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Sex expression in monoecious cucumbers micropropagated *in vitro*

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Abstract

The effects of plant growth regulators (PRGs) on the induction of flowering and sex expression in micropropagated cucumbers are presented. The highest number of male flowers (6.0 ± 0.7 per plant) was produced by cv. Kmicic F1 on the Murashige and Skoog (MS) medium supplemented with 4.0 μM kinetin. The highest number of female flowers (3.1 ± 0.3) was also observed in cv. Kmicic F1 on either control (PRG-free) medium or medium supplemented with 6.4 μM indole-3-acetic acid (IAA). The MS medium supplemented with 4.4 μM benzyladenine inhibited flower formation. The highest percentage of flowering plantlets (67.5 ± 7.5) was observed on the control MS medium after 16 weeks of culture. Female-to-male flower ratio was influenced by the culture media and changed during cultivation. The highest pollen viability (60 - 70 %) was observed in anthers of plants cultured on the control medium and the medium with IAA.

Additional key words: auxins, *Cucumis sativus*, cytokinins, female-to-male flower ratio, *in vitro* flowering, pollen viability.

Introduction

Cucumber *(Cucumis sativus* L.) belongs to the *Cucurbitaceae* family in which almost every type of sex expression (monoecy, dioecy, and hermaphroditism) is represented and staminate (male), pistillate (female), and bisexual (perfect) flowers can be produced (Malepszy and Niemirowicz-Szczytt 1991, Miao *et al*. 2011). Cucurbit accessions are divided into several types depending on the sex and the position of flowers on the plant: androecious (bearing male flowers only), andromonoecious (producing staminate and bisexual flowers on the same plant), gynoecious (bearing female flowers only), hermaphroditic (with perfect flowers only), monoeciuos (with both male and female flowers on the same plant), and trimonoecious (with male, female, and bisexual flowers on the same plant) (Whitaker 1930, 1931, Atsmon *et al*. 1968, Malepszy and Niemirowicz-Szczytt 1991). In *C. sativus*, the most common type of sex expression is monoecy but gynoecious, hermaphroditic, or andromonoecious accessions also exist (Tanurdzic and Banks 2004). In monoecious cultivars, flower buds differentiate in the leaf axils of main shoots. In their early developmental stages, buds contain stamen and pistil primordia which later develop into either male or female flowers (Iwahori *et al*. 1970). Male flowers develop at the lower nodes, followed by female flowers at the higher nodes. The number of nodes to the first female flower and total number of female flowers are both reliable indices of sex expression *ex vitro* (Yamasaki *et al*. 2001).

The sexual expression in certain monoecious species can be influenced by age, injury, disease, irradiance, day length, temperature, or soil fertility (Heslop-Harrison 1957, 1972, Limerk 1959, Lange 1961, Lockhart 1961, McArthur 1977, Neelu 1997). In cucumber, sex expression is determined genetically (Kater *et al*. 2001, Yamasaki *et al*. 2001), however, several environmental factors including nitrogen nutrition, day length, and temperature influence flower sex (Matsubara 1977, Chailakhyan 1979, Freeman *et al*. 1980, Malepszy and Niemirowicz-Szczytt 1991). Sex expression in developing cucumber floral buds can also be altered by chemical treatments. For example, applications of silver nitrate, silver thiosulfate, maleic hydrazide, or giberelins caused increased production of male flowers (Wittwer and Bukovac 1958, Choudhury and Patil 1962, Clark and Kennedy 1969, Heslop-Harrison 1972, Beyer 1976, Hidayatullah *et al*. 2009) whereas applications of 2-chloroethylphosphoric acid, gaseous ethylene, and ethrel caused increased production of female flowers (Iwahori *et al*. 1969, Matsubara 1977, Kshirsagar *et al*. 1995, Yamaski *et al*. 2001). The majority of the above

