hekatech) was used to determine the content of total nitrogen (N) in roots and shoots as well as N and sulfur (S) in soybean nodules by chromatographic separation of the oxidation gases. The principle of analysis in this machine is based on dynamic flash combustion followed by gas chromatography separation of the resultant gaseous species [32]. Due to the partially very small quantities of nodules, mixed samples rather than four replicates were used to determine their N and S contents. For this purpose, the samples were ground to a particle size of $\leq$0.2 mm, the material was filled into 3 $\times$ 6 mm tin capsules (IVA Analysentechnik, Meerbusch, Germany), and then weighed with a balance (Mettler Toledo XA 105 Dual Range, Greifensee, Switzerland) with an accuracy of ±0.01 mg. The amount weighed was based on the carbon/nitrogen ratio of the plant material, which ranged from 2.8 to 3.2 mg for soybean. For the determination of S content, about 7 to 10 mg vanadium pentoxide was added to each sample prior to analyses.

By pyrolysis, a part of the shoot dry matter of the individual plots was broken down in order to carry out a nutrient analysis (Mg, Na, S, Ca, K and P). For this purpose, two portions (duplicate determination) of approximately 1 g of each plant sample were placed in porcelain crucibles and heated in a muffle furnace at 600 °C for four hours to completely oxidize all organic carbon to CO_2. 5 mg of the plant ash was weighed, and exactly 10 mL nitric acid (HNO_3, 2 Vol.%) was added and shaken until the samples were completely dissolved. The samples were measured by ICP OES (spectrometer Optima 4300 DV, Perkin Elmer Company, Waltham, MA, USA) under radial observation. Each sample was measured three times and the mean value was calculated per sample.

2.4. Statistical Analysis

Statistical analyses were conducted using the statistical analysis system (SAS) program version 9.3 of SAS Institute Inc., Cary, NC, USA (2013). The data for all sites were created as a two-level three-factor split system, with inoculation of intercrop and inoculation of main crop together forming the large plot (main unit) and the intercrops being the subplots. The applied control in Eb 18_19 can only be accounted for with the inoculation variant uninoculated/uninoculated and uninoculated/inoculated and was therefore evaluated as a two-factor split system. Intercrop evaluation was also three-factorial, although only uninoculated and inoculated variants can be distinguished. Analyses of variance and mean, as well as regression analyses, were performed. In each case, the data sets were statistically tested for outliers (CUTOFF = 3.0000), which rarely occurred and were only considered if they had a decisive influence on the significance. Differences between treatments were significant or not at $p = 0.05$ for all analyzed data sets.

To calculate significant differences between means, t-test was applied following the method of Munzert [33] for split plot (and unbalanced) designs. As far as significant variances ($p \leq 0.05$) could be observed, the appropriate standard error of difference of the pairwise means was used for calculation the Confidence Limits (upper and lower CL value), that is LSD 5% = $(CL_{upper} - CL_{lower})/2$.

3. Results

The individual presentation per site and year was chosen to illustrate the results of the present study. This is due to the fact that significant interactions between factors were very often observed when evaluating across years and sites, and also that in some cases an orthogonal data set was not available. With the applied evaluation procedure, significant interactions occurred only in rare cases. A supplementary overview of the statistical results for the *p* and N values can be found in Table A1.

3.1. Evaluation of Intercrop

Intercrop emergence and growth was very different in the three years and sites, making it difficult to compare the individual trials. In Pi, high dry matter yield of the catch crop could be documented in both years, which significantly differ from each other (Figure 1). In both years, inoculation of the catch crop in Pi had a positive effect on DM yield. Inoculation showed a significant difference only in Pi 17_18, which was reflected in a 1.26 t ha^{-1} higher mean plant biomass of the intercrop. Further, there was a high proportion of weeds at the Pi site, some of which had a higher dry matter than the intermediate crops (Figure 1, Table S1). In Eb 18_19, on the other hand, very poor intercrop growth was recorded due to low water availability from July to September (Table 2, Figure 1), far below the level at the Pi site. In Pa, the average dry matter yield of the intercrops (Figure 1) was in all cases lower than in Pi because of late sowing, with significant differences between individual intercrops in all three trial years. The buckwheat sole crop had the highest average dry matter yield per hectare at 1.6 tons and the soybean sole crop had the lowest at 0.9 tons. Figure 2A shows a uniform distribution and thus a weak relationship (R^2 = 0.38) between intercrop nodule growth and dry matter and harvested soybean intercrop. Intercrop inoculation showed no significant effect on intermediate soybean yield at the Pa site in all three years of the experiment, but the uninoculated plots always had the lowest dry matter yield. On the other hand, inoculation at Pi 2017 resulted in significantly higher nodule number, N and DM yield of soybean intercrop (Table 4, Figures 1 and 3).

