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Response of *Silybium marianum* L. plants to foliar application of algae, chitosan and effective microorganisms.

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A field experiment was conducted at Faculty of Agriculture, Cairo University during 2014/2015 and 2015/2016, seasons to study the effect of algae, chitosan and effective microorganisms on the vegetative growth, flowering, seed yield and chemical composition of *Silybium marianum* L. plant. The data showed that all bio-stimulants treatments increased the plant height, stem thickness and the formation of branches and leaves/plant, the high level of chitosan and low level of effective microorganisms (EM) were the most effective, resulting the tallest plants with thickest stems, highest No. of branches leaves/plants. Algae treatment at low significantly increased the plant height and stem thickness. All treatments increased the averages of fresh and dry weights of leaves/plant, and the heaviest fresh and dry weights was recorded with EM 5 ml/l followed by chitosan 200 ppm. Spraying *Silybium* plant with two levels EM, high level chitosan and algae chitosan, significantly increased the production of inflorescences/plant. Foliar spray of chitosan at 200 ppm, EM at 10 ml/l and algae at 5.0 g/l, induced the largest inflorescences. The treatments of EM at 5 ml/l, and algae at 5 g/l, induced the heaviest weight of inflorescences. Treating plants with the high level of EM in the first season and chitosan in the second one produced the highest number of seeds/inflorescence. The treatments of EM and high level chitosan were the most effective in increasing the weight of seeds/inflorescence. The seeds production per plant was the highest with E.M 5 ml/l. Raising chitosan or algae to high level significantly increased the production of seeds/plant. A pronounced increase in the chlorophyll-*a* and -*b* and Chl-*a*+*b*, obtained with chitosan and algae at 2.5 g/l, the maximum content of carotenoids was recorded with chitosan. The low level of chitosan or E.M induced the highest carbohydrates content in leaves. Spraying plants with EM at low concentration, markedly increased the contents of N, P, K and Mg followed by chitosan, and algae at high level 5.0 g/l, had a marked effect on increasing P and Mg from the obtained results it can be concluded that EM 5 ml/l followed by chitosan were the most effective treatments for increasing the seeds production of *Silybium marianum* L. plant

Keywords: *Silybium marianum* L., chitosan, algae, effective microorganisms.

INTRODUCTION

Milk thistle plant (*Silybium marianum* L.) Gaertn, belongs to family Asteraceae, it is native to Southern Europe, North Africa and Western Asia; Milk thistle is an annual plant reaches a height of 150-200 cm and has large leaves and pink-purple or white flowers (Frohne, 2010). The *Silybium marianum* L. herb is high in a chemical compound known as silymarin, the active agent in its liver-protective abilities and improve the overall functioning of the liver and is often used to reduce cirrhosis of the liver, chronic liver inflammation, damage done to the liver by alcohol and other intoxicants.(Ramasamy and Agarwal, 2008). The

