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Nitrogen Nutrition Optimization in Organic Greenhouse Tomato Through the Use of Legume Plants as Green Manure or Intercrops

Anastasios Gatsios [1], Georgia Ntatsi [1,2,*], Luisella Celi [3], Daniel Said-Pullicino [3], Anastasia Tampakaki [4], Ioannis Giannakou [5] and Dimitrios Savvas [1,*]

[1] Department of Crop Science, Laboratory of Vegetable Crops, Agricultural University of Athens, 11855 Athens, Greece; gatsios@aua.gr
[2] Institute of Plant Breeding and Genetic Resources ELGO-DEMETER, 57001 Thessaloniki, Greece
[3] Department of Agricultural, Forest and Food Sciences, University of Torino, 10095 Grugliasco (TO), Italy; luisella.celi@unito.it (L.C.); daniel.saidpullicino@unito.it (D.S.-P.)
[4] Department of Crop Science, Laboratory of General and Agricultural Microbiology, Agricultural University of Athens, 11855 Athens, Greece; tampakaki@aua.gr
[5] Department of Crop Science, Laboratory of Agricultural Zoology and Entomology, Agricultural University of Athens, 11855 Athens, Greece; giannakou@aua.gr
* Correspondence: ntatsi@aua.gr (G.N.) dsavvas@aua.gr (D.S.); Tel.: +30-210-529-4510

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Abstract: In the present study, in addition to farmyard manure (FYM), cowpea was applied as green manure and faba bean as an intercrop in an organic greenhouse tomato crop, aiming to increase the levels of soil N. Three experiments (E1, E2, E3) were carried out, in which legumes were either noninoculated or inoculated with rhizobia alone or together with plant growth, promoting rhizobacteria. Inoculation of legumes with rhizobia considerably increased N_2 fixation in E1 but had no impact on N_2 fixation in E2 and E3. In E1, the application of cowpea decreased yield because it imposed a stronger nematode infection as the cowpea plants acted as a good host for *Meloidogyne*. However, in E2 and E3 the nematode infection was successfully controlled and the legumes significantly increased the tomato yield when inoculated in E2, irrespective of legume inoculation in E3. The total N concentration in the tomato plant tissues was significantly increased by legume application in E2 and E3, but not in E1. These results show that legumes applied as green manure can successfully complement N supply via FYM in organic greenhouse tomato, while legume inoculation with rhizobia can increase the amounts of nitrogen provided to the crop via green manure.

Keywords: Cowpea; faba bean; BNF; organic; rhizobia; PGPR; root-knot nematodes

1. Introduction

Increased consumers awareness relating to food safety is one of the drivers that has led to an appreciable expansion of the agricultural land area treated according to organic farming practices in the last two decades. This is clearly reflected in the increase of the total organically cultivated area worldwide, from 11 million hectares in 1999 to 69.8 million ha in 2017 [1]. Organic farming systems rely on environmentally friendly practices, such as crop rotation, maintenance and enhancement of soil microbial activity, soil fertility and biodiversity, and nourishing plants primarily through the soil ecosystem, while excluding the use of synthetic chemicals [2]. Calcium (Ca), magnesium (Mg), and sulfur (S) micronutrients are normally available at sufficient levels in arable soils. Furthermore, the supply of phosphorus (P) and potassium (K) does not pose serious difficulties in organic farming systems, since these nutrients are constituents of nonsynthetic organic or inorganic materials, such as

bone meal, rock phosphate, potassium magnesium sulfate, and dolomitic lime, which are permitted as fertilizers in organic farming [3]. However, inorganic nitrogen (N) fertilizers of natural origin that are compatible with certified organic production are rare. Consequently, the availability of N to plants in organic agriculture strongly depends on supply and recycling of organic residues. Animal manure originating from organic or free-range husbandry is a common form of organic residues as a source of N in organic agriculture. However, according to the relevant European Union legislation (EU Directive 889/2008) [4], the amount of N exogenously supplied to an agricultural ecosystem through animal manure should not exceed 170 kg/ha per year. This amount may be sufficient for open-field crops but is insufficient for greenhouse tomato crops, due to both the length of the cultivation period and the high amounts of fruit removed from the field through harvesting. Indeed, tomato fruit production of 20 kg/m^2, which is a reasonable yield outcome in organic tomato greenhouses [5], removes about 240 kg N ha^{-1} yr^{-1} through harvesting, assuming a fruit dry matter content of 6% and a N concentration of 20 mg g^{-1} dry weight, as reported by Colla et al. [6]. Thus, in addition to animal manure, other sources of N compatible with organic agriculture are needed to cover the high N needs of tomato when cultivated organically in greenhouses.

Inadequate levels of available N in soil may result in nutrient deficiency in greenhouse tomato crops, which is manifested as stunted spindly growth and yellowing of the older and intermediate tomato leaves [7]. Nitrogen deficiency decreases tomato yields considerably due to reduction in both the number of fruit per plant and the mean fruit size, together with a negative effect on fruit quality [8]. Therefore, N fertilization is of major importance for greenhouse tomato crops and should be carefully managed to optimize both fruit yield and quality. The use of legumes as intercrops or green manure may represent an important source of N in organic agriculture [9,10]. The unique ability of legumes to fix N_2 through symbiosis with rhizobia is of paramount importance for crop N supply in organic agriculture [11]. Nevertheless, the total amount of biologically-fixed N provided to the crop, as well as the timely release of plant-available N originating from biological N_2 fixation (BNF) are crucial factors for the successful application of green manure or intercropping in organic tomato crops grown in greenhouses [3]. To the best of our knowledge, peer-reviewed reports on the use of legumes in organic tomato greenhouses treated according to the relevant EU legislation are not available so far.

Based on these considerations, the present study was designed to test the hypothesis that legumes can be successfully used as an additional nutrient source in organic greenhouse tomato production. More specifically, the objective of this study was to test whether legumes applied as green manure or intercrops, inoculated or noninoculated with rhizobia and plant growth promoting rhizobacteria (PGPR), can successfully complement farmyard manure (FYM) as an N source.

2. Materials and Methods

2.1. Plant Material, Growth Conditions, and Treatments

The research presented in this manuscript includes three individual experiments conducted successively in the same greenhouse. The three experiments (henceforth referred to as E1, E2, and E3, respectively) took place from May 2017 to January 2018 (E1), February 2018 to June 2018 (E2), and June 2018 to January 2019 (E3). The exact dates for each experiment and crop are provided in Table 1. The experiments were carried out in a standard commercial arch type greenhouse covered by low-density polyethylene films, with vertical sidewalls. The geometrical characteristics of the greenhouse were as follows: eaves height = 2.80 m, ridge height = 3.5 m, span width = 7.5 m, length = 44 m, ground area = 330 m^2. The greenhouse was ventilated by side vents (total opening area of 150 m^2), which were opened whenever the greenhouse air temperature exceeded 26 °C. The greenhouse was NNE–SSW oriented, and located in Preveza, northwestern Greece (38°59′29.2″N; 20°45′36.1″E, 5 m a.s.l.). The plot size was 3.75 × 5.00 m (i.e., 18.75 m^2). The soil type was sandy loam. The greenhouse was not cultivated and had remained uncovered for the 13 years prior to the establishment of the current experiments.

Table 1. Dates of crop establishment, commencement of harvesting, and crop termination for the legume and tomato crops in each experiment.

Legume Crop			
	Experiment. 1	**Experiment 2**	**Experiment 3**
Sowing	May 22, 2017	October 26, 2017	June 12, 2018
Full anthesis	July 25, 2017	-	-
Incorporation to the soil	July 30, 2017	January 25, 2018	August 7, 2018
Tomato Crop			
	Experiment 1	**Experiment 2**	**Experiment 3**
Tomato planting	August 2, 2017	February 8, 2018	August 12, 2018
Start of harvesting	October 13, 2017	May 4, 2018	October 17, 2018
Crop termination	January 19, 2018	June 11, 2018	January 20, 2019

During the experimental period, climatic data, particularly air temperature and relative humidity, were collected on an hourly basis. Monthly means of temperature (minimum, maximum, and average) and relative humidity (%) for all experiments are presented in Table 2.

Table 2. Monthly averages for mean, maximum, and minimum daily temperatures (T_{mean}, T_{max} and T_{min}, respectively)and relative humidity (RH_{mean}, RH_{max} and RH_{min}, respectively) inside the greenhouse during the experimental period (2017–2018) in Preveza, Greece.

Month	**T_{mean}**	**T_{max}**	**T_{min}**	**RH_{mean}**	**RH_{max}**	**RH_{min}**
August 2017	27.6	36.5	21.6	74.4	100	28.2
September 2017	22.9	30.4	17.6	88.1	100	47.2
October 2017	19.0	27.5	13.6	90.2	100	47.3
November 2017	15.3	23.3	11.1	97.0	100	69.5
December 2017	11.5	19.9	7.2	97.2	100	74.4
January 2018	12.1	18.9	8.3	87.1	100	69.6
February 2018	13.1	15.7	11.6	87.3	94.4	79.2
March 2018	14.7	20.9	10.5	82.9	93.1	75.1
April 2018	19.3	27.5	13.6	78.1	89.4	68.3
May 2018	23.1	31.0	18.1	70.8	87.1	55.3
June 2018	25.5	33.1	20.1	62.3	84.6	33.9
July 2018	26.7	34.4	21.1	59.5	80.1	30.2
August 2018	28.8	38.3	22.9	61.2	82.9	32.0
September 2018	24.7	33.4	20.6	65.4	84.9	39.6
October 2018	22.4	31.6	18.0	65.2	83.6	37.5
November 2018	17.4	26.9	12.6	77.4	98.8	50.5
December 2018	12.9	24.2	8.1	84.9	100	54.8
January 2019	10.2	22.6	5.5	85.1	99.1	58.5

To test the impact of legumes applied as green manure on N nutrition and yield of organic greenhouse tomato, cowpea was cultivated before the tomato cultivation in summer 2017 (E1) and summer 2018 (E3). Furthermore, on October 2017, faba bean was sown between the tomato rows in E2 and incorporated into the soil together with the tomato residues at crop termination in January 2018, to test whether the legume intercrop could substantially enhance the N availability to the next tomato crop in Spring 2018 (E2). The treatments applied in the three experiments are listed in Table 3. All treatments were applied in the same plots in the three successive experiments.

