Folia Hort. 30(1), 2018, 79-92

DOI: 10.2478/fhort-2018-0009

FOLIA HORTICULTURAE

Published by the Polish Society for Horticultural Science since 1989

ORIGINAL ARTICLE | Open access | www.foliahort.ogr.ur.krakow.pl

Morpho-physiological and biochemical responses of bladder cherry (*Physalis alkekengi* L.) induced by multienzymatic biostimulant, IBA, and citric acid

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ABSTRACT

Plant enzymes, growth regulators and organic acids are the main groups of plant biostimulants (PBs), and their combined use in the final formulation may be important for increasing the quantitative and qualitative composition of plant products. This study aimed to determine the effects of a multienzymatic biostimulant (MB), indole-3-butyric acid (IBA), and citric acid (CA) on the morphological and phytochemical traits of bladder cherry (*Physalis alkekengi* L.). The treatments included different concentrations of MB (0, 0.5 and 1.0%), IBA (0, 25, and 50 ppm), and CA (0, and 500 mg dm^{-3}), which were sprayed four times during the vegetative stage, at 12-day intervals, 35 days after planting. The results showed that the treatments had a significant effect on plant height, stem number, diameter and weight, leaf number and weight, fruit number, diameter and weight, the amounts of total phenols, alkaloids and flavonoids, and on the radical scavenging activity. The most effective formulation for improving the fruit yield of bladder cherry was 1% MB with 50 ppm IBA and 500 ppm CA. However, the best treatment for increasing the total phenolic and alkaloid contents, and radical scavenging activity was 0.5% MB. In general, the maximum values of most traits were obtained by spraying the plants with 0.5 and 1% MB combined with IBA and CA. The concentration of alkaloids, the main pharmaceutical metabolites of bladder cherry, increased as a result of the application of the multienzymatic biostimulant.

Keywords: alkaloid, indole-3-butyric acid, flavonoids, phenols, radical scavenging activity

INTRODUCTION

The genus *Physalis* has great economic importance not only as a food supplier but also as a source of important chemical compounds. The fruits of *Physalis* species contain various phenols, alkaloids, steroids, saponins, fatty acids, and flavonoids (Sharma et al., 2015). Bladder cherry (*Physalis alkekengi* L.) is a perennial herbaceous plant of the Solanaceae family, used in traditional medicine in Iran and many other countries in the world (Kusumaningtyas et al., 2015).

Global demand for medicinal plants is rapidly increasing in the pharmaceutical and food industries. This need requires the use of good agricultural practices (GAP) to guarantee medicinal plant quality and facilitate standardization for herbal drugs (Chan et al., 2012; Muchugi et al., 2008). It has been reported that the use of biostimulants in GAP leads to increased growth and quality of most

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plant species (Asad et al., 2002; Du Jardin, 2015). Plant biostimulants, or agricultural biostimulants, include diverse substances and microorganisms that enhance plant growth. Furthermore, it is documented that they also promote cell respiration, photosynthesis, protein synthesis, water and nutrient uptake, and enzymatic activities (Nardi et al., 2016; Du Jardin, 2015). Plant biostimulants contain a substance/substances and/or microorganisms whose function, when applied to plants or the rhizosphere, is to stimulate the natural processes to enhance nutrient uptake, nutrient efficiency, tolerance to abiotic stresses, and crop quality (Calvo et al., 2014). Biostimulants generally include plant growth regulators, organic acids, and enzymes (Mandal et al., 2007; Du Jardin, 2015). These formulations decrease the need for chemical fertilizers and have the capacity to satisfy the nutritional requirements of plants, which further results in higher yields (Subbarao et al., 2015). Furthermore, there is strong evidence that foliar spraying of biostimulants enhances plant growth, nutrient uptake, and improves the yield and production quality of many crops (El-Desuki, 2004). The most effective biostimulants usually contain several types of bioactive stimulants in their formulations, such as multienzymes. Multienzymatic biostimulants (MB) are new integrated agricultural organic products composed of enzymes, amino acids, polysaccharides, humic acids, phytohormones, and other components, which are usually derived from natural products, and their biological activities occur at limited doses.

Auxins, especially indole-3-butyric acid (IBA), are used as plant growth regulators to improve germination and subsequent growth and development of the plant (Bertoni, 2011). Auxins have an important role in major plant growth and development processes such as expansion, division and differentiation of plant cells, which can lead to organogenesis, apical dominance, tropism phenomenon, and vascular patterning (Mockaitis and Estelle, 2008). The normal growth of a plant requires balancing auxin biosynthesis and transport, as well as managing its storage forms. IBA is a potential storage form for auxin in a variety of plants, which can be synthesized from indole acetic acid (IAA) and converted to IBA by peroxisomal *β*-oxidation (Strader et al., 2010; Enders and Strader, 2015). Therefore, it is postulated that IBA has a crucial effect on the sensitivity, transport, and function of auxin (Epstein and Ludwig-Müller, 1993; Enders and Strader, 2015).

Organic acids are involved in a broad range of physiological processes of plant growth and development (An et al., 2014), and are also considered to alleviate biotic stresses (Jafari and Hadavi, 2012). Endogenous organic acids are the source of both carbon skeleton and energy for cells, and are used in the respiratory cycle and other biochemical pathways. It has been expressed that increasing endogenous organic acids can influence the biomass and yield of crops (Da Silva, 2003).

