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Physiological and metabolic responses in broccoli plants fertilized with green manure

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Abstract

Grasses and legumes affect differently the amount of N in the soil over time, mainly due to the differences in the chemical characteristics of its mass. Changes in the availability of N influence the absorption and assimilation of N, which will influence physiological processes in the plants. The objective of this paper is to evaluate the changes in gas exchange, chlorophyll fluorescence and biochemical activity in leaves of broccoli grown under different green manure amendments. The experiment was designed in a complete randomized block design, using the mass of jack bean (JB) and millet (M) pure and in mixtures and one control, totaling six treatments: Control, 100JB, 75JB25M, 50JB50M, 25JB75M, 100M, with four replicates. The canopy area, N content, gas exchange, and biochemical analyzes were evaluated on the broccoli index leaf 40 days after broccoli transplantation. The higher ratio of jack bean, the higher the canopy area, the N content and the amino acid, protein and malate content, and lower starch content, possibly due to the higher N mineralization in this green manure.

Keywords: aminoacids, *Brassica oleracea var. italica*, *Canavalia ensiformis*, *Pennisetum glaucum*, nitrogen, protein, starch

Introduction

Green manures and cover crops are an alternative to mineral fertilizers to vegetable crops in organic production systems or conventional production without affecting vegetable food production while reducing the risk of environmental pollution from the continued and excessive use of mineral fertilizers (Yao et al. 2018).

Green manure supplies nitrogen (N) to vegetable crops from their mineralization due to the decomposition of the cut or mowed mass (Tei et al., 2017). Legumes are more used as green manure because they accumulate large amounts of N in their mass (Campiglia et al., 2010) and decompose more quickly (Watthier et al., 2020). On the other hand, grasses are mainly used to prevent soil erosion, absorb residual inorganic N and reduce nitrate leaching (Möller et al., 2008; Tosti et al., 2012).

It should also be noted that grasses and legumes affect the amount of N in the soil differently over time, mainly due to differences in the chemical characteristics of its mass (Almagro et al., 2021; Watthier et al., 2020). However, plants have developed sophisticated mechanisms to deal with varying concentrations of N in the soil. In addition to its role as a nutrient, nitrate is a local and systemic signal that coordinates its absorption with growth and development of plant (Alvarez et al., 2012).

Nitrate induces changes in the transcription of genes involved in the absorption of N, assimilation of nitrate, production of reducing equivalents necessary for the metabolism of N and C, in addition to a series of other functions, such as growth regulation and root architecture, growth of leaves and induction of flowering (Undurraga et al., 2017).

Since the incorporation of inorganic N into amino acids requires a C skeleton, a delicate balance between N and C must be achieved by the plant (Alvarez et al.,

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2012). About 75% of N is allocated to chloroplasts, and of these, most are used in the synthesis of components of the photosynthetic apparatus. Furthermore, the Rubisco enzyme, which plays a fundamental role in the assimilation of carbon, is strongly affected by conditions of low availability of N (Ciompi et al., 1996; Kant et al., 2011; Mcallister et al., 2012). However, in conditions of low N availability, the synthesis of carbonated and nitrogenous compounds essential for plant growth, such as amino acids, carbohydrates, proteins and other compounds used in photosynthesis, is largely affected (Nunes-Nesi et al., 2010). Studies on the use of green manure in vegetable cultivation are booming (Peralta-Antonio et al., 2019; Silva et al., 2019; Watthier et al. 2022), however, little or nothing is known about the effects of these fertilizers on photosynthetic rates and the accumulation of carbon and nitrogen compounds. Thus, the objective of this work was to investigate changes in gas exchange, chlorophyll fluorescence and biochemical activity in broccoli leaves fertilized with different green manures.

Material and Methods

General aspects

The experiment was conducted in a greenhouse at the Federal University of Viçosa (UFV), Minas Gerais, Brazil, located at 20°45'14"S and 42°52'53" W, at 650 m.a.s.l., tropical climate, between June and November 2016. The tropical climate is one with an average temperature of above 18 °C and considerable precipitation during at least part of the year. In a greenhouse, it is expected that the average temperature is higher than 18°C as observed in this work. The average temperature and relative humidity in the greenhouse were 25.7 °C and 59.7%, respectively.

Jack bean (*Canavalia ensiformis*, (L.) DC) was used as the legume species and millet (*Pennisetum glaucum* (L.) R. Br.) as the grass species. The green manures were cultivated in the field, with the cutting of the aboveground part of the plants at 10 cm from the ground at 60 and 57 days after sowing, respectively, followed by fragmentation into pieces of 8 cm. The mass was dried by placing in a forced circulation air oven at 65 °C until constant weight for the determination of dry matter (DM). The samples were then ground and the N-total content determined by the Kjeldahl method.

