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Metabolism of excised embryos of *Lupinus luteus* L.

IV. *In vitro* growth as compared with *in vitro* growth of germ axes decotylized at different phases of germination

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Comparative studies on the development of excised (cotyledonless) embryos of yellow lupin *in vitro* and the growth of germ axes *in situ* carried out so far found excised embryos cultivated on Heller's medium for 12 days to develop symptoms of starvation (Czosnowski 1962). The metabolically inactive medium fails to replace the dynamic action of normal cotyledons. In order to gain knowledge on the axis-cotyledon metabolic interrelations the following experiment was designed: primarili axes with cotyledons are cultured on distilled water; later they are decotylized at different periods and planted on Heller's medium with 3% sucrose (Heller 1954). It is assumed that the longer a period of contact between axis and cotyledon, the more factors conditioning normal growth will influence the axis. Further development of germ axes isolated from cotyledons at different phases of germination, is expected to provide some light on the metabolic action of cotyledons on them.

Lengthwise increase, the rise in fresh and dry matter of organs and the changing levels of the main nitrogen fractions were established as the arbitrary criteria for estimating the metabolic rates. Further, detailed comparative analyses of metabolic processes in germ axes during early stages after germination, and in cotyledonless embryos cultured in vitro are left for future research along this line. The experimental design is illustrated in Fig. 1. and discussed below.

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. All experiments were carried out under continuous fluorescent light (1600 lux) at 24°C.

Experiments

D a y 0. Seeds of the weight range of 120—130 mg were sterilized by a 1 min. immersion in 95% per cent ethanol followed by a 20 min. immersion in 0.2 per cent mercuric chloride. Several times rinsed in sterile distilled water, the seeds were placed on moistened filter paper in Petri dishes.

D a y 1. Cotyledonless embryos were excised from part of the seeds and placed on Heller-sucrose agar medium. The culture lasted 25 days. Analyses were made after 4, 10, 17 and 25 days (series *A* on graphs 1—8). Samples for analyses were taken from excised embryos (control, series *F* on graphs 1—8).

D a y 3. The water grown seedling axes were decotylized and planted on Heller-sucrose agar medium. The culture lasted 21 days. Analyses were carried out after 5, 10, 16 and 23 days (series *B* on graphs 1—8). Control samples were taken from 3-day-old seedlings. Henceforth the seedling culture was transplanted from Petri dishes into test tubes containing distilled water and filter-paper discs as a support.

D a y 5. Water-cultured seedling axes were decotylized and placed on Heller-sucrose agar medium. The culture lasted 21 days. Analyses were carried out after 5, 10, 15 and 21 days (series *C* on graphs 1—8). Control samples were taken from 5-day-old seedlings.

D a y 8. Seedlings axes grown on distilled water were decotylized and planted on the Heller-sucrose agar medium. The culture lasted

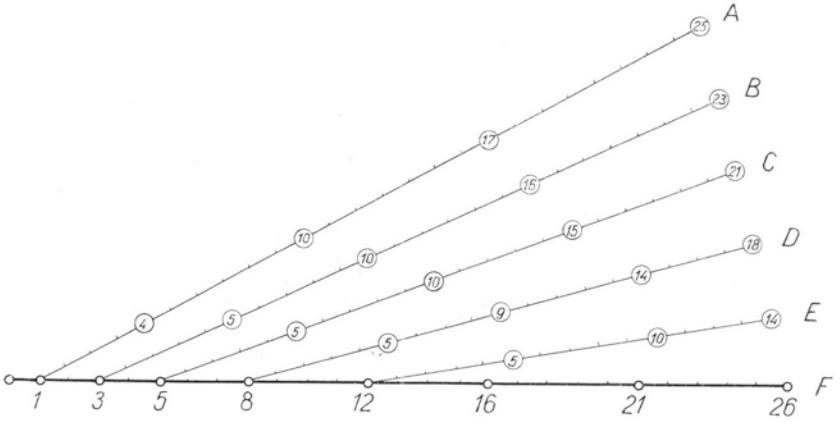

Fig. 1. Diagrammatic representation of the experimental procedure.

Horizontal line (indicated by the letter *F*) — control seedlings cultured for 26 days in distilled water. Slanting lines (*A*—*E*) — experimental series including embryos decotylized after 1 day of seed swelling (*A*), germ axes decotylized after 3, 5, 8 and 12 days (*B*, *C*, *D*, *E*) and cultured on Heller's medium. The figures in the graph indicate days of control culture, or culture on Heller's medium, at which samplings were made. For details see text.

18 days. Analyses took place after 5, 9, 14 and 18 days (series *D* on graphs 1—8). Control samples were taken from 8-day-old seedlings.

Day 12. Water-grown germ axes were decotylized and planted on Heller-sucrose agar medium. The culture lasted 14 days. Analyses took place after 5, 10 and 14 days (series *E* on graphs 1—8). Control samples were taken from 12-day-old seedlings.

Day 16. Control samples were taken from 16-day-old seedlings.

Day 21. Control samples were taken from 21-day-old seedlings.

Day 26. Control samples were taken from 26-day-old seedlings.

Analytical methods

Fresh matter and growth of individual organs were determined immediately after removal from the medium, before freeze-drying; subsequently the dry matter was determined. Analyses for total nitrogen (without NO_3^- were carried out according to Kjeldahl's micromethod, while analyses for soluble nitrogen and those for the others soluble fractions wer performed as follows: 50—60 mg of dry matter was treated with 10 ml H_2O, steam-bathed for 15 min., filled up to 25 ml with 95 per cent ethanol and centrifuged; the supernatant was kept for analyses. Soluble nitrogen was determined in 5 ml samples acc. to Kjeldahl's method.

RESULTS

Length increase (Fig. 2)

Germ axes decotylized after 1, 3 and 5 days (*A*, *B* and *C*) develop on medium in much the same way; the different elements reach more or less the same final sizes. Epicotyls grow steadily to a height of 30—40 mm on the 26th day. Hypocotyls, after 10 days, stop increasing in length and remain at that level until the end; in all three cases the situation is similar. Roots grow steadily until the 20th day; from then onwards the rate of length increase drops.

