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Study of some morphological and chemical characteristics in two varieties of Medicago sativa L. plant with the effect of biofertilizer (AG) and Brassinolide

Wael Shakir Hameed Al-Jboory[1*], Mahir Zeki Faysal Al-Shimary[2]

[1],[2]Department of Biology, Collage of Education Pure Science/ Ibn Al-Haitham, University of Baghdad, Iraq.
Email: wael.biologist91@gmail.com
Email: zakimaher164@gmail.com

***Correspondence author:** Wael Shakir Hameed Al-Jboory (wael.biologist91@gmail.com)

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Abstract

The experiment was carried out in the field of the Botanical Garden belonging to the Department of Biology, College of Education for Pure Science/ Ibn Al-Haitham, University of Baghdad, for the growing season 2021-2022 to study the effect of biofertilizer (AG 8-1-9) in three concentrations (0, 3, 6) mg.L^{-1} and the Brassinolide hormone in four concentrations (0, 2, 4, 6) mg. L^{-1} and their interactions in some morphological and chemical characteristics of two varieties of Medicago sativa plant. The experiment was designed according to Rondomized Complete Blocks Design (R.C.B.D.) with three replicates per treatment. The means were compared using the least significant difference at the probability level (0.05), and the results showed that the effect of Brassinolide in different concentrations led to a significant increase in all studied characteristics, and the concentration of Brassinolide 4 mg.L^{-1} was exceeded in each of the number of branches, the number of root nodes, the content of chlorophyll a and b and the percentage of protein, and the concentration of Brassinolide 6 mg.L^{-1} was exceeded in the height of the plant characteristic. The effect of the biofertilizer in different concentrations led to a significant increase in all the studied characteristics were at a concentration of 3 mg.L^{-1}, except for the height of the plant at the concentration of 6 mg.L^{-1}. All duel interactions had a significant effect on all the studied characteristics with the superiority of the treatment (3, 4) mg.L^{-1} in the number of branches, the number of root nodes and the content of leaves of chlorophyll a and b, the treatment (3, 6) mg.L^{-1} in the percentage of protein and the treatment of (4, 6) mg.L^{-1} in plant height and the superiority of the Indian variety in the number of branches, the number of root nodes and the content of leaves from chlorophyll a and b and the percentage of protein, and the superiority of the local variety in plant height. Triple interactions led to a significant increase in all studied characteristics.

Keywords

Biofertilizer, Brassinolide, Medicago sativa L., Chemical characteristics.

Medicago sativa L. plant is one of the most important leguminous fodder crops and is called the king of fodder for its high nutritional value and multiple uses (green fodder, dress, silage) and is considered a protein source for poultry feed (Fick and Mueler, 1989). Alfalfa or Lucerne is an important leguminous fodder crop in most regions of the world (Pecetti and Tava, 2000). It is a perennial crop that stays in the land for about 4-20 years and according to environmental conditions and the crop management system. The total area of Alfalfa plant cultivation in the world is estimated at 30 million hectares, most of which are located in the United States

and Argentina (Cash et al., 2009). The importance of the Alfalfa plant continues, especially in Europe, through sustainable agriculture, due to its high productivity, as the Alfalfa plant is the largest production of fodder protein among all fodder legumes, as it is the best legume fodder crop, due to its ability to fix nitrogen and tolerate drought, in addition to its ability to interact with soil microorganisms (bacteria) (Moghaddama et al., 2015). Alfalfa plant belongs to the third largest family in the plant kingdom, Fabaceae family (Gepts et al., 2005). It is the fourth most growing crop in North America, USA, after only corn, wheat and soybeans (Zhang et al., 2005). Alfalfa is the first genetically modified feed produced and marketed, although the approval process was complex (Wang and Brummer, 2012).

Biofertilizers are natural materials consisting of bacterial and fungal strains to reduce chemical use in fertilization applications, in addition to that, biofertilizers play a positive role in helping plants because they contain microorganisms capable of processing nutrients and converting them from the unavailable form to the available form through various biological processes, and improving the natural, chemical and biological properties of the soil, as it maintains the balance of nutrients in agricultural land (Raghda'a et al., 2020). Biofertilizers are substances made up of microorganisms that help plants absorb nutrients when applied through seeds or soil, and some microbial processes in the soil accelerate the availability of nutrients in a way that is easier for plants to absorb (Raja, 2013). Biofertilizers are a modern fertilization technique as they are added in several different ways, including spraying them on the plant or irrigation water, treating the seeds with them or adding them directly to the soil. The organism is useful for the plant, the most important of which are bacteria, which have an important role in improving plant growth qualities in addition to their role in the analysis of organic matter and inhibition of the activity of pathogens in the soil, and it also coexists with the root nodes of nitrogen fixation (Woodward, 2003). Brassinolides are a group of phytohormones and are considered the sixth group of phytohormones as well as other phytohormones, which are similar to steroid hormones in animals (Bhardwaj et al., 2006; Arora et al., 2008). Brassinolides (BRs) were discovered 40 years ago, and since then a great deal of work has been done to highlight its role in plant functions (Peres et al., 2019). It was known that it is a steroid hormone specialized in high animals groups only, which is responsible for many biological activities in the animal and in very low concentrations and with a high physiological effect. The idea of the presence of steroid hormones within the plant metabolism was not contained and was excluded because steroid hormones are secreted by especially glands, but the scientist Grove in 1979 discovered the presence of steroid hormones in the foreign rapeseed plant (Brassica napus L.) belonging to the Barssicaceae family, as it was isolated from pollen. The name Brassinolide is derived from the Latin name of the Barssicaceae family (Anjum et al., 2012; Pereira-Netto, 2012). Brassinosteroids are Euphytosteroid hormones with effective effects and very low concentrations and their effect similar to animal steroid hormones has recently been observed (Premalatha et al., 2012; Hussein, 2013) and they are polyhydroxyl steroids that regulate various growth processes such as cell division, cell cycle, elongation, morphology, reproduction and aging (Clouse and Sasse, 1998). The study aims to find out the effect of biofertilizer and the Brassinolide hormone in improving the growth characteristics of two varieties of Alfalfa plant.

