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Some Factors Affecting Root Formation on *in vitro* Regenerated Pea Shoots

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Abstract. The rooting ability of 2 cm long shoots of *Pisum sativum* L., derived from different *in vitro* shoot-tip cultures in two pea cultivars Bohatýr and Kleine Rheinländerin was evaluated. In three mutually independent experiments the full and half-strength Murashige-Skoog medium (containing full or half concentration of macro and microelements), with sucrose concentrations 10—30 g l^{-1}, and with various NAA and IAA combinations, was tested. The variant with half concentration of macro- and microelements, supplemented with 30 g l^{-1} sucrose, and with growth regulators in the quantity of 1 μM proved optimum.

Regeneration of plants in meristem or callus cultures in pea is usually a two-step process initiated by proliferation of buds and shoots and completed by rhizogenesis (GAMBORG *et al.* 1968, KARTHA *et al.* 1974, 1979, MALMBERG 1979, MROGINSKI and KARTHA 1981, RUBLUO *et al.* 1982, 1984, GRIGA *et al.* 1984b, 1986). Root formation is often a restricting factor in achieving regenerants able to be transferred into soil (KUNAKH *et al.* 1984 a, b, c).

In our previous work (GRIGA *et al.* 1984a, b 1986) we formulated a system of clonal propagation *in vitro* in pea, using the culture of shoot apices and axillary buds. However, the frequency of rooting of isolated shoots is relatively the least stable character, which showed up both within one genotype, and — quite distinctly — during evaluating of a greater number of pea genotypes (unpublished results). This fact could considerably restrain potential application of the above technique in pea breeding.

The aim of our present work was to specify in more detail some factors (above all the nutrient ones) affecting the root formation on isolated shoots of peas of different physiological age.

MATERIAL AND METHODS

In the first experiment shoots for testing their rooting ability were produced *in vitro* from *Pisum sativum* ssp. *hortense* A. et G. (the cultivars Bohatýr and Kleine Rheinländerin). The seeds were surface sterilized for 30 min

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in 5 % chloramine B, dipped in 70 % ethanol, and rinsed in sterile water three times. Then they were placed one by one onto filter paper bridges in test-tubes containing the MS medium (MURASHIGE-SKOOG 1962). After achieving the size of 10 nodes the plants were dissected into individual nodes and these were cultured on the MS medium supplemented with 1 μM BAP. Newly produced shoots from these segments, 2 cm in length, with 2 nodes each and bearing a spread leaf, were transferred onto 12 variants of root-inducing medium. We used the MS medium with full or half-strength concentration of mineral salts, with 10 or 30 g l^{-1} sucrose and with three combinations of regulators 0 (control), 1 μM NAA + 1 μM IAA and 3 μM NAA + 2 μM IAA. The completely prepared medium was autoclaved for 15 min at 121 °C and 100 kPa. In each variant 10 tubes in 3 replicates were established. The culture took place at 25 ± 2 °C under 16 h photoperiod, evaluation was made after 4 weeks.

In the second experiment a culture of pea shoot apices was used in the third subculture on a modified MS medium containing 0.1 μM NAA and 20 μM BAP (GRIGA *et al.* 1984 a). For rooting, shoots of the same size as in the first experiment were isolated. They were cultured on 8 variants of the MS medium with full or half concentration of mineral salts, with 10 or 30 g l^{-1} sucrose and with the regulator combinations 1 μM IAA + 1 μM NAA or 3 μM NAA + 2 μM IAA. In each variant 25 tubes in 3 replicates were established. The cultures were maintained at 23 ± 2 °C. Evaluation was made after 3 weeks of culture.

In the third experiment we used a long-term cultured (1 year long) pea culture, cv. Bohatýr, with a good proliferation of multiple shoots, maintained on the same medium as in the second experiment. For rooting shoots of the same size were again isolated. They were placed on 10 variants of root-inducing medium MS, where the MS vitamins had been substituted according to the B5 medium (GAMBORG *et al.* 1968) with full or half-decreased content of MS-mineral salts, 10, 20, and 30 g l^{-1} sucrose, and supplemented with 0, 1, 5 concentrations of μM NAA. In each variant 20 test-tubes in 2 replicates were established. The culture took place under a 16 h photoperiod at 25 °C in the light and at 20 °C in the dark. Evaluation was made after 35 days.

In all three experiments we evaluated the following characters:
frequency of rooting in each experiment variant, number of roots longer than 2 mm produced on each shoot, length of the longest root, length of each shoot, number of nodes in each shoot.

In the first experiment we also evaluated callus-formation on a shoot base.

