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TRACHEARY ELEMENT DIFFERENTIATION AND MORPHOGENETIC CHANGES IN CALLUS DERIVED FROM EMBRYOS OF PEPPER (*Capsicum annuum* L.)

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Abstract. Tracheary elements appear already in the early stages of *in vitro* organogenesis and precede the formation of meristemoids and shoot primordia which further develop into shoots. Thus this process can be considered as an important indicator of potential, organogenic capability of the explants. The aim of this study was to investigate the effect of auxin (IAA), cytokinin (BAP), sucrose and the light conditions (light with 16-h photoperiod, darkness) upon *de novo* differentiation of tracheary elements (TEs) in callus derived from mature embryos of pepper cv. Bryza. Moreover, it was to determine the proliferation ability of the callus cells and changes of callus morphology. TEs were differentiated in the form of single cells, irregular clusters, strands and whirls exclusively with reticulate and pitted thickening of the secondary wall and the clusters occurred the most frequently. Cytokinin used alone, as well as in auxin combination, stimulated xylogenesis with the highest efficiency as well as with regard to the frequency and TE cell number within clusters. The highest number of TEs per cluster was obtained in the case of combination 0.1 mg·dm^{-3} IAA + 0.1 mg·dm^{-3} BAP on the light. Smooth surface, large cohesion cell degree and green colour are the morphological callus features accompanied by the effective TE formation.

Key words: pepper callus, tracheary element differentiation, plant growth regulators, morphology, sucrose, light conditions

INTRODUCTION

Capsicum due to its aroma, taste, high nutrient value (rich sources of beta carotenoids and vitamins C, B-complex, E, minerals) as well as therapeutic properties of capsaicin, is a world wide spread vegetable among other the most important crops [Ochoa-Alejo and Ireta-Moreno 1990]. Since the end of the seventies of the last century, the

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pepper has been the subject of researches on the elaboration of efficient *in vitro* regeneration protocols in order to apply the molecular biology techniques for pepper genetic improvement [Gunay and Rao 1978, Fari 1986, Kim et al. 2009]. Applying, among other things, different pepper cultivars, various types of explants, different media composition and *in vitro* culture conditions, it has been attempted to work out an efficient plant regeneration mainly through direct organogenesis and somatic embryogenesis. Unfortunately those methods are not still satisfactory because *Capsicum* as compared to other species of the *Solanaceae* such as *Nicotiana tabacum*, *Solanum tuberosum, Licopersicon esculentum* appeared to be a recalcitrant plant for the tissue culture techniques [Fari and Andrasfalvy 1994]. Only for some of the pepper genotypes [Szasz et al. 1995, Venkataiah et al. 2003], the efficient way of plant regeneration has been assumed to work out while for many others induced shoot buds elongated into shoot and plants with relatively low efficiency. Considering that, indirect organogenesis with intervening phase of callus could be an alternative for regeneration procedure mentioned above [Diaz et al 1988, Gatz and Tomaszewska 2007]. Callus constitutes the basic object for biotechnology application, it is among other things a potentially good source of the spontaneous variation and a suitable material for the generation and selection of the abiotic stress tolerant lines of plants. First our investigation focused on differentiation callus cells ability towards organogenesis. The cell divisions and the tracheary element (TE) formation in each explant are prerequisite for the organogensis and further plant regeneration. In the present study an attempt has been made to determine auxin IAA, cytokinin BAP, sucrose and light conditions effect upon *de novo* TE differentiation within callus derived from embryos of *Capsicum annuum* L., cultivar Bryza.

MATERIAL AND METHODS

Callus of *Capsicum annuum* L., polish variety Bryza derived from zygotic embryos was applied as plant material in this investigation. To obtain callus, dry seeds were treated with 95% ethanol for 1 minute then were surface sterilized in 4% (w/v) sodium hypochloride solution for 7 minutes, three times rinsed in sterile distilled water and placed onto the moist paper in Petri dishes under sterile conditions for preculture. The seed preculture lasted 3 days and it aimed to achieve the embryos potentially ready for development and their easier isolation from endosperm. For callus induction and proliferation, isolated embryos were placed horizontally on the MS medium [Murashige and Skoog 1962] solified with Difco-Bacto agar (0.8% w/v), complemented with 1 $mg \cdot dm^{-3}$ dichlorophenoxyacetic acid (2,4-D) and incubated for 4 weeks. Subsequently, approximately the seventeen-mg portions of mixed up initial callus (very friable) were transferred on the fresh MS medium with various combination of indole-3-acetic acid (IAA) and 6-benzylaminopurine (BAP) concentrations as well as on MS medium with several sucrose concentrations (% w/v) at constant level of IAA and BAP (tab. 1). Seed preculture and induction callus cultures were maintained at temperature 25º C under the 16-h day light, intensity 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (cool-white fluorescent lamps), and callus cultures for tracheary element (TE) formation were kept additionally on the dark treatment. After one month of callus culture, first morphologic callus features such as the structure

Table 1. Variants of the sucrose, plant growth regulators in the MS medium and light conditions employed for *de novo* tracheary element formation in pepper callus

Tabela 1. Warianty sacharozy i regulatorów wzrostu w pożywce MS oraz warunki świetlne zastosowane do formowania *de novo* elementów trachealnych w kalusie papryki

Light (16 h photoperiod) / Darkness Światło (16 godz. fotoperiod) / Ciemność									
MS + 0.1 (mg·dm^{-3}) IAA + sucrose (% w/v) MS + 0,1 (mg·dm^{-3}) IAA + sacharoza (% w/v)					MS + 0.1 (mg·dm^{-3}) BAP + sucrose (% w/v) MS + 0,1 (mg·dm^{-3}) BAP + sacharoza (% w/v)				
1	2	3	4	5	1	2	3	4	5
MS (3% w/v sucrose) / MS (3% w/v sacharoza)									
IAA (mg·dm^{-3}) + 0.1 mg·dm^{-3} BAP					BAP (mg·dm^{-3}) + 0.1 mg·dm^{-3} IAA				
0.005	0.01	0.1	1	10	0.005	0.01	0.1	1	10

