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PhD Thesis

Legumes crop for a sustainable agriculture: study of soil fertility, greenhouse gas emission, carbon sequestration and nutritional status of the crops

Virginia Sánchez Navarro

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Supervisors:
Juan A. Fernández Hernández
Raúl Zornoza Belmonte

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Scientific production

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- ✓ Sánchez-Navarro, V., Zornoza, R., Faz, A., Fernández, J.A. Comparing legumes for use in multiple cropping to enhance soil organic carbon, soil fertility, aggregates stability and vegetables yields under semi-arid conditions., 2019. Scientia Horticulturae. 246, 835-841.
- ✓ Sánchez-Navarro, V., Zornoza, R., Faz, A., Fernández, J.A., 2019. Comparison of soil organic carbon pools and microbial activity in two horticultural multiple cropping systems under Mediterranean conditions. Scientia Horticulturae.With editor.
- ✓ Sánchez-Navarro, V., Zornoza, R., Faz, A., Fernández, J.A., 2019. Does the use of cowpea in multiple cropping with a vegetable crop improve soil quality and crop yield and quality? A field study in SE Spain. European Journal of Agronomy. Acceptable after further revision.
- ✓ Sánchez-Navarro, V., Zornoza, R., Faz, A., Fernández, J.A., 2019. A comparative greenhouse gas emissions and enzyme activities analysis of legume and non-legume crops grown in organic and conventional management. Scientia Horticulturae. Under review.
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Congress communications

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- ✓ Sánchez-Navarro, V., Zornoza, R., Faz, A., Fernández, J.A. Influencia de la inoculación con *Burkholderia* sp. 38 y hongos micorrícicos arbusculares en la producción y calidad de haba (*Vicia faba*) y en la fertilidad del suelo. 38º Congreso Argentino de Horticultura. 5-8 October 2015. Bahía Blanca, Argentina. "Poster".
- ✓ Sánchez-Navarro, V., Zornoza, R., Faz, A., Fernández, J.A. Carbon dynamics and aggregation in a *Vicia faba* crop: influence of management practice and cultivar. European Geosciences Union General Assembly. 17-22 April 2016. Vienna, Austria. "Poster".
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Resumen

Gracias a su habilidad para fijar el nitrógeno atmosférico, los sistemas de cultivo basados en leguminosas son considerados como una estrategia sostenible para reducir el aporte externo de fertilizantes nitrogenados, disminuyendo así las emisiones de gases de efecto invernadero. Además, la inclusión de leguminosas en rotación puede dar como resultado la mejora de la calidad y fertilidad del suelo, y la mejora del rendimiento de los cultivos posteriores. Esta contribución depende de las especies de plantas, las propiedades del suelo, las condiciones climáticas o las prácticas de manejo. Además, la introducción de bacterias fijadoras de nitrógeno en el suelo podría ser una práctica efectiva para mejorar la eficiencia del nitrógeno, mientras que la inoculación con micorrizas está relacionada con el aumento del crecimiento de las plantas, la absorción de nutrientes inmóviles y la resistencia a patógenos. Estos efectos positivos de los cultivos de leguminosas pueden mejorar la rentabilidad de la agricultura orgánica, que tiende a tener rendimientos más bajos en comparación con la agricultura convencional.

En base a rotaciones de cultivos basados en leguminosas, establecimos un experimento con tres objetivos diferentes. En primer lugar, nuestro objetivo fue evaluar el efecto de dos especies de leguminosas diferentes (caupí, poco común en el área de estudio, y haba, tradicional en el área de estudio) sobre el carbono orgánico del suelo (COS), la agregación del suelo, la fertilidad del suelo y los rendimientos de los cultivos posteriores de hortalizas (brócoli y melón, respectivamente) cultivados bajo prácticas de manejo convencionales y orgánicas después de dos ciclos de cultivo de rotación. Los resultados mostraron que el cultivo previo de caupí, en comparación con el cultivo de haba, era una alternativa más efectiva en términos de producción sostenible para aumentar el COS, la fertilidad del suelo y el rendimiento de los cultivos posteriores mediante procesos de rizodeposición. En el cultivo de brócoli, la práctica de manejo convencional se relacionó positivamente con los depósitos de carbono y nitrógeno. La práctica de manejo orgánico se relacionó con mejoras en la estructura del suelo, pero también con el rendimiento del cultivo. En segundo lugar, nuestro objetivo fue evaluar el efecto de dos cultivares de caupí (Feijão frade de fio preto y Feijão frade de fio claro) en la fertilidad del suelo, el rendimiento, la calidad del cultivo y la composición nutricional del cultivo de brócoli posterior cultivado bajo prácticas de manejo convencional y orgánica durante tres ciclos de rotación de cultivos, mientras que las tasas de fertilización se redujeron un 20% en comparación con el cultivo de brócoli. Los resultados mostraron que el cultivo de caupí era una buena

estrategia para la diversificación de cultivos y para reducir la dependencia de fertilizantes nitrogenados, ya que contribuyó a un aumento del fósforo disponible del suelo mientras que mantuvo el rendimiento y la calidad del cultivo de brócoli después de tres rotaciones de cultivos bajo ambas prácticas de manejo. La práctica de manejo convencional aumentó el diámetro y los rendimientos de las pellas de brócoli. La práctica de manejo orgánico mejoró la estructura del suelo y la actividad microbiana. En tercer lugar, nuestro objetivo fue evaluar el efecto de diferentes especies de leguminosas (caupí, poco común en el área de estudio, y haba, tradicional en el área de estudio) en los depósitos de carbono en el suelo, el contenido de nitrógeno y actividades enzimáticas del suelo de los cultivos posteriores de hortalizas (brócoli y melón, respectivamente) cultivados bajo prácticas de manejo convencional y orgánica después de dos ciclos de rotación de cultivos. Los resultados mostraron que el cultivo previo de caupí en comparación con el cultivo de haba fue mejor para aumentar el COS, el nitrógeno y las actividades enzimáticas del suelo del cultivo posterior de hortalizas debido a procesos de rizodeposición. En el cultivo de brócoli, la práctica de manejo convencional aumentó el COS. La práctica de manejo orgánico se relacionó con un mayor secuestro de carbono. El cultivar de la leguminosa afectó el contenido de nitrógeno en el suelo y la actividad deshidrogenasa. Además, también evaluamos el efecto de un cultivo de leguminosa y no leguminosa (haba y brócoli) durante dos años sobre el rendimiento del cultivo, las emisiones de gases de efecto invernadero (GEI) (N_2O, CO_2 y CH_4) y las actividades enzimáticas del suelo, cultivados bajo prácticas de manejo convencional y orgánica. El año de cultivo afectó las emisiones de GEI, el rendimiento de los cultivos y las actividades enzimáticas. El cultivo de haba mostró las mayores emisiones de GEI, mientras que el cultivo de brócoli mostró mayores actividades enzimáticas en el suelo. La práctica de manejo convencional resultó en mayores rendimientos para ambos cultivos, mientras que la práctica de manejo orgánico condujo a mayores emisiones de N_2O y CO_2 y actividades enzimáticas del suelo en ambos cultivos. El rendimiento de los cultivos estuvo relacionado con menores emisiones de GEI y mayor actividad enzimática.

Finalmente, establecimos un experimento adicional para evaluar la efectividad de diferentes tratamientos de inoculación con los géneros *Rhizobium* y *Burkholderia* (BFN) y / o hongos micorrícicos arbusculares (HMA) a través de inoculación individual y dual en la nutrición vegetal, la fijación biológica de nitrógeno (FBN) y el rendimiento y calidad de dos cultivares de haba (Muchamiel y Palenca), con un 20% de disminución en la tasa de fertilización en comparación con los cultivos no inoculados durante dos años. Los resultados mostraron que la composición nutricional de la planta no se vio afectada por el tratamiento de inoculación o el menor aporte de fertilizantes. La FBN se vio afectada por el cultivar, con valores más altos en la parte aérea del cultivar Muchamiel. La inoculación dual en comparación con la inoculación individual mostró un mayor contenido de nitrógeno en la parte aérea de ambos cultivares de haba. La inoculación con bacterias pertenecientes al género *Burkholderia* (*B. cenocepacia*) en comparación con *Rhizobium* mostró un mayor contenido de nitrógeno en la raíz. Aunque no hubo diferencias en el contenido de nitrógeno en las partes aéreas con la inoculación, el contenido de proteína en el grano fue mayor después del tratamiento de inoculación, lo que sugiere una mayor eficacia en la asimilación de nitrógeno después de la inoculación.

Abstract

Thanks to their ability to fix atmospheric N, legume-based cropping systems are considered as a sustainable approach to reduce external input of N fertilizers, decreasing overall greenhouse gas emissions. Furthermore, inclusion of legumes in multiple cropping can result in the improvement of soil quality and fertility and the enhancement of the yield of subsequent crops. This contribution depends on plant species, soil properties, climatic conditions or management practices. In addition, the inclusion of N-fixing bacteria to the soil could be an effective practice to improve N efficiency while mycorrhiza inoculation is linked to plant growth increase, the uptake of immobile nutrients and the resistance to pathogens. These positive effects of legume crops can enhance the profitability of organic farming, which tends to have lower yields compared to conventional farming.

Using legume-based multiple cropping, we established one experiment with three different objectives. Firstly, we aimed to assess the effect of two different legume species (cowpea -unusual in the study area- and fava bean –traditional in the study area) on soil organic carbon (SOC), soil aggregation, soil fertility and crop yields of subsequent vegetable crops (broccoli and melon, respectively) grown under conventional and organic management practices after two multiple cropping cycles. The results showed that previous cowpea, compared to fava bean crop, was a more effective alternative in terms of sustainable production for increasing SOC, soil fertility and crop yield of the subsequent vegetable crop by rhizodeposition processes. In broccoli crop, conventional management practice was positively linked to C and N pools. Organic management practice was linked to improvements is soil structure but also crop yield. Secondly, we aimed to assess the effect of two cowpea cultivars (Feijão frade de fio preto and Feijão frade de fio claro) on soil fertility, yield, crop quality and nutritional composition of subsequent broccoli crop grown under conventional and organic management practices during three multiple cropping cycles, while fertilization rates were reduced 20% compared to broccoli crop. The results showed that cowpea crop was a good strategy for crop diversification and for reducing N fertilizer dependency, since it contributed to an increase of soil available P while it maintained crop yield and quality in broccoli crop after three multiple cropping under both management practices. Conventional management practice increased broccoli head diameter and yields. Organic management practice improved soil structure and microbial activity. Thirdly, we aimed to evaluate the

effect of different legume species (cowpea -unusual in the study area- and fava bean – traditional in the study area) on soil C pools, N content and soil enzyme activities of subsequent vegetable crops (broccoli and melon, respectively) grown under conventional and organic management practices after two multiple cropping cycles. The results showed that previous cowpea compared to fava bean crop was better for increasing soil organic C (SOC), N and soil enzyme activities of the subsequent vegetable crop due to rhizodeposition processes. In broccoli crop, conventional management practice increased SOC. Organic management practice was linked to higher C sequestration. Legume cultivar affected soil N content and dehydrogenase activity. In addition, we also assessed the effect of a legume and non-legume crop (fava bean and broccoli) during two years on crop yield, GHG emissions (N_2O, CO_2 and CH_4) and soil enzyme activities, grown under conventional or organic management practices. Crop year affected GHG emissions, crop yield and enzyme activities. Fava bean crop showed the highest GHG emissions, while broccoli crop showed higher soil enzyme activities. Conventional management practice resulted in higher crop yields for both crops, while organic management practice led to higher N_2O and CO_2 emissions and soil enzyme activities in both crops. Crop yield was related to lower GHG emissions and higher enzyme activities.

Finally, we established an additional experiment to assess the effectiveness of different inoculation treatment with *Rhizobium* and *Burkholderia* genera (NFB) and/or arbuscular mycorrhiza fungi (AMF) through individual and dual inoculation on plant nutrition, BNF, and crop yield and quality of two fava bean cultivars (Muchamiel and Palenca), with 20% decrease in fertilization rate compared to non-inoculated crop during two seasons. The results showed that nutritional composition of plant was not affected by inoculation treatment or lower input of fertilizers. BNF was affected by cultivar, with higher values in shoot of Muchamiel cultivar. Dual compared to individual inoculation showed higher N content in shoot for both fava bean cultivars. Inoculation with bacteria belonging to *Burkholderia* genus (*B. cenocepacia*) compared to *Rhizobium* showed a higher N content in root. Although there was no difference in N content in shoots with inoculation, protein content in grain was higher after inoculation treatment, suggesting higher efficiency in N assimilation after inoculation.

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Chapter 1

Introduction and objectives

1.1. Benefits of legume production for human consumption

Legumes belong to the family *Fabaceae*, which is considered the third largest group of plants on the planet. The world has lived an increase in the production of legumes of 31% between 1990 and 2014, with a total production in 2014 of 77.6 million tonnes (FAO, 2016). Pea (*Pisum sativum* L.), fava bean (*Vicia faba* L.), and cowpea (*Vigna unguiculata* (L.) Walp.) are three of the most important legume crops grown around the world. However, soybean is the top legume crop globally, representing 50% of the world's legume crop growing area (Herridge et al., 2008). Among the most important features of legumes is their ability to fix atmospheric nitrogen (N), an important milestone with a special relevance to the soil enrichment. Legumes influence our world through nutrition, health, climate change, biodiversity and food security (FAO, 2016). Nutritionally, they are consumed as a perfect complement to cereals mainly because they are rich in protein and essential amino acids (FAO, 2016). In addition to this, they provide carbohydrates, micronutrients and dietary fibre of high quality (Muehlbauer and McPhee, 1997). In health terms, these are able to control the weight through their low fat and high fibre content. They reduce risk of heart diseases due to high doses of potassium and fibre; potassium helps to reduce the blood pressure while fibre reduces LDL colesterol (Anderson et al., 1999; Afshin et al., 2014; Anand et al., 2015). In turn, they prevent anomalies related to nervous system due to their content of folic acid, they avoid the iron deficiency and alleviate the food allergies, since they are exempt from gluten (Messina, 1999; Blancquaert et al., 2010; Miñarro et al., 2012). In this sense, legumes are postulated as a reality for the global food in the future, since they double the protein content compared to cereals, and they grow in several types of productive lands in the world, with a great crop yield as well as their seeds can be retained for a long period without loss of of properties. Furthermore, legumes play a major role in mitigating climate change, since they improve absorption of carbon in soil, and thus reduce CO_2 emissions (FAO, 2016). In turn, their ability to fix atmospheric N through biological nitrogen fixation (BNF) process allows them to be cultivated with a low input of N fertilizers (Turpin et al., 2012). This biological ability to fix N in the soil has positive implications on biodiversity of productive land, since the legumes active microorganisms linked to BNF. These microorganisms infect their roots, and structures known as nodules are formed (Voisin and Gastal, 2015; Aschi et al., 2017). These nodules give shelter to bacteria, which receive energy, while the plant takes the N from the atmosphere and it is transported to the soil

(Rascio and La Bocca, 2013). Legume crop residues possess a biochemical composition significantly different from the rest of crops, with higher N content, and thus they are used as green manure, which allows to generate higher crop yields in subsequent multiple cropping, improve the soil biodiversity by catalyzing the development of microorganisms involved for the improvement of the soil structure and nutrients availability (Franke et a., 2018).

1.2. Characteristics of fava bean (*Vicia faba* L.)

The *Vicia L.* genus is distributed throughout the Mediterranean regions of Eurasia, America and Africa. Origin of fava bean crop is scarce and disputed (Shiran et al., 2014). It is a cool season-crop, which is capable of growing in different soil and agro-climatic conditions. In cool-temperate areas, fava bean is sown in spring to avoid frost damage (Sallan et al., 2015), while in warm-temperate areas, it is sown in autumn (Bilalis et al., 2003). This is used for food, feed and green manure purposes. In the human diet, it has a great importance due to its high fiber and protein concentration along with low fat content in comparison to soybean. Protein content in dry seeds ranges from 17.6 and 34.5 % (Duc et al., 2015). Fava bean has a high efficiency to establish simbiosis with nitrogen fixing bacteria (NFB), which results in BNF. Its N_2 fixation capacity reaches up to 200 kg ha^{-1} (Neugschwandtner et al., 2015). BNF acts as a N sustainable source, which can replace or complement mineral fertiliser inputs in arable lands as well as it increases soils biological activity. Most of nitrogen fixed by legumes is harvested by seed yield or fed to animals, although legumes can deposit significant N amounts in the soil (Fustec et al., 2010; Jensen et al., 2012). In addition, the benefits related to BNF of legumes, including their crop in rotation, multiple cropping or intercropping legumes with non-fixing crops such as cereal or horticultural (Jensen et al., 2010).

1.3. Characteristics of cowpea (*Vigna unguiculata* (L.) Walp.)

The *Vigna* genus includes more than 200 species mainly found in Africa and Asia (Fery, 2002). Origin of cowpea is presumed to have occurred in Africa, in the sub-Saharan territory (Coulibaly et al., 2002; Smykal et al., 2015). It is a warm-season crop, well adapted to semi-arid and subtropical climates, and it is cultivated throughout the Mediterranean Basin. Its cultivation in temperate areas lasts from spring to autumn. This legume has a great agronomic interest due to its resistence to acidity, drought and high temperatures (Ehlers and Hall, 1997; Hall, 2011). Cowpea is mainly used for human food but also for fodder (Tarawali et al., 1997a). Protein content in dry seeds is around of 17.4-31.7% (Antova et al., 2014; Dominguez-Perles et al., 2016; Gonçalves et al., 2016). Regarding human consumption, dry grain is the most important part, although leaves, immature pods and green seeds are also consumed (Singh et al., 2003). Cowpea is considered a promiscuous species for its ability to nodulate with several bacterial species and leave a net gain of N in the field. In addition, when this is inoculated with NFB, it can fix approximately 145 and 224 kg ha^{-1} of N which can potentially be used by intercrops, multiple crops or rotated crops (Creamer et al. 2000; Clark, 2008). Its N_2 fixation capacity is up to 40-80 kg ha^{-1} (Quin, 1997). This is mainly nodulated by bacteria belonging to the genus *Bradyrhizobium* (Allen and Allen, 1981; Jordan, 1982; Thies et al., 1991).

1.4. Benefits of the use of legumes in crop rotations or multiple cropping

The intensification of conventional agricultural practices affects agroecosystem sustainability through loss of soil organic matter (SOM), erosion, soil and groundwater pollution, greenhouse gas emissions (GHG) and low biodiversity (Tilman et al., 2001). Thus, diversification of crops with rotations or multiple cropping plays an important role, since the mineralization of preceding crop residues can release important quantity of nutrients, which maintain soil fertility while create suitable habitats for soil biota in subsequent crops (Askegaard and Eriksen, 2007; Raphael et al., 2016). Higher legume residue inputs and lower rates of decomposition under cool and dry conditions allow to persist the effect of legume crop for years after its crop (Grant et al., 2016). In addition to this, plant roots release substances containing C coming from photosynthesis such as simple sugars, organic acids, phenolics, inorganic compounds, exoenzymes or root border cells (Marschner, 1995; Dakora and Phillips, 2002; Nguyen, 2003; Paterson, 2003), but also substances containing N such as NO_3^-, NH_4^+ and amino acids, whose content is higher in root exudates of legumes than non-legumes. This is due to their ability of N_2-fixation in symbiosis with NFB (Rovira, 1956; Hale et al., 1978; Brophy and Heichel, 1989; Wacquant et al., 1989). Rhizodeposition depends on biotic factors such as plant species, genotype, physiological status, fertilizer application, soil microorganims and N_2-fixation or photosynthetic capacity of the plant (Van der Krift et al., 2001; Nguyen, 2003), but also abiotic factors such as drought, soil texture, anaerobic conditions, light intensity, atmospheric CO_2 concentration or nutrient deficiency (Rovira, 1956; Merckx et al., 1985; Lynch and Whipps, 1990; Whipps, 1990a; Whipps, 1990b; Nguyen, 2003; de Graaf et al., 2007). The amount of carbon exuded by the plant can be determined in the absence of soil such as sterile hydroponic culture (Nguyen, 2003). Legumes, through their ability to symbiotically fix atmospheric N_2 after their association with NFB, are considered essential to reduce the use of N fertilizers, while they influence soil microbial communities directly and SOM dynamics (Stevenson, 1982; Voisin and Gastal, 2015). In turn, enhancing SOM plays a part in the reduction of GHG emissions mainly due to carbon storing in soils, but also to changes in soil structure (Mutegi et al., 2010; Powlson et al., 2011). Legumes with a high harvest N index have a low contribution to the soil N content, despite the non-harvest residue are incorporated into the soil (Senaratne and Hardarson, 1988). However, the effects of legumes crop on soil physical and chemical properties are manifested after a long period (Yusuf et al., 2009). In addition, N contribution of legumes to subsequent crops depends on the legume species, but also if legume seed is harvested or returned to the soil as a green manure crop (Peoples et al., 2009; St Luce et al 2013).

1.5. Biological nitrogen fixation

Nitrogen is present in atmosphere in the form of diatomic molecule (N_2), but its structure makes N_2 molecule inert. Then, Prokaryotic microorganisms as diazotrophs fix atmospheric N_2 in the form of ammonia (NH_3) (Riggs et al., 2001; Galloway et al., 2008). BNF process begins with the exchange off signals between the bacterium and plant. Plant root continuously exudes flavonoids into the rhizosphere, with a higher concentration in the presence of specific NFB (Zuanazzi et al., 1998)**.** The flavonoids activate the expression of nod genes in NFB, which are the responsable for the synthesis of Nod factors, that are necessary for the initiation of nodules (Dénarié et al., 1996; Peck et al., 2006; Wang et al., 2012)**.** Bacteria fix N_2 through a complex enzyme system called nitrogenase, which is formed by two components (dinitrogenase reductase and dinitrogenase metal cofactor) (Kim and Rees, 1994). The chemical reaction of microbial N_2 fixation is:

$N_2 + 8\ H^+ + 8\ e^- + 16\ Mg\ ATP \rightarrow 2\ NH_3 + H_2 + 16\ Mg\ ADP + 16\ Pi$ (Postgate, 1998)

BNF results in a good alternative with regard to N fertilizers, since this process offers a higher efficiency in terms of the utilization of N by the plant, reduction of N leaching along soil and water pollution (Peoples et al., 1995). The NFB-legumes association is highly specific, since each bacteria strain has a host range (Perret et al., 2000). NFB belong mainly to alpha- and beta-proteobacteria. Members of the α-proteobacteria, such as *Rhizobium* and *Bradyrhizobium* are the most studied N-fixing symbionts of legumes, although other genera, such as *Burkholderia* belonging to the β-proteobacteria class, also promote high levels of BNF (Glick, 2012; Nadeem et al., 2014; Zaidi et al., 2015).

In addition to bacteria, arbuscular mycorrhizal fungi (AMF) also represent a significant portion of soil rhizosphere microorganisms involved to BNF. Besides increasing the root surface area, so that the plant can absorb water and nutrients more efficiently, they protect the plant from a variety of stresses such as drought, soil pathogens, salinity and heavy metals. AMF benefit BNF process by increasing uptake of relatively inmobile phosphate ions from non-labile sources (Nadeem et al., 2014). Benefits can be achieved by direct or indirect mechanisms. Direct stimulation involves the production of 1-aminocyclopropane-1-carboxylate (ACC)-deaminase to reduce high levels of ethylene, the production of plant growth regulators such as cytokinins, gibberellins and auxins through BNF, facilitating the uptake of nutrients from the soil, solubilizing minerals like phosphates or modulating phytohormone levels (Glick, 2012; Nadeem et al., 2014). Indirect stimulation is related to the inhibition of pathogens through the synthesis of antibiotic and lytic enzymes, or by increasing the resistance of the host plant against pathogenic organisms. In this context, bacterial genera as *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Microccocus*, *Aereobacter*, *Flavobacterium* and *Erwinia* along with fungi have mobilized poorly available phosphorus (Rodríguez and Fraga, 1999; Barroso and Nahas, 2005; Pandey et al., 2005; Rivas et al., 2006; Ahmad et al., 2008).

After N, P is the most limiting nutrient for crops (Vance et al., 2000). It is vital in the metabolic energy processes in nodules, since this drives symbiotic N_2 fixation into NH_3 (Le Roux et al., 2006; Le Roux et al., 2008; Sulieman et al., 2013), and its subsequent conversion into organic N as amino acid and ureides, since it forms part of ADP and ATP availability (Plaxton and Podesta, 2006). In addition to its role in ATP requirements for nitrogenase function, P is critical in the nodule activity due to its involvement in signal transduction, membrane biosynthesis, and nodule function and development (Ribet and

Drevon, 1995; Al-Niemi et al., 1997). High N_2 fixation, however, requires the presence of adequate numbers of highly effective rhizobia in the soil, legume cultivar, N and P fertilization and soil properties (Schubert et al., 1990; Thies et al., 1991; Adak and Kibritci, 2016, Argaw and Mnalku, 2017).

For many legumes and soils, either the homologous rhizobia are absent, occur in low numbers, or are not highly effective and thus not capable of satisfying the N requirements of the crop. A legume is considered as a traditional crop when the soil contains adequate number of native NFB for an effective inoculation (Thompson et al., 1991). The inclusion of bacteria to the soil is an advisable agricultural practice when this soil does not contain specific NFB able to nodulate the legume that is cultivated by the absence of previous crop of same or symbiotically linked to legume or poor nodulation despite this legume was grown on the land previously, the soil has a low N content (Catroux et al., 2001; Allen and Allen, 1961). Bacteria inoculants must compete with the indigenous populations for nodule occupancy and effective in N_2 fixation, although their effectiveness is difficult to predict. Moreover, selected bacteria are influenced by local abiotic environmental factors (Dowling and Broughton, 1986). On the other hand, native population of NFB generates a competitive barrier to the introduced strains, and thus it leads to a failed inoculation (Thies et al., 1991). In turn, symbiotic effectiveness of native strains may be low as a consequence of their adaptation to agroclimatic conditions (Zahran et al., 1999) or horizontal gene transfer mechanisms, which promote their competitiveness without the need of symbiosis. In addition to this, native strains are widely distributed in the soil while the introduced strains are concentrated around the seed (López-García et al., 2002). Bacteria may be introduced to legumes by inoculation of the seed or soil. Seed may be inoculated prior to sowing or with coating facilities to be sown within a week (Gemmell et al., 2002). Alternative methods to these include direct inoculation of the soil using peat inoculants suspended in water or inoculants formulated as liquids or granules (Brockwell, 1977; Gemmell et al., 2002). The success of an introduced inoculant is depending on inoculant quality, that is the number of viable rhizobia per unit of inoculant and the number of introduced rhizobia required to root infection, because bacteria mobility in soil is limited under field conditions, but also on the effectiveness and infectivity of indigenous NFB (Puppi et al., 1994; Giller, 2001; Rodríguez-Navarro et al., 2010; Ronner et al., 2016). In turn, the ability of NFB strains to nodule and fix N with their specific host legume varies according to legume species and cultivar (Dowling and Broughton, 1986). The survival of these bacterial inoculants in soil is influenced by soil pH, drought, nutrient deficiencies, salinity or temperature (Zahran, 1999; Hungria and Vargas, 2000; Giller, 2001).

1.6. Methodologies for estimating biologically fixed-N

There are different ways for measuring the BNF, such as acetylene reduction assay (ARA), N difference method, ^{15}N enrichment and ^{15}N natural abundance. ARA was used widely in the past, it provides an instantaneous and indirect measure of nitrogenase activity. This method is based on nitrogenase enzyme to reduce acetylene (C_2H_2) to ethylene (C_2H_4) in the presence of high levels of acetylene. The ethylene produced is detected by gas chromatography (Hardy et al., 1968). N difference method is based on the fact that the N_2-fixing plants and reference plants (non N_2-fixing) use exactly the same amount of soil N, thus the difference in uptake of N of the N_2-fixing and reference plants is the amount of N_2 fixed (Unkovich et al., 2008). The use of ^{15}N isotopes to quantify the role of biological N_2 fixation in the N economy of the soil-plant system has become a familiar feature. The ^{15}N isotopic techniques (natural abundance and enrichment) are the most commonly employed because they offer an overall estimate of the contribution of BNF over the entire growth period. The strategy applied in the ^{15}N natural abundance technique is to assume that reference plants, which are unable to obtain N from N_2 fixation, accumulate N only from the soil. If N_2-fixing legumes have significantly lower ^{15}N abundance than the reference plants, then the difference can be interpreted quantitatively to assess the contribution of BNF by bacteria. However, when necessary, the difference between soil N and N_2 is expanded after the incorporation of ^{15}N enriched compounds into the soil. This is the ^{15}N enrichment method, which is currently less used as a consequence of the improvement in the precision of mass spectrometers (Unkovich et al., 2008).

1.7. Conventional and organic farming

Conventional farming has played an important role in the improvement of food to support human demands but it has a strong impact on the environment due to its largely dependent of synthetic fertilizers, pesticides and herbicides (Tu et al., 2006). In this context, organic farming is considered a promising solution, since this is characterized by the ban of chemical pesticides and fertilizers. Organic farming systems rely on ecological practices as biological pest control, the addition of organic matter (green manure including legumes, compost, farmyard manure…), the use of biofertilizers and crop diversification (Fliebach et al., 2007). In addition, organic agriculture provides a higher total C input than conventional agriculture (Gattinger et al., 2013), which is as a consequence of higher external C inputs as organic amendments. However, in this kind of agriculture, the supply of sufficient plant-available N can be a problem, since N availability to plants is dependent on mineralization rates of SOM, and this may result in lower yields, in which case more hectares would be needed to produce the same amount of food as the conventional agriculture (Seufert et al., 2012). On the other hand, the timely delivery of N to the plant may affect legume crops, since the proliferation of N_2-fixing rhizobacteria at the initial cropping stages inmobilizes inorganic N forms (Oberson et al., 2013).

1.8. Greenhouse gas emissions

Intensification of agricultural systems mainly through the increase in the use of mineral N fertilizers has led to highest GHG emissions, particularly nitrous oxide (N_2O). N_2O has a global warming potential 300 times greater than carbon dioxide (CO_2) and is the major contributor to the depletion of the ozone layer in the stratosphere (Ravishankara et al., 2009; Van Beek et al., 2010). Cultivation of legumes is linked to the emission of CO_2, N_2O, and methane (CH_4) from the soil (Forster et al., 2007). Although CO_2 is the main anthropogenic greenhouse gas, the agricultural sector is dominated by N_2O and CH_4 emissions (Schulze et al., 2009). The CO_2 emission from the soil for agricultural use to the atmosphere results from carbon input in the form of crop residues and biomass through the decomposition of their organic matter. This decomposition can be affected by changes that affect soil microbial communities such as substrate quality, soil moisture or temperature as well as changes within soil microbial communities (Ohta, 1990; Lips and Hofstede, 1998; Jug et al., 1999; Chapela et al., 2001). In addition to this, increases of above and belowground biomass production of the crops involve a greater amount of residue returned to the soil and thus an increase in the CO_2 emission (Curtin et al., 2000; Al-Kaisi and Yin, 2005). On the other hand, the increase of the belowground biomass production results in a higher rhizodeposits and root turnover along with the rhizosphere respiration (Amos et al., 2005) but also the increase in soil respiration (respiration from root and microorganims of rhizosphere) (Kuzyakov, 2006).

Emissions of N_2O originate from denitrification and nitrification processes in soils. Denitrification is driven by the presence of denitrifying bacteria sources coming from metabolizable organic carbon, anoxic soil conditions, as well as the presence of nitrate, while nitrification is driven by dissolved ammonium, pH, oxic soil conditions, and, temperature (Tiedje, 1988). Moreover, NFB may also contribute to N_2O emissions in several ways, such as nitrification and denitrification of biologically fixed N (Galloway, 1998), providing N-rich residues for decomposition (Baggs et al., 2000; Huang et al., 2004) and directly by some NFB that are able to denitrify, producing N_2O (O'Hara and Daniel, 1985). N fertilizer applications to soils (organic or mineral), animal wastes and biological N_2 fixation result in N_2O emissions, since this gas is a subproduct of the transformation of N compounds of fertilizers (IPCC, 1996; Firestone and Davidson, 1998). However, contradictory results are reported in literature regarding the increase or decrease of GHG emissions after the aplication of N fertilizers.

The excess in N may also have other negative impact such as nitrate leaching (Oelmann et al., 2007). In this context, the management of cropping systems is considered a powerful tool for the mitigation of GHG emissions in agriculture (Zhong et al., 2009; Rees et al., 2012; Sainju et al., 2012), with the reduction of N fertilizers supplied to crops through multiple cropping systems with legumes or the use of organic fertilizers (Flessa et al., 2002; Burger et al., 2005). In turn, organic fertilizers used in organic agriculture are associated with increased rates of organic matter decomposition, which may enhance N_2O and CO_2 emissions. CH_4 has a global warming potential 25 times greater than CO_2 (IPCC 2007). Soils used for crop are minor sources of CH_4, except after application of manure or other organic materials under flooded conditions (Johnson et al., 2007; Dendooven et al., 2012). CH_4 emission originates mainly from the enteric fermentation in ruminant animals, flooded rice fields, and animal waste processing in anaerobic environments (IPCC, 1996), although agricultural practices influence CH_4 atmospheric concentration by affecting its consumption in aerated soils (Prather et al., 1995).

1.9. Relevance of the work

This doctoral thesis emerged with the aim of promoting and extending the sustainable protein legumes crop, resolving the problem of the deficit of protein sources, which currently exists in Europe, as well as reducing the dependence on the consumption of imported legumes such as soybean. Soybean can enter European market without tariffs, which reduces the profitability of local production. However, a large proportion of soybean crops introduced into Europe are genetically modified, which makes that there is a growing need to stimulate local production. In addition, there is a need to find out sustainable farming practices including legumes to reduce the dependance on external N inputs which can provoke soil, water and atmosphere contamination. Thus, fostering the use of legume crops in horticulture can be an effective solution to enhance the European protein crops while contributing to long-term sustainability of the agro-ecosystems.

1.10. Objetives

The main objective of this thesis is to assess the sustainability of the inclusion of legume crops in multiple cropping with horticultural crops to promote soil quality and fertility and to enhance the biological nitrogen fixation efficiency and the use of biologically-fixed N by the plant. In addition, the added value of the legume crop will be evaluated in subsequent non-legume horticultural crops grown in multiple cropping to assess if there are benefits in crop yield and quality.

