

UDC 582.739 : 581.143 = 20

GROWTH REGULATORS REQUIREMENT FOR
THE INITIATION OF *VICIA FABAE* CALLUS
TISSUEBRANKA PEVALEK, SIBILA JELASKA, DRAŽENA PAPEŠ AND
ZVONIMIR DEVIDE

(Department of Botany, Faculty of Science, University of Zagreb)

Received October 4, 1979

Introduction

A number of synthetic nutrient media for *Vicia faba* callus growth has been described in the past few years.

Venketeswaran (1962) obtained the callus induction on hypocotyl explants of mature broad bean embryos using the medium after Bonner and Devirian (1939), with addition of yeast extract and coconut milk. Grant and Fuller (1968) maintained less than 1% of cultures initiated from embryo radicle primary explants on Bonner-Devirian's medium, modified after Venketeswaran (1962) with the addition of yeast extract. Mitchell and Gildow (1975) studied the effect of a series of nutrient media on callus induction in hypocotyl fragments of mature broad bean embryos (without radicle and plumule). The only callus induction they obtained was on the SH medium (Schenk and Hidelbrandt 1972). Cionini et al. (1978) induced the initial callus culture on explants of immature cotyledons, by means of a modified SH medium, with the addition of auxin or auxin and kinetin.

In our initial investigations the tissue of immature broad bean embryo proved suitable for callus induction (Papeš et al. 1978, Jelaska et al. 1978, Pevalek 1979). In the work for the present paper we used shoot tip tissue and hypocotyl fragments without meristem tips of immature embryo, as well as vegetative and generative buds of young plants. We studied the action of various combinations and concentrations of some growth substances.

Material and Methods

A) Material

Tissue explants of broad bean immature embryos (*Vicia faba* L.) were used. The material dealt with was: plumule tips (size: 0.5—1.0 mm), specified as A-segments, hypocotyl fragments without meristem tips, specified as B-segments, and generative buds of 18 day old plants (size: 0.3—0.5 mm).

B) Methods

Aseptic techniques of plant tissue culture were used.

Unripe legumens with seeds were sterilized for 1 minute with 70% ethanol, which was followed by a 30 min sterilization with a 3% water solution of Halamid (Pliva — Zagreb). Buds of young plants were sterilized for 10 minutes only with a 3% halamid solution, followed by four washes in sterile water, each wash lasting five minutes.

Single explants were inoculated onto a nutrient medium. Two basic media were used: RM-1962 (Murashige and Skoog 1962) and B5 (Gamborg, Miller and Ojima 1968) with the addition of 3% sucrose and 0.9% agar. The following growth substances were added to the basic medium in various concentrations and combinations: 2,4-dichlorophenoxyacetic acid (2,4-D) in concentrations 0.01 — 1.0 $\mu\text{M l}^{-1}$, indolyl-3-acetic acid (IAA) in concentration 5.0 $\mu\text{M l}^{-1}$, α -naphthaleneacetic acid (NAA) in concentration 1.0 $\mu\text{M l}^{-1}$, benzyladenine (BA) in concentrations 2.5 — 5.0 $\mu\text{M l}^{-1}$ and kinetin (k) in concentrations 0.02 — 5.0 $\mu\text{M l}^{-1}$.

The cultures were kept in a temperature-controlled room at 27°C under an illumination of 16 hours daily by fluorescent lamps (IPR 40 W, 220 V, 4500°K), at the intensity of 500 lx.

Each experiment was carried out in 6 — 12 replicates and the results were scored after two or four months.

Results

1) Explants of immature embryos

The results obtained are shown in Tables 1 and 2, and the new calluses morphology in Figs. 4 and 5.

On media with 1.0 $\mu\text{M l}^{-1}$ 2,4-D the explants did not form any callus, regardless of the kinetin concentrations present. The optimal concentration of 2,4-D for callus formation was 0.3 $\mu\text{M l}^{-1}$. Various concentrations of kinetin added to the above mentioned concentration (0.3 $\mu\text{M l}^{-1}$ 2,4-D) did not essentially influence the possibility of callus formation. The most suitable combinations were: 0.3 $\mu\text{M l}^{-1}$ 2,4-D with 1.0 $\mu\text{M l}^{-1}$ kinetin, and 0.1 $\mu\text{M l}^{-1}$ 2,4-D with 1.0 $\mu\text{M l}^{-1}$ kinetin. The percentage of cultures which formed the callus was the same for both segments in the first combination (66.7%), with the average fresh weight of 1994.5 mg in the A-segment, and 4658.0 mg in the B-segment. In the second combination the corresponding figures were: 50% for both segments, with 2745.0 mg for the A-segment, and 4302.0 mg for the B-segment.