Germ axes decotylized after 8 and 12 days (*D* and *E*) develop on media much more abundantly than seedlings separated earlier. In the first place, epicotyls and roots attain after 26 days the standard size, much exceeding that in cases *A*, *B* and *C*. Hypocotyls of axes decotylized after 12 days are twice the size of those in cases *A*, *B* and *C* and nearly the control size. Non-decotylized seedling axes grown in distilled water (*F*) are more exuberant than decotylized axes transplanted on media — whichever the time of separation. During the first 12 to 13 days the entire seedling grows rapidly; later the growth rate falls to the level of embryos isolated after 1, 3 and 5 days.

In general, during the first 12 days non-decotylized seedlings exhibit a highest growth rate. Decotylizing and transplantation into media, mainly when performed until the 8th day, brings down the growth rate

of axes. Axes separated from cotyledons after the 8th day grow at first more slowly than control seedlings of the same age; later they grow more rapidly than control plants and reach their size on the 26th day.

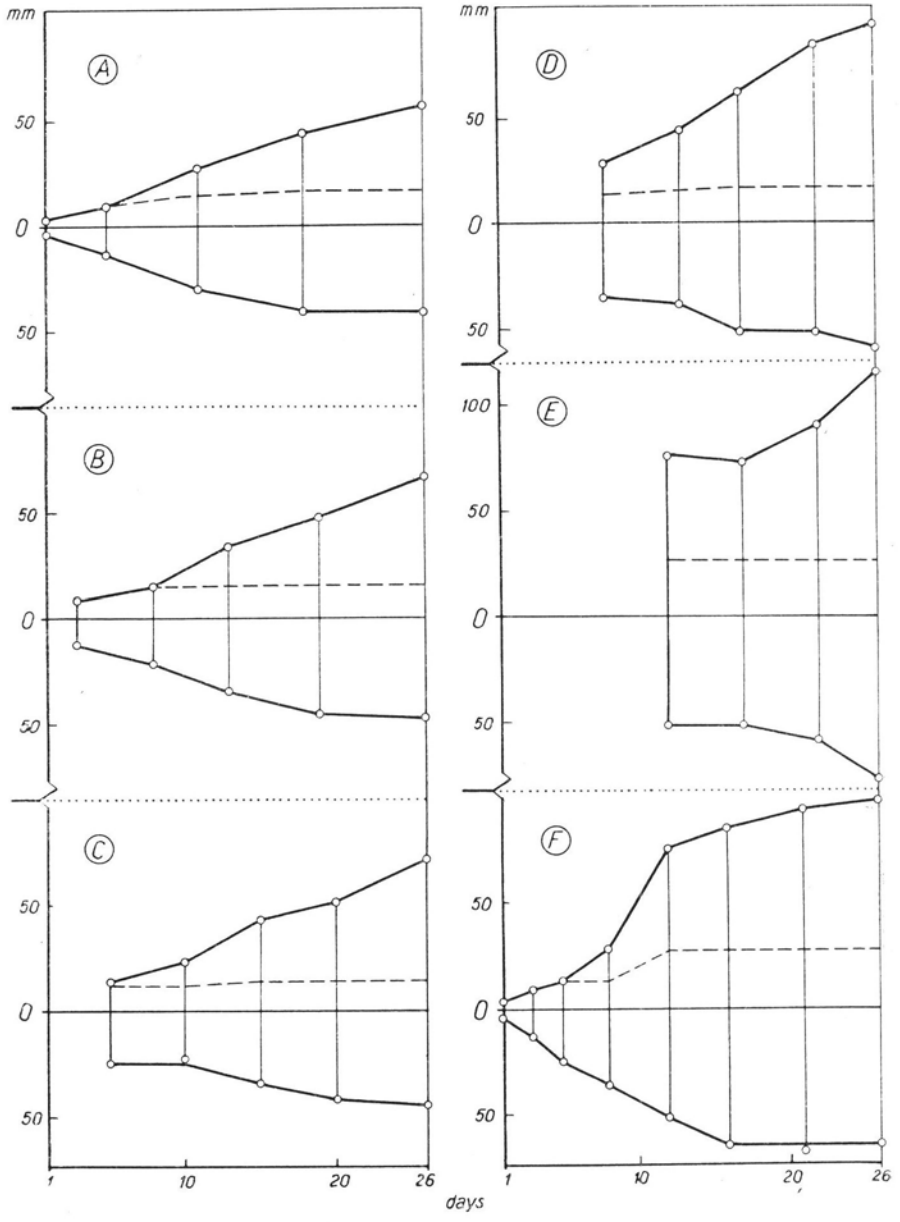

Fig. 2. Length increase (in mm), series *A*—*F*.

Above "O" level = shoot system (below the broken line—hypocotyl, above — epicotyl). Below "O" level = roots.

Fresh matter (Fig. 3)

The level of fresh matter of epicotyls and roots rises at the same rate in axes decotylized after 1, 3 and 5 days (*A*, *B* and *C*). The later axes are transplanted the greater their initial mass, so that final values

