International Journal of Horticultural Science and Technology
Journal homepage: https://ijhst.ut.ac.ir

Interaction of Nitrogen and Ethephon on Female Flower Formation and *Cs-ACS2* Gene Expression in Cucumber (*Cucumis sativus* L.)

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ARTICLE INFO

Article history:

Received: 29 May 2024,
Received in revised form: 26 February 2025,
Accepted: 10 March 2025,

Article type:

Research paper

Keywords:

Aminocyclopropane-1-Carboxylate Synthase gene families (*Cs-ACS2*),
Cucumber,
Ethylene,
Female flower,
Nitrogen

ABSTRACT

Cucumber (*Cucumis sativus* L.) has long served as a model plant for studies on sex expression and floral development. This study investigated the effects of exogenous ethephon (0.5 mM) and urea (2 and 4 g L^{-1}) on female flower production in cucumber plants. The results showed that the highest number of female flowers was observed in plants treated with 4 g L^{-1} of urea. Ethephon treatment reduced the total number of flowers and decreased the male-to-female flower ratio, thereby promoting female flower development. The highest catalase activity was recorded in plants treated exclusively with ethephon, whereas the greatest peroxidase activity was observed in the control plants. In ethephon-treated plants, the expression level of the *Cs-ACS2* gene in female floral organs increased by approximately 4.5- to 6-fold, while in leaf tissues, it increased by 1.5- to 3-fold. Given that ethephon treatment resulted in the highest expression of *Cs-ACS2*, it can be inferred that ethephon promotes female flower formation through upregulation of this gene. In contrast, the effect of nitrogen appears to be independent of the *Cs-ACS2* expression pathway. These findings provide valuable insights for future research on the physiological mechanisms underlying flower induction in cucumber plants.

Introduction

Cucumber (*Cucumis sativus* L.), a monoecious plant, is considered one of the most economically important vegetable crops (Martínez et al., 2014). It has long served as a model species for the study of sex determination (Li et al., 2012). Although sex expression in cucumber is genetically controlled, it is also influenced by various external factors, including plant hormones and environmental conditions. Among these, phytohormones—particularly ethylene—and environmental cues such as low temperature, high light intensity, short day length, and low nitrogen availability, are known to promote female flower formation (Lou et al., 2023).

Nitrogen (N) is an essential macronutrient for plant growth and development. It is well documented that enhanced vegetative growth and adequate photosynthetic capacity contribute significantly to flowering and fruit formation (Kumar et al., 2013). Andrean et al. (2017) examined the effects of nitrogen and potassium fertilizers at four levels on the yield characteristics of Italian zucchini. They reported significant increases in leaf number and total flower count at the highest levels of nitrogen and potassium, although the number of female flowers increased only slightly. Due to its high nitrogen content (46% by mass) and low cost, urea is

the most widely used nitrogen fertilizer in global agriculture, accounting for approximately 50% of total nitrogen fertilizer consumption (Lupini et al., 2017). Ma et al. (2019) demonstrated that urea enhances the expression of genes involved in nitrogen metabolism, leading to more efficient amino acid synthesis and improved plant growth. Furthermore, elevated nitrogen levels—often achieved through urea application—are associated with an increased production of female flowers, which are critical for fruit development.

In many Cucurbitaceae species, the production of female flowers is primarily regulated by the hormone ethylene (Boualem et al., 2015). Ethephon, an ethylene-releasing compound, is widely used to stimulate female flower production in cucurbits (Hidayatullah et al., 2009). Arabsalmani and Rashidi (2017) investigated the effects of ethephon at four concentrations (0, 100, 200, and 300 ppm) across three developmental stages (3-leaf, 6-leaf, and early reproductive growth) in cantaloupe. They found that the highest yield was achieved with 100 ppm ethephon applied at the 3-leaf stage.

1-Aminocyclopropane-1-carboxylic acid (ACC) is a key precursor of ethylene biosynthesis, and the expression of ACC synthase (ACS) genes plays a central role in regulating ethylene production in plants (Saito et al., 2007). The polymorphic ACS genes are associated with female flower development and are conserved across several cucurbit species. For example, *Cit-ACS4* in watermelon, *Cm-ACS7* in melon, *Cs-ACS2* in cucumber, and *Cp-ACS27A* in squash have all been linked to enhanced female flower production (Manzano et al., 2016). Specifically, *Cs-ACS2* not only increases ethylene biosynthesis but is also positively regulated by ethylene itself, creating a self-amplifying feedback loop (Li et al., 2012).

