INDUCTION OF SHOOT BUDS, MULTIPLICATION AND PLANTLET FORMATION IN SEEDLING EXPLANTS OF BELL PEPPER (*CAPSICUM ANNUUM* L., CV. BRYZA) IN VITRO

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ABSTRACT

In vitro shoot bud induction and multiplication as well as plantlets formation from different parts of 21-d old seedlings (shoot tip, cotyledonary node, distal part of cotyledon, acropetal section of hypocotyl) of *Capsicum annuum* L., cv. Bryza were compared. During 4 weeks of primary explant culture on initiation media, first shoot bud primordia appeared; they reminded leaf primordia and subsequently some of them underwent enlargement, some developed into leaves and leaf-like structures (mainly on cotyledon explants). The highest number of shoot bud primordia was noted on cotyledonary node explants, but they were smaller than those on the remaining types of the explants. The best response of shoot regeneration showed cotyledon explants on which most of shoot buds were formed in each from four treated passages. From shoot buds on elongation media after 4 weeks of culture rooted rosettes of leaves were achieved, and the extension of the culture time to eight weeks without subculture caused that the rosettes developed into plantlets. Throughout four successive passages plantlets were obtained from cotyledon and shoot tip explants.

KEY WORDS: *Capsicum annuum* L., seedling explants, shoot regeneration, plantlets.

INTRODUCTION

Pepper is one of the most important vegetable crops in the world (Fari 1986). In Poland the consumption of pepper in last years has significantly increased. On the one hand it is related to Polish cultivars of sweet pepper which have been coming into the market, on the other hand to certain changes of nutrition custom, for the benefit of increase of vegetable and fruit consumption.

New cultivars of pepper are the effect of long and arduous breeding work. Now the application of molecular biology technique creates wide and simultaneously faster possibilities for improvement of important economic plants through genetic transformation. However, success in obtaining transgenic plants depends considerably on their efficient regeneration system (Zhu et al. 1996). Plant regeneration system of pepper is still not so efficient as in other members of the Solanaceae family (Manoharm et al. 1998).

On regeneration capabilities of pepper, like in other plants, endogenic factors such as explant type (Agrawal et al. 1989; Ebida and Hu 1993), its position (Fari and Czako 1981; Gatz and Rogozińska 1994), genotype (Diaz et al. 1988; Ochoa-Alejo and Ireta-Moreno 1990; Christopher

and Rajam 1996) as well as age and developmental stage (Ramirez-Malagón and Ochoa-Alejo 1996) have significant influence. Moreover exogenous factors such as proper combination of plant growth regulators in medium (Gunay and Rao 1978; Phillips and Hubstenberger 1985), the length of photoperiod, temperature and number of subcultures (Phillips and Hubstenberger 1985) also perform an important role in pepper regeneration.

The aim of the present study was to compare the abilities of different explant types of Polish pepper cultivar cv. Bryza for shoot bud induction, multiplication and plantlets formation.

MATERIALS AND METHODS

Seeds of bell sweet pepper (*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, sterile water and placed on diluted (1:1) mineral Murashige and Skoog (1962) medium (MS) solidified with 0.8% (w/v) of Difco Bacto-agar. The pH was adjusted to 5.8 before autoclaving. Four types of explants: shoot tip, cotyledonary node, distal part of cotyledon and acropetal section of hypocotyl were excised from 21-days-old sterile pepper seedlings as shown in Fig. 1. These

Abbreviations:

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

Fig. 1. Type of explants (dotted area) excised from 21-d old pepper seedling cv. Bryza: ST – shoot tip (approximately 3 mm in length), CN – cotyledonary node (approximately 2 mm in length), C – distal part of cotyledon (1 cm in length), H – acropetal section of hypocotyl (5 mm in length)

explants were placed horizontally onto control medium – MS as well as on initiation medium – MS supplemented with vitamins, as described by Phillips & Collins (1979), and plant growth regulators (PGR). For shoot tip and cotyledonary node explants were used $5.7 \mu\text{M}$ IAA + $22.0 \mu\text{M}$ BA, $5.7 \mu\text{M}$ IAA + $44.0 \mu\text{M}$ BA respectively, and for cotyledon and hypocotyl explants $0.57 \mu\text{M}$ IAA + $44.0 \mu\text{M}$ BA according to preliminary studies (data unpublished).