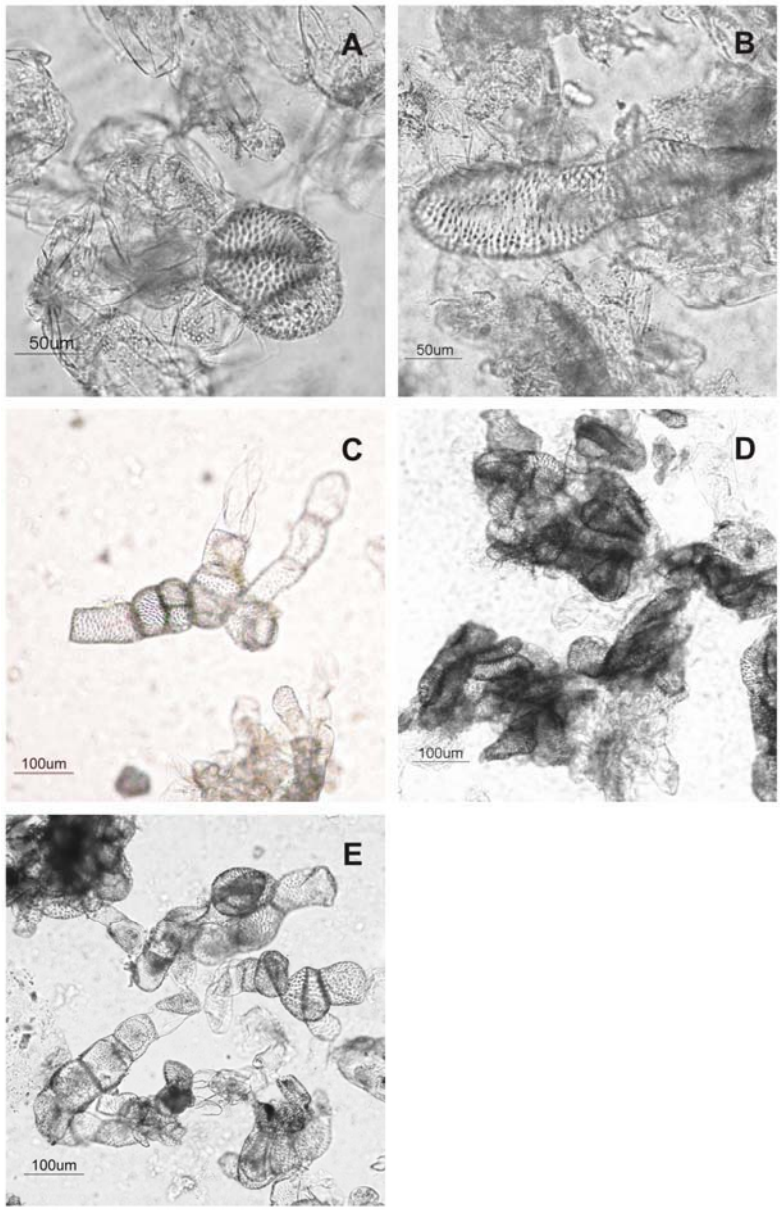
of the surface, friability and coloration were visually determined, subsequently the calli were measured to evaluate the fresh weight and at the end handmade slides without staining for each callus were made to the TE searching. The microscopic slides by cutting longitudinal sections from compact callus and by squashing soft and friable ones were prepared. Tracheary elements were counted in the field of light microscope vision at 100-fold magnification, the TE mean number was estimated on the base of 10 random counting the areas for each callus. Observed TEs were classified to one of the four groups as single cells, clusters of cells, strands and whirls. Moreover, the cell number within TE clusters was evaluated in the manner mentioned above. Each variant consisted of twenty replicates and all experiments were repeated twice. The data were analysed by analysis of variance (ANOVA MANOVA) using Tukey`s multiple range test at 5% level of significance.

RESULTS

The tracheary element differentiation from the pepper callus cells varied in a view of the quality and quantity features depending on the applied plant growth regulators (PGRs), sucrose concentration and the light conditions. That differentiation has been accompanied by changes of callus morphology and its growth rate.

Characteristic of tracheary elements (phot. 1). Tracheary elements (TEs) differentiated from callus cells occurred in a few forms taking into consideration the amount and mutual arrangement of the cells. They were single cells, irregular concentration of the cells – clusters, rarely strands or whirls. The amount and contribution of individual TE forms in regard to the total number of differentiated TEs as well as frequency of their occurrence were varied and depended on the kind, concentration variant as well as combination of examined factors. Most of the observed TEs were characterised by reticulate and pitted thikenings of secondary cell wall.

The effect of sucrose with stable IAA and BAP level (fig. 1, 2). The applying of sucrose in different concentrations at the constant level of auxin (0.1 mg·dm^{-3} IAA) effected on the frequency of TE differentiation (fig. 1A). With the increase of sucrose



Phot. 1. The forms of tracheary element organization in the *Capsicum annuum* callus: single cells with pitted (A) and reticulate (B) thickenings of secondary wall; clusters forming branched (C) or compact (D) structures; strands (E)

Fot. 1. Formy organizacji elementów trachealnych w kalusie *Capsicum annuum*: pojedyncze komórki z jamkowatymi (A) i siatkowatymi (B) zgrubieniami ściany wtórnej; skupienia formujące rozgałęzione (C) lub zwarte (D) struktury; pasma (E)

