REGENERATION, VERNALIZATION AND FLOWERING IN *LUNARIA ANNUA* L. IN VIVO AND IN VITRO

Regeneratie, vernalisatie en bloei in Lunaria annua *L. in vivo en in vitro*

R. L. M. PIERIK

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWKUNDE
OP GEZAG VAN DE RECTOR MAGNIFICUS, IR. F. HELLINGA,
HOOGLERAAR IN DE CULTUURTECHNIEK,
TE VERDEDIGEN TEGEN DE BEDENKINGEN
VAN EEN COMMISSIE UIT DE SENAAT
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN
OP WOENSDAG 21 JUNI 1967 TE 16 UUR

DOOR

R. L. M. PIERIK

H. VEENMAN & ZONEN N.V. – WAGENINGEN – 1967

STELLINGEN

I

Het is niet aannemelijk, dat het verdwijnen van de bloeitoestand direct veroorzaakt wordt door de reductiedeling en/of de bevruchting.

Dit proefschrift

II

Bloei-inductie in ongedifferentieerde weefsels, die afkomstig zijn van koudebehoeftige planten met een jeugdfase, is niet mogelijk.

Dit proefschrift

III

Er dient meer aandacht te worden geschonken aan de rol, die micro-organismen en virussen bij de groei en morfogenese van hogere planten kunnen spelen.

IV

Ten onrechte wordt bij het kankeronderzoek met dierlijke en menselijke weefsels in Nederland de plantaardige weefselkweek te weinig of in het geheel niet betrokken.

V

Bij het samenstellen van voedingsmedia voor de steriele kweek van delen van hogere planten zou het nuttig zijn om gewasanalyse als hulpmiddel te gaan gebruiken.

VI

In vitro kultuur van haploide cellen tot weefsels en planten dient door de plantenveredelaar onverwijld ter hand genomen te worden.

VII

De bruikbaarheid van de spruitvormingstoets met *Cardamine pratensis* L. wordt door de ontwerpers sterk overschat.

P. Paulet et J. P. Nitsch
Bull.Soc.Bot.France *106*, 1959:425–441.

VIII

Bij de steriele kweek van cellen, weefsels en organen van hogere planten valt te veel de klemtoon op groeiregulatoren en veel te weinig op de fysische groeifactoren.

IX

Callus, in steriele kweek verkregen, moet in veel gevallen als pathologisch weefsel worden beschouwd.

X

De serie „Mededelingen van de Landbouwhogeschool te Wageningen" beantwoordt niet volledig aan de eisen, die uit het oogpunt van de publiciteit mogen worden gesteld.

Proefschrift van R. L. M. Pierik
Wageningen, 21 juni 1967.

VOORWOORD

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De steun van mijn vrouw in ons gezin en bij mijn werk vanaf het begin tot aan de voltooiing van dit proefschrift, zou ik niet hebben kunnen missen.

CONTENTS

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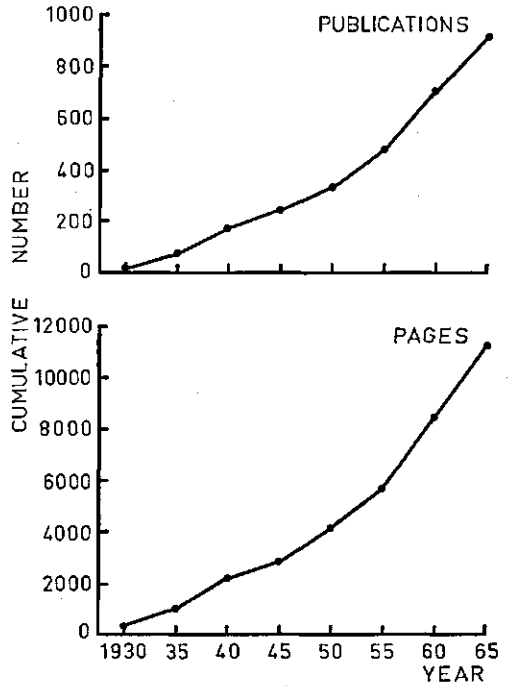
1. GENERAL

1.1. INTRODUCTION

The influence of environmental factors, especially temperature and photoperiod, on the transition from the vegetative to the generative state has occupied numerous research workers for many years. When the present author started to study the process of vernalization, he was confronted with an enormous mass of literature which constitutes the condensation of earlier work. WELLENSIEK (128, 1965) estimated the amount of literature on vernalization and arrived at 720 papers containing 10702 pages. Consulting my own literature system at the end of 1965, I arrived at a number of 916 publications containing 11222 pages. See graph 1. Reference is made to several extensive reviews which have been published: AUGSTEN (8, 1964), CHOUARD (14, 1960), DAVID (19, 1946), HÄNSEL (35, 1953), LANG (58, 1965), LYSENKO (60, 1954), MATHON and STROUN (65, 1962), MURNEEK and WHYTE (74, 1948), NAPP-ZINN (76, 1961), PURVIS (91, 1961) and WELLENSIEK (128, 1965). In the following chapters only those publications will be cited which have a direct bearing on my investigations.



GRAPH 1.
Cumulative number of publications and pages published on vernalization.

Former work with *Lunaria annua* L. has almost exclusively been done by WELLENSIEK (119, 120, 122, 125, 127), who used intact plants and leaf cuttings. The present investigations are a continuation of the work carried out by WELLENSIEK.

1.2. Scope of the investigations

Lunaria annua L. is characterized by a juvenile phase, an absolute cold requirement for flowering and a high regeneration ability in vivo and in vitro. An attempt was made to answer the following questions:

1. Is it possible to change the biennial flowering habit into an annual one by induction of a mutation? (chapter 3)
2. What is the influence on flowering of the light conditions before, during and after vernalization and of temperature in the early and later stages of development? (chapter 4)
3. Is juvenility a character occurring in the whole plant or is it localized? (chapter 5)
4. What is the influence on flowering of root and sprout regeneration before and during vernalization of leaf cuttings, especially of cotyledons? (chapter 6)
5. Is flower induction possible in isolated vegetative tissues without buds or sprouts before the vernalization? (chapter 8)
6 Which factors effect flower bud initiation and/or the possible disappearance of the flowering condition in isolated tissues of generative plants without pre-existing buds or sprouts? (chapter 9)

2. MATERIAL AND METHODS

Plant material. – All experiments have been done with plants, derived from seeds as used in the earlier work on *Lunaria*.

Sowing. – The seeds have been sown in wooden boxes under glass plates and the seedlings were transplanted in pots with fertile soil.

Age. – The age of the plants is the time which has elapsed since the plants have been sown until the beginning of a certain treatment. Consequently, plants of the same age grown in different seasons are not fully comparable owing to differences in growth conditions.

Growth conditions. – Before and after vernalization the plants have been grown in the greenhouse at approximately 20 °C which temperature was not constant and depended on the weather conditions outside. Especially in the summer the temperature was often higher than 20 °C. Plants were grown under long-day conditions, obtained by extending the natural days with fluorescent light, intensity 40.000 erg $\varnothing$ cm^{-2} sec^{-1}. The light intensity has been measured by means of a spherical light meter according to WASSINK and VAN DER SCHEER (118).

Leaf cuttings. – Leaf cuttings consisted of a petiole and a leaf blade and have been placed in a cutting-bed with fertile soil covered with pure sand. The cutting-bed has been covered with glass plates to increase the humidity. After 2–3 weeks the cuttings have been transferred from the cutting-bed to pots with fertile soil. The rooting-capacity of leaf cuttings was roughly scored by immersion of the rootsystem with intact soil into a calibrated glass cylinder filled with water and measuring the change in volume.

Phytotron. – In the phytotron, earlier described by DOORENBOS (21), plants could be grown in long day under strong fluorescent light (Philips TL 40 W/55, maximal intensity 48.000 erg $\varnothing$ $cm^{-2}sec^{-1}$, or under natural daylight both at temperatures of 9°, 12°, 15°, 18°, 21° or 24 °C. The plants could also be placed at approximately 22 °C under 8 different light conditions:

daylength 16 hrs, relative light intensities: 25 %, 50 %, 75 % or 100 %;
daylength 12 hrs, relative light intensity: 100 %;
daylength 8 hrs, relative light intensities: 50 %, 75 % or 100 %.

Number of plants. – Unless otherwise stated the number of plants and cuttings per treatment is 20.

Vernalization. – Vernalization took place in a room at a controlled constant temperature of 5 °C under long-day conditions (16 hrs, low light intensity, fluorescent light) or under different light conditions: short day (fluorescent light or incandescent light), long day (fluorescent light or incandescent light) and continuous incandescent light.

Abbreviations.

CD	: continuous dark
CL	: continuous light
2,4–D	: 2,4–dichlorophenoxyacetic acid
EMS	: ethyl methane sulphonate
GA_3	: gibberellic acid (contains also approximately 10% of GA_4 and GA_7)
IAA	: indole-3-acetic acid
LD	: long days (16 hrs)
NAA	: naphthalene-acetic acid
PV	: plant vernalization
SD	: short days (8 hrs)
SD 4901*:	6–benzylamino-purine
SD 8339*:	6–benzylamino-9-(tetrahydro-2-pyryl)-purine

* We appreciate the gift of these phytokinins from Dr. J. VAN OVERBEEK, Shell Agriculture Research Division, Modesto, California, U.S.A.

3. ANNUAL FLOWERING IN *LUNARIA ANNUA* L.

3.1. SURVEY OF EXISTING MATERIAL

The genus *Lunaria*, belonging to the family of the *Cruciferae*, consists of three species (HEGI, 37): *L. annua* L., *L. rediviva* L. and *L. telekiana* Jáv. Confusion was caused by MOENCH (69, p. 261) by speaking of *L. biennis* Moench instead of *L. annua* L. In the present study the original name *L. annua* L. has always been used, which does not imply, however, that this species is annual. The equivalents for *L. annua* L. in the different languages are: biennial honesty (English), monnaie du pape (French) and Mondviole (German).

Although *Lunaria annua* L. was generally considered a biennial plant species, HAGEMANN (32) demonstrated this for the first time experimentally. WELLENSIEK (119) extended these observations and came to the same conclusion. According to CHOUARD (personal communication) a non-cold-requiring *L. annua* exists. For that reason nearly all botanical gardens in Western-Europe were asked for *L. annua* L. (= *L. biennis* Moench) seeds in order to find a non-cold-requiring honesty. In 1962, 1963 and 1964 seeds have been received from 48 different places located in 17 different countries, which are listed below:

Austria	Frohnleiten	Eastern Germany	Leipzig	Norway	Oslo
	Graz		Oberholz	Poland	Lodz
	Vienna	Western Germany	Bonn		Toruń
Belgium	Brussels		Bremen	Spain	Barcelona
	Ghent		Erlangen		Madrid
Czechoslovakia	Liberec		Essen	Sweden	Stockholm
Denmark	Århus		Frankfort		Uppsala
	Copenhagen		Giessen	Switzerland	Bern
England	Cambridge		Göttingen	The Netherlands	Leiden
	Glasgow		Hamburg		Wageningen
	Leicester		Heidelberg	U.S.S.R.	Kaunas
	London		Marburg		
	St. Andrews	Italy	Bologna		
Finland	Helsinki		Firenze		
France	Dyon		Palermo		
	Nancy		Torino		
	Paris		Trieste		
	Rouen	Monaco	Monaco		
	Tours				

Seeds from the above cited origins were sown in the greenhouse in March 1964 and planted out in the field in the beginning of June 1964. No plants, however, flowered in 1964. It was concluded that an annual *Lunaria annua* most probably did not exist. From another experiment the conclusion was drawn that there are no significant differences in the time required for flower bud initiation after vernalization between plants of 17 different origins. The physiological characteristic of an absolute cold requirement for flowering in *Lunaria*

annua was a generally occurring phenomenon. The same was found to be true for *L. rediviva.*

3.2. The induction of annual flowering by ethyl methane sulphonate

The purpose of this experiment was an attempt to induce artificially annual flowering in the biennial honesty. Ethyl methane sulphonate (EMS) was chosen as an agent because of its high mutagenicity (Heslot cs., 42). On the 4th of July 1963 dry undamaged seeds of *Lunaria annua* L. were immersed in EMS solutions of concentrations 0.00, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.60, 0.80 and 1.00 per cent for 24 hrs at a constant temperature of 24°C; 500 seeds per treatment were used. EMS was dissolved in tap water without the use of a detergent. After the EMS treatment the seeds were rinsed for 5 minutes under a continuous flow of tap water and sown in wooden boxes filled with fertile soil. Periodically the number of germinated seeds was determined. On the 25th of August 1963 all viable plants were planted out in the field, where natural vernalization during the winter of 1963/1964 followed. On the 2nd of May 1964 the number of surviving plants was counted. As self-pollination generally occurs in *Lunaria annua*, the plants were not embagged; this does not imply, however, that cross-pollination had not taken place at all. On the 10th of August 1964 the numbers of plants with proliferations and the numbers of seeds per plant were counted, while the seeds were harvested per plant. The most important data obtained in 1963 and 1964 are summarized in table 1.

Table 1. Effect of EMS treatment in M_1 on % of germination, % planted in the field on 25/8/63, % of hibernated plants on 1/5/64, % of hibernated plants with proliferations (prol.), complete sterility (ster.) and 50 or more seeds (seeds).

% EMS	% germination on		% planted on	% hibernated plants on	% hibernated plants with		
	15/7/63	16/8/63	25/8/63	1/5/64	prol.	ster.	seeds
0.00	58	83	85	48	0	0	100
0.05	50	84	77	19	2	2	96
0.10	38	83	78	21	2	1	98
0.15	23	87	81	23	8	4	95
0.20	15	82	72	32	11	6	83
0.25	9	85	75	22	22	11	58
0.30	3	81	66	30	33	12	40
0.35	2	85	75	23	62	21	13
0.40	1	81	69	24	68	20	5
0.60	0	71	50	2	100	100	0
0.80	0	39	7	0	–	–	–
1.00	0	4	0	0	–	–	–

Table 1 shows that the higher the EMS concentration, the slower the germination speed; at the highest EMS concentration the ultimate germination per-

centage is even very low. The percentages of viable plants planted in the field on 25/8/63 are also very low at the highest EMS concentrations. After natural vernalization the percentage of hibernated plants was drastically decreased at increasing EMS concentrations. Vegetative and/or generative proliferation and sterility strongly increased at increasing EMS concentrations.

On the 12th of April 1965 alltogether 291 M_2-lines were sown in the greenhouse. Totalized over all lines per EMS treatment, seed germination was retarded and even inhibited at increasing EMS concentrations. On the 10th of May 1965 approximately 10.000 viable plants were planted out in the field, where special attention was paid to possible flowering mutants. M_2-line no. 325 from EMS treatment 0.30% consisted of 38 seeds from which only 12 viable plants were obtained, one of which came into flowering. This flowering mutant was transferred to the greenhouse and selfed: sterile flowers occurred only on the top. The mutated plant also exhibited chlorophyll deficiency which resulted in poor growth and few seeds. In November 1965 a number of 50 seeds could be harvested. Vegetative propagation by means of leaf cuttings only resulted in two very small plants both flowering shortly after sprout formation. One leaf cutting after selfing produced 2 seeds, so that alltogether 52 seeds were obtained as M_3.

M_3 seeds obtained after selfing the M_2 of line no. 325 were sown in the greenhouse in February 1966. Seed germination was markedly retarded as compared with control seeds. From 52 seeds 22 viable plants were obtained which all flowered in 1966. The growth of these plants was rather poor, probably due to chlorophyll deficiency. The first visible flower bud was observed 8 weeks after sowing.

3.3. Discussion

It seems reasonable to conclude from 3.1 that *L. annua* L. is a typical cold requiring plant species. The supposition that an annual *Lunaria* existed should, therefore, most probably be rejected. From a physiological point of view, it is now comprehensible that Moench (69) rejected the name *L. annua* L. and replaced it by *L. biennis* Moench although this is incorrect for the plant taxonomist.

The mutagenic influence of EMS on *Lunaria* is in general the same as summarized by Gaul (24) for other plants. EMS appeared to be a very effective mutagen inducing especially a high percentage of chlorophyll mutations and a rather high degree of M_1 sterility which is probably carried over in the succeeding generation.

Schmalz, Misra and Lutkov (cf. Napp-Zinn, 76, p. 50) earlier demonstrated that flowering mutations could artificially be induced in cold requiring plants by Röntgen irradiation. Spontaneous annual mutations in plants with a cold requirement for flowering were observed by Walkof (116) in cabbage and by Wellensiek in brussels sprouts (121). The present work demonstrates that annual flowering can also be induced by EMS.

The inheritance of the flowering habit has not been studied. But most probably the difference between annual and biennial flowering will be monogenic,

while biennial flowering will be dominant over annual flowering. The rapid flowering, 8 weeks after sowing, might indicate that the juvenile phase for flowering, which existed in the biennial material of origin, is shortened or disappeared.

4. VERNALIZATION EXPERIMENTS WITH INTACT PLANTS

4.1. INTRODUCTION

According to WELLENSIEK (119, 120, 127) *Lunaria annua* is a plant with an absolute cold requirement for flowering. From the moment of sowing the plants pass through a juvenile phase lasting approximately six weeks. This juvenile phase is defined (WELLENSIEK, 124) as the period of vegetative growth during which flower formation is impossible. Adult *Lunaria* plants, however, respond to the cold treatment with flowering. In the transitory stage between juvenile and adult the percentage of flowering increases and the time required for budding decreases when age increases. Seed vernalization alone had no visible effect. However, when seed vernalization is followed by plant vernalization, the former appears to have exerted a positive effect on flowering. Flower bud formation may take place during a prolonged cold treatment, but proceeds much faster at higher temperatures after vernalization. Grafting-experiments have shown that in *Lunaria annua* a floral stimulus occurs which can be translocated from a generative donor to a vegetative receptor: buds from the receptor may give rise to flowering shoots. GA_3 treatments result in stem elongation, but no flowering occurs. GA_3 application before vernalization only hastens flower bud initiation.