The specific objectives of the doctoral thesis are to:

- Asses the improvement of soil quality and fertility after growing legume crops in multiple cropping with non-legume horticultural crops under conventional and organic management practices compared with non-legume monocultures.
- Quantify the improvements in the crop yield and quality of subsequent horticultural crops in multiple cropping with legumes compared to monocrop.
- Quantify the GHG emissions (N_2O, CO_2 and CH_4) in legume and non-legume horticultural crops under conventional and organic magament practices, and then be able to asses the effect of management practice and cultivation type.
- Determine if legumes monocrop or multiple cropping favors carbon sequestration in soil.
- Asses the biological nitrogen fixation by different legume cultivars.

- Asses the effect of the selection of N-fixing bacteria and arbuscular mycorrhiza fungi on improving biological nitrogen fixation in the legume crop.

Chapter 2

Comparing legumes for use in multiple cropping to enhance soil organic carbon, soil fertility, aggregates stability and vegetables yields under semi-arid conditions

Comparing legumes for use in multiple cropping to enhance soil organic carbon, soil fertility, aggregates stability and vegetables yields under semi-arid conditions

Abstract

Including legumes in multiple cropping systems may be regarded as a sustainable way to improve soil quality and fertility for subsequent crops. Improvements in soil quality depend on inherent soil properties, climatic conditions, adopted management practices, type of fertilization (organic, chemical, legumes or biological). Hence, the aim of this study was to compare the effect of two legume species (cowpea and fava bean) on soil organic carbon (SOC), total nitrogen (Nt), NO_3^-, NH_4^+, available P, soil aggregate stability and the subsequent yields of two vegetable crops (broccoli and melon) grown under conventional or organic systems after two multiple cropping cycles. A comparison of a broccoli monoculture, broccoli grown after cowpea (multiple cropping), a melon monoculture and melon grown after fava bean (multiple cropping) showed that the broccoli / cowpea double cropping was significantly more effective for increasing SOC and Nt than melon/fava bean double cropping. For the cowpea/broccoli multiple cropping, conventional management contributed to increasing SOC and Nt, while organic management increased available P, aggregate stability and crop yield, although this effect was cultivar dependent. The effect of management practice was not significant for the fava bean/melon except as regard crop yield, the melon yield being greater than monocrop under conventional management. Thus, the use of cowpea in multiple cropping was better for increasing SOC, soil fertility and crop yield of the subsequent crop than the use of fava bean, probably due to rhizodeposition processes. Hence, this crop could regarded as a viable alternative for sustainable crop production under semi-arid conditions.

Keywords: Soil fertility, legume, broccoli, melon, soil organic carbon, soil nitrogen.

2.1. Introduction

Maintaining current crop yield, sustaining soil quality and delivering ecosystem services are encouraged with an adoption of legume in rotation (different crops in the same area in different years) and multiple cropping (different crops in the same area in different seasons within the same year) (Yusuf et al., 2009; St Luce et al., 2015). Crop rotation and multiple cropping have long been recognized as a way of influencing soil physical properties through the reduction of soil erosion, improvements in soil structure and enhanced permeability. The same practice also improves soil fertility as a consequence of increased soil microbial activity and a higher organic matter content (Bullock, 1992; Karlen et al., 1994).

Positive effects of legume rotations and multiple cropping on subsequent crops yield have been reported by several authors (Shah et al., 2003; Smith et al., 2016), and it is primarily due to their ability to fix atmospheric nitrogen (N) through biological nitrogen fixation (BNF), and thus provide extra available N (Unkovich et al., 2008). N_2 fixation in legumes mainly occurs by symbiotic association with nodulating bacteria known as rhizobia, including bacterial genera such as *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Bradyrhizobium* (Unkovich et al., 2008). Such legume-rhizobia symbioses can benefit not only the host crop but may also have positive effects for subsequent crops due to a reduction in the amount of N fertilizers required by BNF, increasing subsequent crop yields through improved soil quality and fertility and controlling weeds (Díaz-Ambrona and Mínguez, 2001; St Luce et al., 2015). In turn, a reduction in the use of N fertilizers reduces farmers’ costs, and the environment risks linked to the release of greenhouse gas emissions (Jensen et al., 2012). Crop rotation/multiple cropping also offers diversified cropping systems, which means greater market opportunities in the face of low commodity prices and even reducing exposure to adverse climatic factors (Zegada-Lizarazu and Monti, 2011).

The establishment of an effective symbiosis between nitrogen-fixing bacteria and legumes commonly grown in a particular area provide suitable habitat for soil microorganisms. This is achieved through processes that influence nutrient cycling, such as the mineralization of preceding legume crop residues after harvest (Arcand et al., 2014) and the release of root exudates during plant development, which tend to be N-rich in legumes plant species (Fustec et al., 2010). Thus, both processes may influence the quantity, quality and distribution of soil organic matter and, therefore, sustain soil quality and fertility. After N, phosphorus is the most limiting nutrient for crops (Vance et al., 2000). This element can affect BNF directly, since it is involved in the development and functioning nodules (Pacyna et al., 2006; Sulieman and Schulze, 2010). The integration of legumes in rotation or multiple cropping provides available C and N sources, and thus promotes the abundance of gram-negative bacteria such as those belonging to *Rhizobium* genus (Voisin and Gastal, 2015; Aschi et al., 2017). Strains from the genera *Rhizobium* are phosphate-solubilizing bacteria that mobilize inorganic P through the production of organic acids, making it available for plants (Rodríguez and Fraga, 1999). In addition, species belonging to *Leguminosae* family contain secondary plant products with allelopathic potential (Rice, 1984; Razavi, 2011). These compounds are released through residues crop decomposition, root exudation or volatilisation from the aboveground plant parts to suppress the growth and size of several plant species (Akemo et al., 2000; Jabran et al., 2015). As a consequence, the use of legumes in rotations or multiple cropping could contribute to reduce weed incidence in crops (Razavi, 2011).

The influence of legumes on soil fertility is normally evident after a long period (Yusuf et al., 2009; Kirkegaard and Ryan, 2014). However, the choice of legume that will result in sustainable improvements in crop productivity is a complex matter since the selected legume has to be well adapted to local biophysical constraints such as soil type or climatic conditions, and to the particular cropping system (e.g. organic or conventional) (Peoples et al., 2001; Peoples et al., 2009). In addition, a given legume's ability to fix N_2 is limited by the amount of effective rhizobia in the soil or specific rhizobial strains that will form an effective symbiosis (Unkovich et al., 2008).

With regard to management practices, organic farming is associated with a higher external carbon input compared with conventional practices (Tuomisto et al., 2012; Gattinger et al., 2013). The application of materials of organic origin to the soil results in an improvement in soil structure (Blair et al., 2006; Tejada et al., 2008). However, at the same time, organic farming is related to lower crop yields, mainly due to a lower nitrogen use efficiency, which is limited by mineralization-immobilization processes (Mallory and Griffin, 2007; Seufert et al., 2012; Alaru et al., 2014).

In line with the benefits reported in literature about the use of multiple cropping, mostly under organic management practices, we designed a two-year field experiment with two vegetable crops with different harvesting season - melon (summer) and broccoli (winter) - cultivated as monocrops or grown after legumes (fava bean and cowpea) under conventional and organic management practices in order to compare the benefits of including legumes in multiple cropping with regard to vegetable species. The use of different management practices allowed their impact on SOC, soil fertility, aggregates stability and yield to be compared in these vegetable, which, to the best of our knowledge, has not previously been investigated. We hypothesized that the traditionally cultivated legume in the region (fava bean) may promote higher increases in SOC, soil fertility, aggregates stability and crop yield in a subsequent vegetable crop (melon) as a result of BNF than cowpea that has been never cultivated, due to a selection of symbiotic microorganisms for the fava bean host. Hence, the main objectives of this study were to: i) assess the effect of a preceding legume crop, considering two different legume cultivars as well as two different management practices, on SOC, soil aggregation, soil fertility and the yield of vegetable crops; and ii) to ascertain whether any such effects depend on the specific legume species.

2.2. Materials and methods

2.2.1. Study site and experimental design

This study was carried out in Cartagena, southeast Spain (37º 41` N 0º 57` E), at the "Tomás Ferro" Experimental Agro-Food Station of the UPCT. In this station, previous crops were always developed under conventional management practices. However, for this study, we established different plots to assess conventional and organic management practices. As a consequence, organic management was firstly implemented in the study area with this experiment, with the aim of assessing its effect. Nonetheless, to avoid negative effects of the use of pesticides/herbicides in the organic plots by dispersion from the conventional plots, pests and diseases in all plots were controlled as in the organic management. The field experiment was designed in a complete randomized block with four replications, using plots of 10 m^2. The area is characterized by a semi-arid Mediterranean climate, with a mean annual temperature of 18 ºC and mean annual rainfall of 275 mm. The soil was a *Haplic Calcisol* (IUSS, 2014) with a clay loam texture. Soil characteristics are shown in Table 2.1. The inclusion of legumes in multiple cropping with two traditional vegetables in the region (broccoli and melon) was studied during two years. The monthly precipitations during the two years that the field experiments lasted are shown in Figure 2.1. Legume residues were removed from the field and so not applied in the soil as green manure.

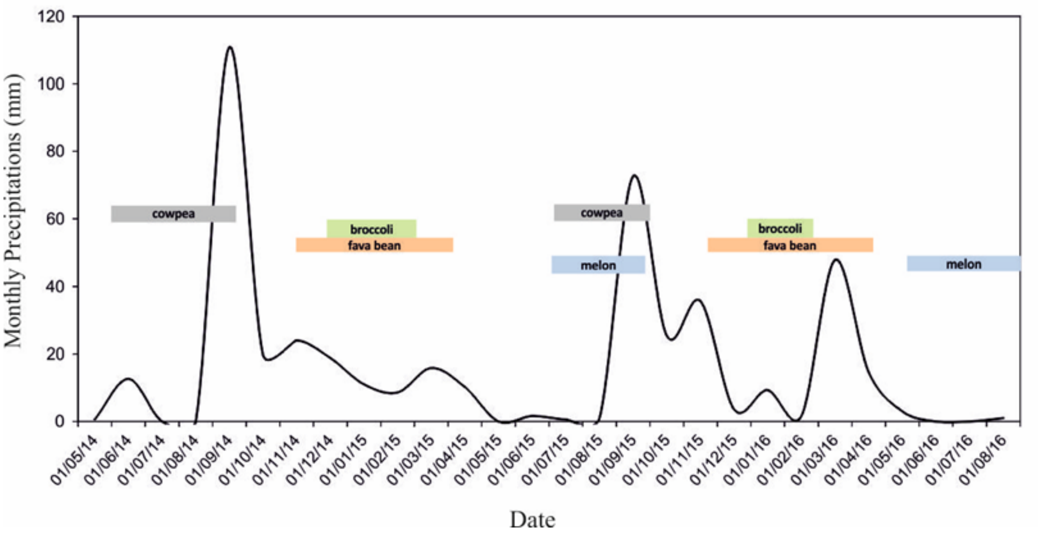


Figure 2.1: Monthly precipitations from the beginning of the experiment to the end of the second melon crop cycle.

Table 2.1: Main soil characteristics. Values shown are mean ± standard deviation (n=4).

Parameter^{a}	
pH	8.4±0.1
EC (µScm^{-1})	343±72
SOC (g kg^{-1})	12.8±0.3
Bulk density (g cm^{-3})	1.0±0.0
CEC (cmol$_{+}$ kg^{-1})	4.2±1.1
$CaCO_3$ (%)	30.2±1.2
Clay (%)	34.5±0.16
Silt (%)	21.3±1.06
Sand (%)	44.2±0.92
Aggregates stability (%)	7.3±0.6
N_t (g kg^{-1})	0.9±0.1
NO_3^- (mg kg^{-1})	150±5
NH_4^+ (mg kg^{-1})	7.7±2.7
Available P (mg kg^{-1})	25.5±3.0

^{a}:EC: electrical conductivity; SOC: soil organic carbon content; CEC: cation exchange capacity; Nt: total nitrogen.

2.2.1.1. Crop 1: cowpea-broccoli multiple cropping system

We assessed the effect of two local Portuguese cultivars (Feijão frade de fio preto (FP) and Feijão frade de fio claro (FC)) of cowpea (*Vigna unguiculata* (L.) Walp.) grown with broccoli as multiple cropping on SOC, soil fertility, aggregates stability and yield, compared with a broccoli monocrop. The broccoli monocrop was left fallow during the cowpea season (no cultivation of cowpea). Cowpea was grown during two summer seasons (29/05/2014-13/08/2014 and 03/06/2015-14/09/2015). Cowpea is not normally cultivated in the region. After each cowpea crop, the soil was prepared to cultivate broccoli (*Brassica oleracea* L. var. italica) cv Parthenon with only a surface tillage (0-20 cm) in the same furrow direction. The broccoli crop was grown during the successive two winter seasons (13/11/2014-26/02/2015 and 1/12/2015-24/02/2016).

Both crops were established under drip irrigation with two management practices: conventional and organic. Cowpea seeds were sown and broccoli plants were planted with a spacing of 100 cm between rows and 20 cm between plants (5 plants m^{-2}). No herbicide treatment was applied, and the crops were kept free of weeds through hand-hoeing when necessary. In the cowpea crop, 30 kg ha^{-1} of N and 2.4 kg ha^{-1} of P_2O_5 were applied as ammonium nitrate (33.5% N) and monoammonium phosphate (61% P_2O_5, 12% N) in the conventional practice, and using a commercial organic fertilizer (Bombardier, Agroquímicos los Triviños, Spain; 10.7% w/v N, 0.7% w/v P_2O_5) in the organic practice. In broccoli crop, 250 kg ha^{-1} N, 100 kg ha^{-1} P_2O_5 and 300 kg ha^{-1} K_2O were applied by fertigation as ammonium nitrate (33.5% N), monoammonium phosphate (61% P_2O_5, 12% N) and potassium sulphate (50% w/v K_2O, 18% S) in the conventional practice, and using two commercial organic fertilizers (Heronatur 4-2-8 and Heronatur 7-2-4; Herogra Fertilizantes, Spain; 4% w/v N, 2% w/v P_2O_5 and 8% w/v K_2O, and 7% w/v N, 2% w/v P_2O_5 and 4% w/v K_2O) in the organic practice.

2.2.1.2. Crop 2: fava bean-melon multiple cropping system

We assessed the effect of two local Spanish cultivars (Muchamiel (M) and Palenca (P) - of fava bean (*Vicia faba* L.)) grown with melon as multiple cropping on SOC, soil fertility, aggregates stability and yield, compared with a melon monocrop. The melon monocrop was left fallow during the fava bean season (no cultivation of fava bean). Fava bean was grown during two winter seasons (24/10/2014-02/03/2015 and 05/11/2015-13/04/2016). Fava bean is a traditional legume crop in the region. After the fava bean crop, the soil was prepared to grow a melon *Cucumis melo* L. cv. Hidalgo with only a surface tillage (0-20 cm) in the same furrow direction. The melon crop was grown during the successive two summer seasons (15/06/2015-08/09/2015 and 13/06/2016-23/08/2016).

Both crops were established under drip irrigation with two management practices: conventional and organic. Fava bean seeds were sown with a spacing of 100 cm between rows and 40 cm between plants (2.5 plants m^{-2}) while melon plants were planted with a spacing of 200 cm between rows and 120 cm between plants (0.8 plants m^{-2}). No herbicide treatment was given, and the crops were kept free of weeds through hand-hoeing when necessary. In the fava bean crop, 20 kg ha^{-1} of N and 1.2 kg ha^{-1} of P_2O_5 were applied as ammonium nitrate (33.5% N) and monoammonium phosphate (61% P_2O_5, 12% N) in the conventional practice, and using a commercial organic fertilizer (Bombardier, Agroquímicos los Triviños, Spain; 10.7% w/v N, 0.7% w/v P_2O_5) in the organic practice. In the melon crop, 200 kg ha^{-1} N, 120 kg ha^{-1} P_2O_5 and 340 kg ha^{-1} K_2O were applied by fertigation as ammonium nitrate (33.5% N), monoammonium phosphate (61% P_2O_5, 12% N) and potassium sulphate (50% w/v K_2O, 18% S) in the conventional practice, and using a commercial organic fertilizer (Espartán Agroindustrial Kimitec S.L, Spain; 3.8% w/v N, 2.9% w/v P_2O_5 and 3.6% w/v K_2O) and potassium sulphate (50% w/v K_2O, 18% S) in the organic practice.

2.2.2. Soil sampling and crop yield

Soil samples (0-20 cm) were collected at the beginning of the experiments before sowing the legumes and upon harvesting each vegetable at the end of the second multiple cropping cycle. Three random soil samples per plot were collected and homogenized to obtain a composite sample. Samples were air-dried for 7 days, sieved < 2 mm and stored at room temperature until analyses, except for NH_4^+ and NO_3^-, where an aliquot of each sample was stored at 4ºC to avoid undesirable mineralization/oxidation processes and sieved < 2 mm previously to analyses, within 4 days from sampling.

Broccoli crop yield was determined by weighing all the heads per plot when the buds of the head were firm and tight, while the melon yield was determined by weighing all the fruits per plot when they were ripe and ready for consumption. With regard to legumes, all the pods in each plot were harvested when the seeds were dried at the end of the crop cycle for cowpea, and were continuously harvested and weighed when the seeds were fresh for fava bean.

2.2.3. Soil analyses

Bulk density was measured using the cylinder method (5 cm diameter x 5 cm high) (Blake and Hartage, 1986); soil pH and electrical conductivity (EC) were measured in deionized water (1:2.5 and 1:5 w/v, respectively); soil texture was determined by the Bouyoucos hydrometer method (Dewis and Freitas, 1970); soil aggregate stability (AS) was assessed with the rainfall simulator method according to Roldán et al. (1994). For equivalent calcium carbonate the volumetric method (Bernard calcimeter) was used (Cobertera, 1993); SOC was determined by the wet oxidation method using $K_2Cr_2O_7$ (Walkley and Black, 1934); total nitrogen (Nt) was analyzed by the Kjeldahl method (Hoeger, 1998); NO_3^- and NH_4^+ were extracted with 2*M* KCl in a 1:10 soil:extractant ratio (Keeny and Nelson, 1982); then, NO_3^- was measured by ion chromatography (Metrohm 861), and NH_4^+colorimetrically (Kandeler and Gerber, 1988); cation exchange capacity was determined using $BaCl_2$ as exchangeable salt (Roig et al, 1980); available phosphorus (P) was extracted according to the Burriel-Hernando method (Díez, 1982) and measured using ICP-MS (Agilent 7500CE).

2.2.4. Statistical analyses

Data were checked to ensure normal distribution using the Kolmogorov–Smirnov test and transformed when necessary to ensure normal distribution. The average value of soil parameters measured at the beginning of the experiment was subtracted from values at the end of the second cycle and divided by the initial values to obtain the increment data (time Δ data) according to the following equation 1:

$$\text{time } \Delta \text{ data} = [(\text{Final value} - \text{Initial value}) / \text{Initial value}] \times 100 \qquad (1)$$

Similarly, the average value of soil parameters and crop yield measured from each vegetable monocrop was subtracted from its respective crop grown after legumes and divided by the monocrop values to obtain the multiple cropping Δ data according to the following equation 2:

$$\text{Multiple cropping } \Delta \text{ data} = [(\text{Legume multiple cropping value} - \text{Monocrop value}) / \text{Monocrop value}] \times 100 \quad (2)$$

Using this approach, the relative increases or decreases for all properties can be used to compare all the properties between both crop systems since the unit can be homogenized to percentage of variation.

The Δ data were submitted to three-way ANOVA to assess the differences among previous legume cultivars, vegetable crop type and management practices at the end of the experiments. Relationships among the Δ data were studied using Pearson's correlations. Multiple linear regression analysis ($Y=m_1X_1 +m_2X_2 +\cdots+m_nX_n +b$) was carried out with the multiple cropping Δ data after two years of multiple cropping with legumes using stepwise and backward methods, with available phosphorus as independent variable and SOC, aggregates stability, NH_4^+ and NO_3^- as dependent variables. Standardized coefficient (β) and partial correlation values were used for the analysis. The β coefficient is the estimated value resulting from the analysis performed on variables that have been standardized to have a variance of 1 in order to determine which of the independent variables has a greater effect on the dependent variable. Therefore, variables with larger β coefficients contribute more to the model. The partial correlation indicates the correlation between the dependent variable and one independent variable when the linear effects of the remaining variables have been eliminated. The unstandardized coefficients (m) were used to fit the values of phosphorus *versus* the values calculated using the regression model. Furthermore, a principal components analysis (PCA) was performed with all Δ data of multiple cropping with regard to monocrops to study the structure of dependence and correlation established among the variables studied in both vegetable cropping systems. Statistical analyses were performed with the software IBM SPSS for Windows, Version 22.

2.3. Results

2.3.1. Changes in soil properties

Variation rate in the studied soil properties with regard to initial value (time Δdata) are presented in Table 2.2 (absolute values are shown in the Table 2.3). The variation of soil properties (Table 2.2) with regard to initial soil characteristics (Table 2.1) was significantly influenced by type of vegetable crops and previous legume cultivars, except ΔSOC in the case of previous legume cultivar (Table 2.2).

Table 2.2: Variation rates (%) ± standard deviation of the studied soil properties in both vegetables (broccoli and melon) after two years of multiple cropping with legumes with regards to initial values at the beginning of the experiments.

Previous legume cultivar[a]	Management practice	ΔSOC	ΔN_t	ΔAS	ΔNH_4^+	ΔNO_3^-	ΔP
				Broccoli			
FP cowpea	Conventional	-2.8±7.4	39.1±3.1	90.8±100.8	-7.1±51.7	162.1±118.8	-28.8±22.1
FC cowpea	Conventional	-3.1±6.1	34.1±8.5	97.9±55.1	-17.1±19.3	60.8±97.5	3.1±14.7
Fallow	Conventional	-19.6±24.3	-91.2±0.4	33.4±37.2	-73.7±5.2	7480±2625	74.3±4.5
FP cowpea	Organic	0.9±16.1	22.5±3.5	157.9±33.3	-78.7±4.0	95.1±82.9	-17.1±9.0
FC cowpea	Organic	-7.5±4.5	31.1±3.0	160.8±83.7	-9.7±27.5	466.0±44.0	-30.9±25.8
Fallow	Organic	-10.9±2.4	-91.2±0.3	44.6±53.6	-34.8±15.2	5926±2664	29.9±34.6
				Melon			
M fava bean	Conventional	-1.6±4.9	12.7±0.6	351.9±33.4	-2.1±5.1	-73.6±2.8	122.7±14.9
P fava bean	Conventional	9.2±9.3	20.2±2.9	399.1±86.7	-0.8±9.0	-76.1±0.1	178.9±31.3
Fallow	Conventional	17.6±8.3	-91.0±0.9	165.3±13.5	281±4.6	-35.2±126	398±0.8
M fava bean	Organic	-0.1±1.9	23.0±1.8	320.5±121.2	-7.3±10.3	-45.2±30.9	161.2±3.1
P fava bean	Organic	2.1±4.5	28.2±6.0	399.1±86.7	-17.6±0.0	-56.6±6.9	116.0±12.8
Fallow	Organic	8.4±6.1	-90.6±0.5	212.7±19.5	600.0±0.7	-43.7±130	416.0±45.2
				F-value[b]			
Vegetable crop type (VCT)		15.4***	34.1***	84.2***	9.1**	39.2***	913.2***
Previous legume cultivar (PLC)		0.1ns	4313.3***	15.8***	10.8***	41.9***	218.6***
Management practice (MP)		0.1ns	0.0ns	1.2ns	2.1ns	0.1ns	2.6ns
VCT x PLC		6.0**	10.8***	2.2ns	2.5ns	30.7***	72.5***
VCT x MP		1.2ns	28.7***	0.8ns	0.0ns	0.5ns	1.8ns
PLC x MP		0.5ns	1.8ns	0.0ns	6.1**	0.4ns	7.9**
VCT x PLC x MP		0.6ns	10.4**	0.8ns	7.5**	0.9ns	3.1ns

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro; M: Muchamiel; P: Palenca.

[b] Significant at ***P < 0.001; ** P < 0.01; *P < 0.05; ns: not significant (P > 0.05).

SOC: soil organic carbon content; N_t: total nitrogen; AS: aggregates stability.

Table 2.3: studied soil properties at the beginning of the experiments and at harvest of both vegetable crops (broccoli and melon) after the second year of multiple cropping with legumes. Values are mean ± standard deviation (n=4).

Previous legume cultivar[a]	Management Practice	SOC (g kg^{-1})	N$_t$ (g kg^{-1})	Aggregates stability (%)	NH$_4^+$ (mg kg^{-1})	NO$_3^-$ (mg kg^{-1})	Available P (mg kg^{-1})
Broccoli							
Fallow (initial)	-	12.86±0.36	0.91±0.03	7.28±0.61	7.70±2.77	150±5	25.48±3.04
FP cowpea	Conventional	12.50±0.95	1.28±0.03	13.90±7.35	7.15±3.98	394±179	18.14±5.64
FC cowpea	Conventional	12.47±0.79	1.23±0.08	14.43±4.02	6.39±1.49	242±146	26.29±3.75
Fallow	Conventional	10.35±3.09	1.13±0.05	17.17±4.79	13.38±0.67	975±337	22.43±0.59
FP cowpea	Organic	12.99±2.07	1.13±0.03	18.80±2.43	1.64±0.31	293±124	21.11±2.31
FC cowpea	Organic	11.90±0.58	1.20±0.03	19.01±6.10	6.95±2.12	852±66	17.59±6.58
Fallow	Organic	11.46±0.31	1.13±0.04	18.36±6.90	8.38±1.95	775±334	16.72±4.46
Melon							
Fallow (initial)	-	12.86±0.36	0.91±0.03	7.28±0.61	7.70±2.77	150±5	25.48±3.04
M fava bean	Conventional	12.66±0.90	1.03±0.01	32.93±3.44	7.54±0.56	39±6	56.76±5.38
P fava bean	Conventional	14.05±1.70	1.10±0.04	36.37±8.94	7.63±0.98	36±0	71.08±11.31
Fallow	Conventional	15.14±1.62	1.15±0.17	34.15±2.47	8.33±0.84	49±23	64.16±0.15
M fava bean	Organic	12.85±0.36	1.13±0.02	30.64±12.49	7.14±1.12	82±66	66.58±1.12
P fava bean	Organic	13.15±0.82	1.18±0.08	36.37±8.94	6.35±0.00	65±15	55.04±4.62
Fallow	Organic	13.95±1.11	1.21±0.09	40.25±3.55	7.24±0.14	90.21±23.70	66.45±8.23

[a]FP: Feijão frade de fio preto; FC: Feijão frade de fio claro; M: Muchamiel; P: Palenca.
SOC: soil organic carbon; N$_t$: total nitrogen.

In terms of ΔSOC, soils under broccoli crop showed higher decreases than melon crop ($P < 0.001$). Soil Nt and aggregate stability (AS) increased after two years in both multiple cropping systems compared with initial values ($P < 0.001$). ΔNt was significantly higher in the broccoli than in melon ($P < 0.001$), but was significantly lower in the monocrop ($P < 0.001$). The melon showed more pronounced increase in ΔAS (320-399%) than the broccoli (91-161%), and was significantly higher in the multiple cropping systems than in the monocrop, underlining the positive effect of the legume in increasing soil fertility and structure. Available N forms (NH_4^+ and NO_3^-) behaved differently according to the type of vegetable crop. The ΔP was significantly different in terms of type of vegetable crop and previous legume cultivars, generally being negative for broccoli in multiple cropping and positive for melon in multiple cropping. The management practice did not significantly influence any of the soil properties studied. The interaction of vegetable crop type with management practice was only significant for ΔN_t ($P < 0.001$).

Multiple linear regression analysis (Table 2.4) showed that the variation in soil available P in both vegetable crops after multiple cropping was positively related to ΔSOC, Δaggregates stability and ΔNH_4^+, and was negatively related to ΔNO_3^- ($R^2 = 0.87$, $F = 34.34$, $P < 0.001$).

Table 2.4: Multiple linear regression model for ΔP in both vegetable crops in multiple cropping (broccoli and melon) after two years of multiple cropping with legumes with regard to initial values at the beginning of the experiments.

Y	*X*	m	Partial correlation	β	R^2	R^2 adj	F value
ΔP	Constant (b)	1.52			0.87	0.85	34.34***
	ΔSOC	2.43	0.50	0.22			
	ΔAS	0.34	0.81	0.57			
	ΔNH_4^+	0.81	0.61	0.28			
	ΔNO_3^-	-0.14	-0.56	-0.29			

m: unstandardized coefficients; β: standardized coefficients. Significant at ***$P<0.001$

P: phosphorus; SOC: soil organic carbon content; AS: aggregates stability

2.3.2. Effect of multiple cropping with legumes on soil properties and crop yield

Table 2.5 shows the variation rate of multiple cropping systems with regard to monocrop (multiple cropping Δdata) in the studied soil properties (absolute values are shown in the Table 2.6). Vegetables significantly influenced the variation in soil properties such as SOC, Nt and NO_3^-. Broccoli grown after cowpea increased the values of SOC and Nt with regards to broccoli monocrop, while both properties decreased in the melon grown after fava bean compared with melon grown as a monocrop ($P < 0.001$). The ΔNO_3^- was significantly lower in both crops in multiple cropping than when grown as monocrops ($P < 0.01$). NH_4^+ also decreased in multiple cropping with regard to monocrop except in the case of broccoli grown after cowpea under conventional management practice, in which case ΔNH_4^+ was positive and significantly different from the values obtained with organic management ($P < 0.01$). Type of vegetable crops, previous legume cultivar or management practice did not influence ΔAS. The interaction of previous legume cultivar with management practice was significant for ΔP. ΔP was highest in broccoli after growing FP compared with monocrop under organic practice; however, ΔP was highest in melon after P fava bean cultivar compared with monocrop under conventional practice ($P < 0.01$). On the other hand, the interaction of type of vegetable crops with management practice significantly influenced ΔSOC, ΔN_t, and ΔNH_4^+ ($P < 0.05$; $P < 0.001$ and $P < 0.01$, respectively).

Table 2.5: Variation rates (%) ± standard deviation of the studied soil properties and crop yield in both vegetable crops (broccoli and melon) at the end of the second crop cycle with regards to broccoli and melon monocrops.

Previous legume cultivar[a]	Management practice	ΔSOC	ΔN_t	ΔAS	ΔNH_4^+	ΔNO_3^-	ΔP	ΔCrop yield
				Broccoli				
FP cowpea	Conventional	20.8±9.2	13.1±2.5	-19.0±42.8	111.6±117.8	-59.5±18.3	-19.1±25.1	-9.6±4.2
FC cowpea	Conventional	20.5±7.6	9.0±6.9	-15.9±23.3	89.0±43.9	-75.1±15.0	17.2±16.7	-0.8±8.6
FP cowpea	Organic	13.3±18.0	-0.4±2.8	2.3±13.2	-80.4±3.7	-62.1±16.1	26.2±13.8	3.3±9.9
FC cowpea	Organic	3.8±5.0	6.6±2.4	3.5±33.2	-17.0±25.3	9.9±8.5	5.2±39.3	4.5±2.7
				Melon				
M fava bean	Conventional	-16.4±4.2	-10.3±0.5	-3.5±7.1	-9.5±4.7	-19.1±8.6	-11.5±5.9	-2.4±9.2
P fava bean	Conventional	-7.2±7.9	-4.3±2.3	6.5±18.5	-8.4±8.3	-26.8±0.3	10.8±12.4	-2.9±0.2
M fava bean	Organic	-7.9±1.8	-6.8±1.3	-23.8±21.9	-1.4±10.9	-8.6±51.7	0.2±1.2	-11.7±1.5
P fava bean	Organic	-5.8±4.1	-2.8±4.5	-9.6±15.6	-12.3±0.0	-27.5±11.7	-17.1±4.9	-11.5±7.5
				F-value[b]				
Vegetable crop type (VCT)		46.2***	86.0***	0.0ns	3.2ns	8.7**	2.3ns	0.1ns
Previous legume cultivar (PLC)		0.0ns	5.1*	0.5ns	0.1ns	0.7ns	0.4ns	0.2ns
Management practice (MP)		1.0ns	3.6ns	0.0ns	15.5**	6.7*	0.3ns	1.0ns
VCT x PLC		2.2ns	1.5ns	0.2ns	0.4ns	5.4*	0.1ns	0.2ns
VCT x MP		5.8*	13.5***	3.7ns	16.4**	4.2ns	2.5ns	0.8ns
PLC x MP		1.3ns	2.5ns	0.0ns	0.9ns	4.6ns	9.8**	2.0ns
VCT x PLC x MP		0.0ns	5.3*	0.0ns	1.7ns	7.7*	0.3ns	2.2ns

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro; M: Muchamiel; P: Palenca.
[b] Significant at ***P < 0.001; ** P < 0.01; *P < 0.05; ns: not significant (P > 0.05).
SOC: soil organic carbon content; N_t: total nitrogen; AS: aggregates stability.

Table 2.6: studied soil properties and crop yield in both vegetable crops (broccoli and melon) at the end of the second crop cycle. Values are mean ± standard deviation (n=4).

Previous legume cultivar[a]	Management Practice	SOC (g kg^{-1})	N_t (g kg^{-1})	Aggregates Stability (%)	NH_4^+ (mg kg^{-1})	NO_3^- (mg kg^{-1})	Available P (mg kg^{-1})	Crop yield (kg ha^{-1})
Broccoli								
FP cowpea	Conventional	12.50±0.95	1.28±0.03	13.90±7.35	7.15±3.98	394±179	18.14±5.64	27200±1261
FC cowpea	Conventional	12.47±0.79	1.23±0.08	14.43±4.02	6.39±1.49	242±146	26.29±3.75	29.850±3295
Fallow	Conventional	10.34±3.09	1.12±0.05	17.17±4.79	3.37±0.67	975±337	22.43±0.59	30100±1559
FP cowpea	Organic	12.99±2.07	1.13±0.03	18.80±2.43	1.64±0.31	293±124	21.11±2.31	23400±3317
FC cowpea	Organic	11.90±0.58	1.20±0.03	19.01±6.10	6.95±2.12	852±66	17.59±6.58	19350±2600
Fallow	Organic	11.45±0.31	1.12±0.04	18.36±6.90	8.37±1.95	775±342	16.72±4.46	22650±4743
Melon								
M fava bean	Conventional	12.66±0.90	1.03±0.01	32.93±3.44	7.54±0.56	39.73±5.99	56.76±5.38	17240±1640
P fava bean	Conventional	14.05±1.70	1.10±0.04	36.37±8.94	7.63±0.98	35.96±0.24	71.08±11.31	17160±40
Fallow	Conventional	15.14±1.62	1.15±0.17	34.15±2.47	8.33±0.84	49.14±23.10	64.16±0.15	17680±1360
M fava bean	Organic	12.85±0.36	1.13±0.02	30.64±12.49	7.14±1.12	82.46±65.95	66.58±1.12	18400±320
P fava bean	Organic	13.15±0.82	1.18±0.08	36.37±8.94	6.35±0.00	65.37±14.90	55.04±4.62	18440±1560
Fallow	Organic	13.95±1.11	1.21±0.09	40.25±3.55	7.24±0.14	90.21±23.70	66.45±8.23	20840±3720

[a]FP: Feijão frade de fio preto; FC: Feijão frade de fio claro; M: Muchamiel; P: Palenca.
SOC: soil organic carbon, N_t: total nitrogen.

