Acta Bot. Croat. 34 (1975) 43—51

NUTRITIONAL AND HORMONAL REQUIREMENTS OF METEOR PEA CALLUS TISSUE

MIRJANA NEŠKOVIĆ and *LJILJANA RADOJEVIĆ*

(Institute of Botany, Faculty of Science and Institute for Biological Research, Beograd)

Received November 22, 1974

Introduction

Tissue culture of Meteor pea was obtained in July 1968 from the proliferation of an immature embryo. The tissue was isolated in the search for a system which might be useful in the study of gibberellin action at the level of unorganized cells. During the preliminary routine experiments aimed at finding the optimal culture medium, it was noted that the tissue was rather specific in its requirements for growth. The present paper reports the observations concerning the effect of mineral salts, carbohydrates, vitamins and hormones.

Callus tissues of different pea varieties have not been very often used in studies of nutrition and morphogenesis. Torrey and Shigemura (1957) obtained a callus tissue from Alaska pea roots using a modified Bonner mineral medium, with sucrose, auxin and vitamins. The root-forming capacity of several strains of that tissue was studied later on in relation to chromosomal constitution (Torrey, 1967). The production of cytokinins was investigated by using higher salt concentrations and a more complex mixture of vitamins and amino acids (Short and Torrey, 1972). Hildebrandt et al. (1963) isolated a callus from the stem tissue, which was growing on White mineral solution, sucrose, NAA, inositol and casein hydrolysate. Bailey (1970) studied the production of pisatin by pea tissue cultivated on a similar medium, containing vitamins, glycine, 2,4-D and coconut milk. Kallak and Yarvekylg (1971) investigated the cytogenetic effect of 2,4-D in a pea callus, derived from cotyledons and grown on the Torrey medium. The influence of environmental conditions, nitrogen nutrition and hormones on shoot formation in a callus tissue obtained from excised field pea apices was studied by Gamborg et al. (1974). Apparently, pea callus tissue can satisfactorily be grown on various media and the precise nutritional requirements were not studied in most cases.

Material and Methods

Green, not fully grown pea pods *(Pisum sativum* cv. Meteor) were opened and the seeds sterilized with 5% calcium hypochlorite and washed with sterile water. Embryos about 3 mm long were taken out of the seeds and placed on the agar medium. Most embryos proliferated in the root region. All tissues referred to in this paper originate from a single embryo.

The isolated embryos were first grown on a medium used previously for pea callus tissue by Torrey and Shigemura (1957). Small proliferations were obtained from the axial tissue, which grew very slowly, so that only a few subcultures could be transplanted. Some of the explants were then transferred onto the medium prepared for wild carrot tissue (Halperin, 1964) and satisfactory growth was obtained. This medium was designated as basal medium (BM) and has been used since then for stock cultures. It contains mineral solution of Murashige and Skoog (1962), 1% agar, 3% sucrose and (in mg l^{-1}): thiamine 3, nicotinic acid 5, adenine 2 and 2,4-D 0.1. After three more transfers, experiments were set up to determine which components of the medium were actually needed. Five mineral solutions were tried:

1. T-medium was a mineral solution used for pea callus tissue by Torrey and Shigemura (1957).
2. H-medium: mineral solution of Heller (1953), which had been proved suitable for many cultivated tissues.
3. W-medium: mineral solution by White (1943) for tomato roots, but also used widely for many tissues.
4. WB-medium was White's solution as modified by Wood and Braun (1961) for *Vinca rosea* tissue; it contains additional amounts of ammonium, nitrate, potassium and phosphate salts.
5. MS-medium: revised medium of Murashige and Skoog (1962) for tissue culture of tobacco.

Organic constituents tested were sucrose, glucose and fructose, vitamins and growth substances. The concentrations are indicated in the text or tables. If necessary, the pH of the media was adjusted with NaOH at 5.8 before autoclaving. Media were autoclaved at 115° C for 25 min.

Experiments were carried out in 100 ml Erlenmayer flasks, containing 40 ml of nutrient agar, with three callus pieces in each. Each series comprised at least five flasks. Experiments were repeated three or more times. Cultures were kept in diffuse light from Sylvania "Gro-Lux" and warm white fluorescent tubes, in a day of 12 hours. Initial weight of the callus pieces at transfers was 20—30 mg. Growth was evaluated as fresh weight after 6—8 weeks.

