

RHIZOGENESIS OF PUMPKIN HYPOCOTYL EXPLANTS

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Introduction

The phenomenon of plant regeneration *in vitro* has been studied extensively. Although tissues of numerous dicotyledonous plant species have been grown in culture, organ formation has been observed in relatively few.

Skoog and his co-workers (Miller and Skoog 1953, 1955; Skoog 1944, Skoog and Miller 1957, Skoog and Tsui 1948) have induced bud and root formation on stem tissue, stem callus and cambium cultures of tobacco and in stem cultures of horseradish. Levine (1950) and Wiggans (1954) have induced organ formation in carrot root-tissue cultures. Other authors have succeeded inducing organ formation on a variety of tissues from a number of plant parts. The present paper is a report on the differentiation of roots of pumpkin hypocotyl explants.

Material and Methods

Pumpkin (*Cucurbita pepo* L.) was used as the experimental plant. Seeds of pumpkin were sterilized with 3% calcium hypochlorite and germinated in test tubes in bidistilled water under light for 10 days, under sterile conditions. Fragments, 1 cm long, of hypocotyls were cut and cultivated individually in test tubes (23 × 200 mm) on 25 ml of culture medium for 1 month. These fragments were implanted horizontally on the surface of medium, and finally the tubes were capped with thin aluminium foil. The basal medium was composed of mineral salts (Heller's solution, 1953; or Murashige — Skoog's complete solution, 1962) + 3% glucose + 0.9% Difco Bacto agar + one of the growth substances, or their combinations.

Growth regulators were: β -indolylbutyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), β -indolylacetic acid (IAA), α -naphthylacetic acid (NAA), kinetin, autoclaved water melon sap and yeast extract (Difco). The pH of the medium was 5.5 or 5.6 before autoclaving. The media were autoclaved at 120° C and 1.5 Atm for 15 minutes.

The cultures were maintained at $26 \pm 1^\circ$ C under artificial light (485 ± 45 lux) from fluorescent lamps IPR 40 W, 220 V (6500 °K) 16 h light and 8 h darkness daily.

Each experiment was made with 20 explants.

Results

In an earlier paper by the author (Jelaska 1973) the unfavourable effect of the Heller's medium on the growth of pumpkin hypocotyl fragment cultivated in vitro has been shown. On Heller's basic medium, without growth stimulators, the occurrence of any smallest roots has never been observed.

In contrast to that hypocotyl explants grew roots on MS-medium even without an addition of growth regulators (Table 1; Fig. 2).

MS-salts, without any additional substances induced roots on explants (on the proximal side), but on this medium they were short and thin. On the complete MS-medium the roots developed vigorously and had a normal appearance (Fig. 2). The effects of the organic additives of the MS-medium were separately investigated and it was shown that casein hydrolizate, pyridoxine-HCl and nicotinic acid affected the root growth. It seems, however, that the mixture of all organic substances gave best results.

The addition of organic substances of the MS-medium was in this case more responsible for the growth and development of the roots than for their induction (Table 1; Fig. 2). The percentage of the induced roots on the complete MS-medium was not considerably different from that on the medium containing only MS-salts.

Table 1. Root formation of pumpkin hypocotyl-explants in dependence of the nutrient medium.

Medium	Number of survived cultures	Cultures with root formation number	%
Heller	4	0	0
Heller + 10^{-6} IAA + 3.10^{-7} kinetin	14	0	0
MS-salts	14	10 thin roots	71
MS-complete	17	13 normal roots	76
MS-complete + 10^{-6} IAA + 3.10^{-7} kinetin	18	3 normal roots	17

In addition the MS-medium, sugar was necessary for the root development. Without the addition of sugar no rootlets were induced on the complete MS-medium (Table 2; Fig. 4). A concentration of 1% of either glucose (Fig. 4) or sucrose allowed root development on a large number of samples. In all further experiments therefore 3% glucose was always added to the medium.

Table 2. The effect of sugar concentration in MS-complete medium on the percentage of rooting pumpkin hypocotyl explants.

