Seed oil quality of GA3 induced flowering evening primrose (*Oenothera biennis* L.)

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Abstract

Gamma-linoleic acid in the seed oil of evening primrose makes it nutritionally and pharmaceutically valuable. If evening primrose wants to be cultivated as an annual plant, sowing time is important. By the late sowing and depends to time most plants do not produce flower stem and stay in rosette stage until next spring. To solve this problem, the present study was performed. Seedling were transplanted to 4 kg plastic pots containing a mixture of leaf compost: local soil: perlite (1:2:1 ratio) and placed in outdoor conditions. Non-vernalized plants were then selected and treated with different temperature regimes (1, 2 and 3 weeks in 4-6ºC) and gibberellic acid (GA3) application in different concentrations of 0, 500, 1000, 2000 ppm, separately. The results showed that although some physiological parameters were affected by low temperature, no flower stem was produced. In contrast, plants that were treated with GA3 produced flower stem. Time of flowering and the number of flowers were significantly affected by the concentrations of applied GA3. Although yield components of GA3 induced plants were higher than that of normal growth plant, the seed yield of them was low. Surprisingly, the gamma linolenic acid (GLA) percentage of seed oil of plants treated with 2000 ppm GA3 was significantly higher than that of non- treated plants. Finally, it can be concluded that, although the application of GA3 guarantees delayed sowing evening primrose flowering and better GLA production, but low seed yield is a subject that cannot be easily ignored in production.

Key words: evening primrose, flowering, gamma linolenic acid, gibberellic acid

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Introduction

Evening primrose (*Oenothera biennis* L.) belongs to the Onagraceae family and is cultivated because of its valuable seed oil. The oil of evening primrose is a rich source of γ-linolenic acid (GLA), a rare beneficial fatty acid (Gimenez et al. 2013; Ghasemnezhad and Honermeier 2007; Murphy et al. 2004). It is a biennial plant and cultivated in eastern and central North America. Despite to the other benefits, evening primrose oil can improve or treat atopic eczema, cardiovascular disease, acne and diabetic. (Park et al. 2014; Bamford et al. 2013; Omran 2012; Zaitone et al. 2011). There are different sources of GLA, but the absorption of this compound as well as the good response of the human body to

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the evening primrose oil, make it the best source (Lapinskas 1993). Evening primrose has some cultivation limitations (Gimenez et al. 2013; Ghasemnezhad and Honermeier. 2007).

Some studies showed that good yielding of seed and oil mostly depend on the environmental conditions of plant growth habitat (Gimenez et al. 2013; Yaniv et al.1989). Evening primrose as a biennial plant needs some requirements like vernalization or long day photoperiods for seed formation (Ghasemnezhad and Honermeier 2007; Liu et al. 2003; Lapinskas 1993; Reekie et al. 1991). Depending on the vernalization demand and variety in Central Europe evening primrose should be cultivated in late summer or at beginning of spring (Ghasemnezhad and Honermeier, 2007). By late sowing the cold temperature requirement does not complete, the plant stays at rosette stage till next winter to flower (Gimenez et al. 2013; Ghasemnezhad and Honermeier, 2007). Some researchers believe that biennial plant must get an optimum size to initiate flowering response to environmental situation (Gimenez et al. 2013; Klinkhamer et al. 1987; Gross 1981; Werner 1975). When environment restricts growth, biennial behaviour will occur and the plant is not permitted to flower and stay in its vegetative phase (Kagaya et al. 2009). Gibberellin (GA) derivatives as a plant hormone involves in the regulation of many phenomena in the plant life cycle. It stimulates seed germination, triggers transitions from meristem to shoot growth as well as from vegetative to flowering phase (Gupta and Chakrabarty, 2013, Sumanasiri et al. 2013). It has been shown that GA3 application under in-vitro conditions plays a key role in flowering (Gantait and Sinniah 2012). The effect of GA3 on flower induction is a multiprocessing that can replace some mechanisms like long day photoperiod, cold requirement and promotes plant to start flowering under unusual conditions (Sumanasiri et al. 2013; Wilkie et al. 2008). It also has been reported that GA3 can start flowering in ornamental plants (Halvey 1990) and substitute cold temperature requirements of plants (Song et al. 2003; Chang and Sung 2000, Christiaens et al. 2012).