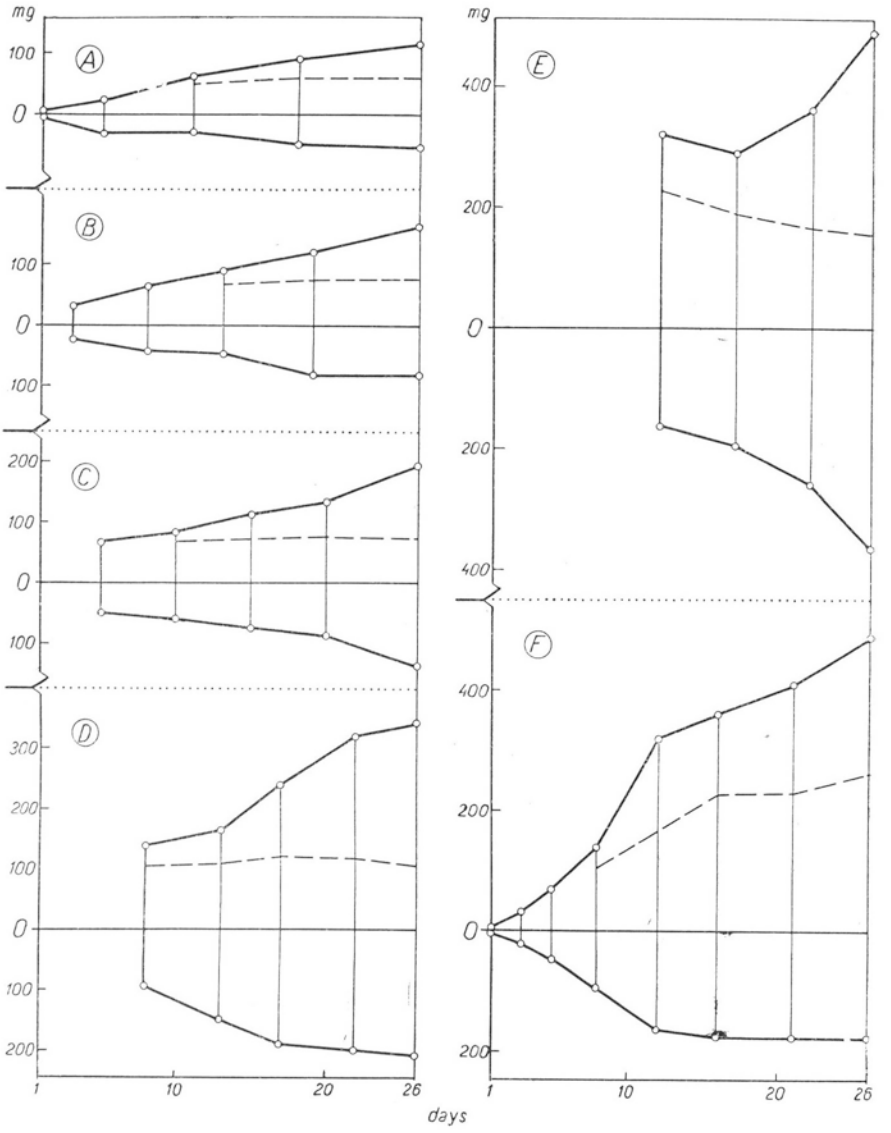

Fig. 3. Fresh weight (mg per organ), series *A*—*F*.
(For further explanation see Fig. 1 and 2.)

of fresh mass were in each case higher. Nevertheless, these values remain much below the controls.

Epicotyls of axes decotylized after 8 days (*D*) increase in weight until the 20th day faster than in cases *A*, *B* and *C*, yet slower than in the controls. Root growth in this series is similar to the control plants.

Fresh matter of epicotyls in axes decotylized after 12 days approaches control values towards the end of the trial; root fresh matter is even

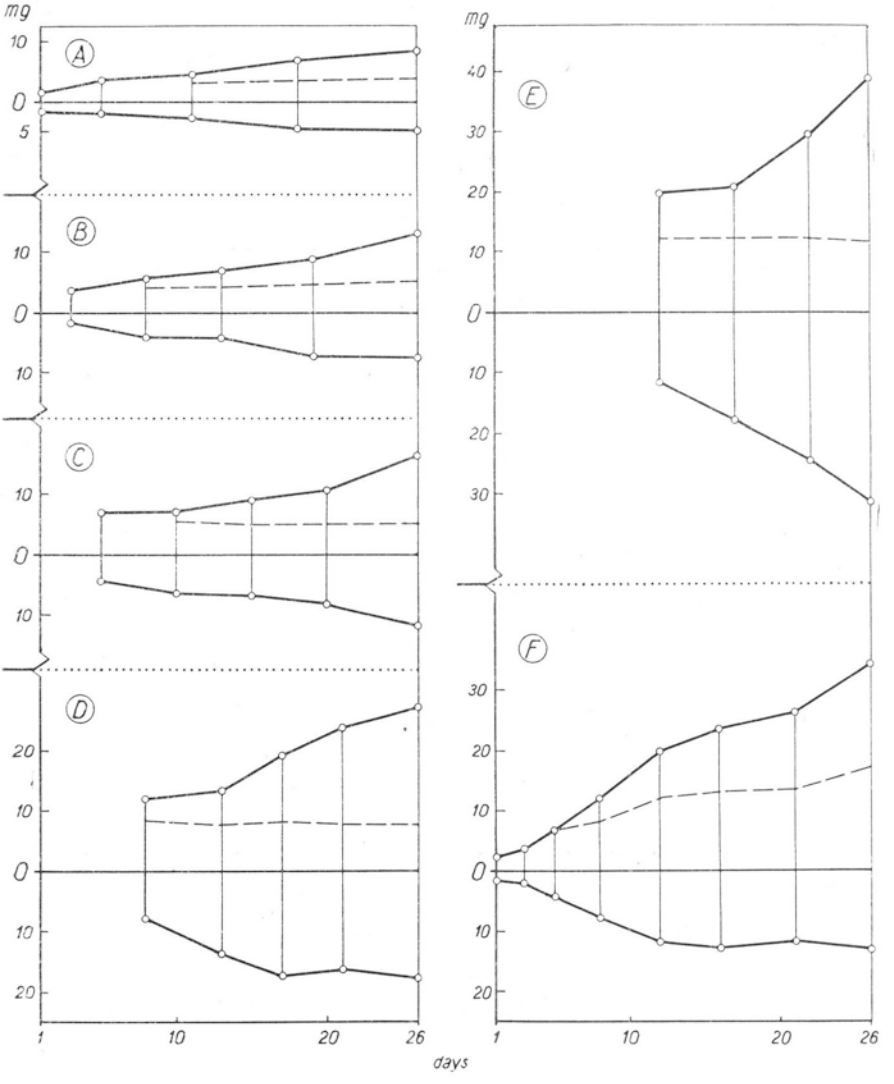

Fig. 4. Dry weight (mg per organ), series *A*—*F*.
(For further explanation see Fig. 1 and 2.)

greater. Fresh matter of hypocotyls increases only slightly in embryos isolated after 1, 3 and 5 days until the 20th day, and then remains on an equal level. In axes isolated after 8 days the hypocotyl fresh matter is at its maximum on the 20th day, henceforth slightly dropping. In germ axes decotylized after 12 days the hypocotyl fresh matter is found to diminish since transplantation onwards. The hypocotyl dry matter's remaining on an equal level indicates the drop to be due to a water-loss.