Throughout evolution, higher plants have developed a range of defense mechanisms that enable them to survive under environmentally adverse conditions. Among these, antioxidant defense enzymes such as catalase (CAT) and peroxidase (POD) play a crucial role in scavenging free radicals, including reactive oxygen species (ROS), thereby mitigating oxidative stress (Lall et al., 2002). However, due to the inherently low resistance of cucumber plants to environmental fluctuations, abiotic stresses often accelerate senescence and inhibit photosynthesis by promoting excessive ROS accumulation (Li et al., 2016). ROS are continually produced as byproducts of natural metabolic processes, including nitrogen metabolism, in plants (Xu et al., 2014).

Ethylene, often referred to as a stress hormone, has been reported to be closely associated with ROS generation, which in turn stimulates ethylene synthesis (De et al., 2003). Jaiswal et al. (1985) demonstrated that female flowers induced by ethephon in cucumber exhibit higher peroxidase activity compared to male flowers.

The primary objective of this study was to investigate the individual and combined effects of ethylene and nitrogen on flowering behavior and flower sex expression in cucumber plants, with the aim of enhancing overall crop yield. Furthermore, the study examined the influence of ethephon and nitrogen treatments on various molecular characteristics to better understand their physiological roles in the flowering process.

Material and methods

Experimental design and statistical analysis

This pot experiment was conducted using a split-plot arrangement based on a completely randomized design with three replications. The main plot included two concentrations of ethephon: 0 mM and 0.5 mM. The subplots consisted of three levels of urea (0, 2, and 4 g L^{-1}), which were applied as foliar sprays at three different growth stages (Table 1). Morphological, phytochemical, and physiological parameters were statistically analyzed using Statistix 8 software. Gene expression levels were evaluated using SAS 9 and GENEX 6 software. Mean comparisons were performed using the Least Significant Difference (LSD) test at 5% and 1% probability levels.

Table 1. Applied treatments in this experiment and their abbreviations.

Treatments	
Control	E0N0
Urea (2 g L^{-1}) without ethephon	E0N1
Urea (4 g L^{-1}) without ethephon	E0N2
Ethephon (0.5 mM) without urea	E1N0
Ethephon (0.5 mM) with urea (2 g L^{-1})	E1N1
Ethephon (0.5 mM) with urea (4 g L^{-1})	E1N2

Plant growth conditions

Homogeneous cucumber hybrid seeds (cv. 'Victor,' purchased from Seminis Co.) were placed in petri dishes containing a sheet of damp filter paper and incubated at room temperature until germination occurred. The germinated seeds were subsequently planted in plastic pots that were 30 cm in length and 32 cm in diameter, ensuring adequate drainage and using a growth medium at a depth of 1.5 cm. The growth medium (Sabz Sinasaran Co., Iran) comprised lotus peat (50%), peat moss (20%), coco peat (10%), vermicompost (15%), sand (2%), perlite (2%), and vermiculite (1%). The physicochemical properties of the growth medium are presented in Table 2. During the growth period, the plants were subjected to a photoperiod of 10 h of darkness and 14 h of light, with average daytime and nighttime temperatures of 29 and 19 °C, respectively.

Table 2. Physicochemical parameters of growth medium.

N (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})	Sand %	Silt%	Clay%	Text soil	O.C%	pH	EC (mS m^{-1})	T.N.V%
7.000	0.043	0.588	49	28	23	Loam	7.2	7.6	3.9	29

Experimental design and treatments

Urea, as a nitrogen source, and ethephon, an ethylene-releasing compound, were applied as foliar sprays at periodic intervals. The first urea treatment was applied when the plants had developed two fully expanded true leaves. Three days later, ethephon was sprayed at a concentration of 0.5 mM. The second urea application was performed five days after the first, followed again by ethephon three days later. This sequence was repeated for a third stage of treatment. The final stage of foliar application was conducted just prior to flowering. Control plants were treated with distilled water at each stage.

