Azotobacter-enriched organic manures to increase nitrogen fixation and crop productivity

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ABSTRACT

The use of fertilizers with beneficial microorganisms has increased in recent years. In this study, the performance was assessed of two manures enriched with *Azotobacter* (BioF1 and BioF2), a non-enriched organic manure (Organ), an inorganic N fertilizer applied at a rate equivalent to the organic manures (MinR1) and applied at twice the rate (MinR2), and a control treatment. A field trial and a pot experiment were carried out both consisting of a sequence of three crops per year [lettuce (*Lactuca sativa*)-lettuce-turnip (*Brassica rapa*)] grown for two years. Above ground dry matter (DM) yield and N recovery were higher in the inorganic fertilized plots in comparison to the organic manured plots. Anion exchange membranes inserted into the soil in short periods during the growing seasons revealed higher soil nitrate levels in the inorganic fertilized treatments. Organic amendments improved performance over time, proving that their fertilizing effect, though modest in the short-term, lasts longer. The biofertilizers containing *Azotobacter* (BioF1, BioF2) increased the bioavailability of N over Organ, by an additional N-fixing value of $11.4\,\mathrm{kg\,ha^{-1}}$ estimated from the six crops of the field experiment ($\sim 5.7\,\mathrm{kg\,N}$ per year). If compared on the basis of the same amount of N recovered, organic amendments produced an average increase of $720\,\mathrm{kg\,DM\,ha^{-1}}$ over the inorganic fertilizer ($\sim 120\,\mathrm{kg}$ per crop) due to a general manuring effect. From the results of these experiments, no beneficial effects on crop growth could be attributed to biofertilizers other than the slight increase in N fixation.

1. Introduction

Many microorganisms have the ability to fix atmospheric dinitrogen (N_2) being collectively known as diazotrophs. Biological reduction of N_2 to ammonia is a highly energy-demanding process mediated by nitrogenase, the key enzyme complex which is only present in the microbes capable of fixing atmospheric N_2. The ecological importance of biological N fixation is enormous. General estimates of N biologically fixed in natural and agricultural ecosystems account for more annual N input than N usage as fertilizer (Cooper and Scherer, 2012; Havlin et al., 2014).

Nitrogen-fixing organisms are all prokaryotes (*Bacteria* and *Archaea*), belonging to hundreds of species, including aerobic, micro-aerobic, anaerobic, and photosynthetic bacteria, and actinomycetes (Russelle 2008). In relation to higher plants, they may be symbiotic, associative or free-living. Symbionts are the most significant N fixers for plants, represented primarily by *Rhizobia* (Probacteria), *Frankia* (Actinomycetes) and *Nostoc/Anabaena* (Cyanobacteria) (Cooper and Scherer, 2012). Nitrogen fixation by *Rhizobia* depends on the establishment of a symbiotic relationship with leguminous plants. This symbiosis plays the largest role in N fixation in agriculture, being responsible for about 50% of total annual biological N fixation worldwide (Havlin et al., 2014).

Free-living N fixers can live in soil, water, rhizosphere and leaf surfaces. Some are photoautotrophs depending on light for energy. Heterotrophic N fixers are another important group of diazotrophs, but they are usually restricted in their fixation capacity by the lack of organic substrates for energy generation (Russelle, 2008; Cooper and Scherer, 2012), resulting in minor benefits to agriculture from their activity. Reported values for such common species as *Azotobacter chroococcum* are typically less than $0.5\,\mathrm{kg\,N\,ha^{-1}\,y^{-1}}$ (Smil, 2001).

The importance of beneficial microbes has been increasingly recognized in recent decades and several microbial inoculants have been used in agriculture as a consequence, not only N_2-fixing bacteria, but also phosphate solubilizers and phosphate scavengers (Sharma, 2005; Gentili and Jumpponen, 2009). These products are usually known as biofertilizers, which can be defined as substances containing living microorganisms which, when applied to seed, plant surfaces, or soil, colonize the rhizosphere or the interior of the plant promoting growth

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by increasing the supply of primary nutrients to the host plant (Banerjee et al., 2009).

Among the heterotrophic free-living N_2-fixing bacteria, *Azotobacter* is the most intensively investigated genera. Apart from its ability to fix atmospheric N, *Azotobacter* is also known to synthesize biologically active growth-promoting substances such as indolacetic acid, gibberellins and B-vitamins in culture media (Sharma, 2005).

