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Effect of bio-fertilizer (AG) and brassinolide hormone on some growth parameters and total chlorophyll of two varieties of Medicago sativa L. plant

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Abstract

The experiment was carried out in the field belonging to 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 bio-fertilizer (AG 8-1-9) in three concentrations (0, 3, 6) mg.L^{-1} and brassinolide hormone in four concentrations (0, 2, 4, 6) mg. L^{-1} and its interactions in some growth parameters and total chlorophyll of two varieties of Medicago sativa plant. The experiment was designed according to Randomized Complete Blocks Design (R.C.B.D.) with three replicates per treatment. The averages were compared using the least significant difference at the probability level (0.05), and the results showed the following:
The effect of brassinolide in different concentrations led to a significant increase in all studied traits, as it exceeded the concentration of 4 mg.L^{-1} of brassinolide in both root length, dry weight, absolute growth rate, total chlorophyll content, nitrogen, phosphorus and potassium percentages. The effect of the bio-fertilizer in different concentrations led to a significant increase in all the studied traits at a concentration of 3 mg.L^{-1}. All duel interactions had a significant effect on all studied traits with the superiority of the treatment (3, 4) mg.L^{-1} in root length, dry weight, absolute growth rate, phosphorus percentage and leaf content of total chlorophyll, treatment of (3, 6) mg.L^{-1} in nitrogen and potassium ratios, and superiority of the Indian variety in all studied traits. Triple interactions led to a significant increase in all studied traits.

Keywords:

Bio-fertilizer, Brassinolide, Medicago sativa plant, growth parameters, total chlorophyll.

Alfalfa (Medicago sativa) plant is one of the oldest perennial leguminous fodder crops in the world, It stays in the soil for a period of 3-4 years and is given a crop of green fodder with protein and nutrients (Al-Taie, 2015). this plant consists of a root system with long roots, leaves, stems, flowers and seeds. It is a perennial flowering plant that is classified as a leguminous crop. The root system of the alfalfa plant is very deep, strong and has several lateral roots, which helps the plant penetrate the soil into deeper layers making it drought tolerant.

The leaves of the alfalfa plant are trifoliate. The middle layer has a short petiole, which is the main difference between alfalfa and clover, with sharply serrated leaflets on the upper third of the leaf edge. Alfalfa flowers may be yellow, blue, white or purple in color. The flowers are collected in a cluster or cluster in the form of inflorescences of the cluster type. Alfalfa is

pollinated by insects, in particular bees. It seeds have a kidney shape, yellowish-brown color and are lightweight (Patra and Paul, 2019). Bio-fertilizers are a modern fertilization technique that is added in several different ways, including spraying them on the plant or irrigation water, treating the seeds with it, 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 characteristics in addition to their role in the analysis of organic matter and inhibition of the activity of pathogens in the soil, and it also symbiosis with the root nodes of nitrogen fixation (Woodward, 2003). The indiscriminate use of mineral fertilizers leads to the deposition of pollutants and minerals and exposes the soil to more qualitative loss, and causes damage to living organisms and the possibility of water pollution, and has negative effects on the health of plant production if reliance on mineral fertilizers in the future, and to get rid of this problem, researchers in countries of the world are moving to modern technologies that are safer and more environmentally friendly that lead to reducing environmental pollution, such as the use of bio-fertilizers. It has a role in providing nutrients to the plant such as nitrogen, phosphorus and potassium, as well as organic acids, secreting some hormones and acids that act as regulators of plant growth, and secreting some antibiotics that participate in resisting some endemic diseases in the soil, and then benefit the plant and work to increase its production (Singh et al., 2020). It has a role in the analysis of organic matter and carbohydrates carrying negative charges, and then improves the characteristics of the soil and increases the stability of the aggregations, and plays a complementary role to add mineral fertilizers, as the role of mineral fertilizers emerges in the early stages of germination, then comes the role of bio-fertilizers, and causes a decrease in the emission of greenhouse gases and the development of low-carbon agriculture, as the manufacture of chemical nitrogen fertilizers produces an emission of nitrous oxide NO, which is one of the greenhouse gases, and reduces the effects stressful to low irrigation water (Fnca, 2014).

Brassinosteroids are steroid compounds with hormonal function have long been known in animals but their presence in plants has not been proven until 1970 Mitchell et al. (1970) was able to discover brassinosteroids by examining approximately 60 species of plant pollen, about half of which caused the growth of bean seedlings and was called growth-promoting substances from different pollen sources (Brassins). A number of researchers at the U.S. Agriculture ministry have been able to from the knowledge of the compound effective in stimulating plant growth within this group has been called Brassinolide (Rao et al., 2002).

Brassinosteroids are true phyto-steroid hormones with effective effect and very low concentrations, and their effect similar to animal steroid hormones has recently been observed, as eating watercress (Eruca sativa) from the Brassicaceae family has an effect on increasing the proportion of male hormones and increasing effectiveness (Premalatha et al., 2012; Hussein, 2013). The study aims to find out the effect of bio-fertilizer and the brassinolide hormone in improving growth parameters and total chlorophyll for two varieties of alfalfa plant.

Materials and Methods

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 bio-fertilizer and brassinolide hormone and their interactions in some growth parameters and total chlorophyll for two varieties of Medicago sativa plant (local and Indian), the experiment was designed according to R.C.B.D. (Randomized Complete Blocks Design) and three replicates for each treatment. Samples were taken from the soil of the field before cultivating for the purpose of estimating the chemical and physical properties as shown in Table (1) according to the methods described by Page et al. (1982) as the analysis was carried out in the central laboratory of the Biology Department, College of Science, University of Baghdad. Tillage, smoothing and leveling operations were conducted, after which the experimental land was divided into

three replicates, each replicate 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 lines is 50 cm, as one experimental unit contains four lines. Neutral compound fertilizer NPK 20:20:20 was added to all experimental units before cultivating, then seeds were planted on 2/11/2021. According to the experiment coefficients, a number of plants (30 plants) were determined for each experimental unit for the purpose of 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 bio-fertilizer, brassinolide hormone and their interactions in some growth parameters and total chlorophyll for two varieties of alfalfa plant, as the treatments were organized in the Randomized Complete Blocks Design. Some growth paremeters have been studied, including:

1. Root length (cm)

The root length of five random plants per experimental unit was measured from its point of contact with the stem to the end of the main root by means of the inserted ruler.