Morphological assessments

Morphological traits assessed included the number of male and female flowers, as well as the male-to-female flower ratio. Flower counts were recorded visually over a 42-day period, from 53 to 95 days after sowing.

Peroxidase activity assay

POD activity was measured using the method of Chance and Maehly (1955). The increase in absorbance at 470 nm, indicating the formation of tetraguaiacol, was recorded over 1 minute using a spectrophotometer. An extinction coefficient of 1.33 mmol^{-1} cm^{-1} was used, and enzyme activity was expressed as units per gram of fresh tissue weight.

Catalase activity assay

CAT activity was assessed by monitoring the decomposition of hydrogen peroxide (H_2O_2) via absorbance changes at 240 nm over a 1-minute period (Aebi et al., 1984). The enzymatic activity was calculated using the molar extinction coefficient of 36.6 mM^{-1} cm^{-1} and expressed as units per gram of fresh tissue weight.

Superoxide dismutase activity assay

SOD activity was determined by measuring the inhibition of photoreduction of nitroblue tetrazolium (NBT). Absorbance of the reaction mixture was recorded at 560 nm using a spectrophotometer. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction (Gentle et al., 2001).

RNA extraction and cDNA synthesis

The expression levels of the *Cs-ACS2* gene were evaluated in the ovary and adjacent leaf tissues at the six- and eight-leaf stages using quantitative reverse transcription PCR (qRT-PCR). Total RNA was extracted using the RNX-Plus kit (CinnaGen Co., Iran). RNA quality was verified by agarose gel electrophoresis and quantified using a Biophotometer (Eppendorf Co.). Genomic DNA contamination was removed using the Thermo RNase-free DNase I kit. To inactivate DNase I, 1 µL of EDTA was added, and the samples were incubated at 65 °C for 10 minutes. First-strand cDNA synthesis was carried out using 2 µL (500 ng) of total RNA, oligo(dT) primers, RevertAid Reverse Transcriptase, and RiboLock RNase Inhibitor (Thermo Scientific, USA), following the manufacturer's instructions. The synthesized cDNA was stored at −20 °C until further use.

Gene expression analysis

Primers for qRT-PCR were designed using Primer3 software after aligning gene sequences obtained from GenBank (NCBI: http://www.ncbi.nlm.nih.gov). Primer specificity was confirmed using BLAST analysis, and synthesis was performed by GenFanAvaran Co., Iran. Details of the primers used are presented in Table 3. *Actin* was used as the reference gene for normalization. qRT-PCR was performed using a Bio-Rad C1000™ Thermal Cycler and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). Each reaction was carried out in a 10 µL volume under standard conditions. Melting curve analysis was used to verify the specificity of amplification and rule out nonspecific products or primer-dimer formations. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 3 minutes; followed by 40 cycles of denaturation at 95 °C for 25 seconds, annealing at 60 °C for 20 seconds, and extension at 72 °C for 25 seconds. Gene expression rates were measured using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The experiments were performed in three replications and the expression levels of the studied genes were finally analyzed by Excel software.

Table 3. The list of primers used in PCR reaction.

Genes	Forward primer (5'→3')	Reverse primer (5'→3')
Cs-ACS2	CCACTCCTTACTATCCTGGATTTGA	CTCCGAGTGGATTTGATGGGT
Actin	TCCACGAGACTACCTACAACTC	GCTCATACGGTCAGCGAT

Results

Flower sexuality

The interaction effects of urea and ethephon treatments on the number of male and female flowers, as well as the male-to-female flower ratio, were found to be statistically significant at the 5% and 1% probability levels, respectively. During the 42-day evaluation period, the total number of flowers ranged from 13.99 to 60.3. The highest mean number of male flowers was recorded in the E0N0 (n = 43.33) and E0N1 (n = 44) treatments. In contrast, the lowest mean number of male flowers was observed in the E1N1 treatment (n = 2.33). The greatest number of female flowers was recorded in the E0N2 treatment (n = 24), which was not significantly different from the E1N0 treatment (Table 3). Conversely, the lowest average number of female flowers (n = 9.33) was observed in the E0N0 treatment, although this value was not significantly different from those observed in the E0N1 and E1N2 treatments (Table 4).