Control plants (*F*) rapidly develop fresh matter until the 12th day. Later the hypocotyl and root fresh matter increases at the same more or less rate as in the *A*, *B*, *C* and *D*.

Similarly to the lengthwise growth, fresh matter of axes cultured on media increases the faster to highest values the later the decotylizing took place.

Dry matter (Fig. 4)

As was the case with fresh matter, the dry matter of germ axes cultured on media attains the highest values the later the decotylization and transplantation. This is most evident in epicotyls and roots. The hypocotyl dry matter remains more or less on the level of the time of separation, differing in this respect from control plants, where the hypocotyl dry matter steadily increases.

Dry matter levels of epicotyls in germ axes of all experimental series are lower than in controls, with the exception of axes isolated after 12 days (*E*), where they are higher than in control plants (*F*) and exhibit a marked tendency to rise. This is even more evident in roots of this series (*E*) whose dry matter level much exceeds that in controls and continues to rise rapidly, while the latter remains unchanged since the 11th day.

Total nitrogen (Fig. 5).

The total nitrogen content in tested germ axes rises only slightly in comparison with control seedlings, and mainly in the epicotyls of axes decotylized at later terms (8 or 12 days, Fig. *D* and *E*). In hypocotyls, the content of total nitrogen drops increasingly as the period from seed swelling to decotylizing extends. In roots, the level of total nitrogen remains unchanged from the time of transplantation.

After separation from cotyledons and transplantation on media the total nitrogen level drops slightly in all tested plants. Evidently it passes into the medium.

Control epicotyls and hypocotyls (*F*) contain larger amounts of total nitrogen than the experimental organs, whichever the period of decoty-

lizing. In hypocotyls the level of total nitrogen rises as the seedlings develop. In roots the nitrogen content increases until the 11th day, and from then remains on an equal level.

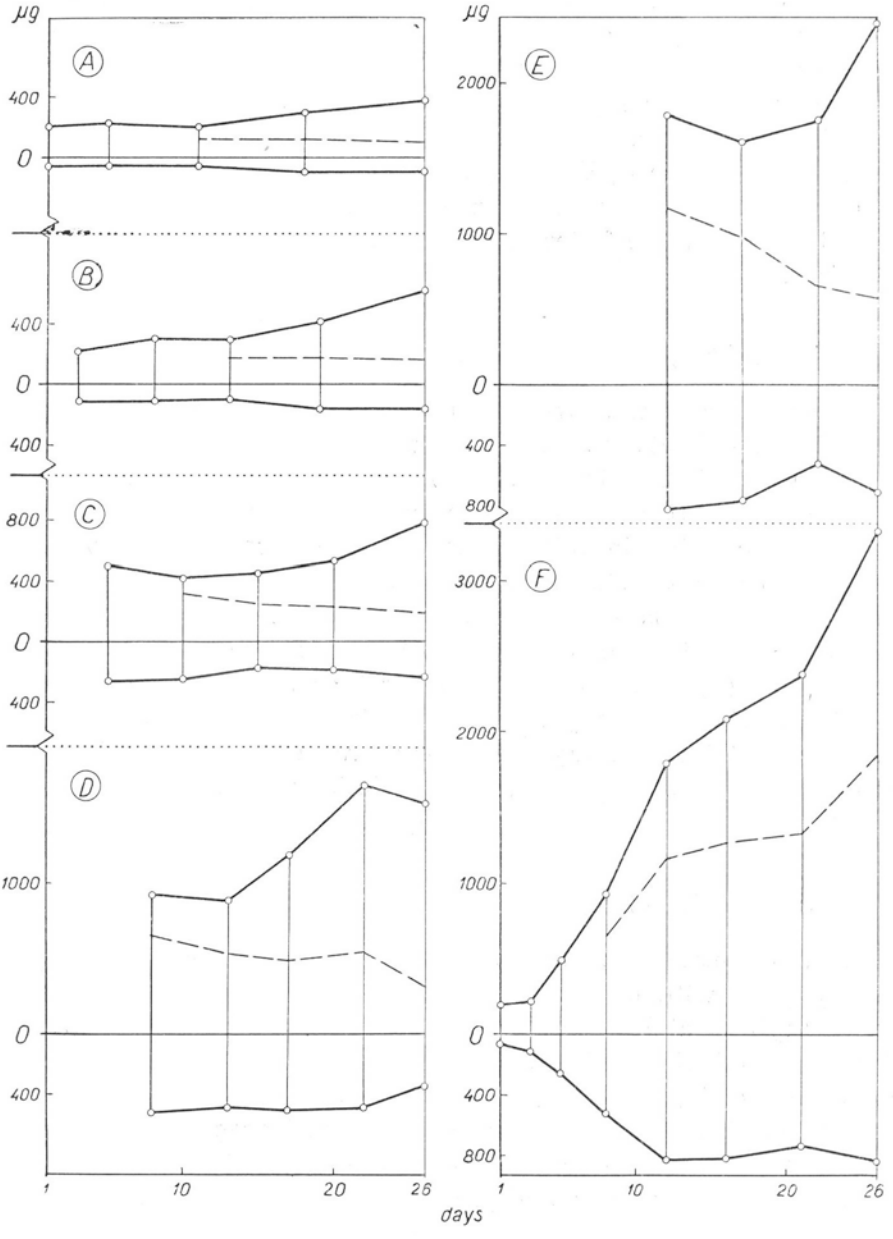

Fig. 5. Total nitrogen (µg per organ), series *A*—*F*.
(For further explanation see Fig. 1 and 2.)

