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Productivity, enzymatic activity and physiological effects in peanut plants associated with arbuscular mycorrhizal fungus and supplemented with seaweed extract

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Abstract
Peanuts cultivation (*Arachis hypogae* L.) has aroused great economic interest. Therefore, use of arbuscular mycorrhizal fungi (FMAs) and natural biostimulants such as soluble seaweed extract (ESA) is spreading in agriculture. These biological agents can stimulate the growth and production of vegetables. The objective of this work was to evaluate the effects on gas exchange, enzymatic activity and production indexes in peanut plants inoculated with mycorrhizal fungi and supplemented with soluble extract of marine algae. The experimental design was completely randomized in a 2x2 factorial scheme, two treatments involving the presence and absence of FMAs and two treatments involving supplementation or not with ESA, T_1 consisted of plants associated to FMAs and supplemented with ESA, T_2, just FMAs associated plants, T_3 plants only supplemented with ESA and T4 control plants (no association with fungi and no supplementation with ESA). The variables evaluated were net assimilation rate of CO_2 (A), stomatal conductance (gs), transpiration (E) and internal carbon concentration (Ci), activities of the antioxidative enzymes sod, pod, cat and proline protective osmolyte as well as yield and yield components. This consortium also increased the distribution of biomass with direct effects on the increase of production. The biological agent and the biostimulant collaborated in the activation and kept active the antioxidative system of the peanut plants even under optimal conditions of cultivation. Management practices that maintain the native community of mycorrhizal fungi, such as ESA application in plants associated to FMAs, would be good biotechnological alternatives for the cultivation of IAC Runner 886 peanuts.

Keywords: Ascophyllum nodosum, arbuscular mycorrhizal fungi, crop wield, gas exchange

Introduction
The peanut crop (*Arachis hypogae* L.) has aroused great economic interest since its seeds constitute an important source of vegetable protein and edible oil (Barbosa; Homem; Tarsitano, 2014) [4].

The planting recommendation for this crop serves several markets and presents great potential for expansion, whether for *in* nature consumption, for the industry, for the recovery of degraded pastures and green manure (Nogueira; Távora, 2005) [18].

The association between arbuscular mycorrhizal fungi (AMF) and arbuscular mycorrhizal (AM) roots (Smith; Smith, 2011) [25] is one of several forms of symbiosis found in nature.

These so-called associations contribute to soil fertility (Almeida *et al*., 2014) [2], promote improvements in plant growth and development and are an option for reducing the supply of fertilizers, as the absorption of elements, especially those with low soil mobility as the P, is facilitated by the hyphae fungal (Fernández-Lizarazo; Moreno-Fonseca, 2016) [6]. Due to the search for organic nutritional sources available to the plants, the use of natural biostimulants based on seaweed extract (ESA) is also increasingly being included in agriculture. Its composition may increase plant growth as the substances present as betaines and compounds with auxin and cytokine action favor the hormonal balance of the plant (Khan *et al*., 2009; Santaniello *et al*., 2017) [14, 24].

The use of techniques that favor the rapid growth of crops with high added value, the maintenance of the native community of mycorrhizal fungi present in the soil, the ability

to select FMAs or efficient combinations of these symbiotic agents by the plant and the consequences of simultaneous root colonization use of biostimulants become important to favor the nutritional status of plants and potential increase in productivity.

The aim of this work was to evaluate the effects on gas exchange, enzymatic activity and production indexes in peanut plants inoculated with mycorrhizal fungi and supplemented with soluble extract of marine algae.

Materials and methods

Installation, location of experiments and cultural dealings

The experiment was developed in an agricultural greenhouse with commercial cultivar* IAC Runner 886 in the Department of Chemistry and Biochemistry of the Institute of Biosciences IB - UNESP, Botucatu Campus, SP. The used soil was characterized as Dark Red Latosol Dystrophic medium texture (Led) with the fertility data presented in the Table 1 and sowing in pots with 30L capacity.

Table 1: Initial chemical characterization of the soil.