In recent decades, inoculants containing N_2-fixing and other beneficial microorganisms have been tested on potted plants, nurseries and field crops. However, the use of selected N_2-fixing bacteria and arbuscular mycorrhizal fungi alone or in combination have shown slight to moderate benefits in increasing crop productivity and a modest capability to reduce the use of mineral fertilizers (El-Sirafy et al., 2006; Aseri et al., 2008; Gupta and Aggarwal, 2008; Jahan et al., 2013; Ansari et al., 2015). The greater benefits to crop growth seem to occur when an inoculant containing N_2-fixing bacteria and other beneficial microorganisms is mixed with organic substrates (Nuruzzaman et al., 2003; Kumar et al., 2007; Singh et al., 2013; Kumar et al., 2014). Kumar et al. (2015) showed that using a biofertilizer in the form of a super granule, containing *Azotobacter chroococcum* and *Bacillus subtilis* entrapped in an organic matrix [consisting of cow dung, rice bran, dried powder of neem leaves, and clay soil in 1:1:1:1 ratio and 25% (w/w) saresh (plant gum of *Acacia* sp.)], the productivity of wheat was clearly higher when compared with the results of the biofertilizer applied alone.

In Portugal, commercial organic amendments enriched with beneficial microorganisms, in particular of the genus *Azotobacter*, have recently appeared on the market. From a theoretical point of view, these organic amendments combine inoculum (N_2-fixing heterotrophic microorganisms) and food supply (organic substrate), thus overcoming the main limitation of fixing N of the free-living heterotrophic N fixers, which can increase the potential for biological N fixation. The weak point in this theory comes from the fact that these microorganisms are ubiquitous in terrestrial ecosystems, that is, under normal conditions they are always present in the soil, and, therefore, the need to apply them with organic fertilizer is questionable. Thus, the major question that arises in this work is whether composted organic amendments enriched with free-living N_2-fixing organisms contribute more N to the plants (through the favourable conditions that are provided to the microorganisms) than composted organic amendments of similar composition but not enriched with such microbes. Additionally, to help assess the performance of the organic amendments, a fertility gradient was created using an inorganic fertilizer applied at the same and double N rate of the organic amendments.

2. Material and methods

This work comprises a field trial and a pot experiment that took place over two years in Bragança, NE Portugal, which included a sequence of six horticultural crops, three per year, namely two spring-summer lettuce crops and a turnip crop in the autumn. In the pot experiment, a seventh unfertilized barley crop was also grown to assess the residual effect of the fertilizers applied to the previous crops.

2.1. Climate and soil

The climate of the region presents Mediterranean characteristics although with some Atlantic influence. The average monthly temperatures of the climatological normal 1971–2000 varied between 21.3 °C (July) and 4.4 °C (January), with the average annual temperature of 12.3 °C. The average monthly precipitation varied between 18.4 mm (August) and 118.6 mm (December) with an annual total of 758.3 mm. Climate records observed during the experimental period at the meteorological station of the Santa Apolónia farm, barely 200 m from the place where the experiments were conducted, are shown in Fig. 1.

The soil used in the pot experiment was collected in the plot where the field trial was carried out. Selected properties of a composite sample

of that soil collected in the 0–20 cm layer are shown in Table 1.

2.2. Experimental design and treatments characterization

The field trial was organized in a completely randomized design with six fertilizer treatments and three replications. The fertilizer treatments consisted of three organic amendments, two enriched with beneficial microorganisms (BioF1, BioF2) and a composted and pelleted organic amendment not enriched (Organ), two treatments of a mineral fertilizer [N:P(P$_2$O$_5$):K(K$_2$O) compound, 15:15:15] applied at a N rate equivalent to that of the organic amendments (MinR1) and at double the rate (MinR2), and a control treatment without N application. The control and the mineral fertilization treatments had the objective of creating an N response gradient to allow comparison of the performance of organic amendments. The organic and MinR1 treatments consisted of 40 kg N ha^{-1} applied to each crop and the MinR2 treatment consisted of 80 kg N ha^{-1} per crop. Taking into account that the organic amendments were composed of different levels of P and K, the rates of these nutrients were matched in all treatments with supplemental applications of superphosphate (18% P_2O_5) and potassium chloride (60% K_2O). In lettuce, each experimental unit consisted of five rows of plants spaced at 50 cm (inter-row) and 30 cm (within row) with 12 plants in each row (2.5 × 3.6 m). Each sample was composed of five random plants harvested inside the plots, excluding the plants of the external rows. In turnip, 10 lines spaced 25 cm apart were sown. The sample consisted of one linear meter of plants.