The PCA performed with Δ values of most studied soil properties and crop yield of multiple cropping systems with regard to monocrops (multiple cropping Δdata; Figure 2.2) showed that 66.3% of the total variation could be explained by the first two PCs. PC1, which explained 36.5% of variation, separated the broccoli crop (positive scores) from melon (negative scores). Cowpea/broccoli multiple cropping system was related to higher increases in SOC and Nt, and higher decreases in NO_3^- (Table 2.7). In the case of broccoli, PC1 also separated the conventional management practice from organic practice, with greater increases in SOC and Nt, and greater decreases in NO_3^- in the conventional management. PC2, which explained 29.8% of the variation, slightly separated management practices in both crops. In broccoli grown after FP, organic management led to higher factor scores, and so was associated with higher increases in P, AS and crop yield. By contrast, in melon, conventional management provided the highest factor scores in PC2.

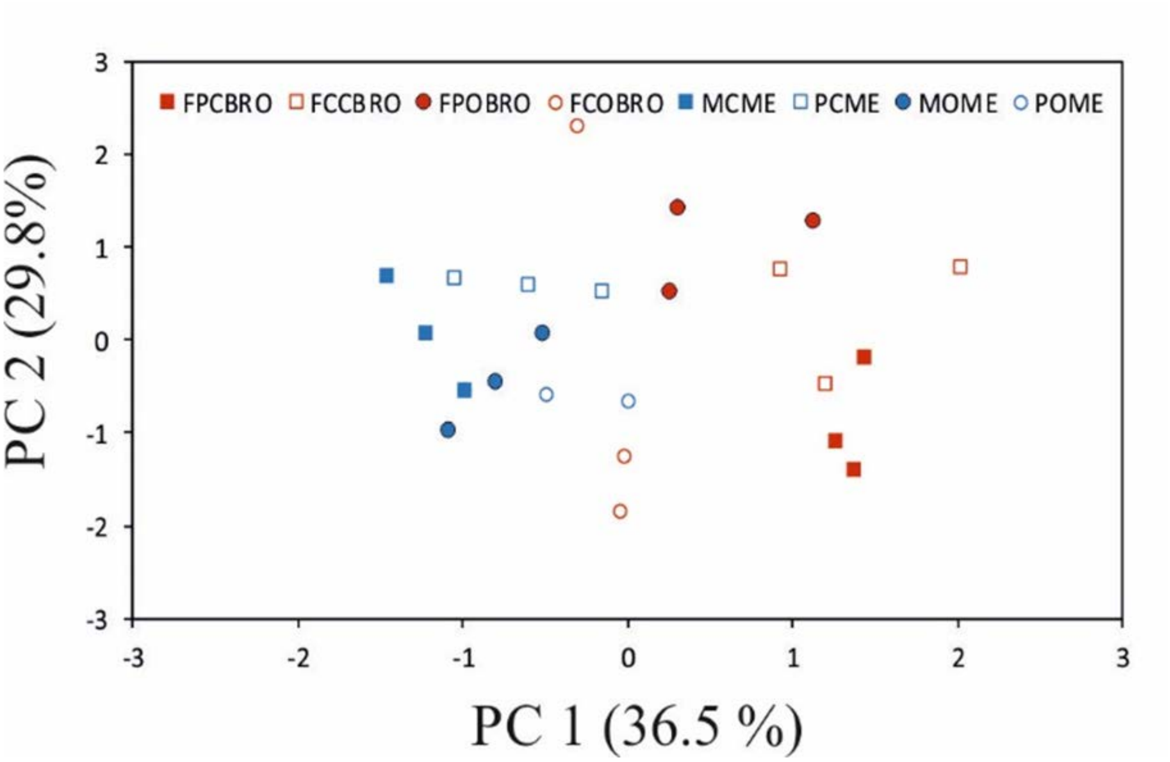


Figure 2.2: PCA factor scores of variations in soil properties and crop yields in vegetable crops grown after grain legumes with regard to monocrops, considering different previous legume cultivars and management practices. Color represents vegetable crop type (red: broccoli crop; blue: melon crop), figure type represents management practice (square: conventional; circle: organic) and figure filling represents previous legume cultivar (filled figure: FP and M in broccoli and melon, respectively; empty figure: FC and P in broccoli and melon, respectively). Broccoli grown after FP cowpea under conventional management practice (FPCBRO), broccoli grown after FC cowpea under conventional management practice (FCCBRO), broccoli grown after FP cowpea under organic management practice (FPOBRO), broccoli grown after FC cowpea under organic management practice (FCOBRO), melon grown after M fava bean under conventional management practice (MCME), melon grown after P fava bean under conventional management practice (PCME), melon grown after M fava bean under organic management practice (MOME) and melon grown after P fava bean under organic management practice (POME).

Table 2.7: Matrix of PCA obtained with variation rates (%) of the most of studied soil properties of vegetable crops in multiple cropping and monocrop.

Variance explained	PC1 (36.5%)	PC2 (29.8%)
ΔSOC	0.940	-0.002
ΔNt	0.862	-0.105
ΔNO_3^-	-0.703	-0.165
ΔAvailable P	0.201	0.835
ΔAggregates stability	-0.160	0.594
ΔCrop yield	0.079	0.837

SOC: soil organic carbon content; Nt: total nitrogen.

2.4. Discussion

There is little information available on the effect of traditional and “non-usual” legumes grown in multiple cropping with vegetable crops under semi-arid conditions including different management practices. In the present case, the introduction of multiple cropping including a legume not commonly grown in the region - cowpea cultivated with broccoli in the same year - performed better than the traditional crop of fava bean grown with melon mainly for improving SOC and total and available N contents. The positive effects of legume-based multiple cropping on soil are related to their ability to fix atmospheric nitrogen, which provides N to the agro-ecosystem, compared to non-legume monocultures. Biologically fixed N is incorporated into the soil through roots, rhizodeposits and the mineralization of above-ground residues after harvest (Laberge et al., 2009). Ball et al. (2005) observed that the introduction of legumes in multiple cropping provided better soil structure, which, in turn, involves better root growth, water and nutrient uptake and thus increased yields (Lipiec and Hatano, 2003).

The increases in SOC and available N associated with a previous cowpea crop compared with broccoli monocrop may be due to a higher release of substances derived from rhizodeposition by cowpea, since there was a lack of nodules in cowpea roots (data not shown). This last observation suggests the absence of specific rhizobial strains that form an effective association with cowpea. The inoculation of rhizobia in cowpea crops where this species has not been previously cultivated may even enhance its positive effects on soil. In fact, rhizobia could increase soil fertility since their symbioses with legume represents an N supply for the plant via BNF. This is translated into a direct increase of nutrients as a consequence of soil organic matter degradation derived from plant residues or root exudation (Hountin et al., 1997). Plant photosyntethically fixed C is the primary source of rhizodeposited C (Nguyen, 2003). Apart from this, legume roots also deposit N-compounds such as NH_4^+ (Brophy and Heichel, 1989). The quantity and quality of root exudates vary according to the plant genetic and growth stage (Jones et al., 2004). In contrast to cowpea, fava bean roots were nodulated (data not shown), which indicates that BNF was taking place. However, SOC and soil fertility in the subsequent melon crop did not much improve compared with the melon monocrop. This may be due to the fact that fava bean removed a higher amount of fixed N in the seed at harvest, which may limit its contribution to positive soil balances (Peoples and Craswell, 1992). This idea was supported by the fact that the N content was significantly higher in fava bean seeds (44 g kg^{-1}) than in cowpea seeds (34 g kg^{-1}) (Table 2.8).

Table 2.8: Nitrogen content in cowpea and fava bean seeds at harvest of the second crop cycle. Values are mean ± standard deviation (n=4).

Previous legume cultivar[a]	Management Practice	N in seeds (g kg^{-1})
FP cowpea	Conventional	31.2±3.1
FC cowpea	Conventional	32.3±0.9
FP cowpea	Organic	38.1±5.7
FC cowpea	Organic	34.5±1.6
M fava bean	Conventional	44.2±2.1
P fava bean	Conventional	43.4±2.0
M fava bean	Organic	44.9±2.9
P fava bean	Organic	44.4±1.5
F-value[b]		12.7***

[a]FP: Feijão frade de fio preto; FC: Feijão frade de fio claro; M: Muchamiel; P: Palenca.
[b]Significant at ***$P < 0.001$.

In addition, the difference between both legumes in terms of improvement of SOC and soil fertility in the subsequent vegetable crop may have been aided by the fact that plant genetics influence the rhizodeposition process (Nguyen, 2003), and cowpea seems to be more active in releasing root exudates than fava bean. Furthermore, precipitations during September 2015 (72.60 mm) after the second cowpea cycle, as well as March 2016 (47.80 mm) after the second fava bean cycle (Figure 2.1), may have affected the nitrate concentration in soil because of leaching processes (Huang et al., 2017), which implies the loss of the main N source for crop nutrition.

The improvement of soil physical properties such as AS after multiple cropping compared to fallow period suggests that diversification of crops plays an important role in terms of quality of soil organic matter (SOM) accumulated in the soil by litter, green manure and rhizodeposition (Raphael et a., 2016). Improvements in SOM quantity and quality may have enhanced the formation of soil aggregates through associations of the sources of carbon with cations and soil particles (Bronick and Lal, 2005). In contrast, fallow periods do not receive any organic matter supply, which results in lower aggregates stabilization.

There was a positive relationship between the variations in soil properties, such as SOC, AS and NH_4^+, with variations in available P. Mechanisms such as the inclusion of diversified cropping systems tend to increase organic matter storage in aggregates, which may also increase P retention and stabilization. In addition, macro-aggregates represent important sites for organic P storage (Nesper et al., 2015). C and N dynamics are associated with P availability through processes as N fixation or C sequestration (Tiessen, 2008). In the former process, P is vital in the metabolic energy processes that drive symbiotic N_2 fixation (Sulieman et al., 2013) while in the latter, it acts as an important structural element in nucleic acid, which regulates energy storage and transfer (Wang et al., 2013a). Thus, this could explain the positive correlation observed between increases in the phosphorus content with increases in SOC and AS.

With regard to management practices, the conventional practice resulted in increased in SOC, N_t and NH_4^+ and declined in NO_3^- content in broccoli grown after cowpea compared with the monocrop (Figure 2.2 and Table 2.5). Conventional management also stimulated higher increases in P, AS and crop yield in melon grown after fava bean compared with the monocrop (Figure 2.2). These results reveal the lower available nutrient contents and subsequent declined crop yields after the use of organic practices in melon, which could be because the supplied N is organically bound and so depends on the mineralization of soil organic matter to become available to the plant. In turn, lower crop yields under organic management practices may lead to lower carbon inputs to the soil from photosynthesis, (Leifeld et al., 2013), and/or higher SOM mineralization by active microbial populations, which can cause positive priming effect by addition of external organic inputs (Blagodatskaya and Kuzyakov 2008). Previous research has shown that in spite of addition of organic amendments into the soil, SOC content does not necessarily increase owing to priming effect, since it is controlled by the amount of organic amendment applied in relation to the soil organic matter (Fontaine et al., 2004; Kuzyakov 2010).

Therefore, this agroecosystem requires a longer period to supply sufficient plant-available N, which implies the need for more land to produce the same amount of food (Seufert et al., 2012). By contrast, organic management led to slight increases in crop yield in broccoli grown after cowpea compared with monocrop (Table 2.5). This may be explained by the fact that cowpea can grow under low-input farming systems and is well adapted to adverse conditions such as high temperatures or drought (Elhers and Hall, 1997; Santiago de Freitas et al., 2012).

2.5. Conclusions

In conclusion, our results showed, contrary to our initial hypothesis, that the inclusion of cowpea, despite being an unusual crop in SE Spain, improved SOC and soil fertility in a subsequent broccoli crop compared with the broccoli monocrop, probably due to active rhizodeposition. The establishment of a traditional crop such as fava bean grown with melon did not greatly improve SOC and soil fertility with regard to the monocrop because a higher amount of fixed N was removed in the seed at harvest. Increases in available phosphorus were directly linked to increases in organic C, N and soil aggregation through N fixation and C sequestration processes. Conventional management was positively related to C and N pools in the broccoli crop. Organic management was related to improvements in soil structure and crop yield in the broccoli crop. Thus, cowpea crop in multiple cropping was seen to represent a good alternative for sustainable production, since it led to improvements in soil structure and subsequent crop yield under organic management.

Chapter 3

Comparison of soil organic carbon pools and microbial activity in two horticultural multiple cropping systems under Mediterranean conditions

Comparison of soil organic carbon pools and microbial activity in two horticultural multiple cropping systems under Mediterranean conditions

Abstract

Multiple cropping with legumes may play an important role in nutrient cycle through soil organic matter dynamic and biological activity, although this contribution depends on the specific plant species. Organic management is in addition expected to decrease the negative impacts of conventional intensive farming through soil C sequestration and activation of microbial populations. Thus, the aim of this study was to compare the effect of different cultivars of two legume species (cowpea –unusual in the study area- and fava bean –traditional in the study area) on C content and pools, N content and soil enzyme activities of subsequent vegetable crops (broccoli and melon, respectively) grown under conventional or organic systems after two multiple cropping cycles. Cowpea/broccoli multiple cropping was significantly more effective than fava bean grown after melon for increasing soil organic C (SOC), N and soil enzyme activities compared with monocrop. For the cowpea/broccoli multiple cropping, organic management contributed to higher C sequestration, while for the fava bean/melon multiple cropping was the conventional management practice that more increased SOC. The legume cultivar significantly affected soil N and dehydrogenase activity. Hence, previous cowpea crop in multiple cropping resulted in a sustainable alternative under semiarid conditions, since it increased soil quality of the subsequent vegetable crop likely due to active rhizodeposition, with more efficiency than fava bean.

Keywords: *Vigna unguiculata*; *Vicia faba*; diversification; management practices; carbon dynamic; nitrogen dynamic.

3.1. Introduction

Diversification of crop along rotations or multiple cropping plays an important role in nutrient cycle through soil organic matter (SOM) dynamic, although this contribution depends on the specific plant species (Raphael et al., 2016; Tivet et al., 2013). In this context, the inclusion of grain legumes in rotation and multiple cropping assumes a good alternative from environment, agricultural and economic viewpoint. Positive effects of legume rotation and multiple cropping are primarily due to their ability to fix atmospheric nitrogen (N) through their association with *Rhizobium* bacteria, and thus provide extra available N, which can lead to a decrease in the use of external fertilizers (Jensen et al., 2012; Unkovich et al., 2008). The establishment of an effective legume-rhizobia symbiosis results in a suitable habitat for soil microorganisms through processes that influence nutrient cycling, such as the mineralization of legume crop residues after harvest (Arcand et al., 2014) or the release of root exudates during plant development, which tend to be N-rich in legumes plant species (Fustec et al., 2010). Both processes influence the SOM content, which not only contributes to improve soil structure and increase soil fertility, but also improves biological properties (Hargreaves et al., 2003; Kaiser et al., 2008). In addition, excessive use of external inputs can decrease soil microbial diversity and biological soil processes (Paul and Clark, 1996). The assay of soil enzyme activities is more sensitive than soil physicochemical properties. They play an important role in soil organic matter decomposition, nutrient availability and soil fertility (Bastida et al., 2008; Nannipieri et al., 2002).

SOM and nutrients content can be enhanced by the management practice due to the introduction of different external inputs into the agro-ecosystem. With this regard, organic management practice is linked to an increase in SOM and microbial activity compared to conventional management (Melero et al., 2006). Agricultural practices such as N fertilization or crop rotations/multiple cropping influence capture and storage of atmospheric C through C sequestration (López-Bellido et al., 2010). Soil organic carbon (SOC) is divided into different pools (recalcitrant and labile). Recalcitrant fraction is composed of organic materials that are highly resistant to microbial decomposition; because of this, it is linked to efficient C storage and sequestration. Labile organic C is composed of those fractions of C easily mineralizable by soil microorganisms (Haynes, 2005; Laganiere et al., 2010). In terms of soil organic carbon decomposition, plants provide C and N compounds through root exudation, which promote soil microbial activity and thus, faster decomposition (Sugiyama and Yazaki, 2012). In addition, root exudates of legumes are especially rich in N (Fustec et al., 2010). However, the increase of N availability in the soil may lead to a reduction in the production of enzymes to mineralize N derived from recalcitrant organic matter (Craine et al., 2007).

A key factor for the sustainable agricultural management is the choice of legume species that result in effective improvements in soil quality and crop production and quality, by active root exudation and efficient soil microbial rhizostimulation. In this sense, the selected legume has to be well adapted to the local biophysical characteristics, since its ability to fix atmospheric N is limited by the amount of effective soil rhizobia or specific rhizobial strains that will form an effective symbiosis (Peoples et al., 2001; Peoples et al., 2009; Unkovich et al., 2008).

In this study we have designed a two-year field experiment with two vegetable crops with different harvesting season - melon (summer) and broccoli (winter) - cultivated as monocrops or grown after legumes (fava bean and cowpea, respectively) under conventional and organic management practices in order to compare the benefits of including legumes in multiple cropping compared to non-legume vegetables. The use of different management practices allows to evaluate their impact on soil C sequestration and microbial activity compared with vegetable monocrops. We hypothesized that fava bean, being a traditional crop in the study region, may stimulate with higher intensity soil microbial populations, with increases in recalcitrant and labile organic carbon pools in a subsequent vegetable crop (melon) as a result of BNF through effective symbiosis with soil microorganisms. Contrarily, unusual legume crops such as cowpea, due to the absence of specific rhizobia strains adapted to the host, should result in lower microbial stimulation and C sequestration. Thus, the main objectives of this study were to: i) assess the effect of a preceding legume crop, considering two different legume cultivars as well as two different management practices, on SOC content and C pools and soil microbial activity; and ii) to ascertain whether any such effects depend on the specific legume species.

3.2. Materials and methods

3.2.1. Study site and experimental design

This study was carried out in Cartagena, southeast Spain (37º 41` N 0º 57` E). The field experiment was designed in a complete randomized block with four replications, using plots of 10 m^2. The area is characterized by a semiarid Mediterranean climate, with a mean annual temperature of 18 ºC and mean annual rainfall of 275 mm. The soil was a *Haplic Calcisol* (IUSS, 2014) with a clay loam texture. The main soil characteristics are shown in Table 3.1. The inclusion of legumes in multiple cropping with two traditional vegetable crops in the region (broccoli and melon) was studied through the establishment of two parallel experiments during two years. The monthly precipitations during the two years that the field experiments lasted are shown in the Table 3.2.

Table 3.1: Main soil characteristics. Values shown are mean ± standard deviation (n=4).

Parameter[a]	
pH	8.4±0.1
EC (µScm^{-1})	343±72
SOC (g kg^{-1})	12.8±0.3
N_t (g kg^{-1})	0.9±0.1
Bulk density (g cm^{-3})	1.0±0.0
CEC (cmol$_+$ kg^{-1})	4.2±1.1
CaCO$_3$ (%)	30.2±1.2
Clay (%)	34.5±0.16
Silt (%)	21.3±1.06
Sand (%)	44.2±0.92
Aggregates stability (%)	7.3±0.6

[a]:EC: electrical conductivity; SOC: soil organic carbon content; Nt: total nitrogen; CEC: cation exchange capacity.

Table 3.2: climatic conditions in the legumes (cowpea and fava bean) and vegetable crops (broccoli and melon) during the two crop cycles.

Crop type	Crop cycle	Rainfall (mm)	Maximum Temperature (ºC)	Mean Temperature (ºC)	Minimum Temperature (ºC)
Cowpea	29/5/14-13/8/14	13.20	28.10	24.13	17.51
	3/6/15-14/9/15	69.60	29.95	25.62	20.33
Broccoli	13/11/14-26/2/16	25.20	18.79	11.85	5.68
	1/12/15-24/2/16	7.25	17.42	12.71	8.84
Fava bean	24/10/14-2/3/15	31.50	19.13	12.55	5.68
	5/11/15-13/4/16	40.45	19.18	13.37	8.32
Melon	15/6/15-8/9/15	67.60	29.95	26.28	20.33
	13/6/16-23/8/16	1.00	28.41	24.92	20.81

3.2.1.1. Crop 1: cowpea-broccoli multiple cropping system

Two local Portuguese cultivars (Feijão frade de fio preto (FP) and Feijão frade de fio claro (FC)) of cowpea (*Vigna unguiculata* (L.) Walp.) were grown during two summer seasons (29/05/2014-13/08/2014 and 03/06/2015-14/09/2015). Cowpea is not normally cultivated in the region. After each cowpea crop, the soil was prepared to cultivate broccoli (*Brassica oleracea* L. var. italica) cv Parthenon with only a surface tillage (0-20 cm) in the same furrow direction. The broccoli crop was grown during the successive two winter seasons (13/11/2014-26/02/2015 and 1/12/2015-24/02/2016). A broccoli monocrop was also used with plots left fallow during the cowpea season (no cultivation of cowpea) to check the effect of multiple cropping with the legume on soil quality and fertility.

Both crops were established under drip irrigation with two management practices: conventional and organic. Cowpea seeds were sown and broccoli plants were planted with a spacing of 100 cm between rows and 20 cm between plants (5 plants m^{-2}). No herbicide treatment was applied, and the crops were kept free of weeds through hand-hoeing when necessary. In the cowpea crop, 30 kg ha^{-1} of N and 2.4 kg ha^{-1} of P_2O_5 were applied as ammonium nitrate (33.5% N) and monoammonium phosphate (61% P_2O_5, 12% N) in the conventional practice, and using a commercial organic fertilizer (Bombardier, Agroquímicos los Triviños, Spain; 10.7% w/v N, 0.7% w/v P_2O_5) in the organic practice. In the broccoli crop, 250 kg ha^{-1} N, 100 kg ha^{-1} P_2O_5 and 300 kg ha^{-1} K_2O were applied by fertigation as ammonium nitrate (33.5% N), monoammonium phosphate (61% P_2O_5, 12% N) and potassium sulphate (50% w/v K_2O, 18% S) in the conventional practice, and using two commercial organic fertilizers (Heronatur 4-2-8 and Heronatur 7-2-4; Herogra Fertilizantes, Spain; 4% w/v N, 2% w/v P_2O_5 and 8% w/v K_2O, and 7% w/v N, 2% w/v P_2O_5 and 4% w/v K_2O) in the organic practice.

3.2.1.2. Crop 2: fava bean-melon multiple cropping system

Two local Spanish cultivars - Muchamiel (M) and Palenca (P) - of fava bean (*Vicia faba* L.) were grown during two winter seasons (24/10/2014-02/03/2015 and 05/11/2015-13/04/2016). Fava bean is a traditional legume crop in the region. After the fava bean crop, the soil was prepared to grow a melon crop (*Cucumis melo L.*) cv Hidalgo with only a surface tillage (0-20 cm) in the same furrow direction. The melon crop was grown during the successive two summer seasons (15/06/2015-08/09/2015 and 13/06/2016-

23/08/2016). A melon monocrop was also used with plots left fallow during the fava bean season (no cultivation of fava bean) to check the effect of multiple with the legume on soil quality and fertility.

Both crops were established under drip irrigation with two management practices: conventional and organic. Fava bean seeds were sown with a spacing of 100 cm between rows and 40 cm between plants (2.5 plants m^{-2}) while melon plants were planted with a spacing of 200 cm between rows and 120 cm between plants (0.8 plants m^{-2}). No herbicide treatment was given, and the crops were kept free of weeds through hand-hoeing when necessary. In the fava bean crop, 20 kg ha^{-1} of N and 1.2 kg ha^{-1} of P_2O_5 were applied as ammonium nitrate (33.5% N) and monoammonium phosphate (61% P_2O_5, 12% N) in the conventional practice, and using a commercial organic fertilizer (Bombardier, Agroquímicos los Triviños, Spain; 10.7% w/v N, 0.7% w/v P_2O_5) in the organic practice. In the melon crop, 200 kg ha^{-1} N, 120 kg ha^{-1} P_2O_5 and 340 kg ha^{-1} K_2O were applied by fertigation as ammonium nitrate (33.5% N), monoammonium phosphate (61% P_2O_5, 12% N) and potassium sulphate (50% w/v K_2O, 18% S) in the conventional practice, and using a commercial organic fertilizer (Espartán Agroindustrial Kimitec S.L, Spain; 3.8% w/v N, 2.9% w/v P_2O_5 and 3.6% w/v K_2O) and potassium sulphate (50% w/v K_2O, 18% S) in the organic practice.

3.2.2. Soil sampling

The soil was sampled at the beginning of the experiments before sowing the legumes and upon harvesting each vegetable crop at the end of the second multiple cropping cycle. Thus, two soil samplings were carried out per crop system. All plots were sampled at 0-20 cm (plough depth). Three random soil samples per plot were collected and homogenized to obtain a composite sample. Samples were air-dried for 7 days, sieved < 2mm and stored at room temperature until analyses. Enzyme activities were measured in air-dried samples since these properties in Mediterranean semiarid soils are medium-term stable in stored air-dried samples (Zornoza et al., 2009a)

3.2.3. Soil analyses

Bulk density was determined using the cylinder method; soil pH and electrical conductivity (EC) were measured in deionized water (1:2.5 and 1:5 w/v, respectively); soil texture was determined by the Bouyoucos method (Dewis and Freitas, 1970); soil aggregate stability (AS) was assessed with the rainfall simulator method according to Roldán et al. (1994); for equivalent calcium carbonate the volumetric method (Bernard calcimeter) was used (Cobertera, 1993); SOC was determined by the wet oxidation method using $K_2Cr_2O_7$ (Walkley and Black, 1934); recalcitrant carbon (RC) and labile carbon (LC) were measured by the method of the double acid hydrolysis (Rovira and Vallejo, 2007); total nitrogen (Nt) was analyzed by the Kjeldahl method (Hoeger, 1998); cation exchange capacity was determined using $BaCl_2$ as exchangeable salt (Roig et al, 1980); β-glucosidase (Glu) activity was based on the determination of *p*-nitrophenol released after incubation at 37 °C with β-D-glucopyranoside (Tabatabai, 1982); β-glucosaminidase (Glm) activity was based on the determination of *p*-nitrophenol released after incubation with *p*-nitrofenil-β-D-glucopyranoside at 37 °C (Parham and Deng, 2000); dehydrogenase (Dhs) activity was determined using *p*-iodo-nitro-tetrazolium chloride as substrate and measuring the absorbance of the iodonitrotetrazolium formazam (INTF) produced (Von Merci and Schinner, 1991); arylesterase (Aryl) activity was based on the determination of *p*-nitrophenol released after incubation with *p*-nitrophenil acetate at 37°C (Zornoza et al., 2009b); cellulase (Cel) activity was assessed by determination of gearboxes sugars using amorphous cellulose as substrate (García-Álvarez and Ibáñez, 1994; Nelson, 1994); urease (Ure) activity was based on the determination of ammonium released after incubation of the soil with urea at 37°C (Nannipieri et al., 1978).

3.2.4. Statistical analyses

The average value of soil parameters measured at the beginning of the experiment was subtracted from values at the end of the second cycle and divided by the initial values to obtain the increment data (time Δ data) according to the following equation 1:

$$\text{time } \Delta\text{data} = [(\text{Final value} - \text{Initial value}) / \text{Initial value}] \times 100 \qquad (1)$$

Similarly, the average value of soil parameters measured from each vegetable monocrop was subtracted from its respective crop grown after legumes and divided by the monocrop values to obtain the multiple cropping Δ data according to the following equation 2:

Multiple cropping Δdata = [(Legume multiple cropping value – Monocrop value) / Monocrop value] × 100 (2)

Using this approach, the relative increases or decreases for all properties can be used to compare all properties between both crop systems since the unit can be homogenized to percentage of variation.

Data were checked to ensure normal distribution using the Kolmogorov–Smirnov test and transformed when necessary to ensure normal distribution. The Δdata of properties following a normal distribution were submitted to three-way ANOVA to assess the differences among previous legume cultivars, vegetable crop type and management practices at the end of the experiments. The Δdata of those properties without normal distribution were submitted to a one-way non-parametric ANOVA (Kruskal-Wallis test) for the factors: legume cultivar, vegetable crop type and management practice. Multiple linear regression analysis ($Y=m_1X_1 + m_2X_2 + \cdots + m_nX_n + b$) was carried out with the time Δdata using stepwise and backward methods, with RC and LC as independent variable and SOC, RC, LC, Nt and enzyme activities as dependent variables. Standardized coefficient (β) and partial correlation values were used for the analysis. The β coefficient is the estimated value resulting from the analysis performed on variables that have been standardized to have a variance of 1 in order to determine which of the independent variables has a greater effect on the dependent variable. Therefore, variables with larger β coefficients contribute more to the model. The partial correlation indicates the correlation between the dependent variable and one independent variable when the linear effects of the remaining variables have been eliminated. The unstandardized coefficients (m) were used to fit the values of RC or LC *versus* the values calculated using the regression model. Furthermore, a principal components analysis (PCA) was performed with all multiple cropping Δdata to study the structure of dependence and correlation established among the variables in both vegetable cropping systems. Statistical analyses were performed with the software IBM SPSS for Windows, Version 22.

3.3. Results

3.3.1. Soil organic carbon content and pools, total nitrogen and enzyme activities after two multiple cropping cycles

Table 3.3 shows the variation rate (Δ values) in SOC, Nt, RC and RL at the end of the second cycle compared to initial values (time Δdata) (absolute values are shown in the Table 3.4). Vegetable crop type only significantly affected ΔSOC, with no significant effect on ΔNt, ΔRC and ΔLC. The other factors had no significant effect on the variation of these properties. The broccoli crop showed significantly higher decreases in SOC than melon crop ($P < 0.001$) after two years of cultivation, mainly under conventional management. The highest SOC decreases were observed in the broccoli monocrops (after a fallow period), with values at the end of the experiment 19.6% lower than initial values.

Table 3.3: Variation rates (%) ± standard deviation of soil organic carbon, total nitrogen, recalcitrant and labile carbon in both vegetable crops (broccoli and melon) after two years of multiple cropping with legumes compared with initial values at the beginning of the experiments.

Previous legume cultivar[a]	Management practice	ΔSOC	ΔN_t	ΔRC	ΔLC
		Broccoli			
FP cowpea	Conventional	-2.8±7.4	39.1±3.1	97.6±8.3	-71.1±7.2
FC cowpea	Conventional	-3.1±6.1	34.1±8.5	80.2±41.5	-59.7±18.5
Fallow	Conventional	-19.6±14.3	-91.2±0.4	-92.2±0.2	-98.3±0.3
FP cowpea	Organic	0.9±16.1	22.5±3.5	138.6±116.4	-47.1±24.8
FC cowpea	Organic	-7.5±4.5	31.1±3.0	123.6±14.4	-90.5±8.8a
Fallow	Organic	-10.9±2.4	-91.2±0.3	-92.7±0.7	-98.3±0.5
		Melon			
M fava bean	Conventional	-1.6±4.9	12.7±0.6	109.3±8.1	-76.9±13.8
P fava bean	Conventional	9.2±9.3	20.2±2.9	80.5±3.7	-51.2±10.0
Fallow	Conventional	17.6±8.3	-91.0±0.9	-91.0±0.3	-97.2±0.5
M fava bean	Organic	-0.1±1.9	23.0±1.8	30.9±22.5	-21.5±18.5
P fava bean	Organic	2.1±4.5	28.2±6.0	65.9±26.7	-29.5±33.7
Fallow	Organic	8.4±6.1	-90.6±0.5	-91.8±0.2	-97.3±0.3
		χ^2 value			
Vegetable crop type (VCT)		14.41***	2.02ns	0.19ns	3.02ns
Previous legume cultivar (PLC)		0.12ns	1.61ns	0.03ns	0.08ns
Management practice (MP)		0.62ns	0.01ns	0.57ns	0.4ns

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro; M: Muchamiel; P: Palenca.

[b] Significant at ***P < 0.001; ns: not significant (P > 0.05). Different letters within each crop type indicate significant differences (P > 0.05).

SOC: soil organic carbon content; N_t: total nitrogen; RC: recalcitrant carbon; LC: labile carbon.

Table 3.4: soil organic carbon, total nitrogen, recalcitrant and labile carbon at the beginning of the experiments and at harvest of both vegetable crops (broccoli and melon) after the second year of multiple cropping with legumes. Values are mean ± standard deviation (n=4).

Previous legume cultivar[a]	Management practice[b]	SOC (g kg^{-1})	N_t (g kg^{-1})	RC (%)	LC (%)
Broccoli					
Initial value at the beginning of the experiment	-	12.86±0.36	0.91±0.03	0.52±0.02	0.76±0.05
FP cowpea	C	12.50±0.95	1.28±0.03	1.03±0.04	0.22±0.06
FC cowpea	C	12.47±0.79	1.23±0.08	0.94±0.22	0.31±0.14
Fallow	C	10.35±3.09	1.13±0.05	1.00±0.02	0.21±0.04
FP cowpea	O	12.99±2.07	1.13±0.03	1.24±0.61	0.41±0.19
FC cowpea	O	11.90±0.58	1.20±0.03	1.16±0.08	0.07±0.07
Fallow	O	11.46±0.31	1.13±0.04	0.93±0.09	0.21±0.06
Melon					
Initial value at the beginning of the experiment	-	12.86±0.36	0.91±0.03	0.52±0.02	0.76±0.05
M fava bean	C	12.66±0.90	1.03±0.01	1.09±0.06	0.18±0.15
P fava bean	C	14.05±1.70	1.10±0.04	0.94±0.03	0.38±0.11
Fallow	C	15.14±1.62	1.15±0.17	1.15±0.06	0.36±0.10
M fava bean	O	12.85±0.36	1.13±0.02	0.68±0.17	0.60±0.20
P fava bean	O	13.15±0.82	1.18±0.08	0.86±0.20	0.54±0.37
Fallow	O	13.95±1.11	1.21±0.09	1.05±0.05	0.35±0.06

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro; M: Muchamiel; P: Palenca.
[b] C: conventional; O: organic.
SOC: soil organic carbon; N_t: total nitrogen; RC: recalcitrant carbon and LC: labile carbon.