Soluble nitrogen (Fig. 6)

The level of soluble nitrogen follows the same general pattern as total nitrogen; the difference though between the experimental and control seedlings is greater on behalf of the latter. Also there is a marked

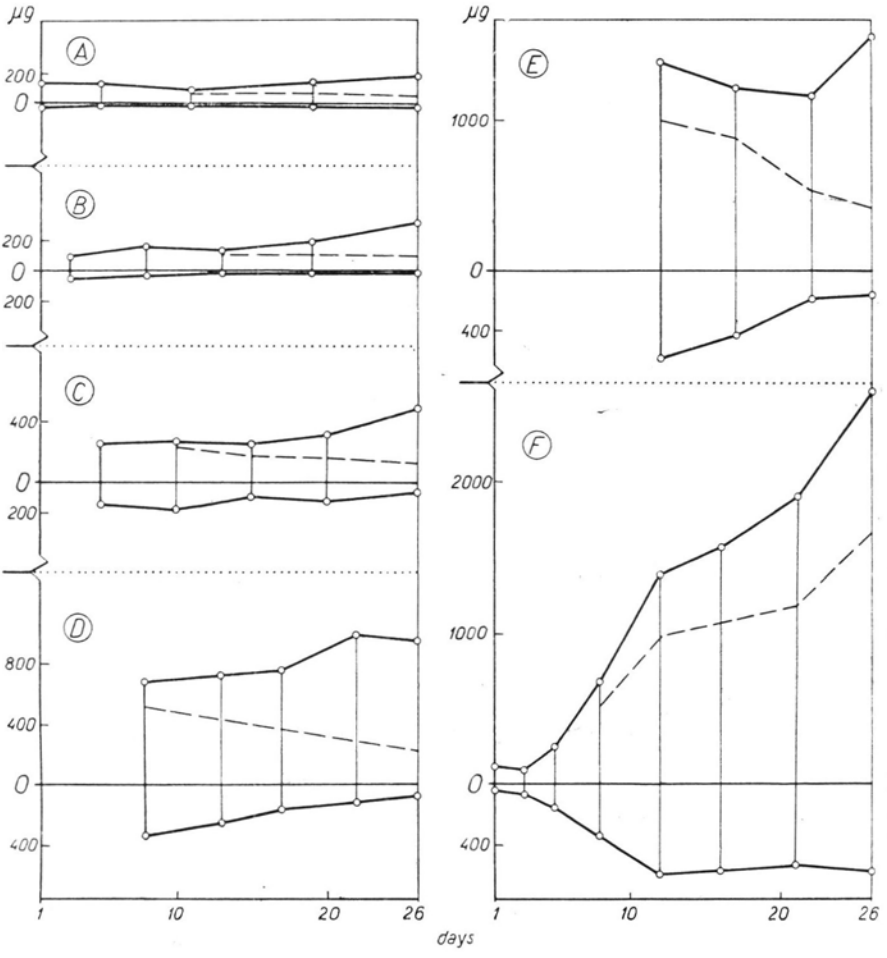

Fig. 6. Soluble nitrogen (µg per organ), series *A*—*F*.
(For further explanation see Fig. 1 and 2.)

tendency to drop in the level of nitrogen in hypocotyls and roots of axes decotylized after 5—12 days (*C*—*E*).

Protein nitrogen (Fig. 7)

The content of protein nitrogen in epicotyls and roots of axes separated after 1, 3 and 5 days steadily rises as the seedlings develop; in hypocotyls it remains on the same level. In germ axes decotylized

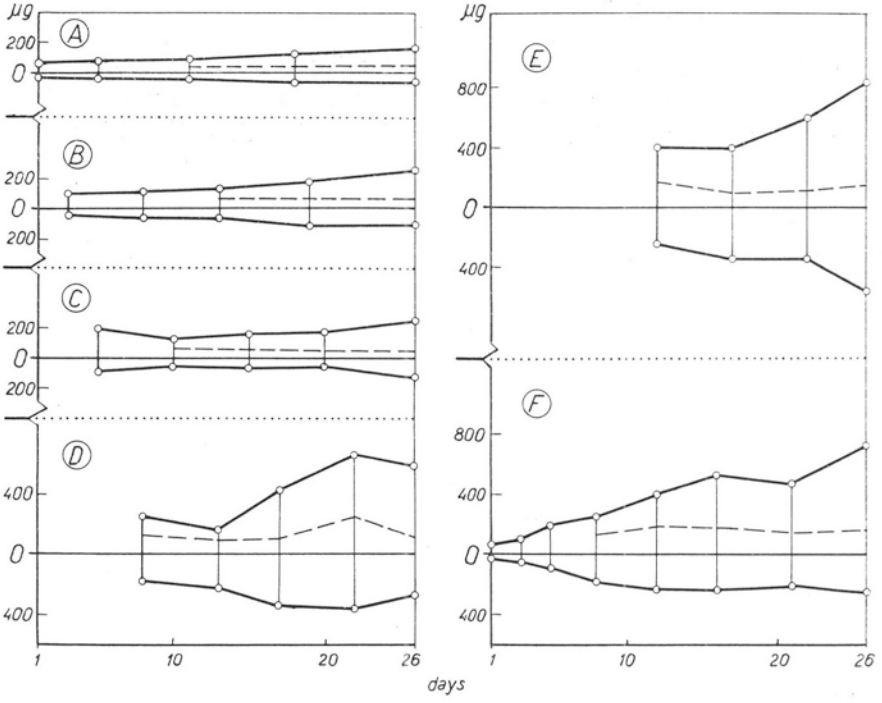

Fig. 7. Protein nitrogen (µg per organ), series *A*—*F*.
(For further explanation see Fig. 1 and 2.)

after 8 and 12 days (*D* and *E*), the increase in the protein nitrogen content is more rapid, similar to that in control seedlings (*F*).