WELLENSIEK and HIGAZY (130), detailed by HIGAZY (43), demonstrated that the juvenile phase in the biennial honesty can be shortened by improving the light conditions before the vernalization. They also demonstrated that suboptimal induction resulted in the exclusive or predominant flowering of lateral buds instead of flowering of terminal buds.

The present chapter gives a brief description of the experiments with intact plants, in so far as they contribute to a better understanding of the work presented in the following chapters.

4.2. THE INFLUENCE OF THE LIGHT CONDITIONS

4.2.1. *Before vernalization*

Plants of 2 weeks old were placed in the phytotron and exposed to 6 different light conditions during 6 weeks, followed by 12 weeks of vernalization and next transferred to the greenhouse. The light conditions were: daylengths of 16, 12 or 8 hrs at 100 % relative light intensity, daylength of 16 hrs at 75 %, 50 % or 25 % relative light intensity. All plants flowered, usually between 16 and 20 days after the vernalization. Hence, the light conditions before the vernalization had no influence on the flowering.

4.2.2. *During vernalization*

Plants of 8 weeks old were vernalized during 12 weeks under 5 different light conditions: incandescent light at daylenghts of 24, 16 or 8 hrs and fluores-

cent light at daylengths of 16 or 8 hrs. After the vernalization the plants were grown in the greenhouse. All plants flowered, usually between 24 and 26 days after the vernalization, so that flowering was not influenced by the daylength and type of light source during vernalization.

In a second experiment the influence of light intensity during vernalization was studied following the same procedure as described earlier (88). Plants of 7 or 8 weeks old were vernalized for 8 weeks at light intensities of 4100, 6700 or 18.000 erg ⌀ $cm^{-2}sec^{-1}$. None of the 7 weeks old plants flowered. For plants of 8 weeks old the percentages of flowering at the different light intensities were 40, 50 and 40% respectively. This indicates that light intensity did not play a role during vernalization.

4.2.3. *After vernalization*

Plants of 8 weeks old were vernalized for 12 weeks. After the vernalization the plants were put in the phytotron under 6 different light conditions: daylengths of 16 or 8 hrs, each combined with 100%, 75% or 50% relative light intensity. All plants flowered. The mean number of days to budding was 48 for all LD-aftertreatments and 58 to 61 for the SD-aftertreatments. We can conclude that flowering is hardly influenced by the light conditions after the vernalization.

4.3. Vernalization in the early stages of development

Since seed vernalization does not lead to complete induction, but interacts with plant vernalization (Wellensiek, 119), it seemed worth while to study whether vernalization of young plants also would interact with vernalization of older plants.

By sowing periodically, plants of 3, 4 or 5 weeks old were obtained. The first vernalization period lasted 0, 3 or 6 weeks. After this vernalization period the plants were all placed in the greenhouse for 4 weeks. The second vernalization period lasted 0, 5 or 10 weeks. After the second vernalization period the plants were replaced in the greenhouse. The dates of sowing were chosen in such a way that the second vernalization period of all treatments ended on the same date; the number of plants per treatment was 10.

In table 2 the average percentages of flowering of 3, 4 and 5 weeks old plants are combined, because no differences could be found between these 3 age-groups. The percentages of flowering are strongly increased by increasing the duration of the 2nd vernalization period. They increased only slightly by increasing the duration of the 1st vernalization period. In conclusion, this experiment shows that vernalization of young plants interacts with vernalization of adult plants only to a small degree.

4.4. Vernalization under artificial and natural conditions

The role of varying low temperatures in the process of vernalization is very obscure. In practice an interrupted cold treatment sometimes resulted in a more

TABLE 2. The effect of two vernalization periods, separated by an interruption of 4 weeks at 20 °C, on the average flowering percentage of plants of three ages.

1st V. period	2nd V. period	% of flowering
0 w.	0 w.	0
0 w.	5 w.	7
0 w.	10 w.	93
3 w.	0 w.	0
3 w.	5 w.	13
3 w.	10 w.	100
6 w.	0 w.	0
6 w.	5 w.	17
6 w.	10 w.	100

complete flower induction than a permanent one (127). This unsolved problem became especially interesting since WELLENSIEK (122, 125) arrived at the concept that dividing cells are needed for the vernalizing action of a low temperature. The purpose of the present experiment was to check which type of vernalization, with constant low temperature (artificial) or with varying low temperatures (natural), was the most efficient one.

By sowing periodically, plants of 6, 8, 10, 12, 14 or 16 weeks old were obtained. Artificial vernalization took place at 5 °C during 0, 4, 6, 8, 10, 12 or 14 weeks. Natural vernalization took place under varying, uncontrolled but relatively low temperatures during the same periods in an unheated greenhouse during the winter of 1963/1964. The dates of sowing were chosen in such a way that the vernalization of all treatments ended on 1/4/64, when all plants were transferred to a greenhouse where the temperature was relatively high.

According to expectation, the percentages of flowering after both types of vernalization increased with increasing durations of the vernalization and with increasing ages at the beginning of the vernalization. We found that the general average percentage of flowering after artificial vernalization was somewhat higher. All plants of 16 weeks old flowered for 100%, even with 4 or 6 weeks of vernalization and independent of the type of vernalization. Incomplete vernalization, resulting in predominant or exclusive formation of laterally flowering branches, was more frequent after natural vernalization.

Table 3 presents the mean numbers of days from sowing to visible flower bud formation in exclusively those treatments where 100% flowering occurred. Independent of the type of vernalization, the mean numbers of days from sowing to visible flower bud formation increased by increasing the age and the duration of the vernalization. In most treatments artificial vernalization resulted in earlier flower bud realization; in a few treatments, especially when the duration of the vernalization was relatively long, the effectiveness of natural vernalization was somewhat higher. The shortest cycles from sowing to budding are found in old plants after short artificial vernalization.

TABLE 3. The mean numbers of days from sowing to visible flower bud formation in treatments with 100% flowering as influenced by the type of vernalization, the age of the plants at the beginning of the plant vernalization (PV) and the duration of PV.

age in weeks	PV in weeks 4	6	8	10	12	14
	artificial vernalization					
10	–	–	–	–	–	–
12	–	–	–	193	205	215
14	–	183	192	201	215	222
16	189	196	204	216	226	230
	natural vernalization					
10	–	–	–	–	–	–
12	–	–	–	203	209	219
14	–	–	203	213	214	219
16	191	208	210	226	226	227

In conclusion, this experiment shows that artificial vernalization was slightly more effective than natural vernalization.

4.5. DEVERNALIZATION

Nothing was known about possible devernalization by high temperature after the vernalization. As temperature plays a decisive role during flower bud realization in vitro, as will be described in chapter 9, an experiment was performed with intact plants.

Twelve weeks old plants were vernalized during 0, 6, 7, 8, 9, 10, 11 or 12 weeks. The dates of sowing were chosen in such a way that the vernalization of all 8 treatments ended on the same date. Immediately after the vernalization 3 post-treatments were given as specified in the legend of table 4. The post-treatments at 18 °C (phytotron) and 22 °C (greenhouse) were given in natural daylight (LD); the post-treatments at 31 °C were given in continuous fluorescent light. The number of plants per treatment was 16.

Table 4 shows that the percentages of flowering are not influenced by the temperature after the vernalization. This indicates that no devernalization occurred even in treatment c where a temperature of 31 °C was applied immediately after the vernalization. With only one exception, the mean numbers of days to budding decreased with increasing duration of the vernalization in all post-treatments and increased by applying a period of 31 °C. The strongest retardation of flowering was obtained when the high temperature was applied immediately after the vernalization.

4.6. FLOWERING AND SEED FORMATION AT LOW TEMPERATURE

Lunaria seeds were sown in the greenhouse on the 9th of August 1964.

TABLE 4. The effect of 3 temperature regimes after the vernalization:

	2w.	2w.	10w.
a =	18°	18°	22°
b =	18°	31°	22°
c =	31°	18°	22°

and of duration of PV in weeks on % of flowering and days to budding.

PV in weeks	% of flowering			days to budding		
	a	b	c	a	b	c
0	0	0	0	∞	∞	∞
6	0	0	0	∞	∞	∞
7	25	33	25	74	68	80
8	100	100	100	65	66	70
9	100	100	100	48	53	64
10	100	100	100	40	43	53
11	100	100	100	29	33	33
12	100	100	100	27	32	36

On the 6th of January 1965 the vegetative plants were placed in LD (fluorescent light) at 5 °C. Flower induction, flower realization, meiosis, fertilization and seed formation took fully place at 5 °C. On the 10th of October 1965 the plants were placed in a heated greenhouse for after-ripening of the seeds, which on the 14th of February 1966 were sown in the same greenhouse. None of the plants flowered.

In conclusion, the disappearance of the flowering condition also takes place at low temperature, so that high temperature devernalization is excluded during both meiosis, pollination and seed formation. This experiment also shows that prenatal vernalization in *Lunaria* is impossible.

4.7. FLOWERING IN VERY OLD PLANTS WITHOUT VERNALIZATION

Per definition plants with an absolute cold requirement will never come into flowering without vernalization. In 4.4 was shown that 16 weeks old plants flowered for 100% after only 4 weeks of vernalization. After this experiment the question arose whether flowering is possible in very old plants without any vernalization at all.

From December 1963 plants were grown in the greenhouse at approximately 20 °C. From the beginning all the plants were manured every three months with a NPK compound fertilizer. The total number of plants studied was 31.

At the end of the experiment in January 1966 some plants had strongly fasciated and 4 plants had flowered; the first visible flower bud was observed in August 1965. Hence, 4 out of 31 (13%) plants flowered without vernalization. It should be stressed that flowering in all cases was rather abnormal, because the first poor flowering was always observed in lateral buds. The realization of flowering was clearly retarded. Finally, however, the terminal buds also flowered.

4.8. Discussion

Light conditions. – The influence of light before, during and after vernalization on flowering of *Lunaria* is very small. For unknown reasons, a promoting effect of additional light before the vernalization (Wellensiek and Higazy, 130) could not be found. The effectiveness of vernalization in *Lunaria* does not increase by increasing the daylength during the cold treatment, as was also demonstrated in *Oenothera biennis* by Picard (84). The reverse was observed for *Matthiola incana* (Tsukamoto and Konishi, 111). Although flowering is promoted in *Cardamine pratensis* (Pierik, 88) by increasing the light intensity during the vernalization, light has no influence on *Lunaria*. In accordance with earlier reports (Barendse, 10, Mathon and Stroun, 65, Napp-Zinn, 76), the role of light during vernalization, as compared in different plants, remains contradictory. A quantitative effect of LD as compared with SD after the vernalization on flowering of the biennial honesty confirms Wellensiek's (119) results. *Lunaria* seems to be one of the few cold requiring plants without an absolute LD-requirement for flowering after the vernalization.

Vernalization of young plants. – As compared with the effect of seed vernalization (Wellensiek, 119), the effect of cold on 3 to 5 weeks old plants was very slight. This may indicate that the sensitivity for cold varies with age, a phenomenon which was earlier described by Napp-Zinn (75) for *Arabidopsis* and by Barendse (10) for *Cheiranthus*. Several authors have shown (cf. Lang, 58) that thermoinduction is fully effective when given during seed development. Prenatal vernalization in *Lunaria*, however, appeared to be impossible.

Varying and constant low temperature. – The influence of varying low temperatures, as compared with constant low temperature during the vernalization on flowering is sometimes positive (Wellensiek, 127, Picard, 84), sometimes negative (Ito and Saito, 49, Purvis, 91, Lang, 58). Although flower realization in *Lunaria* is promoted by constant low temperature treatment, it should be kept in mind that the total quantity of cold was most probably less after varying low temperature treatment; both treatments are, therefore, not fully comparable.

Devernalization. – Realization of flowering in *Lunaria* was retarded when a high temperature was applied after the vernalization. This seems comprehensible, because in nature *Lunaria* prefers a relatively low temperature for flower bud realization. Remarkably, however, no devernalization occurred in suboptimally induced plants, even when high temperature was applied immediately after the vernalization. This is contradictory to the existing literature (Purvis, 91, Barendse, 10) on high temperature devernalization. The possibility cannot be excluded that devernalization would have occurred under other conditions, e.g. SD or low light intensity, than used.

Seed formation at low temperature. – Schwabe (100) concluded that high temperature devernalization during ripening of the seed may be excluded

as the cause of the unvernalized state; the production of unvernalized seeds by a vernalized parent plant must be accounted for by some event in the early origin of the seed, likely to be connected with meiosis and zygote formation. WELLENSIEK (127) supposed that the disappearance of the vernalized condition was caused by devernalization during meiosis. BARENDSE (10) thought that the vernalized condition disappears at least at meiosis but probably earlier. My work demonstrates that high temperature devernalization both during meiosis, pollination and seed formation can be excluded as the cause of the disappearance of the flowering condition.

Flowering without vernalization. – Whether the term 'absolute cold requirement for flowering' can be maintained, is disputable since some flowering was observed in *Lunaria* without any cold treatment. Similar cases were observed: *Dactylis glomerata* (BLONDON, 11) and *Geum urbanum* (CHOUARD, 14). In *Dactylis* and *Geum* good mineral nutrition induced flowering; it is possible that this holds true also for *Lunaria* where a fertilizer was applied. The occurrence of fasciations in old *Lunaria* plants indicates that the physiological condition of the plants is drastically changed, possibly resulting in flowering without any vernalization. That good nutrition is conducive to the appearance of fasciations was earlier demonstrated (cf. GORTER, 29). An indication that the cold requirement for flowering can possibly disappear follows from my observation that the number of weeks cold required for 100% flowering strongly decreased as the age of the plants at the beginning of the vernalization increased. One can imagine that at a certain rather high age a threshold will be reached where no vernalization requirement exists any more.

5. LOCALIZATION OF JUVENILITY

5.1. INTRODUCTION

Flowering of intact plants is often limited by the existence of a juvenile phase, during which no flower formation is possible. Whether juvenility in cold requiring plants is a character of the whole plant or whether it is localized, has been discussed by WELLENSIEK (122, 125, 127), who carried out vernalization experiments with leaf cuttings of juvenile and adult *Lunaria* plants and observed that none of the cuttings of juvenile plants flowered, while cuttings of adult plants flowered. WELLENSIEK (127, p.834) concluded: 'Evidently juvenility is a character of the whole plant, while the condition of the individual leaf is the same as that of the whole plant, no matter what its age'. LANG (58) pointed to two principal, alternative explanations for the inability of juvenile plants to form flowers: (1) the plants may not be able to generate the induced state and to produce flower hormone or (2) their growing points – the sites of actual flower formation – may not be able to respond to the hormone. LANG (58, p.1511) concluded: 'As far as the available evidence goes – and it is not extensive and limited to photoperiodic and dayneutral plants – it is in favour of the first alternative.

In a preliminary paper PIERIK (85) presented evidence that juvenility is not a character of the whole plant. The idea that juvenility in *Lunaria* is located in the bud meristems and not primarily in the leaves will further be developed by presenting studies mainly carried out with leaf cuttings. This technique has also been used by HAGEMANN (32), OEHLKERS (79, 80) and PIERIK (88).

5.2. REGENERATION OF LEAF CUTTINGS

HAGEMANN (32) supposed that regeneration of *Lunaria* leaf cuttings did not strongly depend on the age of the leaf, but my preliminary results (85) oppose this view. WELLENSIEK (122) found that regeneration of *Lunaria* leaf cuttings was much better when the leaves arose from juvenile plants than from adult ones. The questions remain whether regeneration depends on the age of the leaf and whether the regeneration ability is changing during the transition from the juvenile to the adult stage.

Because the regeneration ability is very important in leaf cutting vernalization, regeneration was studied in unvernalized leaf cuttings of *Lunaria* plants of different ages including juvenile and adult ones. By sowing weekly, an age series of 3, 4,... 9 weeks old plants was obtained. Cuttings were simultaneously taken from all leaves on plants of each age group. Each treatment consisted of 10 cuttings. The regeneration at ordinary greenhouse temperatures was studied for $8\frac{1}{2}$ weeks.

Table 5 shows that rooting percentage after 2 weeks first increases and later gradually decreases in both cotyledons and first leaf pairs when the age of the plants increases. Rooting percentage after $8\frac{1}{2}$ weeks is not influenced by the age of the mother plants nor by the type of leaf pair, besides that in cotyledons of

8 or 9 weeks old plants no regeneration occurs. Rooting contents, which gives a rough impression of the total root mass, gradually decreases in cotyledons with increasing age; in the first leaf pair it first increases and later decreases with increasing age. The percentages of sprout regeneration and the mean numbers of sprouts in cotyledons and first leaf pairs first increase and later decrease with increasing age of the mother plants.

TABLE 5. Percentages of rooted cuttings after 2 and 8½ weeks, rooting-contents in cm³ after 3 weeks, % of sprout regeneration after 8½ weeks and mean number of sprouts per cutting after 8½ weeks, in different leaf pairs, as influenced by the age of the mother plants.

leaf pair no.	age of the mother plants in weeks	% rooted after 2w.	% rooted after 8½w.	rooting contents	% of sprout regeneration	number of sprouts
cotyledon	3	80	100	19	60	0.9
	4	100	100	16	70	1.3
	5	40	100	5	70	1.4
	6	70	100	7	30	0.6
	7	50	80	6	60	1.0
	8	0	0	0	0	0.0
	9	0	0	0	0	0.0
1	4	40	50	5	0	0.0
	5	100	100	21	30	0.3
	6	100	100	58	30	0.8
	7	80	100	54	70	1.7
	8	40	100	46	30	0.4
	9	25	100	27	10	0.1
2	7	100	100	66	20	0.3
	8	90	100	91	30	0.3
	9	80	100	74	40	0.8

This experiment shows that very young leaves regenerate moderately, young fully expanding leaves regenerate optimally, whereas regeneration decreases with the further ageing of the leaves. Each leaf pair has its own regeneration optimum, which depends on the age of the plant: cotyledons regenerate optimally when the mother plants are 4 to 5 weeks old, first leaf pairs when the mother plants are 7 weeks old. Although the area of the cotyledons is very small, their regeneration ability is very high as compared with the first leaf pair. There are no indications that the transition from the juvenile to the adult phase in *Lunaria* is directly correlated with the regeneration ability.