The variation in Glu, Dhs, Ure and Cel was significantly influenced by vegetable crop type (Table 3.5), with significantly higher increases of Glu, Dhs, Ure and lower decreases in Cel in the broccoli crop compared with melon ($P < 0.01$). Glm and Aryl were not significantly affected by vegetable crop type. None enzyme activity was significantly affected by legume cultivar or management practice.

Table 3.5: Variation rates (%) ± standard deviation of soil enzyme activities in both vegetable crops (broccoli and melon) after two years of multiple cropping with legumes with regards to initial values at the beginning of the experiments.

Previous legume cultivar[a]	Management practice	ΔGlu	ΔGlm	ΔAryl	ΔDhs	ΔUre	ΔCel
Broccoli							
FP cowpea	Conventional	203.5±81.8	149.8±52.2	-13.6±21.6	1565.3±242.1	3.0±15.4	-78.6±9.4
FC cowpea	Conventional	186.4±57.6	89.8±24.8	-21.4±5.1	1792.2±365.5	143.1±137.6	-85.4±8.6
Fallow	Conventional	-96.5±0.3	-98.8±0.3	526.3±248.8	-93.3±1.5	-96.6±1.0	-88.5±6.5
FP cowpea	Organic	209.4±67.3	60.4±12.1	-29.8±20.2	1681.1±198.2	190.9±214.4	-86.6±6.9
FC cowpea	Organic	341.3±14.6	167.1±45.7	-18.1±17.6	2018.7±383.8	181.2±208.1	-76.3±5.5
Fallow	Organic	-95.7±0.7	-98.8±0.2	850.7±253.2	-93.8±2.5	-97.4±1.1	-94.7±1.8
Melon							
M fava bean	Conventional	0.1±8.9	107.0±58.1	-29.6±12.1	-63.4±8.7	-100.0±0.0	-99.9±0.0
P fava bean	Conventional	-24.0±1.4	119.7±49.6	-44.7±5.3	-42.0±10.7	-100.0±0.0	-99.9±0.0
Fallow	Conventional	-97.9±0.2	-98.7±0.0	899.3±327.4	-99.7±0.0	-100.0±0.0	-99.9±0.0
FP cowpea	Organic	17.5±12.4	83.6±71.6	-55.2±2.5	-56.9±17.3	-100.0±0.0	-99.9±0.0
FC cowpea	Organic	-24.0±1.4	120.9±12.3	-26.2±3.6	-17.3±26.9	-100.0±0.0	-99.9±0.0
Fallow	Organic	-97.2±0.7	-98.8±0.1	433.9±44.6	-99.6±0.0	-100.0±0.0	-99.9±0.0
χ^2value							
Vegetable crop type (VCT)		4.58**	0.03ns	2.02ns	8.10**	30.01***	26.27***
Previous legume cultivar (PLC)		0.40ns	1.76ns	0.65ns	2.25ns	0.09ns	0.08ns
Management practice (MP)		0.46ns	0.06ns	0.16ns	0.16ns	0.06ns	0.53ns

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro; M: Muchamiel; P: Palenca.

[b] Significant at ***$P < 0.001$; ** $P < 0.01$; ns: not significant ($P > 0.05$). Different letters within each crop type indicate significant differences ($P > 0.05$).

Glu: β-glucosidase; Glm: β-glucosaminidase; Aryl: Arylesterase; Dhs: Dehydrogenase; Ure: urease and Cel: cellulase activities.

Multiple linear regression analysis (Table 3.6) showed that the variation in RC for both vegetable crops was positively related to ΔNt, and negatively related to ΔLC (R^2 = 0.86, F = 106069, P < 0.001). ΔLC was positively related to ΔNt, and negatively related to ΔCel (R^2 = 0.71, F = 26.80, P < 0.001).

Table 3.6: Multiple linear regression model for ΔRC and ΔLC in both vegetable crops (broccoli and melon) after two crop years with regard to initial values at the beginning of the experiments.

Y	*X*	m	Partial correlation	β	R^2	R^2 adj	F value
ΔRC	Constant (b)	-18.33			0.86	0.85	106069***
	ΔNt	1.88	0.91	1.10			
	ΔLC	-1.03	-0.55	-0.32			
ΔLC	Constant (b)	-155.41			0.71	0.69	26.80***
	ΔNt	0.78	0.77	1.48			
	ΔCel	-1.11	-0.53	-0.35			

m: unstandardized coefficients; β: standardized coefficients. Significant at ***P<0.001
N_t: total nitrogen; RC: recalcitrant carbon; LC: labile carbon; Cel: cellulase activity.

The PCA performed with time Δdata (Figure 3.1) showed that 77.2% of the total variation could be explained by the first two PCs. PC1, which explained 44.4% of variation, separated multiple cropping (positive scores) from monocrops (negative scores) in both crops. Multiple cropping systems with legume were related to higher increases in Nt, Glm, RL and LC, and higher decreases in Aryl (Table 3.7). PC2, which explained 32.8% of the variation, separated both vegetable crops. Cowpea/broccoli multiple cropping system (positive scores) was related to higher increases in Cel, Dhs, Glu and Ure activities, and higher decreases in SOC.

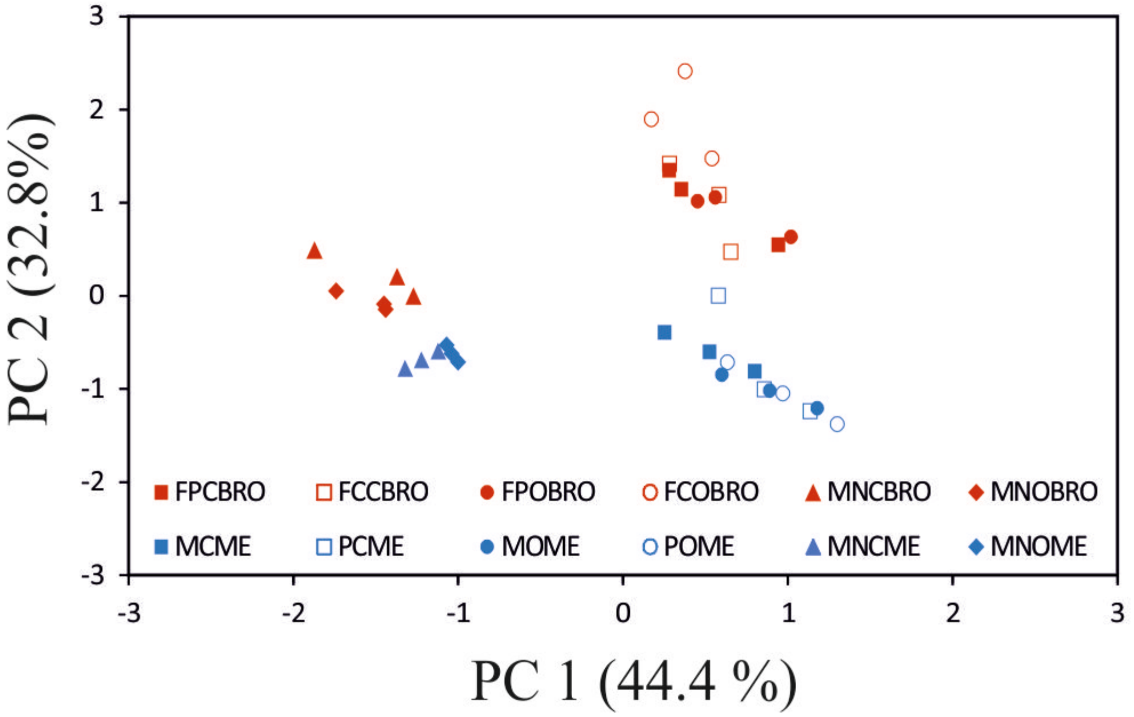


Figure 3.1: PCA factor scores of variations in soil properties in both vegetable crops (broccoli and melon) in multiple cropping with grain legumes compared with initial values at the beginning of the experiments, considering different previous legume cultivars and management practices. Color represents vegetable crop type (red: broccoli crop; blue: melon crop), figure type represents management practice and cropping system (square: conventional multiple cropping; circle: organic multiple cropping; triangle: conventional monocrop; rhombus: organic monocrop) and figure filling represents previous legume cultivar (filled figure: FP and M in broccoli and melon, respectively; empty figure: FC and P in broccoli and melon, respectively). FP: Fio preto cultivar; FC: Fio claro cultivar; M: Muchamiel cultivar; P: Palenca cultivar; C: conventional management; O: organic management, BRO: broccolic crop; ME: melon crop.

Table 3.7: Matrix of PCA obtained with variation rates (%) of the studied soil properties in both vegetable crops (broccoli and melon) after two crop years with regard to initial values at the beginning of the experiments (time Δdata).

Variance explained	PC1 (44.4%)	PC2 (32.8%)
ΔN_t	0.945	0.262
ΔGlm	0.909	0.234
ΔAryl	-0.897	-0.167
ΔRC	0.822	0.370
ΔLC	0.793	-0.305
ΔCel	0.057	0.909
ΔDhs	0.381	0.874
ΔGlu	0.563	0.763
ΔUre	0.304	0.734
ΔSOC	0.234	-0.443

SOC: soil organic carbon content; Nt: total nitrogen; RC: recalcitrant carbon; LC: labile carbon; Glu: β-glucosidase, Glm: β-glucosaminidase; Aryl: Arylesterase; Dhs: Dehydrogenase; Ure: urease and Cel: cellulase activities.

3.3.2. Effect of legume-based multiple cropping against monocrop on organic carbon content and pools, soil nitrogen and enzyme activities

Table 3.8 shows the variation rate of multiple cropping systems compared with monocrops (multiple cropping Δdata) in SOC, Nt, RL and RC (absolute values are shown in the Table 3.4). Vegetable crop significantly influenced the variation in SOC, Nt and RC ($P<0.001$), with highest increases in the cowpea/broccoli multiple cropping system. Previous legume cultivar significantly influenced ΔNt ($P<0.05$), with highest increases in broccoli grown after FP cultivar under conventional practice, and in melon grown after P cultivar under organic practice. The management practice did not significantly influence any of these soil properties. ΔLC was not significantly affected by any factor of study.

Table 3.8: Variation rates (%) ± standard deviation of soil organic carbon, total nitrogen, labile carbon and recalcitrant carbon in both vegetable crops grown after legumes (broccoli and melon) at the end of the second crop cycle with regards to broccoli and melon monocrops.

Previous legume cultivar[a]	Management practice	ΔSOC	ΔN_t	ΔLC (%)	ΔRC (%)
		Broccoli			
FP cowpea	Conventional	20.8±9.2	13.1±2.5	4.4±26.3	2.4±4.3
FC cowpea	Conventional	20.5±7.6	9.0±6.9	45.7±67.1	-6.6±21.5
FP cowpea	Organic	13.3±18.0	-0.4±2.8	90.6±89.5	33.1±64.9
FC cowpea	Organic	3.8±5.0	6.6±2.4	-65.7±32.0	24.7±8.0
		Melon			
M fava bean	Conventional	-16.4±4.2	-10.3±0.5	-50.7±29.5	-5.7±3.6
P fava bean	Conventional	-7.2±7.9	-4.3±2.3	4.5±21.5	-18.7±1.7
M fava bean	Organic	-7.9±1.8	-6.8±1.3	74.7±41.1	-35.1±11.1
P fava bean	Organic	-5.8±4.1	-2.8±4.5	56.8±74.9	-17.8±13.2
		F-value[b]	F-value	F-value[b]	χ^2value
Vegetable crop type (VCT)		46.2***	86.0***	0.0ns	10.4***
Previous legume cultivar (PLC)		0.0ns	5.1*	0.8ns	0.0ns
Management practice (MP)		1.0ns	3.6ns	3.0ns	0.0ns

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro; M: Muchamiel; P: Palenca.
[b] Significant at ***P < 0.001; *P < 0.05; ns: not significant (P > 0.05). Different letter within each crop type indicate significant differences among means (P > 0.05).
SOC: soil organic carbon content; N_t: total nitrogen; LC: labile carbon; RC: recalcitrant carbon.

Table 3.9 shows the variation rate of multiple cropping systems compared with monocrop (multiple cropping Δdata) in the soil enzyme activities (absolute values are shown in the Table 3.10). Vegetable crop significantly influenced ΔGlu, ΔAryl, ΔDhs and ΔCel (P<0.05), with significant general increases in broccoli grown after cowpea. Contrarily, these soil enzyme activities tended to decrease in the melon crop grown after fava bean compared with melon grown as monocrop. Nonetheless, ΔAryl significantly increased in the melon crop grown after fava bean only under organic management practice. Previous legume cultivar only significantly influenced ΔDhs (P<0.01), with highest increases in broccoli grown after FC cultivar, and in melon grown after P cultivar. Management practice significantly affected ΔUre and ΔCel (P<0.05), with highest increases under organic management practice in the cowpea/broccoli system.

Table 3.9: Variation rates (%) ± standard deviation of soil enzyme activities in both vegetable crops (broccoli and melon) at the end of the second crop cycle with regards to broccoli and melon monocrops.

Previous legume cultivar[a]	Management practice	ΔGlu (%)	ΔGlm (%)	ΔAryl (%)	ΔDhs (%)	ΔCel (%)	ΔUre (%)
			Broccoli				
FP cowpea	Conventional	43.3±38.6	13.7±23.8	76.8±44.2	32.5±19.2	15.4±50.9	-41.5±8.8
FC cowpea	Conventional	35.3±27.2	-13.6±11.3	60.8±10.5	50.6±29.1	-21.2±46.4	38.0±78.1
FP cowpea	Organic	18.4±25.8	-25.7±5.6	-5.4±27.2	51.7±16.8	55.2±80.1	118.7±161.3
FC cowpea	Organic	68.9±5.6	23.7±21.2	10.5±23.8	80.5±32.7	175.1±64.1	111.5±156.5
			Melon				
M fava bean	Conventional	-21.3±7.0	-16.5±23.4	-9.6±15.5	-39.1±14.5	-38.6±15.9	0.0±0.0
P fava bean	Conventional	-40.3±1.1	-11.3±20.0	-29.0±6.8	-3.5±17.8	-46.1±33.6	0.0±0.0
M fava bean	Organic	-31.9±7.2	-18.5±31.8	7.6±6.1	-36.2±25.5	-24.1±52.04	0.0±0.0
P fava bean	Organic	-56.0±0.8	-1.9±5.5	77.1±8.8	22.4±39.9	-44.8±22.74	0.0±0.0
		F-value[b]	F-value	F-value	F-value	F-value	χ^2value
Vegetable crop type (VCT)		98.5***	2.0ns	7.4*	41.4***	23.4***	2.2ns
Previous legume cultivar (PLC)		0.0ns	1.8ns	1.9ns	11.2**	0.5ns	1.1ns
Management practice (MP)		0.3ns	0.0ns	0.0ns	3.4ns	10.4**	4.4*

[a]FP: Feijão frade de fio preto; FC: Feijão frade de fio claro; M: Muchamiel; P: Palenca.

[b]Significant at ***P < 0.001; ** P < 0.01; *P < 0.05; ns: not significant (P > 0.05). Different letter within each crop type indicate significant differences among means (P > 0.05).

Glu: β-glucosidase; Glm: β-glucosaminidase; Aryl: Arylesterase; Dhs: Dehydrogenase; Cel: cellulase; Ure: urease.

Table 3.10: Soil enzyme activities at the beginning of the experiments and at harvest of both vegetable crops (broccoli and melon) after the second year of multiple cropping with legumes. Values are mean ± standard deviation (n=4).

Previous legume cultivar[a]	Management practice[b]	Glu (μmol PNP g^{-1} h^{-1})	Glm (μmol PNP g^{-1} h^{-1})	Aryl (μmol PNP g^{-1} h^{-1})	Dhs (μmol INTF g^{-1} h^{-1})	Ure (μmol NH_4^+ g^{-1} h^{-1})	Cel (nmol glucose g^{-1} h^{-1})
			Broccoli				
Initial value at the beginning of the experiment	-	0.20±0.05	0.06±0.02	165±12	0.07±0.05	0.24±0.12	7.96±1.69
FP cowpea	C	0.63±0.17	0.17±0.04	142±35.66	1.13±0.16	0.25±0.04	1.70±0.75
FC cowpea	C	0.60±0.12	0.13±0.02	129±8.51	1.29±0.25	0.60±0.34	1.16±0.69
Fallow	C	0.44±0.05	0.15±0.04	80±32.02	0.85±0.20	0.4±0.13	1.48±0.84
FP cowpea	O	0.65±0.14	0.11±0.01	115±33.35	1.21±0.13	0.72±0.53	1.06±0.55
FC cowpea	O	0.92±0.03	0.18±0.03	135±29.16	1.44±0.26	0.69±0.51	1.88±0.44
Fallow	O	0.55±0.09	0.15±0.02	122±32.59	0.80±0.33	0.33±0.15	0.68±0.23
			Melon				
Initial value at the beginning of the experiment	-	0.20±0.05	0.06±0.02	165±12	0.07±0.05	0.24±0.12	7.96±1.69
M fava bean	C	0.21±0.03	0.14±0.06	116±28	0.02±0.01	0.00±0.00	0.01±0.00
P fava bean	C	0.16±0.00	0.15±0.05	91±12	0.04±0.01	0.00±0.00	0.00±0.00
Fallow	C	0.27±0.04	0.17±0.01	128±60	0.04±0.00	0.00±0.00	0.01±0.00
M fava bean	O	0.25±0.04	0.12±0.07	73±6	0.03±0.02	0.00±0.00	0.00±0.00
P fava bean	O	0.16±0.00	0.15±0.01	121±8	0.06±0.03	0.00±0.00	0.00±0.00
Fallow	O	0.36±0.14	0.15±0.03	68±8	0.05±0.01	0.00±0.00	0.01±0.00

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro; M: Muchamiel; P: Palenca.

[b] C: conventional; O: organic.

Glu: β-glucosidase; Glm: β-glucosaminidase; Aryl: Arylesterase; Dhs: Dehydrogenase; Ure: urease; Cel: cellulase activities; PNP: *p*nitrophenol and INTF: iodonitrotetrazolium formazan.

The PCA performed with Δvalues (Figure 3.2A and B) showed that 72.1% of the total variation could be explained by the first three PCs. PC1, which explained 30.7% of variation, slightly separated the broccoli crop (positive scores) from melon crop (negative scores). Cowpea/broccoli multiple cropping system was related to higher increases in Nt, SOC and Aryl, Glu and Glm (Table 3.11). PC2, which explained 23.4% of the variation, slightly separated management practices in both crops. In broccoli crop, organic management showed higher factor scores, and so was associated with higher increases in Cel and RC, and higher decreases in LC. By contrast, in melon, conventional management provided the highest factor scores in PC2. PC3, which explained 18.0% of variation, separated the broccoli crop under organic management (positive scores) from the rest of treatments. Cowpea/broccoli multiple cropping system under organic management was related to higher increases in Ure and Dhs (Table 3.11).

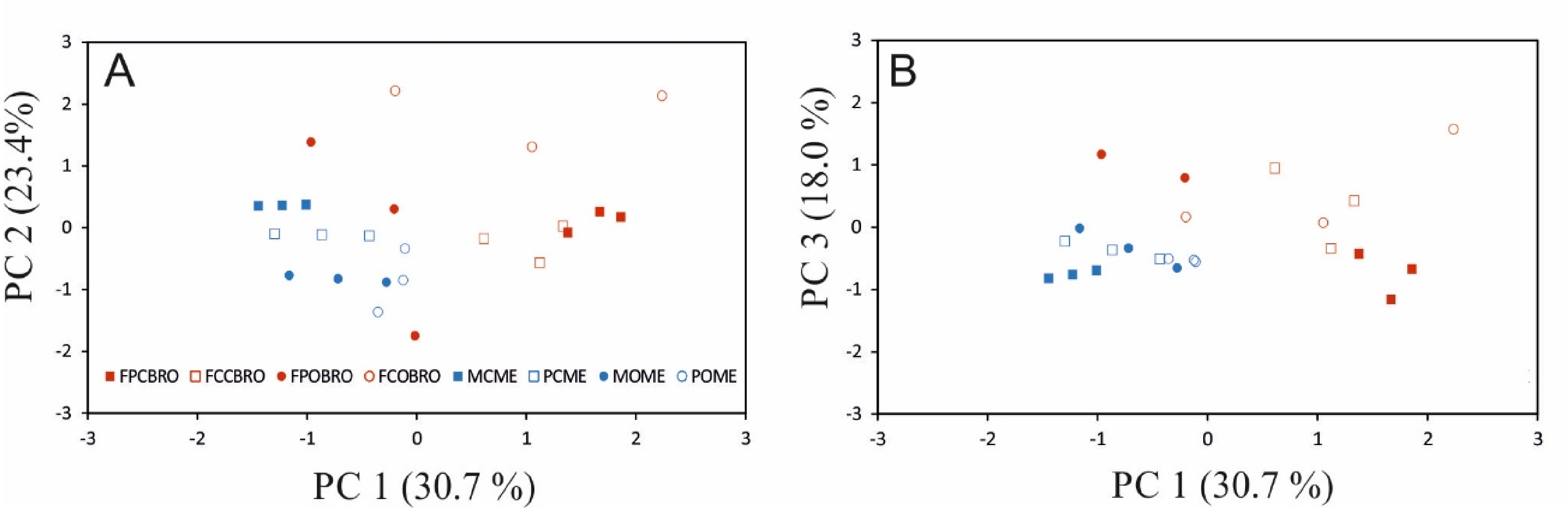


Figure 3.2: PCA factor scores for PC1, PC2 and PC3 of variations in soil properties in vegetable crops grown with grain legumes with regard to monocrops, considering different previous legume cultivars and management practices. Color represents vegetable crop type (red: broccoli crop; blue: melon crop), figure type represents management practice (square: conventional; circle: organic) and figure filling represents previous legume cultivar (filled figure: FP and M in broccoli and melon, respectively; empty figure: FC and P in broccoli and melon, respectively). Legend abbreviations are described in Figure 1 caption.

Table 3.11: Matrix of PCA obtained with variation rates (%) of the studied soil properties in both vegetable crops grown after legumes (broccoli and melon) at the end of the second crop cycle with regards to broccoli and melon monocrops.

Variance explained	PC1 (30.7%)	PC2 (23.4%)	PC3 (18.0%)
ΔN_t	0.904	0.242	0.102
ΔSOC	0.813	-0.124	0.457
ΔAryl	0.718	-0.310	-0.124
ΔGlu	0.645	0.500	0.320
ΔGlm	0.562	0.375	-0.152
ΔLC	0.107	-0.809	0.351
ΔCel	0.192	0.742	0.324
ΔRC	0.072	0.692	0.364
ΔUre	-0.028	0.075	0.870
ΔDhs	0.540	0.312	0.569

SOC: soil organic carbon content; Nt: total nitrogen; LC: labile carbon; Glu: β-glucosidase; Glm: β-glucosaminidase; Aryl: Arylesterase; Dhs: Dehydrogenase; Ure: urease and Cel: cellulase activities.

3.4. Discussion

The comparative study of soil C and N dynamics in two different vegetable crops grown after legume species (traditional and “non-usual”) under semiarid conditions considering conventional and organic management practices is novel. In this respect, the inclusion of cowpea in multiple cropping with broccoli, which has not been previously grown in the study region, led to an improvement in SOC, RC, Nt and enzyme activities compared to the traditional crop of fava bean grown after melon, contrary to our initial hypothesis. Legume crops provide N to the agro-ecosystem through biological N fixation process. In turn, this biologically fixed N is incorporated into the soil through root exudation or by the mineralization of above-ground residues after harvest (Laberge et al., 2009). The increase in SOC, Nt and soil enzyme activities in broccoli grown after cowpea compared to monocrop was not related to higher nodulation in cowpea roots. In fact, there was absence of nodules in cowpea roots in both crop cycles. This finding makes us believe that cowpea is more active in rhizodeposition and rhizostimulation than fava bean, although, as far as we are concerned, there is no previous research to confirm this hypothesis. Plant photosyntethically fixed C is the primary source of rhizodeposited C (Nguyen, 2003), which may be contributing to increase SOC in the cowpea multiple cropping system compared to monocrop. Legume species also provide N-rich root exudates which enhances soil N content and stimulated microbial populations (Fustec et al., 2010). Nonetheless, quantity but also quality of root exudates depends on plant genotype, explaining the differences found with regard to legume cultivars in some enzyme activities, which may be differently stimulated with regards to root exudates quality (Jones et al., 2004). It is well known that plant-derived organic inputs provide substrate for soil microbial communities, reflected in increases in the enzyme activities (Barrios, 2007). The good behavior of cowpea in the study area despite the lack of selected rhizobia strains for nodulation should be related to the physiological features of this species. Cowpea is a species well adapted to stressful environments caused by high temperatures, drought or low fertility, and so a suitable alternative in arid and semiarid regions (Chicoye et al., 2014; Elhers and Hall, 1997), such as that present in SE Spain.

In contrast to cowpea, fava bean roots were nodulated (data not shown), which indicates that BNF was taking place owing to effective symbiosis of the plant with local rhizobia strains. However, SOC, Nt and enzyme activities in the subsequent melon crop did not improve, as a general trend, compared with the melon monocrop. This result may indicate that fava bean removed higher amount of fixed N in the seed at harvest (Peoples and Craswell, 1992), since the N content in seeds was significantly higher in fava bean than in cowpea seeds (N content in fava seed was 29% higher than in cowpea seed) (Table 3.12).

Table 3.12: Nitrogen content in cowpea and fava bean seeds at harvest of the second crop cycle. Values are mean ± standard deviation (n=4).

Previous legume cultivar[a]	Management Practice	N in seeds (g kg^{-1})
FP cowpea	Conventional	31.2±3.1
FC cowpea	Conventional	32.3±0.9
FP cowpea	Organic	38.1±5.7
FC cowpea	Organic	34.5±1.6
M fava bean	Conventional	44.2±2.1
P fava bean	Conventional	43.4±2.0
M fava bean	Organic	44.9±2.9
P fava bean	Organic	44.4±1.5
F-value[b]		12.7***

[a]FP: Feijão frade de fio preto; FC: Feijão frade de fio claro; M: Muchamiel; P: Palenca.
[b]Significant at ***P < 0.001.

With regard to management practices, the organic practice resulted in higher increases in RC, Ure and Cel (Figure 3.2A and B; Table 3.9) and higher decreases in LC (Figure 3.2A) in broccoli grown after cowpea compared with the monocrop. However, conventional management stimulated higher increases in RC and Cel and higher decreases in LC in melon grown after fava bean compared with the monocrop (Figure 3.2A). These results reveal the influence of agricultural management on soil quality, and its interaction with crop type. Both management practices achieved the same results but with different crop systems: organic practice contributed to C sequestration in broccoli but conventional practice did in melon, due to the increase in the recalcitrant fraction and decrease in the labile fraction. This is a promising result for climate change mitigation (Jonhson et al., 2007), and confirms that management practice significantly interacts with crop type and no generalization should be given. Legumes introduction in multiple cropping is efficient to mitigate SOC losses (Plaza-Bonilla et al., 2016). In turn, these crops allow to reduce crop intensification, since the amount of C and N are returned to soil through application of crop residues while also reducing N fertilizers (Sainju et al., 2003). In addition to this, other factor to consider is the C:N ratio, which is low in legumes (Sánchez et al., 2004). However, an easily decomposition of crop residues can also lead to an increase of soil microbial activity and thus, increased SOC decomposition (Kuzyakov, 2010).

There was a positive relationship between the variations in soil properties, such as RC and LC with variations in Nt, as well as a negative relationship between both carbon pools and Cel activity. These correlations are focused on SOC pools, which represent a source of essential elements for biological activity such as nitrogen and optimize soil physical, chemical and biological processes through the improvement of soil aggregation, exchangeable cations or enzymatic activities (Lal, 2014). The incorporation into the soil of crop residues such as legumes, rich in N, normally leads to higher microbial activity and thus, enhanced SOC decomposition (Kuzyakov, 2010; Sánchez et al., 2004), but also may lead to enhanced humification with increases in RC. In this sense, Christopher and Lal (2007) highlighted the importance of N as a limiting component of the humification process that is essential for SOC accumulation. In addition, the activity of soil carbon cycle-related enzymes such as cellulase, which provides metabolic requirements of soil microbial community could be reduced as a result of the increase of available C resources (Veres et al., 2015).

3.5. Conclusions

In conclusion, our results showed, contrary to our initial hypothesis, that the inclusion of cowpea, despite being an unusual crop in SE Spain, improved SOC content and microbial activity in a subsequent broccoli crop compared with the broccoli monocrop, probably due to active rhizodeposition and rhizostimulation. The establishment of a traditional crop such as fava bean in multiple cropping with melon did not greatly improve SOC content and microbial activity compared with the monocrop, likely due to higher amount of fixed N removed in the seed at harvest. Legume genotype had influence on N content and enzyme activities, indicating that rhizostimulation is regulated by genotype. Soil C sequestration was affected by management practice and its interaction with the specific crop type, with enhanced C sequestration under organic management in broccoli and under conventional management in melon. Thus, cowpea crop in multiple cropping represented a good alternative for sustainable production, since it led to improvements in soil quality along with C sequestration under organic management.

Chapter 4

Does the use of cowpea in multiple cropping with a vegetable crop improve soil quality and crop yield and quality? A field study in SE Spain

Does the use of cowpea in multiple cropping with a vegetable crop improve soil quality and crop yield and quality? A field study in SE Spain

Abstract

Information on the effect of cowpea-based multiple cropping in horticulture under arid and semiarid environments are scarce despite the potential for increasing soil quality and fertility. Cowpea is a suitable legume in rotated cropping systems due to its relative tolerance to drought. The main goal of this study was to assess the effect of two cowpea cultivars, Feijão frade de fio preto (FP) and Feijão frade de fio claro (FC), during three multiple cropping cycles, on soil fertility, yield, crop quality and nutritional composition of subsequent broccoli crops grown using mineral and organic fertilizers while decreasing fertilization rates by 20% compared with a broccoli monocrop. A cowpea crop was seen to contribute to increasing subsequent soil available P after three multiple cropping compared to monocrop, using both fertilizers type (an increase of 30 % and 120 % using mineral and organic fertilizers, respectively). The use of mineral fertilizers increased broccoli head diameter (13-41 %) and yields (33-80 %), while the use of organic fertilizers increased soil aggregate stability (23-36 %) and soil enzyme activities (40-110 %). The use of organic fertilizers improved broccoli crop yield with time, with similar values than those obtained from mineral fertilizers at the end of the experiment. Thus, the introduction of cowpea in multiple cropping under Mediterranean conditions was seen to be a good strategy for crop diversification and for reducing current N fertilizer dependency.

Keywords: *Vigna unguiculata*; *Brassica oleracea* var. italica; multiple cropping; fertilizers; carbon dynamics; nitrogen dynamics**.**

4.1. Introduction

Cowpea (*Vigna unguiculata* L. Walp) is a grain legume native of southern Africa, although it is widely consumed all around the word (Singh, 2014). It is consumed for its seeds (green and mature), leaves, green pods or processed products (flour, flavour or paste) (Abudulai et al., 2017; Phillips et al., 2003). Cowpea seeds play an important role in the human diet due to its high nutritional value since it is a rich source of protein, calories, minerals and vitamins (Deshpande, 1992). In addition, cowpea is a species well adapted to stressful environments associated with high temperatures, drought or low fertility, and so is considered a suitable alternative crop in arid and semiarid regions (Chicoye et al., 2014; Elhers and Hall, 1997).

Legumes improve soil fertility through biological nitrogen fixation (BNF), thus reducing the need for N fertilizers (St Luce et al., 2015). BNF occurs through the symbiosis between legumes and rhizobia, which are α− and β-proteobacteria with an ability to fix atmospheric N (Sawada et al., 2003). Cowpea is capable of establishing efficient symbiosis, especially with slow-growing rhizobia belonging to the genus *Bradyrhizobium* (Bejarano et al., 2014; Krasova-Wade et al., 2003), but also, to a lesser degree, with fast-growing rhizobia classified in the genus *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* (Zhang et al., 2007). BNF depends on the host plant genotype, photosynthesis rate and the strain of rhizobia (Gourion et al., 2015). With regard to the latter, the symbiotic performance of rhizobia is directly linked with their population size, survival and effective association with host plant (Sanginga et al., 2000).

The use of legumes in rotation and multiple cropping may regulate C and N storage through the production of belowground biomass, BNF, type and quantity of root exudates and the stimulation of soil microorganisms (Drinkwater et al., 1998). Furthermore, the introduction of legumes in rotation and multiple cropping provides other positive effects, such as inducing the growth of beneficial soil microorganism (Lupwayi and Kennedy, 2007) or reducing the need for pesticides because of crop diversification (Munier-Jolain, 2002). The N contribution of legumes to subsequent crops is difficult to forecast, and depends on legume species and genotypes, since these vary in their ability to biologically fix atmospheric N (Peoples et al., 2009).

The replacement of mineral by organic fertilizers normally contributes to a higher soil organic matter content and increased microbial diversity and activity (Fließbach et al., 2007; Kramer et al., 2006). However, organic crops normally produce lower yields because the availability of sufficient N to the plant is dependent on the mineralization rates of soil organic matter (Seufert et al., 2012) and thus, the crops need more land to produce the same amount (Smith et al., 2007). However, the introduction of a legume in an organic multiple cropping system may provide more available N for successive crops, contributing to maintaining higher crop yields (Shah et al., 2003).

There are very few attempts to show the effect of a suitable legume such as cowpea in a subsequent horticulture crop with the use of mineral and organic fertilizers under arid and semi-arid conditions with the aim of decreasing the external N dependence and increase soil quality. We hypothesized that cowpea would increase soil N availability through BNF, associated to increases in soil organic carbon content and microbial activity as a result of belowground rhizodeposition and phytostimulation. This would result in improved soil quality and higher broccoli crop yields, accompanied by a reduced need for the application of fertilizers. Any such effect may differ with the use of different fertilization strategy (organic or mineral) and the cowpea genotype, since both may influence plant growth, rhizodeposition rates and the efficiency to fix atmospheric N. Thus, the main objectives of the study were to: i) assess the effect of the introduction of cowpea in multiple cropping with broccoli on soil quality and fertility compared with a broccoli monocrop; ii) assess the impact of growing cowpea on broccoli yield, quality and nutritional status; iii) evaluate the influence of the fertilizer type (organic or mineral) on soil quality and broccoli yield; and iv) ascertain whether different cowpea genotypes differently impact the multiple cropping system. With this in mind, a three-year field experiment based on cowpea-broccoli multiple cropping using mineral and organic fertilizers was setup. The adoption of different fertilizer types allowed their impact on soil quality, plant nutrition and crop yield to be compared in an agricultural system under Mediterranean climatic conditions when combined with the introduction of a legume in the crop system.