Materials and Methods

Table (1): Some chemical and physical characteristics of the experiment soil before cultivating.

Characteristic		Value	Unite
Chemical and fertility properties			
pH		7.50	
Electrical Conductivity $EC_{1:1}$		2.3	$dS.m^{-1}$
Organic matter O.M.		2.3	%
Available Nitrogen %		23.3	mg. kg^{-1}
Available phosphorus		14.6	
Available potassium		355	
Dissolved positive ions	Calcium	4.5	$Meq.L^{-1}$
	Magnesium	3.7	
	Sodium	2.43	
Dissolved negative ions	Sulfates	5.22	
	Bicarbonates	1.3	
	Chlorides	4.3	
Physical properties			
Soil separators	Clay	216	g. kg^{-1}
	Silt	420	
	Sand	364	
Soil texture			Loam

The experiment was carried out in the field of the Botanical Garden belonging to the Department of Biology,

College of Education for Pure Sciences - Ibn Al-Haitham, University of Baghdad, for the growing season 2021-2022 to study the effect of biofertilizer and Brassinolide hormone and their interactions in some morphological and chemical characteristics of two varieties of Alfalfa plant Medicago sativa (local and Indian). The experiment was designed according to (Rondomized Complete Blocks Design) R.C.B.D. with three replicates per treatment. Samples were taken from the soil of the field before cultivated for the purpose of estimating the chemical and physical properties as shown in Table (1) according to the methods described in Page et al. (1982). The analysis was carried out in the central laboratory of the Department of Biology, College of Science, University of Baghdad. Plowing, smoothing and leveling operations were conducted, then the experimental land was divided into three repeaters, each repeater contains 24 experimental units, and the area of one experimental unit was 2 m^2 and thus the total number of experimental units is 72 experimental units 2 m^2 as each variety of Alfalfa plant took half the number of these units, and cultivated a plant in the form of straight lines. The distance between each line of the lines is 50 cm, each experimental unit contains four lines.

NPK fertilizer 20:20:20 was added to all experimental units before cultivating, then seeds were cultivated on 2/11/2021 according to the experiment treatments, a number of plants (30 plants) were identified for each experimental unit for the purpose of the study, and all crop service operations were carried out from irrigation, hoeing and weeding whenever needed until the end of the experiment.

The experiment was designed to study the effect of biofertilizer, Brassinolide hormone and their interactions in some morphological and chemical characteristics of two varieties of Alfalfa plant. The treatments were organized in the design of Rondomized Complete Blocks Design (R.C.B.D.). Some morphological and chemical characteristics have been studied, including:

1. **Plant height (cm):** The plant height of five random plants per experimental unit of soil surface up to the highest point of the plant was measured by a measuring machine.
2. **Number of plant branches (branch. plant-1):** The number of plant branches of five plants harvested from the midline of each experimental unit was calculated and then by mean.
3. **Number of root nodes (node.plant-1):** The number of nodes in the main root of five plants harvested from the midline of each experimental unit was calculated and then by mean.
4. **Determination of chlorophyll concentration a, b (mg. g-1 soft leaf weight):** The concentration of chlorophyll a and b was estimated by the Mac-Kinney (1941) and Lichtenthaler (1987) methods.
5. **Estimation of the percentage of protein in the vegetative system (%):** The percentage of protein in the vegetative system was estimated by multiplying the percentage of nitrogen by a constant factor (6.25) according to the method of Vopyan (1984).

Protein percentage = N% Ч 6.25

The results were statistically analysed according to the design and the least significant difference at a probability level of 0.05 (SAS, 2012).

Results and Discussion

Plant height (cm)

The results of Table 2 showed that there is a significant difference between the two varieties of Alfalfa in the characteristic of plant height, the plants of the local variety outperformed the highest mean height of the plant of 93.27 cm compared to the plants of the Indian variety. The difference between the two varieties of Alfalfa plant in the height characteristic can be attributed to the genetic difference between the two varieties because plant varieties differ genetically from each other in the characteristics of vegetative growth, including the characteristic of plant height according to its origin and different geographical areas.

