HISTOLOGICAL CHANGES DURING THE ADVENTITIOUS SHOOT FORMATION IN SEEDLING EXPLANTS OF PEPPER (*CAPSICUM ANNUUM* L.) CULTURED IN VITRO

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ABSTRACT

Adventitious shoots differentiated directly from explant tissue without intermediate callus on all types of examined explants (shoot tip, cotyledonary node, cotyledon and hypocotyl) of *Capsicum annuum* L. cv. Bryza. First cell divisions took place as early as after 3 days of explant culture within epidermal and subepidermal layers of explants, and in the case of cotyledon also within mesophyll cells located near epidermis. Mitotic activity in these layers led to the formation of meristemoids (meristematic centres). In all types of studied explants, meristematic centres appeared approximately at the same time (after about 7 days of culture). In the second week bud primordia began to differentiate from meristematic centres. Subsequently some of shoot primordia developed into leaves and leaf-like structures (mainly on cotyledon explants), and also into adventitious buds with well developed apical meristem and leaf primordia.

KEY WORDS: *Capsicum annuum* L., differentiation, direct organogenesis, leaf-like structures, meristematic centres, shoot primordia.

INTRODUCTION

Genetic engineering techniques provide an increasing number of possibilities of genetic manipulations that can be applied to improve economically important crops. The success in obtaining transgenic plants depends notably on efficient regeneration system of plants via tissue cultures in vitro. As far as the pepper is concerned, the first attempts to develop such a system were unsuccessful (Liu et al. 1990), the obtained transformed shoot buds were unable to elongate into normal shoots. However, recently pepper plant regeneration from the transformed explants has been reported (Zhu et al. 1996; Manoharan et al. 1998).

The regeneration ability of pepper depends on the proper combination of PGRs (Gunay and Rao 1978; Phillips and Hubstenberger 1985), the type of explants (Agrawal et al. 1989; Ebida and Hu 1993), as well as on the explant position (Fari and Czako 1981; Gatz and Rogozińska 1994), or genotype (Diaz et al. 1988; Ochoa-Alejo and Ireta Moreno 1990; Christopher and Rajam 1996). The culture conditions and especially the photoperiod and temperature also appear to be very important factors that affect this crop

plant regeneration (Phillips and Hubstenberger 1985). In pepper the main problem in regeneration processes is not the shoot primordium induction, but their further elongation and development into mature plants (Arroyo and Revilla 1991; Valera-Montero and Ochoa-Alejo 1992; Szasz et al. 1995).

Much valuable information on shoot bud primordia differentiation can be provided by histological analysis of the explant. Up to now, there have been few reports in which histological aspects of shoot bud formation in pepper are raised. These reports concern hypocotyl (Fari 1986) and cotyledon explants (Agrawal et al. 1989; Fraś and Nowak 1995). The aim of the present research was to determine the exact sites of shoot primordia initiation within the explants derived from different parts of pepper seedlings (shoot tips, cotyledonary nodes, cotyledons and hypocotyl), and to describe the early stages of their differentiation.

MATERIALS AND METHODS

Plant material and tissue culture conditions

Seeds of *Capsicum annuum* L., cv. Bryza were obtained from PlantiCo in Świętosławie. The seeds were surface sterilized by immersion in 4% calcium hypochlorite solution for 7 min followed by three washes in double distilled, steri-

Abbreviations:

MS – Murashige and Skoog; IAA – indole-3-acetic acid; BA – 6-benzyladenine; PGRs – plant growth regulators

Fig. 1. Anatomical changes during the adventitious bud formation on the shoot tip explants. (a) First cell divisions in epidermis and subepidermal layers after 3 days of culture; (b) meristemoid stage after 7 days of culture; meristematic centres (arrows) on the explant peripher near the cut surface are visible; (c) shoot primordium (arrow) after 2 weeks of the culture; (d) early stages of shoot bud development, 21 days of culture; (e) callus induction (arrow) on the cut surface of the explant incubated on MS medium without PGRs; a, b, c, e = longitudinal explant stem sections; d = transverse. Bars: Fig. 1a = 10 μm , Fig. 1b, c, d, e = 100 μm .

le water and placed on diluted (1:1) mineral Murashige and Skoog (1962) medium (MS), solidified with 0.8% (w/v) Difco Bacto-agar. Shoot tips (approximately 3 mm long), cotyledonary nodes (approximately 2 mm), distal parts of cotyledons (1 cm long) and acropetal parts of hypocotyl (5 mm long) excised from 3-week-old sterile pepper seedlings were used as explants. The explants were placed horizontally on MS medium without PGRs, as well as on MS medium supplemented with PGRs and vitamins as described by Phillips and Collins (1979). For shoot tip and cotyledonary node explants 5.7 μM IAA + 22.0 μM BA; 5.7 μM IAA + 44.0 μM BA were used respectively, and for cotyledon and hypocotyl explants 5.7 μM IAA + 44.0 μM BA was applied. All cultures were kept at $25 \pm 2^\circ\text{C}$ under 16 h photoperiod (daylight fluorescent illuminations; $35 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Histological analyses

For histological studies, tissue samples were collected from the tested explants after 0, 3, 7, 14 and 21 days of culture. They were fixed in FAA (formaldehyde 5% – acetic acid 5% – 70% ethanol 90%), dehydrated in a graded series of acetone, and embedded in paraffin (Jensen 1962). Tissue samples were sectioned serially both longitudinally and transversally, keeping natural orientation of these explants as in the seedling. The sections (10 μm thick) were stained with hematoxylin (Darlington and La Cour'a 1960).