Layer	pH	MO.	Presin*	H+Al	K	Ca	Mg	SB	CTC	V%
	$CaCl^2$	g dm^{-3}	mg dm^{-3}	mmolcdm-3						
0-15	4.1	12.8	4.8	52.7	0.7	12.2	3.4	16.3	69.0	23.4

The cultural treatments consisted of basic fertilization according to described recommendation by IAC Technical Bulletin 100 (Aguiar *et al*., 2014) [1] for culture. The equivalent of 1.35 g of urea; 1.62 g of KCl and 2.22 g of diammonium phosphate was applied per pot.

Inoculation of arbuscular mycorrhizal fungi (AMF)

For the inoculation of the arbuscular mycorrhizal fungi (AMF), a mixture of 2 isolates was utilized, which included IAC 5 (*Rhizofhagus intraracis*) and IAC 44 (*Claroideoglomus etunicatum*). Both were originally from maize multiplier stocks and had the following structures present: spores, hyphae and pieces of infected roots.

The inoculation procedure of the AMF was performed at the start of the experiment in non-sterile soil, with the application of 100g of the innocuous, equivalent to approximately 5,400 spores per pot and applied at the same time of sowing next to the pit.

Application of soluble kelp extract

Supplementation with algae extract was by the addition of 50 ml of a solution of the commercial extract of *A. nodosum* diluted inconcentration of 0.3% of product via soil. The applications followed the manufacturer's technical recommendations and started at 10 days after planting (DAP) and subsequent weekly applications up to the beginning of plant senescence.

Gaseous exchanges, enzymatic assays, proline content and production components.

For gas exchange, the following variables were evaluated: net assimilation rate of CO_2(A), stomatal conductance (gs), perspiration (E) and internal carbon concentration (Ci) in the second fully expanded leaf near the apical bud. The values were obtained through a portable Mod gas exchange meter. IRGA LI-6400 between 09:00 and 11:00 am. The photosynthetically active radiation was 1000 µmolm-2. Four plants of each treatment were used during the evaluation periods.

For the determinations of the activities of the antioxidative enzymes, extracts were prepared from the maceration of fresh matter of leaves in liquid nitrogen; Thereafter, 4.0 ml of 100 mM potassium phosphate buffer was added; pH 7.0.

The activities of the enzymes catalase (CAT), Superoxide Dismutase (SOD), Peroxidase (POD) and L-proline content were evaluated. The determination of CAT activity was obtained by decreasing the absorbance at 240 nm, due to the consumption of H_2O_2 by the enzyme present in the crude extract, according to Peixoto *et al*. (1999) [20]...

The POD activity was determined by the method of Peixoto *et al*. (1999) [20]; 0.1 mL of the enzyme extract was added to 4.9 mL of potassium phosphate buffer solution (25 mM, pH 6.8) containing 20 mM of Pyrogallol and 20 mM of$_2O_2$. After incubation for 1 minute, the reaction was quenched with 0.5 mL of H_2SO_4 and reading the absorbance at 420 nm. For the calculation of the specific activity of the POD enzyme, a molar extinction coefficient of 2.47 mM-1 was used.

The activity of the SOD enzyme was determined by... And (Giannopolitis; Ries, 1977) [9] the reaction was measured by increasing the absorbance at 560 nm, due to the production of blue formazan, resulting from the photoreduction of p-Nitrobluetetrazolium (NBT).

The activities of the CAT and POD enzymes were expressed in µK at µg Prot-1, and that of SOD in UI $\mu g^{protein-1}$, where UI (enzyme activity unit) is defined as the amount of enzyme required to cause 50% inhibition of NBT photo reduction.

For the peanut yield the following components were obtained: Dry matter of the aerial part (MSPA), dry matter of the root system (MSR), root length (CR), number of pods per plant (NVP), number of grains per pod (NGV), weight of 100 grains (M100) and productivity (PROD).

Experimental design and treatments

The experimental design was completely randomized (DIC) in a 2x2 factorial scheme. Two of the treatments involved the presence and absence of AMF and two treatments involved supplementation or not with ESA, with evaluations carried out in three sampling periods denominated P_1 (pre-flowering), P_2 (reproductive) and P_3 (grain filling) in 4 plants per treatment. The T_1 consisted of plants associated to FMAs and supplemented with ESA, for T_2, plants only associated with FMAs, for T_3 plants only supplemented with ESA and for T_4 control plants (without association with fungi and nor with ESA supplementation).

