ALTERATION OF GROWTH AND FLOWERING OF *Cucumis sativus* L. BY APPLICATION OF SEX STEROIDS *in vitro*

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ABSTRACT

The effect of selected sex steroids on plant morphology, flowering and sex expression in *Cucumis sativus* cultivar Kmicic F1 was investigated. Plants were cultured on the medium with the addition of progesterone, testosterone and estrone at concentrations of 0.1 and 1.0 µM. All tested steroids caused shortening in the main stem over the control, while the number of main stem nodes was similar to the control. Testosterone and progesterone stimulated female flower, decreasing male flower production. Plants cultured on the medium with estrone had shortest stems and the total number of flowers produced was more than four-fold lower compared to the control; however, a shift toward femaleness was observed. Application of the tested sex steroids *in vitro* altered flower morphology. Pollen viability in the anthers of male flowers developed on plants cultured on the media with steroids was decreased, compare to control, suggesting their effect on pollen development.

Key words: cucumber, morphology, *in vitro* flowering, sex hormones, steroids, pollen viability.

INTRODUCTION

Flowering in plants occurs after a vegetative growth and covers the transition of the shoot apical meristem into inflorescence meristem. The switch from vegetative to reproductive development is controlled by multiple pathways responding to different environmental and developmental signals (Simpson and Dean, 2002; Yanovsky and Kay, 2003). Flower development and sex determination are under genetic and environmental control, as well are under the control of various growth regulators and hormones i.e. auxins, cytokinins, and gibberellins (Dellaporta and Calderon-Urrea, 1993; Grant *et al.*, 1994; Lebel-Hardenack and Grant, 1997; Ainsworth, 2000; Mobini *et al.*, 2015), as well as abscisic acid and ethylene (Khryanin, 2002; Vardhini and Rao, 2002).

Sex steroids (androgens, estrogens and progesterone) play a crucial role in the control of reproductive development in mammals. Some of these steroids (especially progesterone) have been also detected in small amounts (µg/kg) in plant tissues, with contents varying between species and organs and changing during development (Zhang *et al.*, 1991; Janeczko and Skoczkowski, 2005; Janeczko *et al.*, 2013). Endogenous sex steroids in plants have been recognized as intermediates in various biosynthetic pathways (Maier *et al.*, 1995; Milanesi *et al.*, 2001). It has been reported that some of the animal steroid hormones applied exogenously have an effect on generative development in plants. Kopcewicz (1970) reported that the cold period necessary for flower induction in *Cichorium intybus* can be replaced by treatment with 17β-estradiol or an estrone. Later, his studies showed that estrogens promoted female flower development in *Ecballium elaterium*, while androgens increased the number of male flowers (Kopcewicz, 1971). Gawienowski *et al.* (1971) observed that the treatment of cucumber (*Cucumis sativus* L.) plants with estradiol increased the number of female flowers. Tanino *et al.* (2002) showed that in *Cucumis melo* estradiol increased the total number of produced flowers. Estrone, estriol and 17β-estradiol induced flowering in winter wheat (Janeczko and Filek, 2002), while androsterone, androstenedione and progesterone induced the generative phase in *Arabidopsis thaliana* (Janeczko *et al.*, 2003). These reports lead us to experiment aiming at evaluation of the effect of animal sex steroids on sex determination in plants. Cucumber is considered as a model plant for studying sex determination (Malepszy and Niemirowicz-Szczytt, 1991), therefore as plant material in this study we chose monoecious cultivar of cucumber, producing both male and female flowers on the same plant. Experiments were designed to assess the effect of progesterone, testosterone and estrone on the morphology and sex expression of cucumber in tissue culture conditions.