RESULTS AND DISCUSSION

Previous studies on plant regeneration in *Capsicum annum* L., cv. Bryza (Gatz 2002), showed that shoot bud in-

Fig. 2. Shoot bud formation on cotyledonary node explants. (a) Anticlinal and periclinal divisions in the epidermal and subepidermal layers after one week of culture; (b) meristematic centres (arrow) within epidermal and subepidermal layers; (c) bud primordium after 7 days of culture; (d) adventitious buds in the early stage of development; (e) adventitious bud with shoot apex (arrowhead) and leaf primordia (*) after 3 weeks of the culture; (f) periderm formation (arrow) on the explant cultured without PGRs; a = transverse sections; b, c, d, f = longitudinal sections. Bars = 100 μ m.

duction took place in all types of studied seedling explants (shoot tip, cotyledonary node, distal part of cotyledon, apical part of hypocotyl) cultured on initiation medium (MS medium with PGRs). Shoot primordia appeared near the cut surface of the explant after about 10 days of culture. During successive days of culture, some of the primordia enlarged, others developed into leaves and leaf-like structures (especially on cotyledon explants). The highest quantity of shoot primordia per explant were noted on the cotyledonary node explants but at the same time these primor-

dia were the smallest of all, and only few of them developed into leaves. As intensive shoot primordia formation as on cotyledonary node explants did not occur on the other explant types. However, taking the further development of induced shoot primordia still on initiation medium into consideration, the best response showed cotyledon explants. Only on these explants almost all of the induced shoot primordia developed into leaf-like structures, leaves and adventitious buds. Generally, in spite of a large number of induced shoot primordia, as early as the first week of

explant culture on initiation media, after their termination, well developed adventitious buds were not obtained except a few ones mainly on cotyledon explants. Subculture of the shoot primordia and leaf-like structure on elongation media (MS with and without PGRs), caused their development into the rosettes of leaves and after extension of the culture time to eight weeks, their conversion into normal plantlets.

In the present investigation histological changes during shoot bud formation on seedling exlants of *Capsicum annuum* L., cv. Bryza were analysed. During month of explant culture on initiation medium shoot primordia, leaf-like structures, leaves and well developed adventitious buds were formed directly (without callus phase) from explant tissue. In spite of the presence of preexisting shoot apical and axilar meristems in shoot tip and cotyledonary node explants, the development of orginal shoots did not take place.

The induction of cell divisions (anticlinal and periclinal) occurred as early as after three days of culture in epidermal and the subepidermal layers of the shoot tip, cotyledonary node and hypocotyl explants (Figs 1a, 2b). In the case of

cotyledon explants, the first sign of mitotic activity was found within mesophyll cells located beneath epidermis. Subsequently with epidermis, mesophyll cells and procambial cells located near the explant cut surface also began to divide (Fig. 3a). The same places of shoot induction also in cotyledon explants of *Capsicum annuum* cv. Mathania and cv. Kujawianka (Agrawal et al. 1989; Fraś and Nowak 1995), *Pseudotsuga menziesii* (Cheah and Cheng 1978), *Vigna radiata* (Mendoza et al. 1993) and leaf explants of *Beta vulgaris* (Detrez et al. 1988) were observed.

Numerous periclinal and anticlinal divisions in epidermis and subjacent layers of studied explants continued and gave rise to meristemoids (meristematic centers) (Figs 1b, 2b, 3b, 4a). These structures were groups of small cells with prominent nuclei and dense cytoplasm (Fig. 3b). Tracheary elements with spiral wall thickenings were found in the neighbourhood of meristematic centres (Fig. 4b) as early as in the first week of culture.

In the second week of culture further extensive proliferation of meristemoid cells resulted in the increase of meriste-

Fig. 3. Shoot formation on cotyledon explants. (a) Mitotic activity occurring within the epidermis, mesophyll (arrowhead) and vascular bundle (arrow) after 7 days of culture (b) meristemoid stage showing meristematic centres (arrowheads) connected with vascular bundles (indicated by arrow); (c) finger-shaped primordia; (d) leaf-like structures; (e) young leaves; (f) group of the elongated cells resembling palisade mesophyll and containing chloroplasts (an arrow) found in the leaf-like structure after 21 days; a, b, f = transverse sections; c, d, e = longitudinal sections. Bars = 100 µm.

