ACTA BIOLOGICA CRACOVIENSIA Series Botanica 47/1: 193–198, 2005

DIFFERENTIATION OF ADVENTITIOUS BUDS FROM *CAPSICUM ANNUUM* L. HYPOCOTYLS AFTER CO-CULTURE WITH *AGROBACTERIUM TUMEFACIENS*

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Received January 2, 2005; revision accepted April 10, 2005

In vitro organogenesis in hypocotyl explants of the pepper cultivar 'Bryza' was induced on MS medium containing 5 mg/l 6-benzyloaminopurine (BAP) and 1 mg/l indole–3-acetic acid (IAA). The hypocotyl explants were then inoculated with *Agrobacterium tumefaciens* LBA4404(pBI121). After 2 days of culture the first cell divisions were observed in the epidermis and cortex. After 6–7 days, numerous adventitious bud primordia appeared in 58.4% of the explants. In further stages of culture, buds developed into shoots in 8.4% of the explants. Histological analysis revealed hypertrophy and the presence of necrotic cells in the cortex. Necrotic changes were also observed in the vascular bundles. It is likely that culture on a selective medium containing kanamycin and co-culture with *Agrobacterium tumefaciens* strongly affected the organization of the hypocotyl meristematic tissue, and in consequence brought about necrosis and isolation of the adventitious buds from the vascular bundles.

Key words: *Capsicum annuum*, genetic transformation, adventitious buds, anatomical structure of hypocotyl, kanamycin.

INTRODUCTION

Pepper is a species highly susceptible to many fungal and viral pathogens. In cultivated varieties or sexually compatible species, genetic transformation is often suggested as a way of introducing resistance. However, genetic manipulation is of limited application in pepper because of the lack of an efficient transformation system (Mihalka et al., 1998; Lee et al., 2004).

Pepper is considered to be recalcitrant in regeneration in vitro and genetic transformation. During the last 30 years, a number of regeneration protocols for various types of pepper have been described. Regeneration in many different cultivars of pepper was reported to take place via shoot organogenesis in cotyledon and hypocotyl explants. However, whole plant regeneration is often limited to the formation of distorted buds or shoot-like structures that do not develop into normal shoots (Valera-Montero and Ochoa-Alejo, 1992; Hyde and Philips, 1996).

During the last few years, transformation using *Agrobacterium tumefaciens* has been reported in sweet and chili pepper (Zhu et al., 1996; Manoharan et al., 1998; Lim et al., 1999; Li et al., 2003; Lee et al., 2004). In most cases, however, the protocols failed to produce results in other laboratories.

The histological aspects of organogenesis in pepper have been studied for the purpose of explaining the obstacles to regeneration (Fari, 1983; Agrawal et al., 1989; Fraś and Nowak, 1995).

The aim of this work was to study adventitious bud differentiation, structural changes in hypocotyl explants after co-culture with *Agrobacterium tumefaciens*, and the explants' response to a selective agent.

MATERIALS AND METHODS

PLANT MATERIAL

The sweet pepper cultivar 'Bryza' was selected for this study because of its reported high regeneration ability (Borychowski et al., 2002). The seedlings used as a source of explants were grown aseptically in vitro. The seeds were surface-sterilized by immersion in 70% ethanol for 60 sec followed by 15 min in 50% sodium hypochlorite (98.5 g/l active chlorine; Chempur), then

PL ISSN 0001-5296

rinsed three times with sterile distilled water. Sterilized seeds were placed in half-strength MS medium (Murashige and Skoog, 1962) and germinated under fluorescent lighting (54 μE, 16 h photoperiod) at 26˚C. Hypocotyls from 10–12-day-old seedlings were used as explants.

BACTERIAL STRAIN AND CULTURE

Agrobacterium tumefaciens strain LBA 4404, harboring the plasmid pBI 121 containing the *nptII* and *uidA* genes, was used in transformation. The bacterial strain was grown in YEB medium (yeast extract, beef extract, peptone) supplemented with 50 mg/l kanamycin (Sigma) and 50 mg/l rifampicin (Polfa). A single colony was transferred to 50 ml liquid YEB medium containing these antibiotics and cultured for 24–48 h at 28˚C until absorbance at 600 nm reached 0.4–0.8. The bacteria were then centrifuged and the pellet was resuspended in MS liquid medium.