	0	1	2	3	4	5	6	% sugar
glucose	0	87,5	57,2	60,0	100	100	41,5	%
sucrose	0	100	71,5	100	50	60	50	%
								(weak roots)

The effect of growth regulators

The effect of some growth regulators on the rooting of explants was investigated. The effects of 2,4-D, NAA, IBA and IAA were examined either alone or in combination with kinetin, yeast extract and water-melon sap, on Heller's or MS-medium.

The addition of 2,4-D to Heller's basic medium increased fresh and dry weight of the explants (Jelaska 1973) and concentrations of 10^{-6} and 10^{-7} induced even rootlet formation on a small number of samples. No combinations of the concentrations of 2,4-D and kinetin (10^{-8} 2,4-D with 10^{-7} , $3 \cdot 10^{-7}$ and 10^{-6} kinetin; 10^{-7} 2,4-D with 10^{-7} , $3 \cdot 10^{-7}$ and 10^{-6} kinetin; 10^{-6} 2,4-D with 10^{-7} , $3 \cdot 10^{-7}$ and 10^{-6} kinetin) resulted in root induction on Heller's medium.

Combinations of a series of concentrations of IAA with kinetin (10^{-8} IAA with 10^{-7} , $3 \cdot 10^{-7}$ and 10^{-6} kinetin; 10^{-7} IAA with 10^{-7} , $3 \cdot 10^{-7}$ and 10^{-6} kinetin; 10^{-6} IAA with 10^{-7} , $3 \cdot 10^{-7}$ and 10^{-6} kinetin) never induced any root formation on Heller's medium (Fig. 1).

The possibilities of rooting of hypocotyl explants were totally different when growth regulators were added to the MS-medium. The addition of IAA (10^{-6}) and kinetin ($3 \cdot 10^{-7}$) to the MS-medium caused vigorous development of the root system on one side of the explants (Fig. 3), while on the other side part of them did not react by rhizogenesis. After three months culturing the last began to produce callus of embryogenic properties (Jelaska 1972, 1973 and 1974).

The addition of 2,4-D in concentrations of 10^{-7} and 10^{-6} to combinations with a series of various concentrations of water-melon sap (10, 15 and 20% vol.) induced extraordinarily vigorous rooting (Fig. 5) which was much stronger than on the MS-medium. In this combination of growth regulators there appeared a certain percentage of explants, which did not develop any roots but formed only callus.

The addition of water-melon sap alone (15% vol.) to the medium of MS-salts gave the same effect as the MS-medium complete, i.e. the root system was as vigorously developed as on the complete MS-medium.

Fig. 1. Explants of hypocotyls cultivated on Heller's basic medium with IAA and kinetin.

(1) Control (Heller's medium).

(2) 10^{-8} IAA + (a) 10^{-7} kinetin, (b) + $3 \cdot 10^{-7}$ kinetin, (c) + 10^{-6} kinetin,

(3) 10^{-7} IAA + (a) 10^{-7} kinetin, (b) + $3 \cdot 10^{-7}$ kinetin, (c) + 10^{-6} kinetin,

(4) 10^{-6} IAA + (a) 10^{-7} kinetin, (b) + $3 \cdot 10^{-7}$ kinetin, (c) + 10^{-6} kinetin.

Sl. 1. Eksplantati hipokotila kultivirani na Hellerovom osnovnom mediju s IAA i kinetinom.

(1) Kontrola (Hellerov medij).

(2) 10^{-8} IAA + (a) 10^{-7} kinetin, (b) + $3 \cdot 10^{-7}$ kinetin, (c) + 10^{-6} kinetin,

(3) 10^{-7} IAA + (a) 10^{-7} kinetin, (b) + $3 \cdot 10^{-7}$ kinetin, (c) + 10^{-6} kinetin,

(4) 10^{-6} IAA + (a) 10^{-7} kinetin, (b) + $3 \cdot 10^{-7}$ kinetin, (c) + 10^{-6} kinetin.

Fig. 2. Explants of hypocotyls cultivated on MS-salts medium (5) and MS-complete medium (6) after 1 month.

Sl. 2. Eksplantati hipokotila uzgajanog na mediju koji sadrži samo MS-soli (5) i na potpunom MS-mediju (6) nakon mjesec dana.