In most plants, dormancy breaking down in productive parts of the plant like flowers will do by reaching their cold requirements and this temperature range is variable from plant to plant (Arora et al. 2003). Some previous research indicated that, cold requirement can be replaced by GA3 treatment (Song et al. 2003; Chang and Sung 2000). As, the most important biochemical and biological process of a plant take place under certain temperature regimes, it plays an important role in plant life cycle (Vaz et al. 2004). In a study it has been showed that, pre-treating of the root of witloof chicory (*Cichorium intybus* L. cv. Tar¬dive d'Anvers), including keeping at 3 °C for 8 weeks, 3 days in complete anoxia at 15 °C, or 4 days in the presence of ethylene (1000 ppm) at 15 °C, induced plants to flower compared with control (Joseph et al, 1985). In present study, the effect of cold temperature and GA3 application on the late planted evening primrose was investigated. The aim of experiment was to find a way how to stimulate flowering in non-flowered evening primrose.

Table 1
Meteorological information during plant growing, Gorgan, 2015-2016

Months	Jan 2015	Feb 2015	Mar 2015	April 2016	May 2016	Jun 2016
Weather factors						
Max. T (˚C)	24.4	22.2	20	37.8	33.4	45.4
Min. T (˚C)	-00.4	-02.0	-02.0	03.4	09.4	17.3
M.S.H (H)	05.2	03.1	03.9	04.9	07.2	08.2
T.R.A (MM)	13.6	18.09	60.6	26.7	07.4	0.00
M. R. H (%)	73	78	77	77	66	53

Max.T= Maximum Temperature, Min.T= Min Temperature, M.S.H= Mean of Sunny Hour, T.R.A= Total precipitation, M.R.H= relative humidity.

Materials and Methods

Present research was done in two different experiments to see how it is possible inducing flower in plants which not produce flower due to late sowing. Study was done in Gorgan University of Agricultural Sciences and Natural Resources, as two different pot experiments.

Experiment 1

The seed samples were obtained from the research field of Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran. Seeds were sown at 14 Jan of 2015. The EC and pH of used soil mixture were, 2.1 days/m and 7, respectively. Plant irrigation was manually done when it was necessary. At four leaf stages, at 25 March of 2015, at four leaves stage, the seedling was transferred to the 25cm diameter pots containing 6kg soil mixture of leaf compost: local soil: perlite (1:2.:1 ratio) and kept under outdoor conditions. Weather information is provided in Table 1.

After 3 months, non-flowered plants were selected and treated with GA3 (Fig I). To stimulate rosette plant flowering, four different levels of GA3 (0, 500, 1000, 2000 ppm) were spread two times one-week interval. The first application was done on 8 Jun 2015 and the next

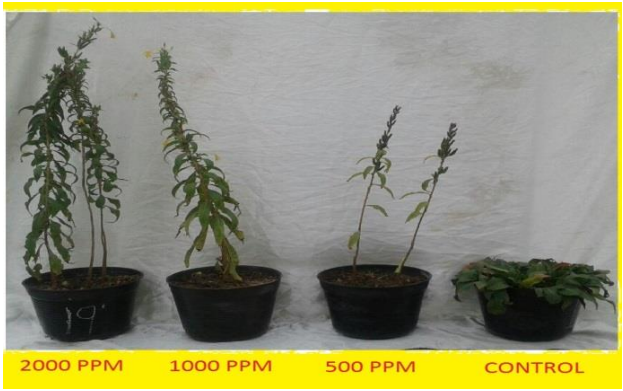

Fig. I. Flower induction in evening primrose using GA3

one week later. For that, approximately 200 ml of GA3 in different concentrations was sprayed on the central part of plant canopy. Morphological changes of experimental plants were inspected every day to record the time of stem elongation and flowering. The length and diameter of the flowering stem as well as the leaf number of the flowering stem, chlorophyll content of leaves, total number of flowers per plant, seed yield, oil content and the fatty acid composition of GA3 treated plants were measured and compared with the normal plants.

