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Original Research

The Influence of Applications of Bio-Inputs Derived from Macroalgae and Bacteria on a *Phaseolus vulgaris* L. Crop

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Abstract

Background: The common bean (*Phaseolus vulgaris* L.), is one of the most relevant legumes worldwide, as a source of protein, fiber, carbohydrates, and biologically important minerals. In recent decades, bean production increased significantly, especially in developing countries, where the availability of animal protein is often in short supply. However, a large portion of this agricultural production has been achieved in an unsustainable manner, through the intensive use of non-renewable agrochemicals, which in both the short and long term negatively affect soil fertility. To address this problem, the use of sustainable and renewable bio-inputs derived from macroalgae, and microorganisms may be amongst solutions required. Extracts of seaweeds have been shown to be biodegradable and non-toxic both for treated plants and consumers. This study aimed to evaluate the influence of the application of three bio-inputs made from different organisms on a common bean variety (BRSMG Realce) by analysing plant physiology and productivity, pod morphology, nutritional and mineral characterization of the bean. The study also aimed to evaluate the length of BRSMG Realce crop life cycle and compare its nutritional value with other commonly consumed varieties. **Methods**: Six treatments were performed: T0 — Control; T1 — Calmar® (soil — 100 kg/ha); T2 — Profertil® (foliar — 0.5%(v/v)); T3 — Albit® (leaf — 0.02%(v/v)); T4 — Calmar® ((100 kg/ha) + Profertil® (0.5%(v/v)); T5 — Calmar® ((100 kg/ha) + Albit® (0.02% (v/v)). **Results**: The leaf chlorophyll index revealed significant increases for T2, T4 and T5, compared to control. In general, the treatments related to the pods morphology showed significant increases in the length/width ratio. In terms of productivity, significant increases were found with T1, T4 and T5. In the analysis of the nutritional value of dried beans there were significant increases in the contents of fiber in T1, protein in T4 and T5 and carbohydrates for T1, T2 and T3. For mineral composition, there were increases in the phosphorus content of T2, T4 and T5 beans. When the cooked beans were analysed, T4 and T1 produced a greater amount of ash and proteins, as compared to control. **Conclusions**: The applications of bio-inputs in the bean crop (*Phaseolus vulgaris* L.) exerted several positive and significant effects, mainly on the CCI, productivity, pod morphology as well as cooked bean nutritional values. It was verified that BRSMG Reakce has the potential to be included in the Portuguese diet.

Keywords: bean BRSMG realce; sustainability; bio-inputs; macroalgae; productivity; nutrition

1. Introduction

Globally, social inequalities are increasingly evident; there is urgent need to discover crops with a lower management cost, higher yields and increased nutritional value. Under these scenarios, the common bean crop (*Phaseolus vulgaris* L.) stands out with untapped potential, as it adapts to a large range of edapho-climatic conditions and produces nutritionally dense foods, rich in protein [1,2]. Another important aspect about the common bean is its ability to establish symbiotic relationships with nitrogen-fixing bacteria of the genus *Rhizobium*. This peculiarity of legumes reduces demands for the use of synthetic nitrogen fertilizers. Such qualities led the common bean to occupy an important role amongst the most economically impactful legumes worldwide. In 2019 approximately 29 million tons were produced, generating approximately 14.5 billion euros in the global economy [3].

However, the apparent successful management of this particular crop and others has been achieved mainly through the over-use of chemical pesticides and fertilizers. This reliance on agrochemicals in contemporary agriculture contributes to the appearance of resistant pests and soil salinization, plus nutritional imbalances (i.e., deficiencies and mineral toxicity) [4–6]. These effects in conjunction with climate change, culminate in desertification, reducing the availability of arable land globally. It is clearly evident that agricultural production needs to become more efficient and must address the nutritional needs of a continuously increasing world population. Therefore, agricultural sustainability emerges as an answer to these and other problems. In recent decades, several bio-degradable products named bio-inputs have been introduced into the agricultural sector in order to reduce the over-use of agrochemicals. Bio-inputs are produced by environmentally friendly methods and use

various macro- and microorganisms as raw materials [7,8]. Several designations have been assigned to these products, such as biostimulants, biofertilizers and biopesticides [9–11].

At the time of writing, for the biostimulant and biofertilizer concepts, there are no globally agreed definitions that make a clear distinction between either. However, the biostimulant concept has been highly debated particularly during last decade and recently, under a new European Union (EU) regulation, the following definition was provided: "*A biostimulant for plants is a fertilizer that stimulates plant nutrition processes independently of the product's nutrient content, with the sole objective of improving at least one of the following characteristics of plant and/or their rhizosphere: (1) nutrient use efficiency; (2) tolerance to abiotic stress, (3) quality features, or (4) availability of confined nutrients in the soil or rhizosphere*" [12]. According to some authors, one of the major differences between biofertilizers and biostimulants lies in their nutritional load, which is higher in the former, making them suppliers of nutrients in forms that can be directly absorbed by plants [10]. Biofertilizers are essentially used to improve soil chemical and biological properties by replacing and/or increasing nutrients available to the plant (in particular, NPK and micronutrients), improving its growth [11,13].