Fig. 4. Adventitious bud formation on hypocotyl explants. (a) Numerous meristematic centres (peripheral part of explant) consisting of the cells with dark stained cytoplasm and large nucleus, 7 days of culture; (b) tracheary elements with spiral wall thickenings (an arrow) near meristematic centres (arrowhead); (c) bud primordium connected with vascular bundle (arrow) of the explant, 14 days; (d) structures resembling early stage of shoot apex with forming leaf primordia (arrowheads), 21 days; a, b, d = transverse sections; c = longitudinal sections. Bars = 100 µm.

moid volume and the formation of shoot primordia (Figs 1c, 2c, 3c, 4c). They could be macroscopically observed as small protrusions of the explant periphery near the cut surface.

Vascular tissues of the explants did not participate directly in initiation of shoot primordia. However, in early stages of shoot bud differentiation, vascular connections between the meristematic centres or bud primordia and the primary vascular system of the explants were observed (Figs 1b-c, 3b, 4c). Researches carried out on other pepper cultivars (Agrawal et al. 1989; Fraś and Nowak 1995) as well as on other plant species, like *Picea glauca* (Rumary et al. 1986), *Camellia reticulata* (San-Jose and Vietez 1992) and *Stylosanthes scabra* (Dornelas et al. 1992), this vascular tissue connects the differentiating shoot primordia with the vascular system of the explant.

In the third week of explant culture, the dome shaped primordia (shoot tip, cotyledonary node, and hypocotyl explants) began to differentiate into structures that resembled the early stage of shoot apex structure. However, they did not yet have distinctly defined apical dome and looked like leaf primordia (Figs 1d, 2d, 4d), whereas the dome shaped primordia on cotyledon explants became transformed into leaf-like structures (Fig. 3d) as well as into leaves (Fig. 3e). Leaf-like structures had different sizes and irregular shape and were often branching in a leaf-like manner (Fig. 3d). Similar structures were observed also on hypocotyl,

young leaf and cotyledon explants of different cultivars of *Capsicum annuum* L., (Liu et al. 1990; Szasz et al. 1995).

Sometimes within leaf-like structures the groups of elongated cells containing chloroplasts were observed. The arrangement of these cells resembled a palisade mesophyll of a leaf (Fig. 3f). Such specified organised groups of cells were observed only within leaf-like structures originating from cotyledon explants. It suggests that these leaf-like structures were more advanced in differentiation of proper leaf tissues than the ones formed on shoot tip and hypocotyl explants.

Apart from shoot primordia, leaf-like structures and leaves in some cases adventitious buds with well-defined structures i.e. with shoot apex, leaf primordia and vascular bundles were observed (Fig. 2e).

On MS medium without plant growth regulators the studied explants enlarged and sometimes on the cut surface either loosely callus tissue (Fig. 1e) or periderm-like tissue (cell arranged in periclinal rows) (Fig. 2f) were formed. The arrangement of callus cells resembling periderm on the cut surface of cotyledon explants of *Capsicum annuum* cv. Kujawianka cultured on MS medium with PGRs was also observed (Fraś and Nowak 1995).

The results obtained in the present studies of *Capsicum annuum* cv. Bryza confirm the general rules of adventitious shoot bud formation in vitro, such as the sites of shoot bud induction as well as their stages of differentiation and de-

velopment. However, further research is required in order to define conditions for efficient conversion of numerous induced shoot bud primordia as well as leaf-like structures (Gatz 2002) into distinct separate shoot buds.

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ZMIANY HISTOLOGICZNE PODCZAS FORMOWANIA PĄKÓW PĘDOWYCH W EKSPANTATACH SIEWEK PAPRYKI (*CAPSICUM ANNUUM* L.)

STRESZCZENIE

Pąki przybyszowe różnicowały się bezpośrednio z tkanek pierwotnych badanych eksplantatów wierzchołków pędu, węzłów liścieniowych, liścieni i hypocotyli *Capsicum annuum* L., cv. Bryza. Pierwsze podziały komórkowe były obserwowane już po 3 dniach kultury w warstwach epidermalnej i subepidermalnej eksplantatów, a w przypadku eksplantatów liścieniowych również w komórkach mezofilu znajdujących się blisko epidermy. Aktywność mitotyczna w tych warstwach eksplantatów prowadziła do formowania centrów merystematycznych we wszystkich typach eksplantatów mniej więcej w tym samym czasie, tj. po około 7 dniach. Zawiązki pąków pędowych zaczynały różnicować się z centrów merystematycznych w drugim tygodniu kultury a w następnym część z nich rozwijała się w liście i struktury liściopodobne (głównie na eksplantatach liścieniowych), jak również w pąki pędowe z dobrze wykształconym merystemem apikalnym i zawiązkami liści.

SŁOWA KLUCZOWE: *Capsicum annuum* L., różnicowanie, bezpośrednia organogeneza, struktury liściopodobne, centra merystematyczne, zawiązki pąków pędowych.