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IN VITRO CLONAL MULTIPLICATION OF *CUCURBITA PEPO* BY SINGLE-NODE CULTURE

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Pumpkin (*Cucurbita pepo* L.) plantlets originated from (6-8 -day-old) seedling shoot tips and somatic embryos — most of them from long-term callus lines, were cloned by nodal explants on agar solidified MS medium without hormones or MS containing IAA and/or BA. The effects of exogenous growt regulators on shoot elongation, root formation and rate of multiplication were investigated. The best development of shoots derived from seedling explants was achieved on MS supplemented with 2.9 μM IAA and 0.22 μM BA, and from somatic embryo shoot explants on MS medium with 5.7 μM IAA. Root formation was obtained on MS without hormones, and MS supplemented with 5.7 μM IAA, or with 2.9 μM IAA and 0.22 μM BA. The last combination of hormones induced callus proliferation at the basal end of shoots but more abundant roots than on medium without BA.

Introduction

Among several possible applications of tissue culture technology the most notable are its use for genetic modification and *in vitro* propagation of plants (Murashige and Huang 1987). *In vitro* propagation is applied in the production of virus-free stock plants, especially of vegetatively propagated, plants, for productions of large number of genetically identical plants, and propagation of large number of plants where seed production is difficult (Bottino 1981, Doré 1987).

Since it insures genetic stability, the production of plants from axillary buds has proved to be the most reliable method of clonal propagation

in vitro (B o x u s 1986/87). In cucurbits, vegetative propagation by axillary buds would be valuable at least for clonal multiplication of triploid plants (watermelon), and rapid propagation of virus-free plants or *in vitro* modified unique plantlets (J e l a s k a 1986). Till now, there are not many reports on axillary bud cultures in cucurbits (H a n d l e y and C h a m b l i s s 1979, B a r n e s 1979, P i n k and W a l k e y 1984).

Plantlets obtained via adventitious bud and somatic embryo formation, generally, are of unicellular origin and therefore they are suitable for breeding investigations such as mutant selection, new genotype cloning, protoplast hybridization etc. (W a n g and V a s i l 1982). Possibility of somatic embryo induction and regeneration of normal plants, however, shows a great variability depending upon a genotype in different plant species (M e z e n t s e v and K a r e l i n a 1982, B o u q u e t et al. 1982, B r o w n and A t a n a s s o v 1985).

Regeneration in *Cucurbita pepo* L. by somatic embryogenesis have been reported previously (J e l a s k a 1974).

In this paper we report a reliable procedure for the microclonal propagation of pumpkin. We paid special attention to shoot elongation, i. e. number of nodes, and root formation what is crucial for a successful microclonal propagation of both plantlets originated from seedling nodal explants and the somatic embryos.

Materials and Methods

Pumpkin seeds were surface sterilized in 2% Izosan (chlorine product, Pliva-Zagreb) for 30 min, followed by a thorough rinse with sterile distilled water. Decoated seeds were set to germinate 6—8 days, under sterile conditions, on wet cotton in test tubes (23 × 160 mm), under light. Explanted seedling shoot tips (5.0 mm) were cultivated in test tubes (23 × 160 mm) on MS (M u r a s h i g e and S k o o g 1962) basal medium supplemented with 3% sucrose, 0.9% agar and 4.4 μM BA (benzyladenine), pH 6.0 before autoclaving. Nodal segments from shoots which developed on MS supplemented with 3% sucrose, 0.9% agar and 4.4 μM BA were subcultured on the same basal medium but: (1) without hormones; (2) with 2.9 μM IAA (indoleacetic acid) + 0.22 μM BA or (3) with 4.4 μM BA. Seven different clones (S_1 — S_7) were examined during three subcultures (3 × 4 weeks).

Also, pumpkin plantlets regenerated from somatic embryos were used as a starting material for investigation of microclonal propagation by single-node explants in pumpkin culture. Different embryogenic callus lines were used as a sources of embryos: long-term lines (20-year-old) MSS, MSS_0, $ž_5b$, $ž_5b_0$, (J e l a s k a 1974) and young lines (10-month-old) NIA, NBA, NDE (J u r e t i ć 1987).