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Abbreviations: BA - 6-benzyladenine; FSR - flower sex ratio; GA_3 - gibberelic acid; IAA - indole-3-acetic acid; IBA -indolebutyric acid; Kin - kinetin; MS - Murashige and Skoog; NAA - 1-naphthaleneacetic acid; PGR - plant growth regulator; Zt - zeatin.
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cited results were obtained using intact greenhouse or field grown plants. *In vitro* culture is useful for investigating the influence of plant growth regulators on the sex modifications without external interference. Earlier studies demonstrated that cucumber flower production is possible under *in vitro* conditions (Rajasekaran *et al*. 1983, Msikita *et al*. 1990, Tisserat and Galltetta 1993, Kiełkowska and Havey 2012), however, no reports on floral sex ratio changes during tissue culture have come to the author attention. The experiments reported here investigated the effects of selected plant growth regulators on *in vitro* micropropagation of cucumber plants and their further effects on sex expression and pollen viability of the regenerated plantlets.

Materials and methods

Experiments utilized commercial seed samples of two monoecious *Cucumis sativus* L. cultivars, Kmicic F1 and Sander F1 (*Polan*, Kraków, Poland). Seeds were soaked in tap water for 1 h, surface-disinfected in 70 % (v/v) ethanol for 5 min, 10 % (m/v) *Chloramine T* (*Bochemie*, Katowice, Poland) for 15 min, and rinsed three times in sterile water for 5 min each. The seeds were then placed into 500 cm^3 plastic culture boxes (*Pakler Lerka*, Krakow, Poland) containing 80 cm^3 of basal Murashige and Skoog (1962; MS) medium (*Duchefa*, Haarlem, The Netherlands). Four-week-old seedlings with two well developed true leaves were used for micropropagation. Stem internode pieces with 2 axillary buds were cut from seedlings and placed into boxes with 80 cm^3 of culture medium consisting of basal MS medium with addition of selected plant growth regulators (PGRs): 4.0, 6.0, and 8.0 µM kinetin (Kin), 4.4 µM 6-benzyladenine (BA), 6.4 and 12.8 µM indole-3-acetic acid (IAA), 2.9 µM gibberelic acid (GA_3), and 4.6 µM zeatin (Zt). PGR-free MS medium was used as a control. All media were supplemented with 90 mM sucrose, adjusted to pH 5.7 - 5.8, and 0.25 % (m/v) *Phytagel* (*Sigma-Aldrich*, Poznań, Poland) was added prior to autoclaving. Culture boxes with stem fragments were placed in a growth chamber and grown under a 16-h photoperiod, irradiance of 100 µmol m^{-2} s^{-1} (*Philips* cool-white fluorescent lamps), and temperature of 26 ± 2 °C. Plants were transferred to fresh media every 4 weeks.

Observations of shoot tips with visible leaf primordia were performed after 4 weeks of culture. The flower sex ratio (FSR) was calculated as the mean number of female flowers divided by the mean number of male flowers over a specified period of time. Data on the total number of flowering plants as well as individual male and female flowers (including flower buds and open flowers) were collected every 4 weeks over the following 16 weeks of the culture. During the flowering phase, pollen from the *in vitro* grown plants was collected, stained with Alexander's dye (Alexander 1969) and observed under a white fluorescent light microscope (*Carl Zeiss*, Göttingen, Germany).

Each treatment was performed in ten culture boxes with five explants per box, for the two cultivars on each of nine media combinations. The experiment was repeated three times. Flowering phase data were taken from a minimum of 10 plants for each cultivar and medium with two replications. 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

Shoot development was influenced by PGRs, no significant cultivar effect was observed. The highest number of developed shoots per explant (3.7 ± 0.2) was observed on the control PGR-free MS medium. These shoots were 10.9 ± 0.4 cm long, with 3 nodes, and established roots after 4 weeks in culture (Table 1). Addition of GA_3 and Zt to the media resulted in development of 2.5 ± 0.3 and 2.6 ± 0.2 shoots per explant, respectively. Shoots cultured on the medium supplemented with GA_3 were similar in height, leaf coloration, and node number to the control. However, the leaf blade margins of GA_3-treated plants were curled downward. In comparison to controls, shoots developed on Zt supplemented medium were much shorter and majority of them had vitreous tissues (data not shown). Lower Kin concentrations (4.0 and 6.0 µM) were less favorable for shoot development than the higher concentration (8.0 µM), however, some plantlets cultivated on high Kin medium suffered from leaf chlorosis whereas others became vitreous. All plantlets developed on media with Kin produced strong root system. On the medium supplemented with 4.4 µM BA short (1.3 ± 0.1 cm) and unrooted shoots along with greenish callus tissue were observed. Media supplemented with IAA induced approximately two shoots per explant. Regenerated plantlets were normal, with green leaves, and well rooted.