The results of Table 2 also show the significant difference between the concentrations of AG fertilizer (0,3 and 6) mg. L^{-1} in plant height. The sprayed plants recorded a concentration of 6 mg. L^{-1} is the highest mean height of 95.62 cm compared to control plants because of spraying the Alfalfa plant with biofertilizer led to a significant increase in the characteristic of plant height and for both varieties, the reason may be due to the positive role of the biofertilizer in the formation of a dense root system, which increases the surface area for water and nutrient absorption (Hartman, 2000b). This is reflected positively in vegetative growth indicators, including plant height, and these results are consistent

with Saaed et al. (2011) and Al-Shamarrey (2007), and may also be the reason for the superiority in vegetative growth indicators (plant height) due to the high ability of Bacillus bacteria to dissolve phosphorus through the secretion of many organic acids in addition to increasing the availability of some nutrients, especially macroelements (Sharma et al., 2012). Thus, this effect is reflected in the increase of the root system and its absorption of nutrients and water, which contributed to the increase in plant growth, through an increase in plant height (Willer and Lernoud, 2019). As for the effect of spraying the growth regulator Brassinolide (BL), the results of Table 2 show significant differences between the sprayed concentrations in the plant height characteristic, the sprayed plants were characterized by a concentration of 6 mg. L^{-1} with the highest mean plant height was 94.49 cm compared to control plants. Al-Saedi, and Al-Mentafji (2016) noted that the treatment of coriander plant with the hormone Brassinolide led to a significant increase in the characteristic of plant height, and Nasralla et al. (2015) also noted that spraying Brassinolide on the vegetative system of the wheat plant led to a significant increase in plant height.

These results are consistent with Sura and Al-Hilfy (2022), they noted that the use of Brassinolide led to a significant increase in plant height, and this is due to the role of Brassinolide in stimulating the processes responsible for cell elongation and division. The cell elongation is controlled by various processes such as coordinated changes in the mechanical properties of the cell wall, biochemical processes and gene expression, because the primary wall in most dicotyledonous and monocotyledonous plants consists of microcellulosic fibers and therefore Brassinosteroids are believed to be involved in the relaxation of the cell wall, so the increase in vegetative growth resulting from the addition of Brassinolide may be due to cell elongation and division (Shahbaz and Ashraf, 2007). It may also be due to the role of Brassinolide in stimulating cell division, especially in the meristem regions at the stem growing apex, which increases the growth of the vegetative population (Zandi, 2012). As for the duel interactions, the results of the same table show a significant interaction between the varieties and spraying with biofertilizer in the characteristic of plant height, The plants of the local and Indian variety sprayed with a concentration of 6 and 3 mg.L^{-1} were achieved the highest interaction values were (101.22 and 94.46) cm with a significant difference between them sequentially compared to control plants.

The interaction between spraying biofertilizer and Brassinolide was also significant in plant elevation, as sprayed plants outperformed at 6 mg.L^{-1} of biofertilizer and 4 mg.L^{-1} of Brassinolide with the highest mean interaction of 99.62 cm compared to control plants. As for the triple interaction between the study factors, the results indicate a significant interaction between the study factors in the plant height characteristic (Table 3), the local variety plants sprayed with a concentration of 6 mg. L^{-1} of Brassinolide and 2 mg.L^{-1} of Brassinolide with the highest mean interaction of 108.50 cm and an increase of 39.08% compared to control plants.

Table 2: Effect of variety, biofertilizer and Brassinolide and their interactions on plant height (cm) of two Alfalfa varieties.

Varity (V)	Biofertilizer Concentrations (AG) (mg L^{-1})	Brassinolide (BL) concentrations (mg. L^{-1})				Effect of dual interaction mean AG Ч V
		0	2	4	6	
Indian	0	69.00	77.31	80.18	85.47	77.99
	3	87.09	93.59	96.95	100.19	94.46
	6	82.70	89.36	94.99	93.03	90.02
Local	0	77.77	84.01	88.50	93.19	85.87
	3	85.55	90.14	99.07	96.20	92.74
	6	93.25	108.50	104.25	98.88	101.22
LSD (0.05)		Triple interaction= 3.75				1.88
Dual interaction VЧBL						
Varity (V)		Brassinolide (BL) concentrations (mg. L^{-1})				Mean variety effect
		0	2	4	6	
Indian		79.60	86.75	90.71	92.90	87.49
Local		85.52	94.22	97.27	96.09	93.27
LSD (0.05)		2.17				1.08
Dual interaction BL Ч AG						
Biofertilizer (AG)		Brassinolide (BL) concentrations (mg. L^{-1})				Mean Biofertilizer effect
		0	2	4	6	
0		73.39	80.66	84.34	89.33	81.93
3		86.32	91.87	98.01	98.19	93.60
6		87.98	98.93	99.62	95.95	95.62
LSD (0.05)		2.65				1.33
Mean Brassinolide effect		82.56	90.49	93.99	94.49	
LSD (0.05)		1.53				

Number of branches (branch. plant^{-1})