5.3. VERNALIZATION OF LEAVES ON YOUNG INTACT PLANTS

HAGEMANN (32), OEHLKERS (80) and WELLENSIEK (125, 127) demonstrated that leaves on intact adult plants could be vernalized. The question remained whether leaves and/or petioles could also be vernalized when present on young plants.

By weekly sowings, an age series of 2, 3 or 4 weeks old plants was obtained. These plants were vernalized for 15 weeks. Immediately after the vernalization, leaf cuttings were made. Different times after the vernalization leaf cuttings were also made from newly formed leaves.

All leaf cuttings regenerated vegetative plants only, even when the leaves were completely primordial during the vernalization, while intact plants also remained vegetative. We conclude that vernalization of petioles and/or leaves on young intact plants is impossible.

5.4. Regeneration and vernalization of shoot and leaf cuttings

As described in earlier work (85), regenerating cells in the neighbourhood of the cut surface of all leaf cuttings could be induced to flowering, even in leaf cuttings of 'juvenile' plants. Juvenility was not located in the petioles of a 'juvenile' plant. However, the terminal meristem of shoot cuttings of 'juvenile' plants could not be induced to flowering. Juvenility was, therefore, supposed to be located in the bud meristems (see photo 1). The regeneration characteristics of leaf cuttings prior to the vernalization were identical with those given in 5.2. An addition is that a clear positive correlation existed between the regeneration ability and the % of flowering in cotyledons and first leaf pairs. This correlation was less clear for the other leaf pairs. The relation between mean number of days to budding after the end of the vernalization and % of flowering totalized over all leaf cuttings is given in table 6, which shows that the percentage of flowering is clearly related to the mean number of days to budding after the vernalization. Sprout formation, although not macroscopically visible, must have taken place during the vernalization, as within 10 days after the vernalization no sprouts could have been formed. This implies that the effectiveness of vernalization is strongly determined by the formation of (pro)meristems during vernalization.

Table 6. Mean number of days to budding after vernalization during 15 weeks and % of flowering.

days to budding	% of flowering
0 – 10	97
11 – 20	52
21 – 30	11
31 – 40	4
41 – 50	0
51 – 130	0

5.5. Vernalization of terminal and lateral buds of young plants

Results of Wellensiek and Higazy (130), Higazy (43) and the present author (see 4.4), demonstrating that suboptimal vernalization in *Lunaria* resulted in

lateral flowering instead of terminal flowering, led me to the following hypothesis: lateral buds are adult at an earlier stage than terminal ones.

By sowing periodically, plants of 5, 6 or 7 weeks old were obtained at the beginning of the vernalization. When the plants were 2 weeks old, all terminal buds were treated with a few drops of GA_3 (conc. 5.10^{-4}), which rapidly resulted in some stem elongation. This elongation made it possible to decapitate the plants just above the cotyledons, resulting in the activation of the two lateral buds in the axils of the cotyledons. The plants in all three age groups were decapitated, either 16, 15 or 14 weeks before the end of the vernalization of 14 weeks or at the end of the vernalization. Control plants were not decapitated. Unvernalized plants remained vegetative.

Table 7 shows that in 5 weeks old plants both lateral and terminal buds are juvenile; in 6 weeks old plants the lateral buds are juvenile and the terminal buds are partly juvenile, partly adult; in 7 weeks old plants the lateral buds are partly juvenile, partly adult, whereas the terminal buds are fully adult. The transition from juvenile to adult in lateral buds of 7 weeks old plants is accelerated by increasing the decapitation time before the end of the vernalization.

Hence, the transition from the juvenile to the adult stage starts in the terminal buds and later procedes to the lateral ones. The hypothesis that lateral buds are adult at an earlier stage than terminal ones must be rejected.

A side-observation was that the developmental pattern of lateral shoots had the same sequence as that of the terminal ones; the first leaf pair of the lateral shoots looked like the cotyledons or had a shape which was intermediate between cotyledon and first leaf pair.

TABLE 7. The influence of the decapitation time of the terminal meristem in weeks before the end of the vernalization during 14 weeks and of the age of the plants on % of flowering.

decapitation time in weeks	% of flowering		
	age of the plants		
	5 w.	6 w.	7 w.
0	0	0	0
14	0	0	0
15	0	0	11
16	0	0	20
–	0	30	100

5.6. AMPUTATION AND VERNALIZATION OF YOUNG PLANTS

The purpose of this experiment was to determine the influence of the cotyledons and the root system on the duration of the juvenile phase and on the initiation of flower buds after the vernalization.

Three weeks old plants, the cotyledons of which were developed only, were given different pretreatments as indicated in table 8. After the amputation, plants

of treatments numbers 1 and 2 (root system not amputated) were grown in the greenhouse for 3 weeks. In treatments numbers 3, 4 and 5 the cuttings were placed in a cutting-bed for 3 weeks in order to obtain root formation. When the control plants (no. 1) were 6 weeks old, the plants and cuttings were divided into 2 groups of 0 or 15 weeks vernalization. At the beginning of the vernalization all cuttings had regenerated roots. No flowering occurred in the unvernalized plants.

Considering the results presented in table 8 we should realize that the control plants were 6 weeks old at the beginning of the vernalization. This explains the 40 % flowering in the control plants, some of which were juvenile, some adult. The transition from the juvenile to the adult phase was partly inhibited in the treatments where the cotyledons were amputated. The amputation of the roots alone promoted flowering slightly; the amputation of the cotyledons and roots, however, promoted flowering clearly when compared with the amputation of the cotyledons alone. Cotyledon cuttings were the most sensitive parts of the plants, as the flowering % is nearly 100. It should be mentioned that 95 % of the cuttings of the cotyledons formed visible sprouts before the end of the vernalization.

TABLE 8. The influence of 15 weeks vernalization on the % of flowering and the mean number of days to budding of intact control plants and amputated young plants.

no.	pretreatment	%	days
1	intact	40	36
2	cotyledons amputated	17	62
3	roots amputated	47	22
4	cotyledons and roots amputated	32	29
5	cotyledon cuttings	90	12

A side-result was made which was reproduced in another experiment. Only in treatments 3 and 4, where the roots were amputated, all non-flowering plants elongated very clearly. This means that the amputation of the roots not only influences the duration of the juvenile phase but also enhances stem elongation.

5.7. DISCUSSION

Regeneration and age. – Regeneration ability in *Lunaria* was found to be strongly correlated with the age of leaves. This evidently is a generally occurring phenomenon, because similar results were e.g. reported for *Begonia* (HEIDE, 39) and *Streptocarpus* (HENGST, 40).

Vernalization of leaf cuttings. – A clear correlation was found, independent of the type of leaf pair considered, between the time of bud initiation in a leaf cutting and flowering. Formation of buds during the vernalization strongly promotes flowering. The high flowering percentage in cotyledon

cuttings, as compared with the other leaf pairs, seems also to be due to the high regeneration ability. The role of regeneration before and during the vernalization on flowering and the possible rejuvenating effect of regeneration will be discussed more extensively in chapter 10.

Localization of juvenility. – In order to explain the results in connection with the localization of juvenility, one should first distinguish between flower induction in a bud meristem and e.g. in petiole tissue without bud meristem. When the induction of flowering is only effectively possible with a bud meristem during the vernalization, as will be proved to be true in chapters 6 and 8, a comparison between the 2 types of induction is impossible. It is, therefore, fully comprehensible that petioles on intact young plants cannot be vernalized (5.3), whereas isolated bud-regenerating petioles of plants of the same age could be induced to flowering (5.4). In 5.4 was also shown that a shoot cutting of a young plant did not come into flowering after vernalization, whereas an isolated leaf cutting from the same plant flowered, after regeneration during 3 weeks prior to the vernalization. This means that juvenility is not a character of the whole plant, but is located in the terminal and in the lateral meristems. WELLENSIEK (122) found that none of the leaf cuttings of 6 weeks old plants came into flowering without regeneration before the vernalization and concluded that those leaf cuttings were juvenile like the mother plants. This conclusion is not necessarily correct, because the regeneration prior to the vernalization was possibly limiting for flowering rather than a juvenile phase.

Terminal and lateral meristems. – According to WANGERMANN (117), KRENKE observed that lateral shoots in herbaceous perennials undergo rejuvenation, i.e. they are at their inception physiologically younger than the node from which they arose. Decapitation experiments with *Cheiranthus allionii* (BARENDSE, 10) indicated that lateral buds from the basal nodes have to reach a certain size in order to make flower formation possible, i.e. possess a juvenile phase. DE CANDOLLE (cf. HAGEMANN, 32) observed that lateral shoots repeat the developmental pattern of the mother plant. All those facts fully agree with my observations in *Lunaria*, where the transition from juvenile to adult takes place in the terminal buds earlier than in the lateral ones. The acceleration of the transition of lateral buds from juvenile to adult seems to be a result of suppression of apical dominance by decapitation. HIGAZY (43), trying to elucidate the phenomenon of predominant flowering in lateral buds after suboptimal vernalization in *Lunaria*, supposed that relatively young plants perceived the low temperature by the leaves only, whereas old plants perceived the low temperature by the growing points. My experiments in 5.3 do not support this view; its seems more reasonable to suppose that vernalization removes apical dominance, resulting in an earlier transition of the lateral buds from juvenile to adult than terminal ones.

Amputation. – The amputation of the cotyledons before the vernalization had a disadvantageous effect on flowering. Similar results have been obtained

by TASHIMA (107), KIMURA (50) and KOJIMA, YAHIRO and INOUE (53) with *Raphanus sativus*. In *Lunaria* the amputation of the cotyledons has primarily a retarding effect on the transition from juvenile to adult and secondarily an unfavourable influence on flower induction. The cotyledons should be considered as an important source of carbohydrates which play a decisive role during the transition from juvenile to adult (WELLENSIEK and HIGAZY, 130) and during flower induction (PURVIS, 91). The amputation of the root system had a promoting effect on both stem elongation and flowering; the roots possibly delay the transition from juvenile to adult by promoting the vegetative growth and inhibiting the generative development. The fastest flower bud initiation and the highest flowering percentage have been observed in cotyledon cuttings. The high regeneration ability of cotyledons possibly shortens the rejuvenation period occurring after regeneration.

DE ZEEUW and LEOPOLD (20) reported that in *Brassica oleracea* the juvenile phase was shortened by NAA. It may be that a high auxin content of *Lunaria* cotyledons also promotes the transition from juvenile to adult in the terminal meristem of young plants and in regenerating cotyledons themselves. When juvenility in the terminal meristem should be considered as an inhibition (WELLENSIEK, 129), the possibility cannot be excluded that juvenile meristems exert an inhibiting influence on cotyledons of intact plants; this inhibition disappears after isolation of the cotyledons.

6. REGENERATION, VERNALIZATION AND FLOWERING OF COTYLEDON CUTTINGS

6.1. INTRODUCTION

OEHLKERS (79) demonstrated that cotyledons of *Streptocarpus* must reach a certain size before they are able to be vernalized. Cotyledon cuttings of *Lunaria*, however, could be vernalized, even when taken from juvenile intact plants (chapter 5).

Isolated cotyledons of *Lunaria* have a high regeneration ability, can easily be cultured and hence lend themselves to physiological studies in connection with flower induction. An additional, but very important advantage of cotyledons is that they occupy little space.

The origin of the experiments with cotyledon cuttings is WELLENSIEK's (127, p. 835) concept: 'Vernalization only takes place when dividing cells are present during the cold treatment'. Further research (PIERIK, 86, 88) with *Lunaria* and *Cardamine* indicated that the effectiveness of vernalization in plants with a juvenile phase strongly depends on the induction of sprouts during the cold treatment. In *Cardamine pratensis* full rejuvenation occurred as a result of regeneration.

The objects of the present chapter are the connections between dividing cells, rejuvenation and flower formation. Preliminary experiments showed that regeneration of both roots and sprouts could strongly be promoted by the application of auxins; GA_3 had an inhibitory effect on root formation, but sometimes promoted sprout formation; phytokinins inhibited primarily both root and sprout formation, although sprout formation was promoted later on.

6.2. DURATION OF VERNALIZATION

Cotyledon cuttings of 3 weeks old plants regenerated during 3 weeks in a heated greenhouse and were vernalized during 0, 5, 8, 11 or 14 weeks. After the vernalization the cuttings were placed in the greenhouse. Visible sprout formation often already took place during the vernalization. The percentages of flowering cuttings with or without sprout formation before the end of the vernalization were 60 and 0% respectively. This indicates that the effectiveness of vernalization depends on the formation of sprouts before and/or during the vernalization.

Table 9 shows that the mean number of sprouts per cutting decreased by the application of a cold treatment. The percentages of flowering cuttings and flowering sprouts rapidly increased with the duration of the vernalization.

6.3. DURATION OF REGENERATION BEFORE VERNALIZATION

Cotyledon cuttings of 3 weeks old plants regenerated during 0, 1, 2 or 3 weeks in the greenhouse. The rooting-capacity was scored by estimating the rooting-

contents in cm^3, just before the vernalization of 10 or 15 weeks. After the vernalization, the cuttings were placed in a greenhouse. No flowering occurred in the unvernalized cuttings.

TABLE 9. The effect of the duration of vernalization on the mean number of sprouts per cutting, % of cuttings with at least one flowering sprout and % of flowering sprouts.

vernalization in weeks	sprouts per cutting	% of flowering cuttings	% of flowering sprouts
0	2,1	0	0
5	1,5	0	0
8	1,6	20	13
11	1,4	78	62
14	1,6	89	68

Table 10 shows that within each duration of vernalization the mean number of days to sprouting decreases and the percentage of flowering increases by increasing the duration of the regeneration. The comparable mean number of days to sprouting and the percentages of flowering are always higher when the duration of the vernalization increases.

TABLE 10. The effect of the durations of regeneration and vernalization on the mean number of days to sprouting after the cuttings were made, % of cuttings with at least one flowering sprout and % of flowering sprouts.

regeneration in weeks	vernalization in weeks	days to sprouting	% of flowering cuttings	% of flowering sprouts
0	10	101	0	0
1	10	97	0	0
2	10	82	22	25
3	10	26	89	64
0	15	120	0	0
1	15	102	67	50
2	15	98	75	60
3	15	35	100	75

The rooting-contents for regeneration periods of 0, 1, 2 or 3 weeks were 0.0, 0.1, 21.0 and 64.3 cm^3 respectively. Hence a correlation exists between the rooting-contents before the vernalization and flowering. In the regeneration group of 3 weeks macroscopically visible sprouts were often formed before the vernalization started, probably as a result of good rooting. The percentages of flowering cuttings with or without visible sprout formation before the end of the vernalization were 100 and 6% respectively. Photo 2 shows the regeneration and flowering of cotyledon cuttings after the vernalization.

6.4. The effect of growth substances

6.4.1. *Indoleacetic acid*

The purpose of the present experiment was to test whether promotion of regeneration by IAA also results in a higher percentage of flowering after vernalization.

Cotyledon cuttings of 3 weeks old plants were placed in IAA solutions of different concentrations at 26°C under continuous strong fluorescent light, where they remained 4 days. Auxin treatment was followed by a regeneration period in a cutting-bed of a heated greenhouse during 10 days. After the regeneration, unvernalized control cuttings remained vegetative. The other cuttings were vernalized during 10 weeks. After the vernalization the cuttings were placed in the greenhouse.

Table 11 shows that the mean number of days to sprouting tends to decrease by increasing IAA concentrations. The mean number of sprouts per cutting is slightly increased by all IAA treatments. The percentages of flowering cuttings and sprouts increase by increasing IAA concentrations, be it somewhat irregularly.

Table 11. The influence of different IAA concentrations and 10 weeks of vernalization on the mean number of days to sprouting after the cuttings were made, the mean number of sprouts per cutting, the percentage of cuttings with at least one flowering sprout and the percentage of flowering sprouts.

IAA concentrations	number of days to sprouting	number of sprouts per cutting	% of flowering cuttings	% of flowering sprouts
0	112	1,1	15	13
5.10^{-8}	104	1,4	45	36
10^{-7}	114	1,3	25	20
5.10^{-7}	106	1,3	20	15
10^{-6}	96	1,2	50	42
5.10^{-6}	94	1,2	72	58
10^{-5}	89	1,3	72	54
5.10^{-5}	91	1,2	86	73
10^{-4}	88	1,3	76	59

The promotion of regeneration by IAA in unvernalized control cuttings was almost the same as that of vernalized ones. The percentages of flowering cuttings of all treatments with or without visible sprout formation before the end of the vernalization were 90 and 44% respectively.

6.4.2. *Gibberellic acid*

Cotyledon cuttings of 3 weeks old plants were placed in GA_3 solutions of different concentrations at 26°C under continuous strong fluorescent light, where they remained 4 days. The GA_3 treatment was followed by a regeneration period during 14 days in a cutting-bed of a heated greenhouse. After regeneration the percentages of rooted cuttings were determined. Unvernalized cuttings

remained vegetative. Other cuttings were vernalized during 10 weeks and afterwards placed in the greenhouse.

Table 12 shows that rooting was hampered by increasing GA_3 concentrations. The mean number of days to sprouting decreased by lower and increased by higher GA_3 concentrations. The mean number of sprouts per cutting tends to decrease, be it irregularly, with increasing GA_3 concentrations. The percentage of cuttings with sprouts is not effected by the lower but gradually decreases with the higher GA_3 concentrations. Flowering was promoted by the lower but hampered by the higher GA_3 concentrations.

TABLE 12. The influence of different GA_3 concentrations and 10 weeks of vernalization on % of rooted cuttings at the beginning of the vernalization, the mean number of days to sprouting after the cuttings were made, the mean number of sprouts per cutting, % of sprout-regenerating cuttings, % of flowering cuttings (at least one flowering sprout per cutting) and % of flowering sprouts.