Table 4. Mean comparison for effects of different treatments on morphological traits in cucumber.

Mean comparison			
Treatments	**Number of male flowers***	**Number of female flowers***	**Male-to-female flower ratio****
E0N0	44.33^{a}	9.33^{c}	4.32^{b}
E0N1	44.00^{a}	15.66^{bc}	5.58^{a}
E0N2	36.00^{b}	24.30^{a}	2.18^{c}
E1N0	10.00^{c}	17.66^{ab}	0.54^{d}
E1N1	2.33^{d}	11.66^{bc}	0.23^{d}
E1N2	7.00^{cd}	16.66^{abc}	0.59^{d}

Columns with at least one similar letter have no significant differences. *, ** significant at $P < 0.05$ or $P < 0.01$, respectively.

Antioxidant enzymes activity

According to the ANOVA results, the interactions between urea and ethephon on peroxidase and catalase enzyme activity were statistically significant at the 5 and 1% probability levels, respectively, while the interaction effects were not significant for superoxide dismutase activity. The highest average peroxidase activity (0.6 u fw^{-1}) was recorded in the control plants, while the lowest activity (0.12 u fw^{-1}) was observed in plants treated with E1N2 (Fig. 1). As shown in Figure 2, the mean catalase activity of the plants treated with E1N0 (0.081 u fw^{-1}) was significantly higher than that of all other treatments applied.

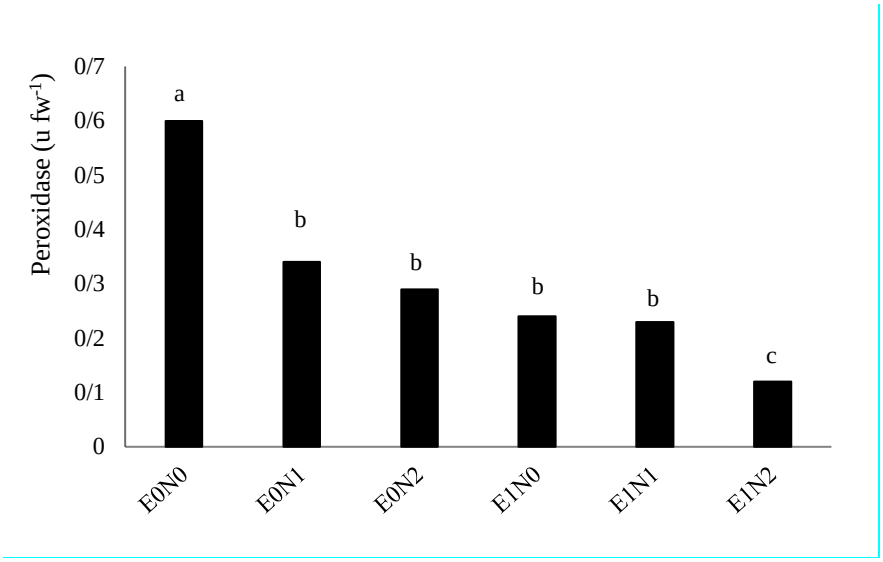

Fig. 1. The interaction effect of ethephon and urea on peroxidase enzyme activity in cucumber E0N0 (control), E1N0 (ethephon 0.5 mM + urea 0), E0N1 (ethephon 0 + urea 2 g L^{-1}), E1N1 (urea 2 g L^{-1} + ethephon 0.5 mM), E0N2 (ethephon 0 + urea 4 g L^{-1}), E1N2 (urea 4 g L^{-1} + ethephon 0.5 mM).