Fig. 3. Explants of hypocotyls cultivated on MS-complete medium with 10^{-6} IAA and $3 \cdot 10^{-7}$ kinetin. Some explants show development of vigorous roots, one explant displays callus only.

Sl. 3. Eksplantati hipokotila uzgajani na potpunom MS-mediju s 10^{-6} IAA i $3 \cdot 10^{-7}$ kinetina. Pojedini eksplantati pokazuju razvitak snažnog korijenja, dok je jedan eksplantat razvio samo kalus.

Fig. 4. Explants of hypocotyls cultivated on MS-complete medium with glucose in various concentrations (%);

(10) 0 glucose, (11) 1%, (12) 2%, (13) 3%, (14) 4%, (15) 5% and (16) 6%.

Sl. 4. Eksplantati hipokotila uzgajani na potpunom MS-mediju s glukozom u različitim koncentracijama (%):

(10) 0 glukoze, (11) 1%, (12) 2%, (13) 3%, (14) 4%, (15) 5%, i (16) 6%.

Fig. 5. The effect of 2,4-D and water melon sap on the explants of hypocotyls. Basic MS-salts medium.

(10) 10^{-7} 2,4-D + 10% vol sap,

(11) 10^{-7} 2,4-D + 15% vol sap,

(12) 10^{-7} 2,4-D + 20% vol sap,

(13) 10^{-6} 2,4-D + 10% vol sap,

(14) 10^{-6} 2,4-D + 15% vol sap,

(15) 10^{-6} 2,4-D + 20% vol sap,

(16) only 15% vol sap.

Sl. 5. Djelovanje 2,4-D i soka lubenice na eksplantate hipokotila.

Osnovni medij MS-soli.

(10) 10^{-7} 2,4-D + 10% vol soka,

(11) 10^{-7} 2,4-D + 15% vol soka,

(12) 10^{-7} 2,4-D + 20% vol soka,

(13) 10^{-6} 2,4-D + 10% vol soka,

(14) 10^{-6} 2,4-D + 15% vol soka,

(15) 10^{-6} 2,4-D + 20% vol soka,

(16) samo 15% vol. soka.

Fig. 6. The effect of 2,4-D ($3 \cdot 10^{-7}$) and yeast extract on the explants of hypocotyls. Basic MS-complete medium.

(1) 0 g/l y. e., (2) 1, (3) 2, (4) 3, (5) 4, (6) 5 and (7) 6.

Sl. 6. Djelovanje 2,4-D ($3 \cdot 10^{-7}$) i ekstrakta kvasca na eksplantate hipokotila. Osnovni medij MS-kompletni.

(1) 0 g/l e. k., (2) 1, (3) 2, (4) 3, (5) 4, (6) 5 i (7) 6.

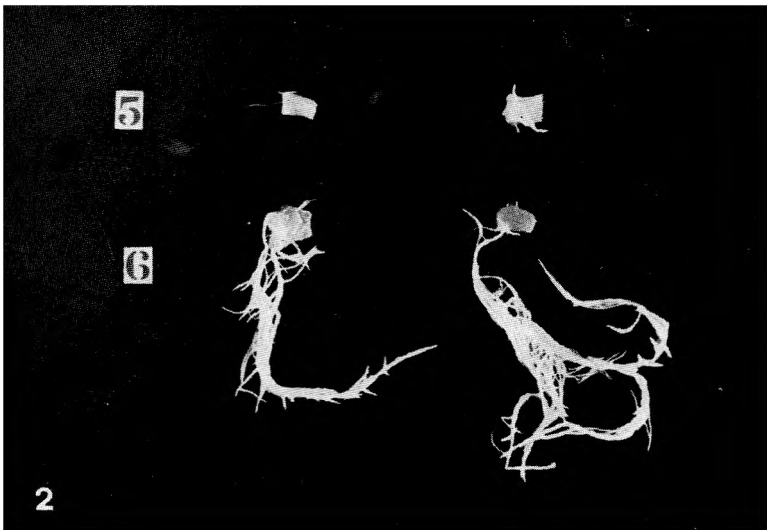
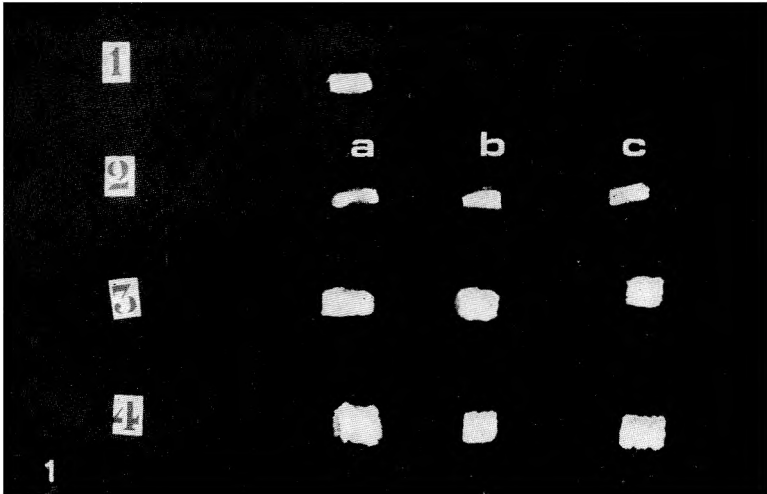