Application of biostimulants in the commercial production of medicinal plants is a viable management practice for the production of these species, increasing biomass production and enhancing the synthesis of secondary metabolites. Studies on the effects of plant biostimulants on the accumulation of secondary metabolites in medicinal plants have been conducted in order to increase the medicinal and trade values of those species (Rafiee et al., 2016). Application of different plant biostimulants at various concentrations and diverse mechanisms of action have resulted in different effects on medicinal plants. Considering the biochemical and pharmacological importance of bladder cherry (*Physalis alkekengi* L.) as a multi-purpose plant, as well as recognizing the biological effects of biostimulants, particularly enzymatic biostimulants, bioregulators and organic acids on the production of metabolites and plant growth, this study focused on the morpho-physiological and biochemical responses of bladder cherry to different formulations containing a multienzymatic biostimulant (MB), indole-3-butyric acid (IBA), and citric acid (CA). So far there has been very little information on the influence of MB, IBA, and CA on the morpho-physiological traits and concentrations of chemical compounds in bladder cherry. The results of this study could be useful in increasing *Physalis alkekengi* L. crop production with reduced fertilizer consumption, and also in providing practical information for the food and pharmaceutical industries.

MATERIAL AND METHODS

In order to evaluate the effects of some biostimulants on the growth and chemical composition of bladder cherry (*Physalis alkekengi* L.), two field experiments were conducted as factorial experiments based on a randomized complete block design (RCBD) with 18 treatments (Tab. 1) and 3 replications at the agricultural system research farm of Medicinal Plant Institute (MPI), ACECR of Karaj in Iran (35°, 90′ N and 50°, 88′ E, 1500 m above sea level) during

Table 1. Composition of the biostimulant formulations used in the experimental treatments

Treatment	MB* (%)	IBA** (ppm)	CA*** (mg dm^{-3})
T1	0	0	0
T2	0	0	500
T3	0	25	0
T4	0	25	500
T5	0	50	0
T6	0	50	500
T7	0.5	0	0
T8	0.5	0	500
T9	0.5	25	0
T10	0.5	25	500
T11	0.5	50	0
T12	0.5	50	500
T13	1	0	0
T14	1	0	500
T15	1	25	0
T16	1	25	500
T17	1	50	0
T18	1	50	500

*MB: Multienzymatic biostimulant; **IBA: Indole-3-butyric acid; ***CA: Citric acid

2015 and 2016. Monthly changes in temperature and precipitation during 2015 and 2016 are presented in Figure 1.

Transplants of bladder cherry were obtained from the gene bank and seed collection of MPI (1341-MPISB), and were transferred into farm plots. Plot size in each replication was 5 m in width and 6 m in length, with 1 m distance between each plot in order to prevent spray drift. Each plot had 12 planting rows spaced 50 cm apart and planting intervals of 25 cm in the rows. The treatments included different concentrations of a multienzymatic biostimulant (MB) at 0, 0.5, and 1.0% of a commercial product called Multienzim

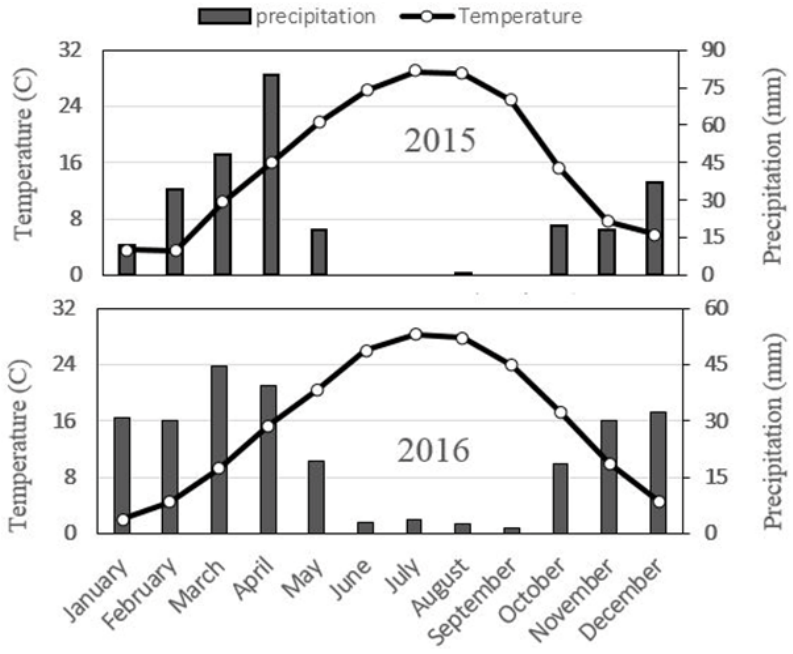

Figure 1. Monthly changes in temperature and precipitation between 2015 and 2016 at the experimental farm

Organik Gübre (MOG), which in Turkish means multienzymatic organic liquid fertilizer, indole-3-butyric acid (IBA) at 0, 25 and 50 ppm, and citric acid (CA) at 0, and 500 mg dm^{-3}. The details of the formulations used in the experimental treatments are given in Table 1. MOG is a liquid multienzymatic biostimulant for use as an organic growth promoter; it is manufactured from fruit juice and crop residues, and contains 18 enzymes (such as alkali proteinase, amylosine, glucamylase, xylanase, lipase, lyase, rubisco, transglutaminase, catalase, lipoxygenase, isomerase, urease, etc.), natural forms of micro- and macronutrients, and vegetable-based vitamins. The product was provided by Azarabadegan Company (West Azerbaijan province, Iran). The MOG liquid biostimulant contained 25% total organic carbon, 4% total nitrogen, 4% K_2O, 1.06% P, 0.42% Fe, 0.16% Cu, and 13% enzymes, with a pH of 6.1. The solutions for all the treatments contained surfactant "Tween 60" at 0.5% (v/v) to improve adhesion. All the chemicals used in the experiment, except the biostimulant, were purchased from Merck (Germany). All of the treatments were performed by spraying four times during the vegetative plant growth, at 12-day intervals, starting on the 35th day after transplanting the bladder cherry plants. The spraying was carried out in such a way that all of the aboveground parts of the bladder cherry plants were covered with the liquid. Motorized backpack-type sprayer with a capacity of 14 dm^3 was used for spraying, and the sprayer nozzle was held 40 cm above the plants.