Results and Discussion

The association of peanut plants with FMAs (T_2) presented higher internal CO_2 concentration (Ci), in the reproductive (P_2) and grain filling (P_3) periods, when compared to the plants submitted to the other treatments. This indicates the intercellular concentration available by photosynthesis and may be influenced by gs and A. (Taiz; Zeiger, 2006) [27]

Among the proposed treatments, the net assimilation values of CO_2(A) and stomatal conductance (gs), after the pre-flowering period were higher in the T_1 plants and it was also observed that the association with the FMAs maintained A throughout the development period of peanut plants. However, there was a decrease of A e gsin the final third of the development cycle of the plants when they were supplemented with ESA.

The influence on stomatal conductance, transpiration and assimilation of CO_2 by ESA is attributed by favouring the

absorption of nutrients promoted by FMAs to the plants, enabling the synthesis of hormones, such as ABA, necessary to promote the susceptibility of plants to the formation of symbiosis, development and functionality of the arbusculus (Kiriachek *et al*., 2009) [15]. As well as the activation of antioxidative, enzymatic and non-enzymatic mechanisms, even in plants not submitted to an abiotic stress condition, these mechanisms being variable according to the involved AMF (Ruiz-Lozano, 2003; Folli-Pereira *et al*., 2012) [23, 7]

However, the effects observed in the gas exchange in plants associated with the FMAs are not always perceptible and are variable throughout the development of plants (Augé; Toler; Saxton, 2015) [3], the responses being differentiated according to the interaction between the FMAs present, the host species and the appropriate edaphoclimatic conditions for the effective establishment of the symbiosis (Fernández-Lizarazo; Moreno-Fonseca, 2016) [6].

Regarding the application of the ESA and its effects exclusively in the gas exchanges, there are still few works found in the literature. The ESA contributes to the photosynthetic mechanism of some plants by the presence of biostimulating molecules, such as: macronutrients, vitamins, organic compounds, carbohydrates and amino acids (Spann; Little, 2011. Xu; Leskovar, 2015) [26, 29]

Its application may stimulate the partial closure of the stomata, thus influencing stomatal conductance and on Ci through gene expression, in particular those promoters of ABA synthesis and responsible for stomatal regulation (Santaniello *et al*., 2017) [24] (Xu; Leskovar, 2015) [29].

The ABA synthesis influenced by the ESA may have contributed to the better establishment in the association of the peanut plants with FMA due to the beneficial effect of this hormone to the process of root colonization (Ludwig-Müller, 2010). The presence of oligosaccharides resulting from the enzymatic degradation of the alginic acid present in the ESA composition also favours the growth of the fungal mycelium of the FMAs (Khan *et al*., 2009) [14].

The development of arbuscular mycorrhizae is a complex process as well as the influence caused by biostimulants such as ESA for symbiotic establishment, whose mechanisms of regulation are very variable (Augé; Toler; Saxton, 2015; Lambais; Ramos, 2010) [3, 16] and the responses obtained by the interaction of these biological agents may be different in plants maintained under favourable conditions or when subjected to some level of stress (Spann; Little, 2011; Xu; Leskovar, 2015) [26, 29].

This potential response observed for the gas exchanges in T_1 and T_2 seems to demonstrate, in the peanut crop, a positive correlation between the morphophysiological attributes of the host and the degree of mycorrhizal dependence (Quilambo *et al*., 2005) [22] being, which can be associated with the rusticity of the material, low nutritional requirement (Folli-Pereira *et al*., 2012) [7] and ecological specificity (Zhu *et al*., 2001) [31].

