Electronic Journal of Polish Agricultural Universities is the very first Polish scientific journal published exclusively on the Internet, founded on January 1, 1998 by the following agricultural universities and higher schools of agriculture: University of Technology and Agriculture of Bydgoszcz, Agricultural University of Cracow, Agricultural University of Lublin, Agricultural University of Poznan, Higher School of Agriculture and Teacher Training Siedlce, Agricultural University of Szczecin, and Agricultural University of Wroclaw.

ELECTRONIC JOURNAL OF POLISH AGRICULTURAL UNIVERSITIES

2002
Volume 5
Issue 1
Series AGRONOMY

TOMASZEWSKA-SOWA M., DROZDOWSKA L., SZOTA M. 2002. EFFECT OF CYTOKININS ON *IN VITRO* MORPHOGENESIS AND PLOIDY OF PEPPER *Capsicum annuum* L. **Electronic Journal of Polish Agricultural Universities**, Agronomy, Volume 5, Issue 1. Available Online http://www.ejpau.media.pl

EFFECT OF CYTOKININS ON *IN VITRO* MORPHOGENESIS AND PLOIDY OF PEPPER *Capsicum annuum* L.

Magdalena Tomaszewska–Sowa[1], Lucyna Drozdowska[1], Maria Szota[2]

[1]*Department of Plant Physiology, University of Technology and Agriculture in Bydgoszcz, Poland*
[2]*Institute of Plant Breeding and Acclimatization in Bydgoszcz, Poland*

ABSTRACT

The study-compared effect of cytokinins on morphogenesis and ploidy of plants regenerated *in vitro* from explants of 'Stanola F_1', ATZ and ATM pepper seeds. The aim of defining the morphogenetic potential of the studied genotypes, half-seed explants consisting the proximal part of the hypocotyl and radicle were put onto the MS medium containing BAP (5.0 $mg \cdot dm^{-3}$), 2iP (2.5 $mg \cdot dm^{-3}$), ZEA (2.5 $mg \cdot dm^{-3}$), TDZ (1.5 $mg \cdot dm^{-3}$), while MS medium without cytokinins constituted the control. After the initiation period, explants were transferred onto the medium without cytokinins. The effect of the growth regulators to the morphogenetic response of explants was estimated based on the number of explants on which adventitious buds and shoots were developing. The cytokinins applied did not show a significant effect on the development of adventitious buds on pepper explants. However, the ability to regenerate shoots increased with subsequent passages. There was observed an effect of BAP, applied in the initial medium, on the number of shoots in 'Stanola F_1'. Similarly there was noted a formation of flower buds *in vitro* on regenerated shoots of all genotypes; the number of flower buds was the greatest in 'Stanola F_1', also due to the effect of BAP in the initial medium. All the plants obtained in the experiment were diploid (2n=24).

Key words: cytokinins, morphogenesis, ploidy, *in vitro* culture

INTRODUCTION

Plant cell, tissue and organ cultures and genetic engineering offer a new tool, which might accelerate the production of peppers with desire quality traits and resistance to biotic and abiotic stresses. The success of these efforts depends on an efficient *in vitro* plant regeneration system and genetic stability of regenerated plants.

Researching the morphogenetic potential of pepper *in vitro*, some authors report on forming leaf-like structures or numerous adventitious buds on explants, which do not develop into normal shoots [1,8, 20,27]. However, *in vitro* plant regeneration was achieved in pepper from hypocotyl, shoot tip, cotyledon and leaf explants [1,4,7,8,10,12,19,20,23,26,28]. Another source of explants was identified in halves of imbibed seeds with the proximal part of hypocotyl and radicle [3,9,21]. The application of that method made it possible to elongate the induced buds and plant development in a relatively short period of time. Unlike other explants tested, seed halves formed adventitious shoots without growth regulators in the medium, although in some genotypes cytokinins increased the regeneration effectiveness [3]. The results of research on the development of efficient and repetitive plant regeneration system and its applications for genetic manipulations call for experimental definition of cytokinin type and concentration in medium for each genotype [2].

The present study aimed at comparing of the effect of various cytokinins on morphogenesis and ploidy of pepper plants obtained *in vitro* from seed explants of 'Stanola F_1' and ATZ and ATM lines.