Results

The callus tissue obtained was a slow growing tissue with average increase in weight of 250—350 mg in the culture period. The tissue was rather friable, composed of small, firm masses, sometimes greenish in colour. By the completion of the experiments described here, the tissue had been subcultured 23 times.

Mineral salts

The requirements for mineral salts were studied by using the five solutions mentioned above. They were supplemented with sucrose and other organic constituents as in the BM. It was found that only two of

them could support the growth of the tissue. These were MS and WB media, the former being much superior. Tissues transferred on T, H or W medium did not grow at all, turned brown and died (Table 1).

Table 1. Growth of Meteor pea callus tissue on different mineral solutions. Results of five separate experiments, each comprising five flasks with three callus pieces. Figures indicate fresh weight in mg per callus, after six weeks of culture.

Mineral solutions				
T	H	W	WB	MS
16.7	15.8	21.4	48.5	231.0
18.3	29.2	17.5	122.4	305.9
14.4	26.0	27.1	101.7	246.8
15.5	14.7	19.6	171.0	245.0
15.6	20.0	21.6	80.2	208.7

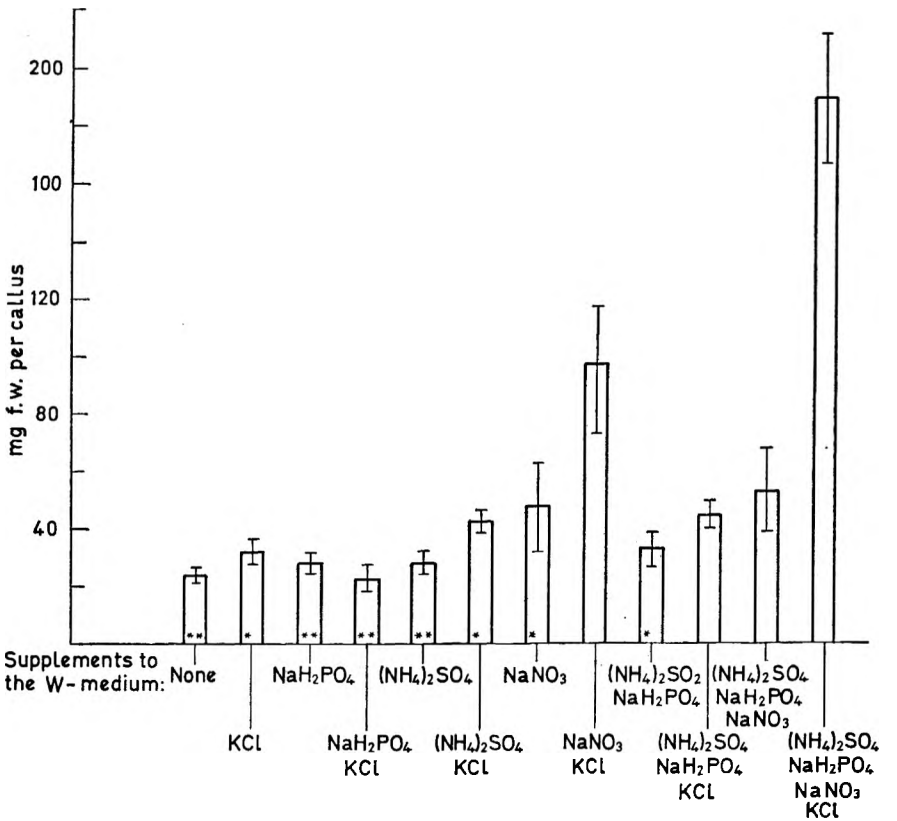

Fig. 1. The growth of Meteor pea callus tissue on White's mineral medium, supplemented as indicated. Amounts of particular salts added same as in the complete WB medium.
** = tissues necrotic
* = tissues partly necrotic

The media used differ greatly from one another in the presence and concentration of almost all salts. As the WB and MS media contain part of their nitrogen in the form of an ammonium salt, it was thought in the beginning that the Meteor callus might be unable to reduce nitrate. However, when in MS medium all the nitrogen was given as nitrate, the tissue was still growing, although at a slower rate.