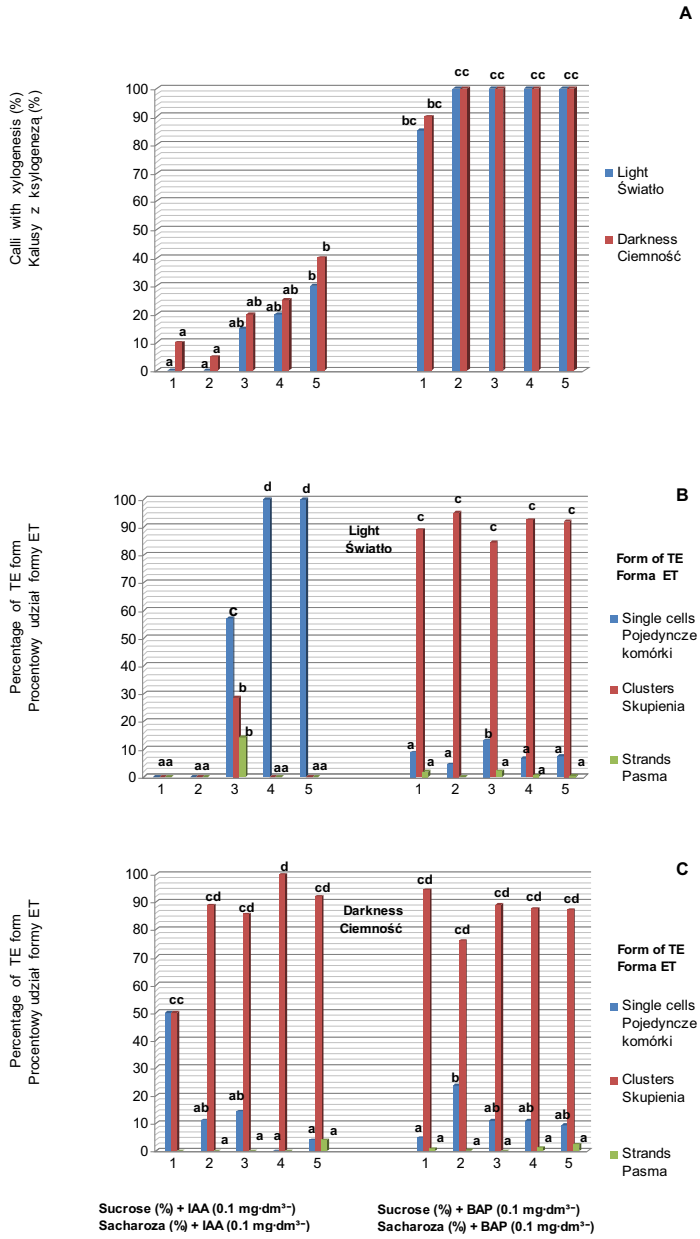


Fig. 1. The effect of sucrose, IAA, BAP and light conditions on the frequency of xylogenesis (A); the percentage of the TE form (B, C). Different letters indicate significant differences at p = 0.05 according to Tukey`s test

Ryc. 1. Wpływ sacharozy, IAA, BAP oraz warunków świetlnych na częstotliwość ksylogenezy (A); procentowy udział danej formy ET (B, C). Różne litery alfabetu oznaczają istotne różnice przy p = 0,05 zgodnie z testem Tukeya

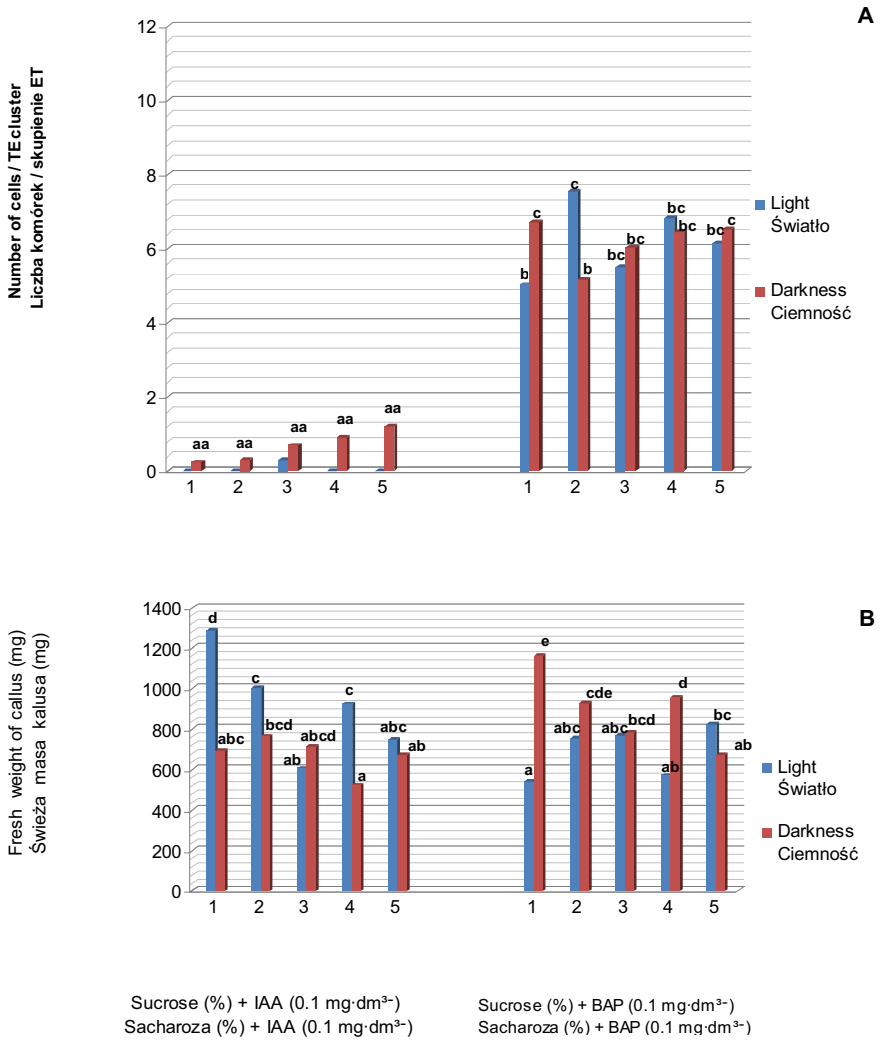


Fig. 2. The effect of sucrose, IAA, BAP and light conditions on the number of the cells within TE cluster (A); the fresh weight (B) of pepper callus after 4 weeks of culture. Different letters indicate significant differences at p = 0.05 according to Tukey`s test

