

Culture of Scots pine callus and its nutritional requirements

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The method of plant tissue culture is useful in solving problems regarding the physiology of growth. It is useful especially in the research of tree physiology, because trees are a difficult material for study owing to their great dimensions and longevity.

Gautheret (1934) in his early work on tissue culture already employed woody species. Simultaneously with the bringing of the method into general use, the number of various plant species maintained in tissue culture constantly increased. The culture of coniferous plant tissues, however, is not undertaken so often nor is it completely elaborated.

From cambial tissues isolated from trunks of 25 forest tree species Jacquot (1959) obtained viable callus cultures of only 7 species. The nutritional requirements of tissues in vitro culture as known up to 1958 were summarized by Gautheret (1959). Besides mineral salts and sugars the nutrient medium usually included vitamins and growth substances. Some workers (Hildebrandt et al. 1956; Bové and Morel 1957; Pelet et al. 1960; Mathes 1964), have grown tissues of various trees on media supplemented with natural products such as coconut milk, malt, yeast and casein hydrolysate.

It is generally recognized that representatives of the genus *Pinus* propagate vegetatively very poorly and it is difficult to maintain callus cultures of pine for indefinite periods on artificial media. However, tissues of *Pinus banksiana* and *P. strobus* (Lowenberg and Skoog 1952), *P. clausa* (Barnes and Naylor 1958), *P. serotina* (Barnes and Naylor 1959), *P. gerardiana* (Konar 1963) and *P. palustris* (Brown and Lawrence 1968) have been cultured with varying success.

The lack of data regarding the culture and nutritional requirements of Scots pine tissue, one of the most common tree species, was a stimulus to undertake this work.

MATERIAL AND METHODS

The pine tissue used in this work was isolated in the spring of 1967, although preliminary research was done on material isolated two years earlier. Young, one-year-old pine (*Pinus silvestris* L.) shoots sterilized by dipping in ethanol and then by flame were cut into 1 cm. sections and put on White medium (Murashige and Skoog 1962, p. 475). The callus which appeared on shoots placed on the medium with auxin was subcultured and thus a tissue culture was obtained. Pieces of callus weighing about 100 mg. were subcultured at four week intervals in test tubes with 20 ml. medium. White and Linsmaier and Skoog (1965) medium was used with a modification in its organic compounds. Inositol and thiamine concentration in the media used was the same as in the Linsmaier and Skoog medium. The sucrose level was lowered to 20 g. per liter and agar to 9 g. per liter. Indoleacetic acid (IAA) however was replaced by 2,4-dichlorophenoxyacetic acid (2,4-D) — 5 mg. per liter and kinetin was replaced by benzyladenine (BA) — 0.2 mg. per liter. The White medium was supplemented with casein hydrolysate (4 g/l).

The tissues were cultured at 25° under weak light of about 250 lux and in experiments on the influence of growth regulators in stronger light of about 3000 lux.

Each series comprised 10 tissues cultured in separate test tubes. After four weeks the fresh and dry weight of the tissues was determined. In experiments on the influence of growth regulators, the culture period was prolonged to 8 weeks. All experiments were repeated at least twice, with analogous results, with the exception of the experiment on the influence of various nitrogen sources (Table 1) which was not repeated.

RESULTS

During the past four years many attempts have been made by the author to grow callus cultures of Scots pine on various kinds of synthetic media. In this paper the results of the influence of various nitrogen sources, several cyclitols and growth regulators on the growth of this tissue are reported.

Influence of various nitrogen sources. In the initial cultures the White medium was used. The growth of the tissue on this medium was rather slow, therefore it was supplemented with casein hydrolysate. The results of one of the experimental replicates are presented in Figure 1. The tissues cultivated on acid casein hydrolyzate, showed better growth than those on enzymatic casein hydrolyzate and exhibited less growth inhibition at higher concentration. A distinct growth optimum occurred when