The pot experiment was also arranged in a completely randomized design with six fertilizer treatments as per the field trial, and with 10 replications (10 pots per treatment). Pots of 3 L in volume were used. The pots were filled with fine (< 2 mm) and dried soil mixed with 50 g of perlite to favour aeration. The fertilizer rate was estimated in order for each pot to receive 0.375 g N pot^{-1}. As in the field trial, the rates of P and K were adjusted with calcium superphosphate and potassium chloride. The N rate applied in the pot experiment was more conservative than that applied in the field to reduce a possible risk of a saline effect on plant growth. In the field, 40 kg N ha^{-1} was used (except MinR2 treatment) and in the pots only the equivalent of 25 kg N ha^{-1} was applied. This rate was estimated considering a planting density of 66,667 plants ha^{-1}. In the pots there was also applied, at a rate of 0.08 g pot^{-1}, a commercial fertilizer formulation consisting of a mixture of macro- and micronutrients containing 10% MgO, 0.3% B, 18.5% SO$_3$, 0.3% Cu, 2% Fe, 1% Mn, 0.02% Mo, and 1.6% Zn. In the pot experiment, a single lettuce and six turnip plants were cultivated per pot.

The commercial organic amendments identified as BioF1 was prepared from sheep manure sterilized and enriched with *Azotobacter* (8.6 × 10^7 micro g^{-1} soil), and containing a total of 8.1 × 10^9 micro g^{-1} soil. The organic amendment identified as BioF2 was prepared from poultry manure sterilized and enriched with *Azotobacter*, and containing similar numbers of microorganisms than BioF1. The non-enriched organic amendment (Organ) is a municipal solid waste obtained from selective collection, composted and pelletized. Other characteristics of the three organic amendments are shown in Table 2.

2.3. Installation and management of field and pot experiments

The experiments were installed from the spring of 2014. The installation of the field trial was preceded by the preparation of the soil and the incorporation of the manures and fertilizers provided in the experimental design. Fertilizers and manures were incorporated into the 0–15 cm layer with a cultivator. Lettuces of cv. 'Maravilha-de-verão' (summer wonder) were obtained from a commercial nursery. At the time of transplantation, lettuces were in the phenological stage 2nd to 3rd true leaf unfolded, close to the end of the lag phase. As previously mentioned, plants were spaced at 50 × 30 cm. During the growing
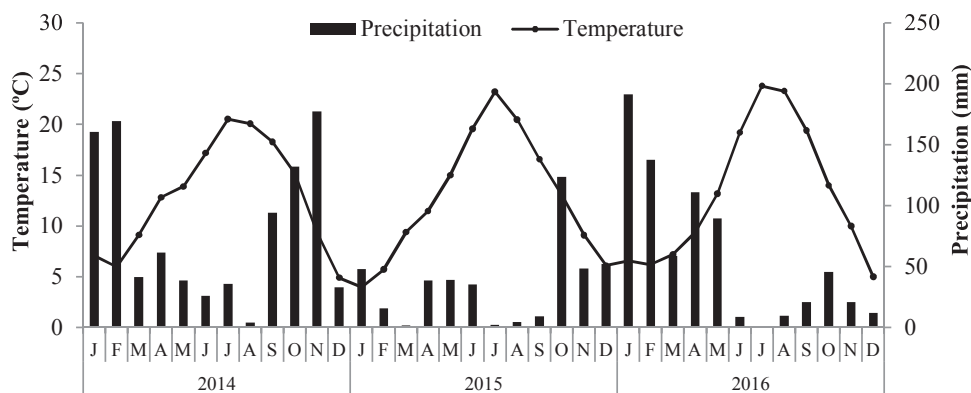
Fig. 1. Mean monthly temperatures and precipitations observed between 2014 and 2016 at the meteorological station of Santa Apolónia farm in Bragança, NE Portugal.

Table 1
Selected properties of the soil used in the field and pot experiments sampled shortly before the trials started at a depth of 0–20 cm.

Soil properties		Soil properties	
Clay (%)	23.9	Extractable P (mg P_2O_5 kg^{-1})[d]	30.0
Silt (%)	21.8	Extractable K (mg K_2O kg^{-1})[d]	61.9
Sand (%)	54.4	Exchangeable bases[e]	
pH$_{H2O}$	6.3	Ca (cmol$_c$ kg^{-1})	10.7
pH$_{KCl}$	5.3	Mg (cmol$_c$ kg^{-1})	3.6
Oxidizable C (g kg^{-1})[a]	8.5	K (cmol$_c$ kg^{-1})	0.3
Total organic C (g kg^{-1})[b]	18.4	Na (cmol$_c$ kg^{-1})	0.4
Extractable B (mg kg^{-1})[c]	0.9	Exchangeable acidity (cmol$_c$ kg^{-1})	0.1

[a] Walkley-Black.
[b] Incineration.
[c] Azomethine H.
[d] Ammonium-lactate.
[e] Ammonium-acetate, pH 7.

Table 2
Selected properties of the three organic amendments used in this study.