SELECTION ON KANAMYCIN

To determine the optimum concentration of kanamycin as a selective agent, concentrations of 0, 50, 60 and 70 mg/l were tested. Hypocotyl explants of cv. 'Bryza' were used for this experiment. The explants were incubated on bud-inducing medium containing the selective agent at the concentrations indicated above. The medium was refreshed every 2 weeks, and after 10 weeks the number of adventitious buds and shoots was determined.

TRANSFORMATION AND PLANT REGENERATION

The hypocotyls were excised from seedlings and inserted vertically apical part down into bud-inducing medium [MS basic medium + 5 mg/l BAP (6-benzyloamino purine) + 1 mg/l IAA (indole–3-acetic acid)] without antibiotics. After two days of preculture the explants were inoculated with *Agrobacterium* suspension for 15 min, blotted dry on filter paper and returned to the same medium for co-culture. After another two days the hypocotyls were placed on the selective medium (MS medium + 5 mg/l BAP + 1 mg/l IAA supplemented with 50 mg/l kanamycin and 300 mg/l timentin) in darkness. Some of the explants were not inoculated. The control explants, not co-cultured, were divided into two groups, one of which was placed on the bud-inducing medium (control I) and the other on a selective medium (control II). After 16 days the explants were transferred to the selective MS medium without growth regulators and cultured in the light at 26˚C.

MATERIAL PREPARATION FOR MICROSCOPIC ANALYSIS

To study the differentiation of adventitious buds, hypocotyl samples were collected and fixed at day 0 and after 1, 2, 3, 4, 5, 6, 7, 15 and 20 days of culture on bud-inducing medium. Each combination consisted of five hypocotyls. Structural observations were carried out on the control explants, not inoculated with *Agrobacterium tumefaciens* and cultured on medium with or without antibiotics, and on hypocotyls co-cultured with *Agrobacterium tumefaciens*. The material was fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h and postfixed in 1% OsO_4 for 2 h. After dehydration in a graded ethanol series, the material was immersed in acetone and then embedded in Epon resin.

Sections 4 μm thick were cut on a Reichert Ultracut E ultramicrotome and stained in a mixture of 1% methylene blue and 1% Azur B. The sections were examined and photographed under an Olympus BX60 microscope with an Olympus PM-C35DX camera.

Mean values and standard errors were estimated for analysis of the data.

TABLE 1. Kanamycin concentration versus bud and shoot development; means ± SE

Concentration of kanamycin [mg/l]	No. of explants	% of explants showing organogenesis (buds)	% of explants producing shoots
0	73	83.6 ± 0.37	11.0 ± 0.31
50	65	23.1 ± 0.42	0.0
60	65	18.3 ± 0.39	0.0
70	60	9.2 ± 0.29	0.0
100	33	0	0.0

RESULTS

GENETIC TRANSFORMATION

In the first phase of the survey, an experiment was performed to determine the most suitable kanamycin concentration for selection of transformed cells (Tab. 1). It was found that a kanamycin concentration of 50 mg/l reduced the amount of explants showing organogenesis to 23.1%, while the respective value for the control (without the selective agent) was 83.6%. Higher concentrations of kanamycin reduced organogenesis even more, resulting in complete necrosis of hypocotyls at a concentration of 100 mg/l. Shoot-producing explants were only observed in the control (11%), whereas a concentration of kanamycin as low as 50 mg/l inhibited shoot production. Therefore the 50 mg/l concentration of kanamycin was chosen as optimal for the selective medium.

The frequency of bud regeneration on hypocotyl explants after co-culture with *Agrobacterium tumefaciens* is presented in Table 2. In the three experiments carried out to obtain transgenic plants, the number of

TABLE 2. Percentage of explants showing organogenesis and producing shoots on selective medium after co-culture with *Agrobacterium tumefaciens* (LBA4404-pBI121); means ± SE

Experiment no.	No. of explants	% of explants showing organogenesis (buds)	% of explants producing shoots	% of total number of regenerated shoots
Inoculated with LBA4404(pBI121), with antibiotic				
I	50	80.0 ± 0.40	18.0 ± 0.38	24.0 ± 0.43
II	50	56.0 ± 0.50	16.0 ± 0.37	26.0 ± 0.44
III	126	50.8 ± 0.50	1.6 ± 0.12	4.0 ± 0.19
Total	226	58.4 ± 0.49	8.4 ± 0.28	13.3 ± 0.34
Not inoculated, without antibiotic (control I)				
I	30	60.0 ± 0.49	23.3 ± 0.42	23.3 ± 0.42
II	52	98.1 ± 0.14	51.9 ± 0.50	82.7 ± 0.38
III	21	85.8 ± 0.35	9.5 ± 0.29	19.0 ± 0.39
Total	103	84.5 ± 0.36	34.9 ± 0.48	52.4 ± 0.50
Not inoculated, with antibiotic (control II)				
I	36	2.8 ± 0.16	0.0	0.0
II	60	90.0 ± 0.30	0.0	0.0
III	28	14.3 ± 0.35	0.0	0.0
Total	124	47.5 ± 0.50	0.0	0.0