Table 3. Description of the treatments in the three experiments.

1st and 3rd Experiment (E1 and E3)		
No.	**Treatment Short Name**	Treatment description
1.	FYM	Farmyard manure (FYM) (considered as control)
2.	FYM + L-NI	FYM and legume (green manure of cowpea) noninoculated
3.	FYM + L-I-Rh	FYM and legume (green manure of cowpea) inoculated with rhizobia (*Bradyrhizobium* sp. VULI11)
4.	FYM + L-I-Rh-PGPR	FYM and legume (green manure of cowpea) inoculated with rhizobia (*Bradyrhizobium* sp. VULI11) and PGPR[1]
2nd Experiment (E2)		
No.	**Treatment short name**	Treatment Description
1.	FYM	Farmyard manure (FYM) only (considered as control)
2.	FYM + L-NI	FYM and legume (faba bean as intercrop [2]) noninoculated
3.	FYM + L-I-Rh	FYM and legume (faba bean as intercrop [2]) inoculated with rhizobia (*Rhizobium* sp. VFBL1)
4.	FYM + L-I-Rh-PGPR	FYM and legume (faba bean as intercrop [2]) inoculated with rhizobia (*Rhizobium* sp. VFBL1) and PGPR [1]

[1] A mix of *Enterobacter* sp. C1.2, *Enterobacter* sp. C1.5, *Enterobacter* sp. C3.1, and *Lelliottia* sp. D2.4. [2] Intercropping was applied in the preceding tomato crop (E1). FYM = farmyard manure; L-NI = legume noninoculated ; L-I-Rh = legume inoculated with rhizobia ; L-I-Rh-PGPR = legume inoculated with rhizobia and PGPR.

In E1, farmyard manure (FYM) originating from free-range cattle farming was applied on July 30, 2017, at a rate of 50 t/ha in all treatments. The FYM contained 0.34% N, 0.15% P, and 0.48% K. This amount of FYM is equivalent to N supply of 170 kg/ha in order to comply with European Union Directive 889/2008. In treatment 1 (FYM), which was considered the control, no other source of N was applied except for FYM. In treatments 2 (FYM + legume noninoculated: L-NI), 3 (FYM + legume inoculated with rhizobia: L-I-Rh), and 4 (FYM + legume inoculated with rhizobia and PGPR :L-I-Rh-PGPR), additional N was provided through green manure by sowing cowpea (*Vigna unguiculata* (L) Walp.) on May 23, 2017, and incorporating it into the soil on July 27, 2017 (i.e., 6 days before planting tomato, which took place on August 2, 2017). In FYM + L-NI, the seeds of cowpea were not inoculated with rhizobia. In FYM + L-I-Rh, the seeds of cowpea were inoculated with *Bradyrhizobium* sp. VULI11 (BV) [12], while in FYM + L-I-Rh-PGPR, the seeds of cowpea were inoculated with a mix of BV and putative plant growth promoting rhizobacteria (PGPR), isolated from cowpea nodules (*Enterobacter* sp. C1.2, *Enterobacter* sp. C1.5, *Enterobacter* sp. C3.1., and *Lelliottia* sp. D2.4. Strains have been characterized by multi-locus sequence analysis (unpublished data). Strains' designations "C" and "D" represent the geographical regions of field-collected cowpea root nodules in Greece, that is Epirus and Crete, respectively, and followed by a lab code number.

In treatments FYM + L-NI, FYM + L-I-Rh, and FYM + L-I-Rh-PGPR, faba bean was sown as an intercrop between the tomato rows at a density of 10.67 plants/m^2 on October 26, 2017. The faba bean plants were intended to be incorporated into the soil as green manure for the next tomato crop (E2) after termination of E1. In the FYM + L-NI treatment, the seeds of faba bean were not inoculated with any rhizobia. In the FYM + L-I-Rh treatment, the seeds of faba bean were inoculated with *Rhizobium* sp. *svmbiovar* (sv.) *viciae* VFBL1, isolated from field-grown faba bean nodules in Greece, while in the FYM + L-I-Rh-PGPR treatment, the seeds of faba bean were inoculated with a mix of VFBL1 and PGPR (*Enterobacter* sp. C1.2, *Enterobacter* sp. C1.5, *Enterobacter* sp. C3.1, and *Lelliottia* sp. D2.4). Upon termination of the tomato crop on January 25, 2018, the faba bean plants and the aboveground parts of the tomato residues were incorporated into the soil. The root residues of tomato were removed and disposed out of the greenhouse because they had been infected by root-knot nematodes (*Meloidogyne* sp.) during E1. Subsequently, on January 29, 2018, FYM was applied again in all four treatments at a rate of 50 t/ha. Finally, on February 8, 2018, new tomato seedlings were planted to establish a spring–summer tomato crop (E2). This crop was terminated on June 11, 2018, and the residues were incorporated again into the soil, including the roots, because the incidence of nematode infection was very low during E2.

On June 12, 2018, cowpea was sown again in the plots of FYM + L-NI, FYM + L-I-Rh, and FYM + L-I-Rh-PGPR, which was intended to be used as green manure for the next tomato crop (E3). Similarly to E1, the seeds of cowpea in E3 were either not inoculated with any rhizobia (FYM + L-NI), inoculated with *Bradyrhizobium* sp. VULI11 only (FYM + L-I-Rh), or inoculated with both *Bradyrhizobium* sp. VULI11 and the same PGPR bacteria as in E1 and E2 (FYM + L-I-Rh-PGPR). In E3, the commercial cowpea cultivar Iron and Clay (Seed Ranch Company, Odessa, TX, USA) was used, which is considered nematode-resistant [13]. The cowpea plants were incorporated into the soil on August 7, 2018 (i.e., 56 days after sowing). On August 10, 2018, FYM was applied again in all treatments at a rate of 50 t/ha. Finally, on August 12, 2018, new tomato seedlings were planted to establish E3. Harvesting of commercially ripe tomato fruit commenced in October 17, 2018, and the crop was terminated on January 20, 2019.

In E1, self-rooted seedlings of the commercial tomato hybrid "Elpida F1" were used to establish the experiment. However, due to a severe infection by root-knot nematodes in E1, the commercial tomato hybrid "Ekstasis F1" was grafted onto the commercial rootstock Maxifort F1 (*Solanum lycopersicum* × *Solanum habrochaites*) was cultivated in E2 and the hybrid "Elpida F1" was grafted onto "Maxifort" in E3. The plant density was 2.13 plants m^{-2} in all three experiments. The tomato and faba bean plants were drip-irrigated with drippers set 50 cm and 20 cm apart, respectively, while the cowpea plants were overhead-irrigated using sprinklers. During the cropping period, no additional fertilizers were provided to the plants in E1 and E3 in all treatments. However, in E2, due to the occurrence of N deficiency symptoms seven weeks after crop establishment, extra fertilization via the drip irrigation system was applied in all treatments at four dates, particularly on April 25 and 29 and on May 2 and 6, using an organic fertilizer based on amino acids, containing 14% N. The total fertilizer application rate was 16 g/m^2 (i.e., 22.4 kg N ha^{-1}) in all treatments.

2.2. Growth, Mineral Analysis, and Nitrogen Fixation by Legumes

In all plots of the cowpea and faba bean crops, root samples were collected from soil cores using a 1 L cylindrical metal auger. All soil samples were placed for 24 h in a "Calgon" solution (dispersing agent) prepared by adding 40 g $(NaPO_3)_6$ and 10 g Na_2CO_3 per 1000 mL of water. Subsequently, the roots were carefully washed out over a sieve and the number of nodules was measured after detaching them from the roots. The root dry weight was also determined after drying the samples for 48 h at 65 °C. To determine the aboveground fresh and dry biomass, shoots from a 1 m^2 area of each plot center were fresh-weighed before their incorporation into the soil, and subsequently tissue subsamples were oven-dried at 65 °C to a constant weight.

In E1, the aboveground part of three cowpea plants per plot was sampled at 23, 35, 52, and 63 days after sowing. In E2, the aboveground parts of three faba bean plants per plot were sampled at 31, 43, 53, and 77 days after sowing. Similarly, in E3, the aboveground parts of three cowpea plants per plot were sampled at 22, 37, and 52 days after sowing. All tissue samples were oven-dried at 65 °C to a constant weight, powdered using a ball mill, and passed through a sieve (0.5 mm). Organic C and total N in plant tissue samples were determined by high temperature combustion using an elemental analyzer (Unicube, Elementar Analysensysteme GmbH, Hanau, Germany). Total P concentrations in plant tissues were determined by ashing at 550 °C for 8 h, dissolving the soluble salts in 4 M HCl, and quantifying P in the extracts using a spectrophotometer (U-2000, Hitachi, Tokyo, Japan) following the molybdate blue method [14]. Potassium was determined in the same aqueous extract using a flame photometer (Sherwood Model 410, Cambridge, UK).