The cultivar and induction media influenced the flower production of micropropagated cucumber plantlets (Table 2). In general, cv. Kmicic F1 produced more flowers than cv. Sander F1. The highest number of male flowers (6.0 ± 0.7 per plant) was produced by cv. Kmicic F1 on medium with 4.0 µM Kin. The highest number of female flowers was also observed in this cultivar on both control medium and medium supplemented with 6.4 µM IAA. On the medium with

Table 1. Effect of plant growth regulators on the formation, length, and node number of cucumber shoots after 4 weeks of culture. Means ± SE, $n = 95$. Means in columns followed by the same letter are not significantly different at $P \leq 0.05$ (HSD test).

PGRs	Number of shoots [explant^{-1}]	Shoot length [cm]	Number of stem nodes [shoot^{-1}]
Control	3.7±0.2a	10.9±0.4a	2.8±0.1a
4.4 μM BA	1.9±0.1bc	1.3±0.1f	0.8±0.1d
2.9 μM GA_3	2.5±0.3b	7.3±1.2abcd	2.3±0.3abc
6.4 μM IAA	2.0±0.2bc	10.3±1.3ab	2.3±0.2abc
12.8 μM IAA	2.1±0.2bc	8.5±0.9abc	2.1±0.2abcd
4.0 μM Kin	1.4±0.1c	6.5±0.5cd	2.5±0.2ab
6.0 μM Kin	2.3±0.2bc	7.2±0.8bcd	2.7±0.2ab
8.0 μM Kin	2.3±0.2b	4.6±0.5de	1.7±0.1bcd
4.6 μM Zt	2.6±0.2ab	2.4±0.2ef	1.2±0.1cd

4.6 μM Zt, only male flowers were observed. Media supplemented with GA_3 yielded 2.8 ± 0.4 male flowers and 1.6 ± 0.3 female flowers in cv. Kmicic F1, however, in cv. Sander F1, this pattern was reversed with 1.5 ± 0.4 male flowers and 2.3 ± 0.4 female flowers per plant. On media containing Zt and GA_3 female flowers were in clusters localized mainly on the upper part of the plant. In contrast, on medium with IAA, female flowers were distributed evenly over the plant similarly to the controls. Shoots cultured on medium supplemented with 4.4 μM BA did not flower.

Time until first flowering was also media dependent (Table 3). During the first 4 weeks of culture, no plants with open flowers were noted with the exception of a few plantlets (2.5 ± 0.5 %) with male flowers cultivated on the medium containing GA_3. After 8 weeks of culture, open flowers were observed in plantlets cultivated on control media and media supplemented with IAA or 8.0 μM Kin, however, flowering was rare, observed in less than 7 % of the plantlets. After 12 weeks of culture, 37.5 ± 12.5 % of the plantlets cultured on medium with GA_3 had open flowers. The plantlets grown on the remaining media flowered rarely (from 11 to 17 %). The highest percentage of flowering plants (67.5 ± 7.5) was observed on the control medium after 16 weeks of culture. At the same time, approximately 50 % of plantlets cultivated on IAA supplemented media flowered. The lowest percentage of plantlets with flowers (19.9 ± 7.4) was scored on the medium supplemented with Zt. Regardless of media, some male flowers became successively smaller in size over time and those last formed did not open.