Several extracts of macro-organisms, particularly seaweeds, have been used as raw materials for various products with biostimulant and/or biofertilizer properties [14]. These extracts are constituted by chemical compounds, such as complex polysaccharides, fatty acids, vitamins, phyto-regulators and mineral nutrients [14–16]. This type of product improves chlorophyll synthesis in plants as well as root development, resulting in greater vegetative growth, flowering and increased fruit quality [17]. Biostimulants also provide plants with an enhanced tolerance to several abiotic stresses, such as salinity, thermal and water stress [18]. The major of seaweed-based biostimulants are extracted from brown macroalgae (e.g., *Ascophyllum nodosum*, *Durvillaea* spp., *Ecklonia maxima* and *Sargassum* spp.,) and from the red macroalgae (e.g., *Kappaphycus alvarezii*, *Gracilaria* spp. and some coralline algae belonging to the family Lithothamniaceae) [4,8,19,20]. Profertil® is a solution with biostimulant effects that can be used in organic farming, it is based on extracts of European *A. nodosum*. The commercial product contains a range of organic compounds, namely: colloids; phytohormones; elicitors; amino acids and various nutrients [21]. Calmar® is a micro-granulated biofertilizer made from extracts of the red macroalgae *Phymatolithon calcareum*.This product comprises is essentially constituted of calcium (Ca) and magnesium (Mg), but it also has a small amount of iron (Fe). In addition, it contains minor amounts of macro- and micro-nutrients such as phosphorus (P), manganese (Mn). The consitutuents presnt on the labels of the three products tested are: Albit® — Total N 7.50%; phosphorus pentoxide 6.0%; K2O 4.50%; MgO 0.60%; sulphate 2.70%; acid poly(3-hydroxybutyrate) (active ingredient) 0.62%; organic matter 20%; pH: 6.5 ± 1. Profertil® Mannitol 0.5%; potassium oxide 3%; alginic acid 1.5%; pH 9–10; organic matter 9%; conductivity 60 ms/cm. Calmar® — mineral matter 95.13%; CaO 77.6%; MgO 12.1%; SiO2 0.3%; K 0.01%; P 0.02%; pH (25 °C): 9.6; raw protein: 4394 mg/kg.

In the last few decades, a large portion of agricultural bio-inputs, with biostimulant properties and/or biopesticides have been produced from microorganisms, such as Plant Growth-Promoting Bacteria (PGPB), fungi and arbuscular mycorrhizal fungi [7,22]. The use of these bacteria in agricultural practices improves plant nutrient acquisition and induces tolerance to biotic and abiotic stress. Amongst the most studied and applied it is worth mentioning the following genera: *Pseudomonas* and *Bacillus* as well as non-pathogenic *Azotobacter*, *Serratia* and *Azospirillum* [23,24]. Albit® is a commercial product based on such bacteria. It is considered a bio-activator with biostimulant properties and its active ingredient is poly(3-hydroxybutyrate) which is synthesized by the bacterial species *Bacillus megaterium* and *Pseudomonas aureofaciens* [25,26].

The aim of this study was to evaluate the potential effects of the three bio-inputs Calmar®, Profertil® and Albit® on a common bean variety (BRSMG Realce) by analyzing the the effects of applications on the plant physiology and productivity, pod morphology, nutritional and mineral characterization of the bean. This study also evaluated the crop life cycle length of BRSMG Realce and compared its nutritional value with other varieties commonly consumed beans.

2. Materials and Methods

2.1 Trial Site and Plant Material

The trial was conducted in an east-west oriented greenhouse, at the Coimbra Agriculture College (ESAC), located in Bencanta in the civil parish of São Martinho do Bispo and Ribeira de Frades (Coimbra) at approximately 21 m above sea level, latitude 40°12′55″N and longitude 8°27′12″W. According to Köppen-Geiger's classification, the climate in this region is categorized as Cool-summer Mediterranean (Csb), with mild, relatively rainy winters and hot, sunny summers. The mean annual temperature is 14.9 °C and the average annual rainfall is 914 mm [27].

The bean seeds used in this trial were the BRSMG Realce variety developed by the Brazilian Agricultural Research Corporation (EMBRAPA) and offered to this trial by the Federal University of Lavras, Brazil. The plant has a type I growth habit, i.e., characterized by having reproductive buds on the main stem and limited or non-existant node and leaf production after flowerings starts, and begins flowering, on average, at 35 days after sowing (DAS). Its pods are green with red stripes which begin to mature, on average, at 83 DAS. Its seeds are beige with stripes and small

red dots [28].

2.2 Trial Establishment

The trial was established as a random block design and consisted of six different treatments (Table 1), each of which was performed in triplicate. Pots of 4.5 L were used to ensure effective control over the variables. Each pot was filled with 3 Kg of the following mixture: 60% soil; 30% sand and 10% composting cow dung. The soil was collected from ESAC's agricultural land and had an approximate pH of 6.4 with an electrical conductivity close to 0.37 ms/cm. Chemically, it had the following constitution: P — 44 mg/Kg; K — 75 mg/Kg; calcium oxide (CaO) — 140 mg/Kg; magnesium oxide (MgO) — 35 mg/Kg. In total, 18 pots (3 per treatment) and 36 plants (2 per pot) were used.

Table 1. Bio-inputs used in each treatment.

Treatments	Bio-inputs
T0	Without application of agricultural bio-inputs
T1	Soil application of Calmar®
T2	Foliar application of Profertil®
T3	Foliar application of Albit®
T4	Application of Calmar® + Albit®
T5	Application of Calmar® + Profertil®

A drip irrigation system was provided. At an early stage of the trial, when the plant's roots did not occupy a large volume, the plants were watered four times a week using 400 mL per pot. The amount of water was adjusted to the needs of the plants according to temperature in the greenhouse, in order to compensate any losses by evapotranspiration.