natural bio-stimulators are extensively used in modern agricultural production of medicinal and aromatic crops to intensify the quality, quantity of these crops (Dhargalkar and Pereira, 2005). In the last few years there has been a growing interest in the use of chitosan, algae and effective-microorganism for enhancing the growth of many economic medicinal crops. The activity of these compounds is often attributed to provide mineral nutrients to plants that improves plant productivity (Yakhin et al., 2016) or to increase plant productivity via phytohormones, which influences the plant's ability to control its hormone biosynthesis. Algae (seaweed), chitosan and EM are the famous natural bio-stimulators. The effect of chitosan on vegetative growth and yield was reported by many authors, Kim et al., (2005) on *Ocimum basilicum* found that 0.5 % chitosan increased weight and height, Lee (2005) on *Glycine max* L. stated that chitosan at 1000 ppm increased plant height, thickness and fresh weight, Boonlertniruni et al., (2008) on rice showed that chitosan application significantly increased rice yield, Dzunga et al., (2011) on coffee mentioned that chitosan increased the mineral uptake and stimulated seedling growth, Yin et al*.,* (2011) on *Origanum vulgare* found that chitosan (50-1000 ppm) promoted plant height growth fresh weight yield, and dry yield. On *Phaseolus vulgaris*, chitosan had a positive effect in enhancing shoot and root length, fresh & dry weights of shoots and leaves area (Sheikha, 2011). On okra plant (Mondal et al., 2012) sprayed plants with chitosan, found that plant height, leaf number ,total dry mass ,growth rate, photosynthesis and yield attributes (number of fruits/plant, fruit yield and fruit size) were increased with chitosan until 125 ppm. On potatoes the best plant vegetative growth (plant height, number and dry weights of leaves and shoots) with chitosan at 5% plus the application of the inorganic fertilizer (Shaheen et al., 2014). On freesia the chitosan resulted in higher chlorophyll content and higher No. of inflorescence shoot and length (Salachna and Zawadzińska, 2014). On mung bean found that branch and leaf number/plant, leaf area/plant, total dry mass/plant, photosynthesis and No. of pods/plant increased significantly with chitosan at 50 ppm (Mondal et al., 2013). On *Ocimum basilicum* stated that foliar application of chitosan at 400ppm increased plant growth (Malekpoor et al., 2016). On *Majorana hortensis* plants sprayed plants with chitosan at 2.5 and 5 g/l, found that the low concentration induced the maximum values of plant height, number of branches, fresh and dry weights of herb, chlorophyll-a and carotenoids contents (El-Khateeb et al., 2017). Massoud et al., (2009) stated that EM improved the nutrient uptake efficiency, enhanced root growth and increased yield. On the other hand, Youssef (2011) on marjoram plants found that application of organic materials with EM produced the maximum No. of branches/plant, herb fresh weight and herb dry weight. Abdou et al., (2011) found that, plants treated with EM and Phosphorus produced the best results of all tested growth and yield parameters. Frąszczak et al., (2012) on *Ocimum basilicum* found a significantly higher macro-element content with the application of EM. The extracts of seaweeds are widely used in horticulture crops to promoting effects and to ameliorate abiotic stresses such as salinity, nutrient deficiency and drought. The chemical constituents of seaweed extract include complex polysaccharide, fatty acids, vitamins, phytohormones and mineral nutrients. A number of seaweeds are reported to possess plant-growth promoting activity and thus they are used in agriculture and horticulture as organic manures and fertilizers (Craigie, 2011). Seaweed extracts are the most widely used seaweed production horticultural crops. Liquid extracts of algae may be applied near the roots or mixing with irrigation water or as foliar sprays on a variety of flower of vegetable crops (Fornes et al., 2002; Selvaraj et al., 2004 and Haider et al., 2012 and Mohamed et al., 2015) sprayed *Ocimum basilicum* with Algreen 600 and found that it significantly increased plant height, number of branches, fresh and dry weight of plant, chlorophyll *a* and *b* and N, P and K contents.

The aim of this study was to investigate the effect of algae, chitosan and EM on *Silybium marianum* L. plants, aiming to improve growth, flowering and seed yield which in turn increases the main component of the plant (silymarin and its derivatives).

MATERIALS AND METHODS

A field experiment was conducted at the Experimental Station, Faculty of Agriculture, Cairo University, Egypt, during the two successive seasons of 2014/2015 and 2015/2016. The aim of this investigation was to study the response of Milk thistle plant (*Silybium marianum* L.) to foliar application of algae, chitosan and effective microorganisms (EM) and their effects on the vegetative growth, flowering, seed yield and chemical composition of the plant.

Table A: The physical and chemical characteristics of the soil.

pH	EC $ds.m^{-1}$	Soluble Cations and Anions (meq/l)							
		Cations				Anions			
		Na	K	Ca	Mg	Cl	HCO_3	CO_3	SO_4
7.5	1.80	0.61	0.52	1.23	0.75	1.4	0.64	0.05	1.26

Particle size distribution			
Total Sand	Silt	Clay	Texture
36.8	36.0	27.2	Clay loam