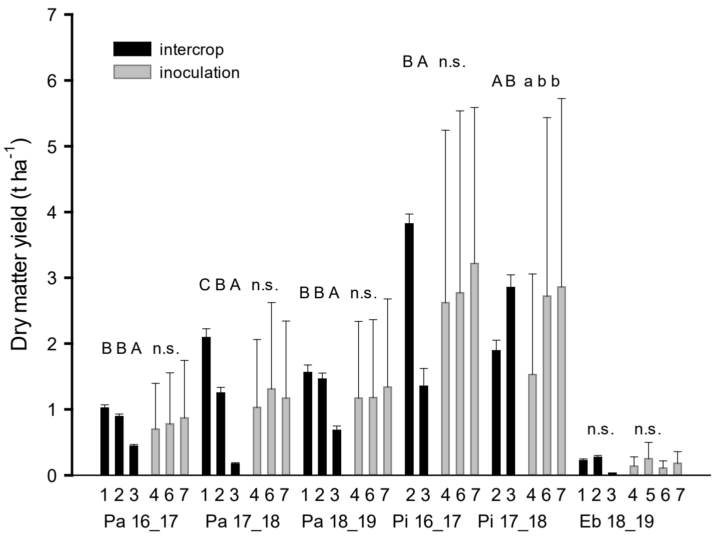

Figure 1. Shoot dry matter yield intercrops. Values followed by different letters are significantly different at LSD 5%. In this context, each site and the type of intercrop as well as inoculation was considered separately. Bars indicate the standard error (SE). Large letters indicate significant differences between the different intercrops, small letters between the inoculation variants. n.s. = not significant; Pa = Pawlowice; Pi = Pillnitz; Eb = Ebersbach; 1 = buckwheat; 2 = buckwheat/soybean; 3 = soybean; 4 = uninoculated/uninoculated; 5 = uninoculated/inoculated; 6 = inoculated/uninoculated; 7 = inoculated/inoculated.

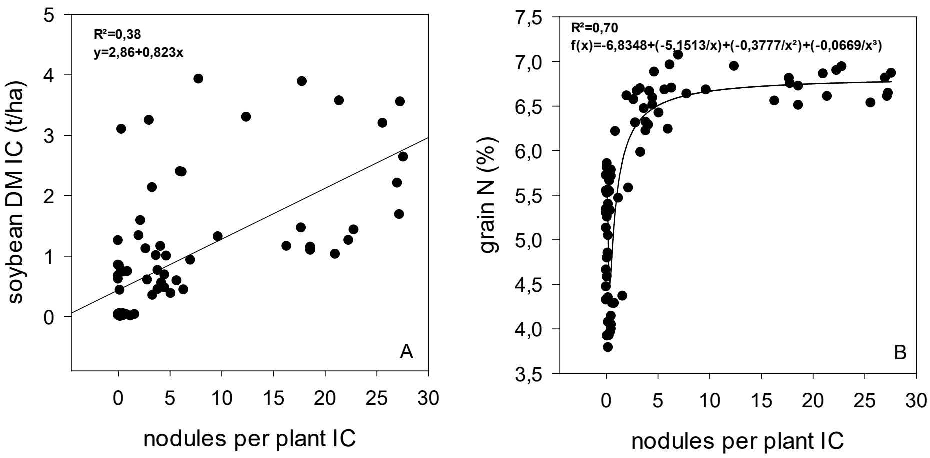

Figure 2. Regression of number of nodules per IC soybean plant on IC soybean DM (t ha^{-1}) (**A**) and number of nodules per IC soybean plant on grain N of MC soybean (**B**); IC = intercrop; MC = main crop; DM = dry matter.

Table 4. Recorded nodule data in soybean intercrop.

	Intercrop	Inoculation	No. of Nodules per Plant		±SE	Weight of Nodules per Plant (mg)		±SE	Nodules_N (%)		±SE	Nodules_S * (%)	± SE
	BS		2.6		0.4	16.99		3.53	5.45		0.19	0.26	0.006
	S		3.9		0.7	26.26		5.49	5.87		0.15	0.25	0.005
Pi 16_17		u/u	0.2		0.0	1.12	A	0.36	5.49		0.34	0.26	0.009
		i/u	3.9		0.3	20.22	B	2.40	5.77		0.09	0.25	0.003
		i/i	5.6		0.4	43.54	C	4.60	5.72		0.13	0.25	0.005
	BS		14.6		1.9	139.67		15.34	5.42		0.11	0.23	0.005
	S		14.9		2.2	150.94		16.48	5.31		0.13	0.23	0.007
Pi 17_18		u/u	2.8	A	0.4	55.87		6.46	5.17		0.15	0.26	0.006
		i/u	19.6	B	0.9	189.9		7.77	5.28		0.09	0.21	0.004
		i/i	21.8	B	1.4	190.15		11.28	5.65		0.09	0.23	0.003
	BS		0.3		0.04	0.56		0.11	-		-	-	-
	S		0.3		0.05	0.76		0.05	-		-	-	-
	B		-		-	-		-	-		-	-	-
Eb 18_19		u/u	0.3		0.03	1.27		0.06	-		-	-	-
		u/i	0.2		0.01	0.13		0.02	-		-	-	-
		i/u	0.5		0.06	0.65		0.06	-		-	-	-
		i/i	0.3		0.05	0.58		0.15	-		-	-	-
	BS		5.5	B	0.7	35.01	B	3.90	5.13	B	0.07	0.21	0.002
	S		3.5	A	0.5	12.82	A	1.70	4.81	A	0.04	0.22	0.002
Pa 17_18	B		-		-	-		-	-		-	-	-
		u/u	0.35	A	0.1	4.05	A	0.80	4.64	A	0.03	0.21	0.002
		i/u	7.00	B	0.4	35.38	B	3.20	5.24	B	0.05	0.22	0.002
		i/i	6.10	B	0.5	32.32	B	3.20	5.03	B	0.07	0.22	0.002
	BS		2.45	A	0.4	7.43		0.001	4.72		0.05	0.21	0.003
	S		3.27	B	0.5	7.69		0.001	4.78		0.10	0.22	0.003
Pa 18_19	B		-		-	-		-	-		-	-	-
		u/u	0.05	A	0.0	0.22		0.000	4.39	A	0.07	0.21	0.004
		i/u	3.68	B	0.3	11.86		0.001	5.10	C	0.10	0.22	0.003
		i/i	4.86	B	0.3	10.60		0.001	4.75	B	0.08	0.22	0.003