After the four spray applications, 83 days after transplanting to the farm, fruits at the stage of full maturity (golden yellow ripe fruit) were collected manually. To record data on the morpho-physiological and biochemical traits, six plants from each experimental unit (plot) were randomly selected, and then average values of the traits were calculated for the two years. In order to measure total dry matter, the plants were divided into stems, leaves and fruits. Then they were placed in an oven at 75°C until constant weight was obtained.

Phytochemical analysis

Extraction

Shade-dried fruit was powdered with an electric mill and stored at room temperature in darkness before it was subjected to the extraction process. The fruit powder (10 g) was macerated with 200 ml of methanol (85%) at ambient temperature for 48 h. The extract was concentrated after filtering through a Whatman filter paper no.1. This extract was used for determining the total concentration of phenolic and flavonoid compounds as well as radical scavenging activity.

Total phenolic content

Total phenolic content was determined by the Singleton and Rossi (1965) method with some modifications. Briefly, 500 µL of diluted extract was mixed with 5 mL of the Folin-Ciocalteu reagent and 4 mL of a sodium carbonate solution (7%). The reaction mixture was brought to 25 mL with distilled water and was incubated for 15 min. in the dark. Then the absorbance was measured at 765 nm. Total phenolic content was calculated according to a gallic acid standard curve.

Total flavonoids

Flavonoid content was estimated by the Ordonez et al. (2006) method. Accurately measured 0.5 ml of the extract was mixed with 1.5 ml of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The mixture was allowed to stand at room temperature for 15 min. The absorbance of the reaction mixture was read at 415 nm with a double beam spectrophotometer (Human crop, Xma-2000). The total flavonoid content was calculated based on a quercetin standard curve.

Radical scavenging activity

DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging was used for an assessment of the antioxidant activity of the extract. The reaction mixture consisted of 2925 µl of a freshly prepared DPPH solution (25 mg dm^{-3}) in methanol and 75 µl diluted extract. After 30 minutes, the absorbance of the reaction mixture was read at 517 nm with a spectrophotometer (Human crop, Xma-2000) against the blank and control (the mixture of methanol and DPPH) (Blois, 1958).

Total alkaloid content

The fruit powder was subjected to extraction with methanol for 24 h in a Soxhlet apparatus. After filtering, the methanolic extract was evaporated to dryness, and the residue was dissolved in 2 N HCl and discoloured with diethyl ether. Then the solution pH was adjusted to 10 with 0.1 N NaOH and washed 3 times with 10 ml chloroform. The chloroform phase was evaporated and the residue was dissolved in methanol. For alkaloid measurements, 1 ml of the extract was mixed with 5 ml of a bromocresol green solution and 5 ml of phosphate buffer. The mixture was shaken and the formed complex was extracted

with chloroform. The volume of the collected complex was brought to 10 ml with chloroform. The absorbance of the complex was read at 470 nm. The alkaloid content was measured against an atropine standard curve (Sharief et al., 2014).

Statistical analysis

Analysis of variance (ANOVA) was performed for each trait included in the field experiments in 2015 and 2016. Bartlett's test was used to assess the homogeneity of variance over the two years. SAS statistical analysis software (ver. 9.2, SAS Institute) was used for ANOVA. Significant differences between the treatment means were determined using Duncan's Multiple Range Test at the 0.05 probability level.

RESULTS

Morphology and growth parameters

The results of means comparisons for each trait were presented based on the overall average for the years, and if the effect of the years was significant then each year was analyzed separately. The effect of the treatments on the traits is shown in Table 2 and 3. The results show that only stem and leaf fresh weight, radical scavenging activity, and flavonoid content were significantly different in the two years as a result of using the various formulations of the biostimulant. MB and IBA had a significant ($p \leq 0.01$) effect on plant height, whereas CA had no significant influence on it. However, their interaction had a significant effect ($p \leq 0.01$) on plant height in both years. Spraying IBA at both concentrations improved plant height compared to the control (71.4 to 75.5% increase), although increasing IBA concentration had led to a lower increase in plant height in 2015. Spraying CA, either alone or in combination with both IBA concentrations and MB, had no significant effect on plant height. The two MB concentrations strongly increased plant height compared to the control; however, the highest plant height was observed for 1% MB (135.6 cm). Overall, the highest plant height was obtained by spraying with a mixture of 0.5% MB and 25 ppm IBA (141.1 cm) (Tab. 2).

Spraying IBA at both concentrations significantly ($p \leq 0.01$) increased stem number, node number, and the main stem diameter compared to the control. However, the lower concentration of IBA was more effective than the higher concentration. Also, the two concentrations of MB increased these traits compared to the control. Spraying with CA increased only the number of stems (43.4%). Spraying CA together with IBA or MB was often ineffective on these features. However, the addition of CA to the mixture of MB and IBA was effective only at the higher concentration of IBA. The highest number of stems (20.2) was recorded for the combination of 1% MB with 50 ppm IBA and 500 mg dm^{-3} CA. The greatest number of nodes and stem diameter were obtained with the mixture of 0.5% MB and 25 ppm IBA (49.1 and 10.05, respectively). However, the lowest values of these traits were observed in the control (Tab. 2 – part 1).