The results of Table 3 showed a significant difference between the two varieties of Alfalfa plant in the characteristic of the number of branches of the plant, the plants of the Indian variety excelled with the best mean number of branches of 19.52 compared to the plants of the local variety, the difference in the two varieties of Alfalfa plant in the characteristic of the number of branches may be due to their genetic differences and the response of each of them to the study treatment. It is also noted from the results of Table 3 the significant difference between the concentrations of biofertilizer AG (0, 3 and 6) mg. L^{-1} in the mean number of branches of the Alfalfa plant, where sprayed plants with a concentration of 3 mg.L^{-1} recorded the best mean characteristic of 19.18 compared to control plants, indicating the superiority of sprayed plants at a concentration at 3 mg. L^{-1} in the number of plant branches characteristic. The use of biofertilizer led to a significant increase in the number of plant branches, the reason may be due to the role of the biofertilizer in increasing the number of Plant branches by its content of bacteria that improve growth and improve evolution, and the formation of branches, and also plays a role in increasing the side buds, which leads to an increase in the number of branches of the plant by increasing the growth of vascular tissue and this is consistent with Shaalan (2004). As for the effect of spraying the growth regulator Brassinolide (BL), the results of Table 3 show significant differences between the sprayed concentrations in the mean number of plant branches, the sprayed plants were characterized by a concentration of 4 mg. L^{-1} with the highest mean characteristic was 19.91 compared to control plants. Salman and Saleh, (2016) noted that the use of the Brassinolide hormone led to a significant increase in the number of plant branches, and that this increase in the number of branches may be due to the role of Brassinolide in stimulating elongation and cell division as it works to regulate the cell cycle in a similar way to the role of cytokines in the cell cycle (Taiz and Zeiger, 2010). As for dual interactions, the results of the same table show that there is a significant interaction between the varieties and the biofertilizer in the mean number of branches of the plant. The plants of the Indian variety sprayed with a concentration of 3 mg. L^{-1} were recorded best mean interaction was 20.98, while the local variety plants sprayed with a concentration of 6 mg. L^{-1} recorded the best mean interaction was 17.59 with a significant difference between them compared to control plants. The interaction between varieties and the spraying of the growth regulator Brassinolide was significant and the Indian and local variety sprayed plants with a concentration of 4 mg. L^{-1} had the best mean interaction (21.60 and 18.39) respectively compared to control plants. The interaction between biofertilizer and Brassinolide was significant in the mean number of plant branches, the sprayed plants with a concentration 3 mg. L-1 of biofertilizer and 4 mg. L^{-1} of Brassinolide were given best mean interaction was 21.24 compared to control plants.

As for the triple interaction between the study factors, the results indicate a significant interaction between the study factors in the characteristic of the number of plant branches (Table 3), the plants of the Indian variety sprayed with a concentration 3 mg. L^{-1} of biofertilizer and 4 mg. L^{-1} of Brassinolide were recorded the highest mean interaction of 23.25 compared to control plants.

Table 3: Effect of variety, biofertilizer and Brassinolide and their interactions on the number of branches (branch.plant-1) of two Alfalfa varieties.

Varity (V)	Biofertilizer Concentrations (AG) (mg L^{-1})	Brassinolide (BL) concentrations (mg. L^{-1})				Effect of duel interaction mean AG Ч V
		0	2	4	6	
Indian	0	13.67	16.63	20.50	19.59	17.60
	3	18.64	21.40	23.25	20.64	20.98
	6	19.83	20.68	21.04	18.36	19.98
Local	0	10.50	15.84	17.16	16.80	15.08
	3	15.12	18.08	19.22	17.32	17.39
	6	16.95	18.25	18.77	16.20	17.59
LSD (0.05)		0.81				0.41
Dual interaction VЧBL						
Varity (V)		Brassinolide (BL) concentrations (mg. L^{-1})				Mean variety effect
		0	2	4	6	
Indian		17.38	19.57	21.60	19.53	19.52
Local		14.25	17.39	18.39	16.71	16.68
LSD(0.05)		0.47				0.23
Duel interaction BL Ч AG						
Biofertilizer (AG)		Brassinolide (BL) concentrations (mg. L^{-1})				Mean Biofertilizer effect
		0	2	4	6	
0		12.08	16.24	18.83	18.19	16.34
3		16.88	19.74	21.24	18.88	19.18
6		18.49	19.46	19.90	17.28	18.79
LSD (0.05)		0.57				0.29
Mean Brassinolide Effect		15.82	18.48	19.91	18.12	
LSD (0.05)		0.33				

Number of root nodes (node. plant-1)

The results of Table 4 indicated that there is a significant difference between the two varieties of Alfalfa plant in the mean number of root nodes, the plants of the Indian variety outperformed the highest mean number of root nodes amounting to 38.00 compared to the plants of the local variety, the difference between the two varieties of Alfalfa plant in the number of root nodes may be due to the variation of the genetic material of both varieties.