The development of axillary buds was induced in some cultures of the A-segment on the medium with $0.01 \mu\text{M l}^{-1}$ 2,4-D and 0.1 or $0.02 \mu\text{M l}^{-1}$ kinetin (Fig. 1).

A shoot from the apical meristem showed prolonged growth in one of the cultures growing on the medium with $0.01 \mu\text{M l}^{-1}$ 2,4-D and $1.0 \mu\text{M l}^{-1}$ kinetin (Fig. 2).

Roots spontaneously developed in two of the cultures growing on the medium with $0.05 \mu\text{M l}^{-1}$ 2,4-D and $0.1 \mu\text{M l}^{-1}$ kinetin (Fig. 3).

2) Explants of undeveloped flower buds

Explants taken from 18 day old plants (0.3–0.5 mm long) were put on the B5 medium with the addition of 3% sucrose and 0.9% agar and various concentrations of BA, NAA and IAA (Table 3).

A vigorous, compact, green callus with very few necroses developed in 41.7% of cultures growing on the medium with $1.0 \mu\text{M l}^{-1}$ NAA with $2.5 \mu\text{M l}^{-1}$ BA. By subculturing one of these cultures the NB 1 callus-line was maintained. It has retained its green colour and compact structure up to now (after 18 months in the culture).

After one month, a callus developed in as many as 66.7% of cases (Fig. 7) on the medium with the addition of $5.0 \mu\text{M l}^{-1}$ BA only, while it became necrotic and died after two months in 41.7% of cultures. In 25% of cultures it survived as a compact, green tissue on the base of developed buds and leaflets.

Table 1. The effect of 2,4-D and kinetin on callus induction in primary explants (A-segment) of immature embryos (basic medium: RM-1962 with 3% sucrose and 0.9% agar) after 4 month culturing.*

2,4-D ($\mu\text{M l}^{-1}$)	Kinetin ($\mu\text{M l}^{-1}$)	Cultures with induced callus %	Average callus fresh weight (mg)
0.01	0.02	0	0
0.01	0.1	33.3	222.5
0.01	1.0	50.0	794.0
0.01	5.0	33.3	941.0
0.1	0.02	0	0
0.1	0.1	16.7	314.0
0.1	1.0	50.0	2745.0
0.1	5.0	33.3	1142.5
0.3	0.02	16.7	576.0
0.3	0.1	66.7	1517.0
0.3	1.0	66.7	1994.5
0.3	5.0	66.7	1040.0
1.0	0.02	0	0
1.0	0.1	0	0
1.0	1.0	0	0
1.0	5.0	0	0

* Each experiment was carried out in 6 replicates.

Table 2. The effect of 2,4-D and kinetin on callus induction in primary explants (B-segment) of immature embryos (basic medium: RM-1962 with 3% sucrose and 0.9% agar) after 4 month culturing.*

2,4-D ($\mu\text{M l}^{-1}$)	Kinetin ($\mu\text{M l}^{-1}$)	Cultures with induced callus %	Average callus fresh weight (mg)
0.01	0.02	0	0
0.01	0.1	0	0
0.01	1.0	50.0	1996.0
0.01	5.0	0	0
0.1	0.02	0	0
0.1	0.1	33.3	2287.0
0.1	1.0	50.0	4302.0
0.1	5.0	50.0	885.7
0.3	0.02	0	0
0.3	0.1	33.3	1373.5
0.3	1.0	66.7	4658.0
0.3	5.0	50.0	1437.3
1.0	0.02	0	0
1.0	0.1	0	0
1.0	1.0	0	0
1.0	5.0	0	0

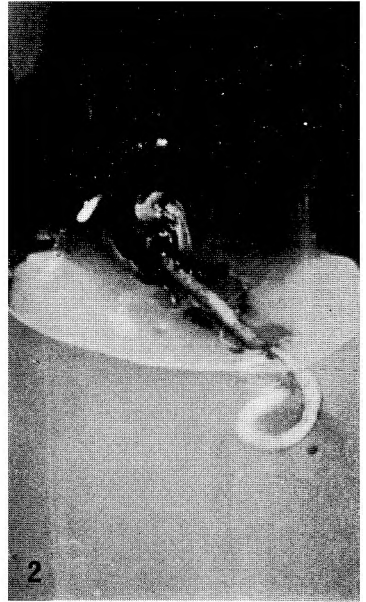
* Each experiment was carried out in 6 replicates.