4.2. Materials and methods

4.2.1. Study site and experimental design

This study was carried out in the same field in Cartagena, south-eastern Spain (37º 41` N 0º 57` E), for three years. The field experiment was designed as a complete randomized block with four replications, and each plot had a size of 10 m^2. Two local Portuguese cultivars (Feijão frade de fio preto (FP) and Feijão frade de fio claro (FC)) of cowpea (*Vigna unguiculata* (L.) Walp.) were grown during spring and summer in 2014 (29/05/14-13/08/14), 2015 (03/06/15-14/09/15) and 2016 (01/06/16-22/08/16). After the cowpea crop, the soil was prepared for a broccoli crop (*Brassica oleracea* L. var. italica) with surface tillage (0-20 cm) in the same direction as the furrows. The broccoli crop was grown during autumn and winter in 2014/2015 (13/11/14-26/02/15), 2015/2016 (01/12/15-24/02/16) and 2016/2017 (27/12/16-29/03/17). Both crops were drip irrigated and two fertilizer types were applied: mineral and organic. A broccoli monocrop was also used as a treatment with plots left fallow (no cultivation of cowpea) to check the effect of multiple cropping with cowpea on soil quality and fertility, soil microbial activity and broccoli yield and quality, using mineral and organic fertilizers.

The soil was a *Haplic Calcisol* (IUSS, 2014) with a clay loam texture, the main characteristics of which are shown in Table 4.1. The mean annual temperature of the study area is 18 ºC and the mean annual precipitation is 275 mm. Annual potential evapotranspiration surpasses 900 mm. The meteorological conditions during the three cowpea and broccoli crop cycles are shown in Figure 4.1. Cowpea seeds were sown and broccoli plants were planted with a spacing of 100 cm between rows and 20 cm between plants (5 plants m^{-2}). No herbicide treatment was provided, and the crops were kept free of weeds through hand-hoeing when necessary.

Table 4.1: Main soil characteristics. Values are mean ± standard deviation (n=4).

Parameters^a	
pH	8.40±0.09
EC (dS m^{-1})	0.34±0.07
SOC (%)	1.19±0.18
Bulk density (Mg m^{-3})	1.01±0.03
CEC (cmol kg^{-1})	4.24±1.10
$CaCO_3$ (%)	30.2±1.2
Clay (%)	34.5±0.16
Silt (%)	21.3±1.06
Sand (%)	44.2±0.92
Nt (%)	0.094±0.007
NO_3^- (mg kg^{-1})	156±45
NH_4^+ (mg kg^{-1})	5.69±1.12
Available P (mg kg^{-1})	30.0±5.4
Exchangeable K (mg kg^{-1})	369±63
Exchangeable Ca (mg kg^{-1})	2726±126
Exchangeable Mg (mg kg^{-1})	606±24
Exchangeable Na (mg kg^{-1})	301±31

^{a}:EC: electrical conductivity; SOC: soil organic carbon; CEC: cation exchange capacity; Nt: total nitrogen.

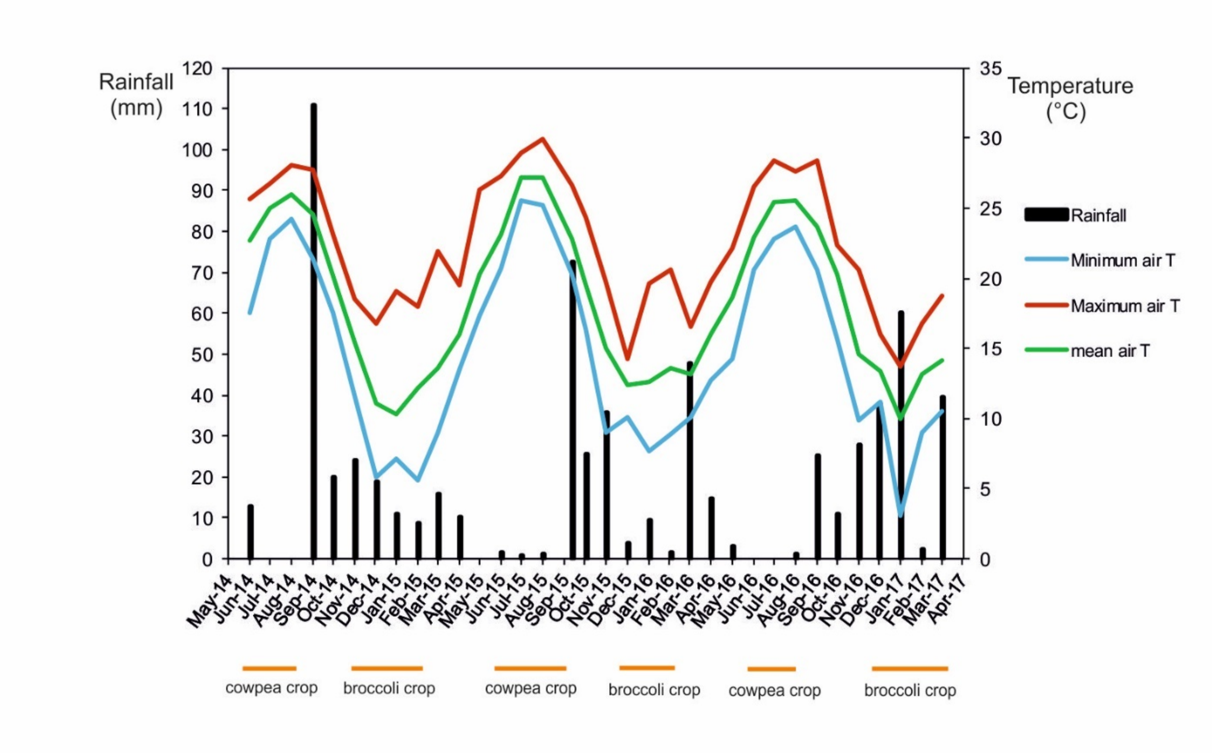


Figure 4.1: Maximum air T^a, mean air T^a, minimum air T^a and rainfall from May 2014 to April 2017.

Every year, a surface application of 16,000 kg ha^{-1} of goat and sheep manure was carried out in all plots before sowing. This manure had the following characteristics: pH = 8.32±0.07; electrical conductivity = 21.2±1.01 mS cm^{-1}; total organic carbon content = 307±11 g kg^{-1}; total nitrogen content = 13.4±0.7 g kg^{-1}; P_2O_5 content = 1.8±0.08 % and K_2O content = 4.6±0.2 %. Fertilizer application in the cowpea plots started between two or three weeks after sowing and continued until harvest. In the cowpea crops, 30 kg ha^{-1} of N and 2.4 kg ha^{-1} of P_2O_5 were applied by fertirrigation as ammonium nitrate (33.5% N) and monoammonium phosphate (61% P_2O_5, 12% N) in the mineral fertirrigation, and using a commercial liquid organic fertilizer (Bombardier, Agroquímicos los Triviños, Spain; 10.7% w/v N, 0.7% w/v P_2O_5) in the organic fertirrigation. Fertilizer application in the broccoli plots started two weeks after planting and continued until harvest. In the broccoli monocrop, 250 kg ha^{-1} N, 100 kg ha^{-1} P_2O_5 and 300 kg ha^{-1} K_2O were applied by fertirrigation as ammonium nitrate (33.5% N), monoammonium phosphate (61% P_2O_5, 12% N) and potassium sulphate (50% w/v K_2O, 18% S) in the mineral fertirrigation, and using two commercial liquid organic fertilizers (Heronatur 4-2-8 and Heronatur 7-2-4; Herogra Fertilizantes, Spain; 4% w/v N, 2% w/v P_2O_5 and 8% w/v K_2O, and 7% w/v N, 2% w/v P_2O_5 and 4% w/v K_2O) in the organic fertirrigation. For broccoli grown after cowpea, the fertilizers were the same as those used for the monocrop, but the application rate was reduced by 20 % to check whether external inputs can be saved by introducing legumes in multiple cropping. Cowpea residues were removed from the field and so not applied in the soil as green manure. The irrigation was established on the basis of evapotranspiration rate, crop coefficient and climatic conditions as rainfalls. The irrigation amount was between 238-277 mm for broccoli crop in each year.

4.2.2. Soil and plant sampling

The soil was sampled at the beginning of the experiments before sowing cowpea and after harvesting the broccoli crop at the end of each multiple cropping cycle during three successive years. All plots were sampled at 0-20 cm (Ap horizon). Three random soil samples per plot were collected and homogenized to obtain a composite sample, which was air-dried for 7 days, sieved < 2 mm and stored at room temperature until analyses, except for NH_4^+ and NO_3^-, where an aliquot of each sample was stored at 4ºC to avoid undesirable mineralization/oxidation processes and sieved < 2 mm previously to analyses, within 4 days from sampling. Enzyme activities were also measured in air-dried samples since this property is medium-term stable in stored air-dried samples of Mediterranean semiarid soils (Zornoza et al., 2009a).

Broccoli crop yield was determined by weighing the heads when the buds of the head were firm and tight. Broccoli head and stem diameter were recorded as crop quality parameters. With regard to cowpea yield, all the pods in each plot were harvested when the seeds were dried at the end of the crop cycle (Table 4.2).

Table 4.2: Cowpea crop yield at harvest of the crop cycle during three years. Values are mean ± standard deviation (n=4).

Previous cowpea cultivar[a]	Fertilizer type	Crop yield (kg ha^{-1})
	2014	
FP	Mineral	2333±133
FC	Mineral	2490±167
FP	Organic	2135±306
FC	Organic	2371±161
	2015	
FP	Mineral	100±10
FC	Mineral	216±144
FP	Organic	121±70
FC	Organic	366±152
	2016	
FP	Mineral	3033±757
FC	Mineral	4166±1171
FP	Organic	2533±321
FC	Organic	3366±1167
	χ^2 value	
Year (Y)		17.31***
Previous cowpea cultivar (PCC)		1.29ns
Fertilizer type (FT)		0.42ns

[a]FP: Feijão frade de fio preto; FC: Feijão frade de fio claro.
[b]Significant at ***P < 0.001; ns: not significant (P > 0.05).

4.2.3. Soil analyses

The following parameters were measured: bulk density by the cylinder method; soil pH and electrical conductivity (EC) in deionized water (1:2.5 and 1:5 w/v, respectively); soil texture by the Bouyoucos method (Dewis and Freitas, 1970); equivalent calcium carbonate using the volumetric method (Bernard calcimeter) (Cobertera, 1993); soil organic carbon (SOC) by the wet oxidation method using $K_2Cr_2O_7$ (Walkley and Black, 1934); aggregate stability (AS) by the method proposed by Roldán et al. (1994) based on the application of a simulated rainfall with known intensity; total nitrogen (Nt) by the Kjeldahl method (Hoeger, 1998); cation exchange capacity using $BaCl_2$ as exchangeable salt (Roig et al, 1980). For its part, NO_3^- was extracted with deionized water in a 1:10 soil:extractant ratio (Keeny and Nelson, 1982) and measured by ion chromatography (Metrohm 861); NH_4^+ was extracted with 2*M* KCl in a 1:10 soil:extractant ratio (Keeny and Nelson, 1982) and colorimetrically measured (Kandeler and Gerber, 1988); available phosphorus (P) was extracted according to the Burriel-Hernando method (Díez, 1982), using Burriel-Hernando solution (0.2g $CaCO_3$, 0.17g Mg CO_3, 5 ml glacial acetic acid and 0.2 ml H_2SO_4 in 2L deionized water) in a 1:25 soil:extractant ratio. Exchangeable Ca, Mg, Na and K were determined in the $BaCl_2$ extract for CEC; P, Ca, Mg, Na and K concentrations were measured using ICP-MS (Agilent 7500CE); β-glucosidase activity was based on the determination of *p*-nitrophenol released after incubation at 37 °C with β-D-glucopyranoside (Tabatabai, 1982); β-glucosaminidase activity was based on the determination of *p*-nitrophenol released after incubation with *p*-nitrofenil-β-D-glucopyranoside at 37 °C (Parham and Deng, 2000); dehydrogenase activity was determined using *p*-iodo-nitro-tetrazolium chloride as substrate and measuring the absorbance of the iodonitrotetrazolium formazam (INTF) produced (Von Merci and Schinner, 1991); arylesterase activity was based on the determination of *p*-nitrophenol released after incubation with *p*-nitrophenyl acetate at 37°C (Zornoza et al., 2009b); cellulase activity was assessed by determination of gearboxes sugars using amorphous cellulose as substrate (García-Álvarez and Ibáñez, 1994; Nelson, 1994) and urease activity was based on the determination of the ammonium released after incubation of the soil with urea at 37 °C (Nannipieri et al., 1978).

4.2.4. Plant analyses

Plant samples were oven dried and ground (A11 Basic, IKA) before incinerating at 500 ºC; the ashes were dissolved in 0.6N HNO_3 and analysed for P, Ca, Mg, Na and K by ICP-MS (7500 CE, Agilent). Nitrogen (N) was determined by the Kjeldahl method (Hoeger, 1998). NO_3^- was extracted with deionized water in a 1:50 plant:extractant ratio (Keeny and Nelson, 1982) and measured by ion chromatography (Metrohm 861); total organic carbon (TOC) was quantified by total combustion.

4.2.5. Statistical analyses

Data were checked to ensure normal distribution using the Kolmogorov–Smirnov test and log-transformed when necessary to ensure normal distribution. Data were submitted to three-way ANOVA to assess the differences related with year, previous cowpea cultivar (fallow, FP and FC) and fertilizer type (mineral and organic) at each sampling time. Furthermore, data were submitted to two-way ANOVA to assess the differences among previous cowpea cultivar and fertilizer type at each sampling time. Those properties without normal distribution were submitted to a one-way non-parametric ANOVA (Kruskal-Wallis test) for the factors: year, previous cowpea cultivar and fertilizer type. Relationships among properties were studied using Pearson's correlations. Statistical analyses were performed with the software IBM SPSS for Windows, Version 22.

4.3. Results

4.3.1. Soil physicochemical properties

As a general pattern, crop year affected most of soil properties. SOC and AS were higher in 2016/17 than in the first two years (Table 4.3). Nt showed significantly lower values during the first year. P content gradually declined from 2014/15 to 2016/17 (Table 4.3).

Table 4.3: Soil organic carbon, total nitrogen, aggregates stability and available phosphorus in soil of broccoli crops grown after cowpea or in monocrop using mineral and organic fertilizers over three years. Values are mean ± standard deviation (n=4).

Previous cowpea cultivar[a]	Fertilizer type	SOC $(g\ kg^{-1})$	N_t $(g\ kg^{-1})$	AS (%)	P (%)
2014/15					
FP	Mineral	11.94±1.21	0.97±0.20	9.81±3.62	28.88±3.70
FC	Mineral	12.25±1.20	0.91±0.17	10.34±2.86	41.00±17.83
Fallow	Mineral	11.70±0.93	0.94±0.10	16.69±2.79	27.76±11.92
FP	Organic	11.22±0.83	0.92±0.11	9.39±0.96	26.22±7.95
FC	Organic	11.87±1.78	0.91±0.05	14.61±2.15	24.57±6.56
Fallow	Organic	12.05±1.25	0.85±0.06	12.45±4.29	27.66±8.34
2015/16					
FP	Mineral	12.50±0.95	1.28±0.03	13.90±7.35	18.14±5.64
FC	Mineral	12.47±0.79	1.23±0.08	14.43±4.02	26.29±3.76
Fallow	Mineral	10.35±3.09	1.13±0.05	17.17±4.79	22.43±0.59
FP	Organic	12.99±2.07	1.13±0.03	18.80±2.43	21.11±2.31
FC	Organic	11.90±0.58	1.20±0.03	19.01±6.10	17.59±6.58
Fallow	Organic	11.46±0.31	1.13±0.04	18.36±6.90	16.72±4.46
2016/17					
FP	Mineral	15.18±1.40	1.17±0.12	34.39±8.58	7.78±0.48
FC	Mineral	16.97±2.04	1.26±0.17	38.93±13.99	8.22±1.28
Fallow	Mineral	17.48±0.90	1.16±0.19	49.43±6.57	6.16±2.88
FP	Organic	15.53±0.65	1.23±0.29	54.04±3.69	13.80±4.17
FC	Organic	15.87±0.36	1.20±0.13	55.48±6.61	7.58±2.52
Fallow	Organic	13.65±1.15	1.20±0.11	58.03±4.36	4.85±0.25
F-value[b]					
Year (Y)		48.41***	26.75***	200.11***	46.35***
Previous cowpea cultivar (PCC)		1.44ns	0.81ns	3.69*	1.09ns
Fertilizer type (FT)		1.64ns	0.71ns	14.53***	2.65ns
Y x PCC		1.30ns	0.17ns	0.78ns	0.65ns
Y x FT		2.19ns	0.40ns	7.98***	1.58ns
PCC x FT		0.48ns	0.08ns	1.77ns	2.92ns
Y x PCC x FT		1.96ns	0.54ns	0.29ns	0.73ns

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro.

[b] Significant at ***P < 0.001; ns: not significant (P > 0.05).

SOC: soil organic carbon content; N_t: total nitrogen; AS: aggregates stability.

The NO_3^- content showed the highest values in 2015/16, while NH_4^+ content was not significantly affected by any studied factor (Table 4.4).

Table 4.4: ammonium and nitrate contents in soil of broccoli crops grown after cowpea or in monocrop using mineral and organic fertilizers over three years. Values are mean ± standard deviation (n=4).

Previous cowpea cultivar[a]	Fertilizer type	NH_4^+ (mg kg^{-1})	NO_3^- (mg kg^{-1})
2014/15			
FP	Mineral	5.21±0.12	42±6
FC	Mineral	6.23±0.91	46±1
Fallow	Mineral	7.28±0.44	34±2
FP	Organic	5.44±1.25	39±6
FC	Organic	6.84±1.03	43±1
Fallow	Organic	6.09±0.73	32±5
2015/16			
FP	Mineral	7.15±3.98	135±49
FC	Mineral	6.39±1.49	126±38
Fallow	Mineral	3.38±0.67	100±3
FP	Organic	1.64±0.31	139±80
FC	Organic	6.95±2.12	99±8
Fallow	Organic	8.38±1.95	138±68
2016/17			
FP	Mineral	29.62±3.13	46±5
FC	Mineral	30.92±10.45	51±8
Fallow	Mineral	40.73±7.21	69±12
FP	Organic	19.64±11.59	16±22
FC	Organic	25.00±6.30	41±5
Fallow	Organic	19.14±4.61	17±25
χ^2 value			
Year (Y)		1.15ns	26.27***
Previous cowpea cultivar (PCC)		2.11ns	1.68ns
Fertilizer type (FT)		0.21ns	2.23ns

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro.
[b] Significant at ***P < 0.001; not significant (P > 0.05).

Exchangeable Mg and K were significantly lower in 2016/17 and 2014/15, respectively (Table 4.5). Previous cowpea cultivar influenced soil exchangeable Ca and AS. The influence of cowpea cultivar on Ca did not follow a clear pattern, but its content tended to decrease with the cultivation of cowpea, compared to broccoli monocrops. Broccoli monocrop showed higher AS than broccoli grown after cowpea. In addition, NO_3^- content tended to be significantly lower in soil cultivated with broccoli in multiple cropping with cowpea compared with the broccoli monocrop, mainly during the last two years. Fertilizer type affected AS and K content, with improved soil structure using organic fertilizers while K content tended to be higher with the mineral fertilizer. According to the two-way ANOVA at each sampling time, it was observed that during 2015/16, fertilizer type significantly affected soil N, with significant lower values using organic than mineral fertilizers ($P<0.05$). The interaction previous cowpea cultivar x fertilizer type was significant for soil P during the last crop cycle, with higher values being obtained in the multiple cropping system compared to the broccoli monocrop with added organic fertilizer ($P<0.05$). The interaction year x fertilizer type was significant with respect to AS and Ca content. AS showed the highest values using organic fertilizers during 2015/16 and 2016/17, while Ca showed the highest values using both fertilizer types during 2014/15. The interaction year x previous cowpea crop was significant for exchangeable cations content (Ca, Mg and K). Ca content after previous FP and FC cultivars was higher during 2014/15 than during the last two years, while in broccoli monocrop, Ca values were relatively stable over time. The interaction previous cowpea crop x fertilizer type was significant for Mg content, which was higher in broccoli crop grown after FP cowpea cultivar using mineral fertilizers during 2015/16. Finally, the interaction of the three factors (year, previous cowpea crop and fertilizer type) was significant for K content, with highest values in broccoli crop grown after FP cultivar using mineral fertilizers during 2015/16 (Tables 4.3, 4.4 and 4.5).

Soil Mg was negatively related with SOC ($r = -0.74$, $P < 0.01$), AS ($r = -0.81$, $P < 0.01$) and NH_4^+ content ($r = -0.76$, $P < 0.01$), and positively related with available P ($r = 0.64$, $P < 0.01$), β-glucosidase ($r = 0.60$, $P < 0.01$), arylesterase ($r = 0.65$, $P < 0.01$) and dehydrogenase ($r = 0.74$, $P < 0.01$).

Table 4.5: Exchangeable calcium, magnesium and potassium in soil of broccoli crops grown after cowpea or in monocrop using mineral and organic fertilizers for three successive. Values are mean ± standard deviation (n=4).

Previous cowpea cultivar[a]	Fertilizer type	Exc Ca (mg kg^{-1})	Exc Mg (mg kg^{-1})	Exc K (mg kg^{-1})
		2014/15		
FP	Mineral	2658±33	612±18	297±26
FC	Mineral	2709±253	625±67	329±57
Fallow	Mineral	2587±47	553±14	350±25
FP	Organic	2518±53	580±7	293±28
FC	Organic	2538±173	599±40	244±21
Fallow	Organic	2552±113	566±13	257±28
		2015/16		
FP	Mineral	2203±106	710±66	853±97
FC	Mineral	2051±29	568±23	651±53
Fallow	Mineral	2520±179	599±59	580±10
FP	Organic	2289±90	590±29	518±21
FC	Organic	2331±50	584±3	545±81
Fallow	Organic	2566±82	629±32	551±93
		2016/17		
FP	Mineral	2333±59	421±15	419±30
FC	Mineral	2382±73	430±19	451±85
Fallow	Mineral	2359±126	407±29	512±42
FP	Organic	2406±87	417±25	419±18
FC	Organic	2374±113	409±16	446±37
Fallow	Organic	2550±206	431±34	447±31
		F-value[b]		
Year (Y)		23.36***	173.79***	175.33***
Previous cowpea cultivar (PCC)		6.22**	2.56ns	0.89ns
Fertilizer type (FT)		1.19ns	2.04ns	32.56***
Y x PCC		4.85**	4.14**	5.04**
Y x FT		5.48**	0.58ns	8.02***
PCC x FT		0.29ns	5.35**	1.35ns
Y x PCC x FT		1.44ns	2.47ns	7.49***

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro.

[b] Significant at ***P < 0.001; ** P < 0.01; ns: not significant (P > 0.05).

Exc Ca: exchangeable calcium; Exc Mg: exchangeable magnesium; Exc K: exchangeable potassium.

4.3.2. Soil enzyme activities

Enzyme activities were significantly influenced by the crop year (Table 4.6 and 4.7). Dehydrogenase, β-glucosidase, β-glucosaminidase and arylesterase activities showed the lowest values during 2016/17. Urease and cellulase showed no activity at any time during the experimental period (data not shown). Previous cowpea crop only affected arylesterase activity. This soil enzyme activity was significantly higher in broccoli grown after FC cultivar. Fertilizer type influenced β-glucosaminidase and arylesterase activities. β-glucosaminidase activity was higher with the use of organic fertilizer. Arylesterase activity showed higher values with application of mineral fertilizers during the first two crop years; however, this activity was higher with the use of organic fertilizer during the last year. The interaction year x previous cowpea crop was significant for dehydrogenase and β-glucosaminidase activities. The interaction year x fertilizer type was significant for β-glucosaminidase and arylesterase activities. β-glucosaminidase activity was higher in broccoli cultivated with organic fertilizers during 2014/15, while arylesterase activity was higher in broccoli cultivated with mineral fertilizers during the first two crop years. The interaction previous cowpea crop x fertilizer type was not significant for any enzyme activity (Table 4.6).

Table 4.6: Dehydrogenase, β-glucosaminidase and arylesterase activities in soil of broccoli crops grown after with cowpea or in monocrop using mineral and organic fertilizers for three successive years. Values are mean ± standard deviation (n=4).

Previous cowpea cultivar[a]	Fertilizer type	Dhs (μmol g^{-1} h^{-1})	Glm (μmol g^{-1} h^{-1})	Aryl (μmol g^{-1} h^{-1})
		2014/15		
FP	Mineral	0.31±0.16	0.13±0.04	156±32
FC	Mineral	0.40±0.14	0.15±0.04	199±8
Fallow	Mineral	0.37±0.10	0.04±0.05	119±45
FP	Organic	0.43±0.24	0.29±0.02	69±16
FC	Organic	0.54±0.31	0.17±0.06	83±20
Fallow	Organic	0.27±0.12	0.17±0.01	90±12
		2015/16		
FP	Mineral	1.13±0.16	0.17±0.04	142±35
FC	Mineral	1.29±0.25	0.13±0.02	129±8
Fallow	Mineral	0.85±0.20	0.15±0.04	80±32
FP	Organic	1.21±0.13	0.11±0.01	115±33
FC	Organic	1.44±0.26	0.18±0.03	135±29
Fallow	Organic	0.80±0.33	0.15±0.02	122±32
		2016/17		
FP	Mineral	0.06±0.01	0.03±0.02	17±12
FC	Mineral	0.05±0.03	0.04±0.01	56±12
Fallow	Mineral	0.08±0.01	0.08±0.02	51±30
FP	Organic	0.04±0.02	0.06±0.01	63±44
FC	Organic	0.07±0.02	0.07±0.03	67±27
Fallow	Organic	0.16±0.01	0.07±0.02	63±7
		F-value[b]		
Year (Y)		204.85***	62.02***	36.26***
Previous cowpea cultivar (PCC)		1.08ns	2.63ns	3.73*
Fertilizer type (FT)		0.92ns	22.19***	4.46*
Y x PCC		5.62***	9.09***	1.28ns
Y x FT		0.36ns	16.57***	17.33***
PCC x FT		0.71ns	0.15ns	2.79ns
Y x PCC x FT		1.86ns	7.37***	2.13ns

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro.

[b] Significant at ***P < 0.001; *P < 0.05; ns: not significant (P > 0.05).

Dhs: Dehydrogenase; Glm: β-glucosaminidase and Aryl: Arylesterase.

Table 4.7: β-glucosidase activity in soil of broccoli crops grown after cowpea or in monocrop using mineral and organic fertilizers for three successive years. Values are mean ± standard deviation (n=4).

Previous cowpea cultivar[a]	Fertilizer type	Glu (μmol g^{-1} h^{-1})
	2014/15	
FP	Mineral	0.21±0.03
FC	Mineral	0.36±0.26
Fallow	Mineral	0.44±0.53
FP	Organic	0.55±0.27
FC	Organic	0.49±0.13
Fallow	Organic	0.34±0.11
	2015/16	
FP	Mineral	0.63±0.17
FC	Mineral	0.60±0.12
Fallow	Mineral	0.44±0.05
FP	Organic	0.65±0.14
FC	Organic	0.92±0.03
Fallow	Organic	0.55±0.09
	2016/17	
FP	Mineral	0.13±0.02
FC	Mineral	0.18±0.08
Fallow	Mineral	0.22±0.15
FP	Organic	0.11±0.03
FC	Organic	0.11±0.04
Fallow	Organic	0.10±0.06
	χ^2 value	
Year (Y)		8.28**
Previous cowpea cultivar (PCC)		0.36ns
Fertilizer type (FT)		0.35ns

[a]FP: Feijão frade de fio preto; FC: Feijão frade de fio claro.
[b]Significant at ** P < 0.01; ns: not significant (P > 0.05).
Glu: β-glucosidase.

4.3.3. Broccoli yield, crop quality and nutritional characteristics

As a general trend, year had a significant effect on the subsequent broccoli yield and quality and on the nutritional characteristics (Tables 4.8 and 4.9). Crop yield and broccoli head diameter were highest during 2015/16, while broccoli stem diameter was higher during 2016/17. Broccoli head nutritional content, was highest during 2014/15. Previous cowpea crop did not affect broccoli yield, crop quality parameters or nutritional characteristics. Fertilizer type significantly influenced crop yield and broccoli head diameter, with higher values for both parameters using mineral fertilizer. In addition, the two-way ANOVA performed separately for each sampling time showed that during 2015/16, N and P content in broccoli head were significantly higher using organic than mineral fertilizers ($P<0.05$). The interaction year x fertilizer was significant for crop yield and broccoli head diameter, and nutrients such as P, with the highest values for both parameters using mineral fertilizers during 2015/16. P content in broccoli head did not follow a clear pattern. In addition, despite crop yield was significantly higher with the use of mineral fertilizers compared to organic fertilizers during the first year, during the last year, crop yield was similar (table 4.8), suggesting that organic fertilizer improved yield in the longer term. However, the interactions of previous cowpea crop with the other factors (year and fertilizers type) was not significant for broccoli crop yield, crop quality or nutritional characteristics, except for P content (Tables 4.8 and 4.9).

Table 4.8: Crop yield and quality of broccoli crops grown after cowpea or in monocrop using mineral and organic fertilizers for three successive years. Values are mean ± standard deviation (n=4).

Previous cowpea cultivar[a]	Fertilizer type	Crop yield (kg ha^{-1})	Broccoli head diameter (cm)	Broccoli stem diameter (cm)
		2014/15		
FP	Mineral	24791±5301	19.46±3.91	3.68±0.17
FC	Mineral	24012±8494	18.42±4.86	3.72±0.40
Fallow	Mineral	29151±4272	22.27±2.49	3.82±0.31
FP	Organic	15537±4052	15.45±1.79	3.47±0.50
FC	Organic	12141±1533	12.39±0.94	3.43±0.06
Fallow	Organic	15992±1324	15.57±1.13	3.59±0.08
		2015/16		
FP	Mineral	27200±1261	22.00±1.00	3.33±0.58
FC	Mineral	29850±2600	23.67±3.21	3.33±0.42
Fallow	Mineral	30100±1559	23.33±2.08	3.35±0.61
FP	Organic	23400±2258	20.67±0.58	3.67±0.48
FC	Organic	19350±5817	20.00±2.65	3.34±0.23
Fallow	Organic	22650±4743	20.33±0.58	3.45±0.68
		2016/17		
FP	Mineral	26263±9277	16.55±3.75	4.04±0.10
FC	Mineral	16607±2269	12.76±1.07	4.01±0.26
Fallow	Mineral	17108±4891	12.90±2.56	4.06±0.16
FP	Organic	20978±1941	19.85±10.30	3.88±0.18
FC	Organic	23591±2028	15.45±0.75	4.02±0.19
Fallow	Organic	16524±5056	12.14±31.70	3.86±0.21
		F-value[b]		
Year (Y)		8.11***	18.37***	10.33***
Previous cowpea cultivar (PCC)		0.99ns	1.44ns	0.04ns
Fertilizer type (FT)		25.14***	5.54*	0.61ns
Y x PCC		2.11ns	2.37ns	0.28ns
Y x FT		8.05***	5.35**	0.95ns
PCC x FT		0.21ns	0.78ns	0.13ns
Y x PCC x FT		1.90ns	0.14ns	0.18ns

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro.
[b] Significant at ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$; ns: not significant ($P > 0.05$).

Table 4.9: Nutrients content of broccoli heads in broccoli crops grown after cowpea or in monocrop using mineral and organic fertilizers for three successive years. Values are mean ± standard deviation (n=4).

Previous cowpea cultivar[a]	Fertilizer type	N (g kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	K (mg kg^{-1})	P (mg kg^{-1})
2014/15						
FP	Mineral	49.65±2.88	14706±6210	2973±707	48972±8399	9046±298
FC	Mineral	48.78±6.21	7326±1020	2791±321	47105±6150	6558±1295
Fallow	Mineral	47.21±1.15	10909±1544	3096±826	50921±11729	7712±2151
FP	Organic	47.93±5.84	7526±605	2610±646	44982±12079	7138±899
FC	Organic	45.86±3.01	9408±5560	3381±687	54071±6770	7958±2018
Fallow	Organic	51.15±4.67	9318±3378	2747±385	49039±9562	8224±1022
2015/16						
FP	Mineral	40.06±3.11	5828±1635	2371±426	41432±4843	5415±1134
FC	Mineral	39.51±0.84	4447±847	1945±321	34230±5170	4686±1004
Fallow	Mineral	41.80±2.97	6228±1186	2523±397	41200±3865	6359±1019
FP	Organic	45.37±4.24	5796±457	2504±119	41396±2321	7714±917
FC	Organic	46.09±5.31	5781±924	2356±157	48667±5555	7116±246
Fallow	Organic	44.94±3.44	5210±940	2283±261	43297±3923	6827±760
2016/17						
FP	Mineral	37.70±0.29	2728±330	1045±162	23753±2064	4449±458
FC	Mineral	44.44±4.79	4425±859	2392±56	40075±1011	7893±208
Fallow	Mineral	42.43±9.89	4993±942	2447±266	42207±4583	8713±1317
FP	Organic	46.89±5.50	3374±40	2098±49	36650±1057	6738±241
FC	Organic	39.44±7.91	3687±196	2153±64	37509±3575	6691±284
Fallow	Organic	42.86±1.79	4125±215	2208±91	39050±619	7271±503
F-value[b]						
Year (Y)		9.02***	64.68***	22.29***	20.42***	8.43***
Previous cowpea cultivar (PCC)		0.22ns	2.07ns	2.56ns	3.34ns	3.00ns
Fertilizer type (FT)		2.64ns	1.35ns	0.59ns	2.87ns	3.61ns
Y x PCC		0.05ns	2.59ns	0.86*	1.27ns	3.69*
Y x FT		1.41ns	1.35ns	0.37ns	0.84ns	4.45*
PCC x FT		1.12ns	1.56ns	2.70ns	1.67ns	1.49ns
Y x PCC x FT		1.68ns	2.52ns	3.09*	2.90ns	5.13**

[a] FP: Feijão frade de fio preto; FC: Feijão frade de fio claro.

[b] Significant at ***$P < 0.001$; *$P < 0.05$; ns: not significant ($P > 0.05$).