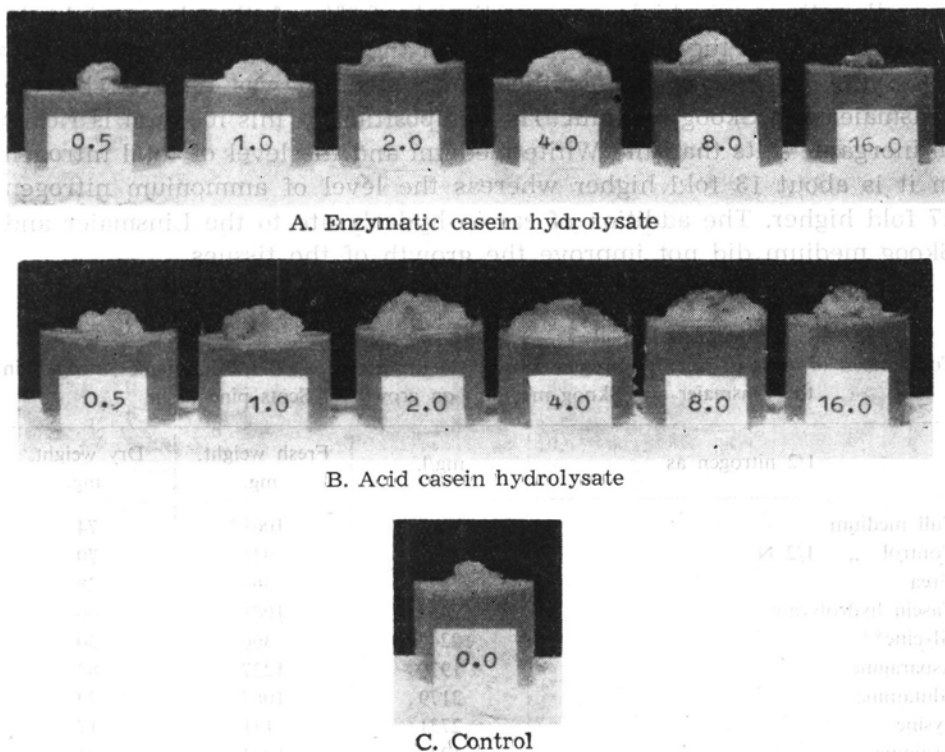


Fig. 1. Comparison of the effects of enzymatic and acid casein hydrolysates (g/l) on growth of Scots pine tissue cultivated on White medium

Fot. K. Jakusz

4 g/l casein hydrolysate was used. Attempts to eliminate the casein hydrolysate from the White medium showed that a good substitute for it were the following amino acids: arginine, glutamine, tryptophan and tyrosine. Applied separately instead of the amino acid mixture in a concentration of 0.5, 1.0, 2.0 and 4.0 mMoles, arginine and tryptophan could successfully replace the casein hydrolysate in the first two tyrosine in the first three and glutamine in all the concentrations tested. An increase of the inorganic nitrogen level to the concentration present in Linsmaier and Skoog medium strongly inhibited the growth of the tissues. The higher level of nitrate and ammonium salts added to White medium cannot replace the casein hydrolysate. The growth is determined not only by a high nitrogen level but also by its form and composition as well as by the relation of the individual compounds in the medium. As regards the tissue requirements for nitrogen nutrition, it seemed of interest to determine their nitrogen level. Parallel comparative analyses were carried out on tissues with intensive cell division, namely on September buds. It was found, that the total nitrogen level

in callus tissues is higher amounting to 6.3% of the dry weight in comparison with the 4.4% of nitrogen in the dry weight of buds.

In the latter experiments the White medium was replaced by the Linsmaier and Skoog medium. The composition of this medium is richer in inorganic salts than the White medium and the level of total nitrogen in it is about 13 fold higher whereas the level of ammonium nitrogen 27 fold higher. The addition of casein hydrolysate to the Linsmaier and Skoog medium did not improve the growth of the tissues.

Table 1

Comparison of various organic nitrogen sources (in the presence of 1/2 inorganic nitrogen in the Linsmaier and Skoog medium) on growth of Scots pine tissue

1/2 nitrogen as	mg/l.	Fresh weight, mg.	Dry weight, mg.
Full medium		1061*	74
Control „ 1/2 N		948	70
Urea	895	366	28
Casein hydrolysate	2458	1081	86
Glycine**	2242	366	30
Asparagine	1970	1227	82
Glutamine	2179	1067	79
Lysine	2731	131	17
Arginine	1242	1094	74
Phenylalanine	4926	163	17
Tyrosine	5404	215	27
Tryptophan	6091	186	30

* The fresh weight of the tissue before planting was about 100 mg.