2.3 Crop Establishment and Life Cycle Monitoring

The sowing of the bean crop took place on 24th of February 2020. Two seeds were placed on the soil surface per pot, spaced 10 cm apart. The gap between pots was 50 cm, the density of plants was 200 000 per hectare, and was determined using the methodology proposed by Lee & Herbek [29]. After sowing, at the beginning of each stage development, the plants were monitored according to the Gepts & Fernandez scale [30].

2.4 Treatments

The Calmar fertilizer® was applied in the trial 0 DAS at the dosage of 100 kg/ha. To that end, 2 g of product were applied in the soil surface of each pot in T1, T4 and T5 treatments.

Approximately seven weeks later, foliar treatments with Profertil® and Albit® started. Both products were diluted following the supplier's instructions, i.e., 5 mL of Profertil® to one litre of water and 0.2 mL of Albit® to another litre of water. Afterwards, using 500 mL sprayers, the Profertil® diluted solution was applied in T2 and T5 and the Albit® diluted solution in T3 and T4. In total, 3 foliar treatments were applied between 9 and 10 AM; the first was applied at 47 DAS, when the plants reached their reproductive phase (pre-flowering). The second was applied during the pod development stage, at 61 DAS. The final application was at 76 DAS during the pod-filling stage. Approximately six days later, the plants reached their final stage of maturity with almost no leaves, consequently, the foliar treatments ended.

2.5 Weed Control, Evapotranspiration and Supplementary Fertilization

During the trial, the crop was maintained by removing weeds from the pots by hand, so these did not compete with the beans.

Evapotranspiration depends on the crop coefficient (Kc), in turn, this depends on the vegetative and reproductive phases of the bean life cycle. Usually, Kc hits its maximum value when a crop reaches the flowering stage. During this stage, approximately 49 DAS, the watering plan was changed to twice a day. Every day, approximately 300 mL of water were administered per pot by the irrigation system, at 9 AM and 5 PM, totalling a daily volume of 600 mL. The implementation of this plan significantly reduced the gap between irrigations, and because the pots did not have a saucer, some essential nutrients may have been lost to leaching. In order to restore some of these, at 64 DAS, the NPK ENTEC© 13-10-20 (Eurochem – DEIBA) complex granular fertilizer was applied in the soil surface of every pot.

2.6 Quantification of the Chlorophyll Content Index (CCI) of the Bean Leaves

A CCM-200 (Opti-Sciences) chlorophyll content meter was used to quantify the CCI of the leaves. Measurements were made at 70 DAS for all treatments during the pod-filling, reproductive stage. One plant was randomly selected per treatment, of which, a fully expanded leaf was chosen to sample. Then, three readings (n = 3) were performed in distinct regions of the leaf: central, proximal (leaf base) and distal (leaf apex).

2.7 Harvesting, Counting of Pods and Beans, Pod Morphology and Bean Mass Determination

On the 4th of June 2020, 101 DAS, the greenhouse trial ended, and all the pods were harvested and grouped according to their treatment and left to dry at room temperature. Three days later, the pods were counted by plant/treatment. The observations of the pod morphology consisted in measuring pod the length, width, and the ratio between both. A calliper was used to measure the length and width of the pods according to Ningsih *et al.* [31] methodology. The beans were removed from the pods, counted, and placed according to their treatment in small aluminium packages. Later, the bean mass in each treat-

ment was recorded using an analytical balance (PB3002-S Mettler Toledo).

2.8 Sample Preparation and Yield (kg/ha) Determination

Before the dry bean laboratory analyses, 20 beans from each treatment were randomly selected and placed in separate vials. Those beans were ground according to the Association of Official Agricultural Chemists (AOAC) 922.02 method [32] by using a coffee mill (KG39 – De-Longhi) and a 0.5 mm sieve, later, the ground bean samples were placed back in their correspondent vials. In order to analyse beans nutritional value and mineral characterization, the ground samples needed to be dried. During this process the moisture content corresponding to the samples of each treatment was determined in triplicate (n = 3), according to the method 930.04 of AOAC [32], by drying 2 g of ground sample at 105 ± 5 °C over a period of 2 h in an oven (Memmert, Büchenbach, Germany). After getting moisture analyses values and the mass of dry beans for each treatment, their yield was obtained using the method described by Lee & Herbek [29] extrapolated to kg/ha. For the cooked bean analyses, a sample of 15 beans of the two treatments with higher yield (kg/ha) were randomly selected and soaked in water for 12 h, then they were cooked on a pressure cooker during approximately 18 min. Afterwards, using a pestle and mortar, the beans were partially crushed. The method applied for the cooked beans moisture analysis was the same applied for ground bean [32].