Fig. 1—2. — Sl. 1—2.

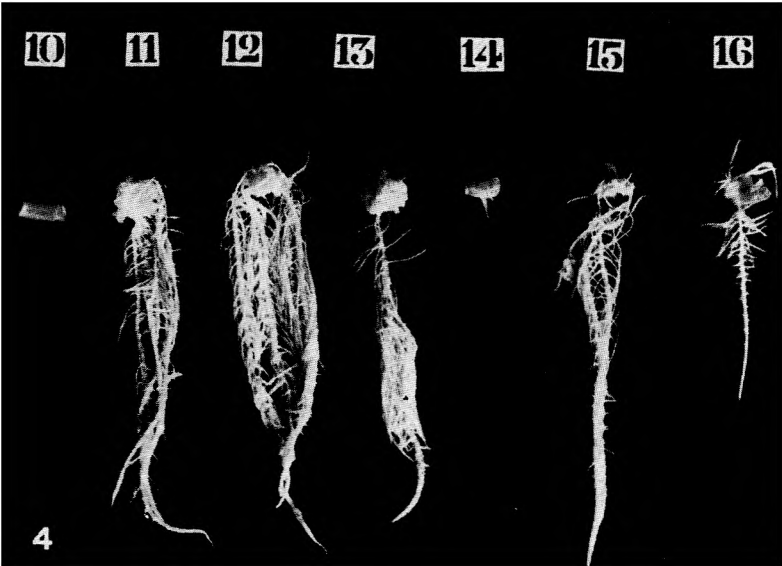
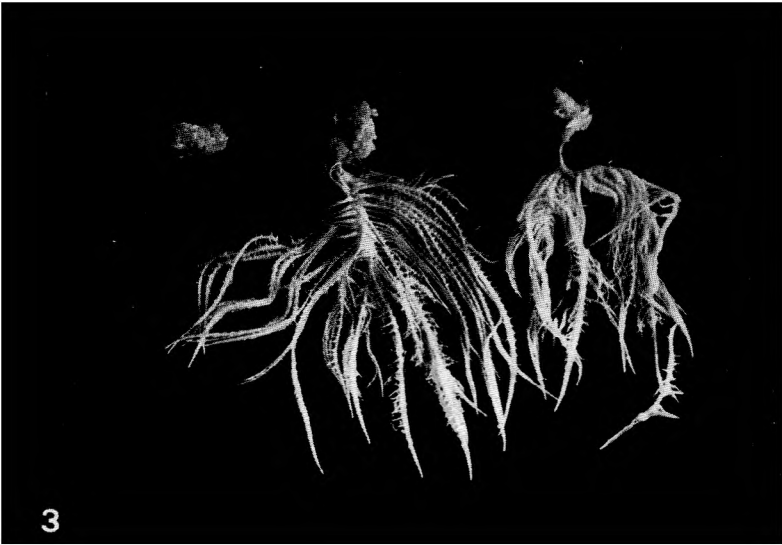


Fig. 3—4. — Sl. 3—4.