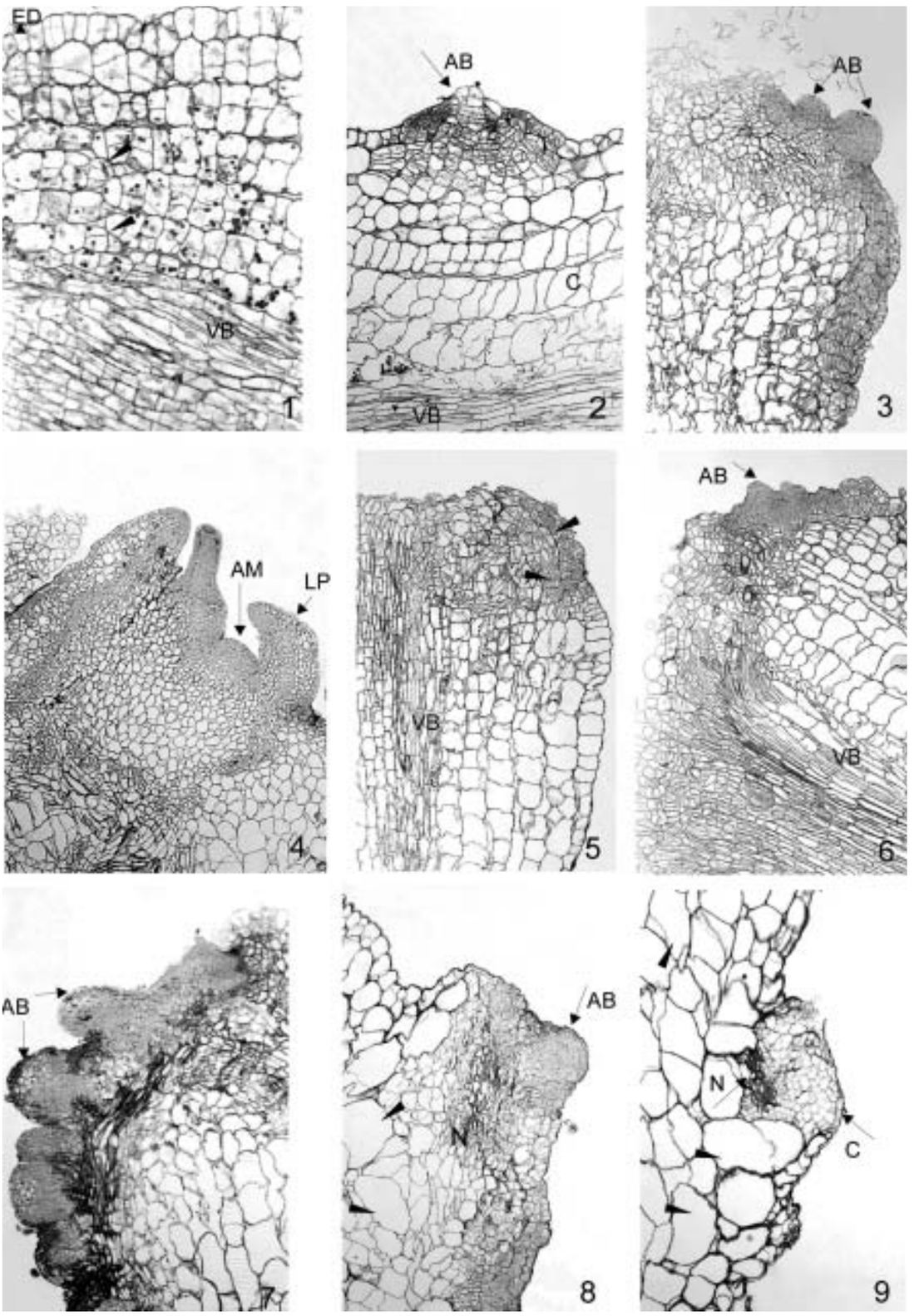
explants showing organogenesis varied, ranging from 50.8% to 80.0%. The percentage of explants producing shoots was much lower, from 1.6% to 18.0%. Buds developed on apical parts of the hypocotyls in direct contact with the medium. The majority of the adventitious buds did not form shoots. They either produced leaf-like structures, callused, or aborted. In the three experiments, 30 shoots were obtained from 19 hypocotyl explants. These shoots were rooted and transferred to soil. Molecular analysis of the obtained shoots will be carried out at a later date.