2.9 Analyses of Dry and Cooked Bean Nutritional Value

The nutritionally analyses of the dry bean, consisted in the determination of protein, ash, fat, and fibre contents of the samples previously prepared corresponding to the different treatments. All these analyses were performed in triplicate (n = 3) according to the methods described by the AOAC [32]. The crude protein was determined by a macro-Kjeldhal method (N × 6.25) by digesting 0.5 g of sample in a digestor (VELP Scientifica, Usmate Velate MB, Italy) and distilled by a Kjeldhal tube distiller (VELP Scientifica, Usmate Velate MB, Italy). The ash content of the dry bean was obtained by incinerating 2 g of sample at 550 ± 15 °C in a muffle furnace (INDUZIR, Portugal) for 2 hours. The crude fat was determined by the extraction of 2 g of sample with diethyl ether using a Soxhlet device (Behr Labor Technik, Dusseldorf, Germany). The crude fibre was determined by using the fraction of the dry bean remaining after digestion of 2 g of sample with 12.5 g/L sulfuric acid using a fibre extractor (Labconco raw fibre extractor, United States of America (USA)) followed by vacuum filtration (General Electric, Boston, MA, USA) and digestion of the same sample with sodium 12.5 g/L accompanied by a new vacuum filtration, using the same equipments. The total carbohydrates (g/100 g) were calculated according to Saupi *et al.* [33]: 100 – (% fat +% protein +% fibre +% ash). The energetic value (Kcal/100 g) of the dried beans was calculated using the Atwater factors: 9 Kcal/g of fat, 4 kcal/g of protein and 4 Kcal/g of carbohydrate, as described by Giuntini *et al.* [34]. The methods applied for the analysis of ash and protein contents of dry bean were the same applied for cooked bean [32], however, in this case, the analyses were only performed in duplicate (n = 2).

2.10 Dry Bean Mineral Characterization

The dry bean mineral characterization reads, and measurements were performed in triplicate (n = 3). In each analysis, 0.5 g of ground sample were taken from the vials corresponding to the different treatments and submitted to the drying process at 105 ± 5 °C, later, the samples were incinerated at 550 ± 15 °C on a muffle furnace (INDUZIR, Portugal) for 14 h. After cooling, the samples in the crucibles were digested with hydrochloric acid 20% (m/v). Afterwards, crucibles were placed in a water bath (Memmert, Büchenbach, Germany) for 30 min at 100 °C. The samples were filtered to 50 mL volumetric flasks and the remaining volume was filled with distilled water, in order to obtain a saturated solution.

Quantification of Zn, Fe, Mn, and Cu was made directly from the saturated solution by flame atomic absorption spectrometry (PerkinElmer PinAAcle 900 T, Waltham, MA, USA) adopting the methodology of Lucas & Sequeira [35]. To quantify Ca, Mg and K, the solution was diluted (1:10) and then 2.5 mL of 0.75% strontium chloride added to each sample, this reading was performed using the same methodology [35].

For the determination of P, 5 mL of saturated solution and 10 mL of ammonium molybdate-vanadate were pipetted in to 50 mL volumetric flasks. The remaining volume was filled with distilled water. The measurements of P were made by spectrophotometry (Pye-Unicam SP6-350 visible spectrophotometer) adopting the methodology of Ribas *et al.* [36].

2.11 Statistical Analysis

The Shapiro-Wilk test was performed for the normal distribution of the dry bean analyses obtained data. Later, that data were treated using a one-factor analysis of variance (one-way ANOVA) to evaluate the effect of treatments on the parameters studied. The significance of differences between evaluated mean values was determined using the Tukey test, at a significance level of $p < 0.05$. The software used was the IBM SPSS® Version 25 for Windows (IBM, Chicago, USA). In the analysis of the cooked beans, as the obtained data only resulted from two observations per treatment (n = 2), the mean was the only statistical method performed.

3. Results and Discussion

3.1 Phases and Stages of the Bean Plant Development

By monitoring the day that the plants reached each phase and stage of development, it was possible to calculate

their length in days. Those periods are presented in Table 2.

Table 2. Starting dates and duration (days) of bean plant phases and stages of development according to Gepts & Fernandez scale.

Phase	Stage	Starting date	Period (days)
Vegetative	V0	24/02/2020	6
	V1	01/03/2020	5
	V2	06/03/2020	7
	V3	13/03/2020	10
	V4	23/03/2020	18
Reprodutive	R5	10/04/2020	4
	R6	14/04/2020	6
	R7	20/04/2020	12
	R8	02/05/2020	20
	R9	22/05/2020	13

V0 — Germination; V1 — Emergence; V2 — Primary leaves; V3 — First Trifoliate Leaf; V4 — Third Trifoliate Leaf; R5 — Pre-Flowering; R6 — Flowering; R7 — Pod Development; R8 - Pod Filling; R9 — Maturity.

The total length of the crop life cycle was 101 DAS, obtained by adding the number of days involved in the different development stages (Table 2). This value fits into the interval of 65 to 120 days corresponding to the duration of the *Phaseolus vulgaris* L. life cycle [37]. Between 2005 and 2006 in the Minas Gerais State - Brazil, Melo *et al*. [38] made several open field trials using the BRSMG Realce variety and obtained a life cycle of around 83 DAS, 18 days shorter than the present crop that was establish in greenhouse. Amalfitano *et al*. [39] studied broad bean (*Vicia faba* L.) on two farming systems (open field, greenhouse), the results revealed a higher harvest precocity for the crops established in greenhouse compared to open field ones. This fact could indicate that a protected environment can provide a quicker life cycle to the plants, however, that did not happen in the present study when comparing with the trials made by Melo *et al*. [38]. As claimed by Oliveira *et al*. [40] the bean life cycle length may vary according to the edapho-climatic conditions of the producing region. That said, according to the Köppen-Geiger classification, there are clear differences between the climate where the current trial took place and the tropical savanna climate type predominant in the region where the Melo *et al*. [38] trials were carried out. Also, the present culture may have been influenced by the number of sunshine hours, as stated by Song & Jin [41], variations in the number of sunshine hours can affect crops growth process.