The fatty acid composition of the oil was analysed by Varian CP 3800 gas chromatography with a modified method of Court et al. (1993). For that finely ground seeds (0.3 g) were weighed and were put into a 10 ml glass test tube. Then 3 ml diethyl ether was added to the tube and sample was centrifuged at 4000 r.p.m for 10 min. To remove the solution, the samples were transferred to a vacuumed desiccator for 1h. Sodium methylate (2 ml) was added to the test tube for desertification. The mixture was kept in the dark for 30 min. Then 1 ml of isooctane was added to the mixture. As isooctane is not soluble in sodium methylate, two liquid phases were formed in the tube and most part of the fatty acid esters were transferred to the isooctane portion. Isooctane phase was pipetted to a glass vial and became ready for GC analysis. Oil analysis was performed on a Varian CP 3800 gas chromatograph with duel FID detector. For that a Permabond® FFAP column (25 m × 0.25 mm i.d., film thickness 0.25 µm) with CP-SIL 88 foe FAME stationary phase equipped with CS-Fused-Silica pre-column was used.

One microliter injection was made with Varian 8200 CX auto sampler. The column oven was temperature programmed from 200 to 240 at 10 °C/min and held to 220 for 1 min. The injector temperature was 280 °C, detector, 280 °C and the flow rate was 2.0 ml/min. Obtained data were statistically analysed using Analysis of Variance Procedure (ANOVA) by the use of SAS program. The experiment was performed as a randomized complete design with 4 treatments and each treatment had five pots (replications) including three plants per pot.

Experiment 2

All applications from seed sowing to treatments application were similar to experiment 1. The non-flowered plants at rosette stage were divided into three groups containing four pots (replications). The first group was kept under normal conditions as control samples.

Second group was kept in a cool chamber (7 ˚C) for 10 days. Third group was kept in the same conditions for 20 days. At the end of the experiment, the proline, phenols and chlorophyll contents of experimental plants were measured to see the physiological response of plants to the treatment. The proline content of the samples was measured according to the methods of Bates et al. (1973). Phenol content was measured based on the proposed method of Ragazzi and Veronese (1973). Chlorophyll content of the leaves was measured using a portable chlorophyll meter (model SPAD-520, Japan). This experiment was done based on the completely randomized design with three treatments (control, 10 days, and 20 days of cold temperature) and four replications.

Results

Experiment 1

Variance analysis of data showed that all of measured parameters involved in flowering were significantly affected by GA3 application (Table 2). The first flower of plants was treated with 500 ppm of GA3 was observed 41 days after treatment application. As the concentration of used GA3 increased, the days needed to flowering decreased. Only 29 days after GA3 application in the concentration of 2000 ppm, the plants start to flower. This time was nearly two weeks earlier than that of plants were spread with 500 ppm of GA3. Although the distance of flowering of plants when treated with 1000 and 2000 ppm GA3 was two days only, but the observed difference was significant statistically (Table 2).

The first flower of plants which were treated with 500 ppm of GA was observed 41 days after treatment application. As the concentration of GA increased, the days needed to plant flowering decreased. Only 29 days after treatment application, the plants start to flower. This time was nearly two weeks earlier than that of plants which were spread with 500 ppm of GA. Although the distance of flowering of plants when treated with 1000 and 2000 ppm GA was two days only, but statistically was significant (Table 2).

As it can be seen in Table 3, non-treated plants did not produce any flower and even flowering stem. Besides the time of flowering, yield components like length, diameter and leaf number of the flowering stem, Chlorophyll content, and total flower were significantly

Table 2.
Results of ANOVA for the effect of GA application on flowering of *Oenothera biennis*

Sources	DF	TF	SPAD	FMS	DFS	LFS
Treatments	1853.07**	598.07**	348.58**	673.27**	0.29**	813.92**
error	0.85	61.85	4.05	3.5	0.002	2.2
cv	3.46	58.59	5.54	11.62	14.39	8.49

FMS: flower number of main stem, DFS: diameter of flowering stem, LFS: length of flowering stem, SPAD: chlorophyll (SPAD values), TF: total number of flower, DF: days to flower

Table 3.
Mean value comparison of the effect of GA on plant parameters of flowering phase of *Oenothera biennis* L.