GA_3 conc.	% of rooted cuttings	days to sprouting	number of sprouts per cutting	% of cuttings with sprouts	% of flowering cuttings	% of flowering sprouts
0	98	92	1.5	100	52	39
10^{-8}	95	72	1.3	100	86	74
5.10^{-8}	95	88	1.4	100	50	38
10^{-7}	90	90	1.3	100	57	50
5.10^{-7}	84	86	1.3	90	67	53
10^{-6}	84	94	1.1	86	37	29
5.10^{-6}	81	105	1.2	85	40	37
10^{-5}	80	100	1.1	76	38	35
5.10^{-5}	56	115	0.8	45	20	24

The percentages of flowering cuttings of all treatments with and without sprout formation during the cold treatment were 96 and 24% respectively. We can conclude that rooting is a prerequisite for sprout formation. GA_3 is able to shift the morphogenetical balance in different directions, depending on the concentration used.

6.4.3. *Phytokinins*

Cotyledon cuttings of 3 weeks old plants were placed in phytokinin solutions of different concentrations at 26°C under continuous strong fluorescent light and kept there for 4 days. The phytokinin treatment was followed by a regeneration period of 10 days in a cutting-bed of a heated greenhouse. After regeneration the percentages of rooted cuttings were determined. Unvernalized control cuttings in the greenhouse remained vegetative. Other cuttings were vernalized during 10 weeks and afterwards placed in the greenhouse..

Table 13 shows that most percentages of rooted cuttings and all mean number of sprouts per cutting decreased, whereas the mean number of days to sprouting increased by phytokinin application. The percentage of cuttings with sprouts

was not affected by phytokinins. The percentages of flowering cuttings and flowering sprouts drastically decreased by phytokinins.

TABLE 13. The influence of phytokinins in two concentrations and 10 weeks of vernalization on % of rooted cuttings before vernalization, the mean number of days to sprouting after the cuttings were made, the mean number of sprouts per cutting, % of cuttings with sprout formation, % of cuttings with at least one flowering sprout and % of flowering sprouts.

phytokinin	conc.	% of rooted cuttings	days to sprouting	number of sprouts per cutting	% of cuttings with sprouts	% of flowering cuttings	% of flowering sprouts
control (water)	0	90	105	1,8	100	23	13
kinetin	10^{-7}	70	109	1,5	100	12	8
kinetin	10^{-6}	43	124	1,2	100	0	0
SD 4901	10^{-7}	35	108	1,3	100	0	0
SD 4901	10^{-6}	17	120	1,5	100	0	0
SD 8339	10^{-7}	96	112	1,3	100	6	4
SD 8339	10^{-6}	43	124	1,4	94	0	0

The percentages of flowering cuttings of all treatments with or without sprout formation during the vernalization were 50 and 0% respectively.

6.4.4. *Indoleacetic acid and kinetin*

The differentiation of roots and buds on tobacco pith tissue could be regulated by the relative amounts of auxin and phytokinin applied (SKOOG and TSUI, 102). The question arose whether the same holds true for *Lunaria* cuttings and whether the application of combinations of growth substances influence flowering after vernalization.

Cotyledon cuttings of 3 weeks old plants were placed in solutions of the growth substances at 26°C under strong continuous fluorescent light where they stayed for 4 days. This treatment was followed by a regeneration period during 10 days in a cutting-bed of a heated greenhouse. The percentages of rooted cuttings were determined at the end of this regeneration period. Unvernalized cuttings remained vegetative in the greenhouse. Other cuttings were vernalized during 10 weeks and afterwards placed in the greenhouse.

Table 14 shows that without kinetin rooting, sprouting and flowering are promoted by increasing the IAA concentration, which confirms the result in 6.4.1. Without IAA, rooting, sprouting and flowering decreased by increasing the kinetin concentration, which confirms the result in 6.4.3. The combination of IAA and kinetin generally resulted in decreased regeneration and flowering as compared with the control without growth regulators, with one exception: IAA 10^{-6} + kinetin 10^{-7}. The inhibiting effect of kinetin on regeneration and flowering tends to be removed in some cases by the addition of IAA.

The percentages of flowering cuttings with or without sprout formation during the vernalization were 95 and 14% respectively.

TABLE 14. The influence of IAA and kinetin in different concentrations and 10 weeks of vernalization on the percentage of rooted cuttings, the mean number of days to sprouting after the cuttings were made, the mean number of sprouts per cutting, the percentage of cuttings with at least one flowering sprout and the percentage of flowering sprouts.

IAA conc.	kinetin conc.	% of rooted cuttings	days to sprouting	sprouts per cutting	% of flowering cuttings	% of flowering sprouts
0	0	86	94	1.5	57	42
10^{-7}	0	94	72	1.3	74	64
10^{-6}	0	100	71	1.3	70	58
0	10^{-7}	84	101	1.2	47	39
10^{-7}	10^{-7}	86	106	1.3	26	21
10^{-6}	10^{-7}	91	96	1.4	62	45
0	10^{-6}	47	115	1.3	0	0
10^{-7}	10^{-6}	73	115	1.4	20	14
10^{-6}	10^{-6}	73	115	1.3	6	4

6.5. DISCUSSION

Regeneration and growth regulators. – The influence of IAA and phytokinins on regeneration of *Lunaria* cotyledons appeared to be quite different from the picture given by SKOOG and TSUI (102) for tobacco pith tissue. Promotion of root formation by auxins resulted in promotion of sprout formation, whereas inhibition of root formation by phytokinins decreased sprout formation. The common view that auxins inhibit and phytokinins promote bud formation (SKOOG and TSUI, 102) is, therefore, not always valid (HAISSIG, 33, WURM, 132). Rooting and shoot formation of *Lunaria* cuttings were inhibited by GA_3, which is in accordance with the literature, cf. MURASHIGE (73).

The regeneration characteristic of *Lunaria* cotyledons is completely identical with that obtained by HARRIS and HART (36) with *Peperomia*, who demonstrated that bud initiation in leaf squares does not occur independently of rooting: when rooting was delayed, bud formation was affected similarly.

Cell division, regeneration and flower induction. – The concept that vernalization only occurs in cells which are in the process of mitosis (WELLENSIEK, 125, 127) was critizized by some authors as to its general occurrence. GRIF (cf. LANG, 58) e.g. showed that any growth activity, including mitosis, is completely suspended at a temperature of -2 °C, although vernalization is still possible at this and at even lower temperatures. BARENDSE (10) could not find mitoses during the vernalization of *Cheiranthus* seeds and concluded that this result opposes the concept of dividing cells as the prerequisite for vernalization. KREKULE (56) supposed that the concept of dividing cells is possibly too much generalized for other plants.

The percentages of flowering cuttings with (+) or without (–) visible sprout formation before and during the vernalization, which are listed below:

	sprouts	
chapter	+	–
6.2.	60	0
6.3.	100	6
6.4.1.	90	44
6.4.2.	96	24
6.4.3.	50	0
6.4.4.	95	14

clearly demonstrate that the formation of sprouts before and during the vernalization markedly promoted the effectiveness of vernalization. Because cell divisions always precede regeneration, they are definitely a prerequisite in order to reach a certain stage in the regeneration process which is necessary for the action of low temperature. The sequence: cell divisions → root formation → sprout formation in leaf cuttings should be considered as inevitable to reach a certain ripeness to flower and/or to escape rejuvenation, so that WELLENSIEK's concept is considered to hold true for *Lunaria*.

7. THE APPLICATION OF STERILE CULTURE TECHNIQUES

7.1. LITERATURE

Although the number of publications devoted to problems connected with flowering is enormous, the reverse is true for in vitro studies on flowering: nearly 80 at the completion of this manuscript. These in vitro studies can roughly be divided into 4 groups: intact plants raised from sterilized seeds; embryos; shoot-tips; tissues without buds or sprouts. The present investigations almost exclusively deal with isolated tissues without buds or sprouts. Hence, only a literature survey in connection with this work will be presented.

SKOOG (see AGHION-PRAT, 5) was the first to observe a small flower bud in regenerating explants of vegetative tobacco plants, but the pioneer work on flower bud formation in isolated *Nicotiana tabacum* tissues was carried out in France by CHOUARD and AGHION (15) and by AGHION (1, 2, 3, 4). In the first publication (15), the existence of a gradient reflecting the ability to form flower buds along the stems of tobacco plants was observed. Later, this gradient of organogenesis was also observed in intact plants (4). The formation of floral buds did not take place in complete darkness or without the addition of sugar to the culture medium (1, 3). A review on flowering in tobacco stem segments was published in 1965 by AGHION-PRAT (5). The influence of different environmental factors on flower bud formation in *Nicotiana suaveolens* was dealt with by PAULET (81).

Cichorium intybus was extensively studied by PAULET (81). The formation of flower buds on excised root tissues from vernalized plants was inhibited by IAA and SD, and was promoted by certain phenolic compounds, GA_3 and LD. No flowering was observed in unvernalized root tissues (82, 83). Flower buds could also be produced on excised tissues from the veins of the leaves, provided the plants were vernalized previously (78). MARGARA (62) observed flower bud formation in stem segments of flowering plants. Several factors affecting flowering on explants of the floral stalks or roots were described by MARGARA cs. (64). Sometimes explants from unvernalized chicory roots formed floral buds (61, 63, 64), while the intact plants remained vegetative. The first report on flower induction in isolated root tissues of vegetative chicory plants after cold treatment was from PIERIK (87).

In vitro control of floral bud morphogenesis in *Ranunculus sceleratus* was described by KONAR and NATARAJA (54, 55), who observed a few cases of flower buds originating from the callus tissue which was derived from floral primordia, but in most cases newly differentiated plants appeared which formed flower buds after 10 weeks of culture. YOKOYAMA and JONES (133) reported that certain ratios of kinetin and IAA induced shoot formation in the dark in callus tissues of *Arabidopsis thaliana;* in the light these shoots produced flower buds.

PIERIK (85, 86) reported preliminarely on the induction and initiation of

floral buds in isolated tissues of *Lunaria*. More extensive data will be described in the following 2 chapters.

7.2. Material and methods

7.2.1. *Plant material*

All plant material was obtained from plants grown in the greenhouse. Petioles of vegetative plants were cut from the youngest leaf pairs, which are capable to regenerate sprouts. Flowering plants were obtained after a long vernalization period; in most experiments plants in full bloom without visible seed and fruit formation were used.

For flower induction experiments vegetative petiole pieces of a uniform lenght (usually 2.5 cm) of adult plants were used. The petiole pieces were always inserted in the medium with their basal ends upwards in order to promote oxygen supply and regeneration (85).

For flower initiation experiments explants from the elongated main axes of generative plants were used, because regeneration of these explants was much faster than of petiole pieces. The explants were always divided into two parts by splitting in longitudinal direction. Explants from the basal to the terminal portion of the axes were isolated in normal position (basal end down). The length of the explants was 2 cm, the diameter depended on the original position of the explant. The explants were always isolated without nodes and buds. All experiments with parts of flowering plants were carried out by varying a single growth factor and keeping all the other growth factors constant.

For each experiment at least 5 different plants in the same developmental stage were used. The explants were distributed at random over the treatments. The number of explants per treatment was at least 12. The ends of the explants were placed in the medium to a depth of about half their length.

The experimental data varied sometimes, depending on the physiological condition of the plant material used, and on the season. Therefore, experiments performed under the same set of environmental growth conditions are not fully comparable still.

Because flowering sprouts were sometimes invisible from outside the test tube, sprouts were always dissected and examined for flower bud initiation at the end of all experiments.

7.2.2. *Sterile culture techniques*

The sterile culture techniques employed were principally the same as those described by Gautheret (25). Tissues of *Lunaria* were grown aseptically on 0.6% Difco Bacto-agar in standard pyrex glass-tubes (height 17.0 cm, diameter 2.2 cm) plugged with cotton and covered by aluminium foil. Before autoclaving, all media were adjusted to pH 6.0 with a few drops of NaOH or HCl solution. All components, including growth substances, coconut milk or plant extracts, were dissolved in hot pyrex-distilled water. Culture tubes contained approximately 20 cm^3 culture solution.

Basic media contained pyrex-distilled water, agar, KNOP's or HELLER's minerals, HELLER's trace elements (for composition cf. GAUTHERET, 25, p.15) and glucose (1 to 3% by weight). In some experiments coconut milk (0 to 15% by volume) of immature nuts was used. Plant extracts were obtained by grinding a weight quantity of frozen leaves or flowers with an equal weight quantity of water; this mixture was filtered under reduced pressure and the filtrate used. The concentration of growth substances is given in weight per volume. All media and added substances or extracts were autoclaved at 112°C (0.6 kg cm^{-2}) during 20 minutes.

Before sterilization of plant parts, leaves were always removed. The plant parts were first immersed in 70% alcohol for a few seconds to remove air and water from the surface, followed by sterilization in calcium hypochlorite solution (5–15 minutes, concentration 50 g/l), washed with sterilized tap water for 80 minutes, finally prepared in a room which was previously sterilized by ultraviolet irradiation and 96% alcohol. Dissection instruments were sterilized by immersion in 96% alcohol, followed by flaming. Sterile, dissected explants were blotted under sterile wetting-paper to remove excess water and cut into segments prior to placing them on the culture medium.

The cultures were usually grown at 25–26°C under a continuous illumination of fluorescent light (Philips TL 40W/29). Care was taken that light intensity in all test tubes was nearly the same, 37.000 erg ⌀ cm^{-2} sec^{-1}, by placing the test tubes in holes of wooden blocks which were placed between two light sources. Experiments at 5°C were performed in LD under the same type of illumination as in the growth chamber at 25–26°C.

8. THE INDUCTION OF FLOWERING IN VITRO

8.1. Introduction

The purpose of this chapter is to study whether induction of flowering by cold is possible in isolated tissues. The first question which had to be answered was whether flower induction would be possible in isolated tissues in which a pre-existing sprout was present at the beginning of the cold treatment. When this question was answered positively, flower induction studies were initiated in tissues without pre-existing sprouts when the vernalization started. The outline of these studies is schematically representated in figure 1, which shows that flower induction in vitro was studied with both undifferentiated tissues (callus) and differentiated tissues (petiole pieces). Callus tissues which were obtained by inducing callus formation in isolated petioles of vegetative plants, were subjected to low temperature in order to check whether flowering sprouts could be obtained from the vernalized callus after the cold treatment. Later on flower

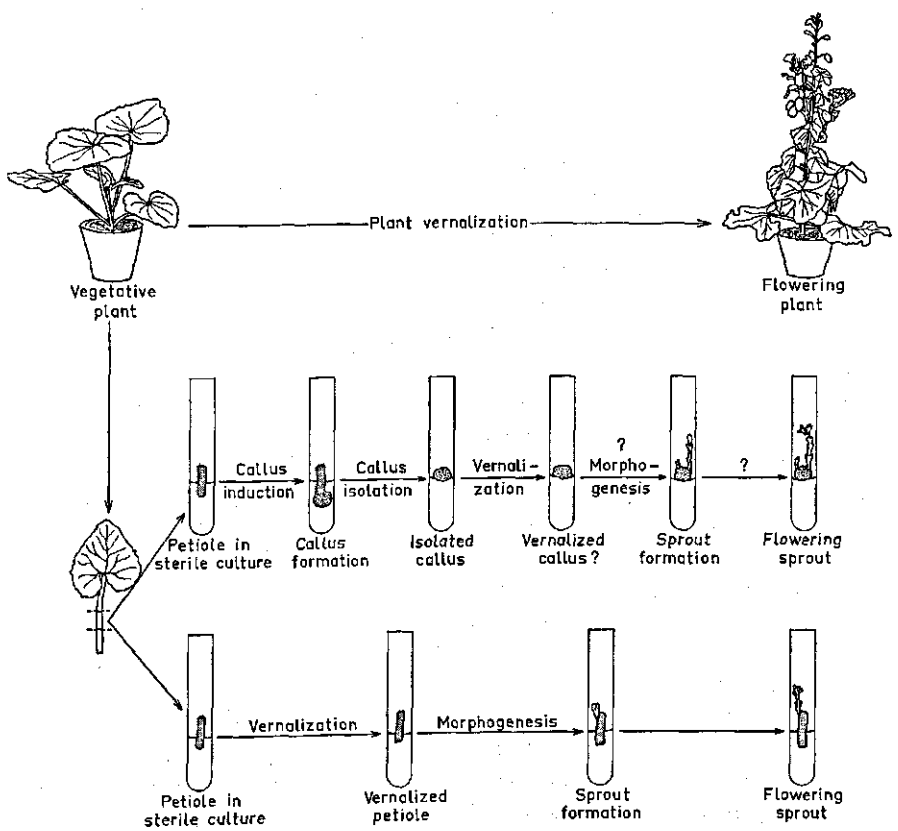


FIG. 1. Schematic representation of flower induction studies with isolated tissues of *Lunaria annua*. Explanation in text.

induction studies were also started with pith tissues. Petiole pieces of vegetative plants were isolated, subjected to low temperature and later transferred to a higher temperature where sprout formation and flowering were obtained.

An essential prerequisite for studies of morphogenesis of the type described above is the formation of buds or sprouts, which sometimes depends on root formation. For that reason special attention has also been paid to possible promotive effects of growth regulators (auxins, phytokinins, GA_3) on root and bud formation.

8.2. Vernalization of juvenile shoot cuttings

In 5.4 was shown that juvenility is located in the bud meristems of young plants. The question arose whether vernalization of shoot cuttings of such plants in vitro is possible.

Shoot cuttings of 3 weeks old plants without roots and cotyledons were isolated in vitro on a basic culture medium: Knop's macroelements, Heller's microelements, glucose 3% and agar 0.6%. Four treatments were given: basic medium (b.m.), b.m. + IAA 10^{-7}, b.m. + kinetin 10^{-7}, b.m. + IAA 10^{-7} + kinetin 10^{-7}. Immediately after isolation the cuttings were vernalized during 0 or 15 weeks. After the vernalization the cultures were placed at 20 °C in continuous fluorescent light.