It is also noted from the results of Table 4 the significant difference between the concentrations of Biofertilizer AG (0, 3 and 6) mg. L^{-1} in the number of root nodes of the Alfalfa plant, as the sprayed plants at a concentration of 3 mg. L^{-1} contained the highest mean in the number of root nodes was 36.16 compared to control plants. This increase in the number of root nodes may be due to the role of the biofertilizer in increasing root growth and the superiority of the fertilizer in increasing the growth of the root system and thus this effect is reflected in an increase in the number of root nodes. As for the effect of spraying the growth regulator Brassinolide (BL), the results of Table 4 show significant differences between the sprayed concentrations in the mean number of root nodes, the sprayed plants were characterized by a concentration of 4 mg. L^{-1} with the highest mean number of root nodes 36.66 compared to control plants. The increase in the root nodes number of two varieties of Alfalfa plant as a result of the addition of Brassinolide may be due to the role of this hormone in increasing root growth and the superiority of the hormone in increasing the growth of the root system and thus this effect is reflected in an increase in the number of root nodes.

As for duel interactions, the results of the same table show a significant interaction between the varieties and the biofertilizer in the mean number of root nodes. The plants of the Indian variety sprayed with a concentration of 3 mg.L^{-1} were given the highest mean interaction of 39.99. While local variety plants sprayed at a concentration of 6 mg. L^{-1} was recorded best mean interaction 33.55 with a significant difference between them compared to control plants.

The interaction between varieties and spraying of the growth regulator Brassinolide was significant and the sprayed Indian and local variety plants with a concentration of 4 mg. L^{-1} were characterized by the highest mean interaction (40.31 and 33.00) respectively compared to control plants. The interaction between biofertilizer and Brassinolide was also significant in the mean number of root nodes; sprayed plants with a concentration of 3 mg. L^{-1} by biofertilizer and 4 mg. L^{-1} by Brassinolide were given highest mean interaction of 37.78 compared to control plants. As for the triple interaction between the study factors, the results indicate a significant interaction between the study factors in the number of root nodes (Table 4). The Indian variety sprayed plants outperformed at a concentration of 3 mg. L^{-1} of biofertilizer and 4 mg.L^{-1} of Brassinolide with the highest mean interaction of 42.39 compared to control plants.

Table 4: Effect of variety, biofertilizer and Brassinolide and their interactions on the number of root nodes (node. plant-1) of two Alfalfa varieties.

Varity (V)	Biofertilizer Concentrations (AG) (mg L^{-1})	Brassinolide (BL) concentrations (mg. L^{-1})				Effect of duel interaction mean AG Ч V
		0	2	4	6	
Indian	0	30.06	35.35	39.04	37.92	35.59
	3	36.72	41.44	42.39	39.41	39.99
	6	38.57	40.47	39.51	35.16	38.43
Local	0	27.98	30.80	31.66	30.66	30.27
	3	31.55	32.49	33.17	32.07	32.32
	6	32.43	34.49	34.18	33.11	33.55
LSD(0.05)		1.11				0.56
Duel interaction VЧBL						
Varity (V)		Brassinolide (BL) concentrations (mg. L^{-1})				Mean variety effect
		0	2	4	6	
Indian		35.12	39.09	40.31	37.50	38.00
Local		30.65	32.59	33.00	31.95	32.05
LSD(0.05)		0.64				0.32
Duel interaction BL Ч AG						
Biofertilizer (AG)		Brassinolide (BL) concentrations (mg. L^{-1})				Mean Biofertilizer effect
		0	2	4	6	
0		29.02	33.07	35.35	34.29	32.93
3		34.14	36.97	37.78	35.74	36.16
6		35.50	37.48	36.84	34.14	35.99
LSD(0.05)		0.79				0.39
Mean Brassinolide Effect		32.88	35.84	36.66	34.72	
LSD(0.05)		0.45				

Leaf content of chlorophyll A (mg.g-1 wet weight)

The results of Table 5 indicated a significant difference between the two varieties of Alfalfa plant in the mean leaf content of chlorophyll a, the plants of the Indian variety outperformed the highest mean leaf content of chlorophyll a was 1.020 mg.g^{-1} wet weight compared to plants of the local variety, the difference in the two varieties of Alfalfa plant in chlorophyll a content may be due to the variation of the genetic material of both varieties.

It is also noted from the results of Table 5 the significant difference between the concentrations of biofertilizer AG (0, 3 and 6) mg.L^{-1} in the leaf content of chlorophyll a in the Alfalfa plant, as the sprayed plants at a concentration of 3 mg. L^{-1} had the highest mean content of chlorophyll a was1.032 mg.g^{-1} wet weight compared to control plants. This increase in the leaf content of chlorophyll a may be due to the role of the biofertilizer containing bacteria by the high ability of Bacillus bacteria to dissolve phosphorus through the secretion of many organic acids in addition to increasing the availability of some nutrients, especially macroelements (Sharma et al., 2012). Thus, this effect is reflected in the increase of the root system and increase its absorption of nutrients and water, which contributed to increasing plant growth, and then increasing the relative density of chlorophyll pigment and this leads to an increase in the content of chlorophyll a as well as the role of the biofertilizer in increasing the absorption of nutrients by increasing the growth of the root system, increasing the availability of these elements and other elements absorbed by the plant, which are involved in the composition of the porphyrin rings involved in the biosynthesis of chlorophylls, the most important of which is magnesium, thus it had an effect on this increase.