MATERIAL AND METHODS

The pepper seeds of ATZ and ATM lines and 'Stanola F_1' cultivar were provided by Nowaczyk collection from the Bydgoszcz University of Technology and Agriculture. The seeds were surface sterilised with 70% ethanol (1 min) and with 5% calcium hypochlorite (7 min) and then rinsed with distilled water. The sterile seeds were placed onto Petri dishes with filter paper moistened with sterilized water. After four-day preculture, imbibed seeds were cut into two parts. The one which contained the proximal part of hypocotyl and radicle was cultured on basal MS medium [17] supplemented with: BAP - 6 – benzylamino purine (5.0 $mg \cdot dm^{-3}$), 2iP - 6γ-γ-dimethyl (allyl) amino purine (2.5 $mg \cdot dm^{-3}$), ZEA – zeatin (2.5 $mg \cdot dm^{-3}$) and TDZ – thidiazuron (1.5 $mg \cdot dm^{-3}$). MS medium without cytokinins constituted the control (0RW). The medium was adjusted to pH 5.6 and solidified with 0,8% agar. 24 explants of each pepper genotype studied were put onto control and tested media.

The initiation was followed by 2 subsequent passages, every 4 weeks, transferring onto MS medium without cytokinins all the explants with buds and shoots differentiated, where 100% stood for the number of differentiating explants in each passage. *In vitro* cultures took 12 weeks under controlled conditions (temperature of 24°C, light intensity of 2000 lux, 16-h photoperiod and RH of 80%). The experiment was carried out in 3 reps.

The morphogenetic response of pepper to cytokinins applied was expressed as a number of explants which formed adventitious buds and where shoots differentiated. After 12 weeks the number of shoots with flower buds developing were also defined as well as the number of flower buds per plant. The results obtained were verified with variance analysis.

The assessment of ploidy level was based on chromosomal analyses. The number of chromosomes in meristematic cells of initiated buds and apical meristems of shoots were counted after 4 weeks off culture initiation and also after 2 subsequent passages with aceto–orcein methods. The plant material was pre-treated at 2°C for 24h and fixed (24h) in acetic acid and ethanol (1:3). The samples were stained with 2% orcein for 24h and macerated while heated slightly over the burner then squashed with the drop of 45% acetic acid and observed under microscope. Mitotic index was defined in randomly sampled 1000 meristematic cells with the formula $IM = n \cdot 100/1000$ (n – the number of cells in respective mitosis stages).

RESULTS

The half-seed explants of ATZ, ATM and 'Stanola F_1' cultured on MS medium control and the one containing cytokinins showed over the first two weeks a development of radicle and hypocotyl elongation. Over subsequent initiation weeks, adventitious buds differentiated around the hypocotyl cut surfaces. A significant effect of cytokinins on adventitious bud development over initiation was not observed, except for slight stimulation of this process due to BAP, 2iP and ZEA application for ATZ and ATM lines (Table 1).

Table 1. Effect of cytokinins and genotype on adventitious buds formation on seed explants (initiation*)

Cytokinin (I)	Genotype (II)					
	ATZ		ATM		Stanola F_1	
	Explants forming adventitious buds					
	number	%	number	%	number	%
0 RW	17.3	72.2	16.7	69.6	21.0	87.5
BAP	21.7	90.4	18.7	77.8	21.3	88.9
2iP	20.3	84.6	19.0	79.2	16.0	66.7
ZEA	21.0	87.5	17.3	72.2	17.3	72.2
TDZ	17.3	72.2	16.0	66.7	17.7	73.6
LSD $_{0.05}$ for I – ns, for II – ns						

*** means for 3 reps, each replicate comprised 24 explants; ns – non-significant difference**

Over the first 4 weeks of culture, the ability of explants to shoot regeneration was inconsiderable. Depending on the genotype, the number of explants with shoots amounted to 0.3 – 5.0 (1.4 - 20.8%) on average. 'Stanola F_1' was better for shoot regeneration then other genotypes (Table 2).