	BioF1	BioF2	Organ
Moisture (%)	15.0	15.0	14.7
Organic carbon (g kg^{-1})	266.8	298.8	309.7
pH (H$_2$O)	7.0	7.0	8.4
Conductivity (mS cm^{-1})	8,7	6.5	3.3
Total nitrogen (g kg^{-1})	21.3	26.2	27.3
C/N ratio	12.5	11.4	11.3
Phosphorus (g P_2O_5 kg^{-1})	25.5	30.0	12.2
Potassium (g K_2O kg^{-1})	17.0	30.0	19.0
Calcium (g CaO kg^{-1})	76.5	42.0	78.2
Magnesium (g MgO kg^{-1})	12.8	3.8	10.0

season the plants were watered as needed to maintain normal growth through a drip irrigation system. In the growing cycle of turnip, sowing was performed after soil preparation and incorporation of fertilizers and manures. The growing season of turnip started after the first autumn rains which meant that an irrigation system was not required. During the growing season all crops were manually weeded by hoe. The dates of sowing of the six crops, lettuce − lettuce − turnip of 2014 and 2015, were, respectively, 30 April 2014, 30 June 2014, 11 September 2014, 24 April 2015, 25 June 2015, and 25 August 2015. The dates of harvest for the same growing cycles were, respectively, 26 June 2014, 10 September 2014, 7 November 2014, 23 June 2015, 21 August 2015, and 30 October 2015.

The pot experiments also began in the spring of 2014. The individual rates of the fertilizers and manures provided in the experimental design as well as the supplements to balance P and K rates among treatments were mixed in the soil with a bucket and a shovel. The fertilizer supplement with micronutrients was applied after installation of the crops with irrigation water. The origin, the cultivar and

the phenological stage of the lettuces used in the pot experiment were the same as reported for the field trial. During the growing season the plants were watered as needed to maintain normal growth. Whenever weeds emerged they were manually removed. Turnip was installed by sowing 10–12 seeds per pot, being then hand-thinned to six plants per pot. The dates of sowing of the sequence of the six crops, lettuce – lettuce – turnip of 2014 and 2015, were, respectively, 2 May 2014, 30 June 2014, 9 September 2014, 23 April 2015, 24 June 2015, and 25 August 2015. Harvest dates for the same crops were, respectively, 13 June 2014, 6 September 2014, 24 October 2014, 16 June 2015, 4 august 2015, and 12 October 2015.

2.4. Assessment of soil N availability

In the pot experiments, soil nitrate levels were monitored during the growing seasons by using anion exchange membranes (AEM). Strips of 1×2 cm of AEM were inserted 4–6 cm into the soil with a spatula and kept there for five days. The AEM were tied with a coloured line allowing for easy identification and removal from the soil. The AEM strips removed from soil were rinsed with distilled water and nitrate ions were then eluted in flasks containing 30 mL 0.5 N hydrochloric acid per AEM strip. The AEM strips were kept in the acid media for 75 min. This analysis was performed on 31 May-5 June 2014, 22–27 September 2014, 16–21 July 2015, 1–6 October 2015, and 3–8 February 2016. This last date of insertion of AEM was carried out long after the last turnip growing cycle and had the objective of assessing the residual effect of the fertilizers and manures on soil N availability.

In March 2016 barley was grown without being fertilized with the same objective, evaluating the residual effect of the fertilizers and manures. The crop was sown on 9 March 2016 and cut on 18 May 2016. Twelve seeds per pot were sown, with the excess of young plants being hand-thinned to 8 plants per pot. Dry matter yield and N recovery in the above ground biomass were used as indicators of soil nitrogen availability.

2.5. Laboratory analysis

Nitrate concentrations in the AEM extracts were determined by UV–vis. spectrophotometry. The strips were regenerated in 0.5 M NaHCO$_3$ before being reused. All tissue samples (lettuce, turnip and barley) were oven dried at 70 °C and ground. Tissue analyses were performed by Kjeldahl (N), colorimetry (B and P), flame emission spectrometry (K) and atomic absorption spectrophotometry (Ca, Mg, Cu, Fe, Zn, and Mn) methods.