4.4. Discussion

The inclusion of a cowpea crop in multiple cropping did not increase, as a general pattern, soil properties or enzyme activities in subsequent broccoli crops. However, there was a 20 % reduction in fertirrigation compared to that supplied to the monocrop with no negative effects on soil nutrient content and crop yield and quality. The previous cultivation of cowpea acts as a source of different types of C through rhizodeposition, which stimulates the growth of rhizosphere microorganisms (Haichar et al., 2008) and facilitates nutrient availability to the plant (Mwafulirwa et al., 2016). Lower values of available P in the broccoli monocrop in the third year could be consequence of a low abundance of phosphate-solubilizing bacteria such as those belonging to *Rhizobium* genus (Rodríguez and Fraga, 1999), meaning a greater need for external inputs, mainly during this last year when soil P content decreased compared to the beginning of the experiment. Phosphorus is often immobilized by precipitation in insoluble forms such as Ca-P or Mg-P, which are solubilized by soil microorganisms (bacteria and fungi) through the release of organic acids, and thus soil availability of P and Mg is increased (Rodríguez and Fraga, 1999; Arcand and Schneider, 2006; Dighton, 2007). We observed no general effects of the specific cowpea cultivar on soil properties or broccoli yield and quality. It has been previously reported that the release of root exudates is controlled by the plant genotype (Merbach et al., 2000; Nguyen, 2003), and so crop genotype could have different effects on soil properties. However, this was not observed in our experiment, rejecting our initial hypothesis. On the other hand, the Ca content was lower in soil cultivated with broccoli in multiple cropping with cowpea compared with the broccoli monocrop. This suggests that cowpea took up this nutrient, which could be reincorporated into the soil through mineralization of cowpea residues if it is used as green manure.

This study confirms that mineral fertilizers normally improve crop yields (Cavigelli et al., 2008; Thorup-Kristensen et al., 2012). The use of organic fertilizers was associated with improvements in soil structure and nutrient cycling through increased microbial activity, as previously reported by several authors (Barto et al., 2010; Zhang et al., 2014). N use efficiency in crops depends on the extent to which fertilizers are degraded and mineralized. The use of mineral fertilizers plays an important role in improving productivity, since it provides a source of readily available N, which is one of the major factors determining crop yields. However, the beneficial effect on crop yield due to the use of N synthetic fertilizers may compromise environmental quality through leaching or denitrification (Chien et al., 2009) if the total soil mineral N available is greater than the crop's ability to take it up.

Previous research has reported an increase in soil total N, nitrate and ammonium after the introduction of legume crops in multiple cropping as a result of BNF, thus contributing to increase soil fertility (Aschi et al., 2017; Biederbeck et al., 2005). However, we observed no such effect of the cowpea crop, or even an increase in the nitrate content, in 2015 in the broccoli monocrop, leading us to reject our initial hypothesis. This may have been due to the lack of nodules in cowpea roots (data not shown), suggesting the absence of specific rhizobial strains that form an effective association with cowpea, which is not commonly grown in our region.

The increase in AS with organic fertirrigation was not related to the increase in SOC, but more likely related to activation of microbial populations, which can be stimulated by organic compounds and the rhizodeposition process (Barros et al., 2007; Hannam et al., 2006), supporting our initial hypothesis. Bacteria, fungal hyphae and plant roots are involved in the formation of stable aggregates through the production secondary metabolites, organic inputs and the exudation that acts as glue between organic and inorganic soil constituents (Jastrow and Miller, 1998; Six et al., 2006). In this sense, Tang et al. (2011) reported the significant influence of bacteria and fungi on soil aggregate stability in agricultural systems. Improvements in soil structure contributes to enhancing soil quality, since it controls soil water retention and movement, aeration, nutrient dynamic, the movement of fauna and root penetration (Bronick and Lal, 2005). Worthington (2001) describes the better quality of organic food compared to conventionally-cultivated foods, which is line with the increase in essential nutrients (N and P) in the broccoli head cultivated under organic farming in this study. Although the increase in N recorded in broccoli heads seems go against the lower N content measured in the soil under organic fertirrigation, it seems to suggest the higher assimilation of available N under organic fertirrigation due to a synergistic effect with active microorganisms.

The inclusion of cowpea, together with the reduction in external fertilizers, can be regarded as a sustainable alternative for saving on external inputs, with no detrimental effects on broccoli crop yield, quality or nutritional characteristics. In this regard, Plaza-Bonilla et al. (2017) also observed that the yield and quality were maintained in wheat crops grown after legumes and cover crops using mineral fertilizers and decreased N fertilizer rates. Although the application of fertilizers is an indispensable agricultural practice for enhancing plant nutrition and increasing crop yield, inorganic fertilizers may contribute to changing the soil environment by depressing soil microbial activity (Ramírez et al., 2012). In addition, N fertilization alters the fungal communities (Paungfoo-Lonhienne et al., 2015) which, together with bacteria, improve soil structure by promoting the formation of soil aggregates (Miller and Jastrow, 2000). Thus, the adoption of multiple cropping systems with legumes may enhance soil quality and biodiversity, while decreasing production costs. Furthermore, multiple cropping also provides diversification, increasing opportunities in the face of low commodity prices and decreasing exposure to adverse climatic factors (Zegada-Lizarazu and Monti, 2011).

4.5. Conclusions

In conclusion, our results show that the inclusion of cowpea in multiple cropping with a broccoli crop, accompanied by a 20% reduction in fertilizer rates, did not improve soil organic matter content or soil structure but maintained high soil nutrient content to promote high broccoli crop yield and quality despite the reduction in external inputs. Mineral fertilizers increased broccoli head quality and led to higher broccoli yields, while the organic fertilizers improved soil structure through the activation of soil microbial populations, and there was a long term effect on crop yield due to the use of organic fertilizers. Thus, the inclusion of cowpea in multiple cropping can maintain horticultural crop yields and quality likely due to activation of microbial populations that solubilize soil nutrients, decreasing production costs by saving on external inputs, widening market opportunities as a result of crop diversification.

Chapter 5

A comparative greenhouse gas emissions and enzyme activities analysis of legume and non-legume crops grown in organic and conventional management

A comparative greenhouse gas emissions and enzyme activities analysis of legume and non-legume crops grown in organic and conventional management

Abstract

Legume crops have been proposed as a way of reducing greenhouse gas (GHG) emissions because of their ability to fix atmospheric N and thus reduce the need for external N fertilizers. Moreover, the establishment of organic agriculture has been proposed as a sustainable strategy to enhance the delivery of ecosystem services, although crop yields are normally lower compared to conventional agriculture. The main objective of this study was to assess the effect of a legume and non-legume crop (fava bean and broccoli) during two years on crop yield, GHG emissions (N_2O, CO_2 and CH_4) and soil enzyme activities, grown under conventional or organic management practices. GHG emissions, crop yield and enzyme activities differed between years. Fava bean generated the highest GHG emissions, while broccoli showed higher soil enzyme activities. Conventional management resulted in higher crop yields for both crops. Organic management led to higher N_2O and CO_2 emissions and soil enzyme activities in both crops, likely due to an increase of soil organic matter mineralization. Crop yield was related to lower GHG emissions and higher enzyme activities. Thus, legume crops may not reduce GHG emissions in all situations, and a thorough assessment should be carried out for each crop and pedoclimatic characteristics. This may be related to the ability of legumes to increase N availability through biological N fixation.

Keywords: *Vicia faba*; *Brassica oleracea var italica*; nitrous oxide; carbon dioxide; methane; soil microorganisms.

5.1. Introduction

The increasing demand for food from a growing human population has led to increase global agricultural land (Wang et al., 2013). Crop production has been related to a source of greenhouse gas (GHG) emissions, including carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) (IPCC, 2007). Although CO_2 is the main anthropogenic GHG, the agricultural sector is dominated by CH_4 and N_2O (Schulze et al., 2009).

N_2O is mainly produced by denitrification under anaerobic conditions or by nitrification under aerobic conditions (Ussiri and Lal, 2013). Both processes may be increased not only by the N fertilization but also by biological nitrogen fixation (BNF) process that takes place in legume crops (Lupwayi and Kennedy, 2007; Carter and Ambus, 2006). It is important to highlight that legume cultivation can also produce N_2O emissions through N released by root exudates during the growing season (Wichern et al., 2008), which may be nitrified and denitrified by soil bacteria (Snyder et al., 2009), but also by the ability of some rhizobia for direct denitrification (O´Hara and Daniel, 1985). In addition, the high microbial activity of the legume rhizosphere may also increase soil organic matter (SOM) mineralization and, as a consequence, CO_2 emissions (Chapela et al., 2001). Rhizosphere is an enzymatic hotspot where microorganisms and living roots produce extracellular enzymes through rhizodeposition (Kuzyakov, 2002; Kuzyakov and Blagodatskaya, 2015). The release of root exudates is depending on plant physiology and root morphology (Nguyen, 2003) and its quality and composition vary between plant species (Aulakh, 2001; Jones et al., 2004).

Thus, the effect of legume cultivation on soil GHG emissions is not totally understood and needs further attention. In this sense, BNF can be an environmentally friendly source of plant available N in the soil, allowing savings in N fertilizers, which are the major GHG emitters in agriculture (Carter and Ambus, 2006). Sources of CO_2 from soil include soil organic matter (SOM), dead plant residues (above and below ground) and organic substances released by living root through exudation (Kuzyakov, 2006). However, the principle source of CH_4 emission from agriculture comes from its production by methanogenesis and its consumption by methanotrophic microorganisms under anaerobic and aerobic conditions, respectively (Dutaur and Verchot, 2007). Thus, there is a need to monitor GHG emissions in legumes and other vegetable crops grown under the same soil, climatic and management conditions to really assess whether legumes efficiently contribute to the reduction or increase of GHG fluxes, and if shifts in emissions are related to the rhizosphere activity.

Cropping system and agricultural management assume a powerful tool for the mitigation of GHG emissions (Sainju et al., 2012). N inputs by fertilization assumes a great influence on GHG fluxes, although this is dependent on fertilizer type (Ding et al., 2007). The N fertilization has a negative impact on the activity of methanotrophic microorganisms, which are responsible for CH_4 oxidation, and, as a consequence, the production of CH_4 is enhanced (Hütsch et al., 1996). Organic fertilizers could be a viable alternative to decrease N_2O emissions in comparison with conventional inorganic fertilizers due to the reduced amount of N availability (Flessa et al., 2002). Nevertheless, the use of organic fertilizers is also associated with increased rates of organic matter decomposition and thus, higher N_2O and CO_2 emissions (Dendooven et al., 2012), mainly by activation of microbial populations and increased enzyme activities (Iqbal et al., 2009; De Forest et al., 2004). Burger et al. (2005) observed the positive relationship between the activity of certain microbial groups and N_2O production in tomato crop grown under organic practices. The adoption of different management practices (conventional vs organic) offers the possibility to assess their influence on crop yield, soil enzyme activities and environmental pollution through GHG emissions.

According to the latter approaches, a winter legume (fava bean) and a non-fixing N vegetable crop (broccoli) were cultivated under conventional and organic management practices during two years. The objectives of this study were to: i) assess the effect of two different crops under two different management practices on soil GHG emissions; and ii) infer if there is a relationship between GHG emissions, soil enzyme activities and crop yield. We hypothesized that the legume-nitrogen fixing bacteria association could increase soil N availability through BNF, that along with the use of N fertilizers may lead to higher GHG emissions, mainly N_2O emissions, compared with the non-N-fixing vegetable crop. The adoption of organic management may reduce N_2O emissions but increase CO_2 emissions by increased soil organic matter mineralization.

5.2. Materials and methods

5.2.1. Study site and experimental design

This study was carried out in Cartagena, southeast Spain (37º 41` N 0º 57` E). The field experiment was designed in a complete randomized block with four replications, and each plot had 10 m^2. A local Spanish cultivar - Muchamiel - of fava bean (*Vicia faba* L.) was grown during two winter seasons (24/10/2014-06/03/2015 and 05/11/2015-13/04/2016). Simultaneously, a local Spanish cultivar -Parthenon - of broccoli (*Brassica oleracea* L. var italica) was also grown during the same winter seasons (13/11/2014-26/02/2015 and 1/12/2015-2/03/2016).

The area was characterized by a semiarid Mediterranean climate with a mean annual temperature of 18ºC and total annual precipitation of 275 mm. The climatic conditions during the two fava bean and broccoli years are shown in Table 5.1. Meteorological data were measured using an automatic weather station located in the experimental field. Soil temperature (T) and moisture (M) were measured using a 5 TM Soil Moisture and Temperature sensor (Decagon Devices, USA).

Table 5.1: Climatic conditions during both fava bean and broccoli crop years

Parameters	Fava bean		Broccoli	
	2014/15	2015/16	2014/15	2015/16
Minimum air T (°C)	5.68	8.32	5.68	8.84
Mean air T (°C)	12.58	13.37	11.85	12.72
Maximum air T (°C)	19.13	19.18	18.79	17.42
Rainfall (mm)	31.50	40.45	25.24	7.25

Both crops were established under drip irrigation with two management practices: conventional and organic. Fava bean seeds were sown with a spacing of 100 cm between rows and 40 cm between plants (2.5 plants m^{-2}) while broccoli plants were planted with a spacing of 100 cm between rows and 20 cm between plants (5 plants m^{-2}). No herbicide treatment was given, and the crops were kept free of weeds through hand-hoeing when necessary. In the fava bean crop, 20 kg ha^{-1} of N and 1.2 kg ha^{-1} of P_2O_5 were applied by fertirrigation as ammonium nitrate (33.5% N) and monoammonium phosphate (61% P_2O_5, 12% N) in the conventional practice, and using a commercial organic fertilizer (Bombardier, Agroquímicos los Triviños, Spain; 10.7% w/v N, 0.7% w/v P_2O_5) in the organic practice. In the broccoli crop, 250 kg ha^{-1} N, 100 kg ha^{-1} P_2O_5 and 300 kg ha^{-1} K_2O were applied by fertirrigation as ammonium nitrate (33.5% N), monoammonium phosphate (61% P_2O_5, 12% N) and potassium sulphate (50% w/v K_2O, 18% S) in the conventional practice, and using two commercial organic fertilizers (Heronatur 4-2-8 and Heronatur 7-2-4; Herogra Fertilizantes, Spain; 4% w/v N, 2% w/v P_2O_5 and 8% w/v K_2O, and 7% w/v N, 2% w/v P_2O_5 and 4% w/v K_2O) in the organic practice.

5.2.2. Soil and plant sampling

The soil was a *Haplic Calcisol* (IUSS, 2014) with a clay loam texture, the main characteristics of which are shown in Table 5.2.

Table 5.2: Main soil characteristics. Values shown are mean ± standard deviation (n=4)

Soil properties[a]	
pH	8.40±0.06
EC (µScm^{-1})	329±48
SOM (%)	2.30±0.10
Nt (g kg^{-1})	0.75±0.05
Bulk density (g cm^{-3})	1.01±0.03
CEC (cmol$_+$ kg^{-1})	7.8±1.2
$CaCO_3$ (%)	30.2±1.2
Clay (%)	34.5±0.16
Silt (%)	21.3±1.06
Sand (%)	44.2±0.92

[a] EC: electrical conductivity; SOM: soil organic matter; Nt: total nitrogen; CEC: cation exchange capacity.

A soil sampling was carried out at harvest for both fava bean and broccoli crops every year. All plots were sampled at 0-20 cm (plough depth). Three random soil samples per plot were collected and homogenized to obtain a composite sample. Samples were air-dried for 7 days, sieved < 2 mm and stored at room temperature until analyses. Enzyme activities were also measured in air-dried samples since these properties in Mediterranean semiarid soils are medium-term stable in stored air-dried samples (Zornoza et al., 2009a).

Fava bean crop yield was determined by continuous collection and weighting of all pods in each plot when the seeds were fresh. Broccoli crop yield was determined by weighing the heads when they reached the marketable size.

5.2.3. Gas sampling

During the two years, gas samples were taken once a week between 9:00 and 13:00 to measure N_2O, CO_2 and CH_4 emissions. The basic experimental procedure used in this study was the static gas chamber technique. The chamber was made of polycarbonate sheets, with a diameter of 30 cm and a height of 37cm, with a septum at the top for sampling**.** The chambers were inserted into the soil to a depth of 10 cm. Gas samples were collected at 0, 30 and 60 min after chamber closure and stored in previously evacuated 10 mL blood containers (Vacutainers, Venojet) at room temperature. N_2O, CO_2 and CH_4 were quantified by gas chromatography (7890B GC Agilent Technologies) equipped with a flame ionization detector (FID) and an electron capture detector (ECD). Average N_2O, CO_2 and CH_4 emissions were calculated and the cumulative values for each gas and treatment were estimated by numerical integration.

5.2.4. Soil analyses

Bulk density was determined by the method of the cylinder; soil pH and electrical conductivity (EC) were measured in deionized water (1:2.5 and 1:5 w/v, respectively); soil texture was determined by the Bouyoucos method (Dewis and Freitas, 1970); for equivalent calcium carbonate the volumetric method (Bernard calcimeter) was used (Cobertera, 1993); soil organic carbon was determined by the wet oxidation method using $K_2Cr_2O_7$ (Walkley and Black, 1934) and then soil organic matter (SOM) was estimated by applying the factor 1.724; total nitrogen (Nt) was analyzed by the Kjeldahl method (Hoeger, 1998); cation exchange capacity (CEC) was determined by the use of $BaCl_2$ as exchangeable salt (Roig et al, 1980); β-glucosidase activity (Glu) was based on the determination of *p*-nitrophenol released after incubation at 37 °C with β-D-glucopyranoside (Tabatabai, 1982); β-glucosaminidase activity (Glm) was based on the determination of *p*-nitrophenol released after incubation with *p*-nitrofenil-β-D-glucopyranoside at 37 °C (Parham and Deng, 2000); dehydrogenase activity (Dhs) was determined using *p*-iodo-nitro-tetrazolium chloride as substrate and measuring the absorbance of the iodonitrotetrazolium formazam (INTF) produced (Von Merci and Schinner, 1991); arylesterase activity (Aryl) was based on the determination of *p*-nitrophenol released after incubation with *p*-nitrofenil acetate at 37°C (Zornoza et al., 2009b).

5.2.5. Statistical analyses

Data were checked to ensure normal distribution using the Kolmogorov–Smirnov test. Soil data were submitted to three-way ANOVA to assess the differences among year, crop and management practice. Crop yield in fava bean and broccoli crops were submitted to two-way ANOVA to assess the effect of year and management practice. Relationships among properties were studied using Pearson's correlations. Multiple linear regression analysis ($Y=m_1X_1 +m_2X_2 +\cdots+m_nX_n +b$) was carried out using stepwise and backward methods, to quantify the contribution of soil enzyme activities and crop yield that might potentially affect GHG. Standardized coefficient (β) and partial correlation values were used for the analysis. The β coefficient is the estimated value resulting from the analysis performed on variables standardized to have a variance of 1 in order to determine which of the independent variables has a greater effect on the dependent variable. Therefore, variables with larger β coefficients contribute more to the model. The partial correlation indicates the correlation between the dependent variable and one independent variable when the linear effects of the remaining variables have been eliminated. The unstandardized coefficients (m) were used to fit the values of cumulative GHG emissions *versus* the values calculated using the regression model. A principal components analysis (PCA) was performed with all data to study the structure of dependence and correlation established among the variables studied in both crops. Statistical analyses were performed with the software IBM SPSS for Windows, Version 22.

5.3. Results

5.3.1. Crop yield

Crop yield for both crops was significantly influenced by year and management practice (Table 5.3). Fava bean and broccoli crops showed higher crop yield during 2015/2016 than in 2014/2015. Moreover, both crop yields were higher under conventional than organic management practice during both years. Fava bean yield was 63% and 31 % higher under conventional management practice during 2014/2015 and 2015/2016, respectively. Broccoli yield was 76% and 33 % higher under conventional management practice during 2014/2015 and 2015/2016, respectively. Thus, differences in yield between conventional and organic management decreased the second year.

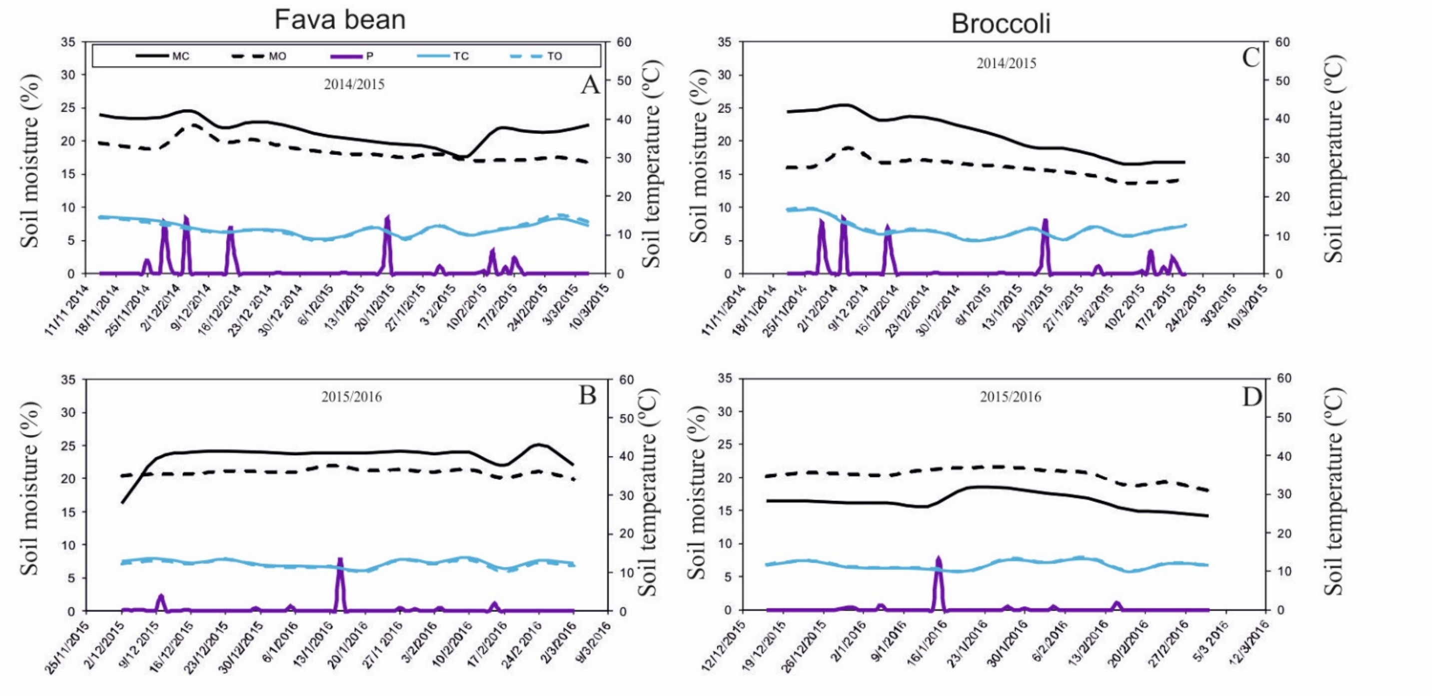
Table 5.3: Crop yield in fava bean and broccoli crops during 2014/2015 and 2015/2016. Values shown are mean ± standard deviation (n=4)

Management practice	Year	Fava bean crop yield (kg ha^{-1})	Broccoli crop yield (kg ha^{-1})
Conventional	2014/2015	15883±3543	24401±6896
Organic		9750±876	13839±1419
Conventional	2015/2016	53000±8887	28525±1212
Organic		40250±6978	21375±3846
F-value[a]			
Year (Y)		97.27***	6.19*
Management practice (MP)		7.58*	14.29**
Y × MP		0.93 ns	0.53 ns

[a] Significant at ***P < 0.001; **P < 0.01; *P < 0.05; ns: not significant (P > 0.05).

5.3.2. Soil moisture and temperature

Year did not affect soil temperature (Figure 5.1). Soil moisture showed higher values in 2014/15 for broccoli and in 2015/16 for fava bean. Crop significantly influenced soil moisture and temperature, with highest values in fava bean crop. Management practice significantly influenced soil moisture, with higher values under conventional than organic management practice for both crops, except for broccoli crop during the last year. The only significant interaction between factors was year x management practice for soil moisture.



Soil temperature

Year (Y) = 1.59 ns
Crop (C) = 7.39*
Management practice (MP) = 0.81 ns

Y × C = 0.23 ns
Y × MP = 0.02 ns
C × MP = 0.52 ns
Y × C × MP = 0.10 ns

Soil moisture

Y = 5.84***
C = 29.22***
MP = 22.36***

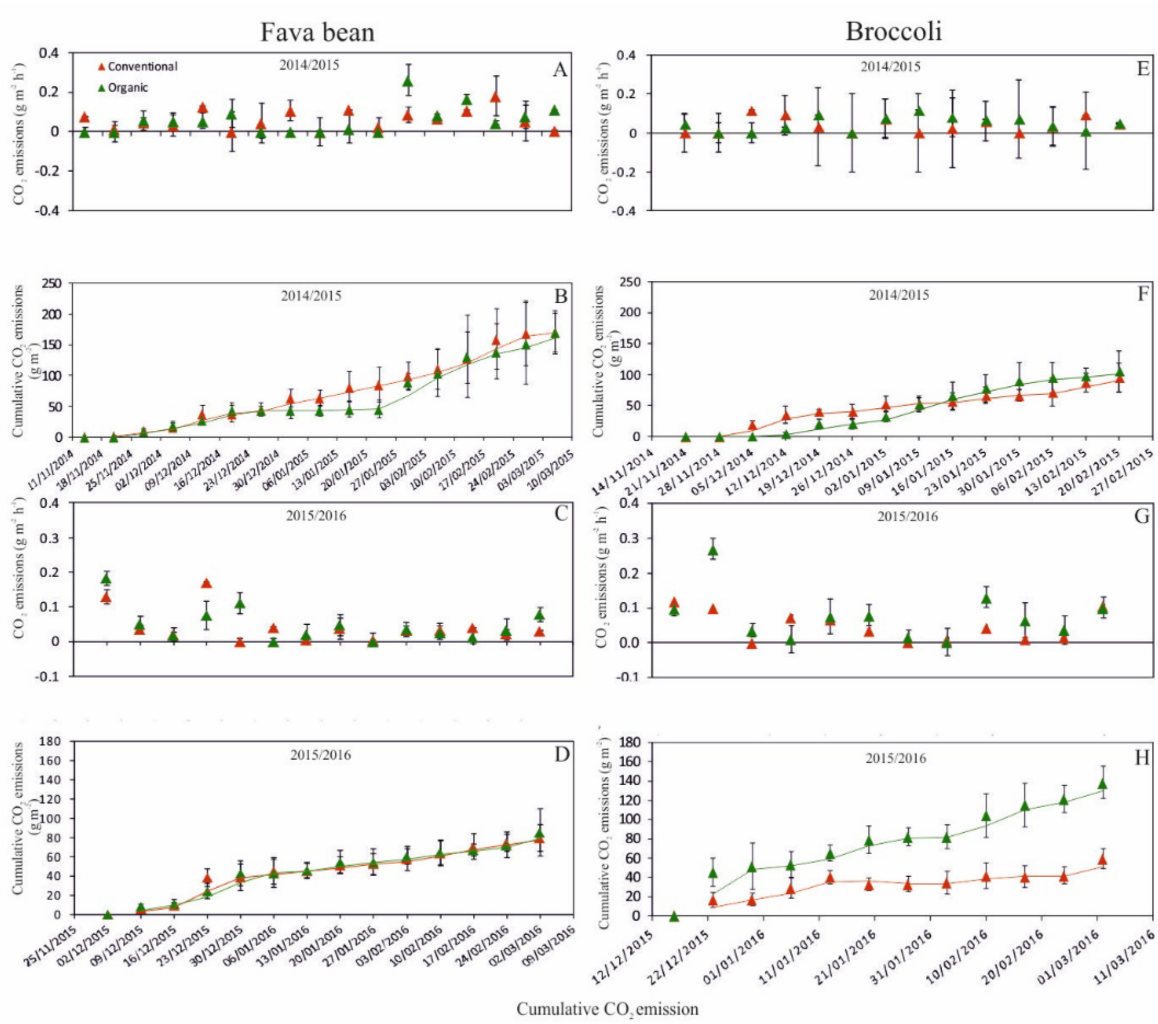
Y × C = 2.16 ns
Y × MP = 11.40**
C × MP = 0.10 ns
Y × C × MP = 0.98 ns

Figure 5.1: Soil moisture, soil temperature and precipitation for both crops during 2014/2015 (A and C for fava bean and broccoli, respectively) and 2015/2016 (B and D for fava bean and broccoli, respectively). MC: soil moisture under conventional management; MO: soil moisture under organic management; P: precipitation; TC: soil temperature under conventional management; TO: soil temperature under organic management. F values and significance of the three-way ANOVA are shown at the bottom of the figure for soil temperature (left) and soil moisture (right). Significant at * P < 0.05, ** P < 0.01 and *** P < 0.001; ns: not significant (P > 0.05).

5.3.3. Soil CO_2 emissions

CO_2 emission rates were similar in both crops during both years regardless of the management practice (Figure 5.2A, C and Figure 5.2E, G). However, organic practice led to higher emission rates during the second year in the broccoli crop. The highest CO_2 emission rate was 0.26 g m^{-2} h^{-1} for fava bean 83 days after the sowing, with no significant differences between organic and conventional management. The highest CO_2 emission rate for broccoli was 0.27 g m^{-2} h^{-1} 21 days after planting under organic management.

Cumulative CO_2 emission was, in average, 175 g m^{-2} and 84 g m^{-2} in fava bean crop during 2014/2015 and 2015/2016, respectively (Figure 5.2B, D). In broccoli, cumulative CO_2 emission was, in average, 110 g m^{-2} and 100 g m^{-2} during 2014/2015 and 2015/2016, respectively (Figure 5.2F, H). Thus, year significantly affected the cumulative CO_2 emission, which was higher during the cycle 2014/2015 in fava bean crop. The cumulative CO_2 emission was significantly affected by crop and management practice. Taking into account the crop, cumulative CO_2 emission was higher in the fava bean than in the broccoli crop during 2014/2015. With regard to management practice, organic practice showed higher cumulative CO_2 emission for broccoli crop during 2015/2016. The interaction between year and crop was significant, with highest values in fava bean during 2014/2015 (Figure 5.2B, D). Cumulative CO_2 emissions were positively correlated with cumulative N_2O emissions ($R = 0.72$, $P < 0.01$) and negatively to crop yield ($R = - 0.60$, $P < 0.01$).



Year (Y) = 23.65***
Crop (C) = 5.30*
Management practice (MP) = 6.33*

Y × C = 15.30***
Y × MP = 2143 ns
C × MP = 3068 ns
Y × C × MP = 4046 ns

Figure 5.2: CO$_2$ emission rates and cumulative CO$_2$ emissions for fava bean crop (left) and broccoli crop (right) during both crop cycles (2014/2015 and 2015/2016). F values and significance of the three-way ANOVA are shown at the bottom of the figure. Significant at ** P < 0.01 and *** P < 0.001; ns: not significant (P > 0.05).

5.3.4. Soil N_2O emissions

N_2O emission rates decreased during the second year under both management practices in both crops (Figure 5.3A, C for fava bean and Figure 5.3E, G for broccoli). The highest N_2O emission rate was 0.26 mg m^{-2} h^{-1} for fava bean in 17/02/2015 under conventional management. For broccoli, the highest N_2O emission rate was 0.27 mg m^{-2} h^{-1} in 21/12/2015 under organic management. As a general pattern, the highest N_2O emissions were observed in fava bean crop compared with broccoli crop.

Cumulative N_2O emission was significantly affected by year, crop and management practice, with significant interactions year x crop and crop x management practice. Cumulative N_2O emission was higher during the cycle 2014/2015 in fava bean crop. With regard to crop, cumulative N_2O emission was, in average for both management practices, higher in fava bean (126 mg m^{-2}) than in broccoli (78 mg m^{-2}) during 2014/2015. Organic management led to highest cumulative N_2O emission in fava bean crop during both years. Cumulative emission was 25% and 63% higher under organic management than under conventional management for 2014/2015 and 2015/2016, respectively. Organic management also led to the highest cumulative N_2O emission in broccoli crop during 2015/2016, with 44 % higher under organic than under conventional management. Cumulative N_2O emissions were positively correlated with Glm ($R = 0.656$, $P < 0.01$), and negatively correlated with Dhs ($R = - 0.657$, $P < 0.01$) and crop yield ($R = - 0.723$, $P < 0.01$).