Plantlets regenerated from somatic embryos (J u r e t i ć and J e l a s k a, in preparation), were cloned by shoot apical tips and axillary buds. Nodal explants (5.0 mm) were planted (individually in 23 × 160 mm test tubes) on MS medium supplemented with 2% sucrose, 0.9% Bacto-agar, 11.4 μM IAA, or one of the next combinations of IAA and BA (μM): a) 2.9 IAA + 4.4 BA; b) 5.7 IAA + 4.4 BA; c) 11.4 IAA + 4.4 BA; d) 2.9 IAA + 2.2 BA; e) 5.7 IAA + 2.2 BA; f) 11.4 IAA + 2.2 BA; g) 2.9 IAA + 0.22 BA; h) 5.7 IAA + 0.22 BA and i) 11.4 IAA + 0.22 BA; pH 6.0 before autoclaving. Shoot elongation and root formation was examined in ten different clones: $ž_4$, $ž_{15}$, $ž_{37}$, $ž_{40}$ $ž_{43}$, $ž_{07}$, $ž_{027}$., $ž_{030}$, MSS_{22} and NIA_{34} during 14 subcultures (4 weeks each).

Cultures were incubated at $26 \pm 1°$ C and illuminated with fluorescent lamps (TEŽ, warm white, 40 W, with a special range of 400—700 nm, 17 Wm^{-2}), and a light dark cycle of 16—8 h.

Results

Multiplication of shoots

a) Cloning pumpkin plantlets from seedling explants

Several clonal populations of pumpkin shoots were established through shoot apical tip culture on MS agar solidified medium with 4.4 μM BA. In order to investigate the influence of exogenous growth regulators on the shoot elongation and root initiation, single-node explants were further subcultured on the next media: (1) MS + 0; (2) MS + 2.9 μM IAA + 0.22 μM BA, and (3) MS + 4.4 μM BA. Seven different clones (S_1, S_2, S_3, S_4, S_5, S_6, S_7) were examined during three subcultures (4 weeks each).

Shoot elongation. The fig. 1 shows the influence of exogenous IAA and BA on shoot elongation. The best results were achieved on MS supplemented with 2.9 μM IAA and 0.22 μM BA, with the average length of shoots from 5.5 cm (clone S_1) to 8.6 cm (clone S_5). Shoot elongation in seven examined clones was significantly lower — from 3.3 cm (clone S_5) to 4.8 cm (clone S_2), on MS with 4.4 μM BA than on MS with 2.9 μM IAA and 0.22 μM BA. On MS without hormones shoot elongation in all clones was the lowest. On the same medium, the mean values of shoot elongation did not differ greatly among clones except one (clone S_5), which stood out evidently. The medium with 4.4 μM BA produced the highest number of nodes.

b) Cloning plantlets generated from somatic embryos

Pumpkin plantlets developed from somatic embryos of embryogenic callus lines ($ž_5b$, $ž_5b_0$, NIA and MSS) were cloned by axillary bud proliferation on MS medium supplemented with 5.7 μM IAA.

Shoot elongation. Shoot development was observed during 14 successive subcultures (fig. 2). During four weeks (one subculture) new plantlets developed from each axillary bud. Each of four curves is related to average length of shoots originated from ten plantlets from the same callus line. Growth maximum was observed in May (5th subculture) and growth minimum in December and January (13th and 14th subculture). The fig. 3 shows the results for ten clones ($ž_4$, $ž_{15}$, $ž_{37}$, $ž_{40}$, $ž_{43}$, $ž_{07}$, $ž_{027}$, $ž_{030}$, MSS_{22} and NIA_{34}) multiplied on the same medium (MS with 5.7 μM IAA). Average length of plantlets (during 14 subcultures) showed a minute differences among the clones, and average number of nodes produced was from 2.5 to 3.7.

Rooting

Root initiation greatly depended upon exogenous growth regulators in medium. On MS with the addition of 2.9 μM IAA and 0.22 μM BA all the seedling origin shoots of seven clones (S_1 — S_7) developed callus and roots at the basal end (fig. 4). On the hormone free MS medium shoots rooted in 100%, although roots were not so abundant as on the medium with IAA and BA. Seven percent of shoots developed both — callus and roots.