The experiment was designed in a randomized complete block, using the mass of jack bean (JB) and millet (M) either alone or in mixtures, totaling five treatments: 100JB, 75JB:25M, 50JB:50M, 25JB:75M, 100M, with three replicates. The 100, 75, 50, 25 and 0 (zero) represent the percentage of N from each species in the treatment. Jack bean contained 3.3% N and millet 1.2% N and the amount of biomass required in each treatment was calculated accordingly (Table 1), providing the same total amount of N in all treatments (9 gpot^{-1}).

Table 1: Jack bean (JBDM), millet (MDM) and total dry matter (TDM) and amount of nitrogen (N) deposited in each pot (70 L) according to the treatment in g pot^{-1}.

Treatments	JBDM (N) (g pot^{-1})	MDM (N) (g pot^{-1})	TDM (N) (g pot^{-1})
100JB*	272.73 (9.0)	0.00 (0.0)	272.73 (9.0)
75JB:25M	204.55 (6.7)	187.50 (2.3)	392.05 (9.0)
50JB:50M	136.36 (4.5)	375.00 (4.5)	511.36 (9.0)
25JB:75M	68.18 (2.3)	562.50 (6.7)	630.68 (9.0)
100M	0.00 (0.0)	745.71 (9.0)	745.71 (9.0)

100% jack bean (100JB); 75% jack bean+ 25% millet (75JB:25M); 50% jack bean+ 50% millet (50JB:50M); 25% jack bean+ 75% millet (25JB:75M); 100% millet (100M). The value in parentheses refers to the amount of N added to the pot.

Broccoli cultivation

Seeds of the BRO 68® single-headed broccoli cultivar were transplanted into pots 33 days after sowing. Irrigation was carried out manually according to the crop's needs, in a closed system, with recovery and return of drained water. After collecting part of the index leaf, recently matured leaves, typically 3-4 nodes down from the growing point (Hartz, 2007), are used for biochemical analysis (item 2.4), the rest of the leaf was dried in an oven at 65 °C, crushed and the total N determined by the Kjeldahl method (Tedesco et al., 1995).

Gas exchange and chlorophyll fluorescence a

Gas exchange and chlorophyll fluorescence were performed using the index leaf at 40 days after transplantation (DAT), a period of greater nutrient absorption by broccoli (Bowen et al., 1999). For that, an open system infrared gas analyzer was used, coupled with a fluorometer (IRGA, Li-cor Inc. LI-6400XT, Lincoln, EUA). The net carbon assimilation rate (*A*N, μmol CO_2.m^{-2}.s^{-1}), stomatal conductance (*gs*, mol H_2O.m^{-2}.s^{-1}), internal CO_2 concentration (*C*i, μmol CO_2.mol^{-1}), transpiration (*E*, mmol.m^{-2}.s^{-1}), minimum fluorescence (*F*0), maximum emitted fluorescence (*F*m) and the other parameters related to fluorescence were determined under controlled CO_2 concentration (400 μmol CO_2.mol^{-1}) and irradiance (1000 μmol photons.m^{-2}.s^{-1} with 10% of blue light) and temperature and ambient water vapor.

Biochemical analyzes

At 40 DAT, on the same leaf used for gas exchange, samples of plant material (~ 25 mg) were collected between 11:00 and 12:00 h and immediately frozen in liquid nitrogen, being stored at -80 °C, proceeding, *a posteriori*, to the extraction and determination of soluble sugars, total amino acids, malate, proteins, and pigments.

The samples were subjected to ethanol extraction, hot, determining, in the fraction soluble in ethanol, the levels of glucose, fructose and sucrose (Fernie et al., 2001), total amino acids and proteins (Cross et al., 2006) and malate (Nunes-Nesi et al., 2007) and the insoluble fraction, the starch contents (Fernie et al., 2001). The chlorophyll contents (*a* and *b*) were determined in the ethanolic extracts, in an ELISA microplate reader (Porra et al., 1989). The total chlorophyll contents (*a* + *b*), as well as the chlorophyll a/b ratio, were calculated (Lichtenthaler & Wellburn, 1983).

Statistical analysis

The data were submitted to analysis of variance and the comparison of averages of green manures with the control carried out using the Dunnett test (p<0,05), with the aid of the SAS 9.0 statistical program. Regression analysis of the effects of legume ratios in the mixture was also performed. The models were selected based on the significance of the coefficients, the determination coefficient and the phenomenon under study, using the OriginPro 7.0 program.