The interaction of MB, IBA, and CA had significantly ($p \leq 0.01$) impacted on the dry and fresh weight of stems and on root fresh weight. The results of means comparison showed that spraying with CA did not produce a significant difference relative to the control. In contrast, the two concentrations of both IBA and MB improved the fresh and dry weight of stems and root fresh weight compared to the control. However, the values of stem and root weight were reduced by increasing the concentration of IBA, which was ameliorated by adding CA to 50 ppm IBA. Adding CA to MB reduced stem number, stem fresh and dry weight, and root fresh weight. Thus, the greatest stem fresh weight, stem dry weight, and root fresh weight were obtained by spraying with a mixture of 0.5% MB with 25 ppm IBA (129.6, 21.66 and 13.82 g, respectively). Their lowest values were obtained in the control (18.9, 4.24 and 2.98 g, respectively) (Tab. 2 – part 2).

The treatments had a significant effect ($p \leq 0.01$) on fruit number, fruit diameter, fruit fresh weight and fruit dry weight. Fruit diameter in the plants treated with IBA and CA was not significantly different from the control (27.14 mm). However, although 0.5% MB had no significant effect on fruit diameter compared to the control, its combination with 500 ppm CA and 50 ppm IBA increased fruit diameter. The use of 1.0% MB increased fruit diameter, but combining it with 500 ppm CA and 50 ppm IBA was more effective. Thus, the greatest fruit diameter was obtained with the formulation of 500 ppm CA and 50 ppm IBA with both concentration of MB (33.9 and 34.3 mm, respectively) (Tab. 2 – part 3).

Spraying with CA increased the number of fruits and fruit fresh weight compared to the control in 2015 and 2016 (an average increase of 40 and 41.4%, respectively) (Tab. 2 – part 3). The use of 25 ppm IBA increased the number of fruits and fruit fresh weight, but the higher concentration had an adverse effect. In addition, CA combined with IBA

Table 2. Effect of the treatments on morpho-physiological traits of *Physalis alkekengi* L. – part 1

Treatment*	Plant height (cm)			Stem number			Node number			Stem diameter (mm)		
	2015	2016	Mean	2015	2016	Mean	2015	2016	Mean	2015	2016	Mean
T1	69.0 j	62.5 c	65.8 h	7.0 i	8.2 l	7.6 j	29.0 h	30.2 g	29.6 j	4.13 i	4.66 ij	4.39 j
T2	64.5 j	71.6 c	68.1 h	13.0 ef	8.8 kl	10.9 gh	31.0 gh	31.0 g	31.0 j	4.4 h	4.99 i	4.69 i
T3	123.0 efg	102.6 b	112.8 fg	16.0 c	11.6 fg	13.8 d	37.0 ef	40.4 cd	38.7 efg	7.4 de	7.23 de	7.32 e
T4	117.0 gh	121.7 ab	119.4 def	5.0 j	9.5 ijk	7.3 j	42.0 cd	41.7 bc	41.9 bcd	4.2 hi	6.16 h	5.18 h
T5	117.0 gh	113.9 ab	115.5 efg	14.0 de	9.1 jkl	11.5 fgh	36.0 ef	35.4 f	35.7 hi	6.31 f	7.05 e	6.68 f
T6	119.0 fgh	122.3 ab	120.7 c-f	12.0 fg	11.2 fg	11.6 fgh	34.0 fg	37.2 def	35.6 hi	6.0g	6.63 fg	6.32 g
T7	130.0 cde	128.5 a	129.3 bcd	14.2 de	13.5 d	13.8 d	32.0 gh	39.5 c-f	35.7 hi	6.5 f	6.31 gh	6.41 g
T8	137.0 bc	120.1 ab	128.6 bcd	11.0 g	8.5 kl	9.7 i	37.0 ef	36.6 def	36.8 ghi	6.5 f	6.93 ef	6.72 f
T9	149.0 a	133.1 a	141.1 a	15.0 cd	20.8 a	17.5 c	49.0 a	49.2 a	49.1 a	10.2 a	9.9 a	10.05 a
T10	96.0 i	119.1 ab	107.6 g	10.6 gh	12.9 de	11.7 fg	32.0 gh	36.4 def	34.2 i	3.32 j	4.31 j	3.82 k
T11	123.0 efg	115.2 ab	119.1 def	5.0 j	9.9 hij	7.5 j	39.0 de	35.6 f	41.3 fgh	7.51 d	7.76 c	7.64 cd
T12	146.0 a	128.6 a	137.3 ab	11.0 g	14.9 c	12.9 de	43.0 c	39.6 c-f	43.9 cde	7.2 e	7.89 c	7.55 d
T13	142.0 ab	129.2 a	135.6 ab	3.0 k	13.3 de	8.1 j	48.0 ab	39.9 cde	42.2 bc	7.93 c	7.71 c	7.82 c
T14	132.0 cd	129.7 a	131.1 abc	9.0 h	12.3 ef	10.6 hi	44.0 c	40.4 cd	40.0 bcd	6.3 f	6.40 gh	6.35 g
T15	111.0 h	117.5 ab	114.3 efg	11.0 g	11.0 gh	11.01 gh	44.0 c	36.1 ef	36.2 def	7.51 d	7.70 c	7.61 cd
T16	123.0 efg	119.1 ab	121.1 c-f	14.0 d	10.4 ghi	12.2 ef	36.0 ef	36.4 def	43.5 ghi	7.93 c	7.55 cd	7.74 cd
T17	116.0 gh	132.6 a	124.3 cde	19.3.0 b	18.9 b	19.1 b	42.0 cd	44.9 ab	44.3 bc	7.6 d	7.56 cd	7.58 d
T18	127.0 def	132.1 a	129.6 bcd	21.0 a	19.5 a	20.2 a	45.0 bc	43.6 bc	44.4 b	9.3 b	9.13 b	9.22 b