2.6. Data analysis

Data analysis was carried out using JMP software. The comparisons of the effect of the fertilizer treatments were provided by ANOVA. After ANOVA examination, the means with significant differences ($\alpha = 0.05$)
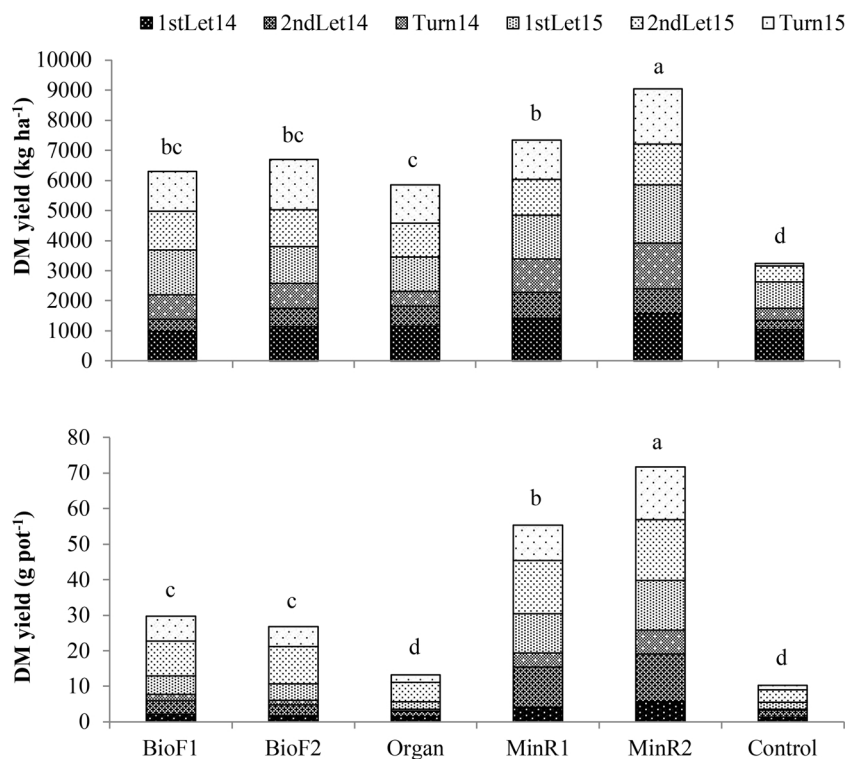
Fig. 2. Dry matter (DM) yield in the first (1st) and second (2nd) growing cycles of lettuce (Let) and turnip (Turn) in the field (above) and pot (below) experiments of 2014 (14) and 2015 (15) as a function of the fertilizer treatments. Letters above the columns is the result of Tukey HSD test (α = 0.05) for the cumulative dry matter yield of the six harvests.

were separated by the Tukey HSD test. An index of N use efficiency (Apparent N Recovery, ANR) was also estimated according to the equation:

Apparent N Recovery (ANR, %) = 100 × (N recovered in the fertilized treatments − N recovered in the control)/N applied as a fertilizer.

3. Results

In the field trial, the highest and lowest mean dry matter yields from the six growing seasons were respectively found in MinR2 and control treatments (Fig. 2). The MinR1 treatment showed higher mean above ground DM yield than all the organic amendments but the differences were not statistically significant when compared to BioF1 and BioF2. The control treatment displayed significantly lower DM yields than any of the other treatments. In the pot experiment, the order of DM yields among treatments was practically the same but with more marked differences. The mineral fertilizers produced significantly more dry biomass than the organic amendments, even the MinR1 treatment. Among the organic amendments, Organ gave significantly lower DM yields than BioF2 and BioF1, with the results of Organ not being significantly different to those of the control treatment.

Nitrogen recovery in the above ground biomass of the six crops grown in the field as a function of the fertilizer treatment is presented in Fig. 3. Nitrogen recovery is the product of DM yield and N concentration in plant tissues. Since N concentration did not greatly change with the fertilizer treatments (data not shown), the pattern of results was quite similar to that of DM yield. However, the differences between fertilizer treatments were clearer. Nitrogen recovery in MinR1 was statistically different to those in BioF1 and Organ. Nitrogen recovery in above ground biomass in the pot experiment also showed a pattern similar to that was observed for DM yield but the differences among fertilizer treatments were higher. As observed for DM yield, organic amendments gave more modest results in the pots than in the field. Among organic amendments, BioF1 gave the higher results and Organ the lower, the latter not being statistically different to those of the

control.

Phosphorus and K recoveries in pot and field experiments (Table 3) followed the patterns of dry matter yield (Fig. 2), since P and K concentration in plant tissues did not significantly vary among fertilizer treatments (data not shown). Therefore, the highest and the lowest values were respectively found in the MinR2 and the control treatments. A similar result of P and K was found for the recovery in plant tissues of Ca and Mg and the micronutrients that were analyzed (data not shown).

The use of AEM during the growing cycle generically showed that nitrate levels in the soil were higher in the fertilizer treatments in those producing more biomass and recovering more N in plant tissues (Table 4). However, 167 days after the last fertilizer application, in February 2016, nitrate levels in the soil reached, for the first time, the highest average value in an organic treatment (BioF1) in spite of the differences for the mineral treatments not being statistically significant.

Barley was grown in the pot experiment to assess the residual effect of six consecutive applications of the fertilizer treatments. The treatment MinR2 recorded the highest average N recovery (Fig. 4). However, MinR1, which received the same rate of N as the organic amendments, gave significantly lower N recovery than BioF1 and Biof2. The control, as expected, displayed the lower average values.