Results and Discussion

N content

There was an increase in the N content of the index leaf as the ratio of jack beans in the mixture increased (Figure 2). The higher N content in the broccoli index leaf with 100JB application is due to the greater mineralization (Lee et al., 2014; Watthier et al., 2020) and availability of this nutrient in the soil solution for absorption by the broccoli roots (Peralta-Antonio et al., 2019). It also partly reflects the leaf's investment in photosynthetic proteins such as Rubisco (Boussadia et al., 2010), since about 50% of the total leaf N in C3 plants is allocated to Rubisco (Kant et al., 2011; Mcallister et al., 2012).

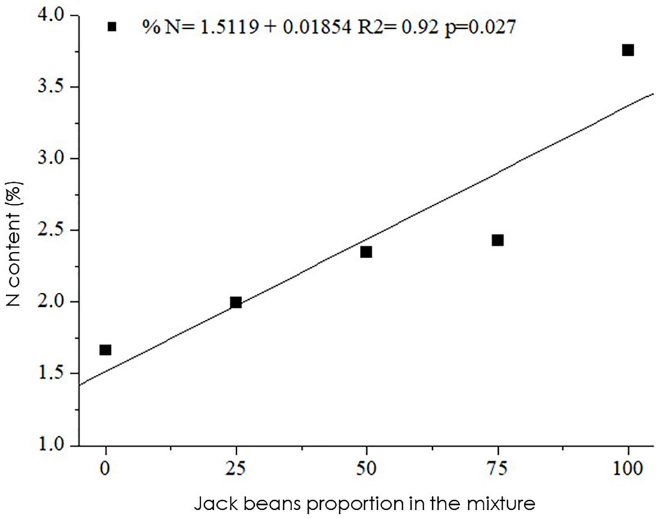

Figure 1: Effect of the jack bean proportion in the mixture under the nitrogen (N) content of the broccoli index leaf at 40 days after transplantation.

Gas exchanges, chlorophyll fluorescence a and pigments

Both the electron transport rate (ETR), the photochemical extinction coefficient (qP) and the quantum yield of photosystem II (ΦPSII) were higher in plants grown with 100JB green manure when compared to the control (Table 2). However, there was no effect of treatments on A, C_i, g_s, E and $F_{v'}/F_{m'}$ (Table 2).

Higher electron transport rates (ETR), photochemical extinction coefficient (qP) and quantum yield of photosystem II (ΦPSII) in plants fertilized with 100JB were observed by analyzing the chlorophyll fluorescence *a*, without changing the efficiency of the ΦPSII open (Fv/Fm`) (Table 2). These results suggest that the absorbed energy was used and/or dissipated efficiently and indicates that the photochemical machinery of the broccoli leaves was more efficient in the 100JB treatment. Together, these results demonstrate that the N mineralized by this green manure positively influenced the photochemical activity in broccoli.

Table 2: Photosynthesis (*A*); internal CO_2 concentration (*Ci*); stomatal conductance (*gs*); electron transport rate (ETR); transpiration (E), photochemical extinction coefficient (qP); quantum yield of photosystem II (ΦPSII) and Fv'/Fm' in broccoli leaves as a function of treatments at 40 DAT.

Treat.	*A*	C_i	g_s	ETR	E	qP	ΦPSII	$F_{v'}/F_{m'}$
	μmo CO_2 m^{-2} s^{-1}	μmol mol^{-1}	-- $mol.m^{-2}.s^{-1}$ --		$Mmol.m^{-2}.s^{-1}$			
Control[1]	25.36	211.76	0.36	171.68	2.94	0.60	0.39	0.63
100JB	26.03	197.28	0.33	195.08*	2.71	0.68*	0.45 *	0.66
75JB:25M	24.92	202.62	0.33	173.42	2.67	0.62	0.40	0.64
50JB:50M	26.55	210.22	0.36	180.56	2.93	0.63	0.41	0.65
25JB:75M	25.68	196.92	0.31	182.57	2.54	0.63	0.42	0.66
100M	25.67	217.91	0.39	176.57	3.12	0.63	0.40	0.64
CV (%)	7.21	8.41	13.12	6.10	10.55	4.88	6.10	1.86
MSD	3.69	34.55	0.09	21.87	0.59	0.05	0.05	2.80

[1] Control; 100% jack bean (100JB); 75% jack bean+ 25% millet (75JB:25M); 50% jack bean+ 50% millet (50JB:50M); 25% jack bean+ 75% millet (25JB:75M); 100% millet (100M). Means followed by * differ from the Control by Dunnett's test (p <0.05).