Means with the same letter in each column are not significantly different at the 5% level of probability.
*For abbreviations see Table 1

Table 2. Effect of the treatments on morpho-physiological traits of *Physalis alkekengi* L. – part 2

Treatment*	Stem fresh weight (g)	Stem dry weight (g)	Root fresh weight (g)	Leaf number			LA (cm^2)	Leaf fresh weight (g)	Leaf dry weight (g)
				2015	2016	Mean			
T1	18.9 i	4.24 i	2.98 i	46.0 l	51.4 j	48.7 l	171.7 efg	14.7 hi	2.21 ij
T2	22.23 i	4.75 i	3.51 ghi	54.0 kl	56.1 j	55.1 l	165.4 g	8.3 j	1.27 l
T3	102.4 b	13.73 def	4.36 efg	200.0 d	201.5 c	200.8 c	185.7 ab	71.7 a	9.29 a
T4	99.6 bc	14.39 cde	3.46 hi	59.0 k	65.3 i	62.2 k	168.0 g	8.9 j	1.26 l
T5	45.0 h	9.32 h	3.59 f-i	109.0 ij	120.7 h	114.9 ij	172.8 d-g	14.6 hi	2.11 ijk
T6	54.4 f	11.54 e-h	4.44 ef	111.2 ij	139.3 g	125.2 h	167.6 g	19.0 g	2.87 gh
T7	67.5 e	13.21 d-g	5.95 d	126.0 gh	152.9 e	139.5 fg	166.4 g	22.2 f	3.32 f
T8	51.4 fg	10.24 h	3.18 hi	140.2 ef	143.6 fg	141.9 fg	165.4 g	15.3 h	2.47 ih
T9	129.6 a	21.66 a	13.82 a	145.0 e	153.8 e	149.4 e	167.1 g	22.6 f	3.15 fg
T10	53.2 fg	10.98 fgh	6.09 d	148.3 e	165.0 d	156.7 d	181.1 a-d	45.1 c	6.43 c
T11	47.7 gh	10.12 h	3.99 fgh	116.0 hi	118.8 h	117.4 i	165.2 g	12.2 i	1.82 jk
T12	68.7 e	15.13 cd	7.12 c	139.0 ef	149.6 ef	144.3 ef	165.7 g	35.8 e	4.70 de
T13	68.2 e	14.05 de	6.02 d	194.0 d	204.6 c	199.3 c	179.2 b-e	47.0 c	5.07 d
T14	76.1 d	13.28 d-g	4.96 e	241.0 b	209.8 c	225.4 b	176.9 cdef	40.7 d	4.64 e
T15	48.8 gh	10.4 gh	4.42 ef	133.0 fg	138.6 g	135.8 g	168.7 f	15.8 h	2.21 ij
T16	48.2 gh	10.98 fgh	4.39 ef	101.0 j	118.1 h	109.5 j	165.6 g	13.3 hi	1.72 k
T17	96.2 c	18.56 b	12.33 b	254.0 a	280.1 a	267.1 a	188.8 a	60.2 b	7.91 b
T18	95.5 c	17.23 bc	12.98 ab	221.0 c	230.5 b	225.7 b	183.8 abc	72.2 a	9.29 a

Means with the same letter in each column are not significantly different at the 5% level of probability. For significant effect of year, the data for each year were analyzed separately.
*For abbreviations see Table 1

Table 2. Effect of the treatments on morpho-physiological traits of *Physalis alkekengi* L. – part 3

Treatment*	Fruit diameter (mm)	Fruit number			Fruit fresh weight (g)			Fruit dry weight (g)
		2015	2016	Mean	2015	2016	Mean	
T1	27.14 de	19.0 gh	19.1 e	19.07 h	190.3 hi	196.9 gh	193.6 hi	18.8 f
T2	27.74 de	29.0 d	24.4 cd	26.71 e	275.4 ef	272.2 ef	273.8 ef	27.01 de
T3	26.69 de	30.0 d	27.3 bc	28.68 d	323.1 d	316.3 d	319.7 c	31.86 c
T4	25.86 def	21.0 fg	24.9 cd	22.96 g	288.3 e	279.6 e	283.9 de	28.29 de
T5	27.08 de	21.0 fg	15.4 gh	18.2 hi	146.1 j	147.5 i	146.8 k	13.69 h
T6	27.89 d	16.0 ij	19.2 e	17.6 hi	199.2 gh	197.9 gh	198.5 gh	19.05 f
T7	23.56 d	28.0 d	19.7 e	23.88 fg	206.0 g	205.5 g	205.8 g	19.84 f
T8	25.05 ef	13.0 k	15.0 h	14.0 k	143.0 j	142.6 i	142.8 k	13.23 h
T9	33.62 ab	25.0 e	27.1 bc	26.06 e	329.5 d	312.7 d	321.1 c	31.74 c
T10	27.74 de	15.0 jk	18.5 ef	16.75 i	181.6 i	188.9 gh	184.9 ij	17.83 f
T11	28.53 cd	23.0 ef	23.4 d	23.24 fg	268.4 f	258.2 f	263.3 f	25.71 e
T12	33.9 a	34.0 c	29.3 b	31.65 c	365.0 c	346.4 c	355.7 b	35.43 b
T13	30.77 c	23.0 ef	26.8 bc	24.94 ef	267.0 f	309.0 d	288.0 d	29.77 cd
T14	31.09 bc	14.0 jk	15.8 fgh	14.93 jk	150.4 j	153.4 i	151.9 k	14.26 gh
T15	27.3 de	18.0 hi	18.3 efg	18.14 hi	180.9 i	185.4 h	183.2 ij	17.58 f
T16	27.67 de	15.0 jk	18.1 efg	16.54 ij	177.2 i	182.5 h	179.9 j	17.26 fg
T17	33.73 ab	37.0 b	40.2 a	38.58 b	563.1 a	526.4 b	544.8 a	55.66 a
T18	34.35 a	44.0 a	42.1 a	43.07 a	523.2 b	561.1 a	542.2 a	57.52 a