MATERIALS AND METHODS

Commercial seed sample of monoecious *C. sativus* cultivar Kmicic F1 (Polan, Kraków, PL) was used. Seeds were soaked for 1 h in tap water, then rinsed in 70% (v/v) ethanol for 5 min, disinfected with 10% (w/v) Chloramine T water solution (Bochemie Poland, Katowice, PL) for 15 min, and washed three times in sterile water for 5 min each. Seeds were then placed in 500 ml plastic culture boxes (Pakler Lerka, Krakow, PL) containing 80 ml of basal MS medium (Murashige and Skoog 1962), prepared using powdered MS salt mixture including MS vitamins (Duchefa, Haarlem, NL). Three-week-old plantlets were used as explants. The following sex steroids were used in the experiments: progesterone (4-pregnene-3,20-dione), testosterone (17beta-hydroxy-4-androsten-3-one) and estrone (3-hydroxy-1,3,5(10)-estratrien-17-one (Sigma-Aldrich, Poznań, PL). The steroid stock solution was prepared by dissolving 1 mg of the steroid in 300 µl of ethanol. To this mixture, 300 µl of Tween-20 and 400 µl of water was added to yield a final concentration of stock solution of 1mg/1ml. The stock solution was added to the culture medium to assess the tested steroid concentrations of 0.1 and 1.0 µM. MS - hormone free medium was used as a control. Control medium was also supplemented with 300 µl of ethanol and 300 µl of Tween-20. All media were supplemented with 90 mM sucrose and adjusted to a pH of 5.7-5.8 and 0.25% (w/v) Phytagel (Sigma-Aldrich, Poznań, PL) was added prior to autoclaving. Culture boxes were placed under a 16 h photoperiod with light supplied by Philips cool-white fluorescent lamps (100 µmol $m^{-2}s^{-1}$) at 26±2°C. Plants were transferred to fresh media every 4 weeks.

For cultured plants, data were collected on main stem height (cm) and the number of nodes on the main stem, the number of lateral branches and the number and sex of flowers. Data on the morphology of flowers were collected during blooming phase and the following traits were analyzed: the number and the length (mm) of sepals, the number and the length (mm) of petals, number of anthers, the length (mm) and diameter (mm) of the ovary. For viability evaluation, pollen was stained with 1% acetocarmine and observed under a white fluorescent light microscope (Carl Zeiss, Göttingen, DE). Additional observations such as position of flowers on the plant, abnormalities in flower development and tendril formation were also collected and reported.

The flower sex ratio (FSR) was calculated as the mean number of female flowers divided by the mean number of male flowers in a specified period of the culture. Data were collected every 4 weeks over the following sixteen weeks of culture.

Single treatment in the experiment consisted of 10 culture boxes with 3 plants per box on each media combination. The experiment was repeated three times. Data were analyzed using a factorial-design ANOVA and mean separations were conducted via Tukey's HSD (Honestly Significant Difference) test at $p \leq 0.05$.

RESULTS

Plant morphology: The plantlets taken as initial explants for culture had approximately 5 cm stems with 2 nodes. After 16 weeks of culture, the length of the main stem of control plants was on average 14.8±0.6 cm with 9.0±0.5 nodes (Table 1). Control plants rarely produced single lateral branches on the main stem; however, tendrils were frequently observed. Supplementation of the culture medium with testosterone and progesterone influenced main stem height and cultured plantlets were approximately 4-4.5 cm shorter than control plants; however the number (8.0-8.9) of main stem nodes per plant was only slightly lower than in control. Plants cultured on the medium with added progesterone and testosterone produced tendrils similarly to control plants; however, plant appearance changed due to the increased production of lateral branches. On the media with testosterone, single plants produced an average of 6-7 laterals, while on the medium with progesterone there were 5-7 laterals produced. The main stem height of the plantlets cultured on the media supplemented with 0.1 µM and 1.0 µM estrone was significantly reduced (6.4±0.5 and 8.3±0.8, respectively) compared to the control. Plants cultured on this media rarely produced lateral branches and did not produce tendrils.

Flowering and sex expression: Flower production was observed in the control and on the media with the addition of steroids (Table 2, Fig. 1). The addition of the tested sex steroids to the culture media caused the production of female, male and a small number of bisexual flowers.

There was no difference in the total number of flowers produced per single plant in the control (12.9±0.6) and plants cultured on the media with 0.1 µM and 1.0 µM of progesterone (13.2±0.9 and 14.2±0.7, respectively). On this media, the mean number of female flowers produced per plant was similar to the control; however, the number of male flowers was lower (Table 2). Control plants produced an average of 6.5±0.4 male flowers per plant, while plants cultured on progesterone-supplemented media produced from 5.1±0.7 (0.1 µM) to 5.6±0.4 (1.0 µM) male flowers per plant.

The number of female flowers produced on plants cultured on the media with testosterone (6.3±0.5 on 0.1 µM and 6.0±0.4 on 1.0 µM of testosterone) was similar to the control; however, the number of male flowers (1.9±0.2 - 2.1±0.2) was over three-fold lower than in the control. The lowest number of flowers produced by a single plant (3.3±0.5 - 3.6±0.4) was

observed on the media supplemented with estrone. Plants cultured on this media mainly produced female flowers.