The seeds of *Silybium marianum* L. plants local cv "Pink- purple" (kindly obtained from the Dept. of Med. and Aromatic Plants, Ministry of Agric., Egypt) were sown on 15th October, 2014, in the open field in clay loam soil (table A) in plots (3x1.8 m) with three rows at 60 cm apart, 50 cm between the seed hills within the row. After month from planting seedlings (15-20 cm length), from 15th November till 15th May 2015, the plants were monthly foliar sprayed, by a hand sprayer until run off point, with the following bio-stimulators: Algae, chitosan and EM using Bio-film at 1 ml/l as a wetting agent. The two levels of these bio-stimulators were chitosan at 100 and 200 ppm, algae at 2.5 and 5 g/l and EM at 5 and 10 ml/l and untreated plants (Control), all bio-stimulators products were obtained from the Union for Agric., Devel. Co., UAP, Egypt. The plants were monthly sprayed with a dose of 2 g/plant of N-P-K fertilizer (Life Green; 20-20-20).The plants were grown under field conditions and irrigated every 7-10 days as the plants needed.

Experimental design:

The layout of the experiment was a complete randomized block design, with 3 replicates (each replicate contained 5 plants). So, the study contained 7 treatments (including the control). The following growth parameters were recorded: Plant height (cm), stem diameter (mm) ,number of branches/plant , number of leaves/plant as well as fresh and dry weights of leaves/plant (g/plant). Flowering and seeds characters were recorded, No. of inflorescences/plant, inflorescence diameter (cm) and weight (g), No. of seeds/inflorescence, seed weight /inflorescence and plant (g). Chlorophyll *a*, *b* and total carotenoids were determined according to (Saric et al., 1967). Total carbohydrates in dried leaves were determined according to (Herbert et al.,1971). Total nitrogen content was determined according to the procedure described by (Koch and McMeekin, 1924). Phosphorus content were determined according to (Troug and Meyer, 1939).The content of potassium and Mg in the leaves was determined by using flame spectrophotometer A646.

Statistical analysis:

Data recorded on vegetative growth, flowering, seeds yield and chemical compositions were statistically analyzed. An analysis of variance (ANOVA) was carried out, and the means of the recorded data were compared using the "least significant difference (L.S.D.)" test at the 5% level, as described by (Snedecor and Cochran 1980).

RESULTS AND DISCUSSION

1. Effect on vegetative growth:

As shown in (Table1), the data indicated that, in both seasons, all bio-stimulant treatments increased the plant height as compared with the control. The high level of chitosan (200 ppm) and the low level of EM (5 ml/l) significantly resulted the tallest plants (208.83, 200.00, 224.33 and 205.00 cm, respectively, against 147.33 and 134.17 cm, for control plants. Spraying plants with the high level of algae was more effective in increasing the plant height than the low level. Concerning the effect of bio-stimulators on thickness of stems, the data generally cleared that (in both seasons), all bio -stimulant treatments increased the thickness of stems, as compared with the control. Chitosan at the high concentration and EM at the lowest one produced the thickest stems, giving 3.20, 3.27, 2.67and 2.70 cm, stem thickness, respectively. The results revealed stem thickness of algae-treated plants showed less responses, as compared with chitosan and EM treatments, however, the differences in stem thickness of algae treated plants was significant as compared with the control. In both seasons, the application of the three bio-stimulators increased the formation of branches/plant, as compared with the control. The number of branches formed on plants was significantly responded to the high level chitosan and the low level of both algae and EM, giving the following number of branches/plant, 7.33, 8.00,

6.33, 7.33, 8.33 and 8.00, respectively.

Table 1. Effect of chitosan, algae and EM on plant height, stems diameter and No. of branches/plant of *Silybum marianum* L. during seasons of 2014/15 (FS) and 2015/16 (SS).

Treatments	Plant height (cm)		Stem diameter (cm)		Number of branches/plant	
	FS	SS	FS	SS	FS	SS
Control	147.33	134.17	1.67	1.80	4.00	4.33
Chitosan(100 ppm)	170.33	178.17	2.50	2.63	4.67	5.50
Chitosan(200 ppm)	208.83	224.33	3.20	3.27	7.33	8.00
Algae (2.5 g/l)	161.33	172.33	2.37	2.60	6.33	7.33
Algae (5.0 g/l)	191.00	184.67	2.30	2.50	5.00	6.00
EM (5 ml/l)	200.00	205.00	2.67	2.70	4.67	5.83
EM (10 ml/l)	185.17	185.33	2.53	2.67	8.33	8.00
L.S.D 5 %	18.66	21.80	0.78	0.42	1.11	1.77

Table 2. Effect of chitosan, algae and EM on No., fresh and dry weights of leaves of *Silybum marianum* L. during the seasons of 2014/2015 (FS) and 2015/16 (SS).