3.2 Quantification of Chlorophyll Content Index (CCI) in Bean Plant Leaves

By analysing the results obtained from CCI readings (Table 3) in the different treatments, it was observed that T2, T4 and T5 differed significantly from the control. Overall, T2 produced the highest mean value of CCI, i.e., about 34% higher than the control, followed by treatments T4 and T5 respectively, where the CCI values increased about 30% as compared to control. There were no significant differences between T3 and T1, compared to control.

Table 3. Chlorophyll Content Index (CCI) measured in bean plants (Chlorophyll Content Meter-200).

	Chlorophyll Content Index
T0 — Control	15.37 $\pm$ 0.31c
T1 — Calmar®	17.57 $\pm$ 0.21bc
T2 — Profertil®	23.30 $\pm$ 0.12a
T3 — Albit®	17.07 $\pm$ 0.12bc
T4 — Calmar® + Albit®	22.06 $\pm$ 0.16ab
T5 — Calmar® + Profertil®	22.03 $\pm$ 0.36ab

The readings were expressed as mean $\pm$ SD (n = 3).
In each column, values with the same letter are not significantly different (Tukey test, $p \geq 0.05$).

The increase of CCI in T4 may indicate a possible positive synergy between the two treatments used, even though when used individually, they did not differ from the control. The increase of CCI found in T5 may be related to the effect of Profertil® and might indicate that this product had a greater impact on the increase and maintenance of chlorophyll in the plant tissue, when compared to Calmar®.

There is a strong relationship between leaf chlorophyll content and nitrogen content, since approximately 70% of the plant total nitrogen is trapped in chloroplast enzymes that participate in the degradation of the molecular structure of chlorophyll [42]. Chlorophyll-measuring devices such as the CCM-200 have been described several times as potentially suitable for assessing total nitrogen in the leaves of many crops [43]. That said, higher values of CCI may be associated with higher levels of N, which could result in greater photosynthetic activity.

In other studies where bio-inputs were used on several crops, similar results were found to the present study. Ali *et al*. [18] and Goñi *et al*. [44] using *Ascophyllum nodosum* formulations in a tomato crop (*Solanum lycopersicum* L.) obtained a higher CCI in the treated leaves as compared to control. The single use of *Phymatolithon calcareum* extracts in tomato (*S. lycopersicum*) [45] and mango crops (*Mangifera indica* L.) [46] did not promote significant changes of CCI on the treated leaves when compared to the control.

Conversely, Gins *et al*. [47] concluded contrary to what occurred in the present study, the CCI recorded in the leaves of Passiflora (*Passiflora incarnata* L.) treated with Albit® were significantly different from their control.

Table 4. Morphology of the pods recorded in each treatment.

	PL (cm)	PW (cm)	L:W (cm)	NBP
T0 — Control	11.12 ± 0.2b	0.94 ± 0.05a	11.87 ± 0.42b	3.72 ± 0.38a
T1 — Calmar®	11.80 ± 0.14ab	0.90 ± 0.01a	13.16 ± 0.15a	4.18 ± 0.19a
T2 — Profertil®	11.93 ± 0.12ab	0.88 ± 0.01a	13.57 ± 0,33a	4.06 ± 0.47a
T3 — Albit®	11.97 ± 0.29a	0.90 ± 0.01a	13.30 ± 0.40a	4.10 ± 0.13a
T4 — Calmar® + Albit®	12.20 ± 0.16a	0.92 ± 0.01a	13.31 ± 0.29a	4.13 ± 0.14a
T5 — Calmar® + Profertil®	12.23 ± 0.40a	0.92 ± 0.01a	13.26 ± 0.57a	4.01 ± 0.08a

The results were expressed as mean ± SD (n = 3).

In each column, values with the same letter are not significantly different (Tukey test, $p \geq 0.05$).

Table 5. Bean plants productivity in number of pods and beans; bean mass; dry bean mass and bean yield (kg/ha) corresponding to each treatment.

	NP	NB	BM (g)	Moisture (%)	DBM (g)	Yield (kg/ha)
T0 — Control	25.67 ± 2.62c	95.00 ± 11.34d	42.64 ± 5.72c	10.91 ± 0.08a	37.99 ± 5.10c	3799
T1 — Calmar®	42.67 ± 1.70a	178.00 ± 2.45a	73.69 ± 7.58[a]	11.11 ± 0.04a	65.60 ± 6.74a	6550
T2 — Profertil®	32.33 ± 6.18bc	128.33 ± 8.26c	48.67 ± 2.92c	10.94 ± 0.01a	43.35 ± 2.60c	4335
T3 — Albit®	36.33 ± 1.25ab	149.00 ± 5.72bc	55.63 ± 2.93bc	11.06 ± 0.05a	49.49 ± 2.61bc	4949
T4 — Calmar® + Albit®	37.33 ± 1.89ab	154.00 ± 3.27b	76.16 ± 1.24[a]	11.05 ± 0.02a	67.75 ± 1.10a	6775
T5 — Calmar® + Profertil®	35.00 ± 0.82b	140.33 ± 1.25bc	69.32 ± 7.80ab	11.13 ± 0.04a	61.60 ± 6.93ab	6160

The results were expressed as mean ± SD (n = 3).