ANR from the field experiment greatly varied among the six growing cycles of lettuce and turnip. The lower values were associated with the organic amendments for which negative values (net immobilization) were often found, and the higher values were usually associated with the application of the mineral fertilizer at the lower rate, MinR1 (Table 5). In general terms, ANR increased in 2015 in comparison with the values found in 2014. When ANR was estimated for the total of the six crops, the higher value was associated with MinR1 (36.6%) and the lower one to Organ (18.6%). ANR found in the pot experiment showed a similar pattern to that observed in the field trial. The lower and the higher values were respectively found in Organ and MinR1 treatments. Total ANR for the six growing cycles varied from 2.4% (Organ) to 33.5% (MinR1).

Based on the field trial, the biofertilizers (BioF1 and BioF2) gave higher average N recoveries than Organ. From the difference of the
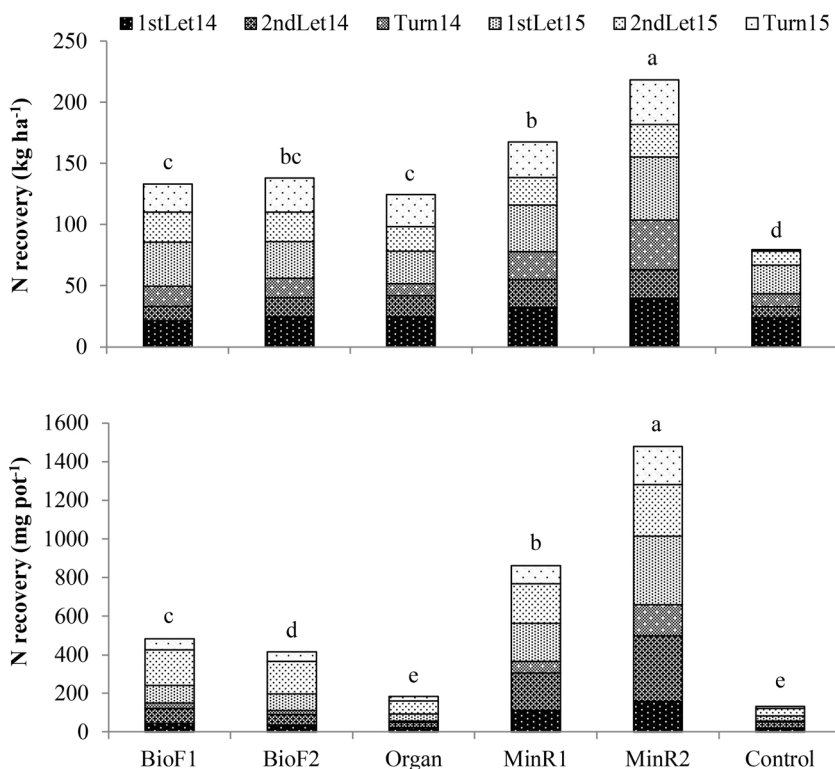
Fig. 3. Nitrogen recovery in the first (1st) and second (2nd) growing cycles of lettuce (Let) and turnip (Turn) in the field (above) and pot (below) experiments of 2014 (14) and 2015 (15) as a function of the fertilizer treatments. Letters above the columns is the result of Tukey HSD test (α = 0.05) for the cumulative N recovery of the six harvests.

Table 3
Phosphorus and K recovery in the six crops grown in the field and pot experiments of 2014 and 2015 as a function of fertilizer treatments. In columns, means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05).

	P recovery		K recovery	
	Field (kg ha^{-1})	Pot (mg pot^{-1})	Field (kg ha^{-1})	Pot (mg pot^{-1})
BioF1	23.0 bc	101.4 c	184.1 bcd	1443.2 c
BioF2	24.5 bc	85.5 d	199.5 bc	1375.0 c
Organ	20.8 c	44.3 e	177.7 cd	639.3 d
MinR1	28.1 b	196.9 b	241.1 b	2181.5 b
MinR2	35.8 a	269.1 a	319.4 a	2924.4 a
Control	12.5 d	37.7 e	128.1 d	483.3 d

Table 4
Nitrate concentration in extracts of anion exchange membranes (AEM) inserted into the soil in the pot experiments as a function of the fertilizer treatments. In columns, means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05).