** All amino acids were of L configuration.

In Table 1 the results of studies on the influence of various nitrogen sources on the growth of the tissues are presented. Their influence was tested by decreasing the nitrogen level in the Linsmaier and Skoog medium to one half, replacing the other half of the nitrate and ammonium nitrogen by an equivalent amount of organic nitrogen. As seen from the table, the lowering the nitrogen level to one half caused an insignificant decrease in tissue growth. Replacement of half of the nitrogen included in the Linsmaier and Skoog medium by an equivalent amount of nitrogen in the form of casein hydrolysate did not cause better growth of the tissues than was observed on a full Linsmaier and Skoog medium. From among the amides, asparagine was found to be a better nitrogen source than glutamine. As regards basic amino acids however only arginine was suitable for the tissues. Urea, glycine and the aromatic amino acids: phenylalanine, tyrosine and tryptophan tested in equivalent nitrogen concentrations strongly inhibited growth. It is interesting to note, that of the amino acids tested asparagine gave the best effects.

similarly as was reported by Brown and Lawrence (1968) who included it in their medium for *P. palustris*.

The problem of nitrogen nutrition of Scots pine tissue is very interesting and demands further detailed studies.

Table 2

Influence of cyclitols on growth of Scots pine tissues cultivated on White medium with the addition of 4 g./l. acid casein hydrolysate

Cyclitols, mM	Fresh weight, mg.	Dry weight, mg.
0	234	28
0.125 pinitol	179	21
0.250	192	23
0.500	164	22
1.000	155	20
0.125 quinic acid	277	31
0.250 „ „	224	26
0.500 „ „	230	27
1.000 „ „	341	36
0.125 shikimic acid	351	39
0.250 „ „	353	39
0.500 „ „	320	36
1.000 „ „	333	38
0.125 myo-inositol	1135	78
0.250 „ „	1153	79
0.500 „ „	1153	78
1.000 „ „	1118	77

Influence of cyclitols. The experiments on the influence of several cyclitols: myo-inositol, pinitol, and quinic and shikimic acid showed, that myo-inositol added to the White medium assured the best growth of the tissues. As seen from Table 2 myo-inositol with in the range of concentrations tested (0.125 — 1.0 μ M) caused an about 5-fold increase in tissue weight in comparison with the control. Pinitol, however, gave only inhibitory effects in all the concentrations tested, quinic acid was without major influence and was stimulatory only at a higher concentration. The growth of the tissues on shikimic acid was by about 50% better than that of the control tissues at all concentrations tested. The above effects of cyclitols were obtained on a White medium enriched with 4 g/l of casein hydrolysate. It is interesting to note the failure of the cyclitols to stimulate spruce tissue growth on media enriched with casein hydrolysate as was found by Steinhart et al. (1962).

Influence of growth regulators. In experiments on the influence of growth regulators on pine tissue growth, the culture time was prolonged to 8 weeks and the tissues were cultivated under light of 3000 lux. There was a distinct decrease in tissue yield in these conditions and the

tissues were more compact and green in colour. However in order to provide optimal conditions for differentiation, the tissues were cultivated in light, which as is known stimulates differentiation.

In the preliminary experiments conducted in the spring of 1965 on the influence of auxins on the growth of pine explants it was found that NAA stimulated proliferation more than IAA, and 2,4-D more than NAA. A similar effect of auxins on the callus tissues was noted. Therefore in further transfers, the tissues were cultivated on media with 2,4-D.