Treatments	Number of leaves/plant		Fresh weight of leaves (g)		Dry weight of leaves (g)	
	FS	**SS**	**FS**	**SS**	**FS**	**SS**
Control	45.00	47.17	619.65	675.95	61.20	66.98
Chitosan(100 ppm)	51.67	54.67	927.96	1284.20	119.84	140.02
Chitosan(200 ppm)	70.33	76.17	1430.25	1573.79	167.63	164.23
Algae (2.5 g/l)	45.33	49.50	909.68	1104.82	112.31	130.88
Algae (5.0 g/l)	52.00	54.67	1217.00	1279.92	138.40	168.13
EM (5 ml/l)	58.33	61.33	1485.74	1611.04	179.74	193.48
EM (10 ml/l)	54.33	52.33	1299.51	889.90	146.47	110.94
L.S.D 5 %	5.87	7.33	44.32	56.15	15.73	24.56

Concerning the effect of the three bio-stimulators on the formation of leaves (Table 2), the data indicated that all bio-stimulants increased the number of leaves/plant. In both seasons, there were huge increases in No. of leaves/ plants with the foliar spray of high level of chitosan (70.33 and 76.17) and the low level of EM (58.33 and 61.33), as compared with the other treatments and control. The effect of algae on the formation of leaves, revealed that the number of leaves formed on the plants treated with high level of algae was significantly higher than the control. The averages of fresh and dry weights of leaves/ plant,in response to the treatments of bio-stimulants (Table 2) showed that, in both seasons, all treatments increased the averages of fresh and dry weights of leaves/plant, compared with the control. Concerning the effect of bio-stimulators on fresh weight the data cleared that (in both seasons), the heaviest weight was obtained with EM at 5 ml/l (1485.74 and 1611.04 g/plant, respectively) followed by chitosan at 200 ppm (1430.25 and 1573.79 g, respectively) against 619.65 and 675.95 g in both seasons, for the control plants. The same trend was recorded for the dry weight of leaves.

Generally, it was found that all bio-stimulants increased the plant height, stem thickness and

the formation of branches and leaves/plant, the high level of chitosan and low level of EM were the most effective, resulting the tallest plants with thickest stems, highest No. of branches leaves/plants. Algae treatment at low significantly increased the plant height and stem thickness. All treatments increased the values of fresh and dry weights of leaves/plant, and the heaviest fresh and dry weights were recorded with EM at 5 ml/l followed by chitosan 200 ppm. In this regard, Yin et al. (2011) on *Origanum vulgare* found that chitosan (50-1000 ppm) promoted plant height growth fresh weight yield, and dry yield. On *Phaseolus vulgaris*, Sheikha (2011) stated that chitosan enhanced fresh and dry weights of shoots and leaves. Shaheen et al., (2014) on potatoes obtained the best plant vegetative growth (plant height, number and dry weights of leaves and shoots) with chitosan at 5%. Malekpoor et al., (2016) on *Ocimum basilicum* using chitosan at 400 ppm and El-Khateeb et al., (2017) on *Majorana hortensis*, using chitosan at 2.5 obtained the highest values of plant height, number of branches, fresh and dry weights. Several workers mentioned that EM significantly increased the growth of plants. Massoud et al. (2009) found that EM treatment improved the growth and increased plant height due to enhancing cell division and/or cell enlargement. Youssef (2011) on marjoram plants found that EM gave the highest No. of branches/plant, herb fresh weight and herb dry weight. Abdou et al. (2011) mentioned that, EM gave the best growth. Seaweed extracts are the most widely used seaweed production to improve the growth of many horticultural crops, including potato, tomato (Selvaraj et al., 2004 and Haider et al., 2012).