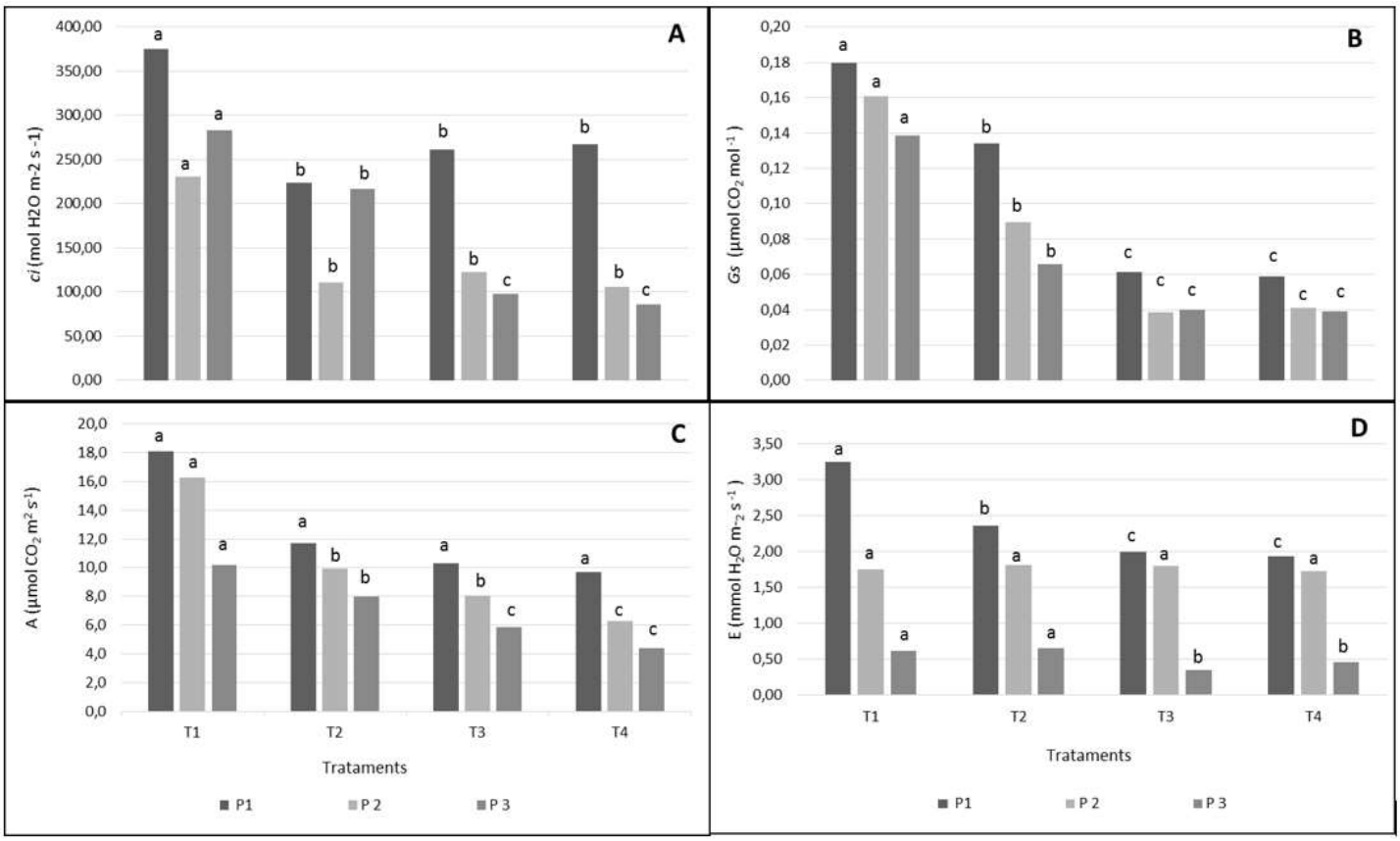

Fig 1: Net assimilation rate of CO2 (A) (µmol CO2 m2 s-1) (A), Stomacal conducance (gs) (mol H2O m-2 s-1) (B), Internal carbon concentration (Ci) (µmol CO2 m-2 s-1) (C), Transpiration (E) (mol H2O m-2 s-1) (D) in cv peanut. Runner 886 associated with FMAs and supplemented with ESA (T_1), only associated to FMAs (T_2), only supplemented with ESA (T_3) and without association with FMAs and ESA application (T_4), evaluated in three plant development periods (P_1, P_2 and P_3). Means followed by different letters between treatments in the same period differed by the Tukey test (p <0.05).