In each column, values with the same letter are not significantly different (Tukey test, $p \geq 0.05$).

3.3 Pods Morphology

The values corresponding to pod length (PL), width (PW), ratio between length and width (L:W) and number of beans per pod (NBP) are presented in Table 4. In a primary analysis of the PL data, it was verified that the highest values were obtained in treatments T5, T4 and T3, these differed significantly from the control, but not between them. There were no significant differences observed between treatments on the PW parameter. The lowest L:W ratio was obtained on T0 which differed statistically from all of the treatments. Regarding the NBP parameter, once again, the control treatment produced the lowest values, however, there were no significant differences between any treatments.

It is notable that the application of bio-inputs in this crop may have contributed to the increase of PL and consequently L:W parameters, compared to control. However, no significant differences were found between T1, T2, T3, T4 and T5. The correlation between NGP and the L:W ratio could be important for estimating bean yield. Although, the Pearson correlation (r = 0.183) between both variables was low according to the scale of Cohen *et al.* [48]. The application of bio-inputs may have induced changes in the L:W ratio, but ultimately did not influence the NBP, since no significant differences were found between control treatment and the others.

In studies carried out by other authors, significant differences were also found in fruit dimensions of treated plants with bio-inputs. Eris *et al.* [49] reported significant increases in fruit length on pepper plants (*Capsicum annuum* L.) treated with an extract of *A. nodosum*. Likewise, Ali [50] used an *Ascophyllum*-based product and obtained significant increases in the length of fava pods (*Vicia faba* L.). Aguiar [51] recorded a significant increase in the diameter of grape berries (*Vitis vinifera* L.) using an extract of *P. calcareum*-based product. Similarly, Amatussi *et al.* [20] also achieved a significant increase in the diameter of tomato fruits (*Solanum lycopersicum* L.) with a *P. calcareum* extract. Morais *et al.* [52] obtained significant increases in the length of strawberry fruit (Fragaria x ananassa Duch.) in plants inoculated with PGPB (*Pedobacter* sp. CC1 and *Bacillus safensis*).

3.4 Bean Plants Productivity

From the results of the bean plants productivity (Table 5), it was observed that T1, T3 and T4 produced the highest values for the NP (number of pods), with an average increase of 34%, as compared to control. As for the NB (number of beans), T1 stands out with a value approximately 47% higher than T0, differing statistically from all other treatments. The results obtained for the NP and NB parameters revealed a strong correlation (r = 0.921) between them, according to Cohen *et al.* [48]. For BM (bean mass) (g), the highest values were recorded in T4 and T1, showing an average increase of around 42% compared to control. It was also possible to verify the existence of a high correlation (r = 0.787) between NB and BM [48]. Given the existence of these correlations, it was expected that treatments with the highest values of NP, NB and BM would correspond to the greatest yields in kg/ha. That was ver-

Table 6. Nutritional composition of dry bean recorded in the different treatments.

	Ash, g/100 g	Fat, g/100 g	Fibre, g/100 g	Protein, g/100 g	Carbohydrates, g/100 g	Energy, Kcal/100 g
T0 — Control	5.63 ± 0.06a	1.02 ± 0.04ab	5.28 ± 0.03bc	23.56 ± 0.08b	64.51 ± 0.14c	361
T1 — Calmar®	5.01 ± 0.06c	0.67 ± 0.03d	6.1 ± 0.06a	22.55 ± 0.04e	65.67 ± 0.06a	359
T2 — Profertil®	5.84 ± 0.07a	0.91 ± 0.03abc	5.23 ± 0.08bc	22.91 ± 0.05d	65.11 ± 0.23b	360
T3 — Albit®	5.32 ± 0.04b	0.81 ± 0.04cd	5.30 ± 0.09b	23.14 ± 0.02c	65.43 ± 0.11ab	362
T4 — Calmar® + Albit®	5.31 ± 0.06b	0.85 ± 0.03bcd	4.78 ± 0.06d	25.19 ± 0.03a	63.86 ± 0.01d	364
T5 — Calmar® + Profertil®	5.60 ± 0.09a	1.07 ± 0.10a	5.02 ± 0.06cd	25.04 ± 0.03a	63.26 ± 0.05e	363

The results were expressed as mean ± SD (n = 3).

In each column, values with the same letter are not significantly different (Tukey test, $p \geq 0.05$).

ified in treatments T4 and T1 that stood out, with a yield 43% higher than control. In general, the treatments subjected to the application of bio-inputs showed significant increases in all parameters.

The application of Profertil® may have inhibited Calmar®, since a higher yield was obtained from the solo application of the latter in T1, when compared to their combination in T5. The clear difference in yields between T1 and T2 (Table 5) was indicative of inhibition or interference between the two products.

In the cultivation of the ground cherry (*Physalis peruviana* L)., Oliveira [53] achieved a higher fruit yield with *P. calcareum* treatments. Russo *et al*. [54] applied Albit® to *P. vulgaris* leaves and reported significant increases in pod productivity as compared to both control and treatment with proline. Tandon & Dubey [17] obtained a higher number of pods and seed yield per plant in soy crop (*Glycine max* L.) when applying a *A. nodosum* commercial extract.