2. Dry weight of the root and vegetative groups (g. plant-1)

Five plants with their roots were extracted randomly from the center of each experimental unit by adding water to the experimental units the day before the plants were taken off, and the root system was separated from the vegetative and then dried in an electric dryer at a temperature of 50-55 °C until the weight of each treatment was established, and the dry weights were recorded for both the root and vegetative total.

3. Absolute growth rate (g. day-1)

The absolute growth rate is the one that expresses the efficiency of the biological activities of the plant over a certain period of time, it was calculated based on the dry weight of both the root and vegetative total and for both harvests by applying the following Hunt (1978) equation:

$$\text{Absolute Growth Rate} = \frac{W2 - W1}{T2 - T1}$$

Whereas :

W_1 Dry weight of the first root and vegetative total (g)

W_2 Dry weight of the second root and vegetative total (g)

T_1 Plant age (day) first.

T_2 Plant age (day) second.

4. Estimation of nitrogen percentage (N%) in the vegetative total

Milled dried specimens were digested according to the method of Gresser and Parson (1979). The percentage of nitrogen in the vegetative total of a plant was estimated by the Kjeldahl method (Jackson, 1958) and the following equation was applied to calculate the nitrogen concentration:

$$N\% = \frac{H_2SO_4 \text{ Volume Consumer} \times \text{Acid Standardization} \times 14 \times \text{Dilution Volume}}{\text{Sample volume taken at distillation} \times \text{Weight of digested sample} \times 1000}$$

5. Estimation of phosphorus percentage (P%) in the vegetative total

Phosphorus was estimated using ammonium molybdate method and according to Olsen and Sommers (1982) by UV-visible spectrophotometer with wavelength 420 nm.

6. Estimation of potassium percentage (K%) in the vegetative total

The concentration of potassium in the vegetative system of the digested sample was estimated by the Atomic Absorption Spectrophotometer of the manufacturer Perkin Elmer Model 5000 according to the method used before by Chapman and Pratt (1961).

7. Determination of total chlorophyll content in leaves

The total chlorophyll content in the leaves was estimated by the Goodwin method (1976).

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

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

Characteristics		Value	Unite
Chemical and fertility properties			
pH		7.50	
$EC_{1:1}$		2.3	$dS.m^{-1}$
Organic matter		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	Sulphate	5.22	
	Bicarbonates	1.3	
	Chlorides	4.3	
Physical properties			
Soil separators	Clay	216	g. kg^{-1}
	Silt	420	
	Sand	364	
Texture			Loam

Results and Discussion

Root length (cm)

The results of Table 2 showed a significant difference between the two varieties of alfalfa in the characteristic of the root length of a plant. The plants of the Indian variety outperformed the best average root length of 23.29 cm compared to the plants of the local variety. The difference between the two varieties of alfalfa in the characteristic of root length may be due to their genetic differences and the response of each to the study treatments. It is also noted from the results of Table 2 the significant difference between the concentrations of bio-fertilizer AG (0, 3 and 6) mg. L^{-1} in the average root length of the alfalfa plant, where sprayed plants with a concentration of 3 mg.L^{-1} recorded the best average trait length of 22.34 cm compared to control plants. Alsalim (2019) noted that the use of biofertilizer led to an increase in the root length of the Vicia faba plant. The superiority of sprayed plants at the concentration is 3 mg. L^{-1} in the root length characteristic of the plant may be due to the role of the bio-fertilizer in the formation of a dense root system, and this leads to a significant increase in the root length of the plant (Hartman, 2000a). It may also be due to the fact that the use of bio-fertilizer leads to an increase in the length of the root of the plant (Guleria et al., 2014; Khan et al., 2015) or to the role of the bio-fertilizer in increasing the growth of the root system of the plant and thus leads to an increase in the root length of the plant (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 average root length of the plant, the sprayed plants were characterized by a concentration of 4 mg. L^{-1} with the highest average trait length of 22.57 cm compared to control plants, and that this increase in root length is due to the role of brassinolide in increasing root growth (Sasse, 1994).

As for the bilateral interactions, the results of the same table show a significant interaction between the varieties and the bio-fertilizer in the average root length of the plant, as the Indian and local variety plants sprayed with a concentration of 3 mg.L^{-1} was recorded best average interaction was 24.68 and 20.00 cm with a significant difference between them respectively compared to control plants. The interaction between varieties and the spray of the growth regulator brassinolide was significant and the plants of the Indian variety sprayed with a concentration of 4 mg.L^{-1} and the local variety with a concentration of 2 mg.L^{-1} were distinguished by the best average interference of (25.25 and 19.93) cm respectively compared to control plants. The interaction between bio-fertilizer and brassinolide was also significant in the average root length of the plant, as the sprayed plants were given a concentration of 3 mg.L^{-1} of bio-fertilizer and 4 mg. L^{-1} of brassinolide best average interaction of 23.34 cm compared to control plants. While the triple interaction between the study factors, the results indicate a significant overlap between the studies factors in the root length trait (Table 2), the Indian variety plants sprayed with a concentration of 3 mg.L^{-1} were exceeded of bio-fertilizer and 4 mg.L^{-1} of brassinolide with the highest average interaction of 26.19 cm compared to control plants.

Table 2: Effect of variety, bio-fertilizer and brassinolide and their interactions on root length (cm) of two varieties of alfalfa plant.