The hypocotyl explants not inoculated with *Agrobacterium tumefaciens* and cultured on medium without antibiotics (control I) showed higher rates of organogenesis (84.5%) and proliferation of shoots (34.9%), resulting in recovery of 52.4%of the shoots, versus 13.3% of the shoots obtained from explants co-cultured with *Agrobacterium tumefaciens*. The control II explants, cultured on a selective medium, showed a lower degree of organogenesis (47.5% of explants), and did not produce shoots.

HISTOLOGICAL ANALYSIS

The development of adventitious buds from hypocotyl explants was observed after a few days of culture on bud-inducing medium. It was manifested in a slight enlargement of the apical part of the hypocotyl. This enlargement was correlated with periclinal cortex cell divisions (Fig. 1). In subsequent days of culture the number of cell divisions in the cortex and epidermis increased. As a result of periclinal and anticlinal cell divisions, meristematic tissue and adventitious bud primordia were differentiated. The process was observed in hypocotyls from both controls (Figs. 2, 3 for control I) and in explants co-cultured with *Agrobacterium* (Fig. 6). It was possible to observe ramification of vascular bundles in meristematic regions (Fig. 5). The meristematic regions were larger than those of inoculated hypocotyls and covered the whole apical part of the hypocotyl in control I.

After two weeks of culture, the apical part of the hypocotyl (control I) enlarged three fold, forming buds visible on the entire apical surface of the explant. Well-developed apical meristems and leaf primordia were noted in longitudinal sections of adventitious buds (Fig. 4). The cells in the differentiating buds were characterized by dense cytoplasm. Histological analysis of inoculated and non-inoculated hypocotyls (control II) revealed cell divisions in the cortex and epidermis. Although the number of hypocotyls capable of bud development was relatively high (Fig. 7), only a small proportion of the buds developed into shoots (Tab. 2). In the hypocotyls that did not produce shoots, the buds showed disturbed cell organization (Fig. 9). Figure 9 shows a structure forming in a hypocotyl co-cultured with *Agrobacterium tumefaciens*, with a group of small non-meristematic cells of the cortex clearly visible. Hypertrophy of cortex cells and necrosis of mesophyll cells was also observed. Necrosis was also seen in vascular bundles connecting the developing bud with the hypocotyl conductive tissue (Fig. 8). The necrotic changes may have isolated the bud from the explant, which could inhibit shoot differentiation.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In the third week of culture, buds differentiated and developed further. The changes were smaller than those observed a week before. At this stage the buds were detached and transferred to regeneration medium without growth regulators, where shoot development occurred.

DISCUSSION

Almost every method of transformation involves a stage of plant regeneration in vitro. To get a positive outcome of transformation, regeneration methods maximizing the number of plants that are transformed independently are needed. An effective regeneration method requires an adequate selective agent that is not too toxic for the plant cells but allows transgenic cell division and differentiation followed by shoot formation.

Most reports on pepper transformation indicate the antibiotic kanamycin as a selective agent, used in a wide range of concentrations. Lee et al. (2004) and Lim et al. (1996) supplemented their selective medium with 100 mg/l kanamycin, while Li et al. (2003) considered a concentration of 50 mg/l to be sufficient for selection using cotyledonary explants. Mihalka et al. (1998) believed that kanamycin resistance is not an optimal selective marker for transgenic pepper production. In their experiments, non-transformed pepper cotyledons could tolerate kanamycin concentrations as high as 150 mg/l. Borychowski et al. (2002) tested the effect of kanamycin on hypocotyls of the pepper cultivar 'Bryza.' All the explants died on medium with a concentration of kanamycin 50 mg/l or higher. In our experiments, hypocotyl explants of the same variety cultured on medium with kanamycin lost their shoot regeneration ability and showed lower frequency of bud development. However, bud formation occurred at 50 mg/l and 70 mg/l concentrations of kanamycin.