Root formation was stimulated by the combination of IAA and vernalization. The growth of the cuttings was promoted by vernalization. None of the cuttings, however, came into flowering, which indicates that juvenility in the terminal meristem is fully maintained in vitro.

8.3. Vernalization of callus tissue

The purpose of this project was to study flower induction in undifferentiated callus tissues and to obtain a flowering sprout from the possibly vernalized callus tissue.

We first tried to induce callus formation in petiole pieces of vegetative *Lunaria* plants, following a procedure which was extensively described earlier (85). Further experiments showed that petioles of rather old vegetative plants could not be used, because enzymatic browning occurred. Therefore, only petioles of young plants were used, in which no browning occurred. Photo 3 shows the formation of callus tissue in isolated petiole segments of young *Lunaria* plants.

Green callus tissues were isolated on different media in order to determine the most favourable set of growth conditions for subculture. The growth of isolated callus tissues was slow under all conditions used. The green colour originally present in the isolated callus gradually disappeared; the tissues lost chlorophyll and turned yellow. This process could not be prevented by adding phytokinins.

A very difficult problem arose when bud formation in yellow-coloured callus tissues turned out to be almost impossible even when phytokinins were added

to the medium: the loss of chlorophyll was accompanied by the loss of morphogenetical potencies.

In several experiments callus tissues were exposed to a cold treatment during a long time in order to make sure that tissues which were possibly vernalized, were obtained. During such a long period, however, the tissues turned yellow, as was also shown for unvernalized tissues. Sprout formation occurred after subculture even on phytokinin containing media only exceptionally; sprout development was always blocked. The sprouts which rarely were formed had quite an abnormal appearance (see photo 4). For those reasons we were forced to discontinue our callus vernalization experiments. The question whether callus vernalization is possible could therefore not be answered.

8.4. Vernalization of pith tissue

Pith tissue was isolated from the subapical region of 6 months old vegetative plants. The basic medium was the same as the one described in 8.2. Several growth regulators, especially phytokinins, were added to the media in different concentrations in order to study their possible effects on sprout formation. The result was disappointing, because all explants completely browned and only one explant formed sprouts.

Although the result of the first experiment was negative, a second one was carried out on the same basic medium, with different kinetin concentrations. Immediately after the isolation of the pith tissue of two years old plants, the explants were vernalized during 0 or 20 weeks. After the vernalization the explants were placed in continuous fluorescent light at 20°C. Both control and vernalized cultures rapidly browned and died almost fully. Only 8% of the unvernalized cultures sprouted. Vernalized explants died without any sprout formation. Hence, the question whether pith tissue vernalization is possible remained unanswered.

A quite remarkable side observation was made in one of the sprout-regenerating unvernalized pith cultures. This explant on the basic medium with kinetin 8.10^{-7} formed one sprout, which came into flowering 4 months after the isolation!

8.5. Vernalization of petioles

8.5.1. *Introduction*

Three types of differentiated tissues of vegetative *Lunaria* plants were used originally in order to study flower induction in vitro: root tissue, leaf blade tissue and petiole tissue. Sterile isolation of root and leaf blade tissue appeared to be impossible owing to internal infections or damage as a result of calcium hypochlorite sterilization. Petiole tissues, however, appeared to be a very suitable material which can easily be sterilized.

8.5.2. *Regeneration of petioles as influenced by the age of the plants, growth regulators and temperature treatment*

In preliminary experiments in vitro (85) the following regeneration characteristics of *Lunaria* petioles were already shown:

- Regeneration is strongly promoted by placing the petiole explants upside down on the medium.
- Root formation is promoted by the combination of low temperatures and IAA.
- Sprout regeneration is promoted by higher temperatures.

Because root and sprout formation play an important role during flower induction, we shall first summarize the results of further regeneration studies, independent of flowering.

The age of the leaf pair plays a decisive role. When petiole explants of different leaf pairs of one plant are isolated, the petioles of the youngest leaves with expanding leaf blades regenerate optimally, petioles of older leaves regenerate moderately, whereas petioles of the oldest leaves regenerate poorly or not. For that reason the youngest leaf pairs have always been used. WELLENSIEK's (122) observation that leaf cuttings of young plants regenerate much better than those of old plants could be confirmed in vitro.

The influence of IAA on regeneration depends on the age of the plant. Regeneration of both roots and sprouts in petioles of young plants at 25°C has been promoted by IAA (see photo 5). Regeneration of roots at 25°C in petioles of old plants, on the contrary, was hardly promoted by IAA, whereas sprout formation was even inhibited. It could be demonstrated that promotion of sprout formation by IAA only occurs via the promotion of root formation, which depends on the age of the plant. Although roots are not a prerequisite for sprouting, root formation generally promotes sprouting.

The phytokinins kinetin, adenine, SD4901, SD8339 usually inhibited root formation and only slightly promoted sprout formation. GA_3 inhibited root formation, whereas the influence of GA_3 on sprout formation was rather obscure; sometimes promotion, but usually a slight inhibition of sprout formation occurred.

The effect of temperature on regeneration was examined in a phytotron experiment. Root formation in petioles of relatively young plants increased with decreasing temperatures. Sprout formation, however, increased with increasing temperatures.

8.5.3. *Vernalization with pre-existing sprouts*

In order to ascertain whether flower induction is possible in isolated petiole tissues with pre-formed sprouts, petiole pieces of 3 cm of 9 months old vegetative *Lunaria* plants were placed on a simple culture medium: agar, KNOP's minerals, HELLER's trace elements, glucose 0.8%. These petioles regenerated a great number of sprouts per explant at 26°C within 10 weeks. After this regeneration, vernalization was applied for 0, 8 or 16 weeks.

Only after a vernalization period of 16 weeks flowering occurred at 26°C. The percentage of flowering sprouts reached the 26. The percentage of explants with at least one flowering sprout reached the 62. This experiment shows that flower induction is possible in explants with sprouts before the vernalization. Flower induction in all pre-formed sprouts, however, seems to be limited when too large a number of sprouts is present per explant.

8.5.4. *Vernalization without pre-existing sprouts*

8.5.4.1. Duration of vernalization

Petiole pieces of 1 cm of 13 months old vegetative *Lunaria* plants were placed on a simple culture medium: agar, KNOP's minerals, HELLER's microelements and glucose 1 %. IAA (4.10^{-7}) was added to the medium to prevent sprout formation before and during the vernalization. The petioles were vernalized during 0, 3, 6, 9, 12 or 15 weeks after a period of 2 weeks at 26°C. No visible sprout formation was observed during vernalization. After the cold treatment the cultures were placed at 26°C.

Table 15 shows that the percentage of rooting and the mean number of root primordia per explant increased by the vernalization. It was not clear whether sprout formation was effected by vernalization. Only after a vernalization period of 12 or 15 weeks some flowering occurred. Although no visible sprout formation occurred before or during the vernalization, flower induction was possible (see photo 7).

TABLE 15. The influence of the duration of vernalization on % of rooting, on the mean number of root primordia per explant, on % of sprouting, on the mean number of sprouts per explant and on % of flowering sprouts of isolated petioles of *Lunaria* at 26°C.

duration of vernalization in weeks	% of rooting	root primordia per explant	% of sprouting	sprouts per explant	% of flowering sprouts
0	52	1,2	62	0,6	0
3	83	3,6	58	0,6	0
6	92	3,3	67	0,7	0
9	100	6,3	50	0,5	0
12	100	3,6	40	0,4	14
15	90	4,9	55	0,6	19

8.5.4.2. The effect of IAA during vernalization

The purpose of this experiment was to examine the influence of IAA in different concentrations during vernalization. Petiole pieces of 3 cm of 13 months old vegetative *Lunaria* plants were placed on a basic culture medium, (agar, KNOP's macroelements, HELLER's microelements, glucose 1 %) containing IAA of concentrations 0, 5.10^{-8}, 10^{-7}, 5.10^{-7}. No sprouts were present before the vernalization during 0 or 16 weeks which was started after a period of 2 weeks at 26°C. After the vernalization the cultures were placed at 26°C.

Table 16 demonstrates that without IAA never rooting occurred in both unvernalized and vernalized cultures. The percentages of rooted petioles, the mean number of root primordia per explant and the mean length of the longest root were slightly promoted by IAA without vernalization, but were strongly promoted by IAA with vernalization. The percentages of sprouting decreased by IAA in unvernalized petioles, but slightly increased by IAA in vernalized petioles. The mean numbers of sprouts per explant decreased by IAA in both unvernalized and vernalized petioles, but were always higher after vernalization. No flowering occurred without vernalization. After 16 weeks of vernalization a relatively high percentage of flowering sprouts occurred on the medium without IAA, whereas almost no flowering was observed with IAA in the medium.

TABLE 16. The influence of vernalization and IAA concentration on % of rooted petioles, on the mean number of root primordia per explant, on the mean length of the longest root, on % of sprouting petioles, on the mean number of sprouts per explant and on % of flowering sprouts.

vernalization in weeks	IAA conc.	% of rooted petioles	root primordia per explant	root length in cm	% of sprouting	sprouts per explant	% of flowering sprouts
0	0	0	0,0	0,0	90	2,8	0
0	5.10^{-8}	13	0,1	0,1	75	1,3	0
0	10^{-7}	13	0,1	0,1	75	1,2	0
0	5.10^{-7}	16	0,1	0,3	27	0,5	0
16	0	0	0,0	0,0	67	4,7	31
16	5.10^{-8}	50	0,7	4,0	67	1,8	0
16	10^{-7}	72	1,0	4,5	86	2,7	6
16	5.10^{-7}	66	3,4	4,7	73	3,0	0

The root formation was completed during the vernalization. The bud formation was inhibited by IAA during the vernalization. Without IAA bud formation already occurred during the cold treatment. The inhibition of bud formation by IAA completely disappeared after the vernalization. The influence of IAA and temperature treatment on regeneration of petioles is shown in photo 6.

Flowering occurred nearly exclusively in those vernalized cultures which regenerated sprouts during the vernalization. The effectiveness of vernalization, therefore, fully depended on the formation of sprouts during the vernalization.

8.5.4.3. The effect of different environmental factors

The purpose of the present experiment was to examine the influence of various other environmental factors (glucose, mineral nutrition, phytokinins, coconut milk) on flowering after petiole vernalization.

Petiole pieces of 2 cm of 14 months old vegetative *Lunaria* plants were placed on 18 different culture media. The petioles were vernalized during 0 or 20 weeks after a period of 2 weeks at 26 °C. The vernalization period was extended until 20 weeks in order to promote possible flower induction. After the vernalization

the cultures were first grown for 4 weeks at 20°C in order to prevent possible devernalization, subsequently at 26°C.

Averaged over all treatments, the percentages of petioles with sprout formation were 39 and 17%, the mean numbers of sprouts per explant were 0.9 and 0.4 and the percentages of flowering sprouts were 0 and 12% for unvernalized and vernalized cultures respectively. The percentage of sprout-forming petioles after vernalization remained very low, so that no complete conclusions could be drawn. Certain points were clear, however. Flowering almost exclusively occurred in those petioles which already formed visible sprouts during the vernalization. Hence, effective vernalization was only possible in sprout meristems. No flowering occurred if the basic culture medium did not contain mineral nutrient salts and/or glucose. Flowering was not influenced by the addition of adenine, kinetin or coconut milk. Flower induction and flower initiation were possible without any root formation (see photo 8).

8.5.5. *Vernalization without preceding regeneration*

8.5.5.1. Introduction

In previous experiments effective vernalization was only possible provided sprouts were formed during the vernalization and a short regeneration period at high temperature was given prior to the vernalization. The question remained whether flower induction is possible without any regeneration before the vernalization.

8.5.5.2. Petioles of young plants

Petiole pieces of 2 cm (the third leaf pair) of 8 weeks old plants were isolated on the following medium: KNOP's macroelements (half strength), HELLER's microelements, glucose 1.5%. Five treatments were given: basic medium (b.m.), b.m. + kinetin 10^{-7}, b.m. + adenine 10^{-7}, b.m. + IAA 2.10^{-7}, b.m. + IAA 4.10^{-7}. Half of the cultures remained at 26°C, the other half was vernalized during 16 weeks immediately after isolation. The cultures were placed at 20°C after vernalization in order to prevent possible devernalization.

The influence of growth substances and vernalization on regeneration was almost the same as described earlier. Adenine and kinetin slightly promoted root formation provided the cultures were vernalized. None of the petioles formed sprouts during the vernalization. No flowering occurred in the sprouts which were formed after the vernalization.

In a second experiment with the same basic medium as described above, petioles of 11 weeks old plants were vernalized during 20 weeks without any regeneration prior to the vernalization. IAA, phytokinins and GA_3 were applied in different concentrations. After the vernalization the cultures were placed at 20°C.

Although none of the petioles formed visible sprouts during the vernalization, some flowering occurred after the vernalization. The percentage of explants with at least one flowering sprout, averaged over all different treatments, ultimately reached 6%. An influence of growth regulators on flowering could not

be detected. In conclusion, this experiment shows that effective flower induction in tissues without sprouts is hardly possible.

8.5.5.3. Petioles of adult plants

Petiole pieces of 3 cm of 17 months old vegetative *Lunaria* plants were isolated on the same medium as described in 8.5.5.2. Five concentrations of kinetin were used: 0 (control), 5.10^{-8}, 10^{-7}, 2.10^{-7}, 3.10^{-7}. Vernalization was given for 0 or 16 weeks immediately after isolation. After the vernalization the cultures were placed at 20°C. No flowering occurred without vernalization.

No sprout formation occurred during the vernalization. The percentages of flowering sprouts for the kinetin treatments were 10%, 17%, 13%, 0%, 0% respectively. This experiment shows that flower induction without any regeneration before the vernalization is possible, be it very ineffective, when the petioles were taken from very old plants (see also photo 9).

8.6. Discussion

Shoot cuttings of young plants. – Shoot cuttings of young *Lunaria* plants could not be induced to flowering in vitro neither in vivo. Juvenility is therefore located and maintained in the meristems. IAA and kinetin had no influence on the transition from the juvenile to the adult phase, because they did not induce flowering. In order to get a better understanding of the environmental factors possibly governing the transition from the juvenile to the adult phase, shoot cuttings lend themselves very well for physiological research in the future.

Vernalization of callus tissue. – The possible induction of flowering in callus or pith tissue seems very attractive because they provide a relatively 'simple system' without bud or root meristems. The realization of this project, pictured in fig. 1, is not as simple as it looks since bud formation was impossible.

The induction of callus tissue in isolated petioles of vegetative plants appeared to depend on an auxin, a phytokinin and coconut milk of immature nuts (85). Coconut milk of mature nuts was much less effective than milk of immature ones which confirms the results of Morel and Wetmore (70) and Cutter (cf. Rappaport, 94). Coconut milk of immature nuts could be replaced by a mixture of 5 vitamins and myo-inositol as was used by Morel and Wetmore (71). Replacement of coconut milk by vitamins seems not so unlikely, since coconut milk contains several vitamins and also myo-inositol (Tulecke cs., 112).

The inability of subcultured callus tissues for bud formation seems to be not so uncommon. Reinert (95) e.g. remarked that the inception of organs occurs most frequently in recently isolated tissues, and that the ability decreases with increasing duration of culture and eventually disappears completely. Similar results have been reported by Vasil and Hildebrandt (113), Gautheret (25), Vasil, Hildebrandt and Riker (114), Hildebrandt, Riker and Muir (44) and Ball (9). No definite conclusions can be drawn about the reason for failure

to induce buds in *Lunaria* callus tissues. It may possibly be connected with the disappearance of chlorophyll after long subculture. Attempts to stimulate chlorophyll synthesis by phytokinins (STETLER, LAETSCH, 105) failed in *Lunaria* callus. Our work demonstrates that the use of callus tissues in morphogenetical studies is not as suitable as originally expected.

Vernalization of pith tissue. – Vernalization experiments with pith tissue were unsuccessful, owing to strong enzymatic blackening which completely blocked any growth including the formation of buds. Prevention of blackening in *Lunaria* pith explants by methods earlier described (REINERT and WHYTE, 96, NICKELL, 77) was impossible; AL-TALIB and TORREY (7), working with *Pseudotsuga*, came to the same conclusion.

That one of the sprout-regenerating unvernalized pith cultures of a very old *Lunaria* flowered, further supports our hypothesis (see also 4.8) that flowering seems possible without any vernalization. Others have found the same for *Cichorium intybus* (NITSCH and NITSCH, 78, MARGARA, 61, 63) cultures in vitro. In isolated *Cichorium* explants, however, flowering seems more likely to be due to genetical variability in the plant material used, whereas in *Lunaria* physiological heterogeneity seems to be the reason of flowering.

Regeneration of petiole segments. – Experiments on the role of the age of the leaf and the plant on regeneration of isolated petioles confirmed our earlier observations in vivo with leaf cuttings. Promotion of root formation by IAA and/or cold generally also promoted sprouting; inhibition of root formation by phytokinins or GA_3, however, did not delay or inhibit sprout formation in contrast with cotyledon cuttings (chapter 6).

The remarkable influence of IAA exclusively at 5 °C, primarily on root initiation but also on root growth in petioles of old *Lunaria* plants, may be explained by: (1) destruction of IAA at 26 °C, (2) the removal of (an) inhibiting substance(s) at 5 °C, (3) the production of (a) substance(s) interacting with IAA. The first explanation, seems not so very likely since a much more stable auxin like NAA was found to act identically, while increased IAA concentrations did not promote rooting. A choice between explanation (2) and (3) is very difficult and cannot be made without further research. When explanation (2) would be true, one can imagine that the production of (a) root-inhibiting substance(s) increase(s) with increasing age of the plants, since petioles of young plants rapidly formed roots at 26 °C with IAA. Explanation (3) confronts us with the postulation of a specific root-forming hormone complex (WENT, BOUILLENNE, cf. GORTER, 28): rhizocaline.