Values followed by different letters are significantly different at LSD 5%. In this context, each site and the type of intercrop as well as inoculation was considered separately. Pi = Pillnitz; BS = buckwheat/soybean; S = soybean; B = buckwheat; u = uninoculated; i = inoculated; SE = standard error; N = nitrogen; S = sulfur; n.s. = not significant; * high number of outliers; normal distribution is not given.

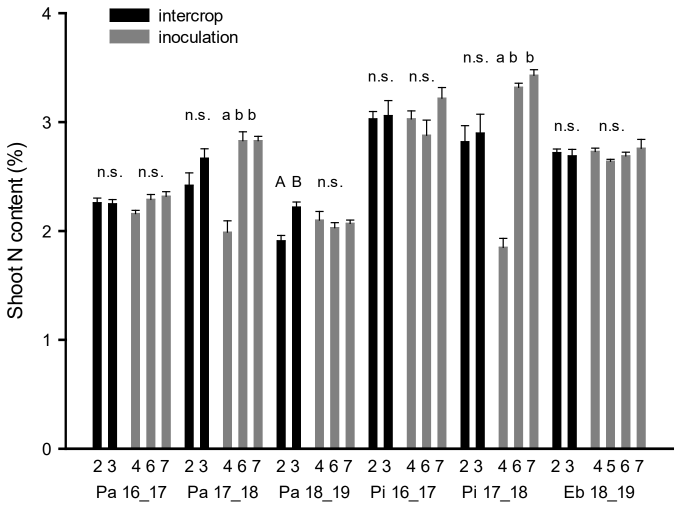

Figure 3. Shoot N content soybean intercrop. For explanations, see Figure 1.

The nitrogen content in the intermediate soybean shoot dry matter was somewhat higher at Pi than at the Eb and Pa sites. However, the inoculation itself only had a significant influence on the N content in Pa 17_18 and Pi 17_18 (Figure 3). In addition, at four of the six sites, the N content in the shoot mass of soybean was lower in the mixture with buckwheat than in sole crop soybean.

The nodule N and S content of intercrop soybean was also higher in Pi than at the Pa site (Table 4). In Pa there was a significant indication that the inoculated soybean had a higher nodule N content. Next, only in Pa 17_18 was there significant evidence that intercropping with buckwheat had a positive effect on soybean nodule N level. At Eb, measurements of the N and S content were not possible due to the very low number of nodules and thus a small amount (<2.8 mg).

3.2. Evaluation of Main Crop Soybean

Grain yield results for the main crop of soybeans are shown in Figure 4. The influence of intercropping on grain yield of soybean is basically opposite in Pi and Pa. In Pa, very consistent results were documented across all three years of the trial. Intercropping soybeans increased the grain yield of the main crop soybeans by an average of 6.7%, when mixed with buckwheat, and by 11.6% in the monocrop soybeans. In addition, double inoculation at the intercrop and main crop stage had a positive effect on the grain yield of soybean. The influence of intercropping and inoculation at the Pi and Eb sites did not give a consistent picture. In Pi, intercropping with buckwheat had a positive effect on soybean grain yield, but yields in Pa and Eb were three to six times higher than in Pi. For site Eb, there was a significant indication for inoculation of the main crop, where the uninoculated variant was also the lowest according to grain yield of soybean. In terms of grain nitrogen content, the intercrop soybean resulted in a higher nitrogen content of the main crop soybean, which was significant only at site Pi 16_17. Inoculation of the intercrop or main crop had a positive and higher effect on grain N content as compared to no inoculation (Figure 5). Additionally, Figure 2B shows a medium correlation (R^2 = 0.7) between the number of nodules per soybean plant to the intercrop and the grain N content of the main crop soybean. In this context, it is worth mentioning that, whenever more than two nodules per plant were documented on the intercrop soybean roots, the grain N content was above 6%. In contrast, the number of nodules per plant of the intercrop had only a weak influence on the number of nodules of the subsequent main crop soybean (R^2 = 0.1). An influence of the number of nodules of the intercrop on the nodule N content of the main crop could not be proven (R^2 = 0.001).