The formation of bisexual flowers was also observed (Table 2). Those flowers were produced in small numbers on plants cultured on the media with testosterone and progesterone. They were not observed in the control or on the medium with estrone. More (0.3-0.4 per plant) of bisexual flowers was observed in plants grown on the media with progesterone, independent of its concentration in the medium. Plants grown on the medium with testosterone produced on average 0.1 of bisexual flower per plant. The majority of bisexual flowers possessed reduced ovary, stigma on a short style and 3-5 greenish or whitish deformed anthers bearing no pollen or pollen with low viability, not exceeding 20%. In some of the flowers, the stigma was whitish, deformed and dwarfed. Due to the overall small number of bisexual flowers and abnormalities in their development, they were excluded from further analyses.

Detailed analysis of flowering in dependency of the culture duration showed a prevalence of male flowers (FSR 0.8) observed in control plants at the 4th week of culture (Fig. 1). Later, a variable proportion of male and female flowers was noted, and a prevalence of female flowers (FRS 1.2) was recorded at the end of the culture. Testosterone caused the predominant production of female flowers (FSR 3.0-4.2) throughout whole culture period; however, in the 12th week of culture, an increase in the number of male flowers independent of the concentration was observed. In that period, some of the newly produced male flower buds, especially on the lateral branches, were very small (0.5-1 mm), did not grow further and did not open. Plants cultured on the media with progesterone at the beginning of the experiment (4th week) produced a similar number of flowers of both sexes (FSR 0.9-1.0); however, the significant prevalence of female flowers was observed later (FSR 1.8-2.3). On this medium, plants produced an average of 3.9±0.5 to 4.9±0.7 male flowers per plant up to the 12th week of culture; however later the number of male flowers increased to 6.3±0.6 on the medium with 0.1 µM and 8.7±0.6 on the medium with 1.0 µM of progesterone.

On the medium with estrone, plants mainly produced female flowers (FSR 1.3-2.9). The mean number of male flowers produced on this medium was low and did not exceed two flowers per single plant throughout the entire culture period. Moreover, on the medium with a higher estrone concentration (1.0 µM), male flower development was delayed and they were observed from the 8th week of culture.

Flower morphology and pollen viability: The steroids used had an effect on flower morphology (Table 3). In the tested populations, there was no variance in the number of sepals (5 sepals in male and female flowers) and number of anthers (5 anthers per flower); those data were excluded from Table 3. In control plants, female flowers first appeared on average on the fourth node and were later localized on the upper nodes, while male flowers were localized evenly over whole plant. Female flowers had an average of five sepals, 4.6±0.2 mm in length, five petals, 4.3±0.1 mm in length, and the ovary, which was 10.9±0.2 x 3.1±0.1 mm in size. Male flowers had five sepals, 4.6±0.1 mm in length, five petals with an average length of 5.8±0.3 mm, and 5 anthers.

Female flowers developed on plants cultured on the media with added testosterone, appeared from the third node, but were most frequently localized on the upper nodes. The number and length of petals of those flowers was similar to that of the control; however, sepals were longer (4.9±0.1-5.4±0.6 mm). The ovaries were shorter than in the control, and were approximately 8.0 mm, irrespective of the testosterone concentration. Male flowers had similar parameters to control flowers; however, they had longer petals (7.3±0.3-8.7±1.7 mm).

Flowers of both sexes developed on plants cultured on the medium containing progesterone were localized evenly over the plant. Female flowers had shorter sepals (3.3±0.3 and 3.7±0.2) and reduced number of petals (4.3±0.5 and 4.4±0.2) in comparison to control flowers. In some of the flowers, petals were fused, while others were deformed and dwarfed. This was also observed in male flowers developed on plants cultured on the media with progesterone.

Female flowers developed on plants cultured on the medium with estrone were localized on the upper nodes, while male flowers were similar to the control and found over the whole plant. In male flowers developed on plants cultured on the medium with 1.0 µM estrone, the mean number of petals in a single flower was 2.3±0.9, while control flowers possessed five petals. Moreover, female flowers which developed on plants cultured on this medium also had smallest ovaries, with a length of 6.7-6.8 mm and diameter of 2.5-3.0 mm.

The highest pollen viability (79.4±2.3) was observed in male flowers of control plants (Table 3). Pollen from male flowers developed on plants cultured on media containing tested steroids had reduced viability. There was no difference in the viability of pollens from male flowers developed on the media with the addition of progesterone and estrone, ranging from 58.8±9.9 to 62.1±6.3%. Lower pollen viability (approximately 50%) was observed in male flowers developed on the medium containing testosterone.