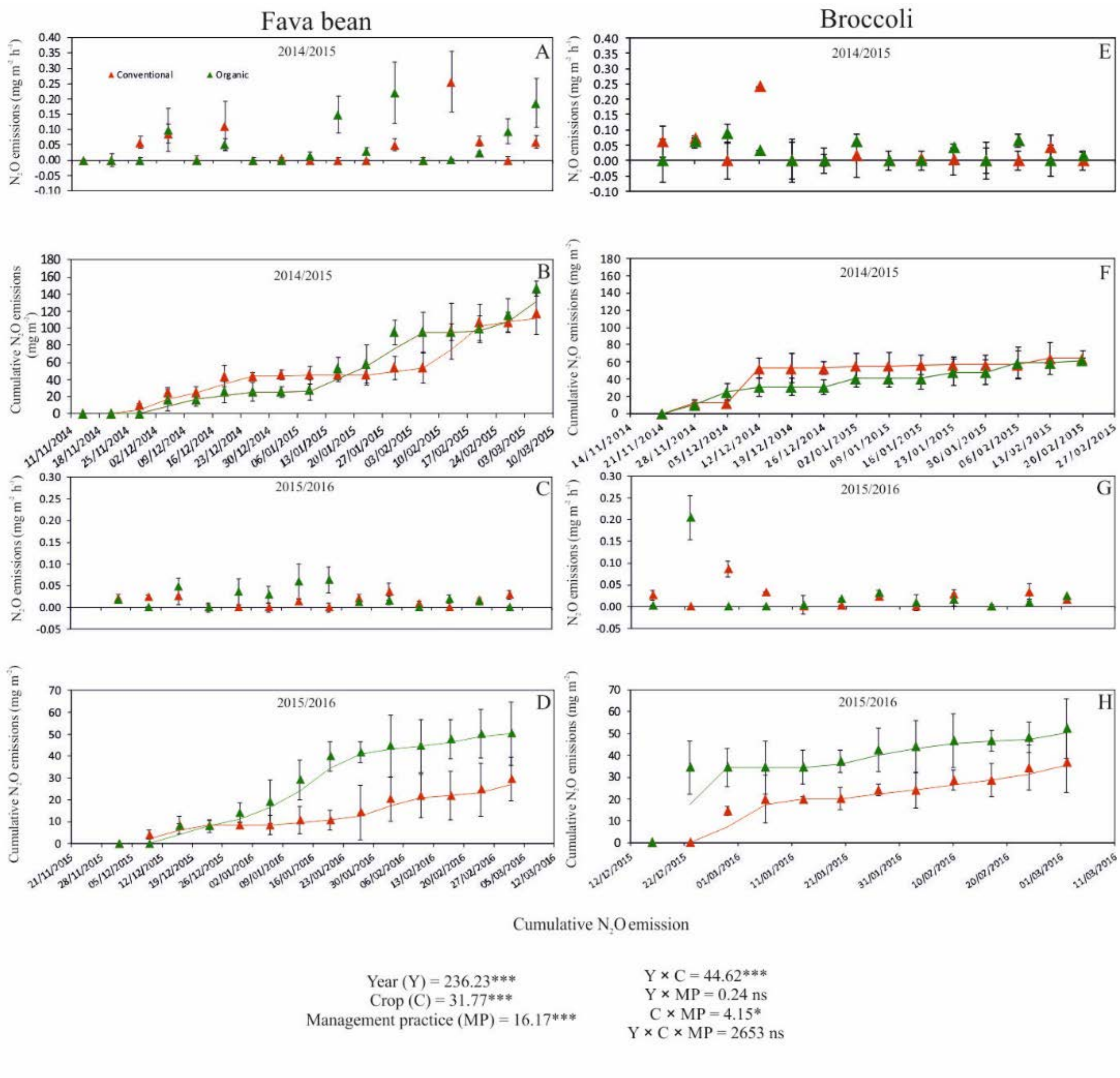


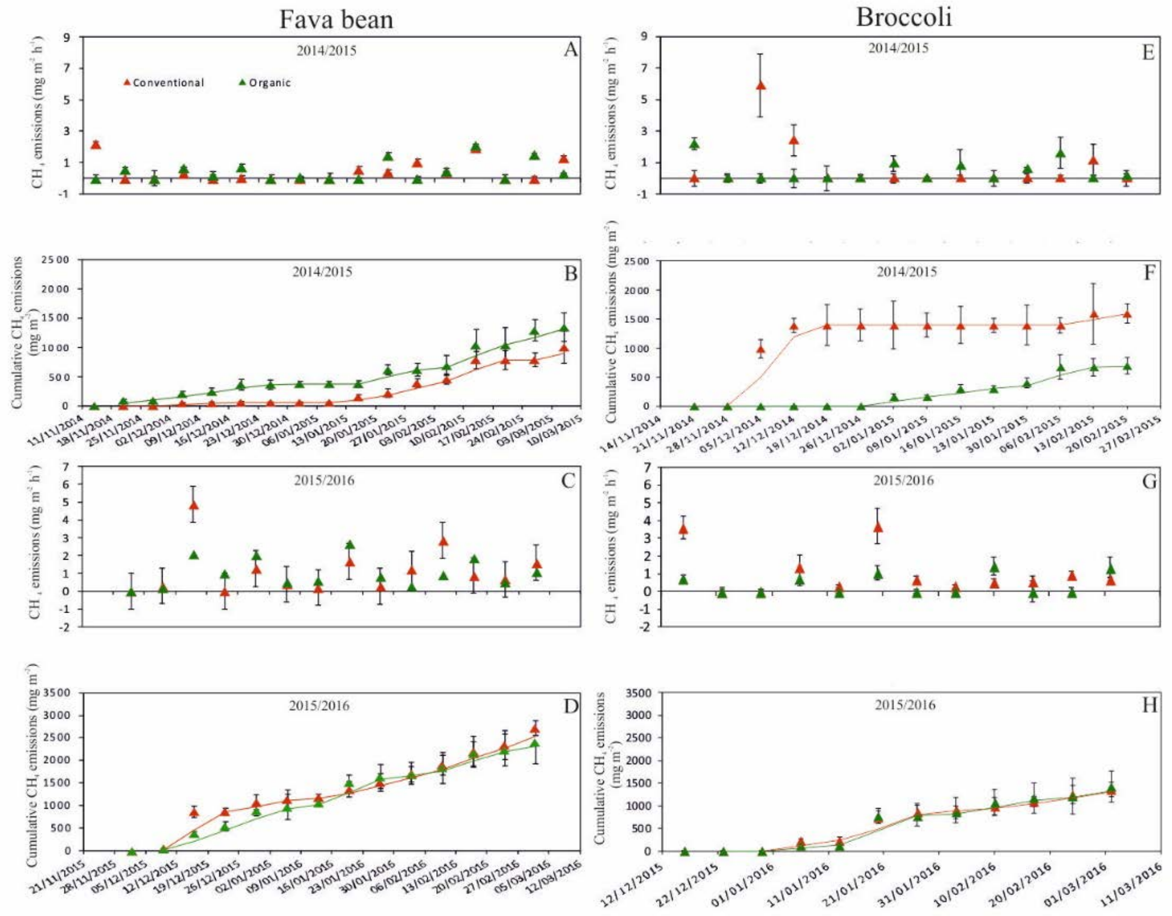
Figure 5.3: N_2O emission rates and cumulative N_2O emissions for fava bean crop (left) and broccoli crop (right) during both crop cycles (2014/2015 and 2015/2016). F values and significance of the three-way ANOVA are shown at the bottom of the figure. Significant at ** P < 0.01 and *** P < 0.001; ns: not significant (P > 0.05).

5.3.5. Soil CH_4 emissions

CH_4 emission rates slightly increased during the second year under both management practices in both crops (Figure 5.4A, C for fava bean and Figure 5.4E, G for broccoli). The highest CH_4 emission rate was 4.9 mg m^{-2} h^{-1} for fava bean in 19/12/2015 under conventional management. In broccoli, the highest CH_4 emission rate was 5.9 mg m^{-2} h^{-1} 22 days after planting under conventional management. In general, CH_4 emission rates were higher in fava bean than broccoli crop during the second year.

Cumulative CH_4 emission was, in average, 1192 mg m^{-2} and 2539 mg m^{-2} in fava bean crop during 2014/2015 and 2015/2016, respectively (Figure 5.4B, D). Cumulative CH_4 emission was, in average, 1499 mg m^{-2} and 1470 mg m^{-2} in broccoli crop during 2014/2015 and 2015/2016, respectively (Figure 5.4F, H). Cumulative CH_4 emission was significantly influenced by year, crop and management practice, and all interactions were significant.

Cumulative CH_4 emission was higher for fava bean than broccoli crop under both management practices during 2015/2016 (2539 mg m^{-2} in fava bean crop and 1470 mg m^{-2} in broccoli crop). In fava bean, cumulative CH_4 emission was higher during 2015/2016 compared to 2014/2015 while in broccoli crop, this emission was higher during 2014/2015 under conventional practice. Organic management led to higher cumulative CH_4 emission during 2014/2015 in fava bean. However, conventional practice showed higher cumulative CH_4 emission during 2014/2015 in broccoli crop (Figure 5.4B, D for fava bean and Figure 5.4F, H for broccoli). Cumulative CH_4 emissions were negatively correlated to Glm ($R= - 0.648$, $P < 0.01$) and positively correlated to crop yield ($R= 0.792$, $P<0.01$).



Cumulative CH_4 emission

Year (Y) = 119.05***
Crop (C) = 39717***
Management practice (MP) = 8241*

Y × C = 129845***
Y × MP = 14420**
C × MP = 9901**
Y × C × MP = 73806***

Figure 5.4: CH_4 emission rates and cumulative CH_4 emissions for fava bean crop (left) and broccoli crop (right) during both crop cycles (2014/2015 and 2015/2016). F values and significance of the three-way ANOVA are shown at the bottom of the figure. Significant at ** $P < 0.01$ and *** $P < 0.001$; ns: not significant ($P > 0.05$).

5.3.6. Soil enzyme activities

Glu, Glm and Dhs activities were significantly affected by year (Table 5.4); Glu and Dhs showed higher activity in both crops during 2015/2016, while Glm activity was the highest during 2014/2015. Glu and Aryl were significantly influenced by crop, with higher activities in broccoli than in fava bean crop. With regard to management practice, Glu and Dhs showed significantly higher activities under organic management, while Aryl showed significantly higher activity under conventional management. The interaction crop x year was significant for Glm, with higher activity in broccoli than in fava bean during 2015/2016. The interaction year x management practice was significant for Aryl, with higher activity under conventional management during 2014/2015 than during 2015/2016.

Table 5.4: Soil enzyme activities in fava bean and broccoli crops during 2014/2015 and 2015/2016. Values shown are mean ± standard deviation (n=4)

Management practice	Year	Glu (μmol PNP g^{-1} h^{-1})	Glm (μmol PNP g^{-1} h^{-1})	Aryl (μmol PNP g^{-1} h^{-1})	Dhs (μmol INTF g^{-1} h^{-1})
		Fava bean			
Conventional	2014/2015	0.196±0.009	0.367±0.084	123±12	0.367±0.261
Organic		0.401±0.149	0.234±0.149	82±2	0.657±0.126
Conventional	2015/2015	0.425±0.137	0.102±0.015	109±38	1.214±0.124
Organic		0.475±0.080	0.113±0.044	88±21	1.310±0.199
		Broccoli			
Conventional	2014/2015	0.287±0.125	0.139±0.022	177±19	0.358±0.103
Organic		0.521±0.093	0.229±0.037	77±17	0.487±0.081
Conventional	2015/2016	0.616±0.126	0.148±0.020	136±22	1.210±0.200
Organic		0.784±0.057	0.144±0.014	125±28	1.327±0.147
		F-value[a]			
Crop (C)		16.59***	2.13 ns	9.46**	0.38 ns
Year (Y)		26.33***	18.77***	0.00 ns	140.67***
Management practice (MP)		14.16**	0.11 ns	22.59***	5.51*
C × Y		2.74 ns	8.48**	0.15 ns	0.50 ns
C × MP		0.72 ns	3.86 ns	1.81 ns	0.27 ns
Y × MP		1.62 ns	0.21 ns	8.99**	0.59 ns
C × Y × MP		0.26 ns	5.01*	3.77 ns	0.46 ns

[a] Significant at ***P < 0.001; **P < 0.01; *P < 0.05; ns: not significant (P > 0.05).

Glu: β-glucosidase activity, Glm: β-glucosaminidase activity, Aryl: Arylesterase activity, Dhs: Dehydrogenase activity.

5.3.7. Interrelationship between GHG emissions, soil enzyme activities and crop yield

Multiple linear regression analysis (Table 5.5) showed that the cumulative N_2O emissions were positively related to the cumulative CO_2 emissions, and negatively to crop yield and Glu activity ($R^2 = 0.77$, $F = 22577$, $P < 0.001$). Cumulative CH_4 emissions were positively related to crop yield and negatively to Glm activity ($R^2 = 0.69$, $F = 23.53$, $P < 0.001$).

Table 5.5: Multiple linear regression models for GHG emissions in fava bean and broccoli crops during 2014/2015 and 2015/2016.

Y	*X*	m	Partial correlation	β	R^2	R^2 adj	F value
Cumulative N_2O emission (mg m^{-2})	Constant (b)	107.97			0.77	0.74	22577***
	Crop yield (kg ha^{-1})	-0.001	-0.64	-0.51			
	Glu activity (µmol PNP g^{-1} h^{-1})	-69.45	-0.59	-0.36			
	Cumulative CO_2 emission (g m^{-2})	0.25	0.45	0.32			
Cumulative CH_4 emission (mg m-^{2})	Constant (b)	1.3×10^6			0.69	0.66	23.53***
	Crop yield (kg ha^{-1})	0.03	0.62	0.68			
	Glm activity (µmol PNP g^{-1} h^{-1})	-1.9×10^6	-0.30	-0.41			

Glu: β-glucosidase activity, Glm: β-glucosaminidase activity.

The PCA performed with the studied cumulative GHG emissions, soil enzyme activities and crop yield of both crops showed that 76.4% of the total variation could be explained by the first three PCs. PC1, which explained 38.8 % of variation, separated crop cycle 2015/16 (positive scores) from crop cycle 2014/15 (negative scores) (Figure 5.5A). In addition, for year 2014/15, PC1 separated broccoli from fava bean. This PC was related with cumulative N_2O and CO_2 emissions, Glu, Glm, Dhs and crop yield (Table 5.6). PC2, which explained 24.1 % of variation, separated the fava bean crop (positive scores) from broccoli crop (negatively scores) (Figure 5.5A and B). Moreover, PC2 slightly separated the conventional practice (higher scores) from the organic practice (lower scores). This PC2 was related with higher values of soil moisture and temperature, and cumulative CH_4 emissions (Table 5.6). PC3, which explained 13.5 % of variation, separated the organic (negatively scores) from conventional management practice (positively scores) for both crops during 2014/15 (Figure 5.5B). Organic practice showed lower Aryl activity (Table 5.6).

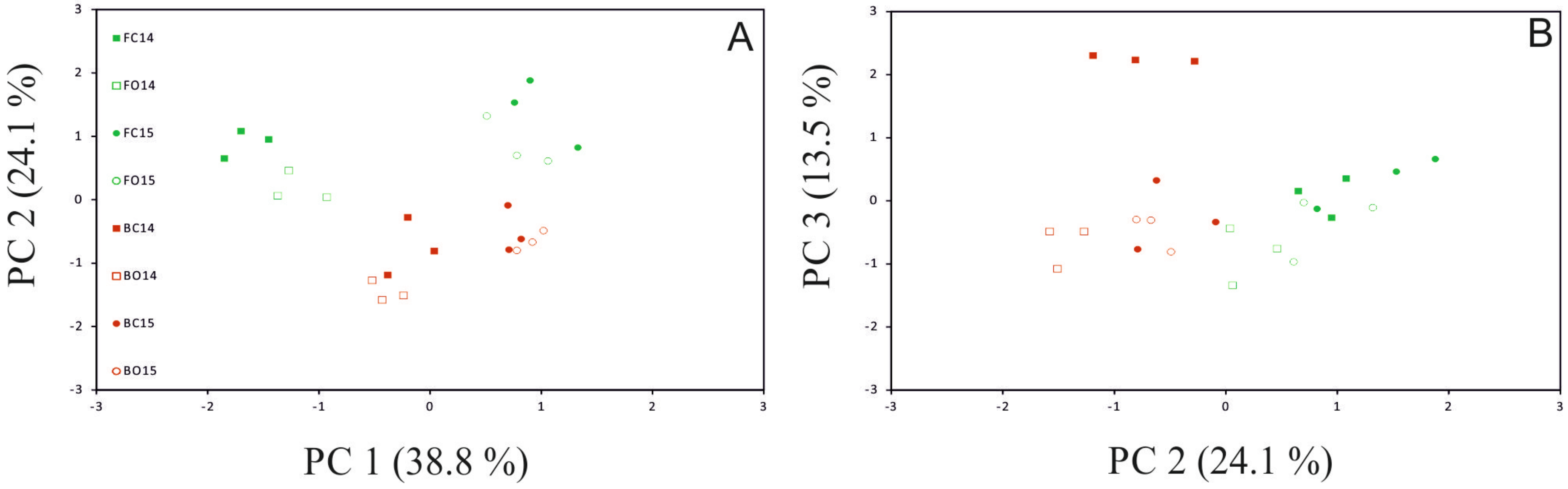


Figure 5.5: PCA factor scores of variations in GHG emissions, soil enzyme activities and crop yield in fava bean and broccoli crop, considering different year, crop and management practices. Color represents crop (green: fava bean crop; red: broccoli crop), figure type represents year (square: 2014/2015; circle: 2015/2016) and figure filling represents management practice (filled figure: conventional management practice; empty figure: organic management practice). F: fava been; B: broccoli; C: conventional management, O: organic management: 14: 2014/2015 crop cycle; 15: 2015/2016 crop cycle.

Table 5.6: Matrix of PCA obtained with variation rates (%) of GHG emissions, soil enzyme activities and crop yield of fava bean and broccoli crops during two years.

Variance explained	PC1 (38.8%)	PC2 (24.1%)	PC3 (13.5%)
Cumulative N_2O emissions (mg m^{-2})	-0.916	-0.079	0.083
Dhs activity (µmol INTF g^{-1} h^{-1})	0.803	0.124	0.342
Cumulative CO_2 emissions (g m^{-2})	-0.780	0.022	0.253
β-Glm activity (µmol PNP g^{-1} h^{-1})	-0.767	-0.153	0.033
Crop yield (kg ha^{-1})	0.693	0.654	-0.096
β-Glu activity (µmol PNP g^{-1} h^{-1})	0.970	-0.499	0.450
Soil temperature (ºC)	0.017	0.804	0.289
Soil moisture (%)	0.057	0.802	-0.340
Cumulative CH_4 emissions (mg m^{-2})	0.591	0.632	-0.082
Aryl activity (µmol PNP g^{-1} h^{-1})	0.107	0.031	-0.863

Dhs: Dehydrogenase activity; Glm: β-glucosaminidase activity; Glu: β-glucosidase activity; Aryl: Arylesterase activity.

5.4. Discussion

This study showed how year, crop and management practice affect crop yield, greenhouse gas fluxes and soil enzymatic activities of legume and non-legume crops. Crop yield and soil enzyme activities for both crops as well as CH_4 emissions for fava bean increased during the second year, while CO_2 and N_2O emissions decreased for fava bean crop. In turn, the increase of crop yield and soil enzyme activities was linked to lower GHG emissions (CO_2 and N_2O). Despite the fact that the decomposition of biomass by soil microorganisms results in CO_2 emission from soil through microbial respiration, the negative relationship between soil enzyme activities and GHG emissions can be explained because a higher proportion of C is retained in the soil through the formation of stable organic matter. This fact can occur when C inputs from photosynthesis exceed C losses through soil respiration, resulting in soil C sequestration (Lal, 2004). In turn, a higher C retention in soil could reduce N_2O emissions, since available carbon controls denitrification rates and thus N_2O production (Beauchamp et al., 1989; Mathieu et al., 2006). The negative relationship between crop yield and N_2O and CO_2 emissions, as well as the negative relationship of soil enzyme activities with N_2O and CH_4 emissions may indicate that high crop yield is related to a more efficient soil microbial community in the use of C and N, which is not lost by rapid mineralization, and favours plant nutrient uptake. Tautges et al. (2016) also observed a positive relationship between soil fungal and bacterial abundance and crop yield in a pea crop, concluding that increased fungi and bacteria sizes may be providing a benefit for plants by greater nutrient availability.

Ecological factors such as soil moisture and temperature can be affected by different crops mainly due to vegetation attributes such as density and height (Özkan and Gökbulak, 2017). In this sense, higher soil moisture in fava bean compared with broccoli crop may be favoring anaerobic conditions, which are required for methanogenesis, explaining higher CH_4 emissions in the legume crop. Legume crops can reduce the use of external N inputs by biological N fixation, and consequently decrease N_2O emissions. However, legume-N-fixing bacteria symbioses may also produce N_2O emissions through the nitrification and denitrification of biologically fixed N (Galloway, 1998) or by N-fixing bacteria that are able to denitrify (O´Hara and Daniel, 1985). This process could explain higher N_2O emissions in fava bean than broccoli crop during 2014/2015. This is opposite to the lower contribution of N_2O emissions linked to N fixed through biological nitrogen fixation confirmed by Zhong et al. (2009). In addition, higher N_2O emissions in soil cultivated with legumes can be due to the release of root exudates during the growing season, which tend to be N-rich in legumes plant species (Fustec et al., 2010). Increased CO_2 emissions in fava bean compared to broccoli may be explained by an increase of C compounds released from root exudation or crop residues decomposition, which may also increase SOM mineralization. However, broccoli crop showed higher soil hydrolytic enzyme activities, which should be related to higher CO_2 emissions than in fava bean. This contradictory result may be indicating that other enzymes and biochemical processes may be taking place in soil to favour increased GHG emissions in fava bean crop, not identified with the studied indicators.

With regard to management practice, the use of conventional management led to higher crop yield. These results revealed that organic practice could be associated with a low N use efficiency, possibly due to a slow mineralization of applied organic inputs and the lack of synchrony between supplied and required N (Alaru et al., 2014). Organic practices have been considered as a possible strategy for reducing GHG emissions through C sequestration (Dalgaard et al., 2001; Flessa et al., 2002; Diacono and Montemurro 2010). However, crops grown under organic management in this experiment showed higher N_2O and CO_2 emissions and soil enzyme activities, which seems to indicate that organic fertilizers activated microbial populations, which favored the mineralization of the organic compounds (Kuzyakov, 2006). These results contradict those obtained by other researchers, which observed reduction of GHG emissions in soils cultivated under organic practices (Diacono and Montemuro, 2010; Abalos et al., 2016).

5.5. Conclusions

In conclusion, the results based on the crop yield, GHG emissions and soil enzyme activities in legume and non-legume crops under conventional and organic practices during two years showed that second year offered lower N_2O and CO_2 emissions for fava bean crop together with an enhancing crop yield and higher levels of enzyme activities for both crops. Fava bean crop released more N_2O, CO_2 and CH_4, while CO_2 emissions were directly related to N_2O emissions. The adoption of conventional management practice resulted in higher crop yields for both crops, while organic practice led to higher N_2O and CO_2 emissions as well as soil enzymatic activities. These results support our initial hypothesis concerning the legume-nitrogen fixing bacteria (NFB) association could increase soil N availability through BNF, that along with the use of N fertilizers can lead to higher GHG emissions, mainly N_2O emission, compared to the non-N-fixing vegetable crop. The adoption of organic management increased N_2O and CO_2 emissions possibly due to an increase of soil organic matter mineralization.

Chapter 6

Influence of different nitrogen-fixing bacteria and arbuscular mycorrhiza on biological nitrogen fixation and yield, quality and nutritional characteristics of a fava bean crop

Influence of different nitrogen-fixing bacteria and arbuscular mycorrhiza on biological nitrogen fixation and yield, quality and nutritional characteristics of a fava bean crop

Abstract

The introduction of nitrogen-fixing bacteria (NFB) into the soil is an advisable agricultural practice to improve N efficiency in the agro-ecosystems. Furthermore, arbuscular mycorrhizal fungi (AMF) inoculation have positive effects for the symbiotic plant by enhancing nutrient, water uptake, and tolerance to biotic and abiotic stresses. Hence, the aim of this work was to study plant nutrition, biological nitrogen fixation (BNF), and crop yield and quality of two fava bean cultivars (Muchamiel and Palenca) inoculated with NFB and/or AMF during two seasons. Both cultivars were inoculated with *Rhizobium* and *Burkholderia* genera NFB and/or AMF through individual and dual inoculation treatments, and fertigated with 20% decrease in fertilization rate compared to non-inoculated crop. Nutritional composition in plants was not significantly affected by inoculation treatments and the reduction in the use of fertilizers. BNF was influenced by cultivar, with higher values in shoot of Muchamiel. Protein content in grain was 17% higher after inoculation in Muchamiel cultivar during second season, showing a higher efficiency in the assimilation of N by the plant with inoculation. Dual inoculation showed higher N content in shoot (106 % and 24 % in Muchamiel and Palenca, respectively) compared to individual inoculation. The inoculation with *Burkholderia cenocepacia* showed 20-29% higher N content in roots compared to *Rhizobium leguminosarum*. Thus, the use of inoculation techniques in fava bean resulted in an environmental friendly alternative, reducing the input of fertilizers at the same time as it maintains crop yield and quality, with increases in grain protein content.

Keywords: *Vicia faba; Rhizobium* sp.*; Burkholderia* sp.*;* biological nitrogen fixation*;* plant nutrition.

6.1. Introduction

The alarming increase of the worldwide population leads to greater fertilizer requirements to reach the required food production. The application of nitrogen-fixing bacteria (NFB) in plant cultivation is one of the most promising methods for an effective management of nitrogen fertilizers and increasing agricultural productivity (Lugtenberg et al., 2002). This is an important option for enhancing biological nitrogen fixation (BNF) in crop production systems, since it is a good alternative to i) reduce external inputs of fertilizers; ii) offer a higher efficiency in the utilization of N by the plant; and iii) reduce N leaching along soil profile and avoid water pollution (Peoples et al., 1995; Giller, 2001). BNF is affected by many factors including weather, water availability, soil pH, phosphorus content and management practices (Hungria et al., 2000; Vance et al., 2000). In this process, Rhizobia are bacterial symbionts of legumes that fix atmospheric N in specialized structures on plant roots called nodules. In exchange, bacteria receive carbon compounds derived from photosynthesis (Laranjo et al., 2014). In this group, most bacteria belong to the α-class of proteobacteria (*Bradyrhizobium, Mesorhizobium, Rhizobium,* and *Sinorhizobium),* although other bacteria from the β- and *γ*-class of proteobacteria can be found, such as *Burkholderia* and *Enterobacter*, respectively (Vandamme et al., 2002; Benhizia et al., 2004; Glick, 2012; Zaidi et al., 2015; Nadeem et al., 2014).

In addition to NFB, arbuscular mycorrhizal fungi (AMF) also play an important role in the BNF process, since they act as phosphate solubilizing microorganisms that are able to mineralize organic P and make available inorganic P (Sharda and Koide, 2010). P is considered the less available nutrient to plants, which is related to nodule formation and functioning, and it is essential for the action of the nitrogenase enzyme in the BNF process (Al-Niemi et al., 1997; Shen et al., 2011). Legumes often need inoculation techniques when they are grown in regions outside their areas of diversity, where they have not been traditionally grown or have not been grown for a long time (Brockwell et al., 1995). In addition to this, the inclusion of NFB into the soil is advisable when the legume is cultivated in a soil with an absence of previous crop of same or symbiotically linked microorganisms (Catroux et al., 2001). NFB may be introduced to legumes by inoculation of the seed or the soil at sowing. Seeds may be inoculated by farmers immediately prior to sowing or by local seed merchants, while soil is inoculated using inoculants suspended in water or formulated as liquids or granules (Deaker et al., 2004).

The survival of introduced bacteria species is depending on factors such as desiccation, the toxic nature of soluble seed coat exudates and unfavorable temperatures (Date, 1968; Vincent et al., 1962). In turn, tripartite symbiosis (NFB-AMF-legumes) against non-inoculated control plant or plant inoculated with AMF or NFB alone, offers benefits to the plant such as enhanced plant growth, crop yield, phosphorus and nitrogen content (Pacovsky et al., 1986; Azcon et al., 1991). In this respect, research studies such as those carried out by Tajini et al. (2012) and Abd-Alla et al. (2014) showed that dual inoculation improved parameters of nodulation, nitrogenase activity, mycorrhization and nutrients content (N and P) in plant compared with individual inoculation.

Based on these approaches, we designed an experiment involving two fava bean cultivars inoculated with NFB belonging to *Rhizobium* and *Burkholderia* genera, and arbuscular mycorrhizal fungi. The objectives of the study were to assess: i) the effectiveness of such dual inoculation on crop yield and quality; ii) whether the dual inoculation can increase BNF by the crop, and hence root nodulation and the content of grain protein; and iii) the different response of cultivars to dual inoculation. We hypothesized that the tripartite symbiosis between NFB, AMF and the plants would increase crop yield and the protein content of the edible grain through enhanced biological N fixation. This response may be cultivar dependent, since plant´s genetic controls microbial activity in the rhizosphere through root exudation.

6.2. Materials and methods

6.2.1. Study site and experimental design

This study was carried out in Cartagena, southeast Spain (37º 41` N 0º 57` E). The climate of the area is semiarid Mediterranean with a mean annual temperature of 18ºC and annual precipitation of 290 mm. Potential evapotranspiration surpasses 900 mm. The soil of the study site is a *Haplic Calcisol* (IUSS, 2014) with clay loam texture. The field experiment was designed in a complete randomized block with four replications, and each plot had 10 m^2. Two different cultivars (Muchamiel and Palenca) of fava bean (*Vicia faba* L.) were grown under drip irrigation under conventional management practice during two seasons. Both cultivars were subjected to eight treatments: 1. individual inoculation with *Rhizobium leguminosarum* (RL)*;* 2. individual inoculation with *Burkholderia cenocepacia* (BC)*;* 3. individual inoculation with *Burkholderia vietnamiensis* (BV)*;* 4. individual inoculation with AMF (*Rhizophagus irregularis, Claroideoglomus* *etunicatum, Claroideoglomus claroideum* and *Funneliformis mosseae*), 5. combined inoculation of *Rhizobium leguminosarum* with AMF; 6. combined inoculation of *Burkholderia cenocepacia* with AMF; 7. combined inoculation of *Burkholderia vietnamiensis* with AMF; and 8. non-inoculated crop (control). A non-inoculated crop of broccoli (*Brassica oleracea var. italica* L.) was included as the reference non-nitrogen fixing species to assess the BNF, also grown under drip irrigation.

In the first season, sowing took place on 6 November 2015, starting to flower on 10 and 24 February 2016 for Palenca and Muchamiel cultivars, respectively, and harvesting from 28 March to 18 April 2016 for Palenca and from 6 to 25 April 2016 for Muchamiel. Broccoli was planted on 1 December 2015 and its plant was sampled at legume sampling time to assess BNF. In the second season, sowing was established on 9 November 2016, flowering started on 20 and 27 February 2017 for Palenca and Muchamiel cultivars, respectively, and harvesting from 27 March to 24 April 2017 for Palenca and from 3 to 28 April 2017 for Muchamiel. Broccoli was planted on 5 December 2016. With regard to fava bean harvest, all the pods in each plot were continuously harvesting and weighting when the seeds were fresh at the end of the crop cycle. Main weather conditions were a minimum air temperature of 8.3°C, mean temperature of 13.2ºC, maximum temperature of 19.3ºC and rainfall of 9.3 mm for the 2015/16 season, and a minimum air temperature of 6.5°C, mean temperature of 12.7ºC, maximum temperature of 17.1ºC and rainfall of 63.9 mm for the 2016/17 season.

Fava bean seeds were inoculated by adding 2 g of the different nitrogen-fixing bacteria and 4 g of AMF at sowing time. In the control treatment, autoclaved inoculants (121°C for 20 min) were applied at the same rate. The strains of the three nitrogen-fixing bacteria were isolated from the active root nodules of fava bean plants from Portugal and selected by their growth-promoting effect in fava bean plants in previous greenhouse studies. The bacterium was isolated by standard methods (Vincent, 1970) and cultivated and maintained on yeast extract-mannitol (YEM) agar medium consisting of 0.4 g yeast extract, 10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 8 g agar and 0.25% Congo Red, dissolved in 1000 mL deionized water and autoclaved at 121°C for 20 min. Bacterial culture was grown in 250 mL Erlenmeyer flasks containing 100 mL of YEM broth medium, for 3 days at 28°C. The contents of each flask were diluted to 300 mL with sterilized deionized water in order to obtain 10^9 cells per mL, estimated from the absorbance at 600 nm, and mixed with 1 kg of the sterilized carrier (compost soil:vermiculite 1:1 v/v), to give approximately 40% moisture in the inoculant (3×10^9 cells per gram of inoculum). AMF was provided by Symbiom (Czech Republic), and 1g contained approximately 160 spores, i.e. 40 spores of each fungal strain per gram.

The inoculated and non-inoculated seeds were sown with a spacing of 100 cm between rows and 40 cm between plants (2.5 plants m^{-2}). No herbicide treatment was carried out, and the crop was kept free of weeds by hand-hoeing when necessary. Fertilizer application in the fava bean plots started three weeks after sowing, adding 20 kg ha^{-1} of N and 20 kg ha^{-1} of P_2O_5 in the form of ammonium nitrate (33.5% N) and monoammonium phosphate (61% P_2O_5, 12% N), as well as 40 kg ha^{-1} of K_2O in the form of potassium sulphate (50% K_2O, 18% S) as fertigation throughout the crops cycles. Broccoli plants were transplanted two weeks after fava bean sowing, with a planting pattern of 20 cm between each plant × 100 cm of space (5 plants m^{-2}), and fertilized similarly to the fava bean crop by fertigation. The application rate of fertilizers was reduced by 20 % in inoculated crops compared to non-inoculated control to check whether external inputs fertilizers can be saved by introducing inoculation techniques.

6.2.2. Plant sampling

Plant sampling was carried out during fava bean flowering. Three plants per plot were carefully uprooted to obtain unharmed roots, and separated into root, shoot, nodules and seeds in the case of the legumes, and into root and shoot for broccoli to assess biological N fixation. Fava bean yield (kg ha^{-1}) was determined by continuously harvesting and weighting all the pods in each plot when the seeds were fresh. In addition, the following parameters were recorded: protein content in grain (%), number of pods per plant, weight of 100 seeds, pod length and dry weight of nodules.

The plant samples were oven dried and ground (A11 Basic, IKA) before incinerating at 500ºC; the ashes were dissolved in 0.6N HNO_3 and analysed for P, B, Ca, Mg, Na and K by ICP-MS (7500 CE, Agilent). Nitrogen (N) was determined by the Kjeldahl method (Hoeger, 1998). The protein content in grain was derived from the estimated N content by the following formula (AOAC, 1990):

$$\text{Protein content (\%)} = \text{N content (\%)} \times 6.25 \qquad (1)$$

NO_3^- was extracted with deionized water in a 1:50 plant:extractant ratio (Keeny and Nelson, 1982) and measured by ion chromatography (Metrohm 861).

6.2.3. Efficiency of biological nitrogen fixation

In order to determine the efficiency of BNF, the ^{15}N natural abundance method was used. The ^{15}N content of the plant samples was determined in the Stable Isotope Facility of UC-Davis, Davis, CA, USA, by CF-IRMS (Europa Scientific, Crewe, UK). This method is useful when the abundance of ^{15}N in the soil is higher than in atmospheric N_2 (0.3663%). The differences (δ^{15}N) between the ^{15}N abundance in each sample and in the atmospheric N were calculated using the equation 2 (Bedard-Haughn et al., 2003):

δ^{15}N=((Sample atom %N - 0.3663) / 0.3663)×1000 (2)

To calculate the proportion of N derived from air (%Ndfa), it is necessary to know the δ^{15}N of the N_2-fixing legume and the δ^{15}N of the non-fixing reference plant (broccoli) grown in the same soil as the N_2-fixing legume (equation 3) (Unkovich et al., 2008):

%Ndfa= ((δ^{15}N of reference plant - δ^{15}N of legume) / (δ^{15}N of reference plant – B)) ×100 (3)

As ´B´ value we used -0.50, based on ´B´ values for fava bean shoot taken from the literature (Unkovich et al., 2008).