Further experiments were set up to see whether the tissue required a higher concentration of all salts in general, or the stimulation of MS and WB media were due to some particular element. Therefore, the W medium was supplemented with $(NH_4)_2SO_4$, $NaNO_3$, KCl and NaH_2PO_4 separately or combined, so as to obtain the concentration of these salts in the WB medium. The results are shown in Fig. 1. As can be seen, no compound alone could support growth of the tissue. However, when a higher amount of nitrogen, either as ammonium or as nitrate, was added together with KCl, the tissue did not die and some increase in growth was achieved. Yet for the optimal growth on that medium all four salts must be present.

It was shown before (S t r e e t, 1969) that tomato roots grown in W medium produce an increase in pH, causing iron to become unavailable. Therefore we tried to substitute the inorganic iron of the W medium by Na_2Fe-EDTA as in the MS medium, but no growth was induced. When all trace elements of the MS medium were added to the W medium, the tissue did not grow either. Added to the WB medium, the same trace elements caused some increase in weight of the tissue.

Carbohydrates

Sucrose, glucose and fructose were added to the BM in three different concentrations. It was rather surprising that sucrose was the only sugar tested which could support the growth of the tissue (Table 2).

T a b l e 2. The effect of sucrose, glucose and fructose on the growth of the tissue

Sugar	Conc. %	mg f. w. per callus ± S. E.
Sucrose	2	268.3 ± 59.1
	3	417.0 ± 168.4
	4	284.6 ± 58.7
Glucose	3	42.9 ± 10.2
	5	29.9 ± 4.8
	7	18.4 ± 4.4
Fructose	3	40.2 ± 5.1
	5	31.4 ± 17.2
Glucose + fructose	2 + 2	37.7 ± 20.2

Vitamins

The basal medium contains thiamine, nicotinic acid and adenine. In order to test whether these vitamins were really necessary, the tissue was grown on media lacking one of them for several subsequent transfers. The results are shown in Table 3. As can be seen, tissues deprived of thiamine died in the third transfer, while without nicotinic acid their weight was very much decreased in the fifth transfer. Apparently, adenine was not needed, as the tissues could grow in its absence for five passages, with no sign of deficiency. An experiment was run through three transfers with 0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 10.0 mg l^{-1} of thiamine. Best growth was obtained with 1.0 and 3.0 mg l^{-1}, but all the other concentrations gave rather close values.

Table 3. The effect of thiamine, nicotinic acid and adenine on the growth of the tissue (in mg f. w. per callus ± S. E.)

Vitamin omitted from the BM	Transfers				
	1st	2nd	3rd	4th	5th
None	230.8±59.6	237.6±78.2	302.4±38.4	300.4±74.6	295.6±58.8
Thiamine	196.0±45.6	72.1±9.6	22.4±5.1	—	—
Nicotinic acid	285.5±74.0	189.1±84.5	102.6±22.9	79.2±44.7	87.4±46.9
Adenine	308.4±76.5	206.9±59.0	156.3±29.8	285.3±89.6	463.4±102.8

The addition of pyridoxine, pantothenic acid, biotin, riboflavin, inositol and folic acid or their mixture did not improve the growth of the tissue beyond that of controls. Ascorbic acid in concentrations of 1 and 10 mg l^{-1} produced an increase in tissue weight.

Growth substances

The Meteor callus tissue has an absolute requirement for an exogenous auxin. 2,4-D at 0.1 mg l^{-1} induced the optimal response, NAA was less effective, while most tissues were unable to use IAA as an exogenous auxin (Table 4).

Cytokinins were not necessary, although both kinetin and 6-BAP significantly improved growth. Optimal concentration was 0.2 mg l^{-1} (Table 4). Coconut milk (15%) was not superior to these substances. Yeast extract (1%) was beneficial to the tissue.

Gibberellins were not required either, but lower concentrations of GA_3 stimulated growth of the tissue on the BM (Table 4). No significant interaction between different hormones was found.