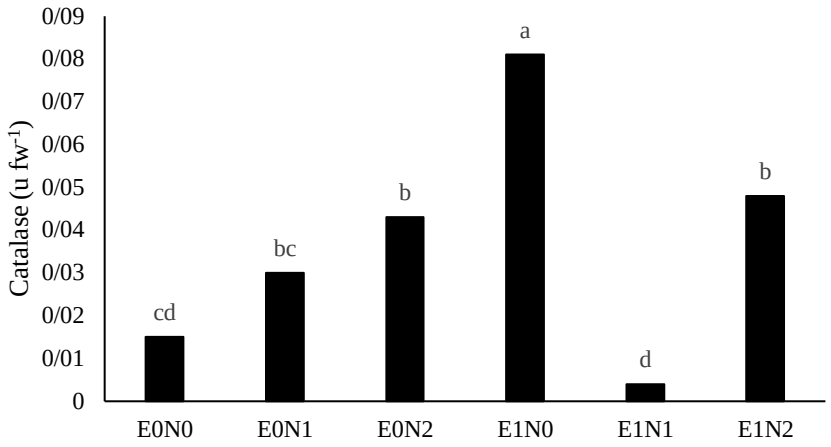

Fig. 2. The interaction effect of ethephon and urea on catalase enzyme activity in cucumber. E0N0 (control), E1N0 (ethephon 0.5 mM + urea 0), E0N1 (ethephon 0 + urea 2 g L^{-1}), E1N1 (urea 2 g L^{-1} + ethephon 0.5 mM), E0N2 (ethephon 0 + urea 4 g L^{-1}), E1N2 (urea 4 g L^{-1} + ethephon 0.5 mM).

The pattern of Cs-ACS2 gene expression

In this study, the expression of the *Cs-ACS2* gene was evaluated in the leaves and flowers of cucumber plants subjected to various treatments. Quantitative curve analysis indicated that the target amplicon was amplified with high efficiency (Fig. 3). Analysis of *Cs-ACS2* gene expression changes in female flowers (Figs. 4 and 5) and in the sixth and ninth leaves (Figs. 6 and 7), using GEN EX software, revealed an increase in expression levels by approximately 4.5 to 6 times in female flowers and 1.5 to 3 times in leaves. The highest expression level of *Cs-ACS2* (4.43 times) was recorded in the E1N2 treatment, both in female flowers and in the sixth leaf. In contrast, the lowest expression levels were observed in the E0N0 (0.96 times), E0N1 (0.99 times), and E0N2 (0.85 times) treatments, all of which belonged to the same statistical group (Fig. 3).

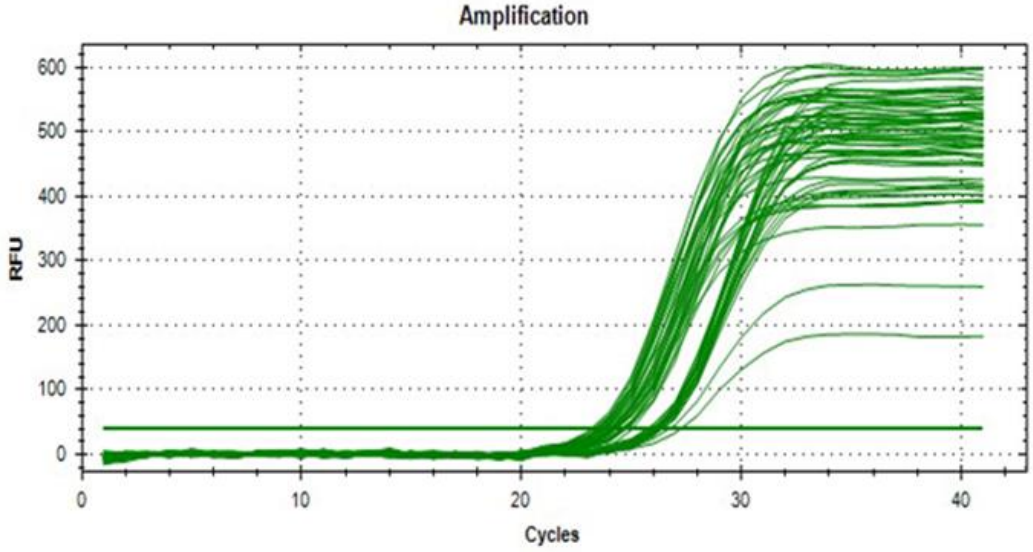

Fig. 3. Fluorescence curve of cDNA samples for the *Cs-ACS2* gene in cucumber plants.