A promotive effect of vernalization on regeneration of roots and sprouts was earlier demonstrated in leaf cuttings (OEHLKERS, 80, WELLENSIEK, 122, 124, HEIDE, 38, 39) and in tuber tissues (SPANJERSBERG and GAUTHERET, 104, DOSTAL, cf. MEEUSE, 66). Because regeneration plays such an important role in vegetative propagation, more attempts should be made to elucidate the role of temperature on regeneration.

Vernalization with pre-existing sprouts. – When petiole segments are

forced to form sprouts before the vernalization (8.5.3), the induction of flowering is possible. This result confirms earlier work with excised shoot tips (BUTENKO and MIN-GUAN, 12, TASHIMA, 107, ISHIHARA, 48, RODRIGUES PEREIRA, 97, 98) and embryos (GREGORY and DE ROPP, 31, GREGORY and PURVIS, 30, PURVIS, 90). PICARD (84), however, did not succeed to vernalize excised terminal buds of *Oenothera biennis* and supposed that for effective vernalization leaves are required, which is in accordance with RODRIGUES PEREIRA (97, 98) and KRUZHILIN and SHVEDSKAYA (57), who observed that the presence of leaves promoted flowering.

Vernalization without pre-existing sprouts. – The conclusions of this chapter are completely identical with those of chapter 6 and will, therefore, only be summarized. The formation of sprouts during the vernalization markedly promoted the effectiveness of vernalization in *Lunaria petioles*. Almost no flowering occurred without sprouts during the vernalization, even not after a very long cold treatment.

No definite conclusions can be drawn concerning the influence of environmental factors on flower induction in vitro since the formation of sprouts during the vernalization rather than the environment as such determines whether flowering occurs. Only in a few cases a conclusion seems justified: flower induction did not take place without minerals and/or glucose in the medium even if sprouts were formed during the vernalization. The importance of sugars during vernalization was earlier shown by PURVIS (90), GREGORY and DE ROPP (31) and SHVEDSKAYA and KRUZHILIN (101). IAA almost completely inhibited flower induction; it seems, however, very likely that this was due to inhibition of sprout formation rather than by a specific action.

9. THE INITIATION OF FLOWER BUDS ON EXPLANTS OF GENERATIVE PLANTS

9.1. INTRODUCTION

Originally it was thought to be easy to obtain flowering on isolated explants from the main axes of flowering *Lunaria* plants, but the reverse appeared to be true. Consequently the questions arose whether the explants were in a flowering condition at the moment of isolation and whether certain environmental factors were limiting for floral initiation.

The purpose of this chapter is an attempt to answer these questions by means of varying one factor which might possibly limit the initiation of flower buds and by keeping all other factors constant. Because (flower) bud initiation has to be considered as a growth process, several factors affecting growth had to be examined. In a later stage, factors possibly more specifically affecting the transition from the vegetative to the generative phase have been studied on explants from the main axes of flowering *Lunaria* plants.

9.2. FACTORS AFFECTING THE INITIATION OF FLOWER BUDS

9.2.1. *The plant*

9.2.1.1. Physiological heterogeneity

We shall first consider the case in which only parts of one flowering plant are isolated. When the main axis of this plant is divided into explants of equal lengths, they generally do not react in the same manner. Usually vegetative sprouts are formed, sometimes flowering occurs (see photo 10). Several reasons can be given for this reaction: the diameter of the axis varies from place to place, the anatomical structure is not the same, the biosynthesis and contents of hormones are different etc. Thus, within one plant physiological heterogeneity may exist.

A much greater problem constitutes the physiological heterogeneity in explants taken from a comparable position of different plants. This heterogeneity even occurs when the plants are in the same stage of development. In order to illustrate this heterogeneity 11 different plants have been chosen in the same flowering stage: elongation 21 cm, all plants with visible flower buds, a few of which were opened. The age at the beginning of the vernalization, the duration of the vernalization, the post-treatment etc. were completely identical. The main axes of all plants have been divided into 12 segments of 1 cm length, each divided into 2 explants by longitudinal splitting, so that every plant gave ultimately 24 explants. The explants have been isolated on a medium with KNOP's macroelements, HELLER's microelements and glucose 2%.

Table 17 shows that the percentages of bud formation and flowering differ from plant to plant, which indicates that a great physiological heterogeneity exists between the different plants.

TABLE 17. Bud formation at different times after isolation and percentage of explants with at least one flowering sprout in explants taken from the main axes of flowering *Lunaria* plants.

plant no.	% of bud formation after		% of flowering after 92 days
	21 days	92 days	
1	13	54	0
2	21	71	13
3	38	71	17
4	66	83	13
5	50	88	13
6	13	62	4
7	38	71	0
8	4	50	4
9	17	62	5
10	21	91	0
11	50	88	13

In order to reduce mistakes owing to this heterogeneity, at least 5 plants were used in all experiments described in this chapter. The explants from these plants were always divided at random over all treatments of a certain experiment.

9.2.1.2. Flowering stage

Preliminary experiments showed that the initiation of flower buds in vitro depended on the flowering stage of the plants used. The percentage of flowering explants derived from plants without opened flower buds was zero or very low (see photo 11). Optimal sprout formation and flowering were obtained when the plants were in full bloom and still without many seeds and/or fruits (see photo 12). Sprout regeneration in explants from plants with seeds and fruits has often been poor and the flowering percentage low, probably owing to poor sprout formation.

This influence of the flowering stage on the initiation of flower buds in vitro was more systematically investigated. Explants of 21 plants in different flowering stages have been isolated on KNOP's macroelements, HELLER's microelements, glucose 1.5% and kinetin 8.10^{-7}. Although the vernalization of all plants used had ended on the same date, the flower bud realization in the greenhouse varied, so that plants in different flowering stages could be used at the same moment. The elongation of the main axes and the opening of the flower buds progressively increased with the advancing flower development.

The results presented in table 18 fully agree with the preliminary observations. Nearly no flowering occurred when all flower buds were closed. Flowering strongly increases when the flower buds open. The percentage of flowering is optimal when all flower buds are open. In conclusion, the flowering stage plays a decisive role in the initiation of flower buds in vitro.

In all experiments to be described in the following paragraphs only plants in

TABLE 18. The influence of the flowering stage of generative *Lunaria* plants on flower bud initiation in their explants.

open flowers	number of explants	number of flowering explants	% flowering explants
none	47	1	2
one	37	0	0
a few	111	8	7
more than half	91	7	8
all	30	6	20

full bloom were used for the isolation of explants. Because regeneration strongly decreases when seeds and fruits are formed, no plants were used in the seed or fruit stage.

9.2.1.3. Position in the plant

In 9.2.1.1 it has been shown that the regeneration of explants from different positions on the main axis is not identical. This point will further be worked out in the following experiment.

Parts of the elongated axes of 2 flowering plants have been isolated on KNOP's macroelements, HELLER's microelements, glucose 1.5% and IAA 2.10^{-7}. Because rooting never occurred without auxins, IAA was added to the medium to promote root formation. The main axes of the plants have been divided into 3 parts of equal length (basal, medial, terminal), each giving 16 explants.

Graph 2 shows that root formation in explants starts from the terminal part and later on from the medial and basal parts of the axes. The percentage of rooted explants at the end of the experiment decreases from the terminal to the basal part of the plant. This result points to the existence of a rooting-gradient.

Bud formation, however, starts at the basal part of the plant. This points to the existence of a budding-gradient in the main axis which is opposite to the rooting-gradient.

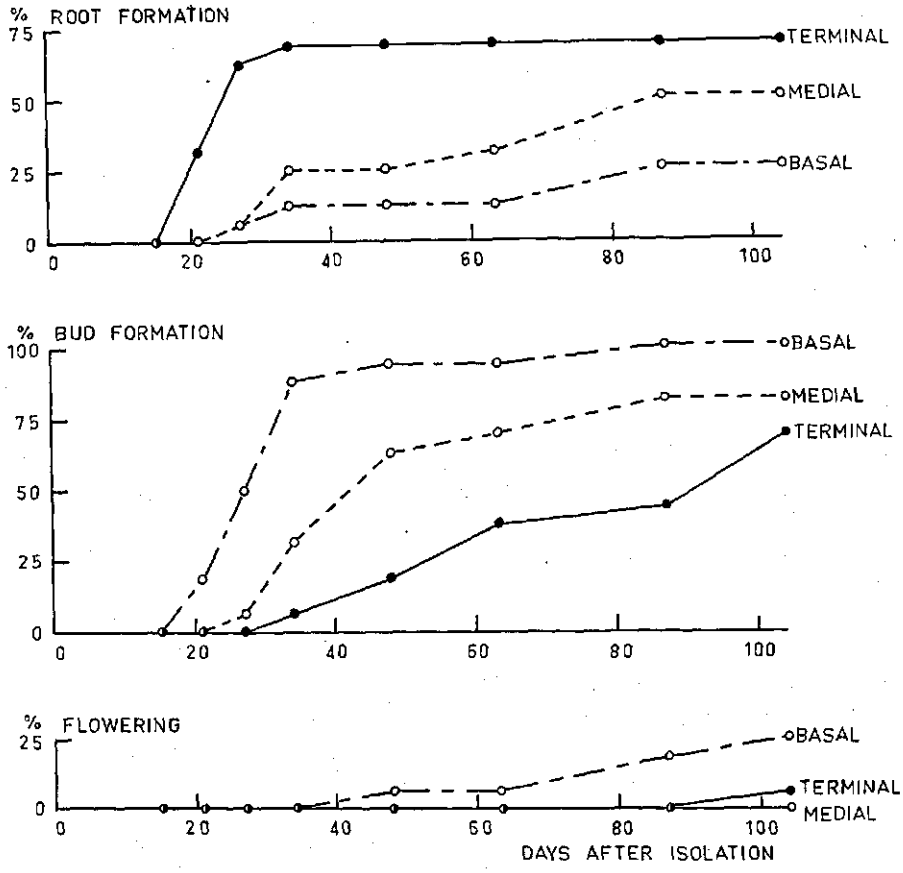
The percentage of flowering explants was rather low, so that no definite conclusions can be drawn on the possible existence of a flowering gradient.

The existence of a budding-gradient is confirmed by the mean numbers of buds per explant which decrease from the base to the top of the plant: 6.8, 3.2, 1.7 respectively. It should be mentioned that a budding-gradient is always observed, even without the addition of auxins or other substances to the culture medium.

In order to determine whether a flowering-gradient in elongated flowering *Lunaria* plants exists, the results of a great number of experiments have been added. As the percentages of flowering explants from basal and terminal parts did not differ significantly, no flowering gradient could be detected.

9.2.1.4. Size of the explant

This experiment has been performed in order to examine the influence of the size of the explant on the initiation of flower buds in vitro. Explants of different



GRAPH 2. Root formation, bud formation and flowering in explants isolated from different parts (basal, medial, terminal) of the main axes of flowering *Lunaria* plants at different times after the isolation.

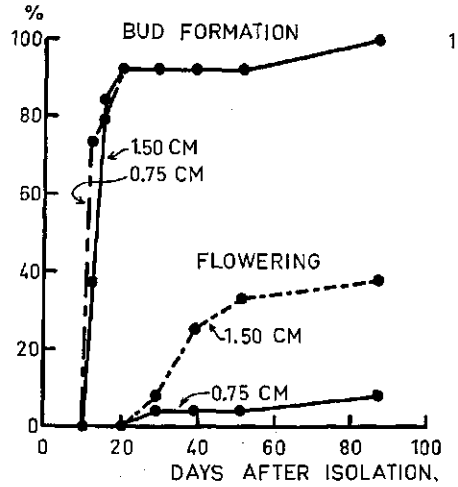
lengths (0.75 cm and 1.50 cm) have been isolated on KNOP's macroelements, HELLER's microelements, glucose 2% and kinetin 8.10^{-7}.

Graph 3 shows that the percentage of budding has not been influenced by the size of the explant; the non-mentioned mean number of buds per explant, however, was higher in the explants of 1.5 cm. The percentage of flowering strongly increased by increasing the size of the explant. We can conclude that the size of the explant is a limiting factor for flower initiation.

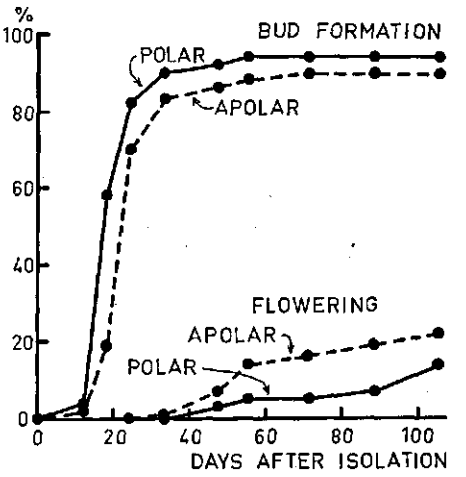
The experiment was repeated. Bud formation increased by increasing the size of the explant. No flowering occurred when the size of the explant was 0.75 cm, whereas 33% flowering occurred when the size of the explant was 1.50 cm.

9.2.1.5. Polarity

The influence of polarity on flower bud initiation was determined as follows. Explants of 7 plants have been placed at random in the medium either polarly



GRAPH 3.
Bud formation and flowering at different times after the isolation on explants from the main axes of flowering *Lunaria* plants, as influenced by the size of the explant.



GRAPH 4.
Bud formation and flowering at different times after isolation of explants from the main axes of flowering *Lunaria* plants, as influenced by the way of placing the explants on the medium: polarly or apolarly.

(basal end down) or apolarly (terminal end down). Each treatment consisted of 80 explants. The medium has been the same as the one described in 9.2.1.1 on p. 43.

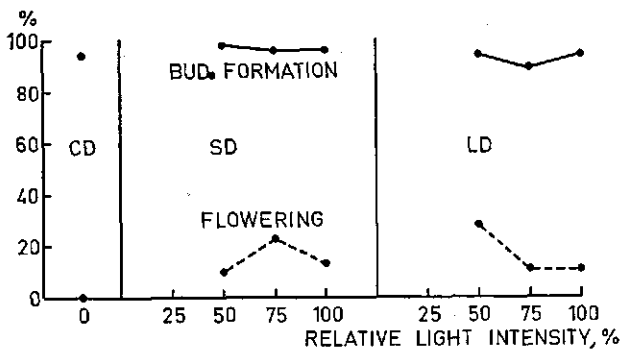
Graph 4 shows that bud formation was slightly promoted by placing the explants polarly. Flowering was slightly promoted by an apolar position of the explants. This experiment was repeated twice with almost identical results.

9.2.2. *Light and temperature*

9.2.2.1. Light intensity and daylength

In preliminary experiments bud formation and flowering had only slightly been influenced by light intensity and daylength. An extensive experiment was carried out in the phytotron with artificial light. Explants of flowering plants have been isolated on the same medium as the one described in 9.2.1.2 on p. 44. The number of explants per treatment was 48. The results are given in graph 5 which shows that bud formation is influenced neither by light intensity nor by daylength (SD versus LD); even in continuous darkness (CD) bud formation nearly reaches the 100%. The time required for bud formation, not mentioned in the table, was longer in darkness than in light.

No flowering occurred in CD which is possibly due to the fact that most sprouts are ultimately dying. The influence of light intensity and daylength on flowering is obscure. The highest percentage of flowering (28%) is reached under LD-conditions and 50% relative light intensity. We can conclude that flowering in complete darkness is impossible and that the light conditions do not markedly influence flowering.

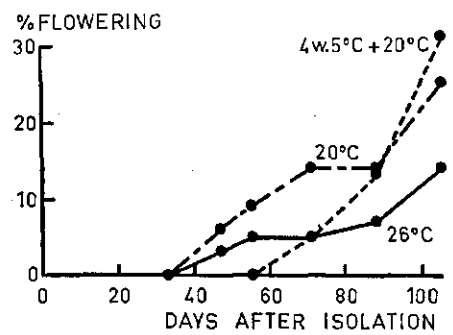


GRAPH 5.
The influence of relative light intensity and daylength on % of bud formation and flowering in explants of flowering *Lunaria* plants. CD = continuous darkness, SD = short day, LD = long day.

9.2.2.2. Temperature

The purpose of the first experiment has been to examine the influence of different temperature treatments immediately after the isolation of the explants. The medium was the same as the one described in 9.2.2.1 on p. 47. Three temperature treatments have been given: continuous 26°C, continuous 20°C, 4 weeks 5°C and afterwards continuous 20°C. The number of explants per treatment was 80.

Graph 6 shows that the percentage of flowering is always lower at 26°C as compared with 20°C. A period of 4 weeks at 5°C followed by 20°C only had a promoting effect on flowering at the end of the experiment. The original retardation of flowering in this treatment is comprehensible, because bud formation was completely blocked at 5°C. We can conclude that flower bud realization tends to be promoted by 20°C in comparison with 26°C.



GRAPH 6.
The influence of three temperature treatments on flower bud initiation in explants from the main axes of flowering *Lunaria* plants.

Some side-observations have been made. At 26°C bud formation usually takes place above and near the surface of the culture medium; at 5° and 20°C, however, nearly all sprouts have been formed in the medium. The development of sprouts and flower buds was much better at 20°C than at 26°C. Flowers quite normally developed and coloured at 20°C (photo 13) as compared with 26°C (photo 12), at which temperature the growth was decreased and flowers did not develop quite well.

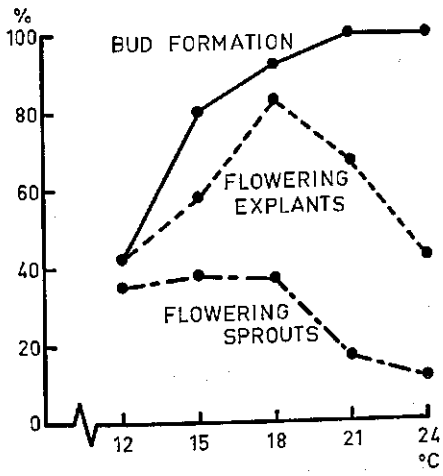
In order to get a more complete picture of the influence of temperature on

flowering, explants have been isolated on the same medium as the one described in 9.2.1.2 on p. 44 at different temperatures in the phytotron (natural daylight): 12°, 15°, 18°, 21° and 24°C. After 11 weeks all the test tubes were placed at 26°C in continuous fluorescent light for another 11 weeks.