GA concentration (mg/l)	0	500	1000	2000
Length of flowering stem(cm)	0	16.6c	23.8b	29.4a
Diameter of flower stem(cm)	0	0.298b	0.48a	0.54a
Leaf number of flowering stems	0	16.0 c	21.6b	26.8a
Chlorophyll content	48.8a	33.2b	31.0b	32.4 b
Total flower number(per plant)	0	10.8b	20.0ab	24.8a
Duration to flower (days)	0	46.0a	31.2b	29.2c

influenced by applied GA3 concentrations. Table 3 shows, plants which were treated with 2000 ppm of GA3 were taller than those of the rest concentrations (16.6, 23.8 and 29.4 cm in 500, 1000 and 2000 ppm of GA, respectively). Plants were treated with 500 ppm were shorter than those plants treated with 2000 ppm of GA3 (Fig I). As can be seen in Fig I, GA3 more than 1000 ppm produced more leaves and flowers. Although the flower stem production taken place by using 500 ppm GA3, the plants seems to be very weak. Furthermore, GA3 also had a significant effect on the stem diameter of plants. Stem diameter of herbaceous plants is an important factor to protect plants against lying down. Thus, plants in which treated with 2000 ppm are stronger than others (see fig 1). Leaf as the main photosynthesis centre, plays an important role not only in plants but also other organisms of the world like Bacteria or Alga. As presented in table 3, plants treated with 500 ppm of GA3 had the lowest number of leaves (16 leaves). Contrasts to that, 26.8 leaves were produced in plants treated with 2000 ppm of GA3. Surprisingly, no significant difference in the content of chlorophyll was observed between different levels of GA3.

As the data show in Table 4, plant height of those plants treated with GA3 was varied. As the concentration of hormone increased from 500 to 1000 ppm, the plant height significantly increased (31.3 cm and 85.1 cm, in 500 to 1000, respectively). Despite to that, control plants were taller than those of plants which were treated with GA3 at 500 ppm. Versus to other treatments, plants which were sprayed with 2000 ppm GA3 tended to produce more side shoots. Both the number and the length of capsules of plants treated with 2000 ppm of GA3 were higher than that of plants treated with 500 ppm of hormone. Compare to control plants, no significant difference was observed in these cases. As it can be seen in Table 4, no significant difference was observed on seed yield of GA3 treated plants.

Results show that from the six main fatty acid constituents of evening primrose, two of them were influenced by used treatments. Seed oil of plants which treated with 2000 ppm GA3, produced the highest GLA percentage. Versus to the expectation this value was even higher than that of normal growth plants. The finding was in front of the LA content. The highest LA rate was observed in control samples and increasing the GA3 concentration tends to reduce the LA percentage.

Experiment 2

All plants treated with cold stress (4-7 ˚C), didn't show any morphological difference compared with the control plants. The rosette phase continued during the experiment and even several months after the end of the experiment. Observations showed that no flower bud and flower stem was produced in the plants which were treated with different cold temperature regimes. Versus to the morphological respond of plant to GA3 application, chemical analysis showed that the contents of proline and total phenol of leaves, two important factors involved in plant response to cold stress, were significantly influenced by the temperature regimes (Table 6).

As it can be seen in table 7, the proline and phenol contents of experimental plants were dramatically increased by increasing the period of treatment. It has been found that the response of the plant to proline production started 4 days after treatment application. Based on the Table 5, the proline content of the plants in which treated with cold temperature for 10 days (10-day group) had 0.71 µg/g proline compared with the control plants (0.23µg/g). When the length of treatment duplicated, the proline content of the samples increased to1.09 µg/g. A similar tendency was also observed in the variation of phenol content. As it can be seen in table 5, when the treatment application duration was increased from zero to 20 days, the proline content increased from 2.23 µg/g in control samples to 2.69 µg/g in the samples which were under the cold temperature (Table 7).

Discussions

Table 4.
Yield and yield components of evening primrose treated with GA compared with control

	Plant height (cm)	Number of side shoots	Number of capsules	Length of capsule (mm)	Seed per capsule	Seed yield (g/plant)
Control	66 b	1.7 b	116.0 a	19.8 ab	133 b	5.2 a
GA 500	31.3 c	2.0 b	42.0 b	16.33 b	143 b	0.83b
GA1000	85.7 a	5.0 b	72.7 ab	19.5 ab	165 b	1.8 b
GA 2000	81.3 a	14.7 a	130.0 a	21.9 a	225 a	2.2 b

Control; normal flower produced plant, PA; palmetic acid, SA; stearic acid, OA;oleic acid, LA; linoleic acid, GLA; gamma linolenic acid

Table 5.
Fatty acid composition of GA induced plants compared to non-treated evening primrose

	PA (%)	SA (%)	OA (%)	LA (%)	GLA (%)
Control	8.0 a	5.8a	25.6a	56.7a	6.7b
GA 500	8.0a	5.7a	21.0ab	59.0a	7.6ab
GA1000	7.8a	5.8a	23.5ab	58.0a	7.2ab
GA 2000	8.0a	5.9a	19.2b	60.4a	8.1a
p-value	0.590	0.895	0.039	0.079	0.016
LSD	0.62	0.768	5.962	4.01	1.052

Control: normal flower produced plant, PA; palmetic acid, SA; stearic acid, OA; oleic acid, LA; linoleic acid, GLA; gamma linolenic acid

Table 6.
Results of ANOVA for the effect of cold temperature on the proline and phenol contents of the evening primrose plant leaves.

