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Metabolism of excised embryos of *Lupinus luteus* L.

III. Comparative study of cultured embryos and normal seedling axes

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Many times it was proved that lupin and other plant embryos decotylised and cultured on synthetic media in vitro develop at a much lower rate than germ axes in situ (F r i e s 1960; in our laboratory too — unpublished data). It was also reported (C z o s n o w s k i 1962) that very early decotylised embryos behave in their osmotic relations quite differently from germ axes united with cotyledons for some time after presoaking. The present announcement gives results of analyses of a few morphological and metabolic parameters, peculiar to the early developmental phases of excised embryos cultured in vitro, and of normal germ axes in yellow lupin.

MATERIAL AND METHODS

Seeds of *Lupinus luteus* var. "Express" originated from the Research Centre of the Institute of Soil Science, Plant Cultivation and Manuring at Przebędowo. A biometric analysis revealed that the majority of seeds fall in the weight range of 120—130 mg. Experiments were performed on seeds of this grade. Seedlings were grown from seeds unsterilized on the surface. A three-day germination on blotting paper soaked with distilled water was continued in plastic germinators with distilled water. The cultures were carried on for 12 days from the seed submersion.

Cotyledonless embryos were taken from seeds sterilized with ethanol and a 0,2 per cent solution of mercuric chloride, after 12 hrs. presoaking in sterile distilled water. They were placed on a sterile agar medium in culture test-tubes.

The medium was composed of:

H e l l e r's solution with trace elements (1954)	1000 ml
Saccharose	30 g
Agar	9 g

Cultures of whole seedlings and of excised embryos were carried out according to two variants:

a) in darkness

b) under continuous fluorescent light (1600 lux). In the latter variant the culture tubes with embryos were inclined at an angle of 45° in order to avoid shading by the cotton plugs. Samples for measuring the lenght and for chemical analyses were taken as follows:

Presoaked seeds after 12 hrs.; seedlings and excised embryos after 3, 7 and 12 days' culture. When the length had been measured seedlings and embryos were dissected into separate organs (root, hypocotyl, cotyledons and epicotyl — the latter if large enough to isolate). Next the fresh matter was weighed and the plant material dried at 80°C.

Dry, ground material was analysed for nitrogen fractions and sugars. Total nitrogen was determined according to the micro-Kjeldahl's method.

Another portion of the dry material was extracted with water on a boiling water-bath for 15 min., and filtered after cooling. The filtrate divided into a few samples was analysed for soluble nitrogen (micro-Kjeldahl), and for the sum of all sugars soluble in the given conditions of extraction. Sugars were determined by the anthrone method. Under our conditions the following operational procedure provided by far the best results: 9 ml of an anthrone reagent is cooled in a test tube in an ice-bath. 1 ml of a sugar solution is added and mixed rapidly; the culture tube is then placed immediately back in the cold bath. When all samples were treated in this manner they were placed at once in a vigorously boiling water-bath. After exactly 10 min. they were immediately transferred back into the ice-bath again. The optical density was read at 620 mμ. The anthrone reagent: 200 mg of anthrone is dissolved in 100 ml sulphuric acid (500 ml H_2SO_4 conc. + 200 ml H_2O).

In view of the applicability of the anthrone method for all sugars soluble under conditions of a 15 min. extraction with water at 100°C — the analytical data are given in micrograms of "glucose" used for plotting the standard photometric curve.

RESULTS

In this part of the paper germ axes are indicated by "*A*", while the excised embryos cultured in vitro by "*B*". The same indications are used in the graphs.

1. Growth in length (Fig. 1 *A.* and *B.*).

A. Growth in light, in particular in darkness, naturally very intensive. Under both luminous conditions the first epicotyl leaves grew rapidly. In the dark, the root stops becoming longer on the 7th day.