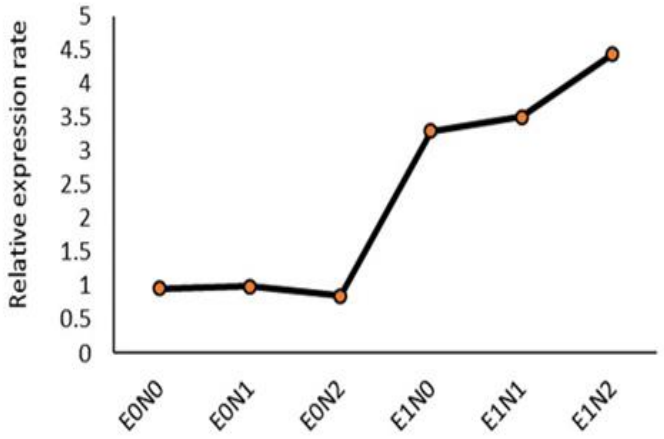

Fig. 4. The interaction effect of ethephon and urea on *Cs-ACS2* gene expression in female flowers next to the sixth cucumber leaf. E0N0 (control), E1N0 (ethephon 0.5 mM + urea 0), E0N1 (ethephon 0 + urea 2 g L^{-1}), E1N1 (urea 2 g L^{-1} + ethephon 0.5 mM), E0N2 (ethephon 0 + urea 4 g L^{-1}), E1N2 (urea 4 g L^{-1} + ethephon 0.5 mM).

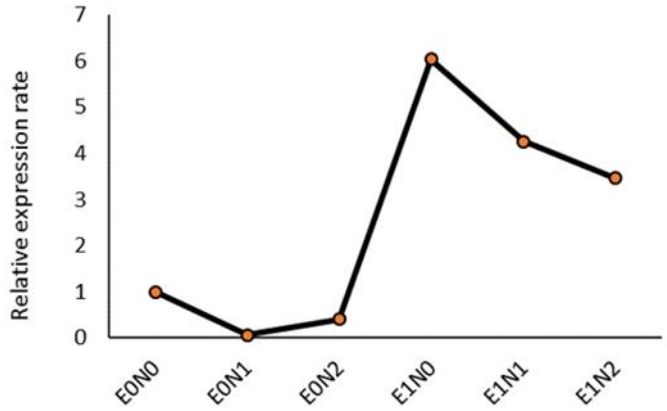

Fig. 5. The interaction effect of ethephon and urea on the expression of *Cs-ACS2* gene in female flowers next to the ninth leaf of cucumber plant. E0N0 (control), E1N0 (ethephon 0.5 mM + urea 0), E0N1 (ethephon 0 + urea 2 g L^{-1}), E1N1 (urea 2 g L^{-1} + ethephon 0.5 mM), E0N2 (ethephon 0 + urea 4 g L^{-1}), E1N2 (urea 4 g L^{-1} + ethephon 0.5 mM).

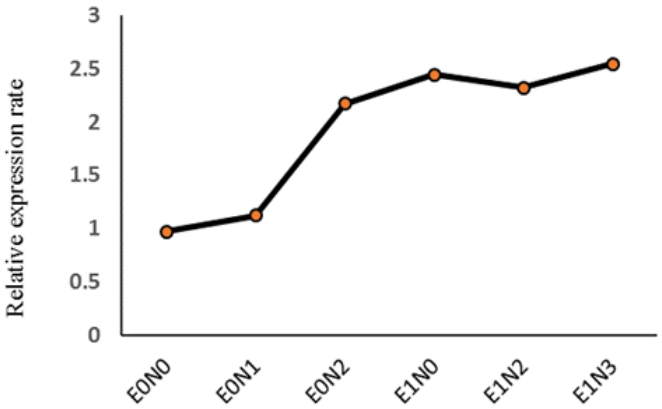

Fig. 6. Comparison of mean ethephon and urea interaction effect on *Cs-ACS2* gene expression in the sixth leaf of cucumber plant. E0N0 (control), E1N0 (ethephon 0.5 mM + urea 0), E0N1 (ethephon 0 + urea 2 g L^{-1}), E1N1 (urea 2 g L^{-1} + ethephon 0.5 mM), E0N2 (ethephon 0 + urea 4 g L^{-1}), E1N2 (urea 4 g L^{-1} + ethephon 0.5 mM).