Table 1. Effect of sex steroids on stem length, number of nodes and lateral branches in cucumber after 16 weeks of *in vitro* culture.

Steroid concentration [µM]		Stem length [cm]		No. of nodes per plant		No. of lateral branches per plant	
Control	0.0	14.8±0.6	a	9.0±0.5	a	0.7±0.1	c
Testosterone	0.1	10.2±0.3	bc	7.6±0.3	ab	5.7±1.1	ab
Testosterone	1.0	10.3±0.2	bc	7.8±0.4	ab	6.7±1.1	ab
Progesterone	0.1	11.6±0.5	b	8.9±0.3	a	4.9±0.5	b
Progesterone	1.0	11.0±0.3	bc	8.0±0.1	ab	6.8±0.5	a
Estrone	0.1	6.4±0.5	d	6.0±0.6	b	0.3±0.2	c
Estrone	1.0	8.3±0.8	cd	7.4±0.8	ab	0.5±0.2	c

Values are means ± SE. Means in columns followed by the same letter are not significantly different ($p \leq 0.05$, HSD)

Table 2. Average effect of sex steroids on flower production in cucumber cultured *in vitro*.

Steroid concentration [µM]		No. of flowers per plant female		male		bisexual		total	
Control	0.0	6.4±0.4	a	6.5±0.4	a	0.0±0.0	c	12.9±0.6	a
Testosterone	0.1	6.3±0.5	a	1.9±0.2	c	0.1±0.0	bc	8.3±0.5	b
Testosterone	1.0	6.0±0.4	a	2.1±0.2	c	0.1±0.0	bc	8.2±0.6	b
Progesterone	0.1	7.7±0.6	a	5.1±0.7	b	0.4±0.1	a	13.2±0.9	a
Progesterone	1.0	8.3±0.5	a	5.6±0.4	b	0.3±0.1	ab	14.2±0.7	a
Estrone	0.1	2.4±0.3	b	1.2±0.2	c	0.0±0.0	c	3.6±0.4	c
Estrone	1.0	2.4±0.4	b	0.9±0.2	c	0.0±0.0	c	3.3±0.5	c

Values are means ± SE. Means were calculated base on all observation periods (4, 8, 12 and 16 weeks) taken together. Means in columns followed by the same letter are not significantly different ($p \leq 0.05$, HSD)

Table 3. Effect of sex steroids on flower morphology and pollen viability in cucumber flowered *in vitro* (±SE).

Steroid concentration [µM]		Female flowers				Male flowers					
		SL	PN	PL	OL	OD	SL	PN	PL	PV	
Control	0.0	4.6±0.2	5.0±0.0	4.3±0.1	10.9±0.2	3.1±0.1	4.6±0.1	5.0±0.0	5.8±0.3	79.4±2.3	a
Testosterone	0.1	4.9±0.1	5.0±0.0	3.8±0.1	8.1±0.2	3.1±0.1	4.3±0.7	5.0±0.0	7.3±0.3	49.7±2.9	e
Testosterone	1.0	5.4±0.6	5.0±0.0	3.8±0.2	8.0±0.8	4.0±0.3	4.3±0.7	5.0±0.0	8.7±1.7	53.0±10.9	cd
Progesterone	0.1	3.3±0.3	4.3±0.5	3.3±0.3	7.8±1.3	3.0±0.6	4.0±1.0	4.5±0.5	6.0±2.0	62.1±6.3	b
Progesterone	1.0	3.7±0.2	4.4±0.2	3.0±0.0	7.7±0.9	3.1±0.4	3.8±0.3	4.3±0.5	6.5±0.9	58.8±9.9	bc
Estrone	0.1	4.9±0.2	5.0±0.0	4.4±0.1	6.8±0.2	3.0±0.1	5.0±0.0	4.2±0.7	4.5±0.4	61.5±6.8	b
Estrone	1.0	3.7±0.3	5.0±0.0	3.8±0.3	6.7±0.5	2.5±0.3	5.0±0.0	2.3±0.9	5.0±0.6	60.8±6.6	b

SL=length of sepal [mm], PN= no. of petals PL= length of petals [mm], OL= length of ovary [mm], OD= diameter of ovary [mm], PV= pollen viability [%]

Values are means ± SE. Means in PV column followed by the same letter are not significantly different ($p \leq 0.05$, HSD)