Verities (V)	Bio-fertilizer concentrations (AG) (mg.L-1)	Brassinolide concentration (BL) (mg.L-1)				Effect of mean bilateral interaction AG ×V
		0	2	4	6	
Indian	0	15.74	21.10	24.64	23.69	21.29
	3	22.72	25.09	26.19	24.71	24.68
	6	23.31	24.73	24.91	22.58	23.88
Local	0	13.48	18.30	19.46	18.98	17.56
	3	18.85	21.22	20.49	19.44	20.00
	6	17.95	20.26	19.70	17.92	18.96
LSD(0.05)		1.01				0.51
Bilateral interaction BL × V						
Verities (V)		Brassinolide concentration (BL) ($mg.L^{-1}$)				Effect of mean variety
		0	2	4	6	
Indian		20.59	23.64	25.25	23.66	23.29
Local		16.76	19.93	19.88	18.78	18.84
LSD(0.05)		0.58				0.29
Bilateral interaction BL × AG						
Bio-fertilizer (AG)		Brassinolide concentration (BL) ($mg.L^{-1}$)				Effect of mean bio-fertilizer
		0	2	4	6	
0		14.61	19.70	22.05	21.34	19.43
3		20.79	23.15	23.34	22.08	22.34
6		20.63	22.50	22.31	20.25	21.42
LSD(0.05)		0.72				0.36
Effect of mean brassinolide		18.67	21.78	22.57	21.22	
LSD(0.05)		0.41				

Dry weight of plant (g. plant-1)

The results of Table 3 indicated that there is a significant difference between the two varieties of alfalfa plant in the average dry weight of the plant, the plants of the Indian variety outperformed the highest average dry weight of 26.34 g $plant^{-1}$ compared to the plants of the local variety, that the difference between the two varieties of alfalfa plant in dry weight may be due to the variation of genetic material for both varieties. It is also reported from the results of Table 3 the significant difference between the concentrations of biofertilizer AG (0, 3 and 6) mg. L^{-1} in the dry weight of the alfalfa plant, as the sprayed plants contained a concentration of 3 mg. L^{-1} is the highest average dry weight average of 25.94 g $plant^{-1}$ compared to control plants. These results are consistent with Al-Qasy and Al-Shammari (2022a) who were recorded that the use of bio-fertilizer led to a significant increase in the dry weight of the fenugreek plant.

Mahmood et al. (2022) also satated that the use of bio-fertilizer led to a significant increase in the dry weight of the plant. The reason for the superiority in dry weight may be due to the bio-fertilizer content of Bacillus bacteria, which have 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). This effect is thus reflected in the increased growth of the root system and its increased absorption of nutrients and water, which contributed to the increase in plant growth, through an increase in the dry weight of the plant. This may also be belonged to the increase in the amount of nitrogen, phosphorus and potassium supplied through the effectiveness of the bio-fertilizer, as it converts these elements into amino acids and compounds that the plant uses in the formation of tissues, and then the formation of vegetative plant growth for the alfalfa plant. It is also consistent with the results Rugheim et al. (2014) in fenugreek plant. The reason for the superiority in dry weight is also due to the role of the bio-fertilizer in increasing vegetative growth, as the bio-fertilizer works to increase and stimulate vegetative growth characteristics as a result of its secretion of chelating compounds (iron carriers, which bind and transport iron in microorganisms) Siderophores (Adesemoye and Kloepper, 2009).

The bio-fertilizer works to stimulate plant growth through the secretion of plant growth regulators, which work in accordance with other mechanisms, including

increasing the availability of nutrients and then absorbing them by the plant and this is reflected positively in increasing plant growth and soft weight (Harman, 2000) and thus increasing the dry weight of the plant. 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 dry weight of the plant, the sprayed plants were characterized by a concentration of 4 mg.L^{-1} with the highest average trait of 26.77 g. $plant^{-1}$ compared to control plants, Nasralla et al. (2015) noted that the use of brassinolide led to significant differences in the dry weight of callus of buckwheat. the increase in average dry weight due to the addition of brassinolide may be due to increased photosynthesis efficiency, which leads to an increase in the net CO_2 represented in the leaf, which is the basic unit of carbohydrate synthesis (Mahgoub et al., 2006). As for bilateral interactions, the results of the same table show a significant interaction between the varieties and the bio-fertilizer in the average dry weight, where the Indian and local variety plants sprayed with a concentration of 3 mg.L^{-1} were recorded best average interaction were 28.43 and 23.44 g.$plant^{-1}$ and a significant difference between them respectively 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 were characterized by a concentration of 4 mg. L^{-1} with the highest average interaction of (29.43 and 24.11) g.$plant^{-1}$ respectively compared to control plants. The interaction between bio-fertilizer and brassinolide was also significant in the average dry weight of the plant; the sprayed plants were given a concentration of 3 mg. L^{-1} of bio-enriched and 4 mg. L^{-1} of brassinolide highest average interaction of 28.62 g.$plant^{-1}$ compared to control plants. While the results of triple interaction between the study factors indicate a significant interaction between the study factors in the dry weight of the plant (Table 3), the Indian variety plants sprayed with a concentration of 3 mg.L^{-1} excelled of bio-fertilizer and 4 mg.L-1 of brassinolide with the highest average interaction ratio of 31.50 g.$plant^{-1}$ compared to control plants.

Table 3: Effect of variety, bio-fertilizer, brassinolide and their interactions on plant dry weight g. plant-1 of two alfalfa plant varieties.