The N derived from the atmosphere in the aboveground part of cowpea and faba plants bean was determined by applying a method based on the natural abundance of ^{15}N in plant tissues relative to the air [15–17]. To apply this method, the stable N isotopic composition of legume tissue samples was determined using an Isoprime 100 continuous flow isotope ratio mass spectrometer coupled to a Vario Isotope Select elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). The δ-values were calibrated relative to air by means of a three-point calibration using standard reference materials IAEA-N1, IAEA-600, and IAEA-N2. Measurement uncertainty was monitored by repeated measurements of internal laboratory standards and standard reference materials. Precision

was determined to be ± 0.19‰ based on repeated measurements of calibration standards and internal laboratory standards. Accuracy was determined to be ± 0.19‰ on the basis of the difference between the observed and known δ values of check standards and their standard deviations. The total analytical uncertainty was estimated to be ± 0.27‰ for $\delta^{15}N$. The $\delta^{15}N$ values were estimated as parts per thousand (‰) deviations relative to the nominated international standard of atmospheric N_2 (0.3663%), using the following equation [18]:

$$\delta^{15}\mathrm{N}(‰) = \left(\frac{\mathrm{atom}\%^{15}\mathrm{Nsample} - 0.3663}{0.3663}\right) * 1000 \quad (1)$$

Subsequently, the proportion of N derived from the atmosphere (%Ndfa) was estimated by substituting the $\delta^{15}N$ (‰) of the N_2-fixing legume and a non-N_2-fixing reference plant grown in the same soil, as calculated using Equation (1), into the following equation suggested by Unkovich et al. [15]:

$$\%\mathrm{Ndfa} = \left(\frac{\delta^{15}\mathrm{N\ of\ reference\ plant} - \delta^{15}\mathrm{N\ of\ legume}}{\delta^{15}\mathrm{N\ of\ reference\ plant} - \mathrm{B}}\right) * 100 \quad (2)$$

where "B" is the $\delta^{15}N$ in shoots of cowpea or faba bean plants grown on an inert medium and starved of N throughout their life, thereby being fully dependent on N_2 fixation. The B values used in the current study were −1.61 for cowpea and −0.50 for faba bean, as suggested by Unkovich et al. [15]. The reference plant used in this study to determine the corresponding $\delta^{15}N$ values was the grass weed *Digitaria sanguinalis* (L.).

The total amounts of biologically-fixed N_2 by cowpea and faba bean per cultivated area unit (BNF, kg/ha^{-1}) were estimated using the following equation [19]:

$$\mathrm{BNF} = \frac{\mathrm{DB} * \mathrm{Nt} * \%\mathrm{Ndfa}}{100} \quad (3)$$

where DB is the total dry biomass of the shoot, Nt is the total N concentration (% w/w) in the aboveground dry biomass, and %Ndfa are the values obtained from (2).

2.3. Tomato Tissue Sampling and Mineral Analysis

To assess the amounts of nutrients removed through harvesting of ripe fruit in the tomato crops, four ripe fruits from the 2nd cluster of 4 plants per plot were collected. The fruits were chopped and oven-dried at 65 °C for at least 3 days to a constant weight. Then, they were powdered using a ball mill, sieved (0.5 mm), homogenized, and chemically analyzed for total N, P, and K, as described above.

To determine the nutritional status of the plants, samples of the youngest fully expanded leaves were collected from all plots in all three experiments. The leaves were washed with distilled water, chopped, and oven-dried at 65 °C for at least 2 days until they reached constant weight, were powdered using a ball mill, and passed through a 40 mesh sieve. Subsequently, 0.5 g of powdered material was dry ashed in a muffle furnace at 550 °C for 5 h, and chemically analyzed for total N, P, and K, as described above.

Due to an unexpected nematode infection by *Meloidogyne* spp. in E1, the severity of the infection was estimated as described by Bridge and Page [20], based on visual observation of the tomato plants. At the termination of the experiment, the root systems of 10 plants from each plot were used for yield determination, which were dug from the soil and indexed for root galls using a 0–10 scale (0 = no root galls, 1 = few small galls, difficult to find, 2 = small galls but main roots clean, …, 10 = all roots severely galled).

2.4. Soil Analysis

Soil samples were collected from the central square of each plot (dimensions 2 × 2.5 m). In each plot, 5 soil cores weighing about 400 g were collected from the root zone of 5 plants at a depth of 0–20 cm. The samples were oven-dried at 40 °C for at least 3 days until their weight stabilized to a

constant level. Subsequently, the samples were sieved (2 mm diameter), homogenized, and analyzed to determine the organic C, total N, NO_3-N, NH_4-N, and plant-available P and K concentrations. Total C and N in soil samples were determined by high temperature combustion using an elemental analyzer (Unicube, Elementar Analysensysteme GmbH, Hanau, Germany). Since soil pH was 7.5 due to the presence of carbonates, organic C was determined in soil aliquots that were pretreated with HCl to remove inorganic C before elemental analysis. To determine the concentration of mineral nitrogen (N-min, i.e., NO_3^-N+ NH_4^+-N) in the soil, each sample of sieved soil was extracted using a KCl solution, as described by Keeney and Nelson [21]. Subsequently, the nitrate and ammonium concentrations in the sample extracts were determined by applying the cadmium reduction to NO_2^- and the indophenol blue methods, respectively [21], using a Spectronic Helios spectrophotometer (Thermo Electron Corporation, Mercers Row, Cambridge CB5 8HY, UK). Plant-available phosphorus was determined using the Olsen method [22] and quantified by molybdate colorimetry [23]. Exchangeable soil K was determined using a flame photometer (Sherwood Model 420, Sherwood Scientific, Cambridge, UK) following extraction with an ammonium acetate solution.

2.5. Tomato Growth and Yield

The impact of the experimental treatments on crop yield was assessed by harvesting all ripe tomatoes from 10 plants of the plot center twice per week, counting them, and weighing them on a commercial scale.

2.6. Statistical Analysis

All experiments were set as randomized block designs with 4 replicates. The data were statistically analyzed by applying ANOVA using the STATISTICA software package, version 12.0 for Windows. A Duncan's multiple range test was performed when the ANOVA was significant at $P < 0.05$ level. Data are presented in graphs as means ± SE of four replicates, or in tables.

3. Results

3.1. Nitrogen Fixation and Aboveground Biomass of Legumes

In Figure 1, the evolution in the percentage of N derived from the atmosphere through BNF (%Ndfa) during the cultivation of cowpea used as green manure (E1 and E3) and faba bean used as intercrop (E2) is shown. The data clearly show that cowpea inoculated with *Bradyrhizobium* sp. VULI11 was capable of biologically fixing appreciably more N_2 than the noninoculated plants in E1, while the inoculation with PGPR together with *Bradyrhizobium* had no additional impact on the %Ndfa. However, unlike E1, in E3 the inoculation of cowpea with *Bradyrhizobium* sp. VULI11 increased the %Ndfa only on one sampling date (37 days after sowing). The inoculation of faba bean with *Rhizobium* sp. VFBL1 in E2 had no impact on the %Ndfa in the shoot tissues (Figure 1B). The combined inoculation of faba bean with VFBL1 and PGPR slightly increased the %Ndfa, but the difference with the measured results in noninoculated plants was significant only in for sampling date (i.e., 54 days after planting).

The inoculation of cowpea seeds with *Bradyrhizobium* sp. VULI11 (BV) in E1 appreciably increased the aboveground fresh and dry biomass of cowpea plants used as green manure, while the inclusion of PGPR (*Enterobacter* sp. C1.2, *Enterobacter* sp. C1.5, *Enterobacter* sp. C3.1. and *Lelliottia* sp. D2.4) to the BV inoculum provided no additional benefits to the plant biomass production (Table 4). Furthermore, inoculating the roots of cowpea only with *Bradyrhizobium* sp. VULI11 (BV) significantly increased the total N concentration in the shoots of cowpea plants. As a result, the total amount of N per cultivated area unit, as well as the net amount of N contributed to the soil by BNF through green cowpea manure, were appreciably enhanced by the inoculation of cowpea with BV in E1. Unlike in E1, in E3 the inoculation of cowpea with BV had no impact on plant biomass, shoot total N concentration, and BNF, which was fully anticipated, given that the %Ndfa was also not influenced by rhizobia inoculation (data shown in Figure 1).

Table 4. Aboveground fresh (FB) and dry biomass (DB), dry matter content (DMC), total N concentration in the aboveground dry biomass, total N content per cultivated area unit, and total amount of biologically fixed N (BNF) per unit area cultivated with a legume (cowpea or faba bean) in three successive experiments (E1, E2, E3) with organic greenhouse tomato. Cowpea (E1 and E3) and faba bean (E2) were either noninoculated, inoculated with *Bradyrhizobium* sp. VULI11 (BV) or *Rhizobium* sp. VFBL1 (RV), respectively, or inoculated with both a rhizobium (BV or RV) and PGPR.

Treatment	FB g/m²	DMC%	DB g/m²	Total Nmg g^{-1}	Total N g/m²	BNF kg/ha
1st experiment						
Cowpea noninoculated	1165 b	9.91 b	117 b	21 b	2.4 b	15 b
Cowpea with BV	2925 a	12.90 a	378 a	30 a	11.1 a	96 a
Cowpea with BV and PGPR	2638 a	12.88 a	339 a	38 a	13.1 a	120 a
Significance of differences	**	**	**	**	**	**
2nd experiment						
Faba bean noninoculated	662	8.23	54.4	35.3	1.93	14.7
Faba bean with RV	691	8.65	59.8	33.7	2.02	15.0
Faba bean with RV and PGPR	723	8.09	58.4	36.9	2.16	16.9
Significance of differences	ns	ns	ns	ns	ns	ns
3rd experiment						
Cowpea noninoculated	3219	9.70	313	35.9	11.3	44
Cowpea with BV	3367	9.54	320	36.3	11.6	50
Cowpea with BV and PGPR	3375	9.73	327	36.3	11.8	50
Significance of differences	ns	ns	ns	ns	ns	ns

In each column, within each experiment, means of different treatments (n = 4) followed by different lower-case letters are significantly different according to Duncan's multiple range test. Significant differences at $p = 0.01$ are denoted by **, while lack of significance is denoted as ns.