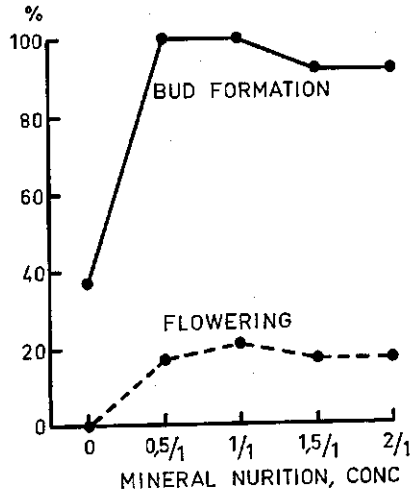
Graph 7 shows that bud formation strongly increased at increasing temperatures. The percentage of flowering explants first increased and later decreased as the temperature increased: the optimal temperature for flowering is 18°C. The percentage of flowering sprouts gradually decreased as the temperature increased from 18° to 24°C.

At the end of the experiment the mean number of buds per explant with increasing temperatures was: 1.4, 3.0, 4.5, 4.3 and 6.3. This means that bud formation also quantitatively increased at increasing temperatures.

In conclusion, this experiment shows that temperature plays a decisive role in the process of bud formation and flowering.



GRAPH 7.
The effect of a temperature pre-treatment during 11 weeks in the phytotron on % of bud formation, % of flowering explants and % of flowering sprouts at 26°C.



GRAPH 8.
The influence of the concentration of mineral nutrition on bud formation and flowering in explants of flowering *Lunaria* plants.

9.2.3. *Basic culture medium*

9.2.3.1. Mineral nutrition

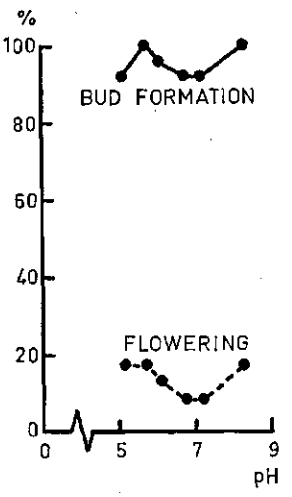
The purpose of this experiment has been to determine the influence of the mineral nutrition on flowering. Explants of flowering plants have been isolated on a medium with glucose 1.5% and kinetin 8.10^{-7}. Different concentrations of mineral nitrition (KNOP's macroelements, HELLER's microelements) have been given: 0 (control), 0.5/1, 1.0/1, 1.5/1 and 2.0/1 strength.

Graph 8 shows that bud formation and flowering strongly increase with mineral nutrition, but that the highest effect has already been reached at 0.5/1. In conclusion, mineral nutrition is required for flowering.

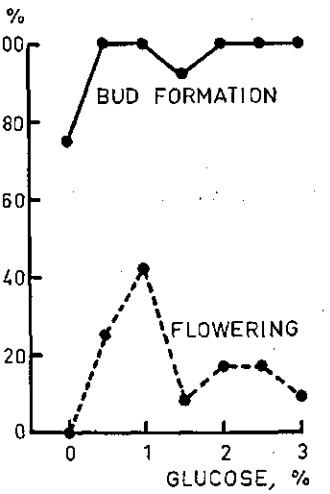
9.2.3.2. Acidity

The influence of the pH on flowering was examined by placing explants on the same medium as described in 9.2.1.2 on p. 44 and by varying the pH of the culture medium.

Graph 9 shows that bud formation and flowering are very irregularly influenced by the pH of the culture medium. A definite conclusion appears not possible.



GRAPH 9.
The influence of the pH on bud formation and flowering in explants of flowering *Lunaria* plants.



GRAPH 10.
The influence of the glucose concentration on bud formation and flowering in explants of flowering *Lunaria* plants.

9.2.3.3. Glucose

The influence of the glucose concentration on flowering was examined by adding different glucose concentrations to a medium of agar, KNOP's macroelements, HELLER's microelements and kinetin 8.10^{-7}.

Graph 10 shows that bud formation is relatively low when no glucose is added to the medium. The glucose concentration does not influence the percentage of bud formation. Flowering is fully suppressed without glucose in the medium. The optimal glucose concentration for flowering is 1 %. It can be concluded that glucose plays a decisive role in the process of flower bud formation.

Some side-observations were that bud formation occurred within the culture medium when glucose was omitted, but over and above the culture medium when glucose was added. Underdeveloped inflorescences were observed only at a glucose concentration of 0.5% (photo 14 compared with photo 12).

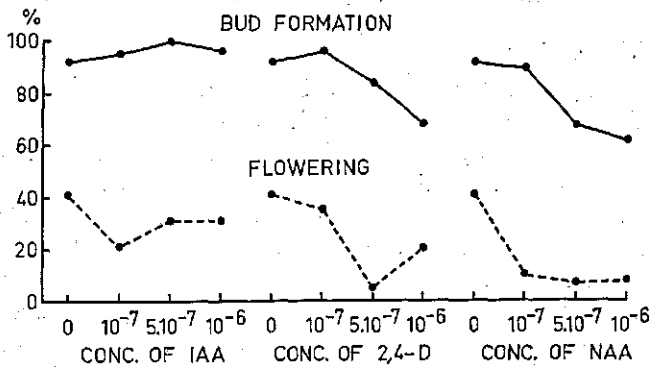
9.2.4. *Growth regulators*

9.2.4.1. Auxins

The purpose of this experiment was to examine the influence of the auxins IAA, 2,4-D and NAA on flower bud initiation. The medium was: agar, KNOP's macroelements, HELLER's microelements, glucose 1.5%. Four auxin concentrations were used: 0 (control), 10^{-7}, 5.10^{-7} and 10^{-6}. The number of explants per treatment was 24.

Graph 11 shows that bud formation was hardly influenced by IAA, but strongly decreased with increasing 2,4-D and NAA concentrations. Flowering only slightly decreased by IAA, but strongly decreased by 2,4-D and NAA. The decrease of flowering by 2,4-D and NAA was caused partly by the inhibition of bud formation and partly by a specific action on flowering.

The mean number of buds per explant was not decreased by IAA, but was strongly decreased with increasing 2,4-D and NAA concentrations.

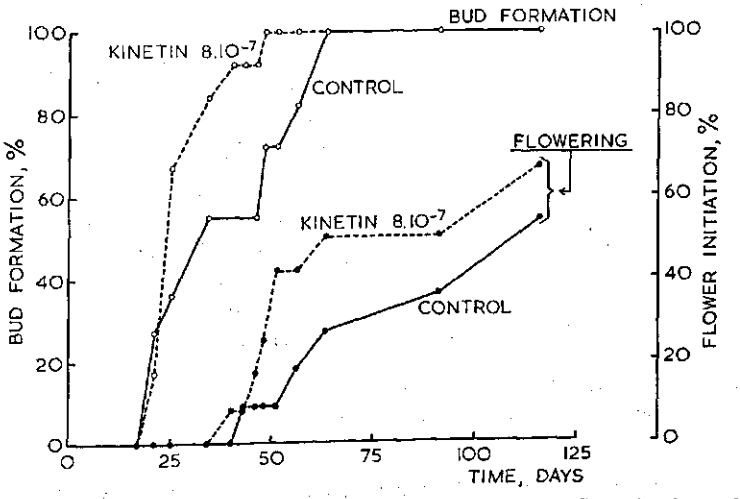


GRAPH 11. The influence of the auxins IAA, 2,4-D and NAA in the concentrations 0, 10^{-7}, 5.10^{-7} and 10^{-6} on bud formation and flowering in explants of flowering *Lunaria* plants.

9.2.4.2. Phytokinins

Explants have been isolated on the same basic culture medium as described in 9.2.4.1 on p. 50 to which 0 or 8.10^{-7} kinetin was added.

Graph 12 shows that the percentage of bud formation only initially increased



GRAPH 12. The influence of kinetin (8.10^{-7}) on bud formation and flowering in explants of flowering *Lunaria* plants.

by kinetin. The percentage of flowering first strongly increased, but later slightly increased by kinetin.

Kinetin increased the mean number of buds per explant from 3.9 to 5.1, whereas the percentage of flowering sprouts was not influenced. We can conclude that kinetin only slightly promoted flowering.

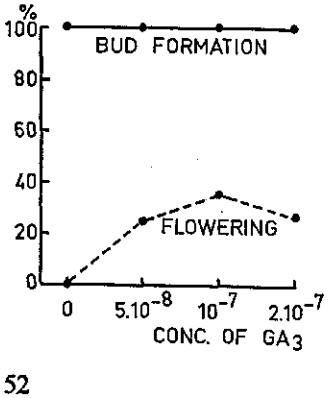
Several experiments were carried out in order to examine the influence on flowering of other phytokinins: adenine, SD 4901, SD 8339. The results were similar to those for kinetin and will, therefore, not be presented.

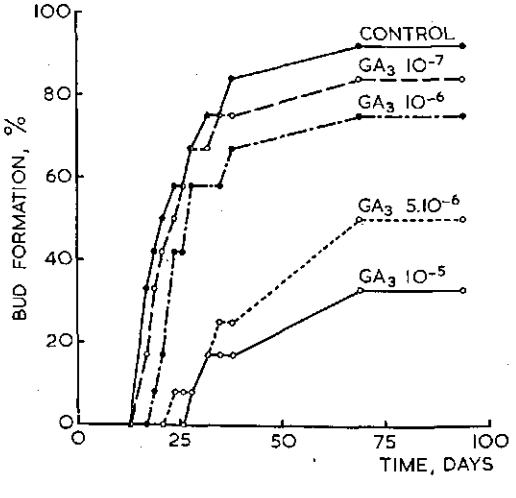
9.2.4.3. Gibberellic acid

Preliminary experiments showed that bud formation strongly decreased by increasing GA_3 concentrations (graph 13). The mean number of buds per explant was only decreased by the three highest GA_3 concentrations.

Because high GA_3 concentrations strongly inhibit bud formation, the influence of GA_3 on flowering has been examined only on media with relatively low GA_3 levels. Explants were isolated on the same basic culture medium as described in 9.2.4.1 on p. 50 with the GA_3 concentrations: 0 (control), 5.10^{-8}, 10^{-7}, 2.10^{-7}. It can be concluded from graph 14 that the percentage of bud formation was not influenced by the GA_3 concentration. The same holds true for the mean number of buds per explant which is not given in graph 14. Flowering increased by all GA_3 concentrations as compared with the control. For unknown reasons no flowering occurred without GA_3 in the medium. We can conclude that GA_3 has a promotive effect on flowering.

GRAPH 13.
The influence of different GA_3 concentrations on bud formation in explants of flowering *Lunaria* plants at different times after isolation.





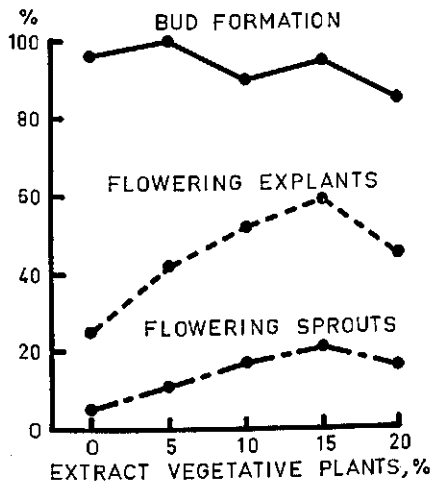
GRAPH 14. The influence of GA_3 on bud formation and flowering in explants of flowering *Lunaria* plants.

9.2.5. *Plant extracts*

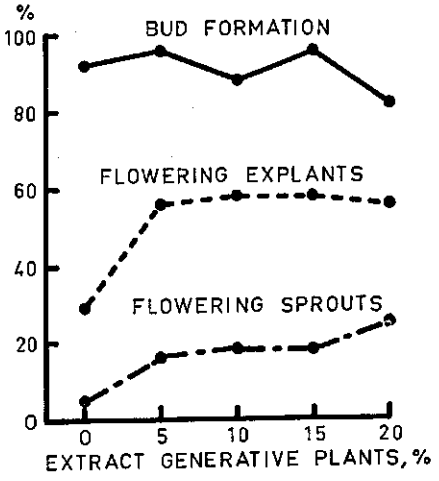
9.2.5.1. Extract from vegetative *Lunaria* plants

The purpose of this experiment was to examine the influence of an extract from vegetative *Lunaria* plants on flowering. The basic medium consisted of KNOP's macroelements, HELLER's microelements and glucose 1.5%. Different concentrations (% by volume) of a crude extract obtained from leaves of vegetative *Lunaria* plants were used.

Graph 15 shows that the percentage of bud formation is only slightly influenced by the addition of the extract. The percentages of flowering explants and flowering sprouts strongly increased at increasing concentrations except the 20% of the extract. We can conclude that the extract from vegetative *Lunaria* plants strongly promotes flowering in vitro.



GRAPH 15.
The influence of a leaf extract from vegetative *Lunaria* plants on % of bud formation, % of flowering explants and % of flowering sprouts in isolated explants of flowering *Lunaria* plants.



GRAPH 16.
The influence of a flower extract from generative *Lunaria* plants on % of bud formation, % of flowering explants and % of flowering sprouts in isolated explants of flowering *Lunaria* plants.

9.2.5.2. Extract from generative *Lunaria* plants

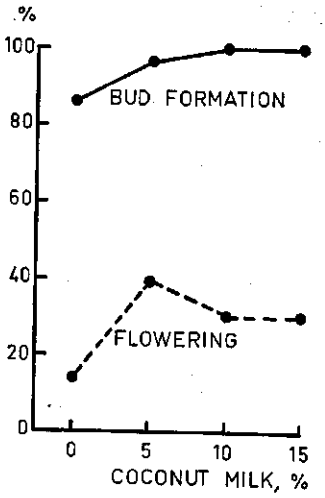
The influence of an extract from generative *Lunaria* plants was examined using the same basic culture medium as described in 9.2.5.1 on p. 53. Different concentrations (% by volume) of crude extract obtained from flowers of generative *Lunaria* plants were used.

Graph 16 shows that the percentage of bud formation is hardly influenced by the addition of the extract. The percentages of flowering explants and flowering sprouts strongly increased by the addition of the extract, but appeared to be independent of the concentration in the range 5–20%. We can conclude that the extract from generative *Lunaria* plants has a promotive effect on flower bud initiation in vitro.

9.2.6. *Coconut milk*

Explants have been isolated on the same basic culture medium as described in 9.2.5.1 on p. 53 with different concentrations of coconut milk added. Coconut milk of mature nuts appeared to be toxic, which was also observed by CUTTER and WILSON (18); milk of immature nuts was not toxic and has therefore been used.

Graph 17 shows that the percentages of bud formation slightly increased by coconut milk. The percentages of flowering also increased by coconut milk of which 5 % seems to be optimal.



GRAPH 17. The effect of different coconut milk concentrations on % of bud formation and % of flowering in explants of generative *Lunaria* plants.

Some side-observations should be mentioned. The mean number of buds per explant strongly increased by increasing concentrations: 2.9, 3.8, 4.7 and 6.2 respectively. Coconut milk of immature nuts, especially the highest concentrations, caused abnormal sprout development and even deformations. The most remarkable morphogenetical action of coconut milk was that occasionally flower buds and/or inflorescences were formed without bracts and leaves. Photo 15 shows the regeneration of such an inflorescence without bracts and leaves as a result of the addition of coconut milk of immature nuts (10%), 2,4-D (4.10^{-7}) and kinetin (8.10^{-7}) to the culture medium.

9.3. DISCUSSION

Heterogeneity and gradients. – The physiological heterogeneity between explants of one plant and between explants of different plants of the same developmental stage, is a very annoying complication from which an escape is impossible. REINERT (95) concluded that the most immediate difficulty to be overcome in the use of tissue cultures for physiological investigations arises from the variability of the cultures.

Heterogeneity finds expression in a rooting-gradient along the main axis of a flowering *Lunaria* plant, which runs opposite to a budding-gradient. It seems very likely to suppose with AGHION-PRAT (4) that such gradients of organogenesis are due to gradients of hormones inside the plant or to a gradient of IAA oxydase activity increasing towards more basal parts or older cells (GALSTON and HILLMAN, 22). In *Lunaria* and *Cichorium* (MARGARA, 62) a flowering gradient, as was demonstrated in tobacco stems (AGHION-PRAT, 4) and *Streptocarpus* leaves (OEHLKERS, 79), could not be found.

Localized regeneration of flower buds or inflorescences without leaves or bracts as was earlier demonstrated by OEHLKERS (79), AGHION (2), KONAR and NATARAJA (54), was exceptionally observed in *Lunaria* explants; for unknown reasons this phenomenon occurred exclusively in *Lunaria* when coconut milk of immature nuts was present in the medium.

Flowering stage. – Flower initiation in isolated explants of generative *Lunaria* plants increased as the stage of flowering of the mother plants increased, with an optimum in the stage of full bloom. The investigations of SMIRNOV (103) with vernalized sugar beet plants in vivo had a similar result as described for *Lunaria*. Since the existence of a floral stimulus in *Lunaria* was demonstrated by WELLENSIEK (123), it seems reasonable to suppose that the translocation of this stimulus is accomplished and/or optimal when the stage of full bloom is reached.

Size of the explant. – As contrasted with bud formation, flowering is fully suppressed when the isolated explant is too small. This points to the quantitative importance of a substratum for the flowering process as recognized earlier by WELLENSIEK (126). In tobacco stem explants (AGHION-PRAT, 5), the size of the explant had not such a decided effect on flower initiation in vitro.

Polarity. – In *Lunaria* flowering was slightly promoted by placing the explants upside down as compared with placing in normal position. MARGARA cs. (64) found that flowering in explants of vernalized chicory roots decreased by placing the explants upside down. A plausible explanation for these deviating results cannot be given.