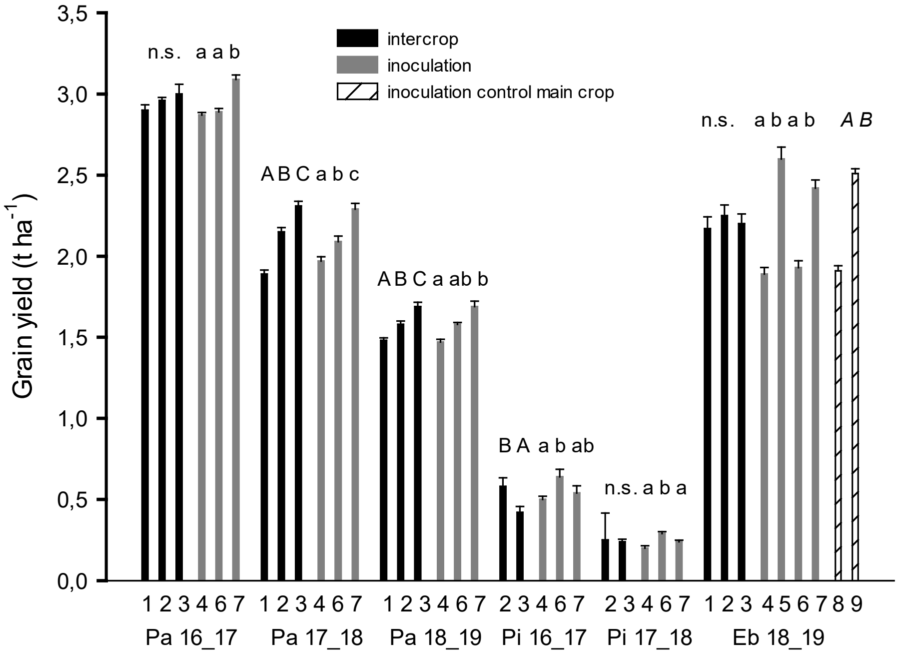

Figure 4. Grain yield (t ha^{-1}) for soybean main crop. For explanations, see Figure 1. Italic capital letters indicate significant differences between the inoculation variant of the main crop. 8 = without inoculation main crop; 9 = with inoculation main crop; n.s. = not significant.

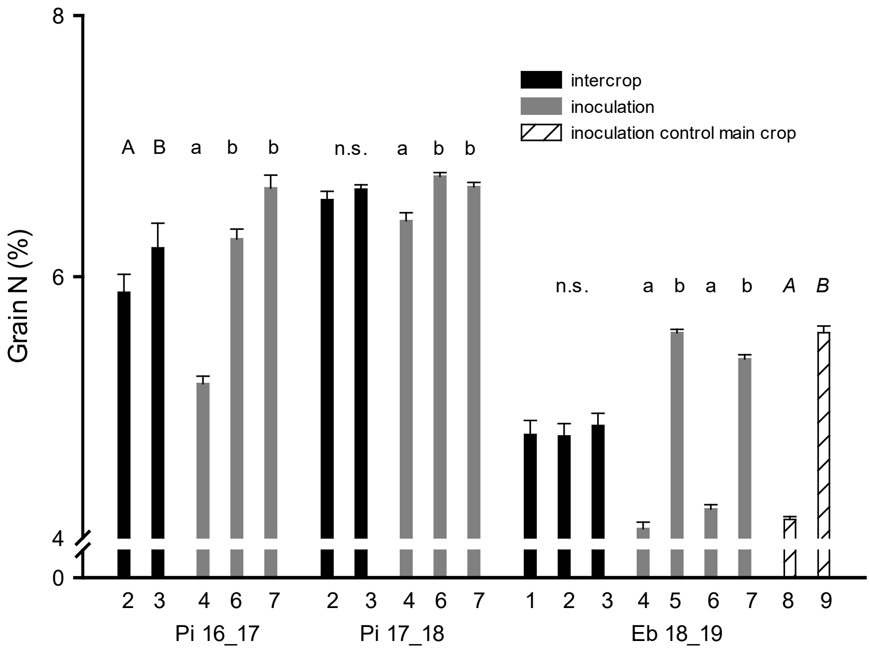

Figure 5. Grain N (%) soybean main crop. For explanations, see Figure 4.

Nodule growth to the soybean main crop at one (Pi 17_18) of three sites showed that the nodule number was significantly increased by inoculation of the intercrop. In Pi 16_17, nodule number was also higher by inoculation of the intercrop compared to the uninoculated variant (u/u), but not significantly. Dual inoculation of intercrops and main crop soybean led to the highest nodules numbers and mass. At site Eb, the influence of intercrop inoculation was not visible due to poor growth of the soybean intercrop. Nevertheless, inoculation of the main soybean crop resulted in a higher number of nodules than without inoculation. The type of intercrop did not affect the nodule number of the soybean main crop (Table 5).

Table 5. Recorded nodule data and shoot nutrient content in the soybean main crop.