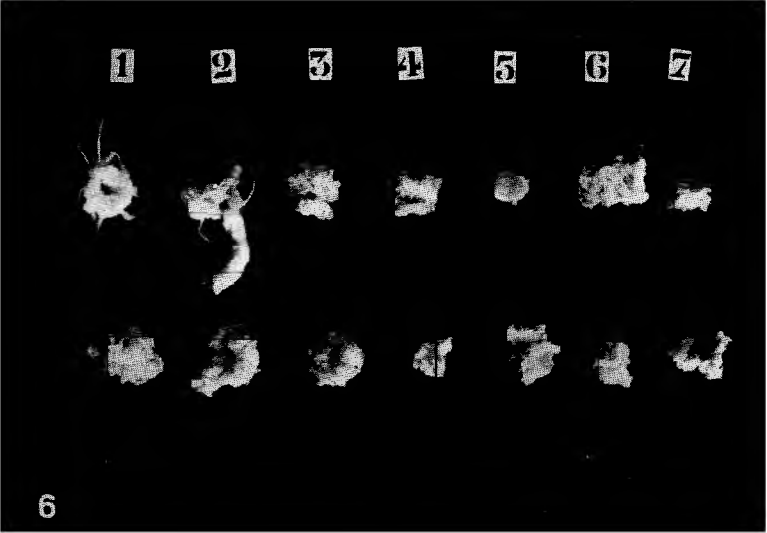
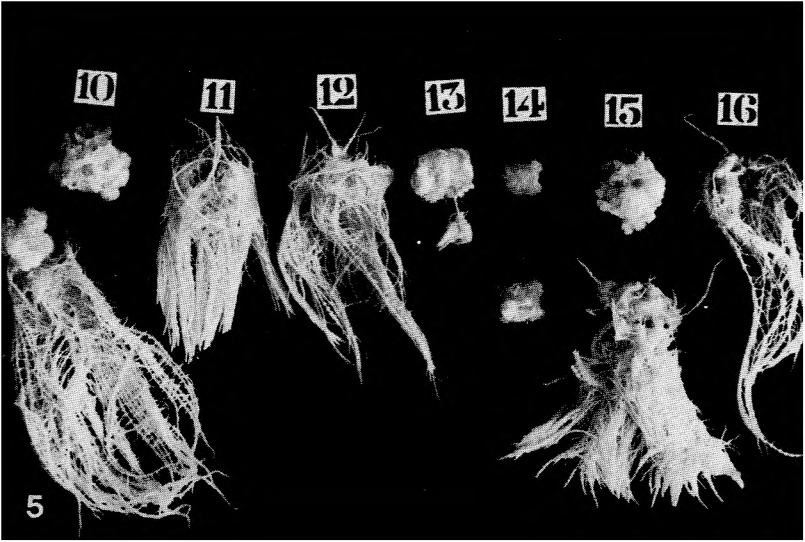


Fig. 5—6. — Sl. 5—6.

The addition of yeast extract in concentrations of 0.5, 1, 2, 3, 4, 5 g/l with 2,4-D ($3 \cdot 10^{-7}$) allowed the development of rootlets at low concentrations (0.5 and 1 g/l), although they were very small and rudimentary (Fig. 6).

The effect of IBA, which was added in concentrations from 10^{-10} to 10^{-5} , on the rooting of explants was somewhat unexpected. At high concentrations it had no toxic effects on the tissue of pumpkin hypocotyls and the percentage of rooted explants was the highest just here. But the roots were not as vigorous as one would expected (Table 3).

Table 3. The effect of IBA on the rooting of hypocotyl explants after one month culturing (MS-complete medium).

IBA	Number of cultures	Cultures with roots Number	%
10^{-10}	15	4	26.6
10^{-9}	17	9	52.7
10^{-8}	17	5	29.4
10^{-7}	19	6	31.5
10^{-6}	15	15	100.0
10^{-5}	10	10	100.0

The action of NAA was examined at concentrations from 10^{-9} to $2 \cdot 10^{-5}$. Higher concentrations of NAA (e.g. 10^{-6}) caused necrosis and decay of the cultured tissue, while low concentrations (10^{-9} and 10^{-8}) were useful for root-growing in explants.