There are two modes of shoot regeneration from in vitro culture of leaf, hypocotyl, and cotyledon explants. Shoots can develop directly from the explant or indirectly through a callus phase. Direct in vitro regeneration was induced with the use of the cytokinin benzylamino-purine (BAP) (Gatz, 1994; Fraś and Nowak, 1995; Li et al., 2003). Kinetin (Fraś and Nowak, 1995) and zeatin (Lee et al., 2004) were used to induce callus capable of bud formation. In the present experiment, adventitious buds were induced on MS medium containing BAP. Buds developed directly from the hypocotyl, without callus formation. Regeneration ability differed significantly between the three experiments. Such variability was also observed in previous experiments (Borychowski et al., 2002), and was attributed to a number of factors, among others the type and size of explants, season, and mode of culture. Lee et al. (2004) claimed that transgenic shoot regeneration in pepper is only possible with an intermittent callus phase. In their experiments, callus consisting of non-differentiated cells was formed during preculture. The use of callus presents some advantages, as it can be more easily inoculated with *Agrobacterium tumefaciens* than the hypocotyl tissue. Lee et al. (2004) never obtained a transformed pepper plant from shoots regenerated directly from explants, but they were the first to achieve pepper transformation via isolation of a callus-mediated shoot. The transformation protocol was published only recently, and has not been confirmed in other laboratories yet.

A review of the few publications available on transgenic pepper development raises the difficulties associated with the introduction of transgenes into pepper cells and subsequent transgenic shoot development. In this study, histological observations were made to shed some light on this problem. In the first week of culture, numerous anticlinal and periclinal cell divisions took place in the epidermis and cortex. As a result, meristematic tissue was developed. These cells were small, with large nuclei and dense cytoplasm. These features are in agreement with histological observations made by others with regard to regeneration from hypocotyls (Fari, 1983) and cotyledons of pepper (Fraś and Nowak, 1995).

Histological analysis of in vitro shoot morphogenesis indicated its subepidermal and/or epidermal origin, but the current study did not determine whether

Figs. 1–4. Histological changes during regeneration from hypocotyl explants after incubation on bud-inducing medium without antibiotics (control I). **Fig. 1.** Explant after 2 days of culture. Arrowheads indicate cortex cell divisions. **Fig. 2.** Explant after 6 days of culture. Group of meristematic cells (marked with arrow) forming adventitious bud. **Fig. 3.** Explant after 7 days of culture. Numerous primordia of adventitious buds (arrows). **Fig. 4.** Adventitious buds developing after 2 weeks of culture on inducing medium. ED – epidermis; VB – vascular bundles; C – cortex; AB – adventitious buds; AM – apical meristem; LP – leaf primordium. Fig. 1 × 130, Figs. 2–4 × 70. **Figs. 5–7.** Longitudinal section of hypocotyl explants inoculated with *Agrobacterium tumefaciens* LBA4404(pBI121). **Fig. 5.** Explants 2 days after inoculation. Arrowheads indicate numerous cortex and epidermis cell divisions. **Fig. 6.** Adventitious bud primordia in explants 5 days after inoculation with *Agrobacterium*. **Fig. 7.** Arrows indicate the development of adventitious buds after 3 weeks of culture. VB – vascular bundles; AB – adventitious buds. Figs. 5–7 × 70. **Figs. 8–9.** Disturbed organization of meristematic tissue and buds formed on hypocotyls treated with kanamycin and *Agrobacterium tumefaciens*. **Fig. 8.** Bud primordium (arrow) after 2 weeks of culture (control II). Arrowheads indicate enlarged cortex cells (hypertrophy) and necrosis of vascular bundles (N). **Fig. 9.** Hypocotyl explant inoculated with *Agrobacterium tumefaciens* LBA4404(pBI121) after 2 weeks of culture with bud primordium (arrow). Arrowheads indicate hypertrophic and necrotic cortex cells. C – cortex; AB – adventitious buds; N – necrotic cells. Figs. 8, 9 × 70.

the buds were formed from a single cortex or epidermal cell or from clusters of cells. It is supposed that the buds on the hypocotyl explants developed from multiple cells of cortex and epidermal origin. Fari (1983) stated that the meristematic tissue from which bud primordia developed could have originated from a single cell in the epidermis. We believe, however, that buds develop more frequently from clusters of epidermal and cortex cells. Wilmink et al. (1995) reported adventitious bud differentiation from clusters of subepidermal cells. Anticlinal and transversal divisions in epidermis cells contribute to the development of the epidermis of differentiating buds. This may reduce the chances of obtaining transformed tissue and result in the development of nontransgenic shoots. The multicellular origin of buds may also bring about the development of chimaeras. Histological analysis of hypocotyls (Fari, 1983) showed that some of the forming buds were missing the tunica and/or corpus. This observation explains the presence of leaf-like buds or callus formation.