Verities (V)	Bio-fertilizer concentrations (AG) (mg.L-1)	Brassinolide concentration (BL) (mg.L-1)				Effect of mean bilateral interaction AG ×V
		0	2	4	6	
Indian	0	16.91	23.39	28.28	26.53	23.78
	3	25.27	28.99	31.50	27.97	28.43
	6	26.87	27.02	28.50	24.88	26.82
Local	0	12.59	22.03	21.46	19.55	18.91
	3	20.20	24.92	25.75	22.89	23.44
	6	22.93	24.19	25.13	18.92	22.79
LSD(0.05)		1.18				0.59
Bilateral interaction BL × V						
Verities (V)		Brassinolide concentration (BL) (mg.L^{-1})				Effect of mean variety
		0	2	4	6	
Indian		23.02	26.47	29.43	26.46	26.34
Local		18.57	23.72	24.11	20.45	21.71
LSD(0.05)		0.68				0.34
Bilateral interaction BL × AG						
Bio-fertilizer (AG)		Brassinolide concentration (BL) (mg.L^{-1})				Effect of mean bio-fertilizer
		0	2	4	6	
0		14.75	22.71	24.87	23.04	21.34
3		22.73	26.96	28.62	25.43	25.94
6		24.90	25.61	26.82	21.90	24.81
LSD(0.05)		0.83				0.42
Effect of mean brassinolide		20.79	25.09	26.77	23.46	
LSD(0.05)		0.48				

Absolute growth rate (g.plant-1.day-1)

The results of Table 4 indicated a significant difference between the two categories of alfalfa in the absolute growth rate of a plant, the plants of the Indian variety outperformed the highest average in the absolute growth rate of 0.1722 g.$plant^{-1}$.day^{-1} and an increase of 8.09% compared to the plants of the local variety. It is also recorded from the results

of Table 4 the significant difference between the concentrations of bio-fertilizer AG (0, 3 and 6) mg. L^{-1} in the absolute growth rate of a plant. The sprayed plants contained a concentration of 3 mg. L^{-1} the highest average absolute growth rate of 0.1695 g.plant^{-1}.day^{-1} with an increase of 14.51% compared to control plants, the increase in the absolute growth rate as a result of the addition of bio-fertilizer is due to its positive role in increasing dry weight as discussed pervious (Table 3) and thus increasing the absolute growth rate of the plant. As for the effect of spraying the growth regulator brassinolide (BL), the results of Table 4 showed significant differences between the sprayed concentrations in the absolute growth rate of the plant, the sprayed plants were characterized by a concentration of 4 mg.L^{-1} with the best trait rate of 0.1750 g.plant^{-1}.day^{-1} and an increase of 54.83% compared to control plants. Al-Shammry and Al-Halfi (2017) observed that spraying the hormone brassinolide led to a significant increase in the absolute growth rate of the coriander plant. This increase in the absolute growth rate is a result of the dry weight increase as discussed pervious (Table 3) and this is positively reflected in the increase in the absolute growth rate. As for bilateral interactions, the results of the same table show a significant interaction between the varieties and the bio-fertilizer in the average absolute growth rate. The Indian and local variety plants sprayed with a concentration of 3 mg. L^{-1} were recorded best average interference was 0.1858 and 0.1532 g plant^{-1}.day^{-1} with significant differences respectively compared to control plants. The interaction between varieties and spraying of the growth regulator was significant and the sprayed Indian and local variety plants were characterized by a concentration of 4 mg. L^{-1} with the highest mean interaction of (0.1923 and 0.1576) g.plant^{-1}.day^{-1} respectively compared to control plants. The interaction of bio-fertilizer and brassinolide was also significant in the average absolute growth rate of a plant, the sprayed plants were given a concentration of 3 mg. L^{-1} of bio-fertilizer and 4 mg. L^{-1} of brassinolide highest average interaction was 0.1871 g.plant^{-1}.day^{-1} 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 absolute growth rate (Table 4), the Indian variety plants sprayed with a concentration of 3 mg. L^{-1} excelled of bio-fertilizer and 4 mg.L^{-1} of brassinolide with the highest average interaction of 0.2059 g.plant^{-1}.day^{-1} compared to control plants.

Table 4: Effect of variety, bio-fertilizer and brassinolide hormone and their interactions on the absolute growth rate g-1.plant-1. day-1 of two alfalfa plant varieties.

Verities (V)	Bio-fertilizer concentrations (AG) (mg.L-1)	Brassinolide concentration (BL) (mg.L-1)				Effect of mean bilateral interaction AG ×V
		0	2	4	6	
Indian	0	0.1105	0.1529	0.1849	0.1734	0.1554
	3	0.1651	0.1895	0.2059	0.1828	0.1858
	6	0.1756	0.1766	0.1863	0.1626	0.1753
Local	0	0.0823	0.1440	0.1403	0.1278	0.1236
	3	0.1320	0.1629	0.1683	0.1496	0.1532
	6	0.1499	0.1581	0.1643	0.1236	0.1490
LSD(0.05)		0.0077				0.0039
Bilateral interaction BL × V						
Verities (V)		Brassinolide concentration (BL) (mg.L^{-1})				Effect of mean variety
		0	2	4	6	
Indian		0.1504	0.1730	0.1923	0.1729	0.1722
Local		0.1214	0.1550	0.1576	0.1337	0.1419
LSD(0.05)		0.0045				0.0022
Bilateral interaction BL × AG						
Bio-fertilizer (AG)		Brassinolide concentration (BL) (mg.L^{-1})				Effect of mean bio-fertilizer
		0	2	4	6	
0		0.0964	0.1484	0.1626	0.1506	0.1395
3		0.1486	0.1762	0.1871	0.1662	0.1695
6		0.1628	0.1674	0.1753	0.1431	0.1621
LSD(0.05)		0.0054				0.0027
Effect of mean brassinolide		0.1359	0.1640	0.1750	0.1533	
LSD(0.05)		0.0031				