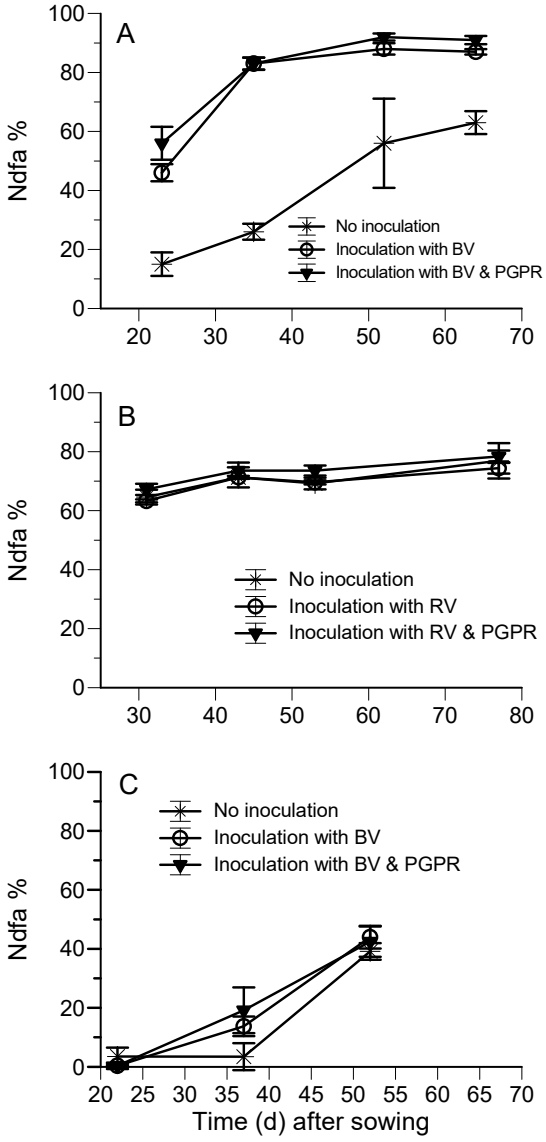

Figure 1. Proportion of N derived from atmospheric N_2-fixation (% Ndfa) in the shoot of cowpea in E1 (**A**), faba bean (**B**), and E3 (**C**) at different growing stages, as influenced by no rhizobia inoculation, inoculation with rhizobia only, or inoculation with a mix of rhizobia and PGPR. The rhizobia inoculum used was *Bradyrhizobium* sp. VULI11 (BV) in cowpea and *Rhizobium leguminosarum viciae* (RV) in faba bean.

Similarly to E3, the inoculation of faba bean with *Rhizobium* sp. VFBL1 in E2 had no impact on plant biomass, shoot total N concentration, or BNF, in agreement with the lack of any impact of inoculation on %Ndfa in this experiment.

3.2. Soil Measurements

Figure 2 shows the evolution of the soil NH_4-N concentrations during organic tomato cultivation in E1, E2, and E3, starting from the day of organic matter (OM, either FYM alone or together with legume fresh biomass) incorporation to the soil. As shown in Figure 2A, the soil NH_4-N concentration was very low before application of OM and increased appreciably 11 days after incorporation of OM (DAIOM) in E1. However, the soil NH_4-N concentration decreased again to 5.1 mg kg^{-1} 74 DAIOM (i.e., 71 days after tomato planting) and further decreased to very low levels at crop termination. The incorporation of cowpea as green manure together with FYM and the inoculation with rhizobia alone or together with PGPR had no significant impact on the soil NH_4-N levels. A sharp increase of the

soil NH_4-N concentration 18 days after incorporation of OM to the soil (FYM alone or together with faba bean and tomato residues from the previous crop), followed by a decrease to almost the initial levels 48 DAIOM was observed also in E2. In E3, the soil NH_4-N also exhibited an increasing peak 41 DAIOM. However, in E3, the starting soil NH_4-N level before OM incorporation to the soil was higher (4.2 mg kg^{-1}) than in E1 and E2, and the decrease in the soil in NH_4-N after the initial increasing peak was not as sharp as in E1 and E2. The treatments in the current study had no impact on the soil NH_4-N for any sampling date or experiment.

In E1, the soil NO_3-N was very low (9.1 mg kg^{-1}) before incorporation of OM to the soil, but increased sharply thereafter to 44 mg kg^{-1} when only FYM was applied, and to 55 to 66 mg kg^{-1} when cowpea was also applied as green manure (Figure 3). Subsequently, the soil NO_3-N decreased slightly on the second sampling date, and increased again on the last sampling date, while the lowest values were recorded consistently in the treatment without green manure application (FYM). The inoculation with rhizobia, alone or together with PGPR, had no significant impact on the soil NO_3-N. Similarly to E1, the lowest soil NO_3-N concentrations were recorded in the treatments of E2 and E3 without green manure application (FYM), with the exception of the last sampling at crop termination in E2. However, the inoculation with rhizobia alone or together with PGPR bacteria had no additional impact on the soil NO_3-N.

As shown in Table 5, the application of cowpea as green manure in addition to FYM increased the total N in the soil to significantly higher levels than the sole application of FYM in E1. However, in E2 and E3, the incorporation of cowpea or faba bean to the soil as green manure had no significant impact on the total N level in the soil. In E1, the total carbon concentration was significantly higher when cowpea was incorporated into the soil as green manure, compared to the other three treatments, regardless of inoculation with rhizobia alone or together with PGPR. However, in E2 and E3, no significant differences in the soil carbon could be found between the tested treatments. The soil P and K concentrations were not significantly influenced by the application of legumes as green manure.

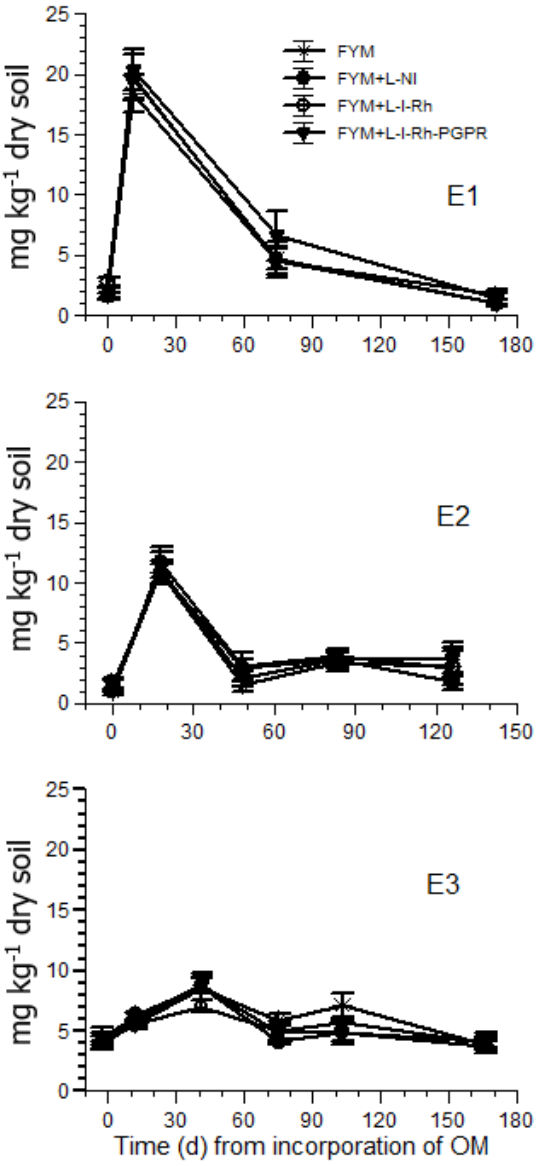

Figure 2. Impact of different organic fertilization treatments on soil NH_4-N concentration at different dates during the cropping period in three successive greenhouse tomato experiments (E1, E2, E3). Note: FYM = farmyard manure; L = legumes; Rh = inoculation with Rhizobia; PGPR = inoculation with PGPR; OM = organic matter (FYM, legume biomass).

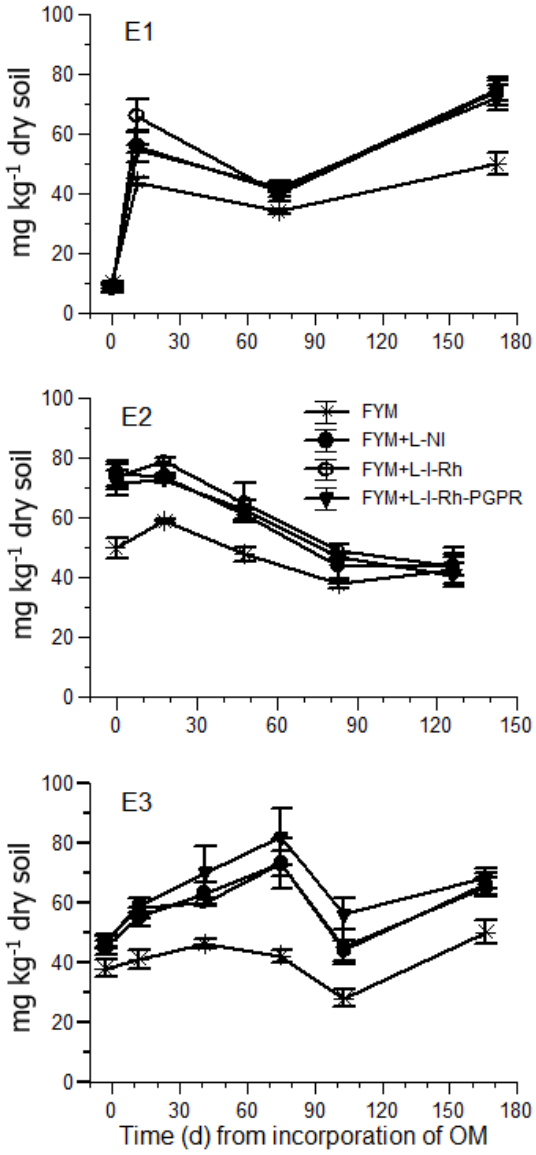

Figure 3. Impact of different organic fertilization treatments on soil NO_3-N concentration at different dates during the cropping period in three successive greenhouse tomato experiments (E1, E2, E3). Note: FYM = farmyard manure; L = legumes; Rh = inoculation with Rhizobia; PGPR = inoculation with PGPR; OM = organic matter (FYM, legume biomass).

Table 5. Impact of different organic fertilization treatments on total organic C and N, and plant-available P and K in the soil at 3 weeks after incorporation of organic matter, in three successive experiments (E1, E2, E3) with organic greenhouse tomato. Note: FYM = farmyard manure; FYM + L-NI = FYM and legume, noninoculated; FYM + L-I-Rh = FYM and legume inoculated with rhizobia; FYM + L-I-Rh-PGPR = FYM and legume inoculated with rhizobia and plant growth promoting rhizobacteria.