Table 3. The effect of BA, NAA and IAA on the callus induction in primary explants of undeveloped flower buds (basic medium: B5 with 3% sucrose and 0.9% agar), after two month culturing.*

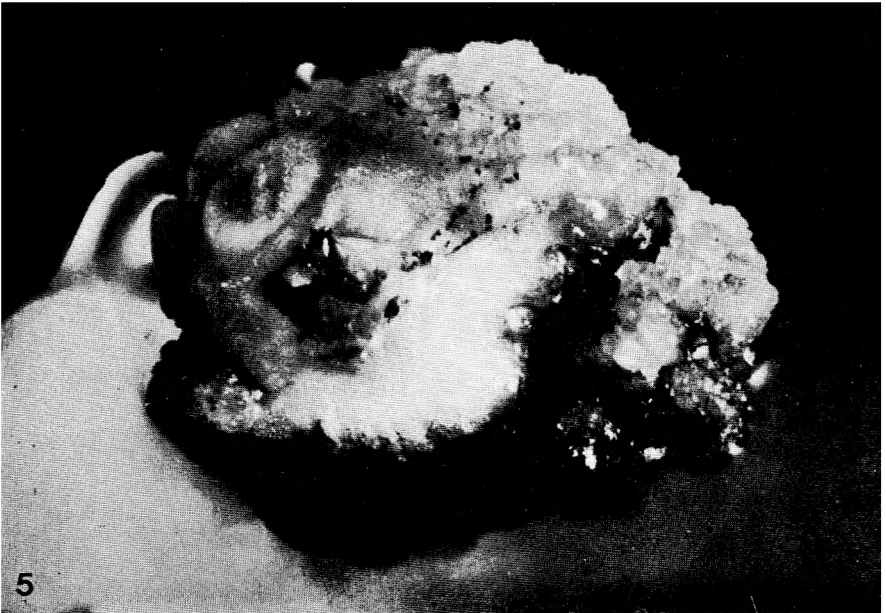
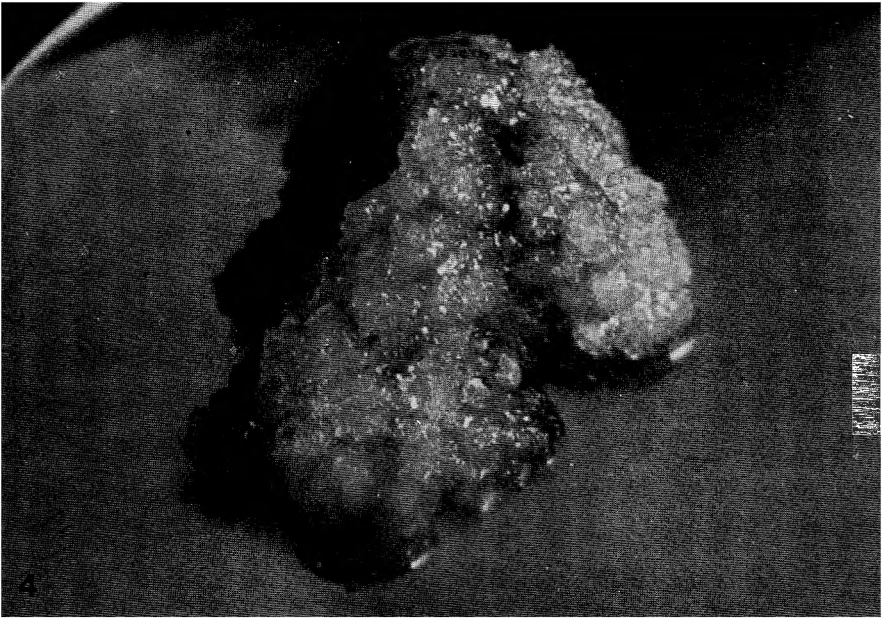
NAA ($\mu\text{M l}^{-1}$)	BA ($\mu\text{M l}^{-1}$)	IAA ($\mu\text{M l}^{-1}$)	Cultures with induced callus %
1.0	2.5	0	41.7
0	5.0	0	25.0
0	5.0	5.0	16.7

* Each experiment was carried out in 12 replicates.