Table 3

Influence of auxins and benzyladenine on growth of Scots pine tissues cultivated on Linsmaier nad Skoog medium

μM		BA							
		0		1		5		25	
		f.wt. mg.	d.wt. mg.	f.wt. mg.	d.wt. mg.	f.wt. mg.	d.wt. mg.	f.wt. mg.	d.wt. mg.
IAA	0	240	37	230	35	235	36	220	34
	1	231	36	284	38	250	32	224	27
	5	223	37	320	44	280	42	275	41
	25	260	41	260	33	270	34	258	32
NAA	0	262	40	244	37	271	41	248	37
	1	313	47	285	44	313	45	253	37
	5	408	58	421	60	351	52	319	47
	25	652	77	677	80	751	86	953	93
2,4-D	0	254	40	250	39	236	39	244	38
	1	340	56	326	52	302	49	327	50
	5	1130	85	1202	103	1157	92	842	86
	25	1249	94	1119	89	1020	86	1491	99

In Table 3 the influence of auxins and their interaction with benzyladenine on the growth of the tissues cultured on the Linsmaier and Skoog medium is presented. As indicated by the results presented in the table, IAA alone caused an insignificant intensification in the growth of the tissues, whereas in interaction with BA the growth effect was somewhat better. NAA stimulated the growth of the tissues more and showed a stronger activity in combination with BA. Of the three 2,4-D was the most active auxin and its interaction with BA was most effective.

During the 8 week culture period no organogenetic differentiation of the tissues was obtained under the influence of the growth regulators tested. Anatomical studies of the material fixed will make it possible to reveal whether histogenetic differentiation has occurred.

The investigations showed that 2,4-D is in Scots pine tissue, similarly

as in many other plants, the most effective of the auxins tested. This applies both to explants and to callus tissues. In the presence of BA alone the tissues showed very poor growth. It could be that the callus tissue synthesized cytokinins in sufficient amounts. The occurrence of endogenous cytokinins in various tissues and organs of Scots pine has been demonstrated (Rogozńska 1967; Rogozńska and Legocki 1969). The addition of BA to the medium containing an auxin, intensified the growth of the tissues.

Table 4
Influence of gibberellic acid on growth of
Scots pine tissues cultivated on Linsmaier
nad Skoog medium

mM GA ₃	Fresh weight, mg	Dry weight, mg
0	1270	77
10 ⁻⁶	1241	71
10 ⁻⁵	1268	73
10 ⁻⁴	1082	68
10 ⁻³	195	21

The experiments dealing with exogenous gibberellic acid showed, that it does not play any important role in the growth of the callus tissue of Scots pine. The highest of the concentrations used was inhibitory to the tissues (Table 4).

DISCUSSION

Investigations were carried out on the nutritional requirements of Scots pine tissue for culture in vitro. In preliminary experiments White medium was applied with the addition of casein hydrolysate. In further investigations Linsmaier and Skoog medium was adopted. This medium worked out especially for tobacco tissue is suitable for tissue culture of many plants, and with some modification also for Scots pine tissue. The growth rate of this tissue is, however, not as fast as that of herbaceous plants for which the media were specially devised, they assure, however, a continuous growth of the tissues.

The results of experiments on the influence of individual amino acids on Scots pine tissue were similar to those obtained by other investigators with tissues and organs of various plant species. The best growth effects were obtained on the Linsmaier and Skoog medium by adding: asparagin, glutamin and arginine. According to Bollard (1959) the arginine effect may be attributed to the guanidine group which may be more readily

utilized by the tissues than the alfa-amino group of most amino acids. The aromatic amino acids applied in equivalent nitrogen concentrations inhibited tissue growth. However, when series of concentrations were applied to the White medium, stimulatory effects with tryptophan and tyrosine were obtained. Glycine and lysine inhibited growth of the tissues probably by blocking various metabolic processes. Inhibitory effects of glycine on the growth of *P. palustris* were reported by Brown and Lawrence (1968). As results from the experiments on nitrogen nutrition, Scots pine tissue requires the bulk of nitrogen in the form of simple inorganic compounds or some other more complex ones.

The role of inositol phosphates as a form of storage of phosphorus and that of inositol phospholipids as structural components of cells is known. Probably cyclitols present as free molecules also have some function in the cell. Some tissues do not require the addition of exogenic cyclitols, in other tissues cyclitols stimulated growth, for still other cyclitols are indispensable. To the latter group belongs the tissue of *Fraxinus* (Wolter 1966) and of the Scots pine tested here. In spruce tissue the myo-inositol enhanced growth by 15—30% (Steinhart 1962). Pinitol, present in some coniferous plants inhibited the growth of Scots pine tissue. On the other hand, in spruce tissue it showed a stimulatory effect, though lower than did myo-inositol (Steinhart 1962). The data reported in this paper demonstrated that Scots pine tissue requires addition of myo-inositol to the medium, and of the three cyclitols tested none could replace it satisfactorily.