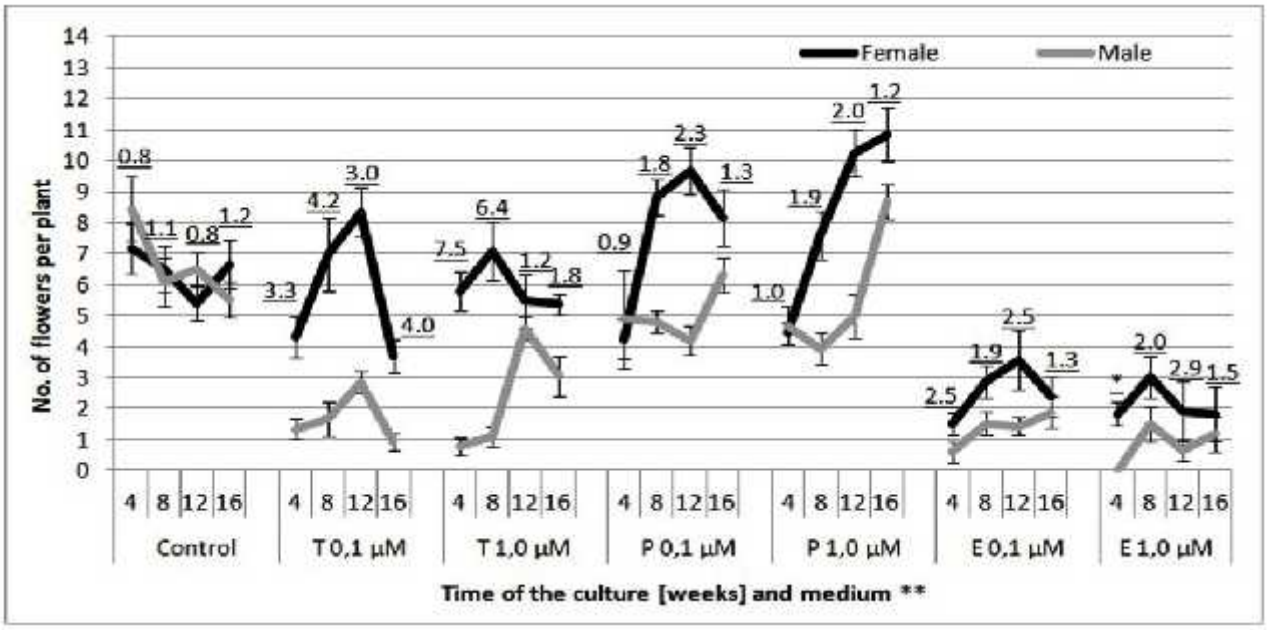

Fig 1. Effect of sex steroids and culture duration on flower production and FSR (female/male) in cucumber cultured *in vitro*.

Values are means ± SE. Underlined values denote FSR at certain periods of culture; * no male flowers developed; ** T - testosterone, P - progesterone, E – estrone

DISCUSSION

In this study, the influence of exogenously applied sex steroids on vegetative development in cucumber cultured *in vitro* was observed. The supplementation of the culture medium with tested steroids caused decrease in the main stem height, while the number of nodes was similar to control. Such results suggest that steroids decreased the length of internodes, in comparison to control plants. Bhattacharya and Gupta (1981) reported that the external application (in lanoline paste) of 0.1 and 0.25 µg per plant of 17β-estradiol increased stem growth but decreased root growth in the dwarf variety of sunflower. The same concentrations of testosterone inhibited both shoot and root growth. Progesterone at a concentration of 0.25 µg per plant promoted shoot growth but inhibited root growth, while at a concentration of 0.1 µg per plant had minor effects on shoot growth. Kopcewicz (1969) observed a stimulatory effect of 17β-estradiol, β-sitosterol and estrone at concentrations of 0.01-10 µg per plant (added as ethanol solution to the pots with sawdust) on the growth of dwarf peas, while cholesterol and testosterone were inactive. In *Medicago sativa*, estrone and 17β-estradiol at concentrations of 0.005-0.5 µg dm^{-3} (dissolved in nutrient solution) stimulated plant growth, but the growth was inhibited at concentrations of 50-500 µg dm^{-3} (Shore *et al.*, 1992). In *A. thaliana* seedlings, growth was stimulated on the media containing 0.01–1 µM of progesterone, while concentrations of 10-100 µM had an inhibitory effect (Iino *et al.*, 2007). Such results suggest that the exogenous estrogens have a greater impact on plant growth compared to androgens and progesterone, and their effect is highly dose-dependent. Our results showed that applied to the culture medium sex steroids in tested concentrations were inhibitory to the vegetative growth of cucumber.