Biochemical results

Compared to the control, there was a higher content of aminoacids and lower starch in plants fertilized with 100JB and 75JB:25M as well as higher protein in 100JB (Table 3). The 75JB:25M, 50JB:50M, 25JB:75M and 100M treatments had lower glucose content and higher fructose content than the Control. No changes in the sucrose content were observed. The malate content in plants grown with 100JB was higher than those grown in the Control (Table 3).

In treatments with higher ratios of jack beans,

Table 3: Protein content (prot.), Starch, malate (mal.), Glucose (glu.), Fructose (fruct.), Sucrose (suc.), Amino acids (aa), total chlorophyll (ChlT) and chlorophyll *a/b* (*a/b*) ratio in broccoli leaves as a function of treatments at 40 DAT.

Treatment	Prot.	Starch	Mal.	Gluc.	Fruct.	Suc.	*aa*	ChlT	*a/b*
	mg.g^{-1} MF	µmol glicose.g^{-1} MF			µmol.g^{-1} MF				
Control[1]	10.65	47.90	10.15	21.48	11.11	4.39	11.30	2401.00	3.70
100JB	15.36 *	20.94 *	22.99 *	21.37	10.83	3.86	24.55 *	2833.11	4.24
75JB:25M	12.04	35.00 *	16.33	16.52 *	26.54 *	3.70	22.68 *	3086.82	4.27
50JB:50M	11.10	38.67	14.45	15.50 *	24.22 *	4.74	14.65	2861.49	4.21
25JB:75M	14.77	51.10	11.77	15.96 *	26.07 *	3.70	16.95	2882.58	3.97
100M	9.23	41.14	18.03	13.47 *	20.78 *	5.27	15.17	2800.54	3.98
CV (%)	17.68	15.95	40.30	12.36	16.54	24.14	22.96	13.94	7.86
MSD	4.29	12.42	12.56	4.28	6.57	2.05	8.02	704.65	0.63

[1] Control; 100% jack bean (100JB); 75% jack bean+ 25% millet (75JB:25M); 50% jack bean+ 50% millet (50JB:50M); 25% jack bean+ 75% millet (25JB:75M); 100% millet (100M). Means followed by * differ from the Control by Dunnett's test (p <0.05).

higher protein content (100JB) and lower starch content (100JB and 75JB:25M) were observed, indicating that carbon skeletons were diverted to the assimilation of N. Both growth and plant development are extremely dependent on the interaction between C and N metabolisms (Nunes-Nesi et al., 2010) and, therefore, the changes observed concerning growth must be associated with changes in the metabolic profile. For this reason, the changes observed in the metabolic profile of broccoli plants explain, at least partially, the interference of the type of green manure and, as a consequence, that N mineralization affects the growth and production of broccoli (Figure 2). In this context, the differences observed in the concentration of amino acids and proteins can be explained by the higher N absorption in plants grown in the 100JB and 75JB:25M treatments, as evidenced by the higher N content in plants fertilized with these green manures (Figure 1). After the absorption of nitrate or ammonium, this is incorporated into amino acids by the enzymes glutamine synthase (GS) and glutamate synthase (GOGAT), forming glutamine, glutamate, and other aminoacids and their metabolites (Undurraga et al., 2017) that are the basis for the accumulation of dry matter in plants (Nunes-Nesi et al., 2010). It is possible to suggest that the expression and/or activity of these enzymes have been affected in plants grown in the 100JB and 75JB:25M treatments. Future studies should focus on how and to what extent these enzymes are directly affected in response to ratios of green manure.