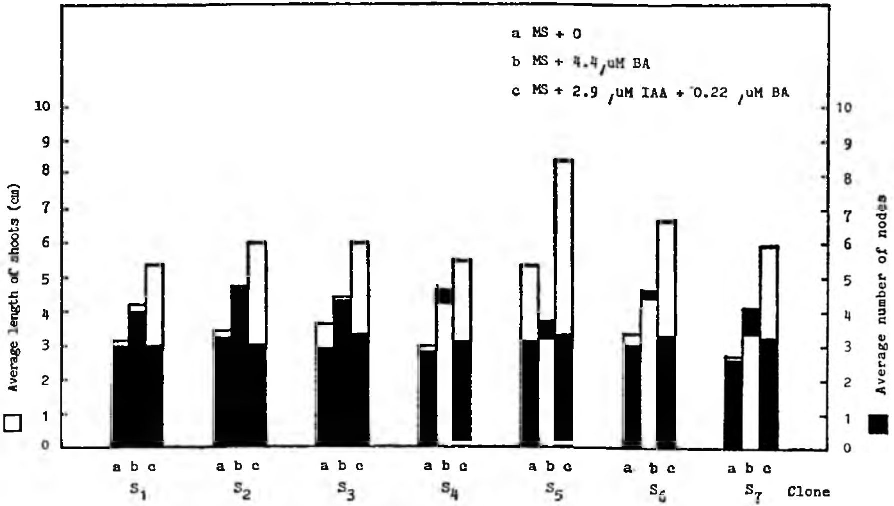

Fig. 1. The influence of IAA and BA on the pumpkin shoot elongation of seven clones (S_1-S_7) derived from seedling explants. On particular treatmant for every clone sample size was 12 cultures. Basal medium: MS + 3% sucrose + 0.9% agar.

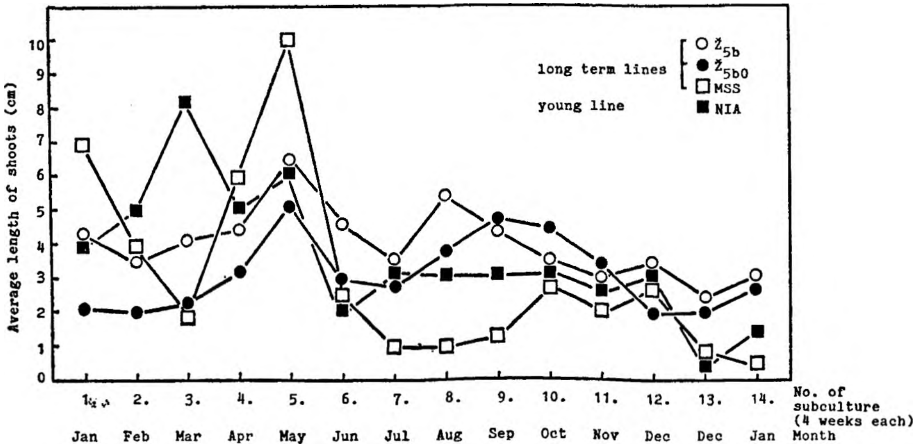

Fig. 2. The effect of year-season on the growth variability in culture of plantlets derived from embryoids in three long-term and one young pumpkin callus line. Basal medium: MS + 2% sucrose + 0.9% agar + 5.7 µM IAA.

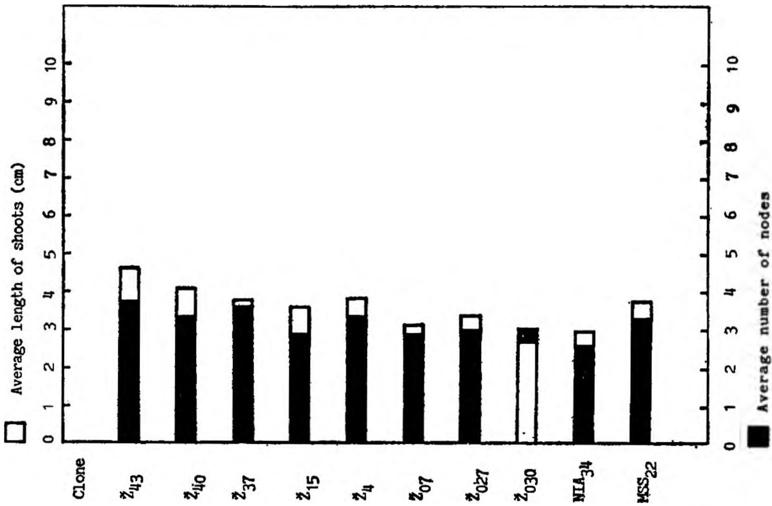

Fig. 3. The influence of the origin (embryoid) of shoots on the number of nodes and the average length of shoots cloned during 14 subcultures (4 weeks each). Basal medium: MS + 2% sucrose + 0.9 agar + 5.7 μM IAA.