Male flowers dominated on the control medium after the first 4 weeks of culture (FSR = 0.1). After 8 weeks, pistillate and staminate flowers were in balance (FSR = 1). During the 12th week of culture, female flowers dominated and by the 16th week, male flowers were once again the most prevalent (Table 4). Similar patterns were observed among plants cultured on the medium supplemented with 6.4 μM IAA, however, under these conditions, female floral dominance lasted until the end of the culturing period. Plantlets cultured on the medium supplemented with 12.8 μM IAA exhibited female flower phase at 8th week of culture but later production of male flowers predominated. Plants cultured on media supplemented with all tested concentrations of Kin produced mainly male flowers. On the medium with GA_3, female flowers dominated (FSR = 1.9) at the 16th week of culture, the earlier stages were accompanied with male flower production. On medium supplemented with Zt, flowering was delayed until the 8th week and later only male flower production was observed.

Table 2. Effect of the cultivar and PGRs [μM] on number of male and female flowers per plant in micropropagated cucumbers after 12 weeks of culture. Means ± SE, $n = 17$. Means in columns followed by the same letter are not significantly different at $P \leq 0.05$.

Sex	Cultivar	Control	4.4 BA	2.9 GA_3	6.4 IAA	12.8 IAA	4.0 Kin	6.0 Kin	8.0 Kin	4.6 Zt
Male flowers	Kmicic F1	5.9±0.5ab	0.0±0.0e	2.8±0.4cde	2.0±0.4de	2.1±0.4de	6.0±0.7a	3.9±0.5abcd	5.0±0.6abc	4.6±0.8abcd
	Sander F1	2.6±0.4de	0.0±0.0e	1.5±0.4de	2.1±0.4de	3.4±0.6bcd	2.6±0.3de	3.4±0.5bcd	2.3±0.6de	1.4±0.3de
Female flowers	Kmicic F1	3.1±0.3a	0.0±0.0f	1.6±0.3cde	3.1±0.5a	2.7±0.5ab	1.2±0.2def	1.7±0.3cd	0.5±0.2ef	0.0±0.0f
	Sander F1	2.4±0.3b	0.0±0.0f	2.3±0.4b	1.9±0.5cb	1.4±0.3cdef	1.4±0.3cdef	1.7±0.4cd	0.5±0.2ef	0.0±0.0f

Table 3. Effect of the PGRs [μM] and duration of culture [week] on the percentage of cucumber (cvs. Kmicic F1 and Sander F1) plantlets with open flowers. Means ± SE, $n = 47$. Means in columns followed by the same letter are not significantly different at $P \leq 0.05$.

Culture	Control	2.9 GA_3	6.4 IAA	12.8 IAA	4.0 Kin	6.0 Kin	8.0 Kin	4.6 Zt
4	0.0±0.0e	2.5±0.5e	0.0±0.0e	0.0±0.0e	0.0±0.0e	0.0±0.0e	0.0±0.0e	0.0±0.0e
8	5.6±0.1e	0.0±0.0e	6.5±0.2e	2.5±0.5e	0.0±0.0e	0.0±0.0e	1.9±0.9e	0.0±0.0e
12	12.1±2.1ed	37.5±12.5bcd	12.5±4.2de	11.5±5.2de	17.4±5.7cde	11.9±1.9de	19.5±1.5cde	11.5±5.2de
16	67.5±7.5a	23.6±1.4cde	53.5±9.0ab	55.0±5.0ab	26.5±4.3bcde	45.2±11.9abc	45.0±5.0abc	19.9±7.4cde

Table 4. Effect of the PGRs [μM] and duration of culture [week] on flower sex ratio (male/female) and pollen viability [%]. Means ± SE, $n = 29$; * - development of male flowers only, ** - no female or male flowers developed.

	Culture	Control	2.9 GA_3	6.4 IAA	12.8 IAA	4.0 Kin	6.0 Kin	8.0 Kin	4.6 Zt
Sex ratio	4	0.1	0.3	0.6	0.3	0.0*	0.1	0.1	0.0**
	8	1.0	0.3	1.0	3.7	0.5	1.0	0.2	0.0*
	12	1.6	0.6	1.4	0.3	0.5	0.9	0.1	0.0*
	16	0.5	1.9	1.6	0.6	0.4	0.2	0.0*	0.0*
Pollen viability		72.4±5.8	51.9±6.6	69.7±6.6	61.6±6.5	50.2±6.0	51.6±9.4	28.7±8.3	22.8±5.6