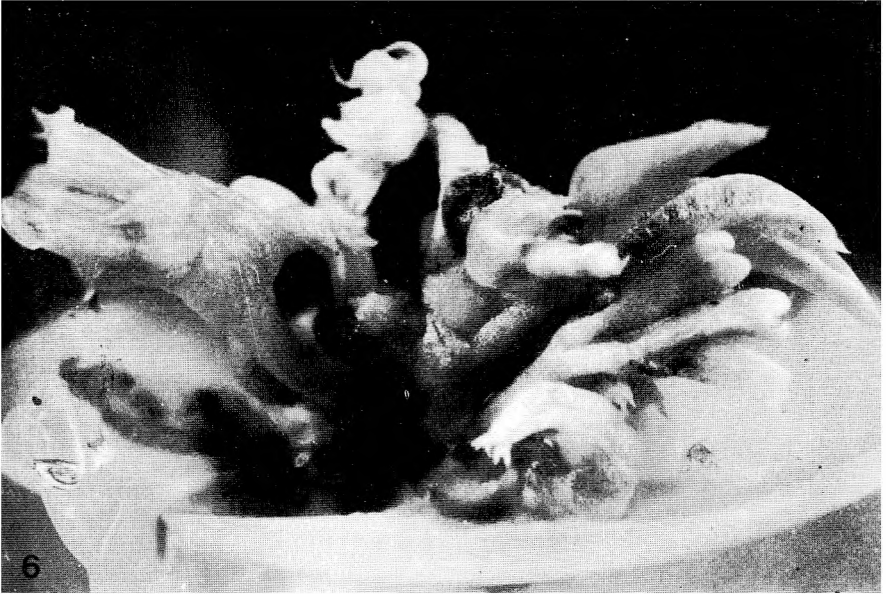
- Fig. 1. Axillary bud on the A-segment, 20 days after inoculation (RM-1962 medium with $0.01 \mu\text{M l}^{-1}$ 2,4-D and $0.02 \mu\text{M l}^{-1}$ kinetin). $\times 5$.
- Fig. 2. Differentiation of rootlets in the primary culture of the embryo plumule tip, 6 weeks after inoculation (RM-1962 medium with $0.05 \mu\text{M l}^{-1}$ 2,4-D and $0.1 \mu\text{M l}^{-1}$ kinetin). $\times 8$.
- Fig. 3. Developed shoot and axillary bud in the primary culture of the embryo vegetative tip, 2 weeks after inoculation (RM-1962 medium with $0.01 \mu\text{M l}^{-1}$ 2,4-D and $1.0 \mu\text{M l}^{-1}$ kinetin). $\times 3$.
- Fig. 4. Callus induced on the B-segment, 45 days after inoculation (RM-1962 medium with $0.01 \mu\text{M l}^{-1}$ 2,4-D and $1.0 \mu\text{M l}^{-1}$ kinetin). $\times 5$.
- Fig. 5. Callus induced on the B-segment, 45 days after inoculation (RM-1962 medium with $0.1 \mu\text{M l}^{-1}$ 2,4-D and $1.0 \mu\text{M l}^{-1}$ kinetin). $\times 5$.
- Fig. 6. Developed inflorescence with buds and leaflets in the primary culture of flower bud, 2 months after inoculation (B5 medium with $2.5 \mu\text{M l}^{-1}$ BA and $1.0 \mu\text{M l}^{-1}$ NAA). $\times 3.5$.
- Fig. 7. Callus and rootlets induced on a flower bud; two month old culture (B5 medium with $5.0 \mu\text{M l}^{-1}$ BA). $\times 4$.



Figs. 1—3.



Figs. 4—5.



Figs. 6—7.

The weakest development of callus was induced by the medium with $5.0 \mu\text{M l}^{-1}$ IAA and $5.0 \mu\text{M l}^{-1}$ BA. One month after the inoculation callus was formed in 16.7% of cultures, but it survived only in 8.3% of cultures in the course of the following two months.

Explants of flower buds developed continuously on the medium with $1.0 \mu\text{ l}^{-1}$ NAA and $2.5 \mu\text{M l}^{-1}$ BA or with $5.0 \mu\text{M l}^{-1}$ BA only. The cultures consisted of developed flower buds and leaflets (Fig. 6).

Rootlets developed on the medium with $5.0 \mu\text{M l}^{-1}$ BA (Fig. 7).