Ryc. 2. Wpływ sacharozy, IAA, BAP oraz warunków świetlnych na liczbę komórek w skupieniu ET (A); świeżą masę (B) kalusa papryki po 4 tygodniach kultury. Różne litery alfabetu oznaczają istotne różnice przy p = 0,05 zgodnie z testem Tukeya

concentration, higher and higher number of the calli differentiating TE was recorded although with relatively low frequency. In contrast to auxin, the sucrose conjunction with cytokinin (0.1 mg·dm^{-3} BAP) did not exhibit the dependence mentioned above but in that case, very high frequency of the calli with xylogenesis at all treatment of sucrose

was noted (fig. 1A). Apart from that, the effect of sucrose concentration on the cell number into cluster of TE was observed. Together with the rise of sucrose level in the medium, containing constant IAA concentration, the number of the cells within clusters gradually increased whereas at the constant BAP concentration that dependence was not observed (fig. 2A). However, the cell number per TE cluster at all variants of sucrose concentration in relation to BAP was several times higher than in the case of IAA. The tested range of the sucrose concentration did not generally effect on the form of differentiated TE in callus cultured on the medium containing both IAA and BAP (fig. 1B-C).

The effect of IAA and BAP combinations (fig. 3, 4). The auxin presence in the combination with constant concentration of cytokinin showed a favourable effect on the frequency of TE differentiation both in the light and dark conditions (fig. 3A). While for BAP concentration variants, only 0.1 and 1.0 mg·dm^{-3} BAP have been found to be more effective to achieve the high frequency of xylogenesis (fig. 3A). A similar tendency with regard to the effect of IAA and BAP combinations occurred in the case of the amount cells within the TE clusters (fig. 4A). The application of IAA with the wide concentration range in the combination with constant BAP level gave lager numbers of cells within TE clusters than *vice versa* – BAP in combination with IAA. None of the applied PGRs effected essentially upon the TE form, only BAP at the highest concentration variants with constant IAA level on the light stimulated more the formation of TEs in single cell form than clusters (fig. 3B).

The effect of the light conditions (fig. 1–4). The effect of the light presence or absence marked first of all in the case when calli were incubated on the MS medium with the studied sugar and constant IAA concentrations. The calli exposured to light stimulated mostly the differentiation of the TE in the single cells form, contrary to the dark variant where the cell cluster formation outweighed (fig. 1B). Although the strands and whirls as the induced TE forms were not numerous within studied callus, they were observed more frequently in the case of the light than in the darkness (fig. 1B-C). In relation to the quantity of the cells in TE clusters, it was larger at the dark condition for variant of sucrose with the constant level of IAA, but at the lowest applied of BAP concentrations (0.005 and 0.01 mg·dm^{-3}) under the light, larger cell quantity within TE clusters has been achieved (fig. 2A).

Morphology of calli differentiating TE (fig. 5, 6; phot. 2). Factors involved in the xylogenesis induction in pepper callus also exerted the effect on callus morphology i.e. surface structure, friability and colour. Plant growth regulators among investigated factors appeared to be the most determining callus morphological features. IAA used alone in the MS medium stimulated soft or fragile callus with nodular surface, whereas BAP was generally favourable for compact, smooth or smooth-nodular callus formation (fig. 5). The application of auxin and cytokinin in their combination at the increasing level of IAA and constant BAP generated generally compact callus with smooth and smooth nodular texture (fig. 6). When the same PGRs in reversal combination of concentrations were used, differences of texture were observed (fig. 6). At a low cytokinin concentrations (0.005 and 0.01 mg·dm^{-3} BAP) the calli were characterized by nodular or smooth-nodular surface and at higher (1 and 10 mg·dm^{-3} BAP) mainly the smooth one. Differences also concerned the degree of cell cohesion, at lower concentration of BAP,