Discussion

The choice of culture media for Meteor pea callus is rather limited by its strict and uncommon nutritional requirements. It apparently has a need for high concentration of mineral salts. In that respect it is different from a callus of Alaska pea which has been maintained in our laboratory for several years on the T medium (Radojević, unpublished), as well as from pea tissues cultivated by other authors mentioned above. Although it is considered that W and T media contain suboptimal amounts of salts (Street, 1969), they still can be used to cultivate many tis-

Table 4. The growth of the callus tissue in the presence of different auxins, cytokinins and gibberellic acid (in mg f. w. per callus ± S. E.)

	Auxins, mg l^{-1}			
	0	0.01	0.1	1.0
2,4-D	21.3 ± 2.3	53.4 ± 13.9	312.0 ± 69.5	103.8 ± 21.8
NAA		18.0 ± 2.8	48.0 ± 4.9	179.2 ± 73.7
IAA		17.6 ± 4.4	34.9 ± 4.3	45.3 ± 8.8

	Cytokinins, mg l^{-1}			
	0	0.02	0.2	2.0
Kinetin	295.6 ± 58.8	542.3 ± 98.0	598.2 ± 99.8	473.6 ± 95.8
6-BAP		549.7 ± 137.7	484.8 ± 79.1	221.0 ± 67.7

	Gibberellins, mg l^{-1}					
	0	0.001	0.01	0.1	1.0	10.0
GA_3	313.5 ± 60.5	366.8 ± 94.4	649.8 ± 92.3	555.5 ± 77.2	425.3 ± 65.5	228.8 ± 61.6

sues. It is surprising that the H medium, which is much richer in all salts, does not support growth better than the former two. Experiments presented in Fig. 1 suggest that potassium has a particular role, since the beneficial effect of any other ion was evident only if the concentration of KCl was also increased. The comparison of macronutrient ions content in the media used, also shows that their ability to support growth may be due to a hisgh potassium content (12.99 mM l^{-1} in WB, 20.06 mM l^{-1} in MS solution), in conjunction to high levels of other ions, particularly nitrate and ammonium.

In experiments with normal tissue of *Vinca rosea* Braun and Wood (1962) showed that high levels of salts could substitute for a number of stimulating substances, such as cytidilic and guanilic acids, asparagine, glutamine, inositol and NAA. They assumed that ions were necessary to activate certain biosynthetic systems in the cells, leading to the elaboration of these substances, and that tissues, requiring more concentrated solutions, possessed lower membrane permeability. Another case of ion-activatable system was found in globular *Capsella* embryos Raghavan and Torrey, 1963), in which a need for certain growth factors (IAA, kinetin, adenine) could be partly substituted by high macronutrient salt concentration. It is possible that in Meteor callus tissue ions are also needed to activate some biosynthetic pathways, essential for growth. However, the attempts to identify these factors by supplementing the W and H media with various known stimulating substances were not successful. There are some indications that the Meteor pea tissue may have rather low membrane permeability. The particular need for potassium in conjunction with other ions could perhaps be explained by the role of potassium in increasing permeability and enabling the cells to take up other ions. There is only one case in which the Meteor tissue attained a higher weight without additional KCl (Fig. 1), when

$(NH_4)_2SO_4$, NaH_2PO_4 and $NaNO_3$ were added, but in this case the concentration of sodium was rather high (26.63 mM l^{-1}). According to Heller (1953), sodium can stimulate the growth of many tissues when the content of potassium is suboptimal, probably by replacing it partly in some non-specific function.

The response of the Meteor tissue to the three sugars tested is difficult to understand. It seems generally accepted that sucrose, glucose and fructose are the best carbon sources for the majority of cultivated tissues (Gautheret, 1959; Hildebrandt, 1962; Street, 1969). In that respect the Meteor pea tissue is similar only to isolated tomato roots (Dormer and Street, 1949), which cannot grow unless sucrose is supplied. A speculation was put forward, that in the tomato roots sucrose may partly serve as substrate for biosynthetic systems other than respiration, in which it is not replaceable by glucose (Thomas et al., 1963). Further work would be needed to check that hypothesis in the case of pea tissue.