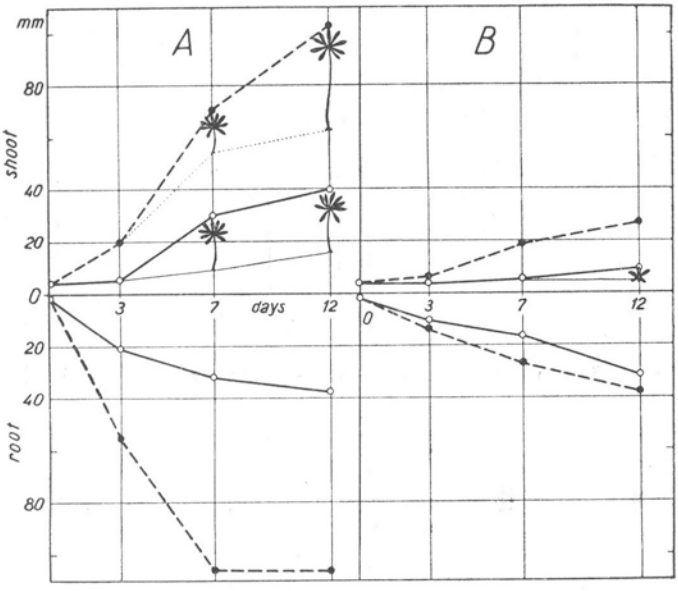

Fig. 1. Increase in length of normal seedling axes (*A*) and excised embryos cultured in vitro (*B*)

Above „O": shoot system, below „O": root system. Solid line — cultures in the light, broken line — cultures in the dark. (The silhouttes represents the length of young leaves emerging from a very short epicotyl)

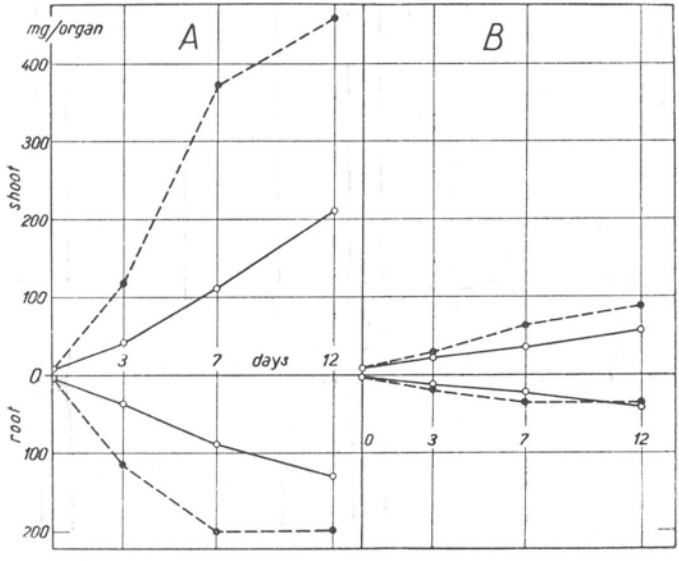

Fig. 2. Fresh weight (mg per organ) of normal seedling axes (*A*) and excised embryos cultured in vitro (*B*). (For further explanation see Fig. 1)

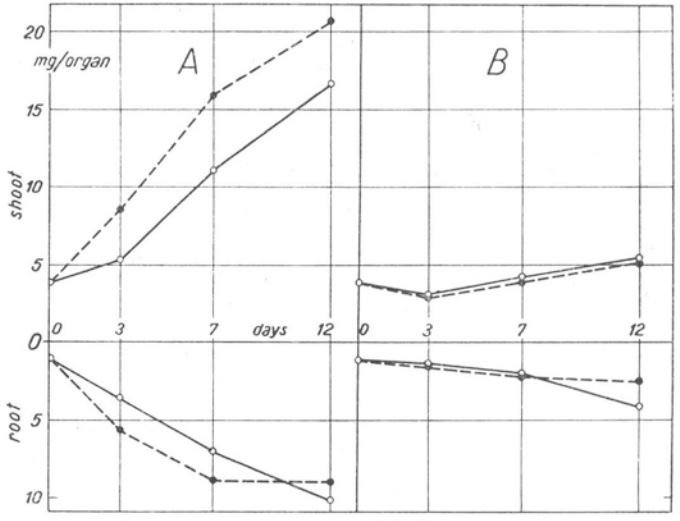

Fig. 3. Dry weight (mg per organ) of normal seedling axes (*A*) and excised embryos cultured in vitro (*B*). (For further explanation see Fig. 1)