Table 2. Effect of cytokinins and genotype on adventitious shoots formation on seed explants (initiation)

Cytokinin (I)	Genotype (II)					
	ATZ		ATM		Stanola F_1	
	Explants forming adventitious shoots					
	number	%	number	%	number	%
0 RW	1.2	6.9	3.0	12.5	4.0	16.7
BAP	2.3	9.7	0.3	1.4	5.0	20.8
2iP	1.0	4.2	1.7	6.9	2.7	11.1
ZEA	1.3	5.6	1.3	5.6	1.3	5.6
TDZ	0.7	2.8	1.0	4.2	1.7	6.9
LSD $_{0.05}$ for I – ns, for II = 1.063						

All the explants with buds and adventitious shoots were subcultured onto the medium without cytokinins. The morphogenetic response depended on the genotype, period of culture and cytokinin type. The cytokinins tested in initial medium increased the explants ability to bud formation in subsequent passages; in ATM (passage I) and 'Stanola F_1' (passage II) revealed a stimulating effect of BAP (Fig. 1). During second passage shoot development was most numerous while the maximum number of shoots (15.7) was formed in 'Stanola F_1' (Table 3).

Fig. 1. Effect of cytokinin, genotype and culture time on the adventitious shoots formation on pepper seed explants

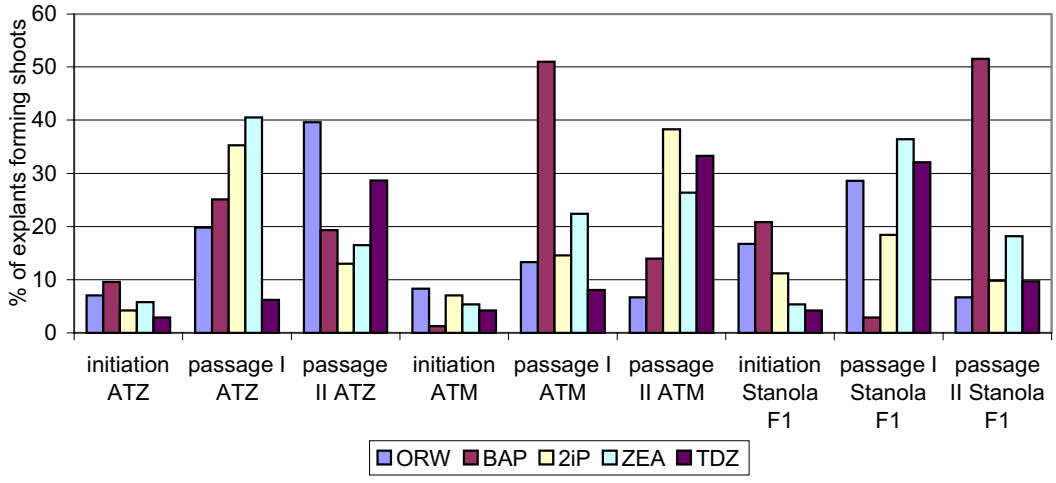
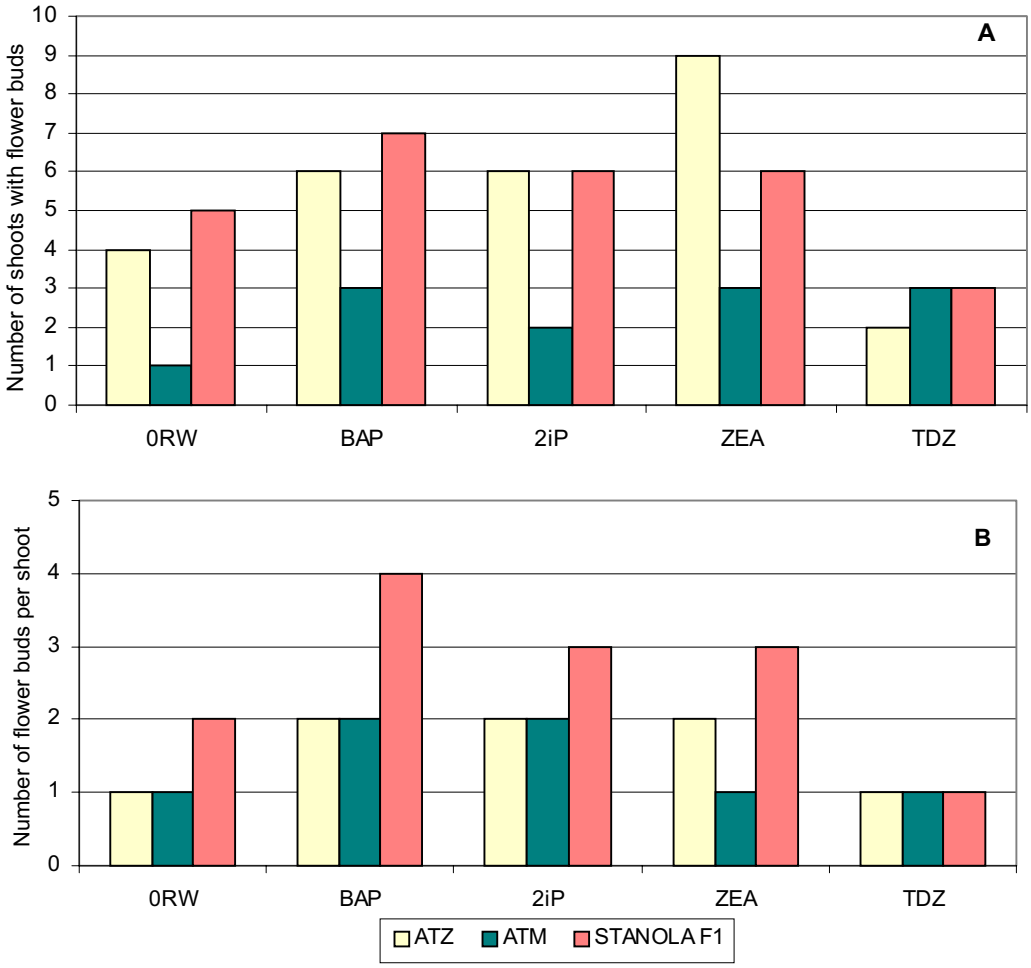