Nitrogen percentage (%) in the vegetative total of the plant

The results of Table 5 indicated that there is a significant difference between the two types of alfalfa plant in the average percentage of nitrogen in the plant, as the plants of the Indian variety outperformed the highest average percentage of nitrogen in the plant was 1.935% compared to the plants of the local variety. It is also noted from the results of Table 5 the significant difference between the concentrations of bio-fertilizer AG (0, 3 and 6) mg. L^{-1} in the average nitrogen content of the alfalfa plant, as the sprayed plants contained a concentration of 3 mg. L^{-1} has the highest average nitrogen content of 1.973% compared to control plants, as reported by Mahmood et al. (2022) that the use of bio-fertilizer led to a significant increase in the percentage of nitrogen in the plant. Allawi and Drefel (2016) also registered that the use of bio-fertilizer led to a significant increase in the percentage of nitrogen in the plant. These results are consistent with AL-Qasy and Al-Shammari (2022b), who noted that the use of biofertilizers led to a significant increase in the protein percentage of fenugreek plants, and this increase in protein percentage is due to the superiority in the nitrogen percentage of the plant. The superiority of sprayed plants in the concentration is 3 mg. L^{-1} may be due to the role of the bio-fertilizer in increasing root growth as discussed pervious (Table 2), which works to increase the plant's ability to absorb nutrients, including nitrogen, it may also be due to the role of the bio-fertilizer in providing nutrients to the plant, including nitrogen (Singh et al., 2020). It may also be attributed to the role of bio-fertilizer in stimulating plant growth by secreting enzymes and growth regulators that help increase the growth of the root system and this leads to an increase in the surface area for absorption of nutrients, including nitrogen and other nutrients (Yadegari, 2008). 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 average percentage of nitrogen in the plant, the sprayed plants were characterized by a concentration of 4 mg. L^{-1} with the highest average trait ratio of 1.971% compared to control plants. Al-Halfi and Al-Shammry (2017) showed that spraying the hormone brassinolide led to a significant increase in the nitrogen ratio of the coriander plant. Plants sprayed with a concentration of more than 4 mg. L^{-1} is due to the role of brassinolide in increasing root growth as discussed pervious (Table 2), which increases the plant's ability to absorb nutrients, including nitrogen. The action of brassinolide is positively reflected in increasing the concentration of nitrogen in the vegetative total (Bera et al., 2008). Verma et al. (2009) reported that a number of growth regulators, including brassinolide, affect the physiological characteristics of crops such as stimulating the absorption of mineral ions, in addition to that brassinolide helps in increasing the absorption of mineral elements from the soil, and this leads to an increase in the nitrogen content of leaves in plants sprayed with brassinolide, which can be due to the high absorption of inorganic nitrogen such as nitrates from the soil and its metabolism (El-Khallal et al., 2009). It may also be due to the role of the hormone brassinolide is one of the steroids that have the ability to retain water as it has the ability to bind with polyamines and lead to an increase in the osmosis of cells in favor of the entry of water and nutrients, as well as that the glucobrasscin compound from the metabolism of brassinosteroids and this compound metabolizes auxin, which increases the growth of the root system and then increases the absorption of nutrients, including nitrogen (Hayat and Ahmad, 2011; Ross and Quittenden, 2016).

As for bilateral interactions, the results of the same table show a significant interaction between the varieties and the bio-fertilizer in the average percentage of nitrogen in the plant, as the Indian and local variety plants sprayed with concentrations of 3 and 6 mg.L^{-1} were achieved the highest average interference ratio of (2.101 and 1.930)% with a significant difference between them sequentially 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 were characterized by a concentration of 4 mg. L^{-1} with the highest average interaction ratio of (2.061 and 1.881) % respectively. The interaction between bio-fertilizer and brassinolide was also significant in the average percentage of nitrogen in the plant, the sprayed plants gave the concentration of 3 mg. L^{-1} of bio-enriched and 6 mg. L^{-1} of brassinolide has the highest average interaction ratio of 2.053% 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 percentage of nitrogen in the plant (Table 5), the plants of the Indian variety sprayed with a concentration of 3 mg. L^{-1} outweighed of bio-fertilizer and 4 mg.L^{-1} of brassinolide with the highest average interaction of 2.252% compared to control plants.

Table 5: Effect of variety, bio-fertilizer, brassinolide and their interactions on the percentage of nitrogen in the vegetative total (%) of two alfalfa plant varieties.

Verities (V)	Bio-fertilizer concentrations (AG) (mg.L-1)	Brassinolide concentration (BL) (mg.L-1)				Effect of mean bilateral interaction AG ×V
		0	2	4	6	
Indian	0	1.612	1.744	1.934	1.827	1.779
	3	1.935	2.052	2.252	2.164	2.101
	6	1.775	1.870	1.996	2.056	1.924
Local	0	1.402	1.574	1.743	1.634	1.588
	3	1.686	1.900	1.850	1.941	1.844
	6	1.778	1.912	2.049	1.982	1.930
LSD(0.05)		0.078				0.039
Bilateral interaction BL × V						
Verities (V)		Brassinolide concentration (BL) ($mg.L^{-1}$)				Effect of mean variety
		0	2	4	6	
Indian		1.774	1.889	2.061	2.015	1.935
Local		1.622	1.795	1.881	1.852	1.788
LSD(0.05)		0.045				0.023
Bilateral interaction BL × AG						
Bio-fertilizer (AG)		Brassinolide concentration (BL) ($mg.L^{-1}$)				Effect of mean bio-fertilizer
		0	2	4	6	
0		1.507	1.659	1.839	1.731	1.684
3		1.811	1.976	2.051	2.053	1.973
6		1.776	1.891	2.023	2.019	1.927
LSD(0.05)		0.055				0.028
Effect of mean brassinolide		1.698	1.842	1.971	1.934	
LSD(0.05)		0.032				

Phosphorus percentage (%) in the vegetative total of the plant

The results of Table 6 indicated a significant difference between the two varieties of alfalfa plant in the average percentage of phosphorus in the plant, the plants of the Indian variety outperformed the highest average percentage of phosphorus in the plant amounting to 0.198% compared to the plants of the local variety. It is also stated from the results of Table

6 the significant difference between the concentrations of bio-fertilizer AG (0, 3 and 6) mg. L^{-1} in the average phosphorus content in the alfalfa plant, as the sprayed plants contained a concentration of 3 mg. L^{-1} highest average phosphorus content of 0.194% compared to control plants. Mahmood et al. (2022) also reported that the use of bio-fertilizer led to a significant increase in the percentage of phosphorus in the plant. Allawi and Drefel (2016) also reported that the use of bio-fertilizer led to a significant increase in the percentage of phosphorus in plants. The superiority of sprayed plants at the concentration 3 mg. L^{-1} may be due to the role of the bio-fertilizer in increasing root growth as discussed pervious (Table 2), which increases the plant's ability to absorb nutrients, including phosphorus, and may also be due to the role of the bio-fertilizer in providing nutrients to the plant, including phosphorus (Singh et al., 2020). It is also due to the role of the bio-fertilizer, which contains Bacillus bacteria, which have a high ability to dissolve phosphorus, as they work to secrete organic acids that affect the lowering of soil pH, which increases the readiness of phosphorus for the plant (Sharma et al., 2012). These bacteria free phosphate ions from insoluble forms of phosphorus and convert them into ions dissolved in soil solution, in addition to increasing the availability of some other secondary elements in the soil (Adesemoye and Kloppr, 2009). It may also be belonged to the role of bio-fertilizer in stimulating plant growth through the secretion of enzymes and growth regulators that help increase the growth of the root system and lead to an increase in the surface area for absorption of nutrients, including phosphorus and other nutrients (Yadegari, 2008).