Treatment	C (%)	N (%)	P (mg kg^{-1})	K (mg kg^{-1})
1st Experiment				
FYM	2.16 b	0.20b	142	1018
FYM + L-NI	2.52 a	0.23a	140	1031
FYM + L-I-Rh	2.48 a	0.23a	146	1073
FYM + L-I-Rh-PGPR	2.44 a	0.22a	132	908
Significance of differences	*	*	ns	ns
2nd Experiment				

FYM	3.20	0.33	188	908
FYM + L-NI	3.10	0.31	215	901
FYM + L-I-Rh	3.50	0.28	201	981
FYM + L-I-Rh-PGPR	3.60	0.29	174	908
Significance of differences	ns	ns	ns	ns
3rd Experiment				
FYM	3.82	0.40	209	763
FYM + L-NI	3.90	0.40	201	646
FYM + L-I-Rh	4.01	0.40	189	722
FYM + L-I-Rh-PGPR	4.13	0.41	218	777
Significance of differences	ns	ns	ns	ns

Means (n = 4) followed by different letters within each column and experiment indicate significant differences according to the Duncan's multiple range test ($p < 0.05$); *significant at $p < 0.05$; ns = not significant.

3.3. Tomato Yield Components

In E1, the application of cowpea as green manure in addition to FYM resulted in lower yield than in the treatment solely with application of FYM, regardless of inoculation with rhizobia alone or rhizobia and PGPR, or no inoculation (Table 6). The higher yield in the FYM treatment was exclusively due to a higher fruit number per plant, while the mean fruit weight did not differ significantly between treatments. In contrast to E1, in E2 the incorporation of faba bean inoculated with rhizobia to the soil in addition to FYM resulted in higher yield than the sole application of FYM, while the inoculation with PGPR provided no additional benefit in terms of yield. In agreement with E2, in E3 the incorporation of cowpea inoculated with rhizobia to the soil in addition to FYM resulted in higher yield than the sole application of FYM. However, in E3, the yield was improved in all treatments when cowpea was applied as green manure in addition to FYM, regardless of rhizobia and PGPR inoculation, compared to sole FYM application. In both E2 and E3, the higher yield provided by application of legumes as green manure compared to sole FYM application was exclusively due to a higher fruit number per plant, while the mean fruit weight did not differ significantly between treatments.

Table 6. Impact of different organic fertilization treatments on tomato yield components in three successive experiments (E1, E2, E3) with organic greenhouse tomato. Note: FYM = farmyard manure; FYM + L-NI = FYM and legume, noninoculated; FYM + L-I-Rh = FYM and legume inoculated with rhizobia; FYM + L-I-Rh-PGPR = FYM and legume inoculated with rhizobia and plant growth promoting rhizobacteria.

Treatment	Total Yield (kg/m²)	Fruit (No Plant⁻¹)	Mean Fruit Weight (g)
1st Experiment			
FYM	7.3 a	16.2 a	211
FYM + L-NI	5.7 b	13.4 b	200
FYM + L-I-Rh	5.6 b	13.2 b	200
FYM + L-I-Rh-PGPR	5.7 b	13.2 b	203
Significance of differences	*	***	ns
2nd Experiment			
FYM	8.3 b	19.1 b	203
FYM + L-NI	8.3 b	19.4 b	201
FYM + L-I-Rh	10.4 a	22.7 a	214
FYM + L-I-Rh-PGPR	9.5 a	21.9 a	204
Significance of differences	*	*	ns
3rd Experiment			
FYM	11.9 b	25.9 b	224
FYM + L-NI	12.7 a	27.2 a	228

FYM + L-I-Rh	12.8 a	27.7 a	223
FYM + L-I-Rh-PGPR	13.1 a	28.3 a	228
Significance of differences	*	*	ns

Means (n = 4) followed by different letters within each column indicate significant differences according to the Duncan's multiple range test ($p < 0.05$); * and *** significant at $p < 0.05$, and $p < 0.001$, respectively; ns = not significant.

3.4. Tomato Tissue Analysis

In E1, the levels of total N and K in the plant tissues of tomato were not influenced by any treatment, while P was significantly lower when FYM application was accompanied by green manure from cowpea inoculated with *Bradyrhizobium* compared to sole FYM application (Table 7). In E2, the sole application of FYM resulted in significantly lower total N levels compared to application of FYM in combination with incorporation of faba bean residues originating from intercropping with the preceding tomato crop. The concentrations of P and K in leaves were not influenced by any treatment in E2. Finally, in E3, the tissue total N concentration was significantly lower in the FYM application, while P and K were not influenced by any of the experimental treatments.

Table 7. Impact of different organic fertilization treatments on leaf N, P, and K concentrations in three successive experiments (E1, E2, E3) with organic greenhouse tomato. FYM = farmyard manure; FYM + L-NI = FYM and legume, noninoculated; FYM + L-I-Rh = FYM and legume inoculated with rhizobia; FYM + L-I-Rh-PGPR = FYM and legume inoculated with rhizobia and plant growth promoting rhizobacteria.

Treatment	N (mg g^{-1})	P (mg g^{-1})	K (mg g^{-1})
1st Experiment			
FYM	13.4	2.84 a	53
FYM + L-NI	14.1	2.48 ab	59
FYM + L-I-Rh	14.1	1.96 b	50
FYM + L-I-Rh-PGPR	12.6	2.14 b	54
Significance of differences	ns	*	ns
2nd Experiment			
FYM	15.0 b	2.25	81
FYM + L-NI	17.1 ab	2.26	83
FYM + L-I-Rh	18.3 a	2.16	87
FYM + L-I-Rh-PGPR	18.3 a	2.26	87
Significance of differences	*	ns	ns
3rd Experiment			
FYM	28.2 b	2.50	117
FYM + L-NI	31.8 a	2.37	113
FYM + L-I-Rh	32.4 a	2.24	113
FYM + L-I-Rh-PGPR	32.6 a	2.42	116
Significance of differences	*	ns	ns

Means (n = 4) followed by different letters within each column indicate significant differences according to the Duncan's multiple range test ($p < 0.05$); * significant at $p < 0.05$; ns = not significant.

3.5. Incidence of Nematode Infection

As shown in Table 8, the root-knot index in E1 was significantly higher when cowpea residues were incorporated into the soil as green manure in addition to FYM, compared to sole application of FYM. The inoculation of cowpea with rhizobia alone or rhizobia together with PGPR had no additional impact on the root galling as indicated by the root-knot index. In E2 and E3, the root-knot index was very low in all plots, without any significant differences between treatments.

Table 8. Effects of organic fertilization treatments on root galling in tomato plants cultivated in a greenhouse following organic farming practices in E1. The root-knot index was estimated according

to Bridge and Page (1982) on scale of 1–10 (1 = no infection; 10 = totally infected). Note: FYM = farmyard manure; FYM + L-NI = FYM and legume, noninoculated; FYM + L-I-Rh = FYM and legume inoculated with rhizobia; FYM + L-I-Rh-PGPR = FYM and legume inoculated with rhizobia and PGPR.

Treatments	Root-Knot Index
FYM	8.30 b
FYM + L-NI	9.18 a
FYM + L-I-Rh	9.32 a
FYM + L-I-Rh-PGPR	9.37 a
Significance of differences	***

Means (n = 4) followed by different letters indicate significant differences according to Duncan's multiple range test ($p < 0.05$); *** significant at $p < 0.001$; ns = not significant.

4. Discussion

As postulated by Atkinson and Watson [24], organic farming is characterized by a complexity of relationships between different system components, and thus the sustainability of the system is dependent upon the functioning of a whole integrated and inter-related system. The results of the present study provide a good example of the complexity of factors governing yield performance in organic crops. Thus, in E1, the tomato fruit yield decreased significantly when cowpea fresh biomass was incorporated into the soil as green manure (GM) in addition to FYM, despite the significantly higher levels of soil NO_3-N compared to sole FYM application, because the local cowpea variety used as GM proved to be a good host of *Meloidogyne incognita*. As a result, the subsequent tomato crop was more severely affected by the root-knot nematode when cowpea fresh biomass grown in the same plots was incorporated into the soil prior to tomato transplanting. Watson et al. [11] already pointed out that despite the benefits obtained from incorporation of green manures on N management, this cultural practice may be associated with disease risks. However, in E2 and E3, the yield was increased by the incorporation of legume biomass to the soil when the nematode infection was effectively controlled. Grafting onto "Maxifort" provides substantial protection against root-knot nematodes [25]. Furthermore, nontoxic agents allowed for organic tomato production, such as *Bacillus firmus* and *Purpureocillium lilacinus* strain 251, can provide additional protection [26,27]. Thus, the use of tomato seedlings grafted onto "Maxifort" and the application of biological control agents against nematodes effectively controlled the nematode infection in E2 and E3, thereby eliminating its interference with crop performance and yield. As a result, the tomato crop benefited from the higher soil NO_3-N levels originating from the legume treatments, as indicated by the significantly higher fruit production, and this effect is reasonable given that N represents the primary nutrient-limiting yield in organic cropping systems [28,29].

In E1, three weeks after incorporation of FYM and legume biomass to the soil, the mean NO_3-N concentrations ranged from 55 to 66 mg kg^{-1} in the plots treated with both FYM and cowpea GM, and from 41 to 46 in the plots receiving only FYM. The NO_3-N levels recorded in all plots treated with cowpea GM at that stage of cultivation are considered adequate for tomato [30,31]. Nevertheless, in October 2017, the soil NO_3-N in E1 decreased to levels close to or below 40 mg kg^{-1} in all treatments, which are considered insufficient for tomato plants carrying a heavy fruit load [8,32]. In agreement with this consideration, N deficiency symptoms were observed in tomato plants by the end of October in E1. However, in E3, which was conducted one year later in the same season, the soil NO_3-N was maintained at sufficient levels for tomato production throughout the cropping period, especially when cowpea GM inoculated with rhizobia and PGPR was applied. In contrast, in the plots treated solely with FYM, the concentration of NO_3-N in the soil ranged within insufficient levels in both E1 and E3 (<50 mg kg^{-1}) according to Sainju et al. [8]. The significant increase of the soil NO_3-N when cowpea was applied as GM compared to sole application of FYM indicates that GM with legumes is an efficient tool to increase the soil N levels in organic tomato crops in greenhouses. However, the benefits of cowpea GM with respect to the soil NO_3-N levels were more profound in E3. This is reasonable, as in organic crops fertility management relies on a long-term integrated

approach [11], because the release of nutrients from organic biomass incorporated into the soil is a long-lasting process exceeding crop life.