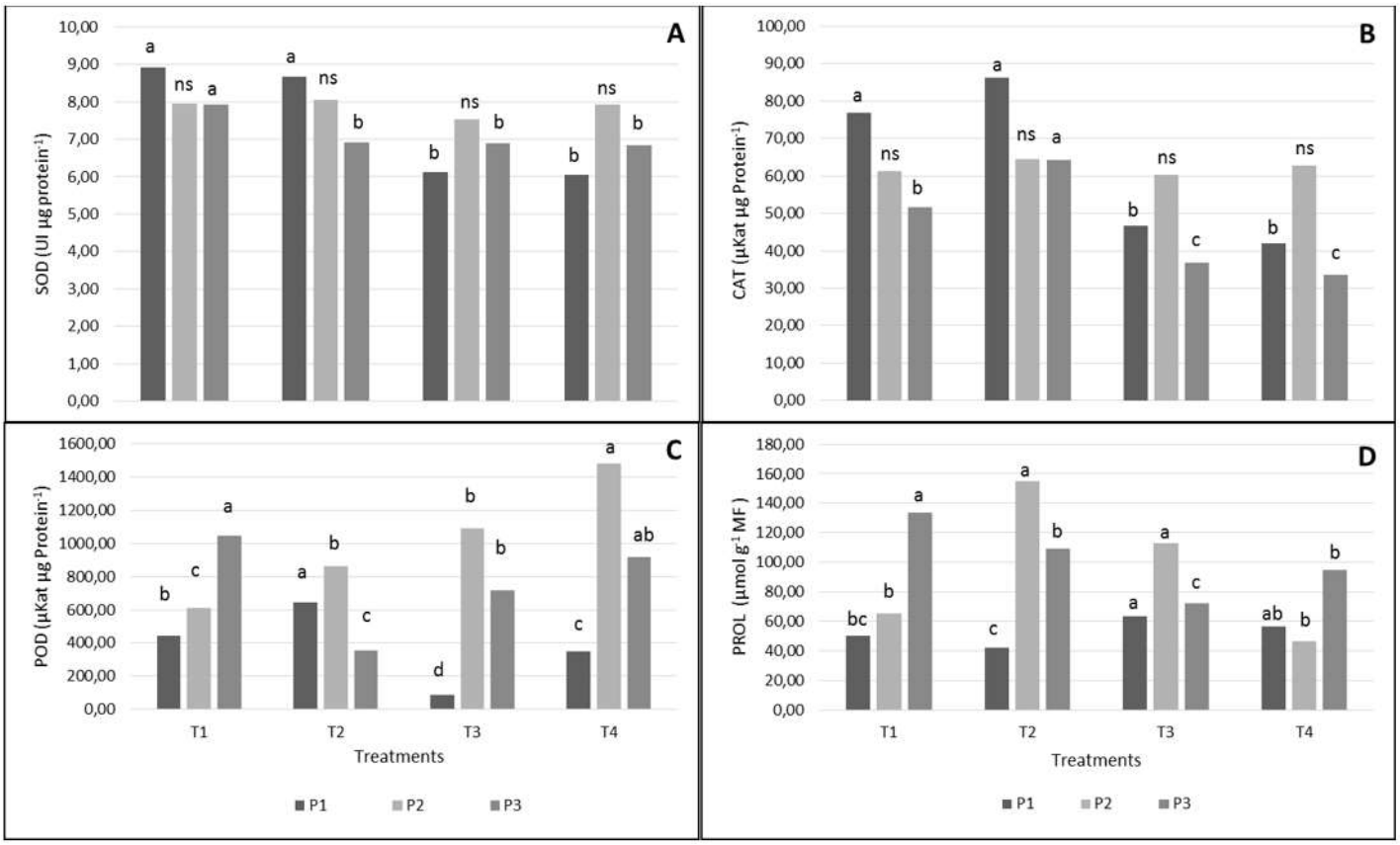

Fig 2: Activities of the antioxidant enzyme: superoxide dismutase (SOD) (A), catalase (CAT) (B), Peroxidase (POD) (C) and Proline content (PROL) (D) in cv peanut. Runner 886 associated with FMAs and supplemented with ESA (T_1), only associated to FMAs (T_2), only supplemented with ESA (T_3) and without association with FMAs and ESA application (T_4), evaluated in three plant development periods (P_1, P_2 and P_3). Means followed by different letters between treatments in the same period differed by the Tukey test (p <0.05).

The possible interaction between the biostimulant and the presence of mycorrhizae was also hypothesized, thus influencing the activation of the antioxidative system of the peanut plants.