	Inter-Crop	Inoculation	No. of Nodules per Plant		±SE	Weight of Nodules per Plant (mg)		±SE	Nodules N [%]	±SE	Nodules S * [%]	±SE	No. of Nodules per Plant m.r.		±SE	No. of Nodules per Plant l.r.	±SE	Shoot_Ca [%]		±SE	Shoot_P [%]		±SE
Pi 16_17	BS		13.0		2.80	143.54		13.75	-	-	-	-	-		-	-	-	1.25		0.014	0.24		0.24
	S		13.8		2.84	132.63		17.95	-	-	-	-	-		-	-	-	1.24		0.016	0.23		0.23
		u/u	2.6	A	0.55	77.78	A	15.53	-	-	-	-	-		-	-	-	1.20		0.018	0.24		0.24
		i/u	7.8	A	0.53	126.09	AB	7.62	-	-	-	-	-		-	-	-	1.26		0.014	0.23		0.23
		i/i	29.9	B	2.12	210.38	B	8.93	-	-	-	-	-		-	-	-	1.22		0.012	0.22		0.22
Pi 17_18	BS		8.5		1.48	29.01		5.09	5.85	0.05	0.38	0.03	-		-	-	-	1.55		0.026	0.24		0.24
	S		11.5		1.39	43.62		5.24	5.77	0.07	0.33	0.03	-		-	-	-	1.65		0.030	0.24		0.24
		u/u	4.4	A	0.72	21.64	A	4.06	6.00	0.06	0.36	0.04	-		-	-	-	1.61		0.042	0.25	B	0.25
		i/u	7.8	B	1.26	34.38	AB	5.76	5.71	0.06	0.31	0.02	-		-	-	-	1.66		0.015	0.24	B	0.24
		i/i	17.8	C	0.56	52.92	B	4.18	5.73	0.05	0.40	0.03	-		-	-	-	1.67		0.027	0.23	A	0.23
Eb 18_19	BS		7.3		1.15	65.65		11.11	-	-	-	-	14.3		2.5	24.0	3.6	1.35	B	0.014	0.19		0.19
	S		6.6		1.05	65.35		10.44	-	-	-	-	13.4		2.0	24.8	3.7	1.27	A	0.010	0.18		0.18
	B		7.7		1.30	70.31		11.38	-	-	-	-	17.1		3.2	21.4	4.1	1.27	A	0.015	0.18		0.18
		u/u	0.1	A	0.02	0.14		0.05	-	-	-	-	0.0	A	0.0	0.2	0.1	1.33		0.018	0.19		0.19
		u/i	16.7	C	1.02	159.69		9.64	-	-	-	-	35.6	C	2.6	50.3	3.4	1.30		0.012	0.18		0.18
		i/u	0.2	A	0.05	1.03		0.38	-	-	-	-	0.0	A	0.0	0.6	0.2	1.25		0.010	0.19		0.19
		i/i	11.9	B	0.72	107.56		6.15	-	-	-	-	24.2	B	1.9	42.4	2.7	1.30		0.009	0.18		0.18
		IMc u	0.1	A	0.02	1.42		0.39	-	-	-	-	0.0	A	0.0	0.4	0.1	1.31		0.005	0.19		0.19
		IMc i	14.4	B	1.15	133.18		12.27	-	-	-	-	29.9	B	2.9	46.4	4.3	1.28		0.015	0.18		0.18
Pa 16_17	BS		-		-	41.87		5.69	6.41	0.05	0.31	0.01	-		-	-	-	-		-	-		-
	S		-		-	54.05		7.89	6.49	0.04	0.30	0.00	-		-	-	-	-		-	-		-
	B		-		-	53.03		6.62		0.13		0.00	-		-	-	-	-		-	-		-
		u/u	-		-	35.87		6.00	6.64	0.04	0.31	0.01	-		-	-	-	-		-	-		-
		i/u	-		-	66.45		7.52	6.33	0.06	0.31	0.01	-		-	-	-	-		-	-		-
		i/i	-		-	46.62		5.84	6.37	0.13	0.30	0.00	-		-	-	-	-		-	-		-

Values followed by different letters are significantly different at LSD 5%. In this context, each site and the type of intercrop, as well as inoculation, was considered separately. Pi = Pillnitz; Pa = Pawlowice; BS = buckwheat/soybean; S = soybean; B = buckwheat; u = uninoculated; I = inoculated; IMc = inoculation main crop; SE = standard error; N = nitrogen; S* = sulfur; Ca = calcium; P = phosphor; m.r. = main root; l.r. = lateral root; * high number of outliers, normal distribution is not given.

In 2019, at site Eb, the number of nodules on the main and secondary roots of the soybean plant was also determined. In this context, a significant effect of inoculation on the main crop was only found in the number of nodules on the main root. Nevertheless, it should be emphasized that the number of nodules on the lateral roots was higher than on the main root. Only two sites (Pi 17_18, Pa 16_17) could be evaluated for nodule N and S content in the main crop, and no significant effects were found. However, it should be noted that at the site Pi 17_18 the contents of N and S were increased compared to those of the intercrop. This is particularly evident in the S content.

To fully assess the effect of the intercrop, an additional nutrient analysis of the soybean shoot mass to the main crop was conducted at the Pi and Eb sites. There was a significant effect only in Pi 17_18 for P content and in Eb 18_19 for Ca content of soybean main crop. In both cases, the nutrient content of main crop soybean was highest in the uninoculated variant (Table 5).

At the Eb site, a separate evaluation was carried out including the applied control (Table S3). The control was conducted for the intercrop as winter fallow and for the main crop soybean uninoculated and inoculated with soybean. A significant difference was observed between the uninoculated and inoculated variants to the main crop soybean for the studied parameters, i.e., grain yield, grain N content, number of nodules (per plant and per plant main root) and SPAD value. The intercrop only had a significant effect on the K and Ca content of the soybean shoot mass to the main crop.

All other data collected did not yield meaningful results, e.g., the SPAD value, and are consequently not discussed in detail. However, the data are listed in full in supplementary materials Tables S1–S3.

4. Discussion

Does an inoculated soybean intercrop increase nodule number, N uptake, and grain yield of the subsequent main soybean crop? The results of the presented field experiments showed that an intercropping with soybean had a positive effect on a subsequent main crop cultivation with soybean.