The effect of sex steroids on the production of flowers was reported for *Ecballium elaterium*, where the application of estrogens (estrone, estriol, 17β-estradiol) considerably affected the total number of flowers and increased the ratio of female to male flowers. On the other hand, androgens (androsterone, androstenedione) did not influence the total number of flowers, but decreased the number of female flowers (Kopcewicz, 1971). This study showed that the steroids used affect flowering and sex determination in cucumber *in vitro*. The total number of flowers produced per single plant cultured on the medium with progesterone was similar to the control; however, the addition of testosterone and estrone caused a decrease in the total number of produced flowers.

In nature, monoecious cumbers first develop male flowers at the lower nodes, later both male and female flowers at various proportions are produced. The proportion of female to male flowers increases as the plants grow older and the plants eventually produce female flowers only. Female flowers are localized at the higher nodes (Yamasaki *et al.*, 2001). In our experiment, control plants exhibited a similar pattern of sex

expression to that observed in nature; however, supplementation of the culture media with the tested sex steroids resulted in variation from this pattern. On the medium with testosterone, irrespective of its concentration, a prevalence of female flowers was observed (FSR>1) throughout the whole culture period. Progesterone added to the culture medium caused a constant increase in the number of female flowers up to the 12th week; however, a subsequent decrease in their number on the medium with 0.1 µM of progesterone was observed. The decrease in the number of female flowers might be partially due to the flower bud abortion observed here in the later culture. Estrone significantly decreased the number of produced flowers compared to the control; however, based on the observed values of flower sex ratio (FSR >1), the conclusion that estrone favors femaleness in cucumber *in vitro* can be made. In this experiment we also observed prevailing number of female flowers after treating the plants with testosterone. The feminizing effect of testosterone is an interesting issue and may be explained by its metabolism to estradiol. Bioconversion of exogenously applied sex steroids has been observed in plants (Hirotani and Furuya, 1974; Young *et al.*, 1977; Young *et al.*, 1979; Janeczko and Skoczowski, 2005).

To our best knowledge there are very few reports on the effect of sex steroids on the flower morphology. Löve and Löve (1945) reported that testosterone caused female flowers in *Melandrium dioecum* to develop rudimentary anthers, and enlarged the anthers in male flowers. In this study, the applied steroids induced changes in the morphology and sex of the developed flowers. We observed a decrease in the length of sepals, number of petals and fusion of petals in flowers of plants grown on the media with steroids. On the medium with estrone, a reduction in the size of ovaries was observed. Testosterone caused the increased production of dwarfed male flowers. Both testosterone and progesterone induced the development of bisexual flowers; however, they were small in number and deformed.

The stimulatory effects of sex steroid treatment on pollen germination have been reported (Löve and Löve, 1945; Ylstra *et al.*, 1995); however, there are no studies on its effect on pollen development and viability. Our previous results showed that control plants flowering *in vitro* exhibited relatively high pollen viability, and that this trait was influenced by media supplements i.e. growth regulators (Kiełkowska, 2013; Kiełkowska and Havey, 2012). This study confirms that finding, as viable pollens were observed in the anthers of plants flowered on the media supplemented with tested steroids; however, these were lower in number compared to the control. The reduced viability of pollens suggests that exogenous steroids also influence the process of microspore- and microgametogenesis. Testosterone was determined in the pistils and pollen of *Lilium davidii* (Zhong-han *et al.*, 1994) and the results suggested that testosterone might be associated with the development of male gametophytes; however, pollen viability in flowers developed on plants cultured on the medium with exogenously applied testosterone was highly reduced in our study.

Conclusion: The effect of progesterone, testosterone and estrone on plant morphology, flowering and pollen viability in monoecious cucumbers cultured *in vitro* was demonstrated. This study showed that selected sex steroids influenced plant morphology and sex expression. Progesterone, testosterone and estrone stimulated female flower production. Tested steroids altered flower morphology, as dwarf and bisexual flowers were noted; moreover the observed decrease in pollen viability suggests their also affect on the process of pollen formation in cucumber.

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Authors' contribution: AK designed the project, supervised lab work and wrote manuscript. IK and AN performed tissue cultures and assisted in statistical analysis.

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