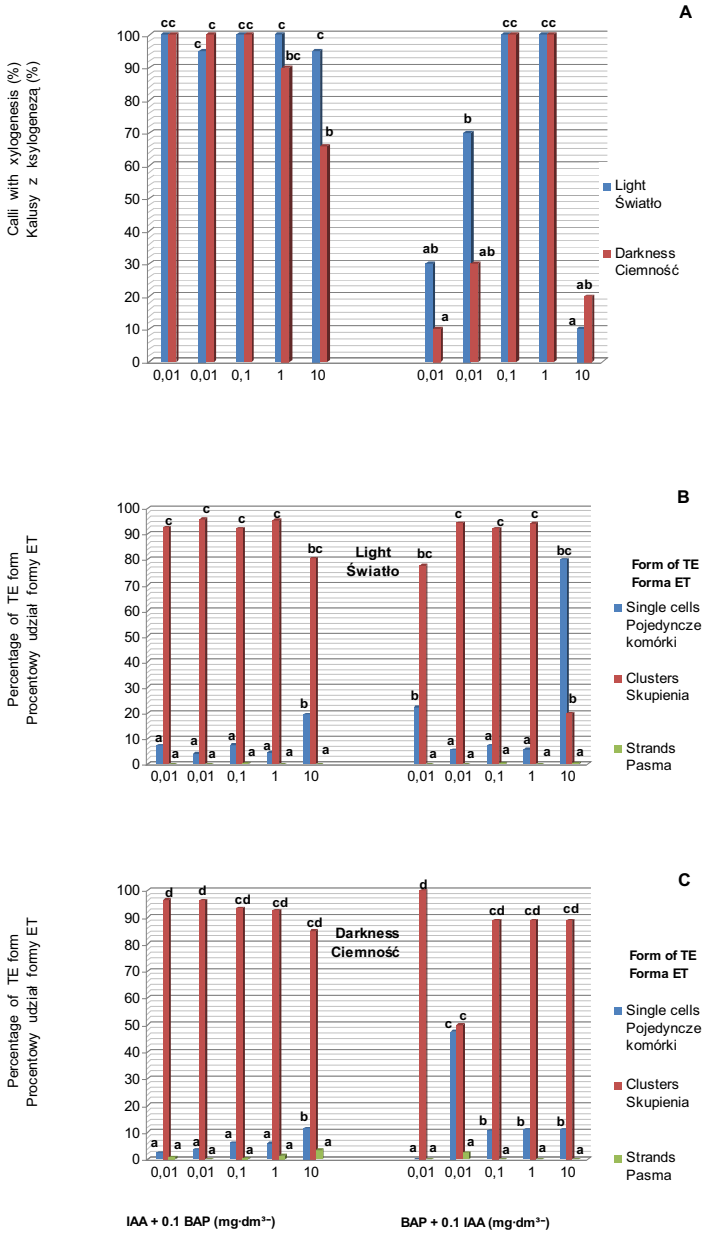


Fig. 3. The effect of IAA and BAP combination and light conditions on the frequency of xylogenesis (A); the percentage of the TE form (B, C). Different letters indicate significant differences at p = 0.05 according to Tukey`s test

Ryc. 3. Wpływ sacharozy, IAA, BAP oraz warunków świetlnych na częstotliwość ksylogenezy (A); procentowy udział danej formy ET (B, C). Różne litery alfabetu oznaczają istotne różnice przy p = 0,05 zgodnie z testem Tukeya

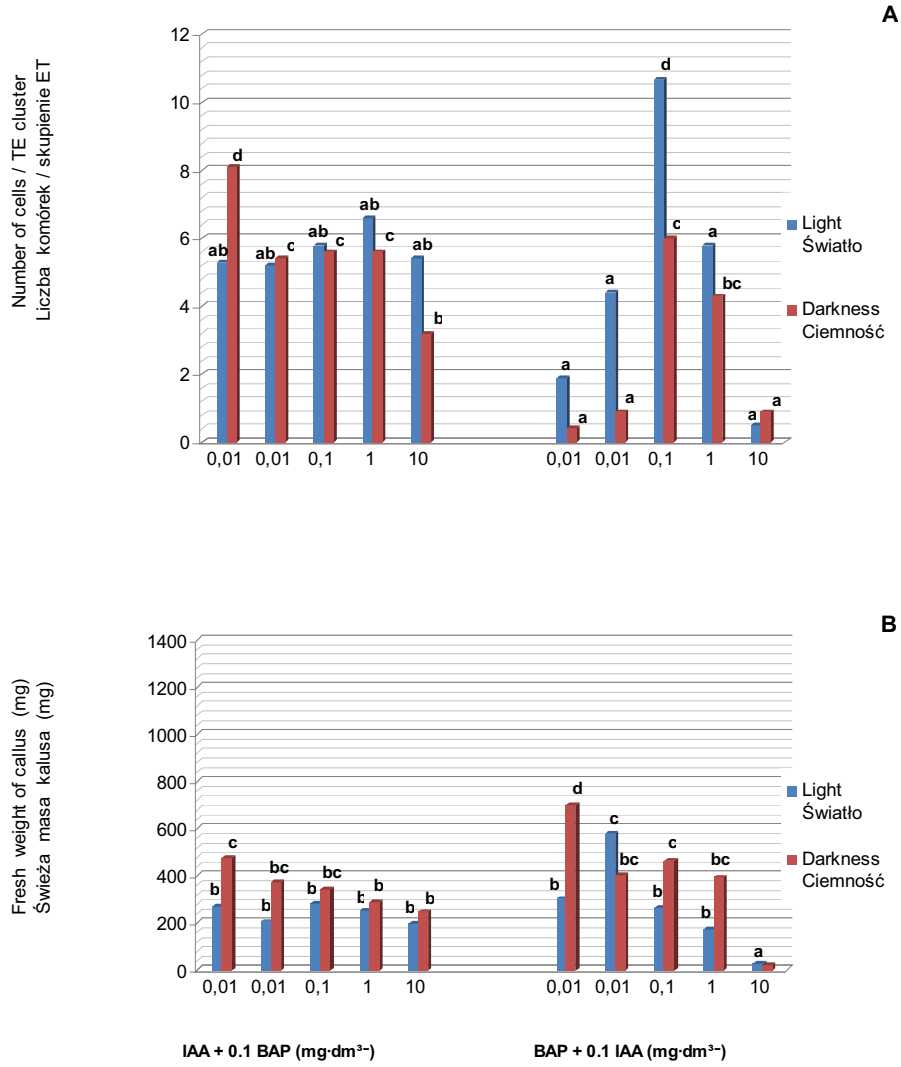


Fig. 4. The effect of IAA and BAP combination and light conditions on the number of the cells within TE cluster (A); the fresh weight (B) of pepper callus after 4 weeks of culture. Different letters indicate significant differences at p = 0.05 according to Tukey`s test

Ryc. 4. Wpływ sacharozy, IAA, BAP oraz warunków świetlnych na liczbę komórek w skupieniu ET (A); świeżą masę (B) kalusa papryki po 4 tygodniach kultury. Różne litery alfabetu oznaczają istotne różnice przy p = 0,05 zgodnie z testem Tukeya