Discussion

The roots developed on the proximal side of the pumpkin hypocotyls when they were cultivated on the MS-complete medium. The MS-salts medium alone stimulated the induction of roots, but the addition of organic substances made the roots robust, long and of natural appearance. The addition of 2,4-D at concentrations of 10^{-7} and 10^{-6} induced rooting of hypocotyl fragments also when they were cultured on Heller's medium, on which alone it had never been induced. The addition of sap of immature water-melon fruits (15% vol.) to the MS-salts medium stimulated vigorous root growth, as did low concentrations of yeast extract (0.5—2 g/l). NAA (10^{-9} and 10^{-8}) and IBA (10^{-6} and 10^{-5}) induced frequent formation of roots, although they were not especially strong.

The absence of sugar (medium MS-complete) completely inhibited rhizogenesis.

On the basis of these data we may say that rhizogenesis of pumpkin hypocotyl fragments is restricted according to the facts known so far by two limiting factors, namely: salts and sugar content in the medium. Neither on Heller's nor on MS-complete medium did the pumpkin hypocotyl fragments ever form any roots without sugar, which is in

good agreement with the data of Gautheret (1969). The presence of some growth regulators in proper concentrations stimulated the development and growth of roots, but for their induction on MS-medium the auxins were not absolutely necessary. The origin of rhizogenesis has been the subject of many investigations. The results have been published in journals, but the most complete surveys are the ones by Dore (1965) and Torrey (1965). The relation of phytohormones to the root induction was studied and discussed by many numerous investigators (see e.g. Champagnat 1961).

The phenomenon of rhizogenesis itself is not rare in tissue culture (Gautheret 1959). What appeared to be most interesting in the course of these experiments is the fact that a small number of explants did not show any root induction even at most favourable conditions for rooting, but after a prolonged culturing revealed another kind of organogenesis, i.e. the embryogenesis and caulogenesis (Jelaska 1974). It is difficult to answer why some explants, cultivated under the same conditions and on the same medium, did not react by a rapid rhizogenesis but began to form embryogenic callus. The assumption that the genetic constitution of the plant plays a significant role in this case seems to be easily acceptable. Further experiments, planned by the author, should elucidate these problems.

S u m m a r y

Hypocotyl fragments of pumpkin were grown on Heller's synthetic medium or MS-medium with 3% glucose and 0.9% Difco Bacto agar and some growth regulators. Heller's basic medium is not suitable for the growth and development of hypocotyls. On this medium the fragments never show any signs of differentiation and hardly survive the culture period of one month.

Moreover, as shown earlier, IAA with kinetin or 2,4-D with kinetin in Heller's medium provoke cell divisions resulting in an increase of fresh and dry weight but never in rooting. In a small number of explants with only 2,4-D at the concentrations of 10^{-7} and 10^{-6} very weak rhizogenesis was induced.

The MS-medium has proved to be much more suitable. The medium consisting of MS-salts and sugar only is sufficient for root induction on the root side of the fragment; for the vigorous root growth, however, the complete MS-medium is necessary. The addition of some substances, e.g. of autoclaved water-melon sap (15% vol.) or autoclaved water-melon sap combined with 2,4-D ($3 \cdot 10^{-7}$) further intensifies the vigorous growth of the roots; low concentrations of NAA, high concentrations of IBA and IAA (10^{-6}) with kinetin ($3 \cdot 10^{-7}$) show the same effect.