6.2.4. Statistical analyses

Data were checked to ensure normal distribution using the Kolmogorov–Smirnov test and ln-transformed when necessary to ensure normal distribution. Data were submitted to three-way ANOVA to assess the differences among season, legume cultivar and inoculation treatment. Data of those properties without normal distribution were submitted to a one-way non-parametric ANOVA (Kruskal-Wallis test) for the factors season, legume cultivar and inoculation treatment. Relationships among properties were studied using Pearson's correlations. Statistical analyses were performed with the software IBM SPSS for Windows, Version 22.

6.3. Results

6.3.1. Plant nutritional characteristics

As a general pattern, most nutrients measured in the different plant parts (seeds, shoot and root) were higher in the second than in the first season (Tables 6.1; 6.2 and 6.3). Legume cultivar did not have a clear influence on the plant parameters analysed, while inoculation treatment significantly affected N in seeds, shoot and root. The inoculation of both fava bean cultivars with *Rhizobium leguminosarum* (RL) led to a significant increase of N in seeds for both growing seasons compared to the inoculated control (Table 6.1). In turn, dual inoculation with RL and AMF significantly increased N concentration in shoot for both fava bean cultivars and seasons compared with the RL individual inoculation (Table 6.2). N content in root was significantly higher after inoculation with *Burkholderia cenocepacia* (BC) than with RL for both fava bean cultivars and seasons (Table 6.3). Seed Mg, Na, K and P concentrations were positively correlated between each other ($R > 0.75$; $P < 0.01$). In shoot, Ca, Mg, Na and P concentrations were positively correlated between each other ($R > 0.73$; $P < 0.01$), while in root, Mg, K and P concentrations were positively correlated between each other ($R > 0.69$; $P < 0.01$).

Table 6.1: Nutrient content in seeds of both fava bean cultivars for two seasons (nitrogen content, nitrate, boron, calcium, sodium, magnesium, potassium and phosphorus). Values are mean ± standard deviation (n=4).

Cultivar (C)	Treatment (T)[a]	N (g kg^{-1})	NO_3^- (mg kg^{-1})	B (mg kg^{-1})	Ca (mg kg^{-1})	Na (mg kg^{-1})	Mg (mg kg^{-1})	K (mg kg^{-1})	P (mg kg^{-1})
2015/2016									
‘Muchamiel’	RL	48.2±0.6	443±6	68.9±4.1	457±320	91.7±52.6	295±146	2919±1283	1090±474
	RL+AMF	47.7±0.9	437±11	64.2±8.9	172±46	81.6±27.2	195±75	2333±838	762±249
	BC	42.4±1.1	438±7	54.9±12.0	166±75	52.2±6.4	184±65	1992±687	760±263
	BC+AMF	42.9±1.6	430±14	68.5±11.1	217±58	84.5±5.7	236±59	2899±598	988±232
	BV	41.6±1.4	440±14	45.3±16.3	186±151	53.6±28.6	208±167	2120±1726	825±658
	BV+AMF	41.6±1.8	451±5	39.8±9.3	117±45	49.8±9.6	114±45	1441±544	501±148
	AMF	44.4±5.1	431±22	59.2±5.4	237±56	79.0±23.4	316±96	2910±727	454±283
	CONTROL	45.7±2.8	442±20	45.8±8.3	176±55	72.5±9.0	217±44	2482±530	883±181
‘Palenca’	RL	45.8±1.8	448±16	42.7±14.5	224±112	71.3±16.5	266±136	2736±492	1035±511
	RL+AMF	49.8±1.3	440±13	49.3±14.3	242±71	96.1±41.2	290±97	3304±1257	1162±324
	BC	44.6±1.9	436±8	35.5±6.1	175±53	52.1±4.3	199±74	2028±719	792±246
	BC+AMF	45.3±0.7	426±17	37.2±9.8	121±56	64.3±26.7	130±55	1673±528	575±201
	BV	46.0±0.8	432±19	42.9±12.5	263±65	87.3±19.8	315±99	3073±848	1261±314
	BV+AMF	46.4±3.4	441±12	62.9±46.9	289±159	113±78	353±201	3979±2330	1398±738
	AMF	47.1±1.0	430±5	29.7±11.7	211±138	65.5±130.9	271±191	2648±1852	1036±732
	CONTROL	44.1±3.9	454±16	58.3±29.8	467±324	162±113	497±330	6243±4142	1785±1101
2016/2017									
‘Muchamiel’	RL	44.3±1.5	786±5	0±0	443±88	194±100	780±296	13820±3735	3836±1060
	RL+AMF	40.3±1.4	605±12	0±0	543±124	193±15	873±251	14764±2411	3976±668
	BC	41.5±2.9	733±13	0±0	343±60	105±43	511±96	10954±1090	2906±391
	BC+AMF	39.7±5.1	529±75	0±0	275±26	117±11	396±62	10101±931	2369±344
	BV	40.8±2.5	611±17	0±0	372±97	134±27	567±153	11609±1961	3206±672
	BV+AMF	41.3±2.9	580±282	0±0	518±101	147±54	757±130	14656±2942	3834±55
	AMF	44.0±5.7	496±16	0±0	347±73	155±13	516±118	10777±1249	2916±329
	CONTROL	42.3±1.7	771±18	0±0	415±241	213±87	660±340	12510±3505	3247±1299
‘Palenca’	RL	46.1±7.8	763±23	0±0	401±71	149±26	654±150	12330±1424	3432±491
	RL+AMF	38.7±1.3	569±16	0±0	378±114	166±44	603±101	12413±1360	3090±378
	BC	44.6±5.4	615±95	0±0	320±81	136±65	448±108	10284±898	2514±480
	BC+AMF	42.0±1.3	516±70	0±0	413±119	151±53	623±203	12691±2199	3225±741
	BV	43.2±3.3	386±321	0±0	316±29	134±20	435±40	10548±796	2656±224
	BV+AMF	39.7±1.5	672±294	0±0	372±101	147±26	531±125	11563±1092	2868±464
	AMF	36.5±2.9	502±53	0±0	444±151	152±50	784±263	13654±3271	3800±964
	CONTROL	38.4±2.9	808±85	0±0	448±140	186±29	744±195	13984±1628	3602±486
		χ^2 value[b]					F value[b]		
Season (S)		24901***	43.78***	81.43***	36.10***	46.94***	97.12***	525.27***	295.87***
Legume cultivar (LC)		1.40ns	0.03ns	0.09ns	0.003ns	2.62ns	0.23ns	0.55ns	0.06ns
Inoculation treatment (IT)		4.81*	1.92ns	0.09ns	3.00ns	3.20ns	1.34ns	0.33ns	0.55ns

[a]Treatment: RL (*Rhizobium leguminosarum),* BC (*Burkholderia cenocepacia),* BV (*Burkholderia vietnamiensis)* and AMF (arbuscular mycorrhizal fungi).

[b]Significant at *P<0.05; ***P<0.001; ns: not significant (P>0.05). Different letters indicate significant differences (P<0.05) among means.

Table 6.2: Nutrient content in shoot of both fava bean cultivars for two seasons (nitrogen content, nitrate, boron, calcium, sodium, magnesium, potassium and phosphorus). Values are mean ± standard deviation (n=4).

Cultivar (C)	Treatment (T)[a]	N (g kg^{-1})	NO$_3^-$ (mg kg^{-1})	B (mg kg^{-1})	Ca (mg kg^{-1})	Na (mg kg^{-1})	Mg (mg kg^{-1})	K (mg kg^{-1})	P (mg kg^{-1})
					2015/2016				
‘Muchamiel’	RL	30.5±6.4	511±40	46.9±11.5	2158±1242	1032±487	584±290	9055±3097	726±192
	RL+AMF	36.7±4.3	454±253	31.6±2.1	3270±779	986±139	939±164	10229±1548	851±145
	BC	27.5±2.3	468±30	21.1±4.0	2457±1144	935±195	732±242	9197±2141	961±233
	BC+AMF	29.7±5.8	460±33	22.3±10.4	2308±1159	1250±176	670±267	8254±2192	599±303
	BV	33.3±3.3	527±50	44.4±7.8	2308±720	1094±561	688±256	9028±4288	842±251
	BV+AMF	20.7±7.4	510±61	50.3±5.4	3143±429	1372±147	961±146	6536±1176	1057±136
	AMF	30.1±2.3	596±130	26.4±5.0	1347±239	795±33	403±83	4375±1085	556±130
	CONTROL	27.4±1.8	646±237	65.1±20.6	4283±743	2182±287	1407±91	16445±2527	1442±166
‘Palenca’	RL	29.9±0.9	610±230	31.5±10.9	2453±1025	929±181	654±234	9406±835	881±257
	RL+AMF	35.5±2.6	476±2	35.8±7.3	4733±1178	1635±249	1186±196	17060±2333	1208±81
	BC	26.4±3.1	640±217	17.3±6.4	2283±1573	897±213	570±233	7223±1159	525±502
	BC+AMF	22.0±9.4	456±12	37.1±23.7	3107±1602	1256±733	826±426	11491±5643	1023±273
	BV	29.9±7.4	446±18	10.4±6.4	1500±546	677±322	443±151	6559±3670	579±867
	BV+AMF	34.5±7.2	468±47	49.3±26.9	4174±2000	1402±712	1085±543	13742±6724	1261±301
	AMF	35.4±2.3	506±7	15.9±7.4	2059±726	976±418	656±232	9059±1900	8634±489
	CONTROL	25.3±9.4	472±4	37.9±16.1	3518±1877	1258±359	940±493	11789±14517	944±277
					2016/2017				
‘Muchamiel’	RL	25.3±3.2	597±3	0.0±0.0	6633±1373	1550±159	1250±291	20623±3343	1538±369
	RL+AMF	35.1±4.2	519±23	0.0±0.0	4552±1389	1680±180	931±219	19849±177	1479±631
	BC	9.8±0.7	491±5	136.0±90.2	23068±6704	2670±674	3576±288	34786±7097	3877±730
	BC+AMF	34.0±1.7	469±160	126.0±24.9	42355±3941	2410±780	3588±852	20314±4004	4046±377
	BV	26.7±2.0	553±46	0.0±0.0	7630±822	1422±293	1520±130	23080±5230	2247±1065
	BV+AMF	37.1±7.2	513±42	0.8±1.4	8728±4136	2018±756	1810±899	29378±12020	2348±143
	AMF	28.4±1.7	525±24	84.8±9.5	46638±7428	2436±319	4413±860	26915±4053	4327±133
	CONTROL	25.9±1.7	628±66	66.9±3.2	47820±2898	2326±340	3810±484	21441±3150	3666±122
‘Palenca’	RL	27.2±3.7	657±113	0.0±0.0	5726±918	1412±91	1035±73	20200±1971	1358±286
	RL+AMF	31.9±2.1	501±26	0.0±0.0	4161±789	1517±415	913±130	19176±1825	1377±883
	BC	11.5±1.3	508±3	57.3±4.4	447001±5374	2551±602	3660±376	22519±2083	4476±758
	BC+AMF	30.0±2.3	494±33	46.0±2.9	47117±5764	2767±177	3713±708	18103±2124	4074±1353
	BV	27.0±2.1	356±44	110.0±23.9	9394±4684	1521±418	2027±1076	26041±9582	2769±959
	BV+AMF	31.4±5.9	490±12	66.6±17.7	11995±6997	1823±631	2876±1612	31782±10733	2519±959
	AMF	25.4±1.1	492±7	0.0±0.0	6993±2508	1430±11	1200±384	20957±3415	1559±299
	CONTROL	26.9±2.8	491±103	0.0±0.0	6545±4187	1589±218	1332±706	26890±4497	2336±851
		χ^2 value[b]				F value[b]	χ^2 value[b]		
Season (S)		3356ns	6.33*	0.33ns	59.87***	48.18***	43.10***	66.03***	66.78***
Legume cultivar (LC)		0.08ns	10.89***	0.72ns	1.42ns	5.77*	3.24ns	0.62ns	1.30ns
Inoculation treatment (IT)		11.60***	3.31ns	5.15*	0.96ns	2.08ns	1.33ns	0.03ns	0.85ns

[a] Treatment: RL (*Rhizobium leguminosarum*), BC (*Burkholderia cenocepacia*), BV (*Burkholderia vietnamiensis*) and AMF (arbuscular mycorrhizal fungi).

[b] Significant at *P<0.05; **P<0.01; ***P<0.001; ns: not significant (P>0.05). Different letters indicate significant differences (P<0.05) among means.

Table 6.3: Nutrient content in root of both fava bean cultivars for two seasons (nitrogen content, nitrate, boron, calcium, sodium, magnesium, potassium and phosphorus). Values are mean ± standard deviation (n=4).

Cultivar (C)	Treatment (T)[a]	N (g kg^{-1})	NO_3^- (mg kg^{-1})	B (mg kg^{-1})	Ca (mg kg^{-1})	Na (mg kg^{-1})	Mg (mg kg^{-1})	K (mg kg^{-1})	P (mg kg^{-1})
2015/2016									
‘Muchamiel’	RL	12.8±0.9	456±55	24.5±2.1	12338±4247	1035±366	803±103	3967±1838	421±112
	RL+AMF	14.5±1.6	729±66	21.3±11.9	3079±1151	1278±773	614±314	5138±2249	410±197
	BC	15.5±0.3	1094±716	35.7±8.9	13544±6189	1932±557	1141±272	7419±3562	740±193
	BC+AMF	14.6±3.7	662±109	14.3±5.1	2570±532	967±536	433±134	3622±1503	280±62
	BV	11.8±0.9	841±141	18.8±4.3	8198±790	903±118	610±57	4096±1218	380±67
	BV+AMF	14.3±2.7	773±153	19.4±8.1	2585±448	1054±391	517±139	4411±2084	374±130
	AMF	12.6±2.5	788±39	28.6±18.4	14207±9458	1364±972	1012±618	4678±2389	557±343
	CONTROL	14.0±0.5	683±227	30.5±0.7	2665±1409	814±636	396±250	3755±2614	283±186
‘Palenca’	RL	8.0±1.7	815±172	22.1±3.2	16048±2743	850±96	897±55	3430±364	397±40
	RL+AMF	11.6±1.5	787±181	22.8±15.4	3102±1552	1662±1542	630±385	7237±5421	471±276
	BC	11.4±2.4	725±111	17.7±12	7282±5415	1309±1072	667±340	5561±4863	444±263
	BC+AMF	13.1±1.7	922±152	16.1±9.6	2340±1238	1271±705	529±238	5337±1454	401±160
	BV	7.5±1.6	844±190	33.3±9.2	15437±4759	752±75	957±257	3661±809	375±12
	BV+AMF	10.1±0.6	781±64	29.4±8.6	4104±1943	1753±398	741±212	7069±1665	497±94
	AMF	7.5±1.7	846±331	43.5±3.0	19044±1815	1004±228	1169±240	3946±1288	436±42
	CONTROL	9.9±0.5	684±184	30.6±19.2	5485±3011	1492±632	1795±310	5847±2348	471±137
2016/2017									
‘Muchamiel’	RL	11.0±0.7	680±57	23.8±5.0	11223±3896	2427±780	1166±310	16856±4059	1112±326
	RL+AMF	10.1±1.2	695±73	31.0±7.5	23672±15915	2506±600	1795±518	13787±1829	999±259
	BC	13.2±0.8	534±33	14.4±0.8	9566±2019	3047±1296	1077±753	15491±1806	1101±77
	BC+AMF	13.4±0.3	646±141	14.3±4.3	17601±8106	2192±319	1357±194	12722±897	834±119
	BV	9.8±0.7	902±74	26.4±5.2	13769±3957	2649±784	1294±404	18360±8763	1209±509
	BV+AMF	11.5±1.3	655±96	27.4±9.4	19758±12867	3244±1136	1832±248	18628±5196	1266±494
	AMF	13.0±1.3	524±3	10.6±3.8	10705±3476	2050±449	969±1044	14013±1607	918±142
	CONTROL	11.8±1.3	573±90	13.0±1.1	13251±4610	2153±522	1227±317	11744±1361	708±99
‘Palenca’	RL	11.4±1.7	624±80	24.2±11.2	12047±2554	2561±283	1111±451	6245±3428	1084±298
	RL+AMF	10.4±1.0	614±75	19.0±1.1	18361±3711	2246±75	1460±327	10775±1733	776±51
	BC	13.3±0.6	566±75	14.0±8.1	13648±6204	2585±854	1446±177	16663±7245	1151±565
	BC+AMF	11.5±2.4	565±163	22.6±0.9	19409±8739	2134±276	1624±937	13609±3630	975±113
	BV	12.1±2.4	579±100	26.6±6.0	15807±2758	3301±194	1839±303	21552±7974	1615±324
	BV+AMF	9.4±1.7	621±115	25.7±8.1	31537±10771	2643±780	2200±338	13351±4240	1095±383
	AMF	12.4±1.0	761±57	40.1±9.6	18207±6951	3207±1164	1731±383	23070±6984	1622±750
	CONTROL	11.5±2.8	791±298	31.7±13.8	21453±12389	1914±1089	1573±786	14954±8481	1216±848
F value[b]			χ^2 value[b]			F value[b]	χ^2 value[b]		
Season (S)		0.15ns	10.63***	1720ns	25.86***	84.28***	56.77***	66.75***	62.97***
Legume cultivar (LC)		1.32ns	0.05ns	0.93ns	4.51*	0.26ns	0.013ns	0.36ns	2.43ns
Inoculation treatment (IT)		6.23***	0.03ns	2.80ns	0.08ns	0.30ns	0.97ns	0.003ns	0.003ns

[a] Treatment: RL (*Rhizobium leguminosarum),* BC (*Burkholderia cenocepacia),* BV (*Burkholderia vietnamiensis)* and AMF (arbuscular mycorrhizal fungi).

[b] Significant at *P<0.05; ***P<0.001; ns: not significant (P>0.05). Different letters indicate significant differences (P<0.05) among means.

6.3.2. Estimates of biologically-fixed N (% Ndfa)

Season significantly affected BNF in roots, with lower values in the second season for both fava bean cultivars. Fava bean cultivar significantly influenced BNF in shoot and root, with higher biologically-fixed N in shoot of Muchamiel than in Palenca cultivar. However, Palenca´s root showed the highest biologically-fixed N in the most of inoculation treatments during the first season, and with dual inoculation during the second season. The inoculation treatment did not significantly affect the BNF in any plant part. So, individual or dual inoculation of N-fixing bacteria and AMF did not improve the biological nitrogen fixation compared with non-inoculated plants (Table 6.4). In addition, BNF was not significantly correlated to plant nutrients, weight of nodules, crop yield and crop quality parameters such as weight of 100 seeds, pod length, number of pods per plant, number of seeds per pod, protein content in grain (data not shown).

Table 6.4: Estimates of N derived from biological nitrogen fixation (% Ndfa) in shoot and root of two fava bean cultivars, using broccoli like non-N_2-fixing reference plant. Values are mean ± standard deviation (n=4).

Cultivar (C)	Treatment (T)[a]	Shoot	Root
2015/2016			
‘Muchamiel’	RL	74.1±10.7	62.8±6.2
	RL+AMF	59.5±23.9	61.2±6.5
	BC	72.4±4.2	63.0±2.5
	BC+AMF	78.2±7.1	61.3±7.8
	BV	76.8±4.2	57.8±6.2
	BV+AMF	64.2±15.2	59.6±2.7
	AMF	74.2±14.0	59.1±0.8
	CONTROL	66.3±9.7	61.8±10.3
‘Palenca’	RL	61.5±3.9	64.3±7.5
	RL+AMF	43.2±8.5	48.6±8.8
	BC	54.3±2.6	66.3±13.1
	BC+AMF	54.5±1.7	82.2±7.6
	BV	43.0±7.6	66.1±1.2
	BV+AMF	68.8±4.7	66.8±13.7
	AMF	60.8±8.7	63.3±5.6
	CONTROL	42.7±16.4	62.4±5.7
2016/2017			
‘Muchamiel’	RL	85.9±5.5	53.3±5.9
	RL+AMF	76.2±3.1	63.8±6.7
	BC	75.3±4.1	56.7±10.7
	BC+AMF	78.0±6.9	37.9±18.1
	BV	85.5±2.7	55.0±12.4
	BV+AMF	77.3±2.6	55.1±10.1
	AMF	84.0±1.1	58.3±1.2
	CONTROL	84.3±2.5	56.6±8.2
‘Palenca’	RL	67.7±20.2	61.7±6.9
	RL+AMF	75.1±7.8	44.1±8.5
	BC	88.6±0.6	47.5±9.6
	BC+AMF	66.2±14.7	51.9±6.5
	BV	76.1±9.6	54.0±4.4
	BV+AMF	80.0±6.3	58.3±7.1
	AMF	86.7±0.2	54.4±6.8
	CONTROL	38.9±7.5	56.7±15.7
F-value[b]			
Season (S)		1.62ns	16.09***
Legume cultivar (LC)		97.30***	34.66***
Inoculation treatment (IT)		0.52ns	1.18ns

[a] Treatment: RL (*Rhizobium leguminosarum)*, BC (*Burkholderia cenocepacia)*, BV (*Burkholderia vietnamiensis)* and AMF (arbuscular mycorrhizal fungi).

[b] Significant at ***P<0.001; ns: not significant (P>0.05). Different letters indicate significant differences (P<0.05) among means.

6.3.3. Crop yield and quality

Season influenced protein content in grain, crop yield, weight of nodules and the number of pods per plant (Table 6.5). Protein content in grain showed the highest values in Palenca cultivar during the first season. Crop yield and the number of pods per plant were the lowest during the second season. However, the weight of nodules in Palenca cultivar was higher in the second season. Fava bean cultivar significantly affected the weight of nodules, the weight of 100 seeds and pod length, which were higher in Muchamiel than in Palenca cultivar during the first season. Inoculation treatment did not significantly influence the weight of nodules, crop yield and crop quality parameters. However, it affected protein content in grain in Muchamiel cultivar, with the highest values after inoculation in NFB and AMF treatments than in control during the second season. There was no correlation between the plant nutrients, BNF, crop yield and crop quality parameters.

Table 6.5: Crop yield, nodules weight, weight of 100 seeds, pod length, number of pods per plant, number of seeds per pod and protein content in grain of the fava bean crop. Values shown are mean ± standard deviation (n=4).

Cultivar (C)	Treatment (T)[a]	Crop yield (kg ha^{-1})	Nodules weight (g)	Weight of 100 seeds (g)	Pod length (cm)	Number of pods per plant	Number of seeds per pod	Protein content in grain (%)
2015/2016								
'Muchamiel'	RL	32541±3295	3.4±0.5	152.4±13.9	37.0±4.5	33±2	7±1	30±0
	RL+AMF	30133±6311	4.9±2.4	178.3±14.9	38.0±0.0	33±5	7±1	26±1
	BC	33333±2480	4.0±1.4	178.7±17.5	36.3±1.1	34±2	6±0	26±1
	BC+AMF	28583±4026	2.6±1.1	165.0±28.8	36.0±3.0	31±1	7±0	28±3
	BV	28166±7381	3.8±0.9	179.9±8.4	36.0±2.0	30±4	7±0	29±1
	BV+AMF	30250±4073	3.5±0.8	158.5±21.7	35.7±0.5	32±7	6±0	28±1
	AMF	27100±7258	4.9±2.1	176.6±28.8	36.7±1.5	29±10	7±0	29±0
	CONTROL	27016±2759	4.8±0.5	137.0±7.3	35.7±3.2	29±6	7±1	29±1
'Palenca'	RL	28866±5957	0.8±0.4	145.6±13.3	27.7±1.5	30±5	6±0	30±1
	RL+AMF	28700±2250	1.7±0.3	168.2±26.4	29.7±2.0	30±1	7±0	27±1
	BC	28633±2321	1.7±0.6	156.8±26.6	28.7±0.5	33±2	7±0	26±1
	BC+AMF	26766±1643	1.7±0.9	114.5±13.5	28.3±2.0	29±3	6±0	28±2
	BV	25616±5247	1.1±0.4	130.0±7.3	28.0±1.7	28±4	7±0	31±1
	BV+AMF	31866±1421	0.7±0.2	144.5±13.1	28.7±1.5	36±2	7±0	28±0
	AMF	23000±4399	0.5±0.5	133.3±7.3	30.0±2.6	22±9	6±0	29±2
	CONTROL	28366±3806	0.9±0.5	100.9±82.2	28.7±0.5	31±7	6±1	27±2
2016/2017								
'Muchamiel'	RL	23672±2797	3.1±0.5	170.7±15.2	37.5±0.5	30±1	7±0	28±1
	RL+AMF	18805±6022	4.8±1.6	183.5±13.1	35.2±3.6	20±7	6±1	26±2
	BC	19857±4828	5.3±2.7	199.6±46.3	35.8±0.7	22±6	7±1	25±2
	BC+AMF	14753±2033	5.8±3.4	169.8±24.4	34.2±2.4	16±2	7±1	27±4
	BV	20379±7372	4.7±1.7	158.8±27.7	34.7±1.2	24±10	7±1	29±5
	BV+AMF	19056±2855	3.7±0.7	154.0±21.2	34.5±2.2	23±2	7±1	28±3
	AMF	24137±2599	6.2±5.3	162.7±15.8	34.3±1.1	26±4	7±1	27±2
	CONTROL	19506±3791	4.3±2.3	176.1±8.0	33.2±1.2	22±5	7±1	23±2
'Palenca'	RL	23117±8825	5.3±0.4	165.5±9.5	29.3±0.7	28±10	7±0	25±1
	RL+AMF	18942±3823	5.0±1.4	178.0±17.7	30.0±1.0	23±5	7±1	25±3
	BC	15732±2727	3.6±0.9	143.3±14.0	30.5±0.8	21±3	6±0	26±2
	BC+AMF	17998±4116	3.0±0.7	166.9±10.6	29.2±2.2	26±3	7±0	26±1
	BV	20770±9163	3.0±1.1	163.5±8.6	28.5±1.3	26±11	7±1	24±1
	BV+AMF	19347±2769	5.2±1.1	143.3±19.6	29.7±2.0	26±8	6±1	26±1
	AMF	20422±5628	5.7±0.5	173.5±34.3	30.8±1.6	23±5	6±1	25±1
	CONTROL	18964±1033	4.8±1.4	185.8±25.2	30.5±0.5	21±3	6±1	24±2
		F-value[b]					χ^2 value[b]	
Season (S)		89.92***	33.88***	8.95**	0.06ns	36.66***	0.23ns	24.90***
Legume cultivar (LC)		0.92ns	20.33***	9.94**	262.16***	0.001ns	3.10ns	1.41ns
Inoculation treatment (IT)		0.59ns	0.43ns	1.27ns	0.25ns	0.88ns	0.52ns	4.81*

[a]Treatment: RL (*Rhizobium leguminosarum),* BC (*Burkholderia cenocepacia),* BV (*Burkholderia vietnamiensis)* and AMF (arbuscular mycorrhizal fungi).

[b]Significant at *P<0.05; **P<0.01; ***P<0.001; ns: not significant (P>0.05). Different letters indicate significant differences (P<0.05) among means

6.4. Discussion

Legume crops, such as fava bean, form tripartite associations with NFB and AMF where the NFB are involved in the fixation of atmospheric nitrogen and AMF improve the ability of the plant to take soil nutrients and phosphorus (Lodwig et al., 2003). The reduction of fertilizers application in inoculated treatments compared to control led to maintenance of crop yield, quality and nutritional characteristics, which can be attributed to minerals solubilization and organic matter mineralization by release of phytohormones, organic compounds or enzymes, making nutrients more available for the plant (Zahir et al., 2004). However, the inoculation with NFB, alone or combined with AMF, did not lead to a higher amount of biologically-fixed N, which could be due to the competition with native microbes or a high N content in soil by applied fertilizers (Puppi et al., 1994; Pennanen et al., 1999). Contrary to the N content in soil and BNF, protein content in grain increased after the inoculation with NFB and AMF. This fact evidences that N assimilation by the plant is enhanced with this inoculation, and the efficiency in protein anabolism is improved. In this context, Zimmer et al. (2016) also observed an increase of protein content in soybean grains after inoculation with *Bradyrhizobium* strains. Symbiotic-nitrogen-fixing rhizobacteria are usually present in soil, and no inoculation is needed if the reservoir in soil is sufficient. NFB are an integral part of the soil microbial community and can remain viable in soil for seasons, even when their legume host is not present (Bottomley, 1992). Hence, the inoculation of NFB would be necessary in soils lacking in a sufficiently high reservoir of NFB or the soil does not contain specific NFB able to nodulate due to the absence of previous legume crops (Catroux et al., 2001).

The specific fava bean cultivar influenced BNF process and parameters linked to crop quality, likely due to rhizodeposition processes since the release of root exudates is controlled by plant genotype (Merbach et al., 2000; Nguyen, 2003), confirming our initial hypothesis about the influence of cultivar on BNF. Plant root exudates modify soil microbial communities, foster beneficial symbioses, and change soil chemical and physical properties (Nardi et al., 2000; Walker et al., 2003). In turn, root exudates interact positively with beneficial microorganisms such as rhizobia and mycorrhiza (Philippot et al., 2013), and thus they would improve their role in improving the mechanisms of plant growth and development (Nadeem et al., 2014). The use of soil inoculation techniques was not followed by an increase of nutrient content in the plant tissues except for N content, despite decreasing fertilizer application by 20%. In addition, the fact that after the inclusion of NFB into the soil, the nutrient content in plant is maintained and N content is increased, it demonstrates an efficient N fixation, uptake and assimilation by plant.

Thus, higher decreases in the amount of mineral fertilizers applied to the soil could be assayed, since N fixation was highly effective in this experiment, which would be an environment-friendly alternative (Carter and Ambus, 2006). On the other hand, there was no clear evidence of the improvement of BNF or crop yield, quality and nutritional characteristics as a result of the dual inoculation compared to individual, and this may be caused by the presence of a reservoir of AMF in soil, which mycorrhized the fava bean roots. In terms of bacterial genus, it was only observed the increase of N content in root for both fava bean cultivars inoculated with bacteria belonging to *Burkholderia* genus (specifically *Burkholderia cenocepacia*) in comparison with *Rhizobium*. Thus, these results evidence the effectiveness β- proteobacteria to make symbiosis with the legume crops, not only α-proteobacteria, which has been the most studied group (Moulin et al., 2001). Contrary to BNF, the inclusion of NFB into the soil resulted in the increase of protein content in grains of Muchamiel cultivar, confirming an increase in the efficiency of assimilation of nitrogen by plant after inoculation. Thus, although BFN (biologically-fixed N) was not significantly affected by inoculation, the efficiency in the N uptake and assimilation into proteins was enhanced, which is very promising to increase the quality of the harvested crops. Nitrogen accumulated in seeds may come from the exogenous N fixed by the plant-bacteria symbiosis or from soil mineral N absorption (Crozat et al., 1994; Sparrow et al., 1995). Thus, these results support higher harvest protein yield with inoculation and confirm our initial hypothesis about the positive effect of the tripartite symbiosis between NFB, AMF and the plants on the protein content of the edible grain.

6.4. Conclusions

In conclusion, our results showed that after two seasons of the introduction of NFB and AMF to the soil along with the decrease in fertilizer rate by 20%, nutritional composition of the plant was not affected. Muchamiel cultivar showed the highest BNF in shoot and crop quality. Individual inoculation with *Rhizobium leguminosarum* increased seed N content. Dual inoculation with *Rhizobium leguminosarum* and AMF compared to individual inoculation increased shoot N content. Inoculation treatment did not influence BNF or crop yield and quality. Furthermore, the inclusion of NFB and AMF increased protein content in the grain of Muchamiel cultivar, confirming increases in the efficiency of plant N uptake and assimilation with inoculation. The inoculation with bacteria belonging to *Burkholderia* compared to *Rhizobium* genera led to an increase of N content in root, suggesting the great potential of β- proteobacteria as plant growth promoting rhizobacteria. The inoculation did not lead to an increase of nutrient content in the plant tissues except for N content, but since fertilizer application was reduced by 20%, these results are highly positive indicating effective N fixation and uptake by plant. This strategy can foster the decrease in the use of external fertilizers, reducing the current environmental impact maintaining high yields and crop quality.

Chapter 7

Conclusions

Conclusions

- ✓ Unusual cowpea crop improved soil fertility and quality in a subsequent broccoli crop compared with the broccoli monocrop, probably due to active rhizodeposition. Traditional fava bean crop did not improve soil fertility and quality in a subsequent melon crop with regard to the monocrop likely because a higher amount of fixed N was removed in the seed at harvest.
- ✓ Previous cowpea crop maintained broccoli crop yield and quality likely through activation of microbial populations that solubilize soil nutrients despite the reduction in external inputs.
- ✓ Legume cultivar influenced soil N content and soil enzyme activities, confirming that plant´s genotype regulates root exudation and so soil microbial activity. Conventional management practice was positively related to C and N pools in the broccoli crop, and to C sequestration in the melon crop.
- ✓ Conventional management practice was related to higher soil C sequestration in melon crop, while organic management practice improved soil structure through the activation of soil microbial populations, soil C sequestration and crop yield in the broccoli crop.
- ✓ Fava bean crop was related to higher N_2O, CO_2 and CH_4 emissions compared to the broccoli crop, likely due to rhizodeposition and the activation of soil microbial populations for N_2O and CO_2 emissions, and anaerobic conditions resulting from higher soil moisture for CH_4 emissions. Organic management practice led to higher N_2O and CO_2 emissions, related to higher soil enzyme activities.
- ✓ The introduction of N-fixing bacteria and arbuscular mycorrhizal fungi to the soil along with the decrease in fertilizer rate by 20% did not affect nutritional composition of the plant. Muchamiel cultivar showed highest biological N fixation in shoot and crop quality. Individual inoculation with *Rhizobium leguminosarum* increased seed N content. Dual inoculation with *Rhizobium leguminosarum* and arbuscular mycorrhizal fungi compared to individual inoculation increased shoot N content.
- ✓ Inoculation treatment did not influence biological N fixation or crop yield and quality. However, the dual inoculation increased protein content in the grain of one cultivar, confirming increases in the efficiency of plant N assimilation with inoculation.

- ✓ The inoculation with bacteria belonging to *Burkholderia* compared to *Rhizobium* genera led to an increase of N content in root, suggesting the great potential of β- proteobacteria as plant growth promoting rhizobacteria.
- ✓ The use of legumes with 20% decrease in fertilizer application, inoculated or not inoculated, led to high quality crops with no difference with the 100% fertilized monocultures. Thus, these results are highly positive indicating effective N fixation, soil nutrients solubilization and uptake by plant. This strategy can foster the decrease in the use of external fertilizers, reducing the current environmental impact maintaining high yields and crop quality.

Chapter 8

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