There were differences in pollen viability depending on the media. On the medium supplemented with both tested concentrations of IAA, pollen viability was high (61.6 ± 6.5 and 69.7 ± 6.6 %), similar to the control (72.4 ± 5.8 %) (Table 4). About half of observed pollen grains in the anthers of plants cultured on media with lower (4.0 and 6.0 μM) concentration of Kin were viable. In media with Zt or 8.0 μM Kin, low pollen viability was observed. Addition of 8.0 μM Kin resulted in occurrence of male flowers with yellow petals and absent or deformed stamens bearing no pollen.

Discussion

Explants cultured on PGR-free control medium produced the highest percentage of shoots. Considering that the explants used possessed two axillary buds, this medium stimulated development of shoots *de-novo*. Media supplemented with 4.6 μM Zt, 8.0 μM of Kin, 6.4 or 12.8 μM IAA, 2.9 μM GA_3, and 4.4 μM BA stimulated development of existing shoot primordia. The lowest number of shoots obtained on explants cultured on media with 4.0 μM Kin indicates that this concentration inhibits cucumber axillary bud development.

In vitro flowering plays an important role in understanding the mechanism of flowering physiology (Scorza 1982, Dellaporta and Calderon-Urrea 1993, Taji *et al*. 2002, Wang *et al*. 2012). Induction of flowering and fruiting *in vitro* depends on content of endogenous growth regulators, sugars, minerals, as well as on phytohormones added into media (Nadgauda *et al*. 1997, Wang *et al*. 2001). Of various phytohormones, role of auxin, ethylene, and gibberellin in regulation of flower sex expression has been widely studied (Pharis and King 1985, Tanimoto 2007, Salman-Minkov *et al*. 2008, Thomas 2008, Makwana *et al*. 2010, Wilmowicz *et al*. 2011). In cucumber, flowering in tissue culture depends on explant type and media composition, and similarly to other species is highly influenced by the types and concentrations of PGRs (Rajasekaran *et al*. 1983, Msikita *et al*. 1990, Tisserat and Galltetta 1993, Ameha *et al*. 1998).

Successful *in vitro* flower formation on media with BA has been reported for bamboo (Nadgauda *et al*. 1997, Singh *et al*. 2000), orchids (Kostenyuk *et al* 1999, Tee *et al*. 2008) cauliflower (Kumar *et al*. 1995), coriander (Stephen and Jayabalan 1998), maize (Mandal *et al*. 2000), and tobacco (Smulders *et al*. 1990). In this study, supplementation of the culture medium with 4.4 μM BA yielded no flower buds development on the micro-propagated shoots. Wang *et al*. (2001) reported that BA promoted male flower formation in bitter melon at concentrations of 1 - 2 μM whereas completely inhibiting flower formation at 4 - 8 μM. These results suggest that in cucumber similarly to bitter melon, 4.4 μM BA inhibits development of flower meristem. Kachonpadungkitti *et al*. (2001) studied the effects of Kin, NAA, IBA, and GA_3 on flower induction in buckwheat. Among the plant growth regulators examined, neither auxins nor GA_3 stimulated flower induction. Only a low concentration of Kin (0.1 μM) was effective in stimulating flower production. Wang *et al*. (2001) reported that MS medium with 8.0 μM Kin promoted female flower formation in bitter melon. In contrast, Wittwer and Aung (1969) showed that Kin inhibited flowering in tomato. In cucumber, all tested concentrations of Kin increased development of staminate flowers whereas pistillate flowers persisted.

To the best of my knowledge, there are no reports about the effect of Zt on the *in vitro* flowering in cucumber. Obtained results suggest, that Zt promotes male flowering in micropropagated cucumber plants and completely inhibits female flower formation. Moreover, the majority of shoots cultured on this medium had vitreous tissues. Kachonpadungkitti *et al*. (2001) reported that micropropagated buckwheat plantlets were vitrified and no flowers were induced from such cultures. In their study, the appearance of vitreous tissues was attributed due to used gelling agents. In this study, the vitrification was most likely due to Zt exposure. However, in both studies, the vitrified shoots did not produced flowers suggesting that vitrification inhibits *in vitro* flower formation.