4.1. Effects of Intercropping with Soybeans on Nodule Development

Contrary to expectations, nodules were documented on the soybean plants at all locations during intercropping, even without inoculation. Nevertheless, the number of nodules was significantly higher after inoculation of the intercrop at four out of five sites than without inoculation. At site Eb, insufficient plant development occurred due to dry growing conditions during intercropping, resulting in reduced or no nodule growth. It had been already shown that *Bradyrhizobium japonicum* is able to survive for a long time under Central European soil and climatic conditions [13,34,35]. Madrzak et al., (1995) and our own preparatory work also demonstrated that rhizobia were present in Central Europe, even though soybeans have not been grown before [12,36]. Further, Narozna et al., (2015) and McDermott and Graham (1989) demonstrated that *Bradyrhizobia* have low mobility in soil [13,37]. Narozna et al., (2015) even attribute the observed short mobility distance to mechanical movement during tillage (e.g., by harrowing) [13]. This contrasts with the study by Ferreira and Hungria (2002), who drew inferences from 40 isolated samples to seven strains in commercially used inoculants, demonstrating spread from soybean growing areas [38]. With the help of seeds or dust arising, rhizobia can apparently reach soils over long distances where no corresponding host had been previously cultivated [39]. Moreover, at four out of five sites, more nodules per soybean plant were counted in the intercropping of sole crop soybean than in the mixture with buckwheat. For the main crop soybean, two of the three locations also showed higher nodule growth when the preceding crop soybean was sown in the field. In these two cases (Pi 16_17, Pi 17_18), there was previously good growth of soybean in the intercrop phase with high nodulation, in contrast to Eb 18_19. Zhang et al., (2011) also documented a higher nodule count in their experiment with pure-seeded soybean compared to the mixture with corn [40]. Furthermore, the nodule number was increased by inoculation. Eb showed a deviation again and the variant with buckwheat as intercrop had a higher number of nodules per plant in the soybean main crop. A dependence of nodule growth on the main crop soybean on soybean dry matter to the intercrop could not be demonstrated (R^2 = 0.08). It can be concluded that not only a high dry matter yield of the soybean intercrop is a success factor for high nodulation in the followed main crop soybean, but other factors (e.g., temperature and water availability) were also important. When comparing the variants with and without inoculation, a significant effect of the inoculant on nodule growth was observed. This is also confirmed by the results of Grossmann et al., (2011) and Zhang et al., (2011) [18,40]. Weaver and Frederick (1972) also demonstrated that the number of nodules on the tap and lateral roots increased when the initial population of rhizobia was low and a high concentration of the inoculant was used [41]. In addition, they were able to show that there is some competition between soil rhizobia and rhizobia supplied by an inoculant and that the inoculant must be applied at a 1000-fold higher soil population concentration (per g of soil) to induce an effect on nodulation.

The hypothesis that intercropping with soybeans increases the number of nodules on lateral roots to the main crop of soybeans cannot be proven statistically. However, it can be shown that, at site Eb, on average of 43% more nodules could be documented on the lateral roots than on taproots if intercropping with soybeans (BS or S) had previously taken place. Considering main and lateral roots separately, the variant with buckwheat seems to have a positive effect on the nodules on the main root. In contrast, the number of nodules on the lateral roots was higher when soybeans were previously planted as an intercrop. This speaks for a better distribution of the bacteria necessary for symbiosis as well as for a generally higher bacterial quantity in the soil. Various studies also conclude that regular cultivation increases the number of rhizobia in the soil. In this context, crop residues and adequate distribution through tillage play an essential role [18,42,43]. In the experimental set-up carried out, the soybean intercrop even remained completely in the field, froze over during the winter, and was completely incorporated into the soil by means of cultivators in the spring. Thus, tillage compensates for the described immobility of rhizobia. McDermott and Graham (1989) reported that, in the absence of a rhizobia population, nodule decline is greater on the lateral roots than on the taproots [37]. Due to difficulties in establishing the intercropping in Eb 2018, the results were not statistically validated, but for the first time they showed a positive effect of the soybean intercrop on the distribution of nodules on the main and lateral roots in the main crop.

Although repeated cultivation of soybean had a positive effect on rhizobial quantity, nodule development in the intercrop had only a weak effect on subsequent nodulation in the main soybean crop and was not reflected in nodule nitrogen content. However, the study showed that there was a strong relationship between the number of nodules in the intercrop and the grain N content of the following soybean main crop. In cases where more than two nodules per plant were found in soybean intercrops, a soybean grain N content of more than 6% could be reached in the subsequent main soybean. This is in line with findings reported by Thilakarathna and Raizada (2017) [44]. A plausible explanation for this is the fact that nodulating soybeans have a longer assimilation time as well as higher assimilation rates of atmospheric nitrogen, and due to the higher chlorophyll content more nitrogen is also available for translocation in the grain as part of chlorophyll degradation [45].

It should be noted that nodules were documented on the soybean plants without inoculation even on the virgin soils, that intercropping with soybean seems to have a positive influence on the distribution of nodules on the main and lateral roots of the succeeding soybean, and that the N content of the grain could also be increased by a low number of nodules per plant in the intercrop of soybean.