Means with the same letter in each column are not significantly different at the 5% level of probability. For significant effect of year, the data for each year were analyzed separately.
*For abbreviations see Table 1

Table 3. Effect of the treatments on the amounts of phenolics, flavonoids and alkaloids, and on radical scavenging activity in *Physalis alkekengi* L. fruit

Treatment*	Total phenolics (mg g^{-1})	Total alkaloids (mg g^{-1})	DPPH scavenging (%)	Total flavonoids (mg g^{-1})		
				2015	2016	Mean
T1	73.6 i	2.06 h	25.05 g	251.2 de	239.1 c	245.2 efg
T2	76.4 ih	2.24 gh	26.14 fg	176.8 h	219.1 de	198.0 j
T3	87.8 cde	3.1 ef	27.70 d-g	291.8 a	208.6 ef	250.2 de
T4	83.9 efg	3.45 de	35.01 c	256.8 b-e	216.3 de	236.6 gh
T5	89.8 abc	3.29 ef	26.77 efg	263.1 bcd	253.1 a	258.1 cd
T6	89.3 bcd	3.28 ef	27.06 efg	296.5 a	239.2 c	267.9 ab
T7	93.5 a	7.14 a	59.02 a	234.8 fg	233.8 c	234.3 h
T8	92.6 ab	5.83 b	38.67 b	265.9 bc	252.7 a	259.3 bc
T9	89.8 abc	4.04 cd	35.54 bc	253.5 cde	213.3 def	233.4 h
T10	87.9 cde	4.02 cd	35.3 c	231.8 fg	241.4 bc	236.6 gh
T11	88.9 bcd	3.40 de	28.76 def	243.1 ef	222.6 d	232.9 h
T12	90.2 abc	3.07 ef	28.61 def	232.1 fg	202.5 f	217.3 i
T13	85.2 def	3.23 ef	26.46 efg	268.5 b	211.1 ef	239.8 fgh
T14	86.2 c-f	4.48 c	28.32 d-g	294.9 a	251.9 ab	273.4 a
T15	82.8 fg	3.56 de	29.66 de	224.1 g	242.3 abc	233.2 h
T16	84.5 ef	3.55 de	28.63 edf	250.4 de	243.1 abc	246.7 ef
T17	80.0 gh	3.01 ef	30.62 d	232.3 fg	163.9 g	198.2 j
T18	84.7 ef	2.74 fg	34.45 c	146.5 i	156.4 g	151.5 k

Means with the same letter in each column are not significantly different at the 5% level of probability. For significant effect of year, the data for each year were analyzed separately.
*For abbreviations see Table 1

reduced their positive effect on fruit number and fresh weight (Tab. 2 – part 3). Both concentrations of MB improved fruit number and fresh weight compared to the control. However, the addition of CA to MB strongly diminished this advantageous effect. The combinations of 25 ppm IBA with 0.5% MB and 50 ppm IBA with 1% MB were more beneficial than spraying each of them alone. The highest number of fruits and fruit fresh weight were recorded for the combination of 500 ppm CA, 50 ppm IBA, and 1.0% MB (43.07 and 542.2, respectively).

Fruit dry weight was increased by spraying with CA (27.01 g) and 25 ppm IBA (31.86 g), while it was reduced by using 50 ppm IBA compared to the control (27.2% reduction). Moreover, spraying with the lower concentration of MB produced no significant difference relative to the control, but increasing the MB concentration to 1.0% caused an improvement in fruit dry weight. Similar to fruit fresh weight, the use of MB with CA diminished fruit dry weight. While this trait increased by adding IBA to MB, the combination of 25 ppm IBA with 1.0% MB was an exception. The highest fruit dry weight was recorded for the combination of 1.0% MB with 50 ppm IBA and CA (57.5 g), whereas the lowest was obtained with the mixture of 0.5 % MB with 500 ppm citric acid (13.23 g).

Using 500 ppm CA reduced leaf fresh and dry weight compared to the control, but it had no significant effect on leaf number and leaf area (LA). At the lower concentration (25 ppm), IBA increased the number of leaves, LA, and leaf fresh and dry weight, whereas the higher concentration (50 ppm) had an adverse effect on these features except the number of leaves. The number of leaves, LA, and leaf fresh and dry weight were improved after spraying with MB at both concentrations, especially at the higher concentration. The greatest number of leaves and LA were obtained in the combination of 1.0% MB with 50 ppm IBA (267.1 and 188.8, respectively). Also, the greatest leaf fresh and dry weight was obtained in the combination of 1.0% MB with 50 ppm IBA and 500 ppm CA (72.2 and 9.29 g, respectively) (Tab. 2 – part 2).