RESULTS

Evaluating the characters, frequency of rooting and number of roots in the first and second experiments by analysis of variance, we found out that the effect of growth regulators, sucrose and mineral salt concentrations is statistically highly significant or significant (Table 1).

As far as root length is concerned, merely different concentrations of growth regulators and sucrose are statistically significant; mineral salt concentration does not affect this character.

For all evaluated characters the optimum root-inducing medium variant is evidently that one containing 50 % of mineral salts and 30 g l^{-1} sucrose.

TABLE I
3-factor analysis of variance. The effect of growth regulators, and sucrose and mineral salts concentrations on the rooting of the pea shoots

Number of experiment	Cultivar	Source of variation	Frequency of rooting		Number of roots		Root Length	
			F	Significance	F	Significance	F	Significance
I	Bohatýr	GR	25.92**	00.71	99.91**	0.19	5.12	8.05
		S	15.48*	1.75	53.41**	0.36	17.40*	1.46
		MS	2.12	21.70	15.24*	1.78	0.60	48.80
	Kleine Rheinländerin	GR	13.24*	1.83	34.26**	0.51	27.73**	0.65
		S	4.19	11.07	29.36**	0.71	68.65**	0.28
		MS	8.56*	4.27	17.69*	1.43	2.36	19.80
II	Bohatýr	GR	115.82*	1.70	0.18	70.66	28.96*	4.07
		S	904.53**	0.83	180.90*	1.39	146.26*	1.52
		MS	29.84*	3.98	14.07	7.16	4.19	17.99
III	Bohatýr	GR	5.80	15.16	11.70	8.70	47.05*	3.05
		S	8.78	10.40	8.17	10.99	11.39	8.47
		MS	0.29	77.45	1.19	38.88	11.92	8.17

GR — growth regulators; S — 10 and 30 g l^{-1} sucrose; MS — full and half concentration of mineral salts.
*$P = 0.05$; **$P = 0.01$.

As far as the regulators are concerned, the results are not quite unambiguous. Maximum rooting frequency in the first experiment, 93.3 % in the cultivar Kleine Rheinländerin and 86.8 % in the cv. Bohatýr, was on the