In E2, a winter legume (faba bean) was applied as the intercrop in the preceding tomato crop to deliver atmospheric N_2 to the soil, since E2 took place during spring–summer, and thus the preceding legume crop had to take place during late autumn and winter. Furthermore, E2 was conducted immediately after the autumn–winter tomato crop of E1 in the same plots, in an attempt to assess whether a legume plant cultivated as an intercrop in an autumn–winter tomato crop is beneficial to a subsequent tomato crop cultivated in spring–summer season in terms of N supply. This would allow for two subsequent organic tomato crops in the same year and concomitantly for an increase in the grower's income. Faba bean was selected to serve the above mentioned role because several studies have shown that the incorporation of legume residues arising from the preceding crop into the soil (including their application as green manure) increases growth and yield of many crops, such as canola, maize, potato. and wheat [33,34]. The results in Figure 3B show that the soil NO_3-N levels in E2 were significantly higher at the beginning of the tomato crop when faba bean biomass originating from intercropping with the previous tomato crop was incorporated into the soil. However, the levels and the difference in soil NO_3-N were similar to those found at the end of the tomato crop in E1 before incorporation of faba bean residues to the soil (Figure 3A). Thus, the large difference in soil NO_3-N between the plots treated solely with FYM and those treated additionally with faba in E2, especially at the beginning of the crop, seem to be directly or indirectly (e.g., by favoring the mineralization of FYM) related to the significant N inputs from cowpea incorporation during the previous cropping period. During the cropping period, this difference tended to decrease and finally diminished by the end of the tomato crop in E2. These results indicate that faba bean did not contribute substantially to the N needs of tomato in E2. As reported by Amanuel et al. [35] and Neugschwandtner et al. [36], faba bean is an efficient N_2-fixing legume plant, as in crops aiming to produce edible pods, this legume plant was capable of contributing from 139 to 210 kg N ha^{-1} and from 63 to 219 kg N ha^{-1}, respectively, through BNF. In agreement with those results, Ntatsi et al. [16] found that faba bean contributed up to 190 kg N ha^{-1} through BNF when cultivated for fresh pod production. However, in the current study, faba bean was cultivated as an intercrop, which dictated a much lower plant density and less light availability than in open-field crops, while it was incorporated into the soil at a much earlier growth stage compared to the studies reported by Amanuel et al. [35], Neugschwandtner et al. [36], and Ntatsi et al. [16]. Therefore, the net contribution of the faba bean intercrop to soil N through BNF in the current study did not exceed 17 kg N ha^{-1}, as shown in Table 4. These results indicate that intercropping of faba bean in a previous tomato crop provides no substantial benefit in terms of N delivery via BNF in a subsequent tomato crop.

The significant increase of N derived from atmospheric N_2-fixation (% Ndfa) in the shoots of cowpea inoculated with *Bradyrhizobium* sp. VULI11 in E1 (Figure 1) show that efficient indigenous rhizobia strains suitable for cowpea were not present in the greenhouse soil used for this experiment. This is corroborated by the appreciably higher number and mean individual dry weight of nodules collected from cowpea plants inoculated with rhizobia compared to those measured in the roots of noninoculated plants. As reported by Soares et al. [37], inoculation of cowpea with *Bradyrhizobium* strains characterized by high nitrogen-fixing capacity in symbiosis with cowpea can substantially increase the ability of this plant species to fix atmospheric N_2. Although cowpea is considered a promiscuous species capable of establishing efficient symbiosis with diverse symbiotic bacteria [38], the most efficient symbiotic relationships are achieved with *Bradyrhizobium* species [39], especially in nonalkaline soils [12]. In the current study, the indigenous *Bradyrhizobium* strain VULI11 [40] was used as inoculum, which exhibited high N_2-fixing ability for cowpea, as confirmed by the results of E1. Nevertheless, in E3 the %Ndfa was similar in inoculated and noninoculated cowpea plants, which indicates that the inoculum applied during E1 was capable of persisting and spreading out throughout the field trial area, and was likely present at high populations in the soil in all plots one year later in E3. The similar %Ndfa values in inoculated and noninoculated cowpea plants in E3 are in line with the similar number of nodules per root segment and individual nodule dry weight, which were measured shortly before incorporation of faba bean to the soil. Thus, inoculation with

Bradyrhizobium sp. did not increase the BNF of cowpea plants and concomitantly provided no benefit to the subsequent tomato crop in E3. These results indicate that inoculation of cowpea with rhizobia is beneficial mainly in fields where this plant had been not cultivated in the recent years, and thus efficient rhizobia strains for cowpea were not present in the soil. Several other investigators found no benefit from rhizobia inoculation of legumes when efficient indigenous rhizobia strains for that particular legume species were present in the soil [41–43]. Furthermore, the significant decrease of the %Ndfa in E3 compared to E1 in the inoculated treatments is ascribed to the notably higher soil nitrate concentrations in E3. Indeed, as shown by other researchers [44,45], high NO_3-N concentrations in the root zone of legume plants are associated with reduced nodulation and N_2-fixation.

The %Ndfa in faba bean fresh biomass ranged from 73% to 78% at crop termination, while inoculation of faba bean with *Rhizobium* sp. VFBL1 had no significant impact on %Ndfa (Figure 1B), or on fresh biomass, tissue N concentration, or BNF (Table 4). The similar %Ndfa, tissue N, and BNF values between treatments in E2 are in agreement with the lack of any significant differences in the number and mean size of nodules, which were high in all treatments. These results indicate that indigenous rhizobia strains that are capable of nodulating faba bean and efficiently fixing atmospheric N_2 were present in the greenhouse soil, and thus inoculation with *Rhizobium* sp. VFBL1 did not provide any benefit to the plants. This finding is in agreement with results found in a previous study [16], in which the %Ndfa in faba bean plants cultivated for fresh pod production in an open field ranged from 79% to 91%, although the plants were not inoculated with rhizobia. Neugschwandtner et al. [36] also found that faba bean was capable of fixing high amounts of atmospheric N_2 (219 kg/ha^{-1} on average), although the plant was not inoculated with any rhizobia.

In nonacidic, oxic topsoils with high microbial activity, NH_4-N derived from organic matter mineralization is rapidly converted into NO_3-N by nitrification. Therefore, whereas similar trends in soil NH_4-N concentrations were observed in all plots in all three experiments, regardless of legume application as green manure or intercrop, differences in soil NO_3-N concentrations between treatments better reflected the differences in net organic N mineralization (under nonleaching conditions typical of the greenhouse environment).

Apart from different N inputs, one of the main factors influencing the N availability for the crop as a result of organic N mineralization is the C/N ratio of the decomposing organic matter [46]. This is because the C/N ratio determines the balance between the rates of microbial N immobilization and mineralization, and therefore the net supply of plant-available N [47,48]. Other factors determining the N mineralization rate, such as the soil type and the soil temperature [49], were similar between treatments at the same time in the experiments of this study. Thus, the higher N supply observed in the plots receiving legume residues applied as green manure together with FYM with respect to those treated only with FYM, as indicated by the generally higher NO_3-N concentrations, may be ascribed to a combination of higher N inputs as well as lower C/N ratio of incorporated organic matter in the former.

The changes in the soil NO_3-N concentration over time seem to be influenced not only by the time and quantity of organic matter incorporation to the soil and the C/N ratio in the organic matter, but also by the soil temperature, which has a direct impact on N mineralization rates [49] and plant uptake. Indeed, as reported by Bhogal et al. [50], the amount of mineralized N is related to thermal time (i.e., the cumulative day degrees above 5 °C). Thus, since the tomato crop in E1 and E3 took place from August to January, it is assumed that the reduction in soil NO_3-N levels at a latter cropping stage was partly due to decreased soil temperature as the crop was aging, which gradually restricted the net N mineralization rates. The partial recovery of the soil NO_3-N in January in E1 and E3 is ascribed to reduced mineral N uptake by the tomato crop due to the low soil temperature. The optimal temperature for the nitrifying bacteria is 41 °C [51], while for N uptake by tomato the optimal level in the roots is about 27 °C [52]. Thus, it seems that the season-related gradual reduction of the ambient temperature in E1 and E3 initially restricted the conversion of organic N to NO_3-N, but in January, the further reduction of the ambient temperature also affected the N uptake, resulting in the small increase of soil NO_3-N at that stage of the crop. In contrast to E1, which was an autumn crop,

in E2, which was a spring–summer crop, the soil NO_3-N tended to decline consistently with time in all treatments, presumably because the net N mineralization rate was lower than the rate of plant uptake. This is reasonable, given the relatively low amount of BNF contributed by the faba bean intercrop (Table 4) and the increasing N needs by the crop with time as the climatic conditions in spring and early summer are favorable for plant growth.

The levels of plant-available P and K in the soil were not influenced by the incorporation of legumes to the soil, and thus their tissue concentrations were not affected by the treatments applied in the current experiments, with the exception of leaf P in E1. From a first approach, the lack of a treatment impact on P and K nutrition is reasonable, given that the legumes used as green manure utilize the available P and K of the soil to grow, and thus their incorporation to the soil does not result in a net input of these nutrients to the soil [53]. In many cases, green manure may result in utilization of plant-available nutrient resources from deeper soil layers [54], or nutrients that might be leached out through rainfall [53] if the field were not cultivated by the green manure crop. However, in the current experiment, both legumes were cultivated for short periods, and thus they had no time to develop a deep root system, while the cultivation of tomato inside a greenhouse prevented any leaching of nutrients via rainfall. On the other hand, the green manure crop may immobilize part of the absorbed P and K for more than one year depending on the weather conditions [55]. Nevertheless, as shown in Table 5, the soil P and K reserves were high in the soil of the greenhouse used in the present experiments, and thus any reduction of their availability due to immobilization in the legume residues incorporated into the soil had no impact on tomato nutrition by K and P.