In general, the activity of source forces, such as

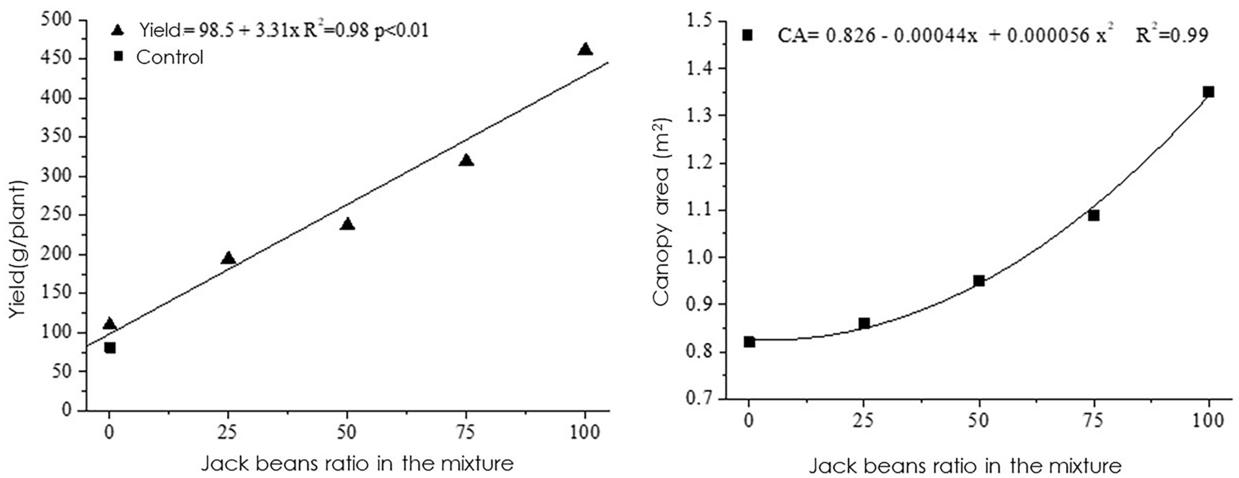

Figure 2: Effect of the proportion of jack bean in the mixture with millet on the yield and canopy area of broccoli plants.

photosynthesis and mobilization of nutrients, are positively regulated under conditions of low sugar content, whereas drain activities (e.g. growth and storage) are positively regulated only when the carbon sources are abundantly available (Taiz et al., 2017; White et al., 2016).

In this context, glucose and fructose were the sugars in greater quantity and differed from control, thus indicating that the higher amounts of glucose in plants fertilized with 100JB were the result of a lower drain force (starch accumulation), which would be associated greater plant growth in this treatment (Figure 2). Furthermore, starch is a transient form of carbon storage that accumulates in the leaves during the day and is remobilized during the night to support growth and metabolism (Stitt & Zeeman, 2012). Genetic and physiological studies have shown that starch plays an essential role in the diurnal distribution of C in many plants (Sulpice et al., 2009). Thus, in a study with 400 accessions of *Arabidopsis*, it was possible to demonstrate that starch and protein correlate with the accumulation of biomass, suggesting that the regulatory network that determines the starch and protein content contributes to the regulation of plant growth and biomass production (Sulpice et al., 2009). Therefore, the low starch content associated with the high protein content seems to have contributed to the greater accumulation of dry matter (207 grams) in broccoli plants fertilized with 100JB, a value 248% higher than the Control. Besides, sucrose is probably being exported from the place of synthesis (leaves) to regions of consumption (stem, roots, vegetative buds and inflorescence) in which it will be used to maintain plant growth. Thus, it is possible to observe a trend in the reduction of sucrose content in plants fertilized with 100JB and 75JB:25M (Table 3). On the contrary, when there is a reduction in growth due to the lower availability of N, the use of assimilates is reduced and thus the production of proteins is less and greater amounts of C can be diverted to the formation of starch (Rufty et al., 1988), as verified in the 100M and control which may indicate that such plants would be, at least in part, under stress (Thalmann and Santelia, 2017).

There was a greater accumulation of malate in broccoli leaves with the 100JB treatment, corresponding to an increase of 226% in relation to the plants grown in the control (Table 3). It has been shown that, just like starch, malate and fumarate accumulate in Arabidopsis leaves during the day and are degraded at night (Pracharoenwattana et al., 2010), functioning as sources of C for plant growth. Furthermore, the accumulation of malate is reduced when the availability of N is very low (Fernie & Martinoia, 2009), thus having a close link between the accumulation of malate and the assimilation of N (Nunes-Nesi et al., 2010). In this context, it is plausible to suggest that the greater growth and production of broccoli plants in the treatments with higher ratios of N provided by the jack bean occurred due, to a large extent, to the greater mineralization of N. Thus, the presence of greater amounts of available N in the soil solution seems to be associated with greater absorption and assimilation of N in nitrogenous and C compounds (e.g., proteins, amino acids, and malate) and a reduction in starch levels culminating in an adequate balance between growth and metabolism.

The present work presents evidence that the type of green manure strongly influences the photochemical efficiency and metabolism in broccoli plants. Although the photosynthetic activity was not drastically affected, the metabolic changes associated with the reduction in N content and the final production of dry matter demonstrate the contribution of green fertilizers with higher ratios of legume in the development of broccoli plants, most likely due to greater mineralization of N. This leads us to suggest that, possibly, it led to greater absorption and assimilation of N in nitrogenous and carbonated compounds in fertilized plants with higher ratios of jack beans. However, future studies must still be carried out to understand how and to what extent N assimilation enzymes are affected in response to different ratios of grass: legume as green manure.

Conclusions

Broccoli plants fertilized with higher proportions of jack beans had greater accumulation of protein, amino acids and malate, resulting in greater growth and yield.

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