Fertilizer treatment	Jun 2014 29 dalfa[a] mg kg^{-1}	Set 2104 18 dalfa	Jul 2015 27 dalfa	Out 2015 42 dalfa	Feb 2016 167 dalfa
BioF1	30.8 bc	67.2 bc	21.5 bc	8.7 ab	18.9 a
BioF2	38.8 abc	43.2 cd	23.6 bc	7.0 b	16.7 ab
Organ	20.9 c	23.7 d	19.3 cd	6.6 b	10.9 bc
MinR1	50.3 a	89.6 b	24.7 b	7.7 b	14.8 ab
MinR2	44.1 ab	164.5 a	31.5 a	11.3 a	15.9 ab
Control	27.4 bc	62.4 bc	15.5 d	5.3 b	7.4 c

[a] dalfa (days after the last fertilizer application).

average of the two biofertilizers to the Organ treatment, an estimate of 11.4 kg N ha^{-1} was found (1.9 kg per crop). This value may represent supplemental biological N fixation due to the addition of microorganisms to BioF1 and BioF2.

The relationship between N recovery and above ground DM yield was linear with a very high coefficient of determination (R^2 = 0.99) when established with the data of control and mineral fertilizer

treatments (MinR1 and MinR2) (Fig. 5). All the organic amendments produced proportionally more dry biomass than the mineral fertilizer per unit of N recovery, since their results appeared positioned over the semi-straight line. The estimates of DM yields of the organic amendments over the mineral fertilizer averaged 720 kg ha^{-1} for the six growing seasons (~120 kg per crop).

4. Discussion

The mineral fertilizer applied at double the rate gave the highest DM yields. Between MinR1 and organic amendments, applied at the same N rate, the former gave higher DM yields and N recoveries. The organic amendments enriched with microorganisms produced more dry biomass and recovered more N than the non-enriched one, whereas the control treatment produced the lowest values. It is well-known that the organic amendments release their nutrients slowly, in the course of the mineralization-immobilization process. Previous researchers have tried to develop decay series for organic manures to forecast the annual rates of mineralization and help farmers to manage these resources (Pratt et al., 1976; Magdoff, 1978; Daudén et al., 2004; Mallory et al., 2010). Thus, the slow-release pattern of organic amendments is probably the main reason why they present lower performances in promoting crop growth in comparison with mineral fertilizers, as has been shown in previous studies (Mataruka et al., 1993; Fauci and Dick, 1994; Rodrigues et al., 2005, 2006).

Beegle et al. (2008) have also stressed that N recovery from manure-amended plots is usually lower than recovery from plots amended with inorganic fertilizers due to the greater denitrification associated with manure-amended plots. In this experiment, ANR was lower in organic amendments in comparison to the mineral fertilizer and the values tended to increase from the first to the sixth growing cycle, stressing in this case the slow release nature of these materials. Low ANR from organic amendments is also a common result in experiments comparing mineral and organic sources of N (Beauchamp, 1987; Bitzer and Sims, 1988; Paul and Beauchamp, 1993; Rodrigues, 2004). However, the reduced ANR from organic manure is not necessarily an effective N loss; it might also be due to the possibility of more N being sequestered in the
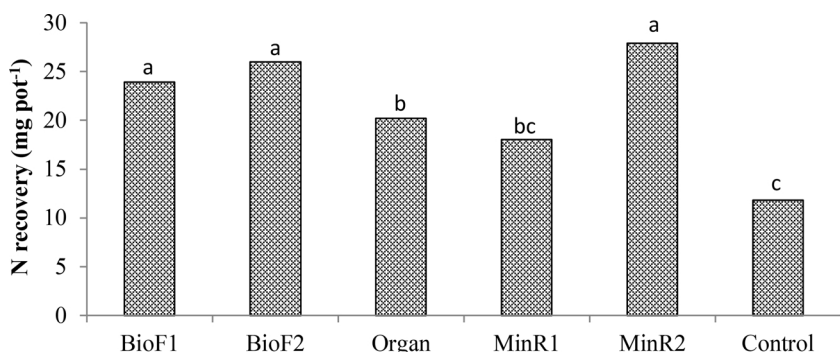
Fig. 4. Nitrogen recovery in barley grown in the pot experiment to evaluate the residual effect of the fertilizer treatments. Letters above the columns is the result of Tukey HSD test ($\alpha = 0.05$).