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 average phosphorus percentage in the plant, the sprayed plants were characterized by a concentration of 4 mg. L^{-1} with the highest average trait ratio of 0.198% compared to control plants. The plants sprayed with concentration 4 mg. L^{-1} is exceed due to the role of brassinolide in increasing root growth as discussed previously (Table 2), which works to increase the plant's ability to absorb nutrients, including phosphorus. The action of brassinolide is positively reflected in the increase in the concentration of phosphorus in the vegetative total (Bera et al., 2008). Verma et al. (2009) reported that a number of growth regulators, including brassinolide, affect the physiological characteristics of crops such as stimulating the absorption of mineral ions, in addition to that brassinolide helps increase the absorption of mineral elements from the soil, and this leads to an increase in the rates of phosphorus in plants sprayed with brassinolide. It may also be belonged to the role of the brassinolide hormone is one of the steroids that have the ability to retain water as it has the ability to bind with polyamines and lead to an increase in the osmosis of cells in favor of the entry of water and nutrients, as well as that the glucobrasscin compound from the metabolism of brassinosteroids and this compound metabolizes auxin, which increases the growth of the root system and then increases the absorption of nutrients, including phosphorus (Hayat and Ahmad, 2011; Ross and Quittenden, 2016).

As for bilateral interactions, the results of the same table show a significant interaction between varieties and bio-fertilizer in the average phosphorus percentage in plants, as the Indian and local variety plants sprayed with concentrations of 3 and 6 mg.L^{-1} were achieved the highest average interaction ratio were (0.219 and 0.182)% with a significant difference between them respectively compared to control plants. The interaction between varieties and spraying of the growth regulator was significant and the sprayed Indian and local variety plants were characterized by a concentration of 4 mg. L^{-1} with the highest average interaction ratio of (0.215 and 0.180) % respectively. The interaction between bio-fertilizer and brassinolide was also significant in the average phosphorus content in the plant, the sprayed plants were given a concentration of 3 mg. L^{-1} of bio-enriched and 4 mg. L^{-1} of brassinolide highest average interference ratio of 0.214% compared to control plants.

While the results of triple interaction between the study factors indicate a significant interaction between the study factors in the phosphorus content in the plant (Table 6). The Indian variety sprayed plants outperformed at a concentration 3 mg. L^{-1} of bio-fertilizer and 4 mg.L^{-1} of brassinolide with the highest average interaction of 0.245% compared to control plants.

Table 6: Effect of Variety, bio-fertilizer and brassinolide and their interactions on the percentage of phosphorus in the vegetative total (%) of two alfalfa plant varieties

Verities (V)	Bio-fertilizer concentrations (AG) (mg.L-1)	Brassinolide concentration (BL) (mg.L-1)				Effect of mean bilateral interaction AG ×V
		0	2	4	6	
Indian	0	0.151	0.173	0.199	0.194	0.179
	3	0.188	0.231	0.245	0.211	0.219
	6	0.197	0.209	0.201	0.176	0.196
Local	0	0.131	0.163	0.168	0.149	0.153
	3	0.154	0.170	0.182	0.166	0.168
	6	0.167	0.197	0.189	0.175	0.182
LSD(0.05)		0.0066				0.0033
Bilateral interaction BL × V						
Verities (V)		Brassinolide concentration (BL) ($mg.L^{-1}$)				Effect of mean variety
		0	2	4	6	
Indian		0.179	0.204	0.215	0.194	0.198
Local		0.151	0.177	0.180	0.163	0.168
LSD(0.05)		0.0038				0.0019
Bilateral interaction BL × AG						
Bio-fertilizer (AG)		Brassinolide concentration (BL) ($mg.L^{-1}$)				Effect of mean bio-fertilizer
		0	2	4	6	
0		0.141	0.168	0.184	0.172	0.166
3		0.171	0.201	0.214	0.188	0.194
6		0.182	0.203	0.195	0.176	0.189
LSD(0.05)		0.0047				0.0023
Effect of mean brassinolide		0.165	0.191	0.198	0.178	
LSD(0.05)		0.0027				

Percentage of potassium (%) in the vegetative total of the plant

The results of Table 7 indicated that there is a significant difference between the two varieties of alfalfa plant in the average percentage of potassium in the plant, the plants of the Indian variety outperformed the highest average percentage of potassium in the plant was 1.105% compared to the plants of the local variety.

It is also noted from the results of Table 7 the significant difference between the concentrations of bio-fertilizer AG (0, 3 and 6) mg. L^{-1} in the average potassium content in the alfalfa plant, as the sprayed plants contained a concentration of 3 mg. L^{-1} highest average potassium content of 1.121% compared to control plants. Mahmood et al. (2022) also showed that the use of bio-fertilizer led to a significant increase in the percentage of potassium in the plant. Allawi and Drefel (2016) also stated that the use of bio-fertilizer led to a significant increase in the percentage of potassium in the plant.