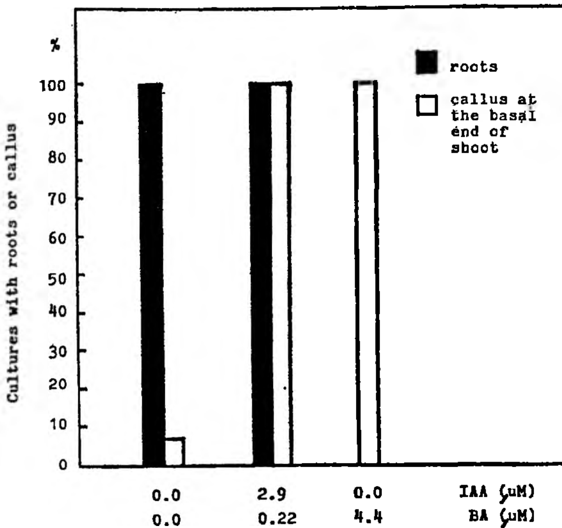

Fig. 4. The influence of IAA and BA on the root initiation and callus formation in pumpkin culture of seedling origin microcloned shoots. The results represent the percentage of cultures in seven clones (S_1-S_7) 12 plants for each clone. Basal medium: MS + 3% sucrose + 0.9% agar.

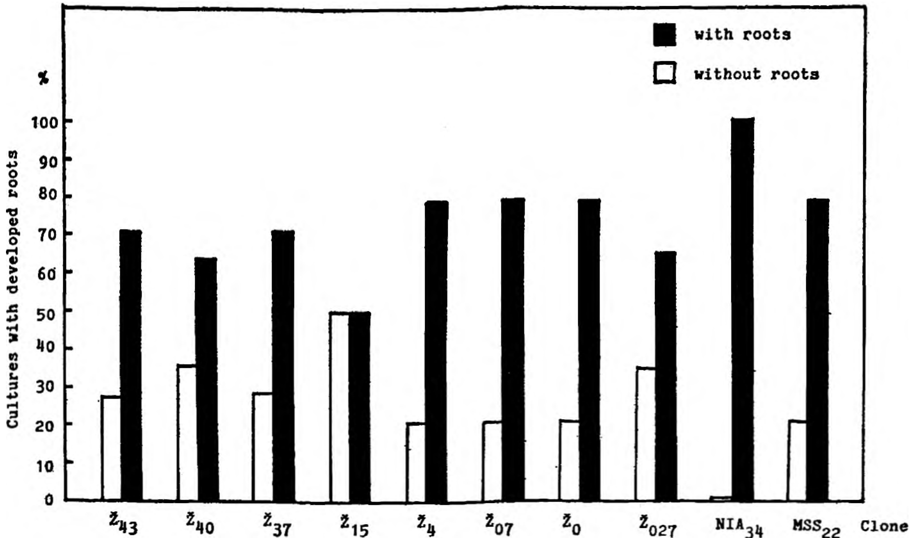

Fig. 5. The influence of the origin (embryoid) of shoots on the root initiation. Basal medium: MS + 2% sucrose + 0.9% agar + 5.7 μM IAA.