Table 3. Number of shoots obtained from seed explants after 4, 8 and 12 weeks of culture

Cytokinin (I)	Genotype (II)								
	ATZ			ATM			Stanola F_1		
	Number of passages (III)								
	initiation	passage I	passage II	initiation	passage I	passage II	initiation	passage I	passage II
0 RW	1.7	2.3	3	2	3.3	8.3	4	6	6
BAP	2.3	7.3	10	0.3	4.7	6.7	5	6.4	15.7
2iP	1	3	9.7	1.7	6.7	8.3	2.7	3	4.3
ZEA	1.4	4.7	8.3	1.3	7	8.7	1.3	6.3	11
TDZ	0.7	1.4	6.3	1	1	5.3	1.6	4.3	6.3
$LSD_{0.05}$ for I = 1.985, for II = 1.894, for III = 1.156, for II/III = 2.135									

The morphological analysis included also the number of flower buds developing on regenerated shoots. The ability of plants to form flower buds was observed following passage II (12-week culture). The highest number of shoots with flower buds was recorded in ATZ line whose explants over initiation were cultured on the ZEA medium (Fig. 2A) and the highest number of flower buds formed per explant – in 'Stanola F_1', which was due to BAP applied in the initial medium (Fig. 2B) (Phot. 1).

Fig. 2. Effect of cytokinins and genotype on the flower bud formation
A - number of shoots with flower buds
B - number of flower buds per shoot



Phot. 1. Flowering plant in culture *in vitro*

Plants regenerated from half-seed explants of the three sweet pepper genotype cultured on the control medium, as well as with cytokinins, were diploids (2n = 24) (Phot. 2), which showed that application of cytokinins, their concentration and culture time did not affect the ploidy of the plant obtained. Also mitotic index expressed as a percentage of dividing cells was defined in meristematic cells of regenerant (Table 4). TDZ and ZEA applied in initial medium increased the value of mitotic index only in ATM line, as compared with the control.

Phot. 2. Mitotical chromosomes in cells of root tips and shoot meristems (2n=24)