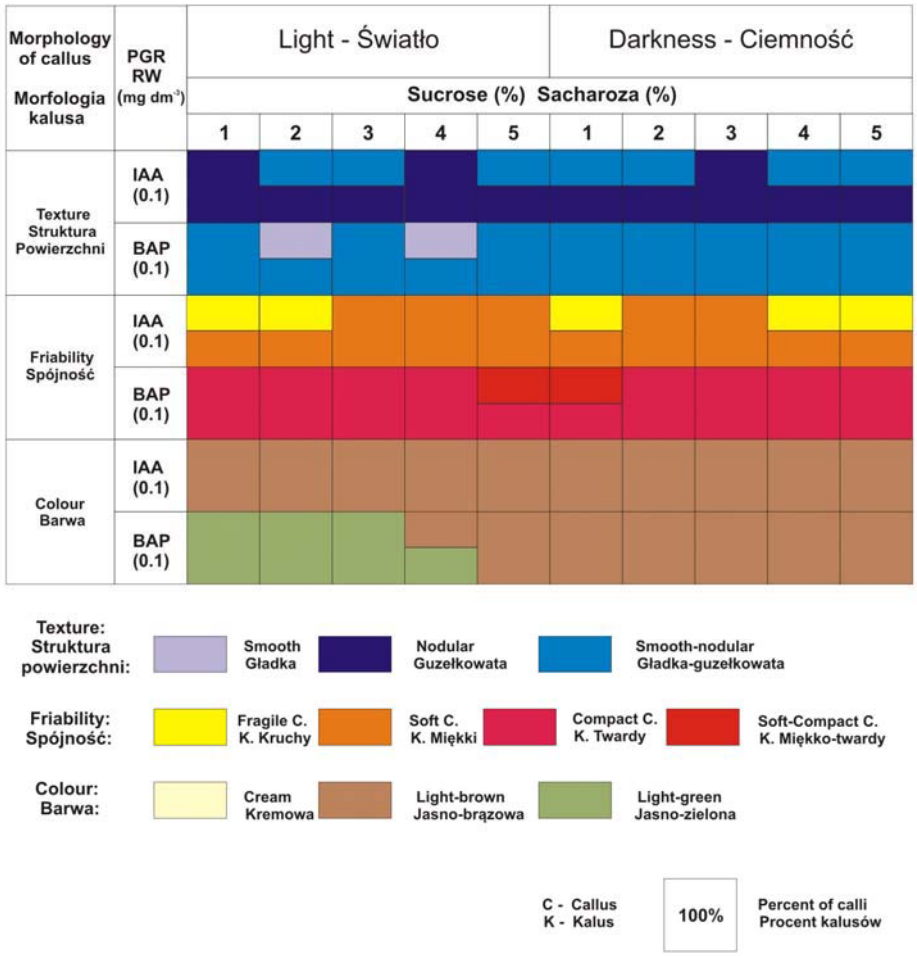


Fig. 5. Morphology of xylogentic callus incubated on MS medium with the different sucrose concentrations at constant IAA or BAP level on the light and in the darkness

Ryc. 5. Morfologia ksylogennego kalusa, inkubowanego na pożywce MS, zawierającej różne stężenia sacharozy przy stałym poziomie IAA lub BAP na świetle i w ciemności

mainly soft and fragile calli were obtained whereas at the higher ones – compact (fig. 6). Three basic colourations of the callus, light brown, light green and cream-coloured have been observed. Light brown callus more often occurred at all variant of examined factors, particularly in the darkness and also in the case of medium supplemented with auxin alone (fig. 5). Cytokinin present at the light participation promoted very often light green callus formation (fig. 5, 6). The morphological features of callus accompaning the effective TE differentiation were following: large degree of the cell cohesion (compact callus), smooth surface and green pigmentation unlike the loosely arranged cells (soft callus), nodular texture and colour other than green.

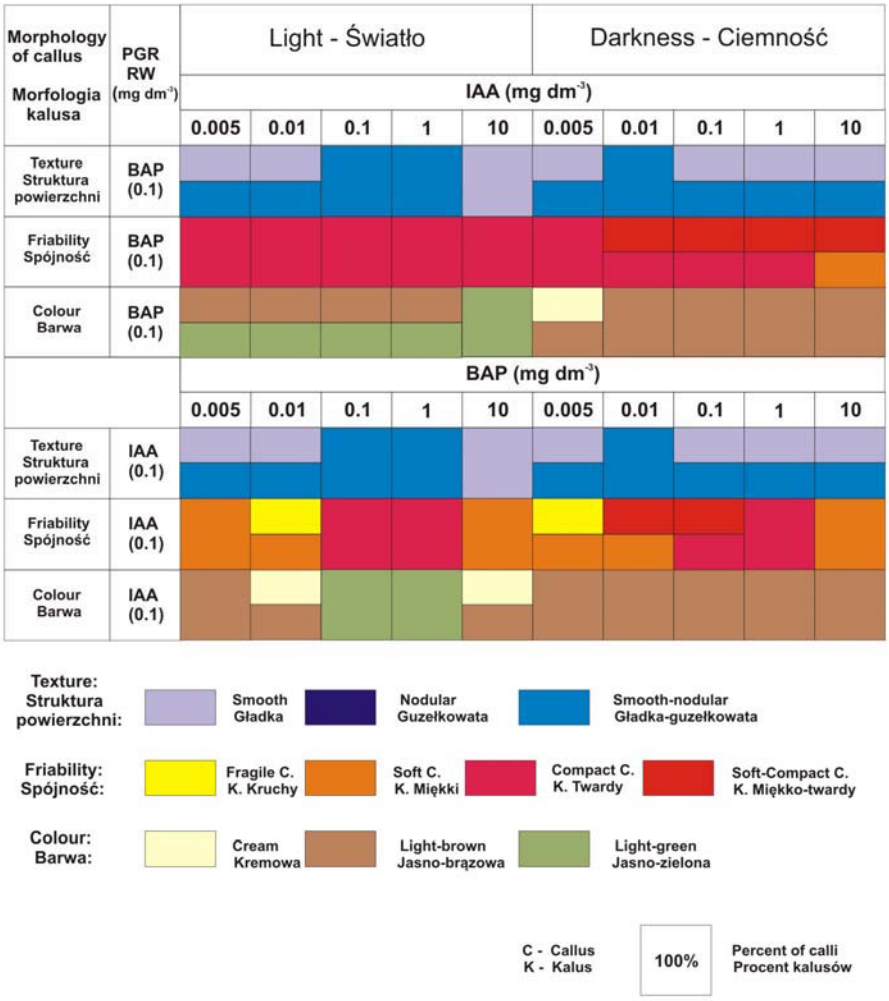
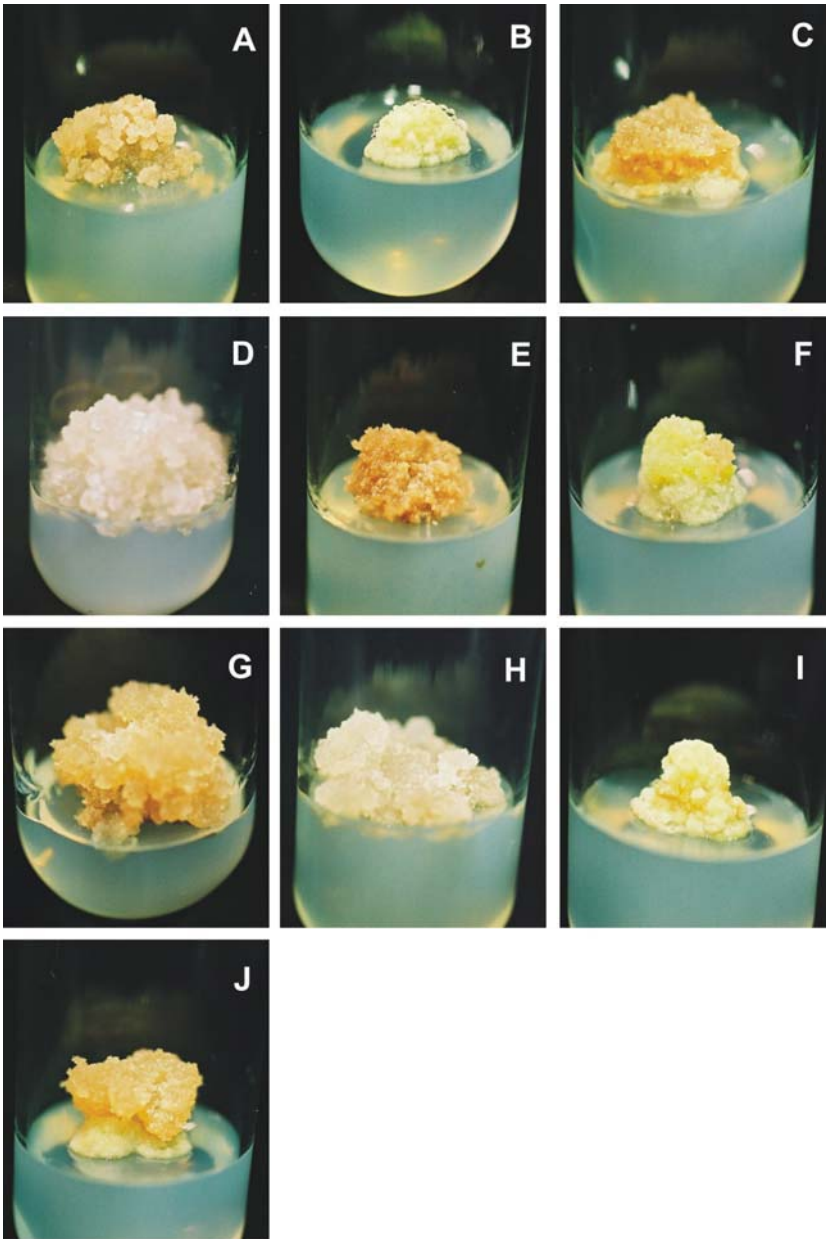