In the presence of FMAs (T_1 and T_2), there was an increase in the activity of the SOD enzyme. However, the supplementation of the plants with ESA did not influence the activity of this enzyme as much in plants associated to FMAs (T_1) as in plants only supplemented with ESA (T_3) in P_1.

No changes in SOD activity were identified in period 3, however, the supplemented plants showed higher activity of the enzyme.

Greater CAT activity was obtained in T_1 and T_2 plants during P_1, and in T_2 treatment plants during P_3. However, no significant influence was observed for SOD and CAT activity by the bioagents during the intermediate period (P_2).

The association of the plants with the FMAs did not promote the greater activities of the POD enzyme in the T_1 and T_2 treatments and the supplementation with the ESA did not promote an increase in the activity of this enzyme, which were the higher values found in the absence of the biostimulant and the mycorrhiza (T_4).

The symbiotic mutualist association between FMA and plants does not fail to represent an invasion to the roots leading to the increase of the activities of antioxidative enzymes such as POD, CAT and SOD by the plant/pathogen interaction (He *et al*., 2007; Folli-Pereira *et al*., 2012) [10, 7].

SOD acts in the activation of other antioxidative mechanisms, from the action of secondary enzymes to dismutation of the reactive oxygen species (ROS). However, information on the activity of the enzyme in plants associated with mycorrhizal fungi are contradictory (Fernández-Lizarazo; Moreno-Fonseca, 2016) [6]. Folli (2012) [7] reported similar behavior for SOD activity in jatropha plants inoculated with G. clarus when identifying that the variation of the activity of the enzyme occurred by the exogenous application of the amino acid glycine, compound also present in the ESA, in the associated plants.

This indicates that other molecules, among them glycinetatin, can modulate the antioxidant activity of enzymes in plants with different levels of tolerance to some stressor agent.

As shown in Figure 2, at P_1, the proline content of the plants under the T_3 treatment differs from the T_1 and T_2 treatment plants demonstrating that supplementation with biostimulants early in the development of the peanut promoted an increase in the proline content. In P_2, maintenance of ESA (T_3) supplementation and the establishment of symbiosis with FMAs also enabled the plants of these treatments to have the greatest accumulation of the osmoregulator. However, the plants of the T_2 and T_3 treatments had the lowest proline content, demonstrating that these osmoregulators were not available in the last evaluation period. It is observed during the same period that the plants associated with FMAs and supplemented with ESA (T_1) accumulated the highest proline content.

The humic acids, present in algae extract, also aid the antioxidative mechanisms of plants, although favourable conditions of cultivation since the accumulation of these non-enzymatic antioxidative compounds, like proline, would cause a decrease of the EROS (Zhang; Schmidt, 1999 Van Oosten *et al*., 2017) [30, 28].

To regulate the activity of antioxidative enzymes in plants associated with FMAs, two mechanisms are proposed: a morphological one consisting of the greater capacity of

absorption of nutrients and water by the fungal hyphae and another physiological by which there is production of enzymatic and non-enzymatic compounds, are induced by symbiosis with fungi especially when the plants are subjected to some stressor agent.

This osmoregulator, as it has greater performance in maintaining the water status of the plant, which was not necessary due to the optimal water conditions promoted by the study, this would possibly justify the increase of some antioxidative compounds of enzymatic origin of the peanut plants.

The variable effect on the biosynthesis of this osmolyte in mycorrhizal plants is reported in the literature mainly when the plants are submitted to saline or water stress (Garg; Baher, 2013) [8]. However, there is not much information on the influence of colonization on this accumulation when the plants are supplemented with a biostimulant.

Since FMAs are complex biological systems and depend on other factors such as soil conditions, species, type or combinations of isolates (Fernández-Lizarazo; Moreno-Fonseca, 2016 Folli-Pereira *et al*., 2012) [6, 7], the supplementation with ESA was positive as the increase in physiological variables and in addition induction of the antioxidative mechanism of the peanut plants were observed. The interaction between these bioagents allowed the peanut plants to have higher photo assimilates production, guaranteeing the formation, functioning and occurrence of the symbiosis.