Discussion

The experiments in the present paper show the possibility of inducing callus formation on young tissue explants of broad bean (*Vicia faba* L.) of various origins. In our experiments the RM-1962 medium with 3% sucrose, 0.9% agar, $0.3 \mu\text{M l}^{-1}$ 2,4-D and $1.0 \mu\text{M l}^{-1}$ kinetin proved to be the most suitable one. Cultures were induced either on vegetative tips or on hypocotyl fragments without meristem tips of immature embryos in 66.7% of explants. Segments of hypocotyl mature embryos of broad bean seeds were used for the initiation of callus cultures on the modified Bonner-Devirian's medium (Bonner and Devirian, 1939) by Venketeswaran (1962). He achieved the best results on the medium with $1.0 \mu\text{M l}^{-1}$ of 2,4-D and NAA, plus 3.0 g l^{-1} yeast extract. Mitchell and Gildow (1975) investigated the effects of a series of nutrient media, including RM-1962, on callus induction in hypocotyl fragments of mature embryos without meristem tips. Callus induction was obtained on the SH medium only (Schenk and Hildebrandt 1972) with the addition of 2,4-D (in the optimal concentration of $2.3 \mu\text{M l}^{-1}$), and of kinetin ($0.05 \mu\text{M l}^{-1}$). In the majority of cultures callus induction was not achieved on RM-1962 medium; and even when the callus developed, it became necrotic and died in the course of two weeks. The experiments of Jelaska et al. (1978, 1980), Papeš et al. (1978) and Pevalek (1979) prove that it is possible to induce the callus on explants of mature and immature embryos of broad bean on the RM-1962 with the addition of 2,4-D and kinetin. In our experiments the callus was induced on explants of an immature embryo on the RM-1962 medium and it survived in 20.83% of cultures. At the optimal concentration of $0.3 \mu\text{M l}^{-1}$ 2,4-D and $1.0 \mu\text{M l}^{-1}$ kinetin the callus developed in 66.7% of cultures. From the results obtained it is clear that the optimal concentration of 2,4-D ($0.3 \mu\text{M l}^{-1}$) for callus induction on primary cultures was considerably lower than the concentrations (0.92 , 1.38 and $2.5 \mu\text{M l}^{-1}$) which proved to be optimal for growth of the maintained callus line (Jelaska et al. 1980). Using Bonner-Devirian's medium modified after Venketeswaran (1962), Grant and Fuller (1968) achieved callus induction on root explants with the addition of $100.0 \mu\text{M l}^{-1}$ IAA and of yeast extract only, but the callus necrotized after 3 weeks. Röper (1978), on the other hand, succeeded in inducing the callus on explants of roots. He maintained two strains on the modified SH medium (Mitchell and Gildow, 1975). Gueuziec et al. (1977) obtained callus induction in 90% of stem explants of 15 day old plants on the RM-1962 medium with $5.37 \mu\text{M l}^{-1}$ NAA and $25.0 \mu\text{M l}^{-1}$ kinetin. Cionini et al. (1978) induced the initial callus culture on explants of immature cotyledons, using the SH medium modified by Mitchell and Gildow (1975) with the addition of auxin only (35.0 — $45.0 \mu\text{M l}^{-1}$ IAA or $2.3 \mu\text{M l}^{-1}$ 2,4-D), or auxin and kinetin (in the concentration of 0.05 — $0.25 \mu\text{M l}^{-1}$).

Conclusion

The possibility of callus induction on broad bean (*Vicia faba* L.) depending on the origin of the explants and on the composition of the medium was studied.

The optimal medium for the callus induction on explants of immature embryos was the RM-1962 medium with 3% sucrose, $0.3 \mu\text{M l}^{-1}$ 2,4-D and $1.0 \mu\text{M l}^{-1}$ kinetin, while the optimal one on explants of generative buds of young plants was the B5 medium with 3% sucrose, $1.0 \mu\text{M l}^{-1}$ NAA and $2.5 \mu\text{M l}^{-1}$ BA.

*

This work was supported by the Research Council of the SR Croatia (SIZ IV).