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References

- Champagnat, P. 1961: Différenciation. Formation des racines et des bourgeons. 839—871. In W. Ruhland (ed.) *Encycl. Plant Physiol.* 14. Springer-Verlag, Berlin.
- Dore, J., 1965: Physiology of regeneration in cormophytes. 1—91. In W. Ruhland (ed.) *Encycl. Plant Physiol.* 15/2. Springer-Verlag, Berlin.
- Gautheret, R. J., 1959: La culture des tissus végétaux. Masson Ed. Paris.
- Gautheret, R. J., 1969: Investigations on the root formation in the tissues of *Helianthus tuberosus* cultured in vitro. *Amer. J. Bot.* 56, 702—717.
- Heller, R., 1953: Recherches sur la nutrition minérale de tissus végétaux cultivés in vitro. *Ann. Sc. Nat. Bot. Biol. Vég.* 14, 1—223.
- Jelaska, S., 1972: Morphogenesis of pumpkin explants cultivated in vitro. Ph. D. — Thesis. Faculty of Sciences, University of Zagreb, 118 p.
- Jelaska S., 1973: The effect of growth regulators on the survival of excised pumpkin hypocotyl-tissue. *Acta Bot. Croat.* 32, 95—100.
- Jelaska, S., 1974: Embryogenesis and organogenesis in pumpkin explants. *Physiol. Plant.* 30, 603—607.
- Levine, M., 1950: The growth of normal plant tissue in vitro as affected by chemical carcinogens and plant growth substances. I. The culture of the carrot tap-root meristem. *Amer. J. Bot.* 37, 445—458.
- Miller, C. O. and F. Skoog, 1953: Chemical control of bud formation in tobacco stem segments. *Amer. J. Bot.* 40, 768—773.
- Miller, C. O. and F. Skoog, 1955: Regulation of growth in tobacco tissue culture. *Plant Physiol.* 30, Suppl. XXXV.
- Murashige, T. and F. Skoog, 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15, 473—497.
- Skoog, F., 1944: Growth and organ formation in tobacco tissue cultures. *Amer. J. Bot.* 31, 19—24.
- Skoog, F. and C. Tsui, 1948: Chemical control of growth and bud formation in tobacco stem segments and callus cultured in vitro. *Amer. J. Bot.* 35, 782—787.
- Skoog, F. and C. O. Miller, 1957: Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp. Soc. exp. Biol.* 11, 118—131.
- Torrey, J. G., 1965: Physiological bases of organization and development in the root. 1296—1327. In W. Ruhland (ed.) *Encycl. Plant Physiol.* 15/1. Springer-Verlag, Berlin.
- Wiggans, S. C. 1954: Growth and organ formation in callus tissue derived from *Daucus carota*. *Amer. J. Bot.* 41, 321—326.

SADRŽAJ

RIZOGENEZA NA EKSPLANTATIMA HIPOKOTILA BUNDEVE

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Fragmenti hipokotila bundeve veličine 1 cm bili su uzgajani na osnovnom Hellerovom mediju ili MS-mediju uz dodatak 3% glukoze, 0,9% Difco Bacto agara i različitih regulatora rasta.

Osnovni Hellerov medij nije bio pogodan za rast i razvitak eksplantata hipokotila bundeve. Na ovom osnovnom mediju, bez regulatora rasta, fragmenti nisu pokazivali nikakvu diferencijaciju, pa ni rizogenezu, i nisu preživjeli period kultiviranja od mjesec dana. Dodatak 2,4-D, IAA i kinetina produžio je preživljenje eksplantata preko mjesec dana, no niti najrazličitije kombinacije 2,4-D i kinetina, a tako ni IAA i kinetina, nikada nisu potakle stvaranje korijena. Jedino je na manjem broju kultura uočena pojava slabih korjenčića nakon dodatka same 2,4-D u visokim koncentracijama.

MS-medij bio je mnogo pogodniji. Sama podloga MS-soli uz dodatak 3% glukoze bila je dovoljna da se korijenje inducira na proksimalnoj (korijenskoj) strani eksplantata. Bujno korijenje naraslo je međutim na kompletnom MS-mediju. Bez dodatka šećera na kompletnom MS-mediju nije bilo moguće izazvati rizogenezu. Dodatkom izvjesnih supstancija rasta pojačao se broj induciranog korijenja i njegov razvoj. Tako je npr. dodatak od 15% soka lubenice osnovnom mediju MS-soli djelovao poput dodatka aminokiselina i vitamina iz kompletnog MS-medija. Dodavanjem 2,4-D u koncentracijama 10^{-6} i 10^{-7} te NAA u nižim koncentracijama (10^{-9} i 10^{-8}), kombinacija IAA (10^{-6}) i kinetina ($3 \cdot 10^{-7}$) te IBA u visokim koncentracijama (10^{-6}) kompletnom MS-mediju postignut je bujni rast korijenja na eksplantatima hipokotila bundeve.

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