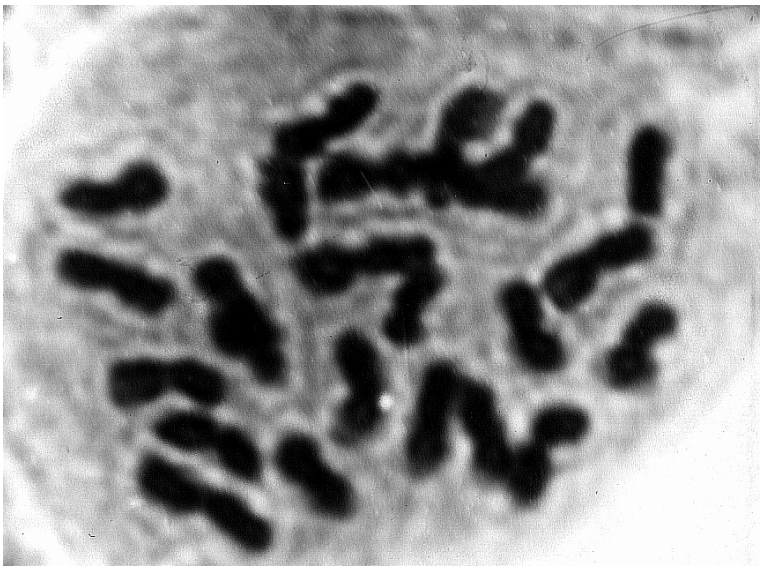


Table. 4. Effect of cytokinin on the mitotic index of apical meristem cells in regenerated plants

Genotype	Mitotic index (%)				
	Cytokinin				
	0 RW	BAP	2iP	ZEA	TDZ
ATZ	7.2	7.6	7.0	7.0	6.9
ATM	7.4	7.7	7.3	7.9	8.1
Stanola F_1	7.7	8.0	7.6	7.8	7.8

DISCUSSION

Halves of imbibing sweet pepper seeds containing proximal part of hypocotyl and radicle provided a source of explants. The experiments carried out resulted in a regeneration of whole plants, which developed flower buds and flowered *in vitro*.

In vitro plant regeneration depends on genotype and explant source. Exogenous growth regulators are important for the expression of this capacity. Additionally the hormonal regulation of morphogenesis depends on the place of their synthesis and transport and so the radicle, which is the place of endogenous cytokinin synthesis, is indispensable to shoot organogenesis, especially when explants are incubated on the medium without growth regulators [3, 9].

The present experiment showed a varied sensitivity of the cultivars tested to cytokinins. Similarly elongation rate for buds and shoots development depended on the pepper genotype which confirm the cross-cultivars differences in the ability to regenerate plants observed also in other experiments [3,4 23]. A varied cultivar response to cytokinins may depend on the activity of enzymes in the metabolism of these phytohormones and exogenous cytokinins added can inhibit the synthesis of endogenous cytokinins and disturb the regeneration process [18]. In the present research all that resulted in a formation of deformed-leaf rosettes, which did not develop shoots, in shoot elongation failure and inhibited root development.

Out of all the cytokinins tested, BAP in the initial medium stimulated the formation and development of adventitious shoots; however the cultivars showed the greatest capacity to regenerate shoots after about 12-15 weeks (passage II). The ability to form buds and adventitious shoots shows the explants competence. However, the factors acting during culture (time, growth regulators) determine explant cells to morphogenetic response. According to Ramage and Leung [19], to induce the regeneration of shoots in ‘Sweet Banana’ pepper a minimum of 8-day culture on BAP is needed. Besides BAP, explants, while acquiring competence, cannot do without carbohydrates (saccharose) which determine the organogenesis [19].

The cytokinins applied in the present experiment, TDZ, as compared with other cytokinins researched, inhibited the induction and elongation of adventitious buds in all the genotypes. In some plants TDZ shows a high effectiveness in stimulating shoot regeneration depending on cytokinins [5, 13]. Similarly in some sweet pepper genotypes recalcitrant to BAP and IAA and in hot pepper TDZ stimulated the regeneration process and bud elongation [15,26].

After 12 weeks of culture flower buds developed on adventitious pepper shoots regenerated *in vitro*, which also depended on the genotype and the type of cytokinins applied in the initial medium. The development of flower buds on pepper microseedlings is quite rare. However, despite the observed flowering in cultures *in vitro*, flower buds were withering very often due to culture conditions and only few developed fruits [15,22,23,24].

Cytokinins contained in the medium stimulated flowering *in vitro* on shoots of varied species of *Passifloraceae* regenerated from leaf explants [25]. Also BAP or KIN stimulated flower bud formation on flowering-induced *Pharbitis nil* explants [11]. Literature and the present results confirm the activity of these growth regulators in plant flowering control. As part of the signalling system, they transfer information to shoot meristems about changes in the morphogenetic program [11,14].