Fig. 6. Morphology of xylogentic callus incubated on MS medium with the combination of IAA and BAP concentrations under the light and dark conditions

Ryc. 6. Morfologia ksylogennego kalusa inkubowanego na pożywce MS z kombinacją stężeń IAA i BAP w warunkach światła i ciemności

Callus growth (fig. 2B, 4B). Like other early mentioned characteristics of callus, its proliferation ability was varied under study conditions. The higher fresh weight increases were achieved when the medium contained various concentration of sucrose at constant level of IAA on the light or BAP in the darkness, than for the IAA and BAP combinations at the constant sucrose concentration (fig. 2B). In both cases the highest callus weight over 1 gram was obtained at the lowest sucrose concentration. Generally



Phot. 2. Morphological features of pepper callus differentiated TEs: surface – nodular (A), smooth (B), smooth-nodular (C); colouration – cream (D), light brown (E) light green (F) friability – callus fragile (G), soft (H), compact (I), soft-compact (J);

Fot. 2. Cechy morfologiczne kalusa papryki z różnicującymi się ET: powierzchnia – guzełkowata (A), gładka (B), gładko-guzełkowata (C); barwa – kremowa (D), jasnobrązowa (E), jasnozielona (F); spójność – kalus kruchy (G), miękki (H), twardy (I), miękko-twardy (J)

there is no distinctive dependence between the fresh weight increase and the efficiency of TE formation, although cytokinin treated calli with the wide range of concentrations at constant IAA level, for which the highest efficiency TE formation occured, showed relatively low fresh weight increase compared with other treatments of PGRs (fig. 4B).

DISCUSSION

One of the aspects of callus regeneration potential is the ability of its cells to TE differentiation. Xylogenesis both in planta and *in vitro* culture conditions is a complex process including among other things hormonal induction, microtubule-oriented secondary cell wall thickening, lignin deposition and programmed cell death (PCD), [Ye and Varner 1993, Roberts and McCann 2000, McCann et al. 2000]. Various factors [Roberts 1983] and hundreds of genes [McCann 1997] are involved in this process but many findings [Twumasi et al. 2009] suggest that plant hormones act all-important role in the induction of TE differentiation. With respect to present investigation on TE formation within *Capsicum annuum* callus also among examined factors, PGRs exerted the greatest effect. Since the beginning of the researches on xylogenesis, auxin has been accepted generally as one of the fundamental inducers of that process [Esau 1965, Aloni 1987]. Into studied pepper callus, auxin also exhibited that ability and its distinguishable influence was shown when IAA was used alone with the combination of the sucrose concentration, and only in the light, it stimulated first of all the single cells of TE in contrast to cytokinin. The reason of that might be the decrease of IAA level under the light caused by its photooxidation. The support of this thesis may be the results achieved under the same conditions but in the darkness where mainly clusters of TEs have been formed.