The superiority of sprayed plants in the concentration is 3 mg. L^{-1} may be due to the role of the bio-fertilizer in increasing root growth as discussed previously (Table 2), which works to increase the plant's ability to absorb nutrients, including potassium, and may also be due to the role of the bio-fertilizer in

providing nutrients to the plant, including potassium (Singh et al., 2020). An increase in the percentage of potassium in the plant may also be due to the microorganisms contained in the bio-fertilizer such as Bacillus bacteria that release potassium from various minerals, as these bacteria release potassium in the soil and make it more ready for the plant, and this is reflected positively on plant growth (Biswas and Basak, 2014). In addition, potassium-releasing bacteria play a role in increasing potassium content accompanied by an increase in phosphorus content and thus an increase in plant biomass compared to control factors (Zhang et al., 2004). It may also be belonged to the role of bio-fertilizer in stimulating plant growth through the secretion of enzymes and growth regulators that help increase the growth of the root system and lead to an increase in the surface area for absorption of nutrients, including phosphorus and other nutrients (Yadegari, 2008). As for the effect of spraying the growth regulator brassinolide (BL), the results of Table 7 show significant differences between the sprayed concentrations in the average percentage of potassium in the plant, the sprayed plants were characterized by a concentration of 4 mg. L^{-1} with the highest average trait ratio of 1.117% compared to control plants. Al-Halfi and Al-Shammry (2017) indicated that spraying the hormone brassinolide led to a significant increase in the potassium content of the coriander plant. Plants sprayed with a concentration of more than 4 mg. L-1 is due to the role of brassinolide in increasing root growth as discussed previously (Table 2), which works to increase the plant's ability to absorb nutrients, including potassium.

The action of brassinolide is positively reflected in an increase in potassium concentration in the vegetative total (Bera et al., 2008). Verma et al. (2009) reported that a number of growth regulators, including brassinolide, affect the physiological characteristics of crops such as stimulating the absorption of mineral ions, in addition to that brassinolide helps increase the absorption of mineral elements from the soil, and this leads to an increase in the percentage of potassium in plants sprayed with brassinolide. It may also be due to the role of the brassinolide hormone is one of the steroids that have the ability to retain water as it has the ability to bind with polyamines and lead to an increase in the osmosis of cells in favor of the entry of water and nutrients, as well as that the glucobrasscin compound from the metabolism of brassinosteroids and this compound metabolizes auxin, which increases the growth of the root system and then increases the absorption of nutrients, including potassium (Hayat and Ahmad,2011; Ross and Quittenden, 2016). As for bilateral interactions, the results of the same table show a significant interaction between varieties and bio-fertilizer in the average percentage of potassium in the plant, as the Indian and local variety plants sprayed with concentrations of 3 and 6 mg.L^{-1} were achieved the highest average interaction ratio of (1.208 and 1.062)% with a significant difference between them sequentially compared to control plants. The interaction between varieties and spraying of the growth regulator was significant and the sprayed Indian and local variety plants were characterized by a concentration of 4 mg. L^{-1} with the highest average interaction ratio of (1.185 and 1.049) % respectively. The interaction between bio-fertilizer and brassinolide was also significant in the average potassium content of the plant, the sprayed plants gave the concentration of 3 mg. L^{-1} of bio-fertilizer and 6 mg. L^{-1} of brassinolide highest average interaction ratio of 1.180% 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 percentage of potassium in the plant (Table 7), the plants of the Indian variety sprayed with a concentration 3 mg.L^{-1} outweighed of bio-fertilizer and 4 mg.L^{-1} of

brassinolide with the highest average interaction of 1.298% compared to control plants.

Table 7 Effect of variety, bio-fertilizer and brassinolide hormone and their interactions on the percentage of potassium in the vegetative total (%) of two varieties of alfalfa plant.

Verities (V)	Bio-fertilizer concentrations (AG) (mg.L-1)	Brassinolide concentration (BL) (mg.L-1)				Effect of mean bilateral interaction AG ×V
		0	2	4	6	
Indian	0	0.874	0.966	1.112	1.052	1.001
	3	1.113	1.177	1.298	1.244	1.208
	6	1.023	1.077	1.146	1.179	1.107
Local	0	0.717	0.872	0.966	0.906	0.865
	3	0.935	1.036	1.053	1.116	1.035
	6	0.985	1.059	1.128	1.076	1.062
LSD(0.05)		0.047				0.023
Bilateral interaction BL × V						
Verities (V)		Brassinolide concentration (BL) ($mg.L^{-1}$)				Effect of mean variety
		0	2	4	6	
Indian		1.003	1.074	1.185	1.159	1.105
Local		0.879	0.989	1.049	1.032	0.987
LSD(0.05)		0.027				0.014
Bilateral interaction BL × AG						
Bio-fertilizer (AG)		Brassinolide concentration (BL) ($mg.L^{-1}$)				Effect of mean bio-fertilizer
		0	2	4	6	
0		0.795	0.919	1.039	0.979	0.933
3		1.024	1.107	1.176	1.180	1.121
6		1.004	1.068	1.137	1.128	1.084
LSD(0.05)		0.033				0.017
Effect of mean brassinolide		0.941	1.031	1.117	1.095	
LSD(0.05)		0.019				

Total chlorophyll content of the leaves (mg.gm-1 wet weight)