Productivity and yield components

The MSPA and MSR variables (Table 3) of the plants associated to mycorrhizal fungi and supplemented with ESA showed the best results, demonstrating that the presence of both biological agents and biofertilizers can improve the growth and development characteristics of the peanut. Supplementation with organic fertilizers in plants associated with FMAs, in some cases, are shown to be more efficient for root growth characteristics (Paszt *et al*., 2015. Pereira *et al*., 2013) [19, 21]

These results demonstrate that there are benefits related to the use of commercial extracts of algae, such as those obtained from *A. Nodosum*. This effect may be associated with the active compounds of the extract that act synergistically in different concentrations (Hernández-Herrera *et al*., 2014) [11]. Also, the direct effect of the use of this extract, with application via soil, was verified as this agent altered the microbiological community of the rhizosphere (Craigie, 2011) [5].

The chemical composition of seaweed extract (*A.* nodosun, in the case of the present work) is rich in amino acids that participate in the synthesis of active compounds and stimulate the growth of aerial and root parts of plants. The humic substances present in these extracts can influence the fertility of the soil, which, by releasing the nutrients, improves its physical-chemical and biological condition.

Table 3: Mean values of shoot dry matter (MSPA), dry matter root system (MSR), root length (CR) and number of pods per plant (NVP) of peanut due to absence or presence of mycorrhizae and ESA.

Variables	ESA	FMAs	
		Presence	Absenteeism
MSPA (g)	Presence	10.63 aA	2.17 bA
	Absenteeism	5.74 aB	2.53 bA
MSR (g)	Presence	0.80 aA	0.84 aA
	Absenteeism	0.54 aB	0.32 bB
CR (cm)	Presence	21.00 aA	21.12 aA
	Absenteeism	17.00 bB	21.25 aA

Means followed by lower case letters in the row and upper case in column differ from treatments, according to the Tukey test (P<0.05).

The complex biological system formed by FMAs, when supplemented with ESA, presented higher NVP and NGV values (Table 4). Some production indicators may be led to increase or decrease depending on the crop, in response to host interaction and fungi. However, in cultivated plants, there is often no need for the application of biostimulants, as their effects may not be evident in favourable environments. Thus, for supplementation with ESA, the productive aspects of the crop should be considered such as M100 and PROD (Table 4), especially when it is associated with FMAs.

Table 4: Average values of number of grains per pod (NGV), mass of one hundred grains (M100G) and productivity (PROD) of peanut due to absence or presence of mycorrhizae and ESA.

Variables	ESA	FMAs	
		Presence	Absenteeism
NGV	Presence	2.68 aA	2.56aA
	Absenteeism	1.91 bB	2.29 aB
NVP	Presence	13.00 aA	5.75 bA
	Absenteeism	3.75 ab	3.25 aB
M100G (g)	Presence	21.99 bA	68.82 aA
	Absenteeism	34.32 aA	27.40 aB
PROD (kg.ha)$^{-1}$)	Presence	1021.98 bA	1255.98 aA
	Absenteeism	331.99 aB	264.59 aB

Means followed by lower case letters in the row and upper case in column differ from treatments, according to the Tukey test (P <0.05).

The IAC Runner 886 peanut material presented, for both growth and production variables, to be dependent on FMA colonization (HIPPLER; MOREIRA, 2013). This dependence may be associated with the rusticity of the material or its low nutritional requirement.

For the peanut crop, the critical moments that can impact the production are of the flowering and the filling of grains (Jongrungklang *et al*., 2011) [13]. In these periods, there was still the supply of biostimulant supplementation, thus benefiting the increase of photoassimiliados that were destined to the reproductive organs, and reflected in the production indexes.

Conclusion

The association with arbuscular mycorrhizal fungi in an individual or combination matter, combined with ESA supplementation promoted an increase in the net assimilation rate of CO_2(A), Gs and Ci. This consortium also increased the distribution of biomass with direct effects on the increase of production.

The biological agent and the biostimulant also collaborated in the activation and kept the antioxidative system of the peanut plants active, even under optimal conditions of cultivation.

In view of these observations, managements that maintain the native community of mycorrhizal fungi, such as ESA application in plants associated to FMAs, would be great biotechnological alternatives for the cultivation of IAC Runner 886 peanuts.

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