The increase of the soil organic C, total N, and plant-available P concentrations in E2 compared to E1 and their further increase in E3 indicate that the organic fertilization practices applied in the current study were capable of increasing the soil fertility as they increased the reserves of organic matter, characterized by a low C/N ratio. This is in line with the suggestions of Janzen et al. [56] and Watson et al. [11] that the primary advantage of organic management practices is the long-term replenishment of stable organic N reserves in the soil. The present study further showed that FYM is capable of increasing the soil P reserves in organic tomato crops. On the other hand, the reduction of the soil K in E2 compared to E1 and the further decrease of soil K in E3 show that in the long term, the K requirements of greenhouse organic tomato cannot be addressed merely by organic fertilization treatments [57]. This finding stresses the necessity to apply inorganic forms of K compatible with organic agriculture in organic greenhouse tomato, such as potassium and magnesium sulphate.

5. Conclusions

The current study revealed that incorporation of cowpea as summer green manure in the soil in addition to farmyard manure (FYM) can increase both the total N and the nitrate concentrations in the soil, thereby resulting in higher fruit yield in organic tomato crops.

In contrast, intercropping of a legume such as faba bean with greenhouse tomato and incorporation to the soil at the end of the tomato crop hardly provides any benefit to the subsequent tomato crop, and therefore it is not recommended for greenhouse production of organic tomato. The main constraint of this practice is the limited area available for the faba bean intercrop, which compromises the amount of atmospheric N_2 fixed biologically and delivered to the subsequent crop after N mineralization.

Despite the benefits obtained from incorporation of green manures on N availability, this cultural practice may be associated with diseases risk, as indicated by the stronger nematode infection in the plots accommodating cowpea as green manure in E1.

Furthermore, inoculation of the legumes used as green manure with specific rhizobia strains, especially when the legume is sown for the first time in the particular soil, can promote the nodulation of rhizobia, and hence increase BNF efficiency and N inputs to the soil, thereby improving crop yield.

The gradual increase of the soil C and total N in all plots over the whole experimental period strongly supports the notion that the major benefit of organic farming practices in greenhouse tomato crops is the maintenance of soil fertility for long-term crop productivity and sustainability.

Author Contributions: D.S. and A.G. conceived and designed the experiments. A.G., G.N., L.C., D.S.P., A.T., and I.G. performed the experiments and the analyses. A.G. and D.S. analyzed the data. D.S., A.G., and G.N. wrote the paper. A.G, G.N., L.C., D.S.P., A.T., I.G., and D.S. reviewed the paper. All authors have read and approved the manuscript.

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References

1. Willer, H.; Lernoud, J. *The World of Organic Agriculture: Statistics and Emerging Trends 2019*; Research Institute of Organic Agriculture (FiBL), Frick, and IFOAM-Organics International, Bonn, Germany; 2019.
2. Kremen, C.; Miles, A. Ecosystem Services in Biologically Diversified versus Conventional Farming Systems. *Ecol. Soc.* **2012**, *17*, doi:10.5751/ES-05035-170440.
3. Gatsios, A.; Ntatsi, G.; Tampakaki, A.; Celli, L.; Savvas, D. Assessing the possibility to use legume plants as cover crops or intercrops in organic tomato production to optimize NUE. In Proceedings of the 30th IHC, Istanbul, Turkey, 12–16 August 2018. Acta Hort. (in press).
4. European Commision. Commission regulation (EC) no 889/2008 of 5 September 2008 laying down detailed rules for the implementation of council regulation (EC) no 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and control. Official Journal L 250 , Brussels Belgium 2008.
5. Tittarelli, F.; Båth, B.; Ceglie, F.G.; García, M.C.; Möller, K.; Reents, H.J.; Védie, H.; Voogt, W. Soil fertility management in organic greenhouse: An analysis of the European context. *Acta Hortic.* **2017**, 113–126, doi:10.17660/ACTAHORTIC.2017.1164.15.
6. Colla, G.; Mitchell, J.P.; Poudel, D.D.; Temple, S.R. Changes of Tomato Yield and Fruit Elemental Composition in Conventional, Low Input, and Organic Systems. *J. Sustain. Agric.* **2002**, *20*, 53–67, doi:10.1300/J064V20N02_07.
7. Needham, P. Nutritional disorders. In *UK Tomato Manual.* Grower Books; London, UK, 1973.
8. Sainju, U.M.; Dris, R.; Singh, B. Mineral nutrition of tomato. *Food Agric. Environ.* **2003**, *1*, 176–184.
9. Lenzi, A.; Antichi, D.; Bigongiali, F.; Mazzoncini, M.; Migliorini, P.; Tesi, R. Effect of different cover crops on organic tomato production. *Renew. Agric. Food Syst.* **2009**, *24*, 92–101, doi:10.1017/S1742170508002445.
10. Fatima, T.; Teasdale, J.R.; Bunce, J.; Mattoo, A.K. Tomato response to legume cover crop and nitrogen: Differing enhancement patterns of fruit yield, photosynthesis and gene expression. *Funct. Plant Biol.* **2012**, *39*, 246, doi:10.1071/FP11240.
11. Watson, C.A.; Atkinson, D.; Gosling, P.; Jackson, L.R.; Rayns, F.W. Managing soil fertility in organic farming systems. *Soil Use Manag.* **2002**, *18*, 239–247, doi:10.1111/J.1475-2743.2002.TB00265.X.
12. Tampakaki, A.P.; Fotiadis, C.T.; Ntatsi, G.; Savvas, D. Phylogenetic multilocus sequence analysis of indigenous slow-growing rhizobia nodulating cowpea (*Vigna unguiculata* L.) in Greece. *Syst. Appl. Microbiol.* **2017**, *40*, 179–189, doi:10.1016/J.SYAPM.2017.01.001.
13. Harrison, H.F.; Thies, J.A.; Fery, R.L.; Smith, J.P. Evaluation of cowpea genotypes for use as a cover crop. *HortScience* **2006**, *41*, 1145–1148.
14. Olsen, R.; Sommers, L.E.; Phosphorus, A.L.; Page, R.H.; Miller, D.R.K.E. Methods of Soil Analysis. In *Part 2 Chemical and Microbiological Properties*; ASA: Madison, WI, USA, 1982; pp. 403–427.
15. Unkovich, M.; Herridge, D.; Peoples, M.; Cadisch, G.; Boddey, B.; Giller, K.; Alves, B.; Chalk, P. Measuring plant-associated nitrogen fixation in agricultural systems. In *Measuring Plant-Associated Nitrogen Fxation in Agricultural Systems*; Australian Centre for International Agricultural Research (ACIAR) Canberra Australia 2008; pp. 132–188, ISBN 978-1-921531-26-2, doi:10.1071/AR9940119.
16. Ntatsi, G.; Karkanis, A.; Yfantopoulos, D.; Olle, M.; Travlos, I.; Thanopoulos, R.; Bilalis, D.; Bebeli, P.; Savvas, D. Impact of variety and farming practices on growth, yield, weed flora and symbiotic nitrogen fixation in faba bean cultivated for fresh seed production. *Acta Agric. Scand. Sect. B Soil Plant Sci.* **2018**, *68*, 619–630, doi:10.1080/09064710.2018.1452286.
17. Ntatsi, G.; Karkanis, A.; Yfantopoulos, D.; Pappa, V.; Konosonoka, I.H.; Travlos, I.; Bilalis, D.; Bebeli, P.;

Savvas, D. Evaluation of the field performance, nitrogen fixation efficiency and competitive ability of pea landraces grown under organic and conventional farming systems. *Arch. Agron. Soil Sci.* **2019**, *65*, 294–307, doi:10.1080/03650340.2018.1501155.