Cells and tissues of shoots forming *in vitro*, especially by indirect organogenesis, there can occur caryologic changes in the chromosome number and structure leading to a development of poliploids and aneuploids as well as changes in genome. The kind and frequency of these changes depend on the plant material genotype used as explant as well as culture conditions, especially type and concentration of growth regulators and culture time. The present research showed that shoot regeneration did not involve callus, and adding cytokinins to the initial

medium did not caused on disturbances in mitosis and changes in plant ploidy. Similarly Christopher and Rajam [7], using various red pepper (*C. praetermissum*, *C. baccatum* and *C. annuum)* seedling explants, and low concentrations of growth regulators, obtained diploid plants. However, there were observed also negative effects of growth regulators in media on the chromosome structure and their number in regenerants. A high concentration of cytokinins in the initial medium disturbed mitosis in cells of apical meristems of shoots *C. praetermissum* and *C. annuum*, including chromosome aberrations, delayed chromosomes and anaphase bridges [6].

Changes in the number of chromosomes and their structure due to growth regulators of high concentrations and culture conditions were observed also in other plant species regenerated *in vitro* [16].
Genetic stability of the regenerants obtained and the fact that the regeneration does not involve callus suggest that the application of this method is well justified in micropropagation of valuable sweet pepper genotypes and in genetic manipulations.

CONCLUSIONS

1. The experiment resulted in regeneration of pepper plants which under *in vitro* conditions developed flower buds and flowered.
2. The capacity to regenerate adventitious shoots depended on the genotype, kind of cytokinins and time of the culture.
3. The most numerous adventitious shoots were formed in the second passage. Out of all the genotypes studied, the highest morphogenetic potential was observed in 'Stanola F_1' in which shoot and flower bud formation was due to BAP applied in the initial medium.
4. Shoot regeneration did not involve callus, which decreases the probability of somaclonal variation. Cytokinins present in the initial medium did not disturb in mitosis. Neither did they cause changes in ploidy in regenerants, which suggests that the application of this method is well justified in micropropagation of valuable sweet pepper genotypes and in genetic manipulations

REFERENCES

1. Arroyo R., Revilla M.A. 1991. *In vitro* plant regeneration from cotyledon and hypocotyl segments in two bell pepper cultivars. Plant Cell Rep. 10, 414–416.
2. Auer C.A., Cohen J.D., Laloue M., Cooke T.J., 1992. Comparison of benzyl adenine metabolism in two *Petunia* hybrid lines differing in shoot organogenesis. Plant Physiol. 98, 1035–1041.
3. Binzel M. L., Sankhla N., Joshi S., Sankhla D., 1996. *In vitro* regeneration in chilli pepper (*Capsicum annuum* L.) from half-seed explants. Plant Growth Regulation 20, 287–293.
4. Borychowski A., Niemirowicz-Szczytt K., 1997. Wybrane czynniki wpływające na regenerację *in vitro* pędów z hypokotyli papryki (*Capsicum annuum* L.) [Selected factors affecting the regeneration *in vitro* of shoots from pepper hypocotyls (*Capsicum annuum* L.)]. Zesz. Nauk. AR w Krakowie, Sesja Nauk. 50, 161–166 [in Polish].
5. Bretagne B., Chupeau M. – C., Chupeau Y., Fouilloux G., 1994. Improved flax regeneration from hypocotyls using thidiazuron as a cytokinin source. Plant Cell Rep. 14, 120–124.
6. Christopher T., Rajam M.V., 1994. *In vitro* clonal propagation of *Capsicum* spp. Plant Cell Tiss. Org. Cult. 38, 25–29.
7. Christopher T., Rajam M.V., 1996. Effect of genotype, explant and medium on *in vitro* regeneration of red pepper. Plant Cell Tiss. Org. Cult. 46, 245–250.
8. Ebida A.I.A., Hu C.Y., 1993. *In vitro* morphogenetic responses and plant regeneration from pepper (*Capsicum annuum* L. cv. Early California Wonder) seedling explants. Plant Cell Rep. 13, 107-110.
9. Ezura H., Nishimiya S., Kasumi M., 1993. Efficient regeneration of plants independent of exogenous growth regulators in bell pepper (*Capsicum annuum* L.). Plant Cell Rep. 12, 287–293.
10. Fari M., Andrasfalvy A., 1994. Regeneration and cloning of pepper (*Capsicum* sp.) *in vitro*: a review. HortScience 26 (2), 9-18.
11. Galoch E., Buracka – Łaukajtys E., Kopcewicz J., 1996. Effect of cytokinins on flower differentiation in cultured plantlets of *Pharbitis nil* Chois. Acta Physiol. Plant. 18 (3), 223–227.
12. Gatz A., Rogozińska J., 1994. *In vitro* organogenetic potential of cotyledon and leaf explants of *Capsicum annuum* L. cv. Bryza. Acta Soc. Bot. Pol. 63 (3-4), 255–258.
13. Huettemann C.A., Preece J.E., 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Tissue Org. Cult. 33, 105–119.
14. Kinet J.-M., 1993. Environmental, chemical and genetic control of flowering. Hort. Rev. 15, 279–334.
15. Manoharan M., Sree Vidya C. S., Lakshmi Sita G., 1998. *Agrobacterium* – mediated genetic transformation in hot chili (*Capsicum annuum* L. var. Pusa jwala). Plant Sci. 131, 77–83.
16. Morrison R.A., Konning R.E., Evans D.A., 1986. Pepper. In: Handbook of Plant Cell Culture. Eds. D.A. Evans, W.R. Sharp, V.A. Philip. Vol. 4, 552–573. New York, Macmillan.
17. Murashige T., Skoog F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