2. Effect on flowering:

Data in Table (3) indicated that, in both seasons, the treatments of chitosan, algae and EM, at the two levels, increased No. of inflorescences/plant, as compared with the control. In the first season, the highest No. of inflorescences/plant were recorded with the treatment of EM at the two rates (28.67 and 26.33), followed by the high dose of chitosan (21.33). On the other hand, the lowest No. of inflorescences/plant (16.00) was produced for control plants. During the second season, the highest No. of inflorescences/plant (30.0), was recorded for plants treated with the low dose of EM, followed by high dose algae (5.0g/l) giving 26.00 inflorescences/plant. The largest inflorescences diameter (4.64 cm) in the first season and in the second one (4.31 cm) was obtained with the foliar spray of chitosan at 200 ppm and EM at 10 ml/l. In both seasons, treating plants with algae at 5.0 g/l, significantly increased the diameter of inflorescences as compared with the control. Data on the effect of bio-stimulants on the fresh weight of inflorescence indicated that (Table 3) in both seasons, chitosan, algae and EM each at the two levels, increased the weight of inflorescence as compared with the control. In the first season, the heaviest inflorescence 6.38 was obtained when the plants were sprayed with EM at 5 ml/l, followed by the treatment of algae at 5 g/l, giving 6.72 g of weight of inflorescence. In the second season, the results showed a similar trend as in the first one. It can be concluded that spraying plants with two levels EM, high level chitosan and algae chitosan, significantly increased the production of inflorescences/plant. Foliar spray of chitosan at 200 ppm, EM at 10 ml/l and algae at 5.0 g/l, gave the largest inflorescences. The treatments of EM at 5 ml/l, and algae at 5 g/l, gave the heaviest weight of inflorescences. These findings are in agreement with that reported by Salachna and Zawadzińska (2014) on freesia showed that chitosan resulted in higher No. of inflorescence.

3. Effect on seed production:

As shown in Table 3 and Fig. 1, 2 and 3, treating plants with the high level of EM as well as chitosan produced the highest No. of seeds/inflorescence (109.33 and 100.67); the control plants produced 84.67 seeds/inflorescence. In the second season, spraying plants with chitosan at 100 ppm, followed by 200 ppm, induced the highest number of seeds/inflorescence (114.00 and 109.67 seeds, respectively).Treating plants with the high level of EM as well as chitosan increased the weight of seeds / inflorescence , as compared with the control, in the first season, whereas in the second one, the low and high levels of EM were the most effective in this respect, produced the heaviest weight of seeds/inflorescence (3.09 and 2.77 g), against 2.00 g for the control plants and 2.07 g for plants treated with the low level of chitosan. The response of seeds production per plant to the foliar application of the bio-stimulants, the data (Table 3) indicated that, in both seasons treating plants with all treatments of bio-stimulants (except for the low level of algae) markedly increased the seeds production per plant. The highest value of seeds production per plant was obtained with the treatment of EM at 5 ml/ l, producing 59.06 and 92.70 g seeds/plant, in the

first and second seasons, respectively and control plants produced 33.28 and 30.66 g respectively.

Table 3. Effect of chitosan, algae and EM on diameter and fresh weight of inflorescence (g) and seed production/plant (g) of *Silybium marianum* L. during the seasons of 2014/2015 (FS) and 2015/16 (SS).

Treatments	Diameter of inflorescence (cm)		Fresh weight of inflorescence (g)		Seed production/plant (g)	
	FS	SS	FS	SS	FS	SS
Control	3.73	3.29	4.50	4.33	33.28	30.66
Chitosan (100 ppm)	4.22	3.70	5.80	5.64	35.00	33.31
Chitosan (200 ppm)	4.64	3.84	5.87	5.71	42.87	47.85
Algae (2.5 g/l)	4.35	3.43	6.06	5.16	29.41	28.67
Algae (5.0 g/l)	4.42	3.92	6.72	6.21	31.12	50.18
EM (5 ml/l)	3.84	3.56	6.38	6.63	59.06	92.7
EM (10 ml/l)	4.07	4.31	4.74	5.89	56.35	70.64
L.S.D 5 %	0.70	0.50	0.54	0.48	6.50	7.42