As a result of induction shoot buds in primary explants and their formation in successive passages typical separated shoot buds in explants did not form, therefore equal in size explant pieces with shoot primordia (in the first passage) and with shoot buds (in the next passages) were applied in passages. After 4 weeks of culture on initiation media, pieces with induced shoot primordia, leaf-like structures and leaves were cut from primary explants, and subsequently one part of them was transferred to MS medium with the same combinations PGR as in initiation media (shoot tip – $5.7 \mu\text{M}$ IAA + $22.0 \mu\text{M}$ BA, cotyledonary node – $5.7 \mu\text{M}$ IAA + $44.0 \mu\text{M}$ BA, cotyledon and hypocotyl explants – $0.57 \mu\text{M}$ IAA + $44.0 \mu\text{M}$ BA) to obtain their multiplication. The second part of shoot primordia was placed on elongation media to achieve shoot bud elongation and plantlet formation. After the first passage one part of multiplied shoot buds was transferred again on multiplication media, the second part – on elongation media and this way of treatment was applied up to the fourth passage. MS medium supplemented with IAA ($0.285 \mu\text{M}$) and BA (0.220

Fig. 2. Explants of shoot tip (ST), cotyledonary node (CN), cotyledon (C), and hypocotyl (H) after 4 weeks of culture on: A) control medium – MS without PGR; B) initiation medium – MS complemented with: $5.7 \mu\text{M}$ IAA + $22.0 \mu\text{M}$ BA (ST), $5.7 \mu\text{M}$ IAA + $44.0 \mu\text{M}$ BA (CN), $0.57 \mu\text{M}$ IAA + $44.0 \mu\text{M}$ BA (C, H). Scale bar = 1 cm.

μM) for the first passage and without PGR for the next one was used as elongation media. The time of culture on elongation media was 8 weeks.

All cultures were kept at $25\pm 2^\circ\text{C}$ under 16 h photoperiod (daylight fluorescent illuminations; $35\ \mu\text{mol m}^{-2}\text{ s}^{-1}$). In the case of shoot bud initiation, their multiplication and elongation data consist of twenty four replications in each explant type and passage treatment. This study was repeated twice. The number and length of shoot bud primordia were determined using Stemi 2000-C stereomicroscope (Zeiss). The results were statistically analysed by the least significant difference (LSD) test.

RESULTS AND DISCUSSION

Various explant types of pepper cv. Bryza showed different abilities of shoot bud induction, their multiplication and plant regeneration.

Shoot bud induction

In the first week of explant culture on initiation media (MS medium with different concentration IAA and BA), enlargement of all explant types was observed, and after about 10 days shoot bud primordia began to appear on the cut surface of the explants. Initially the length of these primordia was about 0.2 mm and they looked like leaf pri-

mordia. During the following weeks some of the shoot bud primordia elongated, others developed into leaves and leaf-like structures, while on the explants from control medium shoot bud induction was not observed (Fig. 2).

The highest number of shoot bud primordia per explant was counted on cotyledonary node explants (Fig. 3A), but their dimensions were the least among studied explant types (0.2-1.0 mm in length) (Fig. 4B), and the primordium development into leaves or other structures was observed only after their passage on elongation media. Shoot primordia of similar length (0.3-1.2 mm) were present on hypocotyl explants (Fig. 4D), but their number per explant was six times lower (Fig. 3A). A little larger shoot bud primordia (0.3-2.0 mm in length) than those on hypocotyl explants occurred on shoot tip (Fig. 4A). However, the most intensive growth and development of induced primordia took place mainly on the cotyledon explants. Most of these primordia originated leaf-like structures and leaves (2-10 mm in length) (Fig. 4C). The frequency of shoot bud primordium induction on studied explant types was relatively high, however, in the case of cotyledonary node and cotyledon explants it was the highest since the first week as compared with shoot tip and hypocotyl explants (Fig. 3B).

In many other cultivars of pepper, cotyledon explants showed also higher shoot bud induction abilities than the explants of hypocotyl (Gunay and Rao 1978; Agrawal et al. 1989; Ebida and Hu 1993), shoot tip (Agrawal et al.