3.5 Dry Bean Nutritional Value

The results obtained from dry bean nutritional analyses are presented in Table 6. Regarding the ash content, amongst all of the treatments, T2 and T5 presented the highest values. However, these did not differ significantly from their control. The highest values of fat content were obtained in T5, but these were not statistically significant. In fibre analyses, T1 samples reported significantly higher values than control. The protein content levels obtained for T4 and T5 were highest. Analyses of carbohydrates analysis for T1, T2 and T3 produced the highest values, which were significantly differently. In general, the calorific data were very uniform. In every treatment, there was a decrease in some parameter as compared to control, as was the case of T1 for ash, fat and protein content, T2 for protein content, T3 for ash, fat and protein content, T4 for ash, fibre and carbohydrate content and T5 for carbohydrate content.

A correlation can be established between the amount of protein and total nitrogen present in dry beans samples, since in most foods non-protein nitrogen is represented by a small fraction that is not significant [55]. The common bean establishes symbiotic relations with nitrogen-fixing bacteria, e.g., *Rhizobium* spp. and *Bradyrhizobium* spp. [56]. When successful, this symbiosis allows these bacteria to invade the roots of legumes, forming nodules, stimulating the growth and development of plants, partly due to the absorption of nitrogen and other macro- and micronutrients [57]. There are several studies reporting that these metabolic changes in legumes lead to a nutritional increase of their seeds, mainly in protein content [58,59]. In the present study, by analyzing more thoroughly the protein levels recorded for each treatment, it was demonstrated that for T1, T2 and T3, the protein values were significantly different and lower than the control. This could indicate that the application of bio-inputs impaired, in some way, the biological nitrogen fixation and consequently the symbiotic relation between plants and rhizobium, reducing the number of nodules in the rhizosphere. On the other hand, significantly higher protein content values were obtained in T4 and T5, when compared to T0 which may indicate that the bio-inputs applied favoured the biological nitrogen fixation reaction. Zhai [57] and Khan *et al*. [60] using *A. nodosum* soluble extract in alfalfa (*Medicago sativa* L.) crop reported an increase in the number of nodules in the treated plants compared to the control, suggesting that those extracts can enhance the symbiotic relationship between the plant root and nitrogen-fixing bacteria.

The literature about possible nutritional changes caused by the bio-inputs application in agricultural crops is scarce, however, there are some publications where results relate with the present study. Kocira *et al*. [61] used a commercial product derived from *E. maxima* on *P. vulgaris*, obtaining a higher protein content in treated plants seeds compared to control, as it occurred in the present study in T5 and in opposition to what occurred in T3. In Eryashev *et al*. [62], the use of Albit® in pea (Pisum sativum L.) promoted significant increases in protein content compared to control, as it occurred in T4 and contrary to T3 of the present study.

3.6 Dry Bean Mineral Characterization

The results of the dry bean are presented in Table 7. Regarding the P content, all treatments differed significantly from the control except T1. The highest content was found in T5, i.e., about 16% higher than the control. T3 values were overall the lowest. As for K, a considerably lower value was obtained for T5, which was the only one

Table 7. Dry bean mineral content recorded in the different treatments.

	T0 — Control	T1 — Calmar®	T2 — Profertil®	T3 — Albit®	T4 — Calmar® + Albit®	T5 — Calmar® + Profertil®
P (%m/m)	0.46 ± 0.004c	0.45 ± 0.005cd	0.51 ± 0.009ab	0.41 ± 0.004d	0.51 ± 0.006b	0.55 ± 0.013a
K (%m/m)	1.48 ± 0.03ab	1.51 ± 0.07a	1.37 ± 0.05ab	1.36 ± 0.07ab	1.29 ± 0.07ab	1.19 ± 0.02b
Ca (%m/m)	0.98 ± 0.06a	0.94 ± 0.10a	0.97 ± 0.04a	0.87 ± 0.07a	1.02 ± 0.07a	1.01 ± 0.05a
Mg (%m/m)	0.12 ± 0.01a	0.14 ± 0.02a	0.11 ± 0.01a	0.11 ± 0.02a	0.12 ± 0.01a	0.12 ± 0.01a
Cu (mg/kg)	8.62 ± 0.31a	7.07 ± 0.48a	7.64 ± 0.10a	8.48 ± 0.37a	7.81 ± 0.20a	8.33 ± 0.31a
Zn (mg/kg)	38.04 ± 0.41a	32.28 ± 0.62b	33.96 ± 0.59b	33.23 ± 2.08b	33.29 ± 2.56b	37.32 ± 2.70a
Fe (mg/kg)	80.51 ± 3.21a	63.57 ± 1.39b	63.37 ± 0.92b	58.87 ± 3.68b	64.99 ± 9.84b	59.96 ± 9.15b
Mn (mg/kg)	15.7 ± 0.08ab	13.45 ± 0.28b	15.83 ± 0.11a	16.07 ± 0.89a	14.78 ± 0.01ab	14.12 ± 0.30ab

The results were expressed as mean ± SD (n = 3).

In each row, values with the same letter are not significantly different (Tukey test, $p \geq 0.05$).

to differ statistically from T0. The analysis of Ca, Mg and Cu indicated that the levels achieved by the several treatments did not differ significantly from each other, or from the control. For Zn content, the highest value was obtained in the control followed closely by T5. The remaining treatments were considerably lower, although they did not differ significantly from each other, they differed from T0 and T5. Regarding Fe amount, treatments subject to the application of bio-inputs did not differ statistically from each other, however, they were much lower than T0. For Mn, none of the treatments were significantly different from T0.