Raising the level of chitosan or algae to the high level significantly increased the production of seeds/plant in both seasons, as compared with the low level and the control. Treating plants with the high level of EM in the first season and chitosan in the second one produced the highest number of seeds/ inflorescence. The treatments of EM and high level chitosan were the most effective in increasing the weight of seeds/inflorescence. The seeds production per plant was the highest with EM 5 ml/l. raising chitosan or algae to high level significantly increased the production of seeds/plant. Similar findings were reported by Mondal et al. (2012) on okra plant they found that yield attributes (No. of fruits/plant, fruit yield and fruit size) were increased with the application of chitosan at 125 ppm.

4. Effect on chemical composition.

4.1. Pigment content:

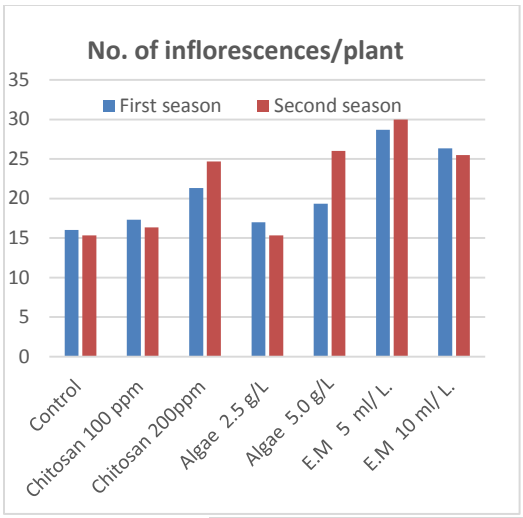
A pronounced increase in the chlorophyll-*a* content was determined in leaves of plants treated with chitosan 100 and 200 ppm, followed by algae at 2.5 g/l, compared to the control (Table 4), and the other treatments had a slight effect on chlorophyll-*a* content. The higher chlorophyll-*b* content was determined with the foliar application of chitosan at low level. The content of Chl-*a*+*b*, recorded the highest value, in both seasons, with both levels of chitosan followed by algae at the high level in the first season, and the low level in the second one. In both seasons, spraying plants with the two levels of EM had a slight effect on increasing the total chlorophylls content, as compared with the control. The content of carotenoids attained the highest value, in both seasons, with the foliar application of chitosan. In conclusion, the highest increases in the chlorophyll-*a* and-*b* and Chl-*a*+*b*, obtained with chitosan and algae at 2.5 g/l, the highest content of carotenoids was recorded with chitosan.

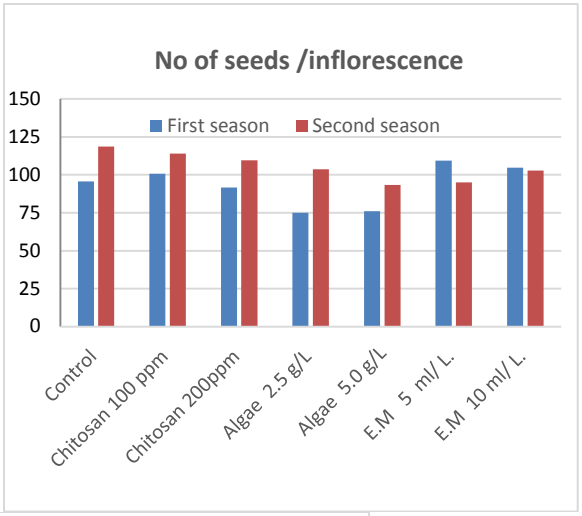
4.2. Total carbohydrates content:

The effects of chitosan, algae and EM treatments on total carbohydrates content in dried leaves of *Silybium marianum* L. plant are shown in Table (4). The data revealed that, treating plants with low level of chitosan or EM, were the most effective treatments in increasing the accumulation of total carbohydrates in the dried leaves and the other treatments had no clear trend, as compared with the control.4.3.