References

- Bonner, J., P. S. Devirian, 1939: Growth factor requirements of four species of isolated roots. *Am. J. Bot.*, 26, 661—665.
- Cionini, P. G., A. Benmici, F. D'Amato, 1978: Nuclear cytology of callus induction and development in vitro. I. Callus from *Vicia faba* cotyledons. *Protoplasma*, 96, 101—112.
- Gamborg, O. L., R. A. Miller, K. Ojima, 1968: Nutrient requirements of suspension cultures of soybean cells. *Exp. Cell Res.*, 50, 151—158.
- Grant, M. E., K. V. Fuller, 1968: Tissue culture of root cells of *Vicia faba*. *J. Exp. Bot.* 19, 667—680.
- Gueuziec, N. N., J. P. Parvais, R. Galzy, 1977: Culture prolongée in vitro de tissus de *Vicia faba* minor. *C. R. Acad. Sc. Paris*, 284, 911—914.
- Jelaska, S., D. Papeš, B. Pevalek, Z. Devidé, 1978: Developmental and karyological studies of *Vicia faba* callus cultures. IVth International Congress of Tissue and Cell Culture, Calgary (Canada), Aug. 20.—25., Abstracts No. 1103, 101.
- Jelaska, S., B. Pevalek, D. Papeš, Z. Devidé, 1980: Developmental aspects of long-term callus culture of *Vicia faba* L. *Protoplasma* (in press).
- Mitchell, J. P., F. E. Gildow, 1975: The initiation and maintenance of *Vicia faba* tissue cultures. *Physiol. Plant.* 34, 250—253.
- Murashige, T., F. Skoog, 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15, 473—497.
- Papeš, D., S. Jelaska, M. Tomaseo, Z. Devidé, 1978: Triploidy in callus culture of *Vicia faba* L. investigated by the Giemsa C-banding technique. *Experientia* 34, 1016—1017.
- Pevalek, B., 1979: Djelovanje fitohormona na rast, morfogenezu i genetsku stabilnost tkiva boba u kulturi in vitro. Magistarski rad, Sveučilište u Zagrebu.
- Röper, W., 1978: Callus cultures and cell suspension cultures of *Vicia faba*. *Cytobiologie* 18, 180—181.
- Schenk, R. V., A. C. Hildebrandt, 1972: Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.*, 50, 199—204.
- Venkateswaran, S., 1962: Tissue culture studies on *Vicia faba* L. I. Establishment of culture. *Phytomorph.*, 12, 300—306.

SAŽETAK

REGULATORI RASTENJA U INICIJACIJI KALUSA VRSTE *VICIA FABA*

Branka Pevalek, Sibila Jelaska, Dražena Papeš i Zvonimir Devidé

(Botanički zavod Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu)

Provedena istraživanja dokazuju da je moguće inducirati i kontinuirano kultivirati kalus boba (*Vicia faba* L.).

Eksplantati tkiva uzimani su iz nezrelih embrija (vegetativni vrškovi klice — segment A i fragmenti hipokotila bez meristemskih vrškova — segment B), te vegetativnih i generativnih pupova biljaka starih 18 dana.

Optimalni medij za indukciju kalusa na eksplantatima tkiva nezrelog embrija bio je RM-1962 medij s 3% saharoze, 03 $\mu\text{M l}^{-1}$ 2,4-D i 1,0 $\mu\text{M l}^{-1}$ kinetina na kojem se kalus razvijao u 66,7% kultura s prosječnom težinom od 1994,5 mg na segmentu A, odnosno 4658,0 mg na segmentu B. Optimalni medij za indukciju kalusa na primarnim eksplantatima nerazvijenih cvjetnih pupova bio je B5 medij s 3% saharoze, 2,5 $\mu\text{M l}^{-1}$ BA i 1,0 $\mu\text{M l}^{-1}$ NAA na kojem se kalus razvio u 41,7% kultura.

U početnim kulturama vegetativnih vrškova embrija u 16,6% kultura na RM-1962 mediju s 3% saharoze, 0,05 $\mu\text{M l}^{-1}$ 2,4-D i 0,1 $\mu\text{M l}^{-1}$ kinetina spontano su se razvili korjenčići. Novostvoreni korjenčić se razvio i na eksplantatu cvjetnog pupa na B5 mediju s 3% saharoze i 5,0 $\mu\text{M l}^{-1}$ BA.

Na eksplantatima vegetativnih vrškova nezrelih embrija mogao se potaknuti razvitak aksilarnih pupova na RM-1962 mediju s 3% saharoze, 0,01 $\mu\text{M l}^{-1}$ 2,4-D i kinetinom u koncentraciji 0,1 ili 0,02 $\mu\text{M l}^{-1}$.

Adress of the authors:
Department of Botany
Faculty of Science, University of Zagreb
Rooseveltov trg 6 P. O. Box 933
YU-41001 Zagreb (Yugoslavia)