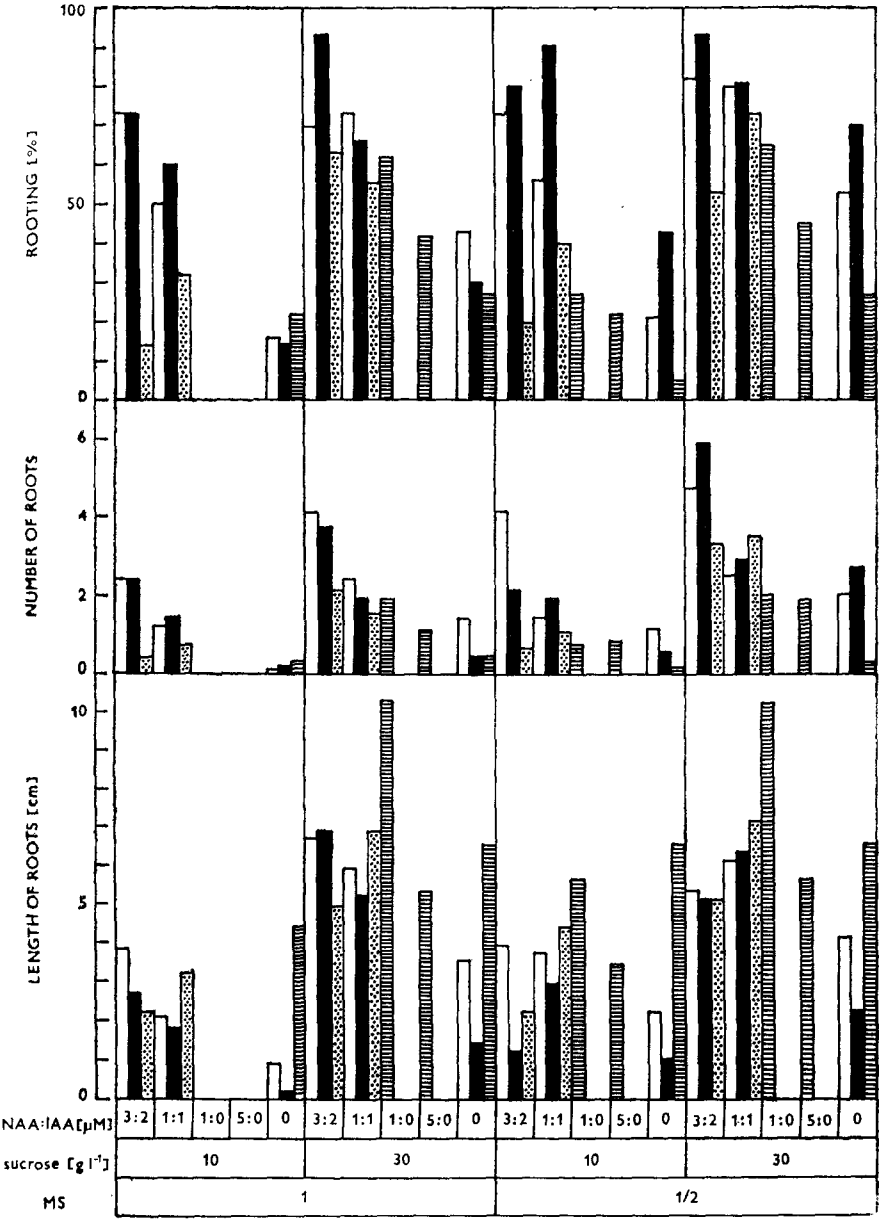

Fig. 1. Frequency of rooting, mean number of roots per shoot and mean length of roots (calculated including the non-rooted shoots in each variant, *i.e.* zero values of the number of roots on a shoot and their length were taken into account) in two pea cultivars (Bohatýr and Kleine Rheinländerin). Cv. Bohatýr: 1st experiment — empty column, 2nd experiment — dotted column, 3rd experiment — dashed column; cv. Kleine Rheinländerin: 1st experiment — full column.

medium variant with 3 μM NAA + 2 μM IAA. In the second experiment, in the cv. Bohatýr, the highest rooting frequency was 73.3 % on the medium with 1 μM NAA + 1 μM IAA. In the third experiment (cv. Bohatýr) maximum 65 % rooting was achieved on the medium variant with 1 μM NAA (Fig. 1).

The characters number and length of roots are very unsteady. The number of roots is 0—17, their length is from 0.2—16.5 cm. These characters vary in the same way as the frequency of rooting — on optimum root-inducing medium variants we also achieved the highest numbers of roots of maximum length.

As far as the shoot length and the number of nodes are concerned, we observed no conclusive differences in any experiment. Generally, it can be said that a good development of shoots corresponds with an optimum development of roots. The variants with a full concentration of MS medium are insignificantly better. A higher concentration of auxins also inhibits the shoot growth.

Maximum shoot length in the first experiment was 7.7 cm and maximum number of nodes was 9. In the third experiment it was 14.5 cm and 12 nodes; this character was not evaluated in the second experiment.

DISCUSSION

In spite of the facts that the experiments were carried out in 3 different laboratories, that we used cultures of different ages, and that the schemes of individual experiments were not completely identical, the effects of the observed factors are in all cases similar.

The stimulating effect of sucrose on root formation is also mentioned by HUNALT and LEROUX (1976) in *Sanseviera trifasciata* (40 g l^{-1}), by DUHOUX and DAVIES (1985) in *Acacia albida* (50—65 g l^{-1}), and MAENE and DEBERGH (1985) in *Cordyline terminalis*, who found out that the function of higher sucrose concentrations is nutrient and not osmotic.

Our further experiments also showed that even a higher concentration of sucrose will be optimal for root formation in pea, namely above 30 g l^{-1} (unpublished).

The stimulating effect of auxins upon root formation is generally known in all plants. KARTHA (1984) uses 1 μM NAA in a 1/2 medium B5 for root induction in pea. He also mentions the fact that a high concentration of salts can inhibit the initiation of roots (KARTHA *et al.* 1974).

An optimum concentration of regulators must be selected with regard to the subsequent transfer into soil, where plants without callus at the base, with normally developed root system survive better (Fig. 2).

In our further experiments the NAA appears more effective than the IAA (unpublished), which can be caused by the fact that the latter is decomposed by the cells.

The lower frequency of root formation in explants in the second and third experiments can be caused by the age of the culture on one hand, and also by a high concentration of the BAP (20 μM) in the previous subculture on the other hand. Low concentrations of exogenous cytokinins have a stimulating effect on root formation (NEMETH 1979), whereas high concentrations even in the previous subculture can have a negative impact.

A deeper knowledge of factors affecting the root induction *in vitro* on regenerated shoots of pea should enhance the efficiency of complete plants during regeneration *in vitro* (in a meristem or callus culture), and in this way bring the explant techniques in pea closer to a potential exploitation in breeding.

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REFERENCES

DUHOUX, E., DAVIES, D.: Caulogénèse à partir des bourgeons cotylédonaires d'*Acacia albida* et influence du saccharose sur la rhizogénèse. — J. Plant Physiol. **121** : 175—180, 1985.

GAMBORG, O. L., MILLER, R. A., OJIMA, K.: Nutrient requirement of suspension cultures of soybean root cells. — Exp. Cell Res. **50** : 151—158, 1968.