4.2. Effect of Intercropping on the Main Crop Soybean

In this context, it was important to investigate to what extent double cropping of soybeans as well as the use of an inoculant has an influence on soybean yield and nutrient content in soybean plants. Therefore, it was evaluated whether the two times uninoculated soybeans (u/u) can achieve the same yield as the one or two times inoculated combination of soybeans (i/u or i/i). It should be noted that the non-inoculated soybean combination always produced the lowest yield and thus inoculation always had a positive effect on growth of soybean main crop. Only at two sites (Pi 16_17, Pi 17_18) was the yield higher if soybean were inoculated once (i/u) than when inoculated twice (i/i). In both experimental years, very low yields were measured, due to heavy weed infestation and drought during the growth of soybean main crop. However, the question posed at the beginning regarding the increase in yield due to indirect inoculation of the soil via a seed-infected soybean intercrop can be answered in the affirmative in this case.

In addition, it should be verified whether the inoculation of the intercrop (i/u) was more effective than the inoculation of the main crop (u/i) and whether there was an additive effect. To answer this question, the trial was expanded and systematically established at the Eb site. However, the growing conditions during summer 2018 were very unfavorable for the establishment of the intercrop. Intercrops responded by lowering and delaying germination in 2018 and were only able to achieve limited shoot growth due to continued drought stress. In particular, a persistently low opening width of the stomata during the course of the day is likely to have greatly reduced the plants' photosynthetic performance, a typical physiological response of plants to drought stress [46]. Consequently, no influence of the intercrop on the main crop soybean could be detected. These issues should be addressed and reexamined in a further study. This could also incorporate the results of Sheteiwy et al., (2021), who demonstrated a positive effect of biofertilizers on plant growth under drought stress [47].

The influence of the intercrop led to opposite results in Pi and Pa. In Pi, the BS mixture had a positive effect on yield formation of the main crop soybean. In Pa, however, the sole crop of intercrop soybean produced the significantly higher yields in the following year. This is related to increased nodulation of the soybean intercrop and consequently increased nitrogenase activity associated with higher nitrogen content in nodules [48]. This not only increased the amount of N, but also the N content in the crop residues, which can then be mineralized in the spring and made available to the main crop, soybean. In addition, Hardarson et al., (1989) described that the placement of the inoculant and the position of the nodules on the root also affect symbiotic N_2 fixation [20]. This could also lead to an increase in N content in the grain of the soybean main crop due to intercropping with soybean, since the rhizobia are already better distributed in the soil due to appropriate inoculation of the intercrop. A better nodulation of lateral roots may also have had a positive effect on N content, as shown at site Eb. This would be consistent with the findings of Hardarson et al., (1989) that nodules formed later contribute significantly to the total amount of N_2 fixed [20].

A complementary and sustainable approach is shown by Muneer et al., (2021) with their study on the production of protein concentrate in catch crops [49]. Here, the advantages of catch crops can be used to generate additional income. Harvesting the catch crop would also make less N available for the following crop, but the soybean can compensate for this by producing highly efficient nodules, thus achieving a doubly sustainable effect.

The analysis of the shoot mass showed a result only for the Ca and P content. In two of the three experimental years, the intercrop BS mixture seems to have a positive effect on nutrient content, although there is no significant evidence for this. The hypothesis that buckwheat would have a positive effect on the yield of the main crop, soybean, due to phosphatases, because more P would then be available for the plants, could not be confirmed [50]. This could be due to insufficient availability of phosphorus in the soils, (P availability between 3.5–8.2 mg $\times$ 100 g^{-1} soil, Table 1). In addition, the uninoculated variants seem to have higher nutrient contents, which can be explained by a generally lower yield and thus a lower nutrient demand. In Pa, a generally higher P content in the soil was also observed, meaning that the effect of buckwheat may not have been visible in the result. Bekere and Hailemariam (2012) documented a significant effect of P fertilization on soybean yield and were also able to demonstrate its importance for nodule development [51]. In their study too, nodules were recorded on virgin soils without inoculation and it was shown that even low P fertilization could increase shoot dry matter as well as the number of pods per plant.

For the time being, a recommendation for action can be derived from the present results with reservations: indications can be derived that intercropping with soybean or buckwheat/soybean can have a positive effect on the subsequent soybean crop. The prerequisite for this is the successful establishment of the catch crop in late summer with appropriate nodulation. Furthermore, it should be noted that no plant diseases were observed on the main crop soybean after the soybean intercrop. In the case of intercropping with soybean before soybean and thus a shortened cultivation break, other effects could occur due to an increased disease pressure. Especially, root-related diseases could then potentially become a problem. Nevertheless, this still needs to be investigated in more detail.

Overall, in order to evaluate the influence of the crop rotation design, environmental factors must of course also be taken into account. Several environmental factors affect nodulation and symbiotic N_2 fixation in soybean, including soil nutrient factors [52]. The dry years 2018 and 2019 had an impact not only on the establishment of the intercrop, clearly visible at site Eb 2018, but also on the yield formation of the main crop itself, for example at site Pi 2018. In addition, soil properties and management methods also play an important role. For example, the study by Zaeem et al., (2019) showed that intercropping with maize under cool site conditions increased forage production and improved soil health. In this context, intercropping resulted in a decrease in rhizosphere soil pH and a significant increase in available phosphorus [53]. These aspects could also be investigated in further studies in combination with buckwheat.