Fig. 3. Shoot bud primordia formation from various parts of 21-day old pepper seedling explants on initiation medium = MS with: 5.7 μM IAA + 22.0 μM BA for shoot tip, 5.7 μM IAA + 44.0 μM BA for cotyledonary node, 0.57 μM IAA + 44.0 μM BA for cotyledon and hypocotyl explants. A – Number of induced shoot bud primordia, leaf-like structures and leaves after four weeks of incubation; B – Percent of explants with regenerating shoot bud primordia (including leaf-like structures and leaves) during four weeks of incubation. Means with different letters are significant at $p = 0.05$ by using LSD procedure.

Fig. 4. Shoot bud primordia morphology of shoot tip (A), cotyledonary node (B), cotyledon (C), hypocotyl (D) explants after 4 weeks of culture on initiation medium – MS supplemented with 5.7 μM IAA + 22.0 μM BA (shoot tip), 5.7 μM IAA + 44.0 μM BA (cotyledonary node), 0.57 μM IAA + 44.0 μM BA (cotyledon and hypocotyl explants) Shoot bud primordia indicated by arrows; in the case of cotyledon explants arrow shows leaf-like structure. Scale bar = 1 mm.

1989; Rogozińska and Tobolewska 1992), leaf (Agrawal et al. 1989; Gatz and Rogozińska 1994), stem and root segments (Agrawal et al. 1989; Ebida and Hu 1995). However, some authors (Christopher and Rajam 1996) reported that among seven studied genotypes of *Capsicum* (*C. praetermissum*, *C. baccatum*, *C. annuum* cvs. G₄, Bhiwapuri, Sweet pepper, Cayenne pepper and Hybrid pepper) on three different media they achieved more shoots from leaf explants than cotyledon or hypocotyl. That difference of shoot organogenesis response of cotyledon explants in a great measure results probably from genotype differences (Ochoa-Alejo and Ireta-Moreno 1990).

Generally after 4 weeks of culture on the studied explants of pepper among numerous primordia, leaves and leaf-like structures, distinctly separated shoot buds can not be observed, except single cases on cotyledon explants.

Shoot bud multiplication

After shoot primordia induction on primary explants, equal pieces of each explant shoot primordia comprising (first passage) and shoot buds (next passages) were isolated and subcultured on fresh media (the same media for induction) in order to multiply them. On these media additional shoot bud formation occurred on all studied explant types during 4 passages. The size of regenerated shoot buds at the end of each passage was bigger than shoot bud primordia on induction media (Fig. 5A). In the case of *C. annuum* cv. Mathania (Agrawal et al. 1989) the size of shoot buds on multiplication medium was the same like on induction media. It results from the fact that in *C. annuum* cv. Mathania well developed shoot buds occurred already on induction media and not only shoot bud primordia or leaf-like structures like it was in the studied explants.

Fig. 5. Structures primary induced on cotyledon explant (mainly leaf-like structures) during the first passage A) on multiplication medium – MS with IAA (0.57 μM) and BA (44.0 μM); shoot buds, bar = 0.5 cm; B) on elongation medium – MS with IAA (0.285 μM) and BA (0.220 μM) after 4 weeks of culture; rosettes of leaves with roots and after 8 weeks (C) on the same medium; seen plantlet with well developed roots. Scale bar (B, C) = 1 cm.

Regarding to pepper cv. Bryza the highest shoot bud formation response exhibited cotyledon explants. It concerned both number of induced shoot buds per explant (Fig. 6A), and the percentage of explants forming shoot buds, although the number of produced shoot buds gradually declined during the successive passages (Fig. 6B). These results are in contrast to the report of Phillips and Hubstenburger (1985), who reported that the best shooting response exhibited shoot tip and cotyledonary nodes as meristematic explants of bell and chile peppers (*Capsicum annuum*). Moreover, in similar incubation environment (temperature 25°C, 16-h photoperiod) and also using the same growth substances (IAA and BA), they obtained formation of shoots only during 2 passages and only from meristematic explants, but from non meristematic explants (distal cotyledon pieces, hypocotyl sections) only in the first passage. Shoot bud regeneration was possible during 4 passages for nonmeristematic explants and during 8 passages for meristematic only at higher temperature (28.5°C) and the extension of photoperiod (from 16-h to continuous light). Unfortunately these authors present data only as percentage of shooting explants, therefore it is difficult to discuss the realistic efficiency of shoot bud multiplication in analysed passages.