Table 5
Apparent N recovery (ANR) in the first (1st) and second (2nd) growing cycles of lettuce (Let) and in turnip (Turn) in the field and pot experiments of 2014 (14) and 2015 (15) as a function of the fertilizer treatments. ANR = (N recovery in fertilized plots − N recovery in control plots)/N applied as a fertilizer x 100.

| Fert. treatment	1stLet14	2ndLet14	Turn14	1stLet15	2ndLet15	Turn15	Total
	%						
Field							
BioF1	− 5.8	7.0	14.0	32.3	32.8	53.5	**22.3**
BioF2	2.1	16.1	13.6	16.6	31.3	66.7	**24.4**
Organ	2.6	20.3	− 3.0	9.4	20.8	61.7	**18.6**
MinR1	22.0	33.8	29.7	37.3	28.1	68.6	**36.6**
MinR2	20.2	17.9	36.7	36.0	18.7	43.7	**28.9**
Pot							
BioF1	6.1	13.0	6.4	17.5	40.5	13.4	**16.2**
BioF2	4.0	6.7	4.0	16.5	36.5	10.3	**13.0**
Organ	0.4	− 1.6	1.0	2.6	8.0	3.7	**2.4**
MinR1	24.4	45.8	14.8	46.9	45.7	23.2	**33.5**
MinR2	18.7	42.5	21.6	45.0	31.6	26.0	**30.9**
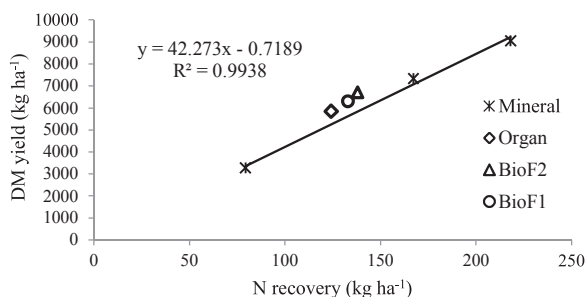
Fig. 5. Relationship between N recovery and dry matter (DM) yield from data of the six growing seasons of the field experiment. Linear adjustment was established from the results of the control and mineral fertilizer treatments (MinR1 and MinR2).

soil in an organic form (Kramer et al., 2002). In the pot experiment the general performance of the organic materials in the production of dry matter and N recovery was particularly low in comparison to the mineral fertilizer treatments. It is possible that the plastic plates that were placed at the base of the pots to avoid loss of water and nitrate leaching, could have created anoxic conditions after the irrigation events. Lack of oxygen in the soil and a fresh organic substrate are conditions particularly favourable to the activity of denitrifying bacteria (Coyne, 2008; Havlin et al., 2014), which may have caused substantial losses of N by denitrification.

The anion exchange membranes, by assessing the nitrate levels in the soil, corroborated the above-mentioned results, and proved to be itself a suitable method of monitoring soil nitrate levels as was shown in previous studies (Qian and Schoenau, 2007; Rodrigues et al., 2010; Arrobas et al., 2011; Arrobas and Rodrigues, 2013). Barley grown without fertilization showed a good performance of the organic

amendments, with results of dry matter yield and N recovery higher than those of MinR1, proving that their fertilizing effect, though modest in the short-term, lasts longer.

The three organic amendments used in these experiments, excluding the microbial properties resulting from the enrichment in *Azotobacter* of two of them, were of similar composition and had very similar C/N ratios (Table 2), which is usually referred to as one of the major factors determining the mineralization rate and the timing of N release (Beegle et al., 2008; Havlin et al., 2014). Among the three organic amendments, the non-enriched in *Azotobacter* consistently yielded the lower values of dry matter, N recovery and soil nitrate levels, although sometimes the differences were not statistically significant. Thus, it seems that the better performance of the amendments enriched with *Azotobacter* can be attributed to supplemental N fixation. On the basis of the average difference between N recovery in enriched with microorganisms and not enriched, an additional N-fixing value of $11.4 \, kg \, ha^{-1}$ during the six growing cycles of the field experiment was estimated (an average of $1.9 \, kg \, N$ per crop or $5.7 \, kg \, N$ per year).

The organic amendments produced more dry biomass than the mineral fertilizer per unit of N recovered. From this index, Organ produced a similar result to the organic amendment enriched in microorganisms. Thus, this result can be justified by a general manuring effect, consisting of the improvement of soil physical, chemical and biological properties, usually recognized in organic materials (Jenkinson, 1988; Weil and Magdoff, 2004). On average there was also observed an increase of $720 \, kg \, DM \, ha^{-1}$ in the six crops ($\sim 120 \, kg$ per crop) due to the manuring effect, and no relevant differences were found between micro-enriched and non-enriched manures. Thus, it seems from this experiment that no beneficial effects on crop growth could be attributed to the biofertilizers other than the increase in N fixation.

5. Conclusions

In the course of these experiments inorganic fertilizer gave higher DM yield and N recovery than the organic manures. However, the differences tended to decrease over time. A manuring effect was observed, associated with all organic fertilizers since they produce more biomass than the inorganic fertilizer per unit of N recovered. The biofertilizers recovered more N than the organic amendment not enriched with microorganisms, which was likely due to an increase in N fixation estimated at $5.7 \, kg \, yr^{-1}$. No beneficial effects of biofertilizers on crop growth were found when compared with the manure not enriched, other than the increase in N fixation.

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