GRIGA, M., TEJKLOVÁ, E., NOVÁK, F. J.: [Hormonal regulation of growth of pea (*Pisum sativum* L.) shoot apices in *in vitro* culture.] In Czech. — Rost. Výroba (Praha) **30** : 523—530, 1984a.

GRIGA, M., TEJKLOVÁ, E., NOVÁK, F. J.: *In vitro* propagation of pea by axillary and adventitious bud technique. — In: NOVÁK, F. J., HAVEL, L., DOLEŽEL, J. (ed.): Plant Tissue and Cell Culture — Application to Crop Improvement. Pp. 507—508. Institute of Experimental Botany, Czechoslovak Academy of Sciences, Praha 1984b.

GRIGA, M., TEJKLOVÁ, E., NOVÁK, F. J., KUBALÁKOVÁ, M.: *In vitro* clonal propagation of *Pisum sativum* L. — Plant Cell Tissue Organ Cult. **6** : 95—104, 1986.

HUNAULT, G., LEROUX, R.: Influence de divers facteurs sur la rhizogenése de tissus d'une monocotylédone (*Sansevieria trifasciata* PRAIN) et d'une dicotylédone (*Pisum sativum* L.). — Actes du 101e Congrès National des Sociétés Savantes, Sciences Fasc. I. Pp. 529—539. Lille 1976.

KARTHA, K. K.: Culture of shoot meristems: Pea. — In: VASIL, I. K. (ed.): Cell Culture and Somatic Cell Genetics of Plants. Vol. 1. Pp. 106—110, Academic Press, Orlando—San Diego—New York—London—Toronto—Montreal—Sydney—Tokyo 1984.

KARTHA, K. K., GAMBORG, O. L., CONSTABEL, F.: Regeneration of pea (*Pisum sativum* L.) plants from shoot apical meristems. — Z. Pflanzenphysiol. **72** : 172—176, 1974.

KARTHA, K. K., LEUNG, N. L., GAMBORG, O. L.: Freeze-preservation of pea meristems in liquid nitrogen and subsequent plant regeneration. — Plant Sci. Lett. **15** : 7—15, 1979.

KUNAKH, V. A., ALKHIMOVA, E. G., VOITYUK, L. I.: [Variability of the chromosome number in callus tissues and pea regenerants.] In Russ. — Tsitol. Genet. **18** : 20—26, 1984a.

KUNAKH, V. A., ALKHIMOVA, E. G., VOITYUK, L. I., ALPATOVA, L. K.: Obtaining of tissue cultures and organogenesis induction in *Pisum sativum* L. — In: NOVÁK, F. J., HAVEL, L., DOLEŽEL, J. (ed.): Plant Tissue and Cell Culture — Application to Crop Improvement. Pp. 135—136. Institute of Experimental Botany, Czechoslovak Academy of Sciences, Praha 1984b.

KUNAKH, V. A., VOITYUK, L. I., ALKHIMOVA, E. G., ALPATOVA, L. K.: [Callus tissue formation and induction of organogenesis in *Pisum sativum* L.] In Russ. — Fiziol. Rast. **31** : 452—548, 1984c.

MAENE, L., DEBERGH, P.: Liquid medium additions to established tissue cultures to improve elongation and rooting *in vivo*. — Plant Cell Tissue Organ Cult. **5** : 23—33, 1985.

MALMBERG, R. L.: Regeneration of whole plants from callus culture of diverse genetic lines of *Pisum sativum* L. — Planta **146** : 243—244, 1979.

MROGINSKI, L. A., KARTHA, K. K.: Regeneration of pea (*Pisum sativum* L. cv. Century) plants by *in vitro* culture of immature leaflets. — Plant Cell Rep. **1** : 64—66, 1981.

MURASHIGE, T., SKOOG, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. — Physiol. Plant. **15** : 473—497, 1962.

NEMETH, G.: Benzyladenine-stimulated rooting in fruit-tree rootstock cultured *in vitro*. — Z. Pflanzenphysiol. **95** : 389—396, 1979.

RUBLUO, A., KARTHA, K. K., MROGINSKI, L. A., DYCK, J.: Plant regeneration from pea leaflets cultured *in vitro* and genetic stability of regenerants. — J. Plant. Physiol. **117** : 119—130, 1984.

RUBLUO, A., MROGINSKI, L. A., KARTHA, K. K.: Morphogenetic responses of pea leaflets cultured *in vitro*. — In: FUIWARA, A. (ed.): Plant Tissue Culture. Pp. 151—152. Jap. Assoc. Plant Tissue Culture, Tokyo 1982.

Fig. 2 at the end of the issue.