Shoot elongation and plant regeneration

In order to obtain complete plantlets of pepper cv. Bryza from induced shoot primordia on primary explants, some of them were transferred to the elongation media. On

these media after a month of culture, shoot primordia developed into the rosettes of leaves (sometimes distorted) with roots (Fig. 5B). Only extension of the culture time to eight weeks on elongation media caused that some of the rosettes converted into normal plantlets (Fig. 5C). Structures resembling rosettes were observed on cotyledon and hypocotyl explants of other pepper species (Arroyo and Revilla 1991). In that case, subculturing on growth regulators free medium, the reduction of cytokinin concentration, addition of giberelic acid or dark and cold treatment did not stimulate shoot elongation. As a result of rosette transfer to soil under greenhouse conditions elongated shoots were obtained. The same procedure of shoot elongation was used for shoot tip, cotyledon hypocotyl and root explants in *C. annuum* cv. California Wonder (Ebida and Hu 1993). Other authors (Szász et al. 1995) report that elongation of shoot buds induced on cotyledon explants of one Hungarian, one hybrid and three Italian varieties of pepper was possible by using medium supplemented with GA₃.

Shoot elongation and plantlets formation in pepper cv. Bryza were achieved during the four passages but only from shoot tip and cotyledon explants, whereas hypocotyl explants showed weak plant regeneration ability and cotyledonary node the weakest in spite of the highest number of induced shoot primordia (Fig. 6C). In other cultivars of pepper (Phillips and Hubstenburger 1985) high frequency of shoot elongation occurred both on the shoot tip and cotyledonary node explants, during 8 passages, however the percentage of elongated shoots rapidly declined. In addi-

Fig. 6. Influence of explant type and passage number on shoot bud regeneration (A, B) and plantlets formation (C). Means with different letters are significant at $p = 0.05$ by using LSD procedure.

tion, shoot elongation occurred at higher temperature (28.5°C) and longer photoperiod (continuous light) than in *C. annuum* cv. Bryza, like in the case of mentioned above shoot bud multiplication.

In conclusion, this study has shown that shoot bud primordia production in seedling explant of pepper cv. Bryza is relatively efficient, particularly on cotyledonary node explants. Among studied explant types the best shoot bud formation as well as plant regeneration responses showed cotyledon and shoot tip explants. Since the efficiency of these processes is poor, therefore further work is required to establish optimum conditions for shoot bud elongation and finally plantlets formation.

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INDUKCJA PĄKÓW PĘDOWYCH, ICH NAMNAŻANIE
ORAZ FORMOWANIE MIKROSADZONEK W EKSPLANTATACH SIEWEK PAPRYKI
(*CAPSICUM ANNUUM* L.) CV. BRYZA IN VITRO

STRESZCZENIE

Porównano zdolności do regeneracji pąków pędowych, ich namnażania i formowania mikrosadzonek 4 typów eksplantatów (wierzchołek pędu, węzeł liścieniowy, dystalna część liścienia, górny odcinek hypocotyła), 21-dniowych siewek *Capsicum annuum* L., cv. Bryza w kulturach *in vitro*. Na eksplantatach pierwotnych jako pierwsze pojawiły się zawiązki pąków pędowych, wyglądem przypominające primordia liściowe. Część z nich w kolejnych tygodniach kultury na pożywce inicjalnej uległa powiększeniu, część rozwinęła się w liście i struktury liściopodobne, te ostatnie zaobserwowano głównie na eksplantatach liścieniowych. Największą liczbę zawiązków pąków odnotowano na eksplantatach z węzłów liścieniowych, lecz jednocześnie były one najmniejsze. Największą zdolność do regeneracji pąków pędowych ujawniły eksplantaty liścieniowe, z których w kolejnych 4 pasażach otrzymywano ich najwięcej. Z pąków pędowych inkubowanych na pożywce elongacyjnej po 4 tygodniach uzyskano ukorzenione rozety liściowe, a wydłużenie czasu kultury do 8 tygodni bez pasażowania spowodowało ich dalszy wzrost i w efekcie otrzymanie ukorzenionych pędów (mikrosadzonek). W ciągu 4 kolejnych pasażów, mikrosadzonki otrzymywano z eksplantatów wierzchołków pędu i liścieni.

SŁOWA KLUCZOWE: *Capsicum annuum* L., eksplantaty siewek, regeneracja pąków, namnażanie, ukorzenianie.