N.P.K and Mg contents:

Concerning the effect of chitosan, algae and EM treatments, on the contents of N, P, K and Mg in the leaves of *Silybum marianum* L., the data in Table (5), indicated that, in both seasons of study, spraying plants with EM especially at the low concentration, showed a marked effect on increasing the contents of N, P, K and Mg followed by chitosan applications. On the other hand, treating plants with algae at high level 5.0 g/l, had a marked effect on increasing the contents of P and Mg in the leaves of Milk thistle plants, as compared with the control.





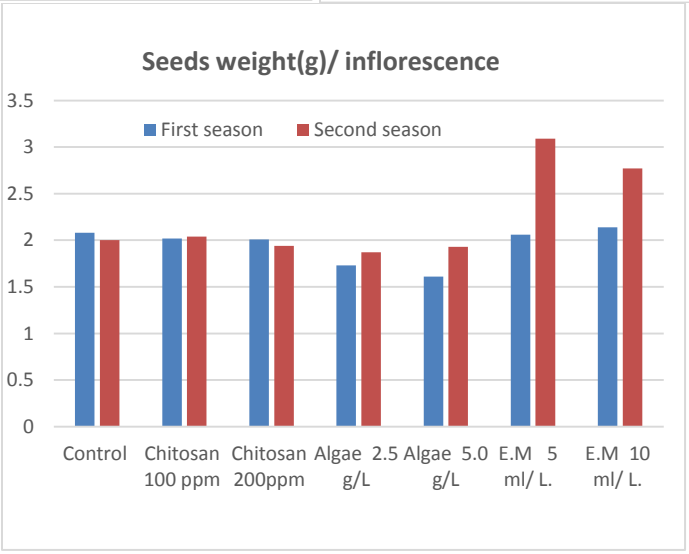


Fig. 1, 2, 3: Effect of chitosan, algae and EM on No. of inflorescence/plant (g) and No. of seed/inflorescence and seed weight/inflorescence (g) of *Silybium marianum* L. during the seasons of 2014/2015 and 2015/2016.

Table 2. Effect of chitosan, algae and EM on No., fresh and dry weights of leaves of *Silybum marianum* L. during the seasons of 2014/2015 (FS) and 2015/16 (SS).

Treatments	Number of leaves/plant		Fresh weight of leaves (g)		Dry weight of leaves (g)	
	FS	SS	FS	SS	FS	SS
Control	45.00	47.17	619.65	675.95	61.20	66.98
Chitosan(100 ppm)	51.67	54.67	927.96	1284.20	119.84	140.02
Chitosan(200 ppm)	70.33	76.17	1430.25	1573.79	167.63	164.23
Algae (2.5 g/l)	45.33	49.50	909.68	1104.82	112.31	130.88
Algae (5.0 g/l)	52.00	54.67	1217.00	1279.92	138.40	168.13
EM (5 ml/l)	58.33	61.33	1485.74	1611.04	179.74	193.48
EM (10 ml/l)	54.33	52.33	1299.51	889.90	146.47	110.94
L.S.D 5 %	5.87	7.33	44.32	56.15	15.73	24.56

It can be mentioned that spraying plants with EM at low concentration, markedly increased the contents of N, P, K and Mg followed by chitosan, and algae at high level 5.0 g/l, had a marked effect on increasing P and Mg In this regard, the promoting effect of chitosan, and EM as well as algae on increasing the uptake and contents of mineral elements, was reported by Yakhin et al. (2016) the stated that the activity of these compounds is often attributed to provide mineral nutrients to plants. Dzunga et al., (2011) on coffee seedlings and El-Khateeb et al., (2017) on *Majorana hortensis*, mentioned that chitosan increased the mineral uptake. Youssef (2011) on marjoram plants found that application of EM induced the maximum N, P and K contents. Mohamed et al., (2015) sprayed *Ocimum basilicum* with algae (Algreen 600) found that it significantly increased the N, P and K contents.

CONCLUSION

From the obtained results it can be concluded that EM 5 ml/l followed by chitosan were the most effective treatments for increasing the seeds production of *Silybium marianum* L. plant**.**

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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