The protein-N to soluble-N ratio (Fig. 8)

In germ axes separated after 1, 3 and 5 days (*A*, *B* and *C*) the protein-N to soluble-N ratio in epicotyls and roots rises until the 12th day, to drop henceforth or remain unchanged. In hypocotyls there occurs a slight but steady rise in the mentioned ratio. A similar rise takes place in axes isolated after 8 and 12 days (*D* and *E*). On the contrary, in control seedlings (*F*) the $N_{prot.}/N_{sol.}$ ratio slightly but steadily falls in hypocotyls as the development proceeds.

In germ axes separated from cotyledons after 8 and 12 days (*D* and *E*) the $N_{prot.}/N_{sol.}$ ratio in roots rapidly rises, while in epicotyls it slightly dwindles. Epicotyls of this series follow the same pattern as controls, while roots differ largely in this respect.

In general, $N_{prot.}/N_{sol.}$ ratio in the tested plants is higher than in controls, whichever the time of sampling and organ.

Figure 9 clearly illustrates the effects of cotyledons on the growth rate of germ axes, on their fresh and dry matter contents and on the level of nitrogen fractions in question. In control seedlings (non

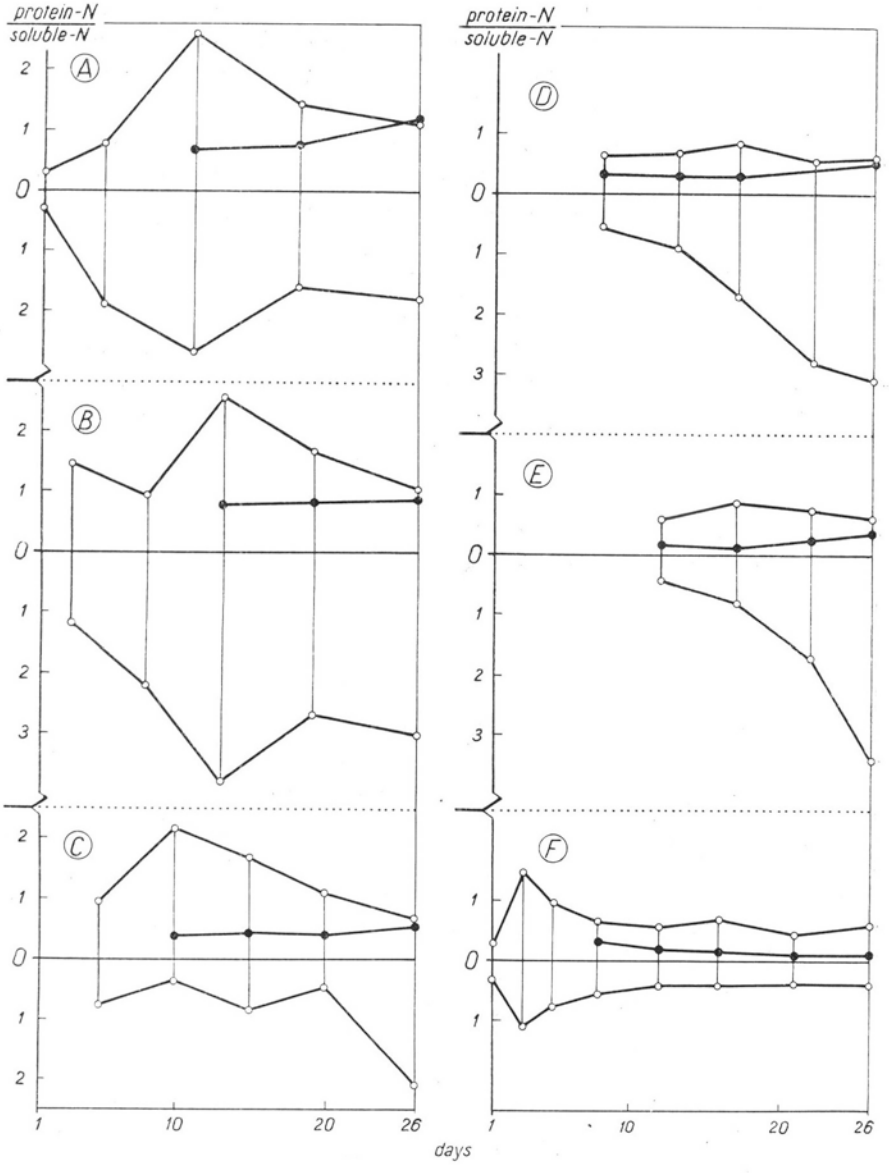

Fig. 8. $N_{protein}/N_{soluble}$ ratio, series *A*—*F*.
Above "O" level = open circles-total shoot system, black circles — values for hypocotyl. Below "O" level = roots.

decotylized, cultured in distilled water) all the tested parameters largely increase until the 12th day; since that time the rate of increase drops to a greater or smaller degree.

Axes decotylized after 1, 3 and 5 days develop less rapidly than control seedlings, and their levels of fresh and dry matter as well as of the nitrogen fractions are lower. Axes separated from cotyledons at the 8th day are of lesser size than control seedlings, while their levels of fresh and dry matter and protein nitrogen surpass those of controls. The same holds true more evidently in axes decotylized after 12 days — where even in size they exceed control plants.

Contents of total nitrogen and soluble nitrogen in separated germ axes are in every case lower than in control plants and in time rise only negligibly or remain on the decotylizing level.

DISCUSSION

Experimental results of the present trial contribute to the evidence in favour of previous findings (C z o s n o w s k i 1962) according to which isolation of yellow lupin embryos from cotyledons and the application of Heller's medium as a substitute bears a greatly unfavourable effect on the embryo development and nitrogen metabolism.

During the present examinations decotylization of embryos or axes took place after 24hrs, 3, 5, 8 and 12 days after the initiation of swelling. It can be regarded as a general rule that the later the separation and transplantation on the medium the more resemblance to the usual pattern (embryos including cotyledons cultured in distilled water) neverthelless there are many deviations from this principle. E.g. the growth rate, fresh and dry matter and the level of protein nitrogen (Fig. 9) in axes decotylized at later periods (8 and 12 days) surpass the respective control parametres. This is most evident in roots.