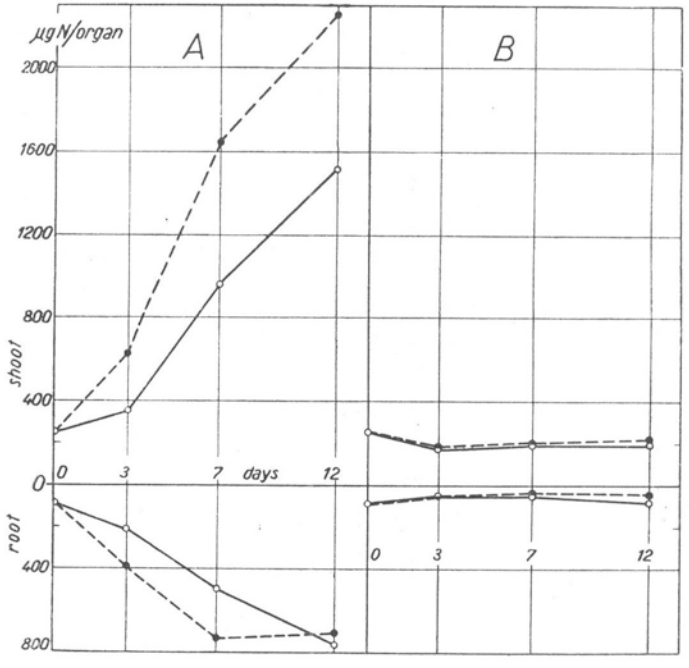

Fig. 4. Total nitrogen (µg per organ) in normal seedling axes (*A*) and in excised embryos cultured in vitro (*B*). (For further explanation see Fig. 1)

B. Growth much weaker than in *A.* Root, both in the light and in darkness, shows no delay in growth.

2. Fresh matter (Fig. 2, *A* and *B*).

A. In the light, continuous increase in amount. In darkness, at first rapid increase, the rate of which however falls. The root under the experimental conditions only grows until the 7th day.

B. Increase in amount of fresh matter very poor in comparison with *A.* Nevertheless, as in *A*, the root fresh matter remains on the same level from the 7th day in darkness.

3. Dry matter (Fig. 3, *A* and *B*).

A. Increase much greater than in *B.*

B. In the 3rd day the quantity of dry matter is smaller than at the beginning of the experiment.

This phenomenon is possibly in part due to exodiffusion (Fries and Forsman 1951). Later the dry matter increase is greatly dependent on the uptake of sucrose from the medium.

4. Total nitrogen (Fig. 4, *A* and *B*).

A. Rapid increase, both in the light and in darkness; nevertheless the nitrogen level in the root slightly falls from the 7th day of growing in distilled water.

B. After 3 days of growth the total nitrogen level falls — probably because of an exodiffusion into medium. Later, a very limited uptake of nitrogen from the medium takes place; in the light, the root draws more than the stem, in the dark the situation is reversed. After 12 days of growth the total nitrogen level per embryo is lower than at the beginning (at the moment of embryo introducing into the culture medium). Embryos cultured on a medium (for composition see above) draw nitrogen from it with great difficulty, so that their economy in respect to this element is on a "starvation" level.

5. Protein nitrogen (Fig. 5, *A* and *B*).

A. This form of nitrogen greatly increases in amount; only roots in darkness start loosing it from the 7th day (culture on distilled water).

B. Similarly to total nitrogen the level of protein nitrogen definitely falls after 3 days of growth. Later the level rises — in darkness slowly, in the light slightly faster; after 12 days the initial level is barely reached.

6. Soluble sugars. (Fig. 6, *A* and *B*).

A. In the dark, until the 3rd day sugars travel rapidly from the cotyledons; later the movement gradually slows down. In the light, at first sugars slowly increase in amount (at a lower rate than in darkness) as the photosynthetic apparatus in the shoot system develops the rate of increase rises.

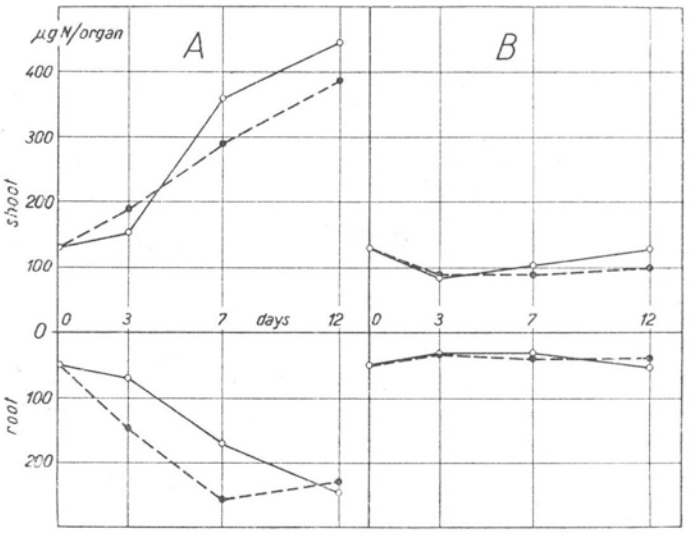

Fig. 5. Protein nitrogen (µg per organ) in normal seedling axes (*A*) and in excised embryos cultured in vitro (*B*). (For further explanation see Fig. 1).