Sources changes	df	Proline	Phenols
Treatments	6	0.32**	0.34**
error		0.008	0.01
cv		11.94	3.98

Table 7.
Mean value comparison of the effect of cold temperature on the proline and phenol content of evening primrose leaves

	Treatments	Proline (µg/g)	Phenols (mg/g)
10 Day Group.	C	0.23 ± 0.33c	2.23 ± 0.17c
	A1	0.69 ± 0.31b	2.33 ± 0.16bc
	A2	0.71 ± 0.33b	2.35 ± 0.18bc
20 Days Group.	B1	0.72 ± 0.27b	2.42 ± 0.18b
	B2	0.75 ± 0.11b	2.45 ± 0.11b
	B3	1.04 ± 0.12a	2.91 ± 0.11a
	B4	1.09 ± 0.12a	2.96 ± 0.12a

C: control, A1: 4 days (10 day group), A 2: 8 days (10 day group), B 1: 4 days (20 day group), B 2: 8 days (20 day group), B 3: 12 days (20 day group), B 4: 16 days (20 day group).

Evening primrose categorized as a biennial plant (Greiner and Kohl 2014). In nature, the seeds germinate late in summer and before winter plant should be formed enough leaves to guaranty itself against cold temperature. The flowering stem appears in spring and the seed formation finished in the next summer (Wagner 2007). When seeds planted late, in most cases plant does not flower and stay in rosette form till next winter (Ghasemnezhad and Honermeier 2007). If evening primrose wants to be a commercial plant, the field occupation should be

reduced. Vernalization demand of evening primrose varied in different varieties. Some varieties like "Anoter" have low vernalization demand and can be used in annual cultivation by planting early in spring (Ghasemnezhad and Honermeier 2007).

It has been reported that evening primrose has different requirement for flower induction and formation (Johnson 2007; Greinerand kohl 2014). As it has been mentioned before, reproduction behaviour of this plant depends on the genotype and temperature (Greiner and Kohl 2014). For vegetative growth, this plant species requires 16 h light and 8 h dark, but this photoperiod is not just for vernalization (Johnson et al. 2009). The best temperature that this plant can complete its life cycle is about 18-22 ˚C and if the temperature rises up to more than 27˚C during vegetative phase, flower induction might be inhibiting by environmental conditions (Gimenz et al. 2013; von Arx et al. 2012). This plant shows different behaviour response to flowering and vernalization requirements (Greiner and Kohl 2014). It has been reported that in some species of Oenothera vernalization happen (Clough et al. 2001; Wilkins and Anderson 2007). It seems that in evening primrose, flower induction depends on the long-day exposure and cold temperature, but the cold-warm temperature is more effective than continual cold (Picard 1965). In addition, in seedling stage a better response to vernalization will occur (Gimenz et al. 2013). When late sowing time in early spring encounters biennial plants to warm temperature and long day, the transition from vegetative to reproductive phase will stop and plant stay in rosette phase till next winter. In this case the total yield of plant reduces extremely. The results of the present experiment showed that GA3 application stimulates flower induction and final yield of even old rosettes. In Arabidopsis it shows that, GA3 application induces flowering via stimulating the expression of gens like AGL20 (Borner et al. 2000) and GAMYB-like genes (Gocal et al. 2001). It had also been shown that GA3 stimulate the photosynthetic activity of plants by fixing CO2 and perhaps activating some enzymes involved in the dark reactions (Kozłowska et al. 2007; Starck et al, 1987). There is an important relation between plant hormones and the activities of genes involved in carbohydrate metabolism (Thomas and Rodriguez, 1994). Results showed that, opposite to the control samples and the samples in which treated with different cold regimes, not only the stem flower appeared in GA3 treated plants, but also the plants produced flower and seeds. In our research, although most yield parameters such as stem length, number of capsules and flowers of GA3 flower induced plants, were higher than that of controls, but the seed yield of normal flowered plants was higher than those of GA3 induced plants. Although flowering and seed production take place in GA3 induced plants, but the plants did not have enough time to produce enough full matured seeds. Results showed that, beside the higher number of seeds per capsule of 2000 ppm GA3 treated plants, the higher number of immature seeds in this treatment is the reason why the seed yield is lower than that of the normal growth plants. This could be the question point of the present study, when do the plants should be sprayed by GA3?