In excised axes cultured in Heller's medium symptoms of nitrogen deficiency were found. The total amount of nitrogen remains on the level reached at time of decotylizing (Fig. 9); from then onwards there only occurs a transport of nitrogen within the axes, from hypocotyls to epicotyls (Fig. 5, *A*—*E*). In control seedlings on the other hand, the content of total nitrogen increases rapidly and continuously, both in epicotyls and in hypocotyls, owing to an accumulation of soluble nitrogen compounds. Germ axes bred on media maintain the same level of soluble nitrogen compounds they carried at time of decotylizing; in isolated axes during further development this fraction has a tendency to diminish in amount (in hypocotyls and roots) (Fig. 6). The protein fraction rises in decotylized axes relatively rapidly. Owing to this situation, the $N_{prot.}/N_{sol.}$ ratio in isolated axes is much higher than in control seedlings.

The relatively intensive protein synthesis in axes cultured on media is presumably due to a large concentration of sugars, easily drawn from the medium, in main by more advanced axes with a well-developed root system. On this basis it is possible to account for the dynamic

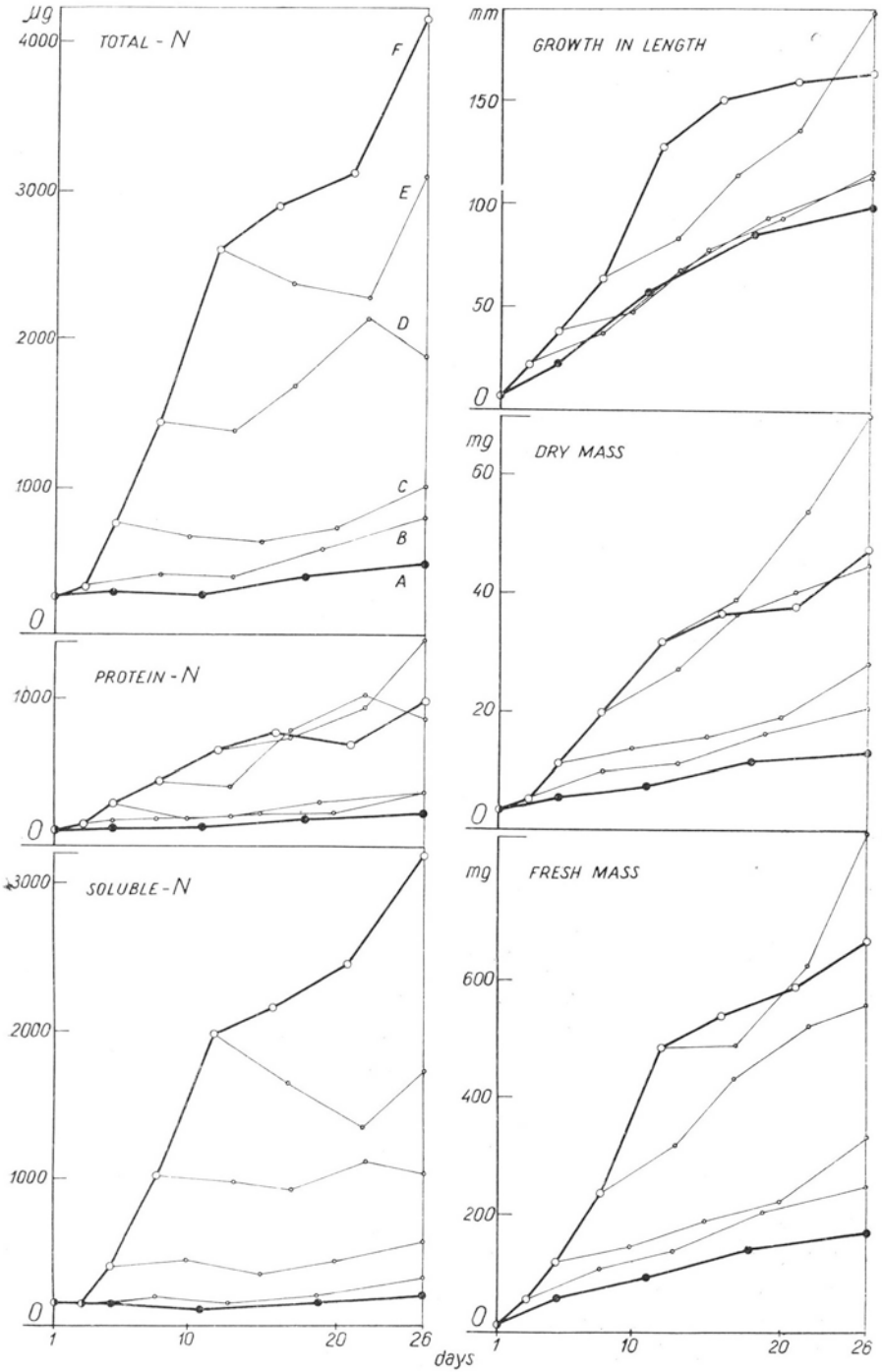

Fig. 9. Graphs summarizing the analytical data for whole axes of the control series F — (open circles), the series A (black circles) and the series B, C, D, E — (thin lines).

rise of the $N_{prot.}/N_{sol.}$ ratio in roots of axes separated from cotyledons after 8 and 12 days (Fig. 8, *D* and *E*), after their transfer to media. In view of the scarce amount of nitrogen and high concentration of sugars drawn through a well-developed root system, there takes place a rapid synthesis of protein and a rise of the $N_{prot.}/N_{sol.}$ ratio. On the other hand in control seedlings the $N_{prot.}/N_{sol.}$ ratio is in every case lower than in decotylized axes: after an initial three-day increase, it drops and becomes stable thus indicating an establishment of equilibrium between the protein fraction and the fraction of soluble nitrogen compounds.