18. Orlikowska T., 1997. Regulatory roślinne w kulturach *in vitro*. W: Regulatory wzrostu i rozwoju roślin [Plant regulators in cultures *in vitro*. In: Plant growth and development regulators]. Red. L. S. Jankiewicz. PWN Warszawa [in Polish].
19. Ramage C.M., Leung D.W.M., 1996. Influence of BA and sucrose on the competence and determination of pepper (*Capsicum annuum* L. var. Sweet Banana) hypocotyl cultures during shoot formation. Plant Cell Rep. 15, 974–979.
20. Ramirez-Malagon R., Ochoa-Alejo N., 1996. An improved and reliable chilli pepper (*Capsicum annuum* L.) plant regeneration method. Plant Cell Rep. 16, 226–231.
21. Rogozińska J., Drozdowska L., 1996. *In vitro* culture of bell pepper (*Capsicum annuum* cv. Bryza) and its greenhouse performance. J. Appl. Genet. 37 (4), 357–366.
22. Rogozińska J., Drozdowska L., 1997. Tworzenie kwiatów i owoców papryki w kulturach *in vitro* [Sweet pepper flower and fruit formation in cultures *in vitro*]. Ogrodnictwo 5, 17–19 [in Polish].
23. Rogozińska J., Tobolewska G., 1992. Genotypic variations in organogenesis of six cultivars of pepper, *Capsicum annuum* L. Gen. Pol. 33, 213–217.
24. Saxena P. K., Gill R, Radashid A., Mehashwari S.C., 1981. Isolation and culture of protoplast of *Capsicum annuum* L. and their regeneration into plants flowering *in vitro*. Protoplasma 108, 357–360.
25. Scorza R., Janick J., 1980. *In vitro* flowering of *Passiflora suberosa* L. J. Amer. Soc. Hort. Sci. 105 (6), 892–897.
26. Szasz A., Nervo G., Fari M., 1995. Screening for *in vitro* shoot-forming capacity of seedling explants in bell pepper (*Capsicum annuum* L.) genotypes and efficient plant regeneration using thidiazuron. Plant Cell Rep. 14, 666–669.
27. Valero-Montero L.L., Ochoa-Alejo N., 1992. A novel approach for chilli pepper (*Capsicum annuum* L.) plant regeneration: Shoot induction in rooted hypocotyls. Plant Sci. 84, 215–219.
28. Zhu Y.X., Ou – Yang W.J., Zhang Y.F., Chen Z.L., 1996. Transgenic sweet pepper plants from *Agrobacterium* mediated transformation. Plant Cell Rep. 16, 71–75.

Magdalena Tomaszewska–Sowa, Lucyna Drozdowska
Department of Plant Physiology
University of Technology and Agriculture
Bernardyńska 6, 85-029 Bydgoszcz, Poland
E-mail: magda@atr.bydgoszcz.pl, drozd@atr.bydgoszcz.pl
Maria Szota
Institute of Plant Breeding and Acclimatization
Department of Genetics and Plant Breeding
Powstańców Wielkopolskich 10, 85-090 Bydgoszcz, Poland
E-mail: mSzota@ihar.bydgoszcz.pl