18. Bedard-Haughn, A.; van Groenigen, J.W.; van Kessel, C. Tracing 15N landscapes: Potential uses and precautions. *J. Hydrol.* **2003**, *272*, 175–190.
19. Collino, D.J.; Salvagiotti, F.; Perticari, A.; Piccinetti, C.; Ovando, G.; Urquiaga, S.; Racca, R.W. Biological nitrogen fixation in soybean in Argentina: Relationships with crop, soil, and meteorological factors. *Plant Soil* **2015**, *392*, 239–252, doi:10.1007/S11104-015-2459-8.
20. Bridge, J.; Page, S.L.J. Estimation of Root-knot Nematode Infestation Levels on Roots Using a Rating Chart. *Trop. Pest Manag.* **1980**, *26*, 296–298, doi:10.1080/09670878009414416.
21. Keeney, D.R.; Nelson, D.W. Nitrogen-inorganic farms. In *Methods of Soil Analysis*; Page, A.L., Miller, R.H., Keeney, D.R., Eds.; American Society of Agronomy: Madison, WI, USA, 1982; pp. 643–698.
22. Olsen, S.R.; Cole, C.V.; Watanabe, F.S.; Dean, L.A. *Estimation of Available Phosphorus in Soils by Extraction with Sodium Bicarbonate*; United States Department of Agriculture: Washington, DC, USA 1954; p. 939.
23. Murphy, J.; Riley, J.P. A modified single solution method for the determination of phosphate in natural waters. *Anal. Chim. Acta* **1962**, *27*, 31–36.
24. Atkinson, D.; Watson, C.A. The research needs of organic farming: Distinct or just the same as other agricultural research? In Proceedings of the BCPC Conference: Pests & Diseases, Brighton, UK, 13 - 16 November 2000; pp. 151–158.
25. Louws, F.J.; Rivard, C.L.; Kubota, C. Grafting fruiting vegetables to manage soilborne pathogens, foliar pathogens, arthropods and weeds. *Sci. Hortic.* **2010**, *127*, 127–146, doi:10.1016/J.SCIENTA.2010.09.023.
26. Giné, A.; Sorribas, F.J. Effect of plant resistance and BioAct WG (*Purpureocillium lilacinum* strain 251) on *Meloidogyne incognita* in a tomato-cucumber rotation in a greenhouse. *Pest Manag. Sci.* **2017**, *73*, 880–887, doi:10.1002/PS.4357.
27. Dahlin, P.; Eder, R.; Consoli, E.; Krauss, J.; Kiewnick, S. Integrated control of Meloidogyne incognita in tomatoes using fluopyram and Purpureocillium lilacinum strain 251. *Crop Prot.* **2019**, *124*, 104874, doi:10.1016/J.CROPRO.2019.104874.
28. Stockdale, E.A.; Rees, R.M. Release of Nitrogen from Plant and Animal Residues and Consequent Plant Uptake Efficiency. *Biol. Agric. Hortic.* **1995**, *11*, 229–245, doi:10.1080/01448765.1995.9754708.
29. Torstensson, G. Nitrogen Delivery and Utilization by Subsequent Crops after Incorporation of Leys with Different Plant Composition. *Biol. Agric. Hortic.* **1998**, *16*, 129–143, doi:10.1080/01448765.1998.9755228.
30. Tang, L.; Chen, Q.; Li, X.; Chen, Y.; Ding, G. Studies on the target value of nitrogen supply for greenhouse tomato growth during autumn-winter season tomato nutrition. *Plant Nutr. Fertil. Sci.* **2005**, *11*, 230–235.
31. Bénard, C.; Gautier, H.; Bourgaud, F.; Grasselly, D.; Navez, B.; Caris-Veyrat, C.; Weiss, M.; Génard, M. Effects of Low Nitrogen Supply on Tomato (*Solanum lycopersicum*) Fruit Yield and Quality with Special Emphasis on Sugars, Acids, Ascorbate, Carotenoids, and Phenolic Compounds. *J. Agric. Food Chem.* **2009**, *57*, 4112–4123, doi:10.1021/JF8036374.
32. Van Eysinga, J.R. *Fertilization of Tomatoes with Nitrogen Tomato Nutrition*; Centre for Agricultural Publishing and Documentation: Wageningen, The Netherlands, 1971.
33. Sincik, M.; Turan, Z.M.; Göksoy, A.T. Responses of Potato (*Solanum tuberosum* L.) to Green Manure Cover Crops and Nitrogen Fertilization Rates. *Am. J. Potato Res.* **2008**, *85*, 390–391, doi:10.1007/S12230-008-9043-1.
34. O'Donovan, J.T.; Grant, C.A.; Blackshaw, R.E.; Harker, K.N.; Johnson, E.N.; Gan, Y.; Lafond, G.P.; May, W.E.; Turkington, T.K.; Lupwayi, N.Z.; et al. Rotational Effects of Legumes and Non-Legumes on Hybrid Canola and Malting Barley. *Agron. J.* **2014**, *106*, 1921, doi:10.2134/AGRONJ14.0236.
35. Amanuel, G.; Kühne, R.F.; Tanner, D.G.; Vlek, P.L.G. Biological nitrogen fixation in faba bean (Vicia faba L.) in the Ethiopian highlands as affected by P fertilization and inoculation. *Biol. Fertil. Soils* **2000**, *32*, 353–359, doi:10.1007/S003740000258.
36. Neugschwandtner, R.; Ziegler, K.; Kriegner, S.; Wagentristl, H.; Kaul, H.-P. Nitrogen yield and nitrogen fixation of winter faba beans. *Acta Agric. Scand. Sect. B Soil Plant Sci.* **2015**, *65*, 658–666, doi:10.1080/09064710.2015.1042028.
37. Soares, B.L.; Ferreira, P.A.A.; de Oliveira-Longatti, S.M.; Marra, L.M.; Rufini, M.; de Andrade, M.J.B.; Moreira, F.M.D.S. Cowpea symbiotic efficiency, pH and aluminum tolerance in nitrogen-fixing bacteria. *Sci. Agric.* **2014**, *71*, 171–180, doi:10.1590/S0103-90162014000300001.
38. Marra, L.M.; Soares, C.R.F.S.; de Oliveira, S.M.; Ferreira, P.A.A.; Soares, B.L.; Carvalho, R.D.F.; de Lima,

J.M.; Moreira, F.M.D.S. Biological nitrogen fixation and phosphate solubilization by bacteria isolated from tropical soils. *Plant Soil* **2012**, *357*, 289–307, doi:10.1007/S11104-012-1157-Z.

39. Thies, J.E.; Bohlool, B.B.; Singleton, P.W. Subgroups of the Cowpea Miscellany: Symbiotic Specificity within *Bradyrhizobium* spp. for *Vigna unguiculata*, *Phaseolus lunatus*, *Arachis hypogaea*, and *Macroptilium atropurpureum*. *Appl. Environ. Microbiol.* **1991**, *57*, 1540–1545.
40. Tampakaki, A.P.; Fotiadis, C.T.; Ntatsi, G.; Savvas, D. A novel symbiovar (aegeanense) of the genus *Ensifer* nodulates *Vigna unguiculata*. *J. Sci. Food Agric.* **2017**, *97*, 4314–4325, doi:10.1002/JSFA.8281.
41. Giller, K.E.; Wilson, K.J. *Nitrogen Fixation in Tropical Cropping Systems*, 2nd ed.; Giller, K.E., Ed.; CAB International: Wallingford, UK, 2001; ISBN 9780851994178, doi:10.1079/9780851994178.0000.
42. Kutcher, H.R.; Lafond, G.; Johnston, A.M.; Miller, P.R.; Gill, K.S.; May, W.E.; Hogg, T.; Johnson, E.; Biederbeck, V.O.; Nybo, B. *Rhizobium* inoculant and seed-applied fungicide effects on field pea production. *Can. J. Plant Sci.* **2002**, *82*, 645–661, doi:10.4141/P01-180.
43. Thapa, S.; Adams, C.B.; Trostle, C. Root nodulation in guar: Effects of soils, *Rhizobium* inoculants, and guar varieties in a controlled environment. *Ind. Crops Prod.* **2018**, *120*, 198–202, doi:10.1016/J.INDCROP.2018.04.060.
44. Saito, A.; Tanabata, S.; Tanabata, T.; Tajima, S.; Ueno, M.; Ishikawa, S.; Ohtake, N.; Sueyoshi, K.; Ohyama, T. Effect of Nitrate on Nodule and Root Growth of Soybean (Glycine max (L.) Merr.). *Int. J. Mol. Sci.* **2014**, *15*, 4464–4480, doi:10.3390/IJMS15034464.
45. Nishida, H.; Suzaki, T. Two Negative Regulatory Systems of Root Nodule Symbiosis: How Are Symbiotic Benefits and Costs Balanced? *Plant Cell Physiol.* **2018**, *59*, 1733–1738, doi:10.1093/PCP/PCY102.
46. Ribeiro, H.M.; Fangueiro, D.; Alves, F.; Vasconcelos, E.; Coutinho, J.; Bol, R.; Cabral, F. Carbon-mineralization kinetics in an organically managed Cambic Arenosol amended with organic fertilizers. *J. Plant Nutr. Soil Sci.* **2010**, *173*, 39–45, doi:10.1002/JPLN.200900015.
47. Bruun, S.; Luxhøi, J.; Magid, J.; de Neergaard, A.; Jensen, L.S. A nitrogen mineralization model based on relationships for gross mineralization and immobilization. *Soil Biol. Biochem.* **2006**, *38*, 2712–2721, doi:10.1016/J.SOILBIO.2006.04.023.
48. Parton, W.; Silver, W.L.; Burke, I.C.; Grassens, L.; Harmon, M.E.; Currie, W.S.; King, J.Y.; Adair, E.C.; Brandt, L.A.; Hart, S.C.; et al. Global-Scale Similarities in Nitrogen Release Patterns During Long-Term Decomposition. *Science* **2007**, *315*, 361–364, doi:10.1126/SCIENCE.1134853.
49. De Neve, S.; Hartmann, R.; Hofman, G. Temperature effects on N mineralization: Changes in soil solution composition and determination of temperature coefficients by TDR. *Eur. J. Soil Sci.* **2003**, *54*, 49–62, doi:10.1046/J.1365-2389.2003.00521.X.
50. Bhogal, A.; Williams, J.R.; Nicholson, F.A.; Chadwick, D.R.; Chambers, K.H.; Chambers, B.J. Mineralization of organic nitrogen from farm manure applications. *Soil Use Manag.* **2016**, *32*, 32–43, doi:10.1111/SUM.12263.
51. Ouyang, Y.; Norton, J.M.; Stark, J.M. Ammonium availability and temperature control contributions of ammonia oxidizing bacteria and archaea to nitrification in an agricultural soil. *Soil Biol. Biochem.* **2017**, *113*, 161–172, doi:10.1016/J.SOILBIO.2017.06.010.
52. Tindall, J.A.; Mills, H.A.; Radcliffe, D.E. The effect of root zone temperature on nutrient uptake of tomato. *J. Plant Nutr.* **1990**, *13*, 939–956, doi:10.1080/01904169009364127.
53. Thorup-Kristensen, K.; Magid, J.; Jensen, L.S. Catch crops and green manures as biological tools in nitrogen management in temperate zones. *Adv. Agron.* **2003**, *79*, 227–302, doi:10.1016/S0065-2113(02)79005-6.
54. Witter, E.; Johansson, G. Potassium Uptake from the Subsoil by Green Manure Crops. *Biol. Agric. Hortic.* **2001**, *19*, 127–141, doi:10.1080/01448765.2001.9754917.
55. Talgre, L.; Lauringson, E.; Roostalu, H.; Makke, A. Phosphorus and potassium release during decomposition of roots and shoots of green manure crops. *Biol. Agric. Hortic.* **2014**, *30*, 264–271, doi:10.1080/01448765.2014.953582.
56. Janzen, H.H.; Bole, J.B.; Biederbeck, V.O.; Slinkard, A.E. Fate of N applied as green manure or ammonium fertilizer to soil subsequently cropped with spring wheat at three sites in western Canada. *Can. J. Soil Sci.* **1990**, *70*, 313–323, doi:10.4141/CJSS90-032.
57. Voogt, W.; De Visser, P.H.E.; Van Winkel, A.; Cuijpers, W.J.M.; Van De Burgt, G.J.H.M. Nutrient management in organic greenhouse production: Navigation between constraints. *Acta Hortic.* **2011**, *915*, 75–82, doi:10.17660/ACTAHORTIC.2011.915.9.