Where lies the reason for a nitrogen deficiency in decotylized germ axes cultured on Heller's medium? For one thing, an ill-developed root system (especially in early isolations) is obviously a handicap as well

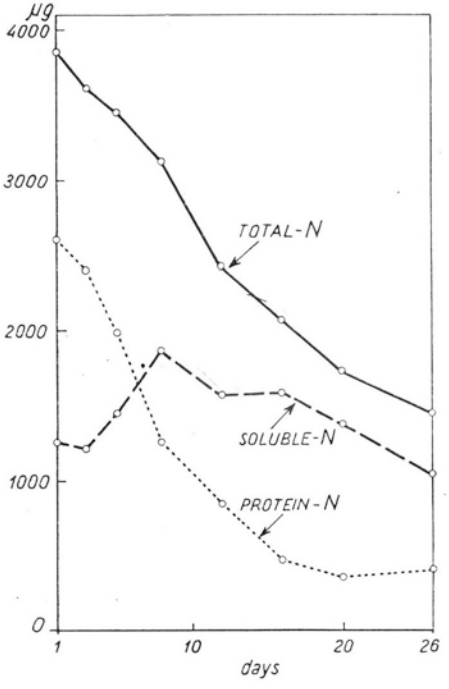

Fig. 10. The levels of total nitrogen, soluble nitrogen and protein nitrogen (µg per organ) in cotyledons — data from the control series (*F*) during 26 days of culture on distilled water.

as the concentration gradient of the medium resulting from the uptake of solutes. Nevertheless sugars are drawn satisfactorily under the same conditions so that the true reason must be found elsewhere.

Determinations of nitrogen fractions within cotyledons of control seedlings (Fig. 10) revealed that for approximately 20 days the protein in cotyledons becomes less, whereas the level of soluble nitrogen compounds remains in general unchanged. The latter exert a continuous "pressure" on the axis of a normal seedling. In terms of quantities, a single pair of cotyledons supplies about 3 mg of soluble nitrogen,

while a portion of Heller's medium alloted to a single germ axis contains 2.1 mg of nitrogen in the form of NO_3^-.

A knowledge of the qualitative and quantitative composition of the soluble fraction of nitrogen compounds in cotyledons and axes may contribute largely to the study of this problem and therefore further analyses are in progress.

SUMMARY

1. Yellow lupin seeds were germinated under sterile conditions in distilled water at 24°C, under continuous fluorescent illumination of 1600 lux.

2. From part of the germinated seeds, embryos after 24hrs, and germ axes after 3, 5, 8 and 12 days, were separated from cotyledons and transplanted in Heller's agar medium, where they were cultured until the 26th day after the initiation of swelling. The remaining lot of germinated seeds were left to develop in distilled water and used as control plants.

3. Every few days (see Fig. 1) measurements were taken of the lenght increase, fresh and dry matter, total and soluble nitrogen contents in different organs of experimental and control plants.

4. As compared with control axes, the length increase, fresh and dry matter, as well as the content of total and soluble nitrogen were lower in isolated axes; the difference was the greater the earlier the decotylization. The content of soluble nitrogen in cultured axes remained on the same level as that reached at time of separation from cotyledons, while in control axes it steadily rised.

5. The $N_{prot.}/N_{sol.}$ ratio is always higher in isolated axes than in axes of control seedlings.

6. In cotyledons of seedlings cultured in distilled water soluble nitrogen compounds remain until the 20th day on a high level; at the same time the content of the protein fraction steadily decreases.

7. It was assumed that a high content of available sugar in the medium accounts for the change in nitrogen metabolism of isolated axes whereby the protein synthesis is increased. The medium which represents a static element as compared with the dynamic nature of cotyledons may induce essential shifts in the metabolic pattern of cultured axes.

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Metabolizm izolowanych zarodków Lupinus luteus L. IV.

Streszczenie

1. Nasiona łubinu żółtego poddano w warunkach sterylnych kiełkowaniu na wodzie destylowanej w temp. 24°C, na ciągłym świetle fluorescencyjnym 1600 lux.

2. Z części wykiełkowanych nasion po 24 godzinach zarodki, a po 3, 5, 8 i 12 dniach osie siewek odseparowano od liścieni i wyszczepiono na pożywkę agarową Hellera z dodatkiem 3% sacharozy, gdzie następował dalszy ich rozwój do 26 dni od daty rozpoczęcia pęcznienia. Pozostała część wykiełkowanych nasion rozwijała się nadal na wodzie destylowanej, stanowiąc kontrolę doświadczenia. Warunki świetlne i termiczne jak w p. 1.

3. Co kilka dni (patrz Fig. 1) oznaczano wzrost na długość, świeżą i suchą masę oraz azot całkowity i rozpuszczalny w poszczególnych częściach roślin doświadczalnych i kontrolnych.

4. Wzrost na długość, świeża i sucha masa oraz zawartość azotu całkowitego i rozpuszczalnego były w izolowanych osiach niższe niż w kontrolnych, przy czym różnica była tym większa, im wcześniej nastąpiło odseparowanie osi od liścieni. Zawartość azotu rozpuszczalnego w hodowanych osiach utrzymywała się na tym samym poziomie co w momencie odizolowania od liścieni, w kontroli natomiast stale wzrastała.

5. Stosunek $N_{białk.}/N_{rozp.}$ w izolowanych osiach jest zawsze wyższy niż w osiach roślin kontrolnych.

6. W liścieniach siewek rozwijających się na wodzie destylowanej utrzymuje się do ok. 20 dnia ten sam wysoki poziom rozpuszczalnych związków azotowych, przy równoczesnym stałym spadku poziomu frakcji białkowej.

7. Wysnuto wniosek, że przyczyną zmiany charakteru gospodarki azotowej w izolowanych osiach, w kierunku wzmożenia syntezy białka, jest duża zawartość przyswajalnego cukru w pożywce. Statyczna, w porównaniu z dynamicznym układem liścieni, pożywka może jednak wywołać zasadnicze przesunięcia w torach metabolicznych hodowanych osi.