The reaction of Scots pine tissue to the three auxins tested is in general agreement with the observations carried out on tissues of other plants. The addition of benzyladenine to the medium containing an auxin enhanced growth, similarly as it was shown for tissues of other woody species (David 1965; Wolter 1966; Rogozińska 1967).

It is not known whether the tissue growth could be further improved by changing the quantitative ratio of organic and inorganic compounds, or by adding specific growth factors, or else by improved external factors such as light, temperature and others. Further research on factors which control the growth of the callus tissue may contribute to the solution of some practical problems regarding vegetative propagation of species rooting with difficulty.

SUMMARY

Isolated callus tissue of *Pinus silvestris* L. has been successfully grown for two years on modified White and Linsmaier and Skoog media. The addition of casein hydrolysate to the Linsmaier and Skoog medium did not improve the tissue growth as it did in the case of the White medium. Casein hydrolysate in the White medium could be satisfactory replaced by some amino acids. By

lowering to one half the inorganic nitrogen level (in the Linsmaier and Skoog medium) and by replacement of the other half with organic nitrogen, a positive effect of asparagine, glutamine, arginine and casein hydrolysate was found. The effects of glutamine, arginine and casein hydrolysate, however, did not exceed those obtained on a full Linsmaier and Skoog medium containing an inorganic source of nitrogen only in the form of ammonia and nitrate salts. Urea, glycine, lysine and aromatic amino acids strongly inhibited the growth of the tissues.

Of the four cyclitols tested myo-inositol was the most active and of the three auxins 2,4-D. It was found that myo-inositol and auxin were essential and that benzyladenine improved the yields.

Gibberellic acid has no stimulatory effects on the growth of Scots pine tissue, under the conditions of the experiments.

On the modified White and Linsmaier and Skoog media the tissues increased their weight about tenfold during the four week culture period, and less so under strong light which inhibited growth of the tissues.

In conclusion, although the growth of the tissues is satisfactory with both media, the author prefers the Linsmaier and Skoog medium because it does not require the addition of organic nitrogen.

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Hodowla i wymagania odżywcze kalusa sosny zwyczajnej

Streszczenie

Wyizolowano tkankę *Pinus silvestris* L., którą udało się utrzymać w trwałej hodowli na zmodyfikowanej pożywce White'a oraz Linsmaier i Skooga. Dodany do pożywki Linsmaier i Skooga hydrolizat kazeiny nie wywierał wpływu stymulującego na wzrost tkanek jak w przypadku pożywki White'a. Hydrolizat kazeiny w tej ostatniej mógł być z powodzeniem zastąpiony niektórymi aminokwasami. Przy obniżonym do połowy poziomie azotu nieorganicznego (w pożywce Linsmaier i Skooga) i zastąpieniu drugiej połowy azotem organicznym stwierdzono dodatni wpływ asparaginy, glutaminy, argininy i hydrolizatu kazeiny. Efekty glutaminy, argininy i hydrolizatu kazeiny nie przewyższały jednak efektów otrzymanych na pełnej pożywce Linsmaier i Skooga zawierającej tylko nieorganiczne źródło azotu w formie soli amonowych i azotanowych. Mocznik, glicyna, lizyna oraz aminokwasy aromatyczne silnie hamowały wzrost tkanek.

Z badanych czterech cyklitolii najaktywniejszy był myo-inozytol, a z trzech auksyn 2,4-D. Stwierdzono, że myo-inozytol i auksyna są niezbędne i że benzyloadenina poprawia wzrost tkanek.

Kwas giberelowy nie wywierał wpływu stymulującego na wzrost tkanki sosny zwyczajnej.

Na zmodyfikowanej pożywce White'a oraz Linsmaier i Skooga tkanki zwiększały swą masę około 10-krotnie w czasie czterotygodniowego okresu hodowli, mniej na silnym świetle, które hamowało ich wzrost.

Tkanki rosły dobrze na obu pożywkach jednak odpowiedniejszą wydaje się pożywka Linsmaier i Skooga, która nie wymaga dodania azotu organicznego.