Vitamins have been generally included into growth media, although in only few cases they were proved to be indispensable. The necessity of thiamine was shown for tobacco (Linsmaier and Skoog, 1965) and *Rumex* virus tumour tissue (Nickell, 1952), while its beneficial effect has been noted many times (Gautheret, 1959). Thiamine seems to be indispensable for the Meteor pea callus, in which deficiency develops slowly, but is invariably evident in the third transfer after thiamine is omitted. It cannot be said with certainty whether the same applies for nicotinic acid. It seems likely that nicotinic acid is synthesized by the tissue, only at a suboptimal level.

The requirement of the Meteor tissue for auxins was invariably shown in all experiments, as the tissues died immediately when subcultured on media lacking auxin. However, the synthetic auxins were markedly superior to IAA, which was actually shown in eight separate experiments during the three years. It is known that the intact dwarf pea plants possess higher peroxidase activity than the tall varieties (Galston and McCune, 1961). As peroxidase is involved in IAA oxidation (Pilet and Gaspar, 1969), it appears that this may be the cause of dwarfness in plants. It should be tested whether the isolated tissues of dwarf pea have retained the capacity of intact plants to destroy IAA.

The great sensitivity of intact Meteor pea plants to gibberellins and the specific action of gibberellins in reversing their dwarf growth habit is a well-known fact. Arney and Mancinelli (1966) presented evidence that the primary effect of gibberellins in the stem of Meteor pea is the stimulation of cell division and not of elongation, as it had been thought before. So the present work was started with the idea that this plant might give rise to tissue cultures which would be more susceptible to exogenous gibberellin treatment and suitable for studies of its action at the cellular level. However, this expectation has not proved correct, since the growth of Meteor callus tissue does not depend on gibberellins. GA_3-induced stimulation of this callus is comparable to its effect found in many other cultivated tissues.

The Meteor pea tissue can also grow without exogenous cytokinins. It probably has the capacity to synthesize its own cytokinins, as already shown for Alaska pea callus tissue (Short and Torrey, 1972). However, this capacity seems to be suboptimal, as the addition of cytokinins markedly stimulated the growth of the tissue. In order to improve its growth, kinetin, 6-BAP, coconut milk or yeast extract are equally well suited.

Summary

A callus tissue was obtained from an immature embryo of Meteor pea (*Pisum sativum* cv. Meteor) and its requirements for optimal growth were investigated. The tissue showed rather peculiar responses in respect to the concentration of mineral salts, to the carbohydrate used and to the kind of auxin supplied to the medium. Of the five mineral solutions tested, only two could support growth, in which the concentration of mineral salts was relatively high. It was not possible to show the specific role for any particular element. However, the stimulating effect of any ion was not evident, unless the content of potassium was increased at the same time. Sucrose was the best carbon source, while the tissue could not grow on media containing glucose or fructose. The addition of thiamine was necessary, while nicotinic acid had a beneficial effect. An absolute requirement for auxin was shown. Only the synthetic auxins could stimulate growth of the tissue, while IAA was almost ineffective. Cytokinins and gibberellic acid were not needed, but they considerably increased the weight of the tissue.

References

Arney, S. E. and *Mancinelli, P.*, 1966: The basic action of gibberellic acid in elongation of "Meteor" pea stem. New Phytol. *65*, 161—175.

Bailey, J. A., 1970: Pisatin production by tissue cultures of *Pisum sativum* L. J. Gen. Microbiol. *61*, 409—415.

Braun, A. C. and *Wood, H. N.*, 1962: On the activation of certain essential biosynthetic systems in cells of *Vinca rosea* L. Proc. natn. Acad. Sci., U.S.A. *48*, 1776—1782.

Dormer, K. J. and *Street, H. E.*, 1949: The carbohydrate nutrition of tomato roots. Ann. Bot. (N. S.) *13*, 199—217.

Galston, A. W. and *McCune, D. C.*, 1961: An analysis of gibberellin-auxin interaction and its possible metabolic basis. In Plant Growth Regulation, Iowa State Univ. Press, Ames, Iowa. 611—625.

Gamborg, O. L. Constabel, F. and *Shyluk, J. P.*, 1974: Organogenesis in callus from shoot apices of *Pisum sativum*. Physiol. Plant. *30*, 125—128.

Gautheret, R. G., 1959: La culture des tissus végétaux. Masson Ed. Paris.