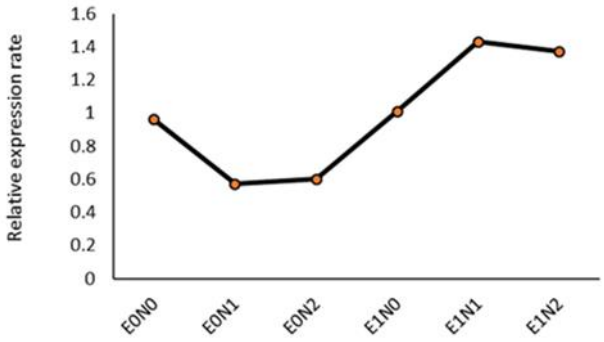

Fig. 7. Comparison of mean ethephon and urea interaction effect on *Cs-ACS2* gene expression in the ninth leaf of cucumber plant. E0N0 (control), E1N0 (ethephon 0.5 mM + urea 0), E0N1 (ethephon 0 + urea 2 g L^{-1}), E1N1 (urea 2 g L^{-1} + ethephon 0.5 mM), E0N2 (ethephon 0 + urea 4 g L^{-1}), E1N2 (urea 4 g L^{-1} + ethephon 0.5 mM).

As illustrated in Figure 5, the highest *Cs-ACS2* gene expression in female flowers, along with the ninth-leaf stage, was observed in E1N0-treated plants (without urea application), with a value of 6.03 times. In contrast, the lowest expression levels were recorded in the control group (0.99 times), E0N1 (0.05 times), and E0N2 (0.4 times), all of which were classified within the same statistical group (Fig. 5). During the sixth-leaf stage, *Cs-ACS2* expression was lowest in plants treated with E0N0 (0.97 times) and E0N1 (1.12 times), with no significant differences between them (Fig. 6). These were followed by E0N2 (2.17 times), E1N0 (2.44 times), E1N1 (2.32 times), and E1N2 (2.54 times) treatments, which exhibited slight numerical differences in gene expression, though these were not statistically significant (Fig. 6). As shown in Figure 7, *Cs-ACS2* gene expression at the ninth-leaf stage was similarly highest in plants treated with E1N1 and E1N2 (1.43 and 1.37 times, respectively), with no significant difference between them. The lowest expression levels at this stage were recorded in E0N1 (0.57 times) and E0N2 (0.6 times), which were also statistically comparable (Fig. 7).

Discussion

Regardless of the nitrogen treatments, the male-to-female flower ratio was significantly lower in ethephon-treated cucumber plants compared to the control, indicating that ethephon promoted the development of female flowers. Although plants treated with E0N2 produced the highest number of female flowers, they also exhibited a high male-to-female flower ratio. This suggests that a high concentration of nitrogen, in the absence of ethylene treatment, can increase the production of both male and female flowers. Among the various plant hormones involved in sex expression in the Cucurbitaceae family, ethylene is well recognized as a key promoter of female flower development (Wang et al., 2010).

Earlier studies reported a negative effect of nitrogen on female flower formation, attributing it to increased auxin biosynthesis and delayed flowering due to excessive vegetative growth (Ito et al., 1960). However, more recent findings suggest that nitrogen nutrition can enhance the production of both male and female flowers and improve the male-to-female flower ratio (Umamaheswarappa et al., 2005), which is consistent with our observations. In general, nitrogen enhances vegetative growth and increases leaf area, which may boost carbohydrate production or alter levels of leaf hormones, potentially contributing to changes in flower sex expression.

In summary, while the highest number of female flowers was recorded under high nitrogen treatment, the male-to-female flower ratio remained significantly high. According to Vaudo et al. (2022), this ratio may increase when the nitrogen-to-phosphorus (N:P) ratio deviates from the optimal value of 4, emphasizing that nutrient balance is more critical than the concentration of any single element. In our study, phosphorus was not a variable; however, elevated nitrogen levels likely altered the N:P ratio, though not beyond the critical threshold.