The results of Table 8 indicated a significant difference between the two varieties of alfalfa plant in the average total chlorophyll content of the leaves, the plants of the Indian variety outperformed the highest average total chlorophyll leaf content of 1.502 mg. g^{-1} Wet weight compared to plants of the local variety. The difference in the two varieties of alfalfa plant in the total chlorophyll content may be due to the variation of the genetic material of both varieties. It is also noted from the results of Table 8 the significant difference between the concentrations of bio-fertilizer AG (0, 3 and 6) mg. L^{-1} in the total chlorophyll content of the leaves in the alfalfa plant, as the sprayed plants contained a concentration of 3 mg. L^{-1} had the highest average total chlorophyll content of 1.511 mg. g^{-1} wet weight compared to control plants. These results are consistent with Al-Qasy and Al-Shammari (2022a) who they showed that the use of bio-fertilizer led to a significant increase in the leaf content of total chlorophyll of the fenugreek plant. Al-Dulaimi and Al-Rawi (2020) also confirmed that the use of bio-fertilizer led to a significant increase in the leaf content of chlorophyll. This increase in the total chlorophyll content of the leaves may be due to the role of the bio-fertilizer containing bacteria through 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 in the growth of the root system and increase its absorption of nutrients and water, which contributed to increasing plant growth, and therefore increasing the relative density of chlorophyll pigment and this leads to an increase in the total chlorophyll content as well as the role of the enriched in increasing the absorption of nutrients through increasing root growth (Table 2), increasing the availability of these elements and other elements absorbed by the plant and involved in the composition of porphyrin rings involved in the biosynthesis of chlorophylls, the most important of which are magnesium has thus had an effect on this increase. These results are consistent with Husain et al. (2021) in fenugreek plants. It may be due to the role of a bio-fertilizer that leads to increased nutrient availability, photosynthesis activity and total chlorophyll formation, as well as nitrogen in the plant which ultimately leads to improved plant elevation in fenugreek plant (Raiyani et al., 2018). The absorption of nitrogen and magnesium leads to an increase in the total chlorophyll content in the leaves of the plant, which leads to an increase in metabolic processes and biosynthesis, and these results are consistent with Jha and Trivedi (2021) in the cowpea plant. As for the effect of spraying the growth regulator brassinolide (BL), the results of table 8 show significant differences between the sprayed concentrations in the average leaf content of total chlorophyll, the sprayed plants were characterized by a concentration of 4 mg. L^{-1} with the highest average total chlorophyll content of 1.568 mg.g^{-1} wet weight relative to control plants. Sura and Al-Hilfy (2022) observed that the use of the hormone brassinolide led to a significant increase in the total chlorophyll content of the leaves. Al-Shimary and Al-Jboury (2017) also reported that the use of brassinolide led to a significant increase in the total chlorophyll content of the leaves of dill. Al-Halfi and Al-Shammry (2017) also noted that spraying the hormone brassinolide led to a significant increase in the leaf content of the total chlorophyll of the coriander plant. Al-Saedi, and Al-Mentafji (2016) also noted that the treatment of coriander plant with the hormone Brassinolide led to a significant increase in the leaf content of the total chlorophyll of the coriander plant. The increase in the total chlorophyll content of the leaves of two varieties of alfalfa plant as a result of the addition of brassinolide may be due to the role of brassinolide in inhibiting the enzyme chlorophyllase responsible for the depletion of chlorophyll, which led to the accumulation of chlorophyll in the leaves (Fariduddin et al., 2003). This finding is consistent with Kandil et al. (2007) found. Brassinosteroids increase the chlorophyll content in the leaves (Swamy and Rao, 2009). The spray of brassinolide has increased the total chlorophyll content of the plant and it is believed that the hormone has a role in stimulating the metabolism of the enzyme NADH-protochlorophyllid reductase and then increasing the content of chlorophyll, it is believed that brassinolide has an important role in increasing the effectiveness of the enzyme Rubisco by increasing the ability of its fixation in plastids by increasing the concentration of thiamine, which enters the synthesis of the enzyme acetyl Co-A, and brassinolide is an anti-accumulation effect of abscisic acid and reduce plant aging and leaf decomposition, and the hormone also works on protection of plastids from the influence of free oxidative radicals (Sharma, 2011; Renu et al., 2014). As for bilateral interactions, the results of the same table show a significant interaction between the varieties and the bio-fertilizer in the average total chlorophyll content, as the Indian and local variety plants sprayed with a concentration of 3 mg.L^{-1} were given the highest average interaction of (1.610 and 1.413) mg. g^{-1} wet weight and has a significant difference between them respectively. The interaction between varieties and spraying of the growth regulator was significant and the sprayed Indian and local variety plants were characterized by a concentration of 4 mg. L^{-1} with the highest mean interaction was 1.684 and 1.452 mg.g^{-1} wet weight respectively compared to control plants. The interaction between bio-fertilizer and brassinolide was significant in the average total chlorophyll content of the leaves, as the sprayed plants were given a concentration of 3 mg. L^{-1} of bio-fertilizer and 4 mg. L^{-1} of brassinolide has the highest average interference of 1.643 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 total chlorophyll content of the leaves of the plant (Table 8), the Indian variety sprayed plants outperformed at a bio-fertilizer concentration of 3 mg. L^{-1} and brassinolide at concentration of 4 mg.L^{-1}

with the highest average interaction trait of 1.769 compared to control plants.

Table 8: Effect of variety, bio-fertilizer, brassinolide and their interactions on the total chlorophyll content of leaves (mg.g-1 wet weight) of two alfalfa plant varieties.

Verities (V)	Bio-fertilizer concentrations (AG) (mg.L-1)	Brassinolide concentration (BL) (mg.L-1)				Effect of mean bilateral interaction AG ×V
		0	2	4	6	
Indian	0	0.931	1.232	1.600	1.532	1.324
	3	1.396	1.663	1.769	1.612	1.610
	6	1.469	1.589	1.683	1.546	1.572
Local	0	0.771	1.169	1.392	1.364	1.174
	3	1.284	1.449	1.517	1.401	1.413
	6	1.339	1.391	1.448	1.303	1.370
LSD(0.05)		0.059				0.029
Bilateral interaction BL × V						
Verities (V)		Brassinolide concentration (BL) ($mg.L^{-1}$)				Effect of mean variety
		0	2	4	6	
Indian		1.265	1.495	1.684	1.563	1.502
Local		1.131	1.336	1.452	1.356	1.319
LSD(0.05)		0.034				0.017
Bilateral interaction BL × AG						
Bio-fertilizer (AG)		Brassinolide concentration (BL) ($mg.L^{-1}$)				Effect of mean bio-fertilizer
		0	2	4	6	
0		0.851	1.200	1.496	1.448	1.249
3		1.340	1.556	1.643	1.507	1.511
6		1.404	1.490	1.566	1.424	1.471
LSD(0.05)		0.042				0.021
Effect of mean brassinolide		1.198	1.415	1.568	1.460	
LSD(0.05)		0.024				

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