It has long been known that, in oilseed crops (e.g. oilseed rape, sunflower and flax) the extent of desaturation in the fatty acid composition of the seed oil is inversely related to the prevailing temperatures during seed maturation (Canvin, 1965). It seems that this is true in evening primrose too, as noted by Levy et al (1993). At warmer altitudes plants tend to produce oil with lower gamma-linolenic acid content (Simpson and Fieldsend, 1993). On the other hand, in some studies, it has been described that low temperature increases the percentage of GLA (Levy et al. 1993; Fieldsend and Morison, 2000; El-Hafid et al. 2002). Sumitomo (2009) showed that there is a linkage between GA3 and cold temperature on flowering and the response of short day chrysanthemum to cold temperature was substituted by GA3. But, if the plant does not response to cold temperature, it does not respond to GA3 too (Sumitomo et al. 2009). Our finding was quite different. Plants which did not answer to cold temperature during a certain period of time, were strongly influenced by GA3 application and flowered even with the lowest concentrations of this compound (500 ppm). Results showed that although rosette plants produced flower stem and flower, but the seed

yield and other measured components were strongly influenced by the concentration of GA3. In another study, it has been reported that, applying of GA3 on *Bleamcanda chinensis*, increased some factors like plant height, flower number, leaf width, flower length, and rhizome weight and reduced the needed time of flowering (Bhuj et al. 1998). Our result showed that after applying GA3, evening primrose showed a significant difference with untreated plants and parameters like length of the flower stem and total flower increased. This observation is in accordance with some previous studies (Sumanasiri et al. 2013; Bhuj et al. 1998). The content of chlorophyll of plants which were treated with GA3 was significantly lower than that of treated plants. This difference is explainable by the growing phase of the plant. Plants which are at their vegetative phase have more assimilation than their reproductive phase.

Similar to our finding it has been reported that Chlorophyll content of leaves was decreased by increasing GA3 concentrations (Monselise and Halevy 1962). The lower chlorophyll content of GA3-induced plants can be explained by the mechanism of the effect of GA3. When plant treated with the external hormone, the balance of phytohormone and carbohydrate shift through reproductive phase. GA3 by affecting Phospho-synthase activity, enhances carbohydrates production (Wardlaw, 1990). This could be the reason why, the chlorophyll production capacity of leaves reduces. Vaz et al (2004) showed that treating the plant with different temperature regimes significantly differs the flowering potency of the plant (Vaz et al. 2004). Finding of Christiaens et al (2012) showed that applying cold treatment cause progressive reduces with time to flowering in *Helleborus niger* and *H. x ericsmithai* (Christiaens et al. 2012). It has been also showed that, cold temperature on corms of (*Liatris spicate* L.) 'Gloriosa' increased percentage of flowering and with an increasing cold period, this effect increased (Waithaka and Wanjao 1982). The finding of us in this area does not support the results of other researchers (Christiaens et al. 2012; Waithaka and Wanjao 1982). The reason why in this experiment plants did not produce flower might be referring to the time of treatment application

Conclusion

Although the application of GA3 on late planted evening primrose in winter induces flowering, but the seed and oil yield as well as the oil quality as important parameters in evening primrose cultivation should be noticed. Lower seed yield and higher oil quality which found in this research led us to more investigation. Versus to the finding of the other researchers, no response of plants to cold temperature means the age of plant in treating time is important. It means that plants should be at their seedling stage to influence by cold temperature. Thus, based on the finding of the present study it can be concluded that by GA3 application of the evening primrose plant in which does not receive enough cold temperature, it is possible to induce flower production of plant and force it to act as an annual plant. By this, the occupation time of the field reduces to one growing season and economically helps the farmer. For further investigation, it suggested that the response of the plant to cold temperature evaluate in different stages of plant development to be sure whether the flowering of evening primrose influence by cold temperature or not.

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