As for the effect of spraying the growth regulator Brassinolide (BL), the results of Table 5 show significant differences between the sprayed concentrations in the mean leaf content of chlorophyll a, the sprayed plants by a concentration of 4 mg. L^{-1} were characterized by the highest mean chlorophyll a content was 1.032 mg.g^{-1} wet weight compared to control plants. Sura and Al-Hilfy (2022) noted that the use of Brassinolide led to a significant increase in the total chlorophyll content of the leaves, and therefore this increase occurred as a result of the significant increase in the rest of the pigments, including chlorophyll a. Al-Jumaily and Al-esawi (2016) also noted that the use of Brassinolide led to a significant increase in the leaf content of chlorophyll of the plant, and Al-Shimary, and Al-Jboury (2017a) also noted that the use of Brassinolide led to a significant increase in the content of the leaves of total chlorophyll of the dill plant and therefore this increase occurred as a result of the significant increase in the rest of the pigments, including chlorophyll a. The increase in the leaf content of chlorophyll a of two Alfalfa varieties as a result of the addition of Brassinolide may be due to the role of Brassinolide in inhibiting the chlorophyllase enzyme responsible for the depletion of chlorophyll, which led to the accumulation of chlorophyll in the leaves (Fariduddin et al., 2003). Brassinosteroids increase the chlorophyll content in the leaves (Swamy and Rao, 2009). As for duel interactions, the results of the same table show a significant interaction between the varieties and the biofertilizer in the mean chlorophyll a content. The plants of the Indian and local variety sprayed with a concentration of 3 mg. L^{-1} had the highest mean interaction of (1.103 and 0.961) mg. g^{-1} wet weight with a significant difference between them respectively. The interaction between varieties and spraying of the growth regulator Brassinolide was significant and the sprayed Indian and local variety plants at a concentration of 4 mg. L^{-1} were characterized by the highest mean interaction of (1.161 and 0.993) mg. g^{-1} wet weight respectively compared to control plants.

The interaction between biofertilizer and Brassinolide was also significant in the mean leaf content of chlorophyll a. The sprayed plants at a concentration of 3 mg. L^{-1} of biofertilizer and 4 mg. L^{-1} of Brassinolide had the highest mean interaction of 1.133 mg.g^{-1} wet weight compared to control plants.

As for the triple interaction between the study factors, the results indicate a significant interaction between the study factors in the leaf content of chlorophyll a of the plant (Table 5). The Indian variety sprayed plants at a concentration of 3 mg. L^{-1} of biofertilizer and 4 mg.L^{-1} of Brassinolide were outperformed with the highest mean interaction characteristic of 1.224 mg.g^{-1} wet weight with an increase of 39.08% compared to control plants.

Table 5. Effect of variety, biofertilizer and Brassinolide and their interactions on the leaf content of chlorophyll a (mg. g-1 wet weight) of two Alfalfa varieties.

Varity (V)	Biofertilizer Concentrations (AG) (mg L^{-1})	Brassinolide (BL) concentrations (mg. L^{-1})				Effect of duel interaction mean AG Ч V
		0	2	4	6	
Indian	0	0.571	0.821	1.097	1.042	0.883
	3	0.934	1.147	1.224	1.106	1.103
	6	0.992	1.088	1.163	1.053	1.074
Local	0	0.454	0.782	0.943	0.927	0.776
	3	0.863	0.995	1.042	0.945	0.961
	6	0.907	0.952	0.994	0.879	0.933
LSD(0.05)		0.045				0.023
Duel interaction VЧBL						
Varity (V)		Brassinolide (BL) concentrations (mg. L^{-1})				Mean variety effect
		0	2	4	6	
Indian		0.833	1.019	1.161	1.067	1.020
Local		0.741	0.910	0.993	0.917	0.890
LSD(0.05)		0.026				0.013
Duel interaction BL Ч AG						
Biofertilizer (AG)		Brassinolide (BL) concentrations (mg. L^{-1})				Mean Biofertilizer effect
		0	2	4	6	
0		0.513	0.801	1.020	0.985	0.830
3		0.899	1.071	1.133	1.026	1.032
6		0.950	1.020	1.079	0.966	1.004
LSD(0.05)		0.032				0.016
Mean Brassinolide Effect		0.787	0.964	1.077	0.992	
LSD(0.05)		0.018				

Leaf content of chlorophyll b (mg.g-1 wet weight)