In conclusion, in the field trials conducted, soybean was grown as an intercrop for the first time to investigate the effects of inoculation and intercrop type on the subsequent main crop, soybean. In the process, soybean sole crop as an intercrop at the Pawlowice site and the buckwheat–soybean mixture at the Pillnitz site proved to be advantageous intercrops. A general recommendation cannot be derived from the field experiment, as the results are very site-specific. However, it was found that a basal population of rhizobia was present at all sites, even when soybeans were grown on virgin soils. Furthermore, inoculation of the main crop soybean with *Bradyrhizobium japonicum* was always associated with a positive effect on yield and the intercrop soybean increased the N grain content in the following main crop soybean.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12040467/s1, Table S1: Results soybean intercrop; Table S2: Results soybean main crop; Table S3: Results Eb 18_19 soybean main crop.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Tests of Fixed Effects, *p*-values.

		Main Crop Soybean															Intercrop Buckwheat/Soybean									
	Effect	Yield (t ha^{-1})	Grain N (%)	No. of Nodules per Plant	Weight of Nodules per Plant (mg)	Nodules_ N (%)	Nodules_ S* (%)	No. of Nodules per Plant m.r.	No. of Nodules per Plant l.r.	SPAD	Shoot_ Mg (%)	Shoot_ Na (%)	Shoot_ S* (%)	Shoot_ Ca (%)	Shoot_ K (%)	Shoot_ P (%)	DM (t ha^{-1})	Weed DM (t ha^{-1})	Weed_N (%)	B_N (%)	S_N (%)	S_N (kg ha^{-1})	No. of Nodules per Plant	Weight of Nodules per Plant (mg)	Nodules_ N (%)	Nodules_ S*(%)
Pi 16_17 N = 24	Inoculation	0.00	0.00	0.00	0.04						0.06	0.04	0.01	0.57	0.99	0.58	0.47	0.37			0.29	0.88	0.00 *	0.00	0.85	0.93
	Intercrop	0.03	0.03	0.76	0.48						0.47	0.62	0.93	0.76	0.18	0.26	0.00	0.01			0.91	0.07	0.04 *	0.20	0.23	0.83
	Inoculation × Intercrop	0.25	0.59	0.52	0.04						0.17	0.94	0.55	0.38	0.24	0.43	0.37	0.05			0.36	0.53	0.24	0.67	0.06	0.91
Pi 17_18 N = 24	Inoculation	0.02	0.00	0.00	0.02	0.17	0.11			0.28	0.85	0.59	0.68	0.68	0.08	0.04	0.01	0.21	0.64	0.92	0.00	0.00	0.00	0.00 *	0.34	0.06
	Intercrop	0.65	0.28	0.12	0.08	0.62	0.49			0.41	0.50	0.91	0.32	0.17	0.78	0.88	0.00	0.51	0.59	0.77	0.39	0.00	0.83	0.58 *	0.44	0.75
	Inoculation × Intercrop	0.14	0.21	0.39	0.13	0.90	0.68			0.70	0.96	0.68	0.20	0.78	0.68	0.11	0	0.79	0.65	0.82	0.28	0.03	0.99	0.88	0.07	0.19
Eb 18_19 N = 56	Inoculation	0.00	0.00	0.00	0.00 *			0.00	0.00 *		0.87	0.99	0.75	0.35	0.12	0.74	0.93 *	0.45	0.42	0.33	0.31	0.02	0.73	0.28		
	Intercrop	0.89	0.63	0.81	0.95 *			0.62	0.86 *		0.37	0.83	0.83	0.01	0.12	0.15	0.00 *	0.43	0.38	0.18	0.83	0.39	0.82	0.70		
	Inoculation × Intercrop	0.67	0.94	0.48	0.88			0.11	0.71	0.66	0.83	0.17	0.59	0.22	0.88	0.55	0.11	0.50	0.82	0.40	0.85	0.71	0.12	0.76		
Pa 16_17 N = 36	Inoculation	0.00			0.53	0.28	0.93										0.29	0.66			0.58	0.21				
	Intercrop	0.07			0.65	0.54	0.47										0.00	0.09			0.96	0.00				
	Inoculation × Intercrop	0.10			0.98	0.79	0.94										0.79	0.14			0.15	0.09				
Pa 17_18 N = 36	Inoculation	0.00															0.43	0.22	0.29	0.08	0.02	0.38	0.00	0.00	0.02	0.86
	Intercrop	0.00															0.00	0.00	0.05	0.04	0.19	0.00	0.02	0.00	0.01	0.43
	Inoculation × Intercrop	0.56															0.36	0.07	0.28	0.29	0.09	0.77	0.22	0.02	0.05	0.59
Pa 18_19 N = 36	Inoculation	0.01															0.25	0.96	0.07	0.84	0.94	0.00	0.00 *	0.00	0.00	0.81
	Intercrop	0.00															0.00	0.00	0.43	0.26	0.01	0.00	0.04 *	0.83	0.83	0.39
	Inoculation × Intercrop	0.08															0.04	0.2	0.75	0.78	0.1	0.05	0.09	0.94	0.08	0.58

m.r. = main root; l.r. = lateral root; DM = dry matter; B = buckwheat; S = Soybean; N = nitrogen; S* = sulfur; Mg = magnesium; Na = sodium; Ca = calcium; K = potassium; P = phosphorus; * high number of outliers, normal distribution is not given.

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