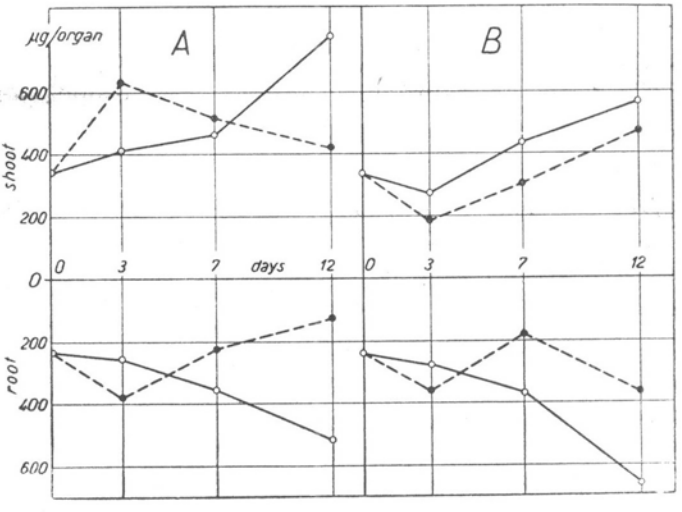

Fig. 6. Soluble sugars (µg per organ) in normal seedling axes (*A*) and in excised embryos cultured in vitro (*B*). (For further explanation see Fig. 1).

B. After a three-day culture in the light, the level of sugars continually rises. In darkness, the sugar level rises visibly only for 7 days from the culture initiation. On the 12th day the total amount of sugars per embryo exceeds, the initial amount, thus indicating a rapid uptake of sucrose from the medium.

In general, it was found that the level of soluble sugars was more or less the same in germ axes and cultured embryos. Owing, however,

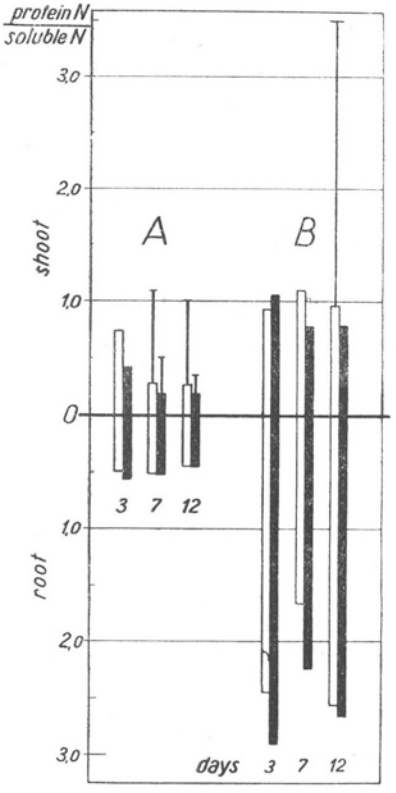

Fig. 7. *A* — $N_{protein}/N_{soluble}$ ratio in normal seedling axes after 3, 7 and 12 days of culture. *B* — $N_{protein}/N_{soluble}$ ratio in excised embryos after 3, 7 and 12 days of culture in vitro. Above "O" level: shoot system, below "O": root system. The "flagstaffs" indicate the $N_{protein}/N_{soluble}$ ratio in young epicotyl leaves

White columns — light cultures; black columns — dark cultures

to a slow growth rate and low level of fresh and dry matter in cultured embryos, the concentration of sugars in their tissues is much higher than in germ axes.

7. Protein nitrogen to soluble nitrogen ratio (Fig. 7, *A* and *B*).

A. The ratio of protein nitrogen to soluble nitrogen ($N_{prot.}/N_{sol.}$) fluctuates at about 0.5 in roots (during a 12 days' culture) — both in the light and in darkness. In hypocotyls, as the time goes on the ratio in question falls, remaining only slightly higher in the light. The $N_{prot.}/N_{sol.}$ ratio in young leaflets fluctuates in the light at about 1.0, while in darkness between 0.35 and 0.50.