Taken together, the results indicated that none of the treatments where bio-inputs were applied contributed to mineral enrichment of the dry bean, since only T2, T4 and T5 recorded significantly higher values than control for P content. The most evident difference for these data was associated with the Fe content in the control. Comparing this value with the reference content for the most consumed bean varieties in Portugal, as described in PortFIR website [63], it is noted that this amount of Fe is above the normal composition of the dried bean, since the usual values for the varieties of cowpea, red and white, are given as approximately 52, 64, and 61 mg/kg, respectively.

An excess of this micronutrient may be associated with stress caused by Fe deficiency in control plants. Faced with an Fe deficiency, dicots stimulate responses aimed to improve the Fe accumulation from the soil. Fe deficiency can induce a 5–10-fold increases of Ferric-chelate reductase activity [64]. The function of this enzyme is to reduce Fe (3^+) to Fe (2^+), so that it can be transported by iron-regulated transporters into the cells [65]. In the study of Cohen *et al*. [64], it was described that Fe deficiency induced the expression of the iron carrier gene, which could facilitate the transport of divalent cations such as Cd (2^+) and Zn (2^+), besides Fe (2^+). This may also be an explanation for the higher levels of Zn obtained in T0, as compared to other treatments. Another hypothesis for this imbalance could reside in the fact that these treatments prevent the uptake of iron, however, there is a large number of studies in which the use of bio-inputs in agricultural crops promoted a greater iron assimilation and other micronutrients, compared with control [66–68].

Some studies with similar analyses were found, i.e., Amatussi [45] that used a commercial product based on *P. calcareum* in a *S. lycopersicum* crop, found that the micronutrient content did not differ significantly from the relevant control. Di Stasio *et al*. [69] used two commercial extracts of *Ascophyllum nodosum* in *S. lycopersicum*, achieving increased P levels as compared to control, agreeing with the observations in this trial in T2 and T5. However, these authors also obtained increases in levels of K, Ca, Mg, Zn and Fe. Petropoulos *et al*. [70] used a commercial product containing the *Bacillus subtilis* bacteria and *A. nodosum* extracts in a greenhouse study of *P. vulgaris* reporting increases in Cu and Ca content in dry beans as compared to control and other treatments. Their report did not concur with the present trial, since there was no statistical difference between any of the treatments for both elements.

3.7 Cooked Bean Nutritional Value

From the cooked bean analysis (Table 8), it is noticeable that the water content was higher in T0, followed by T4 and T1. Samples belonging to T1 and T4 produced approximately 47 and 37% more ash than the control. Regarding the protein content, T4 and T1 were approximately 17 and 12% higher than control. These results indicate that the application of bio-inputs in this crop can reduce cooked bean water content and increase its ash and protein value.

Table 8. Nutritional composition of cooked bean in T0, T1 and T4.

	Moisture, g/100g	Ash, g/100 g	Protein, g/100 g
T0 — Control	61.93	0.97	8.74
T1 — Calmar®	54.72	1.84	9.9
T5 — Calmar® + Profertil®	57.28	1.55	10.57

The results were expressed as mean between the two observations made for each laboratory analysis (n = 2).

Nutritional values of the T0, T1 and T4 cooked bean were compared with PortFIR website [63] reference values for some commonly consumed bean varieties in Portugal (Table 9).

Table 9. PortFIR reference values for cooked white bean, cowpea and butter bean.

	White Bean	Cowpea	Butter Bean
Moisture, g/100 g	69.6	66.2	68.6
Ash, g/100 g	2	1.4	2
Protein, g/100 g	6.6	8.8	7.8

Source: PortFIR (2021).

It was observed that the T0 water content was lower than the three varieties presented above, its protein content was below the reference value for the cowpea and above the value for the butter and white beans, respectively. The ash content in the T0 was lower than the three bean varieties. The T1 water content was lower than the three bean types in Table 8, however, its protein content was higher. Regarding the ash content, T1 was only superior to the variety of cowpea. Doing the same comparison, the T4 treatment behaved in the same way as T1. By these results, it can be stated that the BRSMG Realce variety has the potential to be included in the Portuguese diet, since after the cooking process, it has similar nutritional values to the usually consumed bean varieties.

5. Conclusions

Based on the results obtained in this study, it can be concluded that applications of bio-inputs in the bean crop (*Phaseolus vulgaris* L.) exerted several positive and significant effects, mainly on the CCI, productivity, pod morphology as well as cooked bean nutritional values. However, the treatments did not influence several parameters of the dry bean nutritional and mineral analyses. Overall, it can be assumed that the treatments, i.e., T1 (Calmar®), T4 (Calmar® + Albit®) and T5 (Calmar® + Profertil®) had a greater influence on the studied parameters, showing that the individual application of *Phymatolithon calcareum* extracts via soil were more effective than foliar treatments for this particular bean crop. It was furthermore demonstrated that the life cycle length for BRSMG Realce crop was within the *P. vulgaris* normal range, however, further greenhouse and open fields trials in different areas of Portugal are incentivized in order to understand whether this variety can effectively adapt to the Portuguese climate. It was also verified that BRSMG Reakce has the potential to be included in the Portuguese diet.

Future studies are encouraged in order to understand the modes of action and mechanisms of these products, including better determination of rates and timings and the best return on effort and investment for the farmer and in turn the consumer.

Author Contributions

PCM and KB designed the research study. BM performed the research. ATC provided help and advice on this study. BM and KB analyzed the data. BM and KB wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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