The results of the histological analysis presented here confirm the abnormal organization of the meristematic tissues and buds formed on hypocotyls treated with kanamycin and co-cultured with *Agrobacterium tumefaciens*. Structural changes were observed in explants from the kanamycin control treatment and in the explants that underwent genetic transformation. Most probably, necrosis of the cortex and vascular bundles, hypertrophy of the cortex cells, and the nonmeristematic nature of the cells from which bud primordia developed, were responsible for the tissue degeneration and subsequent poor development of the buds. A more accurate histological analysis at the ultrastructural level should provide more in-depth information on the processes that take place in cells undergoing genetic transformation.

ACKNOWLEDGEMENTS

This work was supported by the Polish State Committee for Scientific Research (KBN grant no. 3 PO6A 006 24)

REFERENCES

AGRAWAL S, CHANDRA N, and KOTHARI SL. 1989. Plant regeneration in tissue cultures of pepper (*Capsicum annuum* L. cv. *mathania*). *Plant Cell, Tissue and Organ Culture* 16: 47–55.

BORYCHOWSKI A, NIEMIROWICZ-SZCZYTT K, and JĘDRASZKO M. 2002. Plant regeneration from sweet pepper (*Capsicum annuum* L.) hypocotyls explants. *Acta Physiologiae Plantarum* 24/3: 257–264.

FARI M. 1983. Histological analysis of adventitious shoot bud formation in pepper (*Capsicum annuum* L.) hypocotyls explants cultured in vitro. *Capsicum Newsletter* 2: 69–71.

FRAŚ A, and NOWAK K. 1995. Response of cotyledon explants of *Capsium annuum* L. cv Kujawianka to chosen plant growth regulators in *in vitro* culture. *Acta Societatis Botanicorum Poloniae* 64: 5–11.

GATZ A. 1994. Tworzenie kalusa i organogeneza z liścieni papryki w kulturach *in vitro*. *Zeszyty Problemowe Postępów Nauk Rolniczych* 414: 379–386.

HYDE C, and PHILLIPS G. 1996. Silver nitrate promotes shoot development and plant regeneration of chili pepper (*Capsicum annuum* L.) via organogenesis. *In Vitro* 32: 72–80.

LEE YH, KIM HS, KIM JY, JUNG M, PARK YS, LEE JS, CHOI SH, HER NH, LEE JH, HYUNG NI, LEE CH, YANG SG, and HARN CH. 2004. A new selection method for pepper transformation: callus-mediated shoot formation. *Plant Cell Reports* 23: 50–58.

LI D, ZHAO K, XIE B, ZHANG B, and LUO K. 2003. Establishment of highly efficient transformation system for pepper (*Capsicum annuum* L.). *Plant Cell Reports* 21: 785–788.

LIM HT, LEE K, YOO YS, and YANG DC. 1996. Plant regeneration of hot pepper and expression of mouse adenosine-deaminase gene via *Agrobacterium*-mediated transformation. *Hortscience* 31: 572.

LIM HT, LEE GY, YOU YS, PARK EJ, and SONG YN. 1999. Regeneration and genetic transformation of hot pepper plants. *Acta Horticulturae* 483: 387–396.

MANOHARAN M, VIDYA CSS, and SITA GL. 1998. *Agrobacterium*-mediated genetic transformation in hot chilli (*Capsicum annuum* L. var. *Pusa Jwala*). *Plant Science* 131: 77–83.

MIHALKA V, SZASZ A, FARI M, and NAGY I. 1998. Gene transfer in pepper: comparative investigation on tissue culture factors and vector system. *Xth EUCARPIA Meeting on Genetics and Breeding of Capsicum and Eggplant*. Avignon, France: 9–11.

MURASHIGE T, and SKOOG F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiologia Plantarum* 15: 473–479.

VALERA-MONTERO L, and OCHOA-ALEJO N. 1992. A novel approach for chili pepper (*Capsicum annuum* L.) plant regeneration: shoot induction in rooted hypocotyls. *Plant Science* 84: 215–219.

WILMINK A, VAN DE VEN BCE, CUSTERS JBM, NOLLEN Y, and DONS JJM. 1995. Adventitious shoot formation in tulip: histological analysis and response to selective agents. *Plant Science* 110: 155–164.

ZHU YX, OU-YANG WJ, ZHANG YF, and CHEN ZL. 1996. Transgenic sweet pepper plants from *Agrobacterium*- mediated transformation. *Plant Cell Reports* 16: 71–75.