B. Here the ratio of these forms of nitrogen is much higher than in germ axes. In roots of cultured embroys it was over 2.0, in the hypocotyl — about 0.9. These values indicate an equilibrium in favour of a protein synthesis. A highest ratio of 3.5 was found in young epicotyl leaflets.

DISCUSSION

The above given experimental data provide evidence in favour of the following situation: embryos detached from the cotyledons and cultured on H e l l e r 's medium containing, sucrose reveal — in comparison with normal germ axes cultured on distilled water — an unexpectedly low value of all examined parametres except of soluble sugars (sucrose in the medium). Nitrogen metabolism is extremely low: after a 12 days' culture the levels of total and protein nitrogen scarcely amount to the initial value at the time of separating from the cotyledons. Excised embryos take up nitrogen from the medium at a very slow rate. Sucrose, on the contrary, is absorbed very rapidly and sugar concentration in tissues of excised embryos is much higher than in germ axis tissues in situ.

On grounds of this situation, high $N_{protein}/N_{soluble}$ ratios in excised yellow lupin embryos and low ratios in the germ axes are considered an interesting phenomenon (Fig. 7). Excised cultured embryos are provided with sugars abundantly, while the amounts of taken-up nitrogen are very limited. There is an evident correlation of the latter with the metabolic equilibrium changing towards protein synthesis. Nevertheless, absolute quantities of proteins in cultured embryos are several times lower than in germ axes. All these findings indicate a difference between excised embryos and germ axes in their metabolism over the 12-day period of culture. Cotyledons seem not only to transmit storage materials to the germinating axial organs but also to exhibit a specified dynamics in putting them in motion. It is not only a question of the quality and quantity of food, but also a problem of sequence in which they are passed to the growing portions of the seedling. When compared with the very active cotyledon metabolism a synthetic medium is a static and inactive arrangement in respect to the cultured embryo.

In some cases it is easy to obtain full growth in a plant from an embryo excised from a mature seed and cultured on a simple, synthetic medium (e.g. in radish — unpublished data of our laboratory). One can also promote a full growth in cultured, immature embryos of e.g. rice — also on inorganic media with glucose (B o u h a r m o n t 1961; here further references). Very often however, it is very difficult to

breed a plant fit for further growth in soil — starting from excised embryos. It is difficult even when media are strengthened by a supply of e.g. casein hydrolizate, nucleic acid derivatives, vitamines or growth substances — as was the case in yellow lupin. There must, therefore, exist plants whose embryos, even when immature, exhibit a considerable metabolic independence. In other plants, a substitution of developing organs of the seed or of cotyledons by an inert medium, lacking metabolic potentialities, is always a drawback. Possibly, long-term experiments aimed at promoting a full plant growth, initiated in an excised embryo with a limited metabolic independence, should be preceeded by comparative analyses of growth and of certain metabolic aspects of embryos cultured in vitro and normal seedlings in situ. Results of such trials could provide grounds for preparing media imitating conditions in developing fruits or in active cotyledons. It may e.g. be necessary to change, in some cases, the qualitative composition of the medium, the concentration of its components, physical properties like the osmotic value, light conditions etc. during the time of culture. This kind of experimental work, together with a penetrating analysis of the influence of trifle changes in the chemical equilibrium in media (F r i e s 1954, 1960) should be of interest not only for physiologists but for geneticists and breeders as well.

SUMMARY

1. Measurements of increase in length, in fresh and dry matter amount, analyses of total, protein and soluble nitrogen as well as of soluble sugars were carried out; *A* — in germ axes of yellow lupin (cultured on distilled water). *B* — in cotyledonless embryos cultured on H e l l e r's medium. The cultures were maintained for 12 days — in the light and in darkness.

2. Excised embryos grew at a much slower rate than germ axes; the fresh and dry matter were on a much lower level. The content of various forms of nitrogen per organ in excised embryos was the same after 12 days of growth as at the time when they were isolated from soaked seeds. The nitrogen uptake from the medium by embryos was very limited. On the other hand sucrose was drawn rapidly and the sugar concentration in tissues of cultured embryos was much higher than in seedling tissues.

3. The $N_{protein}/N_{soluble}$ ratio in cultured excised embryos is much higher than in germ axes.

4. It is concluded that the metabolism of excised embryos during the 12 days culture is very different from the metabolism of germ axes in situ. The results are discussed.

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