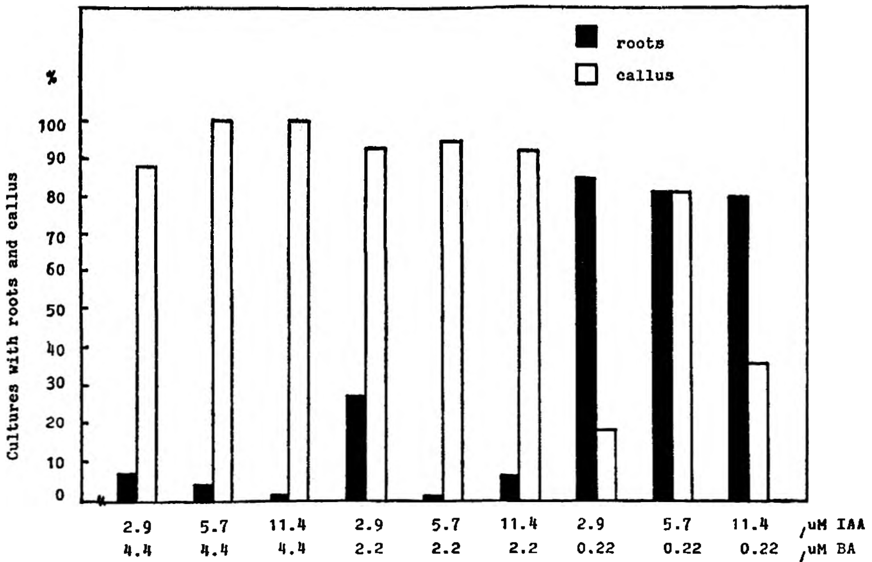

Fig. 6. The influence of IAA and BA on the root initiation and callus proliferation at the basal end of the pumpkin cloned shoots derived from somatic embryos. Basal medium: MS + 2% sucrose + 0.9% agar.

On the embryoid origin shoots the roots were formed on MS medium supplemented with 5.7 μM IAA. The fig. 5 shows differences in rhizogenic potential among ten different clones. Clone NIA_{34} rooted the best (100% of cultures), while clone $ž_{15}$ gave only 50% rooted cultures. The fig. 6 shows that the rooting initiation depended upon a combination of concentrations of exogenous IAA and BA added to the MS medium. On the medium with the lowest concentrations of IAA (2.9 μM) and BA (0.22 μM) the shoots were rooted the best (85% of cultures).

Discussion and Conclusions

The difference in average length of pumpkin plants among clones on the medium MS with the addition of 2.9 μM IAA and 0.22 μM BA was negligible (fig. 1 and 3), regardless of the origin of explants (young seedlings or somatic embryos). In all the clones tested, lower growth rate (shoot elongation) was observed on MS medium without hormones than on the media supplemented with exogenous growth regulators (fig. 1).

The influence of the year-season was tested in cultures of plantlets cloned from somatic embryos. Cultures showed a considerable seasonal growth variability (fig. 2) in spite of the fact that the most of them originated from long-term embryogenic callus lines, which the last 20 years have been growing continually under artificial conditions of the culture-room.

Optimal root induction was obtained when shoots grew on MS medium containing 2.9 μM IAA and 0.22 μM BA (fig. 4 and 6). In cultures of different clones derived from seedlings, root formation was better (100% rooted cultures) in comparison with the clones from somatic embryo origin (85% rooted cultures). However, in shoot cultures (cultured on the MS medium with 5.7 μM IAA) derived from somatic embryos, rhizogenic potential depended on a plant genotype; rooting ranged from 50% of cultures in genotype $ž_{15}$ to 100% of rooted cultures in genotype NIA_{34}. Pink and Walkey (1984) reported the best rooting of pumpkin (*Cucurbita pepo* 'Cinderella') plantlets on MS medium with high concentration of IAA (45.7 μM) and without cytokinin. In our experiments, the low concentration of BA (0.22 μM) in combination with 2.9 or 5.7 μM IAA stimulated root development.

The data presented in this paper, confirmed the possibility of successful clonal propagation by axillary shoot development of pumpkin plants derived either from seedling shoot tips or somatic embryos. Shoot growth and root formation displayed no considerable difference due to the origin of explants.

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SAŽETAK

KLONSKO RAZMNOŽAVANJE BUNDEVE (*CUCURBITA PEPO* L.) KULTUROM NODALNIH SEGMENATA *IN VITRO*

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Kloniranje biljaka bundeve (*Cucurbita pepo* L.) u uvjetima *in vitro* postignuto je na krutoj MS hranidbenoj podlozi bez regulatora rastenja ili uz dodatak IAA i BA u niskim koncentracijama. Za početne eksplantate korišteni su nodalni segmenti biljčica razvijenih iz vegetacijskih vrškova 6—8 dana starih klijanaca isklijavanih u uvjetima *in vitro* ili iz somatskih embrija. Najbolji rast izdanaka i kompletnih biljaka postignut je na podlozi s dodatkom 2,9 μM IAA i 0,22 μM BA, bez obzira na različitu izvornost eksplantata.

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