The results also indicated that both ethylene and nitrogen treatments led to a reduction in peroxidase activity. Ranjbar and Ahmadi (2016) reported similar findings in the miniature rose cultivar 'Sanaz-e-Zard,' where peroxidase activity decreased following ethylene application. Although peroxidase plays an essential role in detoxifying hydrogen peroxide and mitigating oxidative damage in peroxisomes and mitochondria, elevated enzyme levels do not always reflect stress conditions. Under severe stress, enzyme synthesis may be inhibited, or subunit modifications may occur, leading to reduced enzymatic activity (Shah et al., 2001).

Kalantar Ahmadi et al. (2015) reported that the highest catalase activity was observed under moderate drought stress combined with a nitrogen level lower than the recommended rate. This

indicates that stress conditions, coupled with limited nitrogen availability, can lead to elevated catalase activity—a finding that aligns with our observations. Although both peroxidase and catalase enzymes scavenge H_2O_2, catalase is primarily responsible for its removal and plays a more significant role in mitigating oxidative stress (Kuk et al., 2003). Catalase exhibits a high affinity for H_2O_2 and does not become saturated at any physiological concentration (Blokhina et al., 2010). Therefore, the E1N0 treatment can be considered a stress-inducing condition, as ethylene functions as a stress hormone and, in this case, was applied in the absence of sufficient nitrogen.

In the present study, the highest expression levels of the *Cs-ACS2* gene were observed in female flowers adjacent to the sixth and ninth leaves of plants treated with E1N1 and E1N2, respectively. *Cs-ACS2* is preferentially expressed in the developing ovary (Wang et al., 2010) and is known to enhance ethylene biosynthesis, which in turn stimulates its own expression through a positive feedback mechanism (Yamasaki et al., 2003). Ethephon, a widely used plant growth regulator, releases ethylene upon application and is commonly used to manipulate sex expression in cucurbits (Yousefzadeh et al., 2013). However, its effects vary depending on plant species, concentration, timing, and duration of application (Khuankaew et al., 2009).

Saito et al. (2007) examined the role of *Cs-ACS2* in female flower bud development in monoecious cucumber plants and found that *Cs-ACS2* mRNA accumulates at the two-sex stage specifically beneath the pistil primordia. In floral buds destined to become female flowers, the gene continues to be expressed in the central ovary, where the zygote and placenta develop.

Nitrogen is one of the most essential nutrients for plant growth and plays a central role in metabolic processes, including those involved in ethylene biosynthesis. The interaction between nitrogen and ethylene affects various physiological processes, such as gas exchange, root architecture, and the development of leaves, fruits, and flowers (Lupini et al., 2017; Ma et al., 2019). High nitrate concentrations have been shown to upregulate the expression of *ACS* and *ACO* genes, leading to increased ethylene production in *Arabidopsis* (Khan et al., 2015).

Although *Cs-ACS2* expression was strongly upregulated in response to ethephon treatment, it did not significantly increase with nitrogen application alone. This suggests that ethephon promotes the conversion of male to female flowers through the *Cs-ACS2* gene expression pathway. In contrast, the effect of nitrogen on female flower production may operate through an alternative, *Cs-ACS2*-independent regulatory mechanism.

Conclusion

Our findings demonstrated that nitrogen application increased the number of cucumber flowers, regardless of flower sex. A high nitrogen supply, in the absence of ethephon, had a positive effect on female flower production and simultaneously increased the number of male flowers. Conversely, ethephon treatment decreased the total flower number and reduced the male-to-female flower ratio, thereby favoring female flower formation. According to the results, ethephon application resulted in the conversion of male flowers into female flowers through the *Cs-ACS2* gene expression pathway. However, the increase in female flower number associated with nitrogen application appeared to occur via a mechanism independent of *Cs-ACS2* gene expression. Overall, since ethylene was less readily available, more expensive than urea, and also induced stress in the plant, nitrogen treatment could be used as a practical alternative to hormonal treatments for regulating flowering in cucumber plants.

Acknowledgments

This study was conducted using funds provided by Sari Agricultural Sciences and Natural Resources University (SANRU). The authors also wish to thank the laboratory staff of the Genetics & Agricultural Biotechnology Institute of Tabarestan (GABIT) for their technical assistance.

Conflict of Interest

The authors indicate no conflict of interest in this work.

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