Although IAA favoured xylem cell differentiation, the second applied PGRs i.e. BAP promoted the TE formation considerably effectively. The addition of BAP in quantity of 0.1 mg·dm^{-3} to medium, containing auxin in the studied range of concentrations, caused a noticeable increase of TE differentiation frequency of the calli. Whereas IAA addition in the same concentration to the medium with BAP concentration treatments reduced the frequency of xylogenesis occurring at the lower concentrations (0.005 and 0.01 mg·dm^{-3} BAP) where auxin to cytokinin ratios were as 20:1 and 10:1 respectively to the advantage of the auxin. A similar tendency could be observed with relation to the cell number within TE cluster. Generally the IAA presence in combination with BAP did not increase the efficiency of TE formation except the variant when IAA and BAP were applied in equal proportions (0.1 mg·dm^{-3} each), and for that the highest number of cells into TE cluster was noted and also in the case of 0.1 mg·dm^{-3} IAA with1.0 mg·dm^{-3} BAP. More profitable effect of BAP than IAA upon both frequency and the amount of differentiating callus cells into TEs as well as xylogenetic effect could be achieved by BAP alone, independently of IAA, which indicates that cytokinin may play an essential role during TE differentiation within *Capsicum annuum* callus.

Likewise in other species cultured *in vitro*: into cultured *Glicyne max* cells of cotyledonary origin [Fosket and Torrey 1969], in cultured tuber tissue of *Heliantus tubero-*

sus [Minocha and Halperin 1974], into isolated pith cylinder of *Lactuca sativa* [Warren-Wilson et al. 1982] indispensability of cytokinin apart from auxin for xylogenesis was reported. Cytokinin was necessary not only for induction of TEs but also for their progression [Fakuda and Komamine 1985, Church and Galston 1988]. Cytokinin was also needed for the induction of TE formation even in the system of *Zinnia* cell suspension culture in which individual mesophyll cells undergo differentiation into TEs without intervening cell division, it shows that cytokinin itself is prerequisite for the xylogenesis induction [Fakuda and Komamine 1980]. Recently the quickly induced TE system in *Arabidopsis* with the high efficiency based on auxin, cytokinin and brassinosteroid stimulation has been reported [Pesquet et al. 2010].

Carbohydrates also may be a factor stimulating TE formation especially in the auxin presence. The effect of sucrose concentration in the examined pepper callus was noted in the case of the sucrose conjunction only with constant level of IAA, when the increase of sucrose concentration up to 5% caused the increase of xylogenesis frequency and a slight increase of the cell number of TE cluster. Also in tuber explants of *Heliantus tuberosus* [Phillips and Dodds 1977], among several carbon sources, sucrose and glucose appeared to be better at TE formation than fructose, arabinose, soluble starch, manitol, sorbitol or trechalose. Sucrose in 2% concentration was the most effective in stimulating both growth and TE differentiation. However, a further concentration increasing up to 10%, on the one hand supported cell division, on the other hand progressively reduced TE number. Differences in the requirements of the optimal sucrose level for xylogenesis can be considerable between species for instance in *Lactuca sativa* 0.2%, in *Coleus* 0.6–1.0% and in *Nicotiana* 3% [Warren-Wilson et al. 1994] as well as 6% in *Daucus* and *Glicyne* [Aloni 1980] and the highest 10% in *Heliantus tuberosus* [Minocha and Halperin 1974]. The role of sucrose in the xylogenesis might consist in the stimulation of ethylene production which seems to contribute to TE formation as a hidden promoter [Meir et al. 1985, Kuriyama and Fakuda 2000].

Applying transgenic plants, suitable mutants and also synchronous system with the high frequency of TE formation will help to extend the knowledge of the regulation mechanism of TE differentiation.

CONCLUSIONS

1. Among studied factors cytokinin (BAP) applied alone or in the auxin (IAA) combination revealed the best capability for *de novo* TE formation in the wide range of its concentrations.

2. Different forms of the spatial organization of TEs such as single cells, clusters, strands and whirls always with reticulate or pitted thickenings of secondary cell wall were observed. Clusters occurred the most frequently.

3. Effective TE differentiation took place within calli characterised by smooth surface, large degree of cell cohesion and green pigmentation.

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RÓŻNICOWANIE ELEMENTÓW TRACHEALNYCH ORAZ ZMIANY MORFOGENETYCZNE W KALUSIE POCHODZĄCYM Z ZARODKÓW PAPRYKI (*Capsicum annuum* L.)

Streszczenie. Elementy trachealne pojawiają się już we wczesnych etapach organogenezy *in vitro* i poprzedzają powstanie merystemoidów i primordiów pędowych, które następnie rozwijają się w pędy. Proces ten może być zatem ważnym wskaźnikiem, określającym potencjalne zdolności organogenne eksplantatów. Celem pracy było zbadanie wpływu auksyny (IAA), cytokininy (BAP), sacharozy oraz warunków świetlnych (światło z 16-h fotoperiodem, ciemność) na różnicowanie *de novo* elementów trachealnych (ET) w kalusie pochodzącym z dojrzałych zarodków papryki odmiany Bryza. Ponadto określono zdolność do proliferacji komórek kalusa i jego zmiany morfologiczne. ET różnicowały się w formie pojedynczych komórek, nieregularnych skupień, pasm i wirów wyłącznie z siatkowatymi i jamkowatymi zgrubieniami ściany wtórnej, przy czym najczęściej występowały skupienia. Cytokinina zastosowana pojedynczo i w kombinacji z auksyną stymulowała najefektywniej ksylogenezę zarówno pod względem częstotliwości, jak i liczebności komórek ET w skupieniach. Najliczniej formujące się ET w skupieniu odnotowano w przypadku kombinacji 0,1 mg·dm^{-3} IAA + 0,1 mg·dm^{-3} BAP na świetle. Gładka powierzchnia, duża spójność komórek, zielona barwa to cechy morfologiczne kalusa, towarzyszące efektywnemu formowaniu ET.

Słowa kluczowe: kalus papryki, różnicowanie elementów trachealnych, regulatory wzrostu, morfologia, sacharoza, warunki świetlne

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