The results of Table 6 indicated a significant difference between the two varieties of Alfalfa plant in the mean leaf content of chlorophyll b, the plants of the Indian variety outperformed the highest mean leaf content of chlorophyll b by 0.446 mg.g^{-1} wet weight compared to plants of the local variety, the difference in the two varieties of Alfalfa plant in chlorophyll b content may be due to the variation of the genetic material of both varieties. It is also noted from the results of Table 6 the significant difference between the concentrations of biofertilizer AG (0, 3 and 6) mg. L^{-1} in the leaf content of chlorophyll b in Alfalfa plant, it was noted that the plants sprayed with a concentration of 3 mg. L^{-1} had the highest mean chlorophyll b content of 0.455 mg.g^{-1} wet weight compared to control plants. This increase in the leaf content of chlorophyll b may be due to the role of the biofertilizer containing bacteria by the high ability of Bacillus bacteria to dissolve phosphorus through the secretion of many organic acids in addition to increasing the availability of some nutrients, especially macroelements (Sharma et al., 2012). Thus, this effect is reflected in the increase of the root system and increase its absorption of nutrients and water, which contributed to increasing plant growth, and thus increasing the relative density of chlorophyll pigment and this leads to an increase in the content of chlorophyll b as well as the role of the biofertilizer in increasing the absorption of nutrients by increasing the growth of the root system, the increasing availability of these elements and other elements absorbed by the plant that are involved in the structure of porphyrin rings involved in the biosynthesis of chlorophylls, the most important of which is magnesium, them it had an impact on this increase. As for the effect of spraying the growth regulator Brassinolide (BL), the results of Table 6 show significant differences between the sprayed concentrations in the mean leaf content of chlorophyll b. The sprayed plants at a concentration of 4 mg. L^{-1} were characterized by the highest mean chlorophyll b content was 0.477 mg.g^{-1} wet weight compared to control plants. Sura and Al-Hilfy (2022) noted that the use of Brassinolide led to a significant increase in the total chlorophyll content of the leaves, and therefore this increase occurred as a result of the significant increase in the rest of the pigments, including chlorophyll b. Al-Shimary, and Al-Jboury (2017a) also noted that the use of Brassinolide led to a significant increase in the content of the leaves of the total chlorophyll of the dill plant, and therefore this increase occurred as a result of the significant increase in the rest of the pigments, including chlorophyll b. The increase in the chlorophyll b content of two Alfalfa varieties as a result of the addition of Brassinolide may be due to the role of Brassinolide in inhibiting the chlorophyllase enzyme that responsible for chlorophyll depletion, which led to the accumulation of chlorophyll in the leaves (Fariduddin et al., 2003) and this led to an increase in the content of chlorophyll b. As for duel interactions, the results of the same table show a significant interaction between the varieties and the biofertilizer in the mean chlorophyll b content. The plants of

the Indian and local variety sprayed with a concentration of 3 mg.L^{-1} were given the highest mean interaction of (0.485 and 0.424) mg.g^{-1} wet weight and has a significant difference between them respectively. The interaction between varieties and spraying of the growth regulator Brassinolide was significant and the sprayed Indian and local variety plants at a concentration of 4 mg. L^{-1} were characterized by the highest mean interaction of (0.511 and 0.442) mg.g^{-1} wet weight respectively compared to control plants. The interaction between biofertilizer and Brassinolide was also significant in the mean leaf content of chlorophyll b. The sprayed plants at a concentration of 3 mg. L^{-1} of biofertilizer and 4 mg. L^{-1} of Brassinolide has the highest mean interaction of 0.504 mg.g^{-1} wet weight with an increase of 95.01% compared to control plants. As for the triple interaction between the study factors, the results indicate a significant interaction between the study factors in the leaf content of plant chlorophyll b (Table 6). The Indian variety sprayed plants at a concentration 3 mg. L^{-1} of biofertilizer and 4 mg.L^{-1} of Brassinolide were outperformed the highest mean interaction characteristic by0.541 mg.g^{-1} Wet weight compared to control plants.

Table 6. Effect of variety, biofertilizer and Brassinolide and their interactions on leaf content of chlorophyll b (mg. g-1 wet weight) of two Alfalfa varieties.

Varity (V)	Biofertilizer Concentrations (AG) (mg L^{-1})	Brassinolide (BL) concentrations (mg. L^{-1})				Effect of duel interaction mean AG Ч V
		0	2	4	6	
Indian	0	0.251	0.340	0.481	0.458	0.383
	3	0.410	0.504	0.541	0.485	0.485
	6	0.435	0.477	0.510	0.462	0.471
Local	0	0.203	0.328	0.424	0.407	0.341
	3	0.379	0.437	0.467	0.414	0.424
	6	0.398	0.413	0.436	0.386	0.408
LSD(0.05)		0.021				0.011
Duel interaction VЧBL						
Varity (V)		Brassinolide (BL) concentrations (mg. L^{-1})				Mean variety effect
		0	2	4	6	
Indian		0.365	0.440	0.511	0.468	0.446
Local		0.327	0.393	0.442	0.402	0.391
LSD (0.05)		0.012				0.006
Duel interaction BL Ч AG						
Biofertilizer (AG)		Brassinolide (BL) concentrations (mg. L^{-1})				Mean Biofertilizer effect
		0	2	4	6	
0		0.227	0.334	0.453	0.432	0.362
3		0.395	0.470	0.504	0.450	0.455
6		0.417	0.445	0.473	0.424	0.440
LSD (0.05)		0.015				0.007
Mean Brassinolide Effect		0.346	0.417	0.477	0.435	
LSD (0.05)		0.008				

Percentage of protein in the vegetative system of the plant (%)