The effect of exogenous GA_3 on flower sex depends on the species. GA_3 promotes female flowers in *Zea mays* and *Jatropha curcas* whereas it increases male tendency in *Asparagus officinalis, Cannabis sativa*, and *Spinacia oleracea* (Limerk 1959, Lazarte and Garrison 1980, Pharis and King 1985, Mandal *et al*. 2000, Makwana *et al*. 2010). Bhat *et al*. (2010) reported that GA_3

application increases flowering in *Solanum* and also enhances the rate of flower development. In this study, GA_3 induced formation of both male and female flowers, however, their proportion varied in different cultivars. Galun *et al*. (1963) reported that supplementation of the culture medium with 1.0 mg dm^{-3} IAA has modified flowers of genetically male cucumber strain to phenotypically female flowers. A combination of GA_3 and IAA in the media increased the number of pistillate and hermaphrodite flowers. According to my observations, plants cultured on media with IAA produced both male and female flowers; however, the total number of female flowers was higher on media with lower concentration (6.4 μM) of IAA.

The most intensive blooming phase was observed between 12^{th} and 16^{th} week of culture. This feature appears to be highly explant source-dependant. The time needed for shoot development, plant regeneration, flower meristems development, and, finally, flowering was extended when the development of the shoot primordia was induced *de-novo* (Taji *et al*. 2002, Kiełkowska and Havey 2012).

The PGRs used in this study significantly influenced the FSR of the tested monoecious cucumber cultivars. On the control medium and medium supplemented with 6.4 μM IAA, the female-to-male flower ratio was similar to that reported for *ex vitro* conditions (Whitaker 1931, Lloyd and Webb 1977, Malepszy and Niemirowicz-Szczytt 1991). It was possible to distinguish the initial male phase, later balance between male and female flowers, and finally female phase, when, under natural conditions, pollination, fertilization, and fruit formation would occur. Supplementing the media with GA_3, Kin, Zt, and higher concentration of IAA resulted in deviation of the female-to-male flower ratio during culture. Addition of GA_3 to the culture medium caused extension of the male phase to the 12^{th} week coupled with later production of female flowers. Peterson and Andher (1960) and Jutamanee *et al*. (1994) reported that *ex vitro* application of gibberellins increased male flowering in cucumbers. Asghar *et al*. (1990) and Hidayatullah *et al*. (2009) reported that GA_3 reduced the male-to-female flower ratio in cucumber increasing the number of female flowers. Results presented in this study support findings that gibberellins delay female flower formation in cucumber rather than increasing male flowering (Bukovac and Wittwer 1961). The addition of 12.8 μM IAA resulted in a prolonged male phase and a shortened female phase; however, its lowered concentration increased the number of female flowers in the later time of the culture. Kin in all the tested concentrations promoted male flower formation. This results support the evidence that external regulation of sex expression in cucumber involves either type of tested hormones which determines the female to male ratio.

Pollen viability was tested in this study to evaluate how the selected PGRs influenced the process of pollen formation in light of its potential for artificial pollination or other *in vitro* manipulations. A high percentage of viable pollen obtained from the plants cultured on medium supplemented with IAA suggests that the process of pollen development was not disturbed. The reduced percentage of viable pollen grains observed in plants grown on medium with 4.6 μM Zt and with 8.0 μM Kin suggests that both PGRs at the tested concentrations influenced not only plant morphology (vitreous tissues, chlorosis) but also microsporogenesis.

In summary, flowering and pollen viability of *in vitro* propagated monoecious cucumber cultivars under the influence of a range of PGRs was demonstrated. The floral development in the regenerants varied according to applied growth regulators. Observed deviations from the natural pattern of sex expression in monoecious cultivars allowed to track changes at specific intervals that has not been reported earlier for *in vitro* conditions. These modifications of sex expression by hormone application indicate that the genes required for development of the androecium or gynoecium in cucumber are functional and their expression can be chemically suppressed or enhanced.

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