Amounts of phenolics, flavonoids and alkaloids, and radical scavenging activity

The effect of the treatments on the total phenolic content of bladder cherry fruits is shown in Table 3. It shows that all of the formulations increased the total phenolic content in fruit compared to the control. The highest total phenolic content (93.5 mg g^{-1}) was found for 0.5% MB. All treatments increased the alkaloid content as compared to the control. Similar to the phenolic content, the greatest amount of alkaloids (7.14 mg g^{-1}) was recorded for 0.5% MB. CA and both concentrations of IBA produced no significant difference relative to the control with respect to the radical scavenging activity. However, the combination of 500 ppm CA with 25 ppm IBA significantly increased the radical scavenging activity (a 39.8% increase). Spraying with 0.5% MB induced a higher radical scavenging activity in comparison with 1.0% MB. Thus, the highest radical scavenging activity (59.02%) was achieved when 0.5% MB was used alone. The lowest activity was found in the control (25.05%).

Spraying with 500 ppm CA reduced the total flavonoid content relative to the control in 2015 and 2016 (29.6 and 8.4% reduction, respectively). Spraying with IBA increased the flavonoid content, but only the 50 ppm IBA produced a significant difference in relation to the control (245.2 mg g^{-1}). In contrast, the use of MB decreased the amount of flavonoids, although 1.0% MB produced no significant difference relative to the control (239.8 mg g^{-1}). The positive effect of CA combined with IBA depended on the concentration of IBA. The flavonoid content was increased by mixing the higher concentration of IBA with CA. The combination of MB with CA also improved the flavonoid content. In contrast, the mixture of MB with IBA reduced the flavonoid content. Thus, the maximum flavonoid content (273.4 mg g^{-1}) was recorded for the combination of 1.0% MB with 500 ppm CA, and its minimum (151.5 mg g^{-1}) was obtained with the formulation of 1.0% MB with 500 ppm CA and 50 ppm IBA (Tab. 3).

DISCUSSION

In recent decades, customer interest in more advantageous nourishment and government strategies concentrated on environmentally sustainable agricultural systems have both advanced a fast extension of organic farming (Rigby and Caceres, 2001). One of the recently introduced organic biostimulants is MB (MOG), which is manufactured by using fruit juice and crop residues, and contains various types of enzymes such as alkali proteinase, amylosine, glucamylase, xylanase, lipase, lyase, rubisco, transglutaminase, catalase, lipoxygenase, isomerase, urease, etc. In spite of the fact that the advantageous impact of biostimulants has been demonstrated on a few plants, it appears that in semi-arid areas utilization of biostimulants alone will not be adequate, and

concurrent use of other supplementary resources is unavoidable (Janmohammadi et al., 2014, 2016). This finding substantiates the thoughts of Rayan et al. (2012), who have stated that biofertilizers alone are not very effective on the dry lands of the West Asia-North Africa area. The best biostimulants are those containing multiple stimulants and enzymes (Janmohammadi et al., 2016), such as the formulations of biostimulants used in this experiment.

Analysis of the quantitative traits (plant height, main stem diameter, fresh and dry weight of aerial parts and roots) revealed that using the combination of MB with IBA and CA increased all measured parameters. The same trend was also observed for the bladder cherry fruit and its components (number of fruits per plant, fruit diameter, fruit fresh and dry weight). Plant height in bladder cherry showed no significant response to the spraying with CA, but the use of both concentrations of IBA and MB significantly increased plant height. The increment in plant height produced by spraying with MB might have been due to the positive effect of the enzymes on the increase in the number of nodes. Moreover, IBA belongs to a group of auxins which have an inductive effect on cell growth (Takatsuka and Umeda, 2014). Thus, the combination of 0.5% MB with 25 ppm IBA was the most effective formulation for plant height enhancement. The findings of the current study are consistent with those of Mafakheri et al. (2012), who found that concurrent application of organic fertilizer and biostimulants could significantly improve the height of dragonhead (*Dracocephalum moldavica* L.). Stem fresh and dry weights were increased in response to spraying with both IBA concentrations and MB, because the height, diameter, and the number of nodes of the stem had been enhanced. Applying exogenous enzymes in a liquid form as bioactive stimulants can have a positive effect on plant performance. In this regard, some studies have also been conducted on the use of several enzymatic compounds on *Dracocephalum moldavica* L. (Janmohammadi et al., 2014) and *Solanum tuberosum* L. (Janmohammadi et al., 2016). Alam et al. (2012) showed that spraying IAA, IBA, and naphthalene acetic acid (NAA) on *Catharanthus roseus* L. increased plant dry weight, photosynthetic rate, nitrate reductase and carbonic anhydrase activities, leaf nitrogen and potassium content, seed yield, leaf and root alkaloid content, and the amount of vinblastine. However, it seemed that plant height and stem diameter contributed more to the increment in stem weight than the number of stems. CA, when combined with IBA and MB, could improve stem weight, but the highest value of stem weight was obtained with the formulation of 25 ppm IBA and 5% MB. Moreover, these results are consistent with some reports for other plants (Karima and Abdel Wahed, 2005; Abou Dahab and Abd El-Aziz, 2006). Spraying with CA, both concentrations of IBA and with MB alone helped to increase the number of leaves and their weight, but the right combination of these compounds had the greatest impact.