The results of Table 7 showed that there is a significant difference between the two types of Alfalfa plant in the mean percentage of protein in the plant, the plants of the Indian variety recorded the highest mean percentage of protein in the plant was 12.09% compared to the plants of the local variety. The reason for the increase in the protein content in the plants of the Indian variety is due to their superiority in the percentage of nitrogen in the plant. It is also noted from the results of Table 7 the significant difference between the concentrations of biofertilizer AG (0, 3 and 6) mg.L^{-1} in the percentage of protein in the plant. Plants sprayed at a concentration of 3 mg.L^{-1} had the best protein percentage of 12.33% compared to control plants. The outperformed of plants sprayed by 3 mg. L^{-1} in the percentage of protein belongs to an increase in the percentage of protein when adding the biofertilizer may be due to its superiority in the percentage of nitrogen in the plant. This is consistent with the results of Sabry and Abdal-Latife (2017) when they noted that the use of biofertilizers led to a significant increase in the percentage of nitrogen in the plant and thus leads to a significant increase in the percentage of protein in the plant, and the reason may also be due to the fact that the biofertilizer one of its basic components is amino acids and is the basic synthesis of protein. As for the effect of spraying the growth regulator Brassinolide (BL), the results of Table 7 show a significant difference between the sprayed concentrations in the mean protein percentage in the plant, the sprayed plants at a

concentration of 4 mg. L^{-1} were characterized by the highest mean characteristic percentage by 12.32% compared to control plants. These results are agree with Al-Shimary and Al-Jboury (2017b) when they noted that spraying Brassinolide led to a significant increase in the percentage of protein in the dill plant, due to the increase in the percentage of protein when adding Brassinolide due to its superiority in the ratio of nitrogen and potassium in the plant. As for the duel interactions, the results of the same table show that there is a significant interaction between the varieties and the biofertilizer in the mean protein percentage of the plant. The plants of the Indian variety sprayed with a concentration of 3 mg. $^{L-1}$ were recorded best mean interaction was 13.13%, while local variety plants sprayed with a concentration of 6 mg. L^{-1} ware recorded best mean interaction was 12.06% with a significant difference between them compared to control plants.

The interaction between varieties and spraying of Brassinolide was significant and the plants of the Indian and local variety sprayed with a concentration of 4 mg.L^{-1} were characterized by the highest mean interaction percentage of (12.88 and 11.76) % respectively compared to control plants. The interaction between biofertilizer and Brassinolide was also significant in the mean protein content in the plant. The sprayed plants at a concentration of 3 mg. L^{-1} of biofertilizer and 6 mg. L^{-1} of Biofertilizer were given highest mean interaction percentage of 12.83% compared to control plants. The increase in protein content in Alfalfa plants sprayed with the appropriate concentration of biofertilizer and Brassinolide (individually or in combination with both) is due to their superiority in the concentration of nitrogen in plants, which is the necessary element in the synthesis of amino acids, which are the cornerstone of protein formation (Havlin et al., 2005). As well as increasing the concentration of potassium in the plant as a result of the positive role of this element in activating enzymes that stimulate many physiological processes within the plant system, including enzymes associated with protein metabolism (Malvi, 2011). In addition to its active role in reducing nitrates and converting them into amino acids, which are the basic building block in the formation of proteins (Britto and Kronzucker, 2008). As for the triple interaction between the study factors, the results indicate a significant interaction between the study factors in the protein percentge of the plant (Table 7). The Indian variety sprayed plants with a concentration of 3 mg.L^{-1} of biofertilizer and 4 mg.L^{-1} of Brassinolide were excelled with the highest mean interaction ratio of 14.08 compared to control plants.

Table 7. Effect of variety, biofertilizer and Brassinolide hormone and their interactions on the percentage of protein in the vegetative part (%) of two Alfalfa varieties.

Varity (V)	Biofertilizer Concentrations (AG) (mg L^{-1})	Brassinolide (BL) concentrations (mg. L^{-1})				Effect of duel interaction mean AG Ч V
		0	2	4	6	
Indian	0	10.08	10.90	12.09	11.42	11.12
	3	12.09	12.82	14.08	13.52	13.13
	6	11.09	11.69	12.48	12.85	12.03
Local	0	8.76	9.84	10.90	10.22	9.93
	3	10.54	11.88	11.56	12.13	11.53
	6	11.11	11.95	12.81	12.39	12.06
LSD(0.05)		0.49				0.24
Duel interaction VЧBL						
Varity (V)		Brassinolide (BL) concentrations (mg. L^{-1})				Mean variety effect
		0	2	4	6	
Indian		11.09	11.80	12.88	12.60	12.09
Local		10.14	11.22	11.76	11.58	11.17
LSD(0.05)		0.28				0.14
Duel interaction BL Ч AG						
Biofertilizer (AG)		Brassinolide (BL) concentrations (mg. L^{-1})				Mean Biofertilizer effect
		0	2	4	6	
0		9.42	10.37	11.49	10.82	10.52
3		11.32	12.35	12.82	12.83	12.33
6		11.10	11.82	12.64	12.62	12.05
LSD(0.05)		0.34				0.17
Mean Brassinolide Effect		10.61	11.51	12.32	12.09	
LSD(0.05)		0.20				

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