Halperin, W., 1964: Morphogenetic studies with partially synchronized cultures of carrot embryos. Science *146*, 408—410.

Heller, R., 1953: Recherches sur la nutrition minérale des tissus végétaux cultivés in vitro. Ann. Sci. Nat. Bot. Biol. Vég. *14*, 1—223.

Hildebrandt, A. C., 1962: Tissue and single cell cultures of higher plants as a basic experimental method. In *Paech, K.* and *Tracey, M. V.* (eds.) Moderne Methoden der Pflanzenanalyse. *5*, 383—421. Springer Verl., Berlin.

Hildebrandt, A. C., Wilmer, J. C., Johns, H. and *Riker, A. J.*, 1963: Growth of edible chlorophyllous plant tissue in vitro. Am. J. Bot. *50*, 248—254.

Kallak, H. and *Yarvekylg, L.*, 1971: On the cytogenetic effects of 2,4—D on pea callus in culture. Acta biol. Acad. Sci. Hung. *22*, 67—73.

Linsmaier, E. M. and *Skoog, F.*, 1965: Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. *18*, 100—127.

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

Nickell, L. G., 1952: Vitamin B_1 requirement of *Rumex* virus tumor tissue. Bull. Torrey Bot. Club, *79*, 427—430.

Pilet, P. E and *Gaspar, T.*, 1968: Le catabolisme auxinique. Masson, Paris.

Raghavan, V. and *Torrey, J. G.*, 1963: Growth and morphogenesis of globular and older embryos of *Capsella* in culture. Am. J. Bot. *50*, 540—551.

Short, K. C. and *Torrey, J. G.*, 1972: Cytokinin production in relation to the growth of pea-root callus tissue. J. exper. Bot. *23*, 1099—1105.

Thomas, D. R., Craigie, J. S. and *Street, H. E.*, 1963: The carbohydrate nutrition of the excised tomato roots. In *Maheshwari, P.* and *Ranga Swamy, N. S.* (eds.): Plant tissue and organ culture — a symposium. Univ. Delhi. 26—43.
Torrey, J. G., 1967: Morphogenesis in relation to chromosomal constitution on long-term plant tissue cultures. Physiol. Plant. *20*, 265—275.
Torrey, J. G. and *Shigemura, Y.*, 1957: Growth and controlled morphogenesis in pea root callus tissue grown in liquid media. Am. J. Bot. *44*, 334—344.
White, P., 1943: A handbook of plant tissue culture. Ronald Press. N. Y.
Wood, H. N. and *Braun, A. C.*, 1961: Studies on the regulation of certain essential biosynthetic systems in normal and crown-gall tumor cells. Proc. natn. Acad. Sci., U. S. A. *47*, 1907—1913.

SADRŽAJ

NUTRICIONI I HORMONALNI FAKTORI RASTENJA KALUSNOG TKIVA GRAŠKA

Mirjana Nešković i Ljiljana Radojević

(Institut za botaniku, Prirodno-matematički fakultet
i Institut za biološka istraživanja, Beograd)

Kalusna kultura tkiva graška *(Pisum sativum* L. cv. Meteor) dobijena je od nezrelog embriona i ispitivani su faktori neophodni za njeno optimalno rastenje. Od pet mineralnih rastvora samo dva, koji imaju relativno visoku koncentraciju mineralnih soli, mogu da stimuliraju rastenje. Nijedan elemenat posebno nema u tome specifičnu ulogu. Međutim, stimulativni efekat bilo kog jona opaža se samo ako je u isto vreme i koncentracija kalijuma povećana. Najbolji izvor ugljenika je saharoza, dok na rastvorima sa glukozom ili fruktozom tkiva ne mogu da rastu. Vitamin B_1 je neophodan za rastenje, dok nikotinska kiselina ima povoljan efekat. Takođe je pokazano da je neophodno dodavati auksin. Međutim, samo sintetički auksini mogu da indukuju rastenje, a IAA je skoro sasvim neaktivna. Citokinini i giberelini nisu neophodni, ali znatno povećavaju težinu tkiva.

Dr Mirjana Nešković
Ljiljana Radojević mr biol.
Institut za botaniku PMF i
Institut za biološka istraživanja
Takovska 43
11000 Beograd