MB combined with IBA and CA had distinct effects on fruit setting (fruit number) and fruit development (fruit diameter and fruit fresh weight). These results confirmed the study by Nahed et al. (2010) on the use of multiple amino acids and biostimulant compounds on *Thuja orientalis* L. Likewise, enhancement of fruit dry weight may be ascribed to the increase in fruit diameter. Moreover, it has been reported that the positive effect of biostimulants on plant yield might be due to the stimulating effect of amino acids and enzymes on the growth of plant cells. Also, a positive correlation has been reported between the spraying with biostimulants and enhancements in CO_2 absorption, stomata conductance, and yield production in the tea plant (Kumar and Thomas, 2004; Calvo et al., 2014). These data are in accordance with what was obtained by Neri et al. (2002) and El-Desuki (2004).

Enzymes are involved in several physiological and biochemical processes such as cell growth and expansion, differentiation and development, auxin catabolism, lignification, as well as abiotic and biotic stress responses (Siavoshi and Laware, 2013). For several physiological processes the action of enzymes is essential, e.g. in nutrient uptake, protein turnover, cell division, signal transduction, processing of polypeptide hormones, apoptosis and also in the biological cycle of plants. The biostimulant MB is a mixture of enzymes, therefore it can enhance plant growth, nutrient uptake, and the quantity and quality of yield in many crops (Nardi et al., 2016; Ordonez et al., 2006). Multienzyme complexes offer distinct metabolic advantages to the plant cell, and it seems that the natural function of the cellular plant is obtained from a specific enzyme-enzyme interaction. In addition, organic acids are used in the respiration and other biochemical pathways as a source of carbon skeleton and energy for the plant cells. Thus, it has been expressed that increasing endogenous organic acids can influence the biomass and yield of crops (Da Silva, 2003). Jafari and Hadavi (2012) showed that foliar-applied CA (0.1%, w/v) as

a biostimulant increased growth, biomass production, and essential oil yield in basil (*Ocimum basilicum* L.). IBA is a potential storage form for auxin in a variety of plants. It has been found that spraying with various auxin forms can increase plant dry weight, photosynthetic rate, nitrate reductase and carbonic anhydrase activities, and leaf nitrogen and potassium content (Strader et al., 2010; Enders and Strader, 2015). Therefore, it is postulated that IBA has a crucial effect on the sensitivity, transport, and function of auxin (Enders and Strader, 2015). Our results show that MB, IBA, and CA induced growth acceleration in various plant organs such as the leaf, stem, root, and fruit. This phenomenon may have occurred due to improved sink and source relations, increased water absorption and nutrient uptake, abiotic and biotic stress alleviation, hormonal balancing, etc. (Kauffman et al., 2007; An et al., 2014; Enders and Strader, 2015). The results of this study are in agreement with a study by Kamari et al. (2013) on tomato, which showed that the use of humic acid improved the height, crown diameter, and fruit number of the tomato plant. In addition, the results of this study correspond with those of the experiment on *Vicia faba* L. by El-Ghamry et al. (2009). They reported a positive effect of a mixture of enzymes, humic acid and amino acids on enhancing yield and growth parameters. Also, it has been reported that crop yield can be increased by spraying amino acids at different stages of plant development (Liu and Lee, 2012). Plant enzymes with plant growth regulators as biostimulants are a necessary component for the production of various amino acids in protein synthesis, energy production, maintenance of the structural integrity of bio-membranes, and growth regulation. Moreover, several amino acids serve as precursors for the synthesis of secondary metabolites.

The present study has revealed that MB (0.5%) could enhance the radical scavenging activity and total content of phenolic and alkaloid compounds in bladder cherry fruit. By comparison, CA and the two IBA concentrations increased the total phenolic and alkaloid content. It has frequently been reported that phenolic compounds play a major role in radical scavenging activity (Kauffman et al., 2007; Calvo et al., 2014). The present results are evidence that the change in radical scavenging activity under the various formulations was similar to the change in the total phenolic content. Thus, one can conclude that the radical scavenging activity in bladder cherry is attributed to the total phenolic content. Oxidative stress can cause some diseases, such as chronic diseases and cancers, affecting human health (Kumar and Thomas, 2004; Calvo et al., 2014). Thus, the application of MB is important for increasing phenolic compounds and the radical scavenging activity of bladder cherry. Finally, it is possible that the application of MB has no direct effect on the production of some plant metabolites, but it can cause plant growth with an indirect effect on other metabolic pathways and growth characteristics. Each enzyme in MB will undoubtedly be able to stimulate a particular metabolic pathway. Therefore, it will be important to make use of their multiple application in the cultivation and production of medicinal plant metabolites.

CONCLUSIONS

This study revealed a positive effect of MB, IBA, and CA on the growth traits of the stem, leaf, and fruit. Also, the optimum combination of these compounds can improve the growth parameters of bladder cherry (*Physalis alkekengi* L.) as a medicinal plant. The effectiveness of MB can be due to existence of several major enzymes that can promote the metabolic activities, especially the production of amino acids. MB had the greatest effect on increasing phenolic and alkaloid compounds, and radical scavenging activity. However, the formulation of 1% MB with 50 ppm IBA and 500 ppm CA is recommended for improving the fruit yield of bladder cherry. The multienzymatic biostimulant had the greatest effect on increasing the amounts of alkaloid compounds as the main pharmaceutical metabolites of bladder cherry.

ACKNOWLEDGEMENT

We thank the research group of the Department of Cultivation and Development of Medicinal Plants for making their laboratory facilities and equipment available to us.

FUNDING

The research was supported by the Institute of Medicinal Plants, ACECR.

AUTHOR CONTRIBUTIONS

All of the authors contributed to all aspects of this manuscript, including the development of the ideas, writing, and revisions of the content.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Received September 13, 2017; accepted December 20, 2017