Light. – Several plants, including some SD and many LD plants, raised from seeds, can initiate flower primordia in vitro when cultured in total darkness (cf. SUGINO, 106). When explants of plants like sunflower (HENRICKSON, 41), tobacco (AGHION-PRAT, 5) and *Lunaria* were isolated in vitro, no flower initiation was observed in continuous darkness. Hence, an absolute light requirement for flowering seems to depend on the fact whether the whole plant from seed or an explant is cultured.

Since photoperiodism in *Lunaria* is no factor of direct importance for flower initiation (WELLENSIEK, 119 and chapter 4), it is comprehensible that no day-length effect was found in vitro.

Temperature. – A disadvantageous effect of high temperatures after the vernalization on flowering of in vitro cultivated wheat plants was shown by INOUYE cs. (47). The optimum temperature for the realization of flowering in

Lunaria explants was found to be 18 °C, for bud formation near 24 °C. Since the percentages of flowering sprouts are optimal at 18 °C or lower, below 18 °C bud formation rather than temperature was limiting for flower bud formation. Devernalization at higher temperature cannot be excluded but does not seem very likely since the development of flower buds in vitro also strongly decreased at high temperature. Possibly the increase of the mean number of buds per explant at high temperature effects flowering negatively.

Mineral nutrition. – KIMURA (51) showed that the initiation of flower buds in aseptically cultured plants of *Pharbitis* was possible without mineral nutrients and supposed that the mineral nutrients from the seeds and agar were sufficient for the formation of floral primordia. AGHION-PRAT (5) concluded that minerals are not essential for flowering in tobacco stem sections. Experiments with *Lunaria* stem explants demonstrated that minerals are required to obtain bud formation and flowering in vitro.

Acidity. – An influence of the pH on flowering of *Lunaria* explants was not clear. KIMURA (51) demonstrated that the pH did not strongly affect flowering in vitro of *Pharbitis* seedlings, whereas RODRIGUES PEREIRA (98) showed that the influence of the pH between 4.0 and 6.5 was very small on flowering in vitro of *Iris*. The capability of isolated tissues to change the pH of the medium to more optimal values for growth (GAUTHERET, 26) possibly causes that the influence of the initial pH is small.

Glucose. – The presence of glucose in the culture medium was necessary for flower bud initiation in *Lunaria* explants, although glucose was not necessary for bud formation. This is in harmony with many reports, claiming that sugars are essential for flower bud initiation in vitro: HENRICKSON (41), KIMURA and TAKIMOTO (52), KIMURA (51), AGHION-PRAT (5) and MARGARA (61, 62). Some authors came to the conclusion that sugars only promoted flowering in vitro (PAULET, 81, INOUYE, 45, KIMURA, 50) or had no influence at all (RODRIGUES PEREIRA, 98). Sometimes the effect of sugars seems to be more obvious at high temperatures (INOUYE, 46).

Auxins. – The inhibiting effect of auxins on flower bud formation in vitro was earlier shown by RAGHAVAN and JACOBS (93), RODRIGUES PEREIRA (98), PAULET and NITSCH (82) and MARGARA (61). The present experiments fully confirmed these observations; the inhibiting effect of IAA on flowering of *Lunaria*, however, is weak as compared with 2,4-D and NAA. Their inhibition seems to be correlated with the inhibition of bud formation.

Phytokinins. – The influence of phytokinins on flowering in vitro as compared in different plant species is not very clear. AGHION-PRAT (5) obtained inhibition of flowering by kinetin in tobacco, whereas RODRIGUES PEREIRA (98) concluded that kinetin had no influence on flower initiation in *Iris*. PAULET (81) with chicory and own experiments with *Lunaria* showed that kinetin slightly promoted flower initiation. CHAILAKHIAN (13) demonstrated that isolated stem

tips in LD from the SD plant *Perilla* formed flower buds in the presence of adenine and kinetin.

Gibberellic acid. – Flower bud initiation in *Lunaria* explants was promoted by low concentrations of GA_3. Similar results in vitro with other plants were obtained by PAULET and NITSCH (83), MARGARA cs. (64) and RODRIGUES PEREIRA (98). In tobacco stem segments flowering decreased by GA_3 (AGHION-PRAT, 5). At high concentrations, GA_3 inhibited bud formation in *Lunaria*. This is in accordance with MURASHIGE (72, 73), PAULET (81) and AGHION-PRAT (5). The inhibition of bud formation by high GA_3 concentrations prevented flowering in *Lunaria*. Hence, the effect of GA_3 is indirect.

Plant extracts and coconut milk. – Extracts obtained from vernalized plants had sometimes a weak (PURVIS and GREGORY, 92), in other cases a clear positive effect (TOMITA, 108, 109) on flowering in vitro. Flower bud initiation in *Lunaria* explants was clearly promoted by extracts of both vegetative and generative *Lunaria* plants. This indicates that the plant extracts had a growth promoting influence rather than a specific effect on flowering. This growth promoting influence is most probably due to organic substances, because flowering was not influenced by the concentration of anorganic compounds in the medium. Coconut milk from immature nuts has an identical effect.

General conclusions. – Flower initiation in explants of generative *Lunaria* plants was limited both by the condition of the mother plants and by different environmental factors. Because flower initiation should primarily be considered as a growth process, all factors normally affecting growth also affect flower bud formation. The influence of the condition of the mother plants and of certain growth regulators on flowering in vitro suggest that hormonal factors also play a dominant role. The effect of growth regulators, however, is somewhat more difficult to interpret, since they also strongly affect organogenesis (AGHION-PRAT, 5).

The floral condition appeared to be not so very persistent in isolated regenerating explants. The possibility cannot be excluded that a reversion from the generative to the vegetative phase is promoted by regeneration. The possible disappearance of the floral condition by regeneration of adventitious buds in vivo was earlier demonstrated by CHOUARD (14), MILLER (68) and RUNDFELDT (99).

10. GENERAL DISCUSSION

The application of sterile culture. – The application of sterile culture has been demonstrated to be a valuable and promising expediant for the present and for further research. The most important advantage is that complete small scale conditioning of the environment can be obtained without incurring great expenses. An additional advantage is the easy administration of growth regulators and nutrients to isolated plant parts.

Before proceeding to the discussion of the results, some comment should be made on my in vitro experiments. The induction of flowering by cold in undifferentiated callus and pith tissue cultures would be very attractive, but is most probably not realizable because:

1. Flower induction in plants with a juvenile phase is hardly possible without bud meristems.
2. The formation of buds, which is necessary for the expression of flowering, is very difficult.

The first objection can possibly be removed by working with plant species without a juvenile phase for flowering. The second objection can possibly be solved when more is known about the factors affecting the formation of buds. However, a new complication will possibly be introduced, when auxins will be necessary for callus induction and/or tissue growth, since they usually also have an inhibitory effect on both bud formation and flower bud formation.

Nothing can be said with certainty about the influence on flowering of environmental factors during flower induction, because the formation of buds during vernalization is the master factor for flower induction and not the environment. Sometimes, however, the environment is limiting for bud formation and/or for flower induction.

Because the initiation of flower buds in vitro appeared to depend on a very great number of plant and environmental factors, all possible interactions between those factors could not be studied. This implies that the optimum for flower bud initiation may change under another set of growth conditions than used.

In vitro culture of explants is not the same as the normal culture of intact plants. Hence, one should be careful about the application of the results to intact plants without further research.

We shall now discuss a number of phenomena which have to do with vernalization.

What is juvenility? – In this discussion juvenility will be restricted to herbaceous plants with a cold requirement for flowering. This juvenile phase can be defined as:

1. The period of vegetative growth during which flower induction by cold is impossible. This definition implies that the loss of the cold requirement by mutation automatically means either a shortening or the disappearance of the juvenile phase.

2. The period of vegetative growth during which flower formation is impossible. This definition implies that if a cold requirement for flowering is lost by mutation, the juvenile phase does not disappear yet.
3. The period of vegetative growth during which flower induction and flower formation are impossible.

The existence of many plant species with a cold requirement for flowering but without a juvenile phase, makes it somewhat more likely to accept the second definition. However, it cannot be said whether this definition holds true for all cases. Its validity for *Lunaria* can possibly be answered when further research with the annual *Lunaria* will show whether juvenility and cold requirement for flowering are coupled or not.

Localization of juvenility. – In chapter 5 was demonstrated that juvenility is located in the bud meristems. However, cotyledons without any regeneration before the vernalization did not come into flowering, and regeneration of a bud prior to the vernalization was supposed to be limiting for flower induction. Later, in vivo (chapter 6) and in vitro (chapter 8) experiments clearly demonstrated that buds are indeed necessary for effective flower induction.

Rejuvenation. – The beginning of regeneration was supposed to initiate rejuvenation which is a generally occurring phenomenon both in vivo (6, 14, 23, 27, 32, 38) and in vitro (3, 5, 34, 54, 55, 110, 115, 131). It is difficult to say whether rejuvenation after regeneration is an identical phenomenon as the juvenile phase of an intact plant. The difference between the duration of the juvenile phase of an intact *Lunaria* plant (approximately 6 to 7 weeks) and the duration of rejuvenation in a cotyledon cutting of *Lunaria* (approximately 2 weeks) does not permit the conclusion that the juvenile phase and the rejuvenation are not identical.

My experiments in vivo and in vitro have demonstrated that bud meristems play a decisive role in all developmental stages of a plant with a juvenile phase and an absolute cold requirement for flowering like *Lunaria* because:
1. Juvenility was found to be located in the bud meristems.
2. Regeneration of a bud in parts of vegetative plants inevitably starts with rejuvenation so that the induction of flowering is ultimately possible when buds are present during the vernalization.
3. Bud regeneration in explants of a generative plant should be considered as an event whereby reversion from generative to vegetative can occur.

For these reasons I suppose that juvenility and rejuvenation (regeneration) are identical.

The shortening of the juvenile phase. – It was earlier demonstrated that the juvenile phase for flowering in *Lunaria* can be shortened by applying supplementary light of high intensity (43, 130). In addition, my experiments demonstrated that other means are:
1. Regeneration of cotyledon cuttings of very young plants during two weeks.
2. Amputation of the root system of young plants.

Without further research with other plant species, the general validity of these results remain unknown.

The site of cold action. – Much evidence is presented in literature that the main site of the cold action is the growing-point (16, 17, 67, 84, 89, 103), which is in accordance with my results. However, OEHLKERS (80) and WELLENSIEK (127) indicated that the site of cold action is not restricted to buds. In *Lunaria* indirect flower formation as a result of the translocation of a floral stimulus – rejected by WELLENSIEK – cannot be exluded. This explanation is fortified by in vivo experiments of SMIRNOV (103) with sugarbeets and the experiments described in 9.2.1.2. Another explanation is that the rejuvenation time decreases with increasing age of the plants, which is supported by WELLENSIEK (122) and my own experiments (see 8.5.5.2 and 8.5.5.3); this would imply that in older plants no buds are required for flower induction.

When discussing the role of buds during the vernalization, a clear distinction should be made between plants without and with a juvenile phase for flowering. WELLENSIEK (126), PAULET (81) and PIERIK (87) demonstrated that in a plant without a juvenile phase, like *Cichorium intybus*, flower induction is possible in the absence of buds. This leads me to the hypothesis that no buds are required because the absence of a juvenile phase implies that no rejuvenation occurs. However, in plants with a juvenile phase like *Cardamine pratensis* (88) and *Lunaria annua* rejuvenation occurs. My concept that no rejuvenation occurs in plants without a juvenile phase, would imply that for effective flower induction no cell divisions are required before or during the vernalization.

The concept that vernalization only takes place when dividing cells are present during the cold treatment (127) was criticized by some authors (10, 56, 58, 81), since they did not find mitoses during the vernalization, whereas flowering occurred after the vernalization. Recently WELLENSIEK (129) modified his concept by changing 'dividing cells' into 'cells prepared to divide', attaching more value to the premitotic stages than to the mitosis proper. The conclusion drawn from my work with *Lunaria* was that cell divisions, which always precede regeneration, are definitely a prerequisite in order to reach a certain stage in the regeneration process which is necessary for the action of low temperature. WELLENSIEK's concept of dividing cells is therefore confirmed for *Lunaria* in a modified form.

The disappearance of the flowering condition. – The question 'why does the flowering condition disappear in the newly formed seeds' has never been answered. Some hypotheses can be put forward:

1. Devernalization by high temperature before, during and after meiosis, zygote formation and seed formation.
2. Devernalization by meiosis or by zygote formation (10, 127).
3. Devernalization as a result of plant new-formation (regeneration) which starts directly after zygote formation.
4. Meiosis which selectively takes place in vegetative cells.

Hypothesis 1 can be excluded (SCHWABE, 100 and 4.6 on p. 13), hypotheses

2, 3 and 4 not. I consider hypothesis 3 as the most acceptable one for cold requiring plants with a juvenile phase, because it would involve a similar process as rejuvenation, initiated by regeneration. This might even hold true for plants without a juvenile phase and also for photoperiodically reacting plants. Single cell culture would perhaps enable to prove whether this is true.

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SUMMARY

Investigations in connection with flowering were carried out with *Lunaria annua* L., a species with a juvenile phase, an absolute cold requirement for flowering and a high regeneration ability. The experiments were done in vivo with intact plants and leaf cuttings, and in vitro with excised petiole and stem tissues.

1. Induction of annual flowering. – All plants raised from seeds of different origin had an absolute cold requirement for flowering. An annual flowering mutant was obtained in the M_2 after treating seeds with ethyl methane sulphonate.

2. Vernalization experiments with intact plants. – The influence of the light conditions before, during and after the vernalization and the effect of cold on plants of 3 to 5 weeks old on flowering were very small. Vernalization at continuous low temperature was slightly more effective than vernalization under natural conditions. High temperature devernalization after the vernalization, during meiosis, zygote formation and seed formation could be excluded as the cause of the disappearance of the flowering condition. Flowering was sometimes observed in very old plants without vernalization.

3. Localization of juvenility. – Experiments with shoot and leaf cuttings showed that juvenility is not a character of the whole plant, but is located in all the buds. Terminal buds are adult at an earlier stage than lateral ones.

4. The shortening of the juvenile phase. – The duration of the juvenile phase was shortened by the amputation of the roots and by regeneration of cotyledon cuttings of very young plants, lengthened by the amputation of the cotyledons.

5. Vernalization of leaf cuttings. – The regeneration of leaf cuttings was determined by the age of the leaf and of the plant. The effectiveness of vernalization in leaf cuttings strongly depended on the regeneration of buds before and during the vernalization. When bud regeneration in cotyledon cuttings before and during the vernalization was retarded or promoted by the application of growth regulators, flowering was affected similarly.

6. Induction of flowering in vitro. – Vernalization of shoot cuttings of young plants failed owing to juvenility. Whether vernalization of callus and pith tissues is possible remained unanswered, because attempts to induce sprouts were unsuccessful. Flower induction in petiole segments of vegetative plants was effective, but only when buds were formed before or during the cold treatment and when the vernalization lasted relatively long.

7. Root initiation. – Root initiation in petiole segments of old vegetative plants was markedly stimulated by low temperature and IAA.

8. Initiation of flower buds in vitro on explants of generative plants. – Flower bud initiation on isolated explants of the main axes of flowering plants was influenced by the flowering stage of the mother plants, and by almost all environmental factors normally affecting growth, such as: size of the explant, light, temperature, mineral nutrition, carbohydrates. Auxins generally had an inhibitory, phytokinins a slightly promotive and GA_3 a promotive effect on flower initiation. Extracts of vegetative and generative *Lunaria* plants and coconut milk of immature nuts strongly stimulated flower initiation. A rooting and a budding gradient were observed in the elongated main axes, but no flowering gradient occurred. Physiological heterogeneity in the plant material used was recognized as a complicating factor.

9. General. – In the general discussion the following hypotheses were put forward:

1. Rejuvenation as a result of regeneration is identical with juvenility.
2. Cell divisions are considered to be a prerequisite in order to reach a certain stage in the regeneration process, which is necessary for the action of low temperature.
3. The disappearance of the flowering condition is due to plant new formation directly after zygote formation.

SAMENVATTING

REGENERATIE, VERNALISATIE EN BLOEI IN *LUNARIA ANNUA* L. IN VIVO EN IN VITRO

Er werd bloei-onderzoek verricht met *Lunaria annua* L., een soort met een jeugdfase, absolute koudebehoefte voor de bloei en een sterk regeneratievermogen. De proeven werden in vivo gedaan met intacte planten en bladstekken en in vitro met stukjes bladsteel- en stengelweefsel.

1. Inductie van eenjarigheid. – Alle planten, die verkregen werden uit zaden van verschillende herkomst, bleken een absolute koudebehoefte voor de bloei te bezitten. Door zaden met ethyl methaan sulfonaat te behandelen, werd in de M_2 een bloeimutant verkregen, die eenjarig was.

2. Vernalisatieproeven met intacte planten. – De bloei werd weinig beïnvloed door de lichtomstandigheden vóór, tijdens en na de vernalisatie en evenmin door planten van 3 tot 5 weken oud bloot te stellen aan koude. Vernalisatie bij een constant lage temperatuur was iets effectiever dan vernalisatie onder natuurlijke omstandigheden. Uitgesloten kan worden, dat devernalisatie door hoge temperatuur tijdens de reductiedeling, bevruchting en zaadvorming de oorzaak is van het verdwijnen van de bloeitoestand. In zeer oude planten trad soms bloei op zonder vernalisatie.

3. Localisatie van de juveniliteit. – Proeven met scheut- en bladstekken toonden aan, dat niet de hele plant juveniel is, maar dat de juveniliteit gelocaliseerd is in alle knopmeristemen. Terminale knoppen zijn vroeger volwassen dan laterale knoppen.

4. Verkorting van de jeugdfase. – De duur van de jeugdfase werd verkort door de wortels te amputeren en door cotylstekken van zeer jonge planten te laten regenereren en werd verlengd door amputatie van de cotylen.

5. Vernalisatie van bladstekken. – De regeneratie van bladstekken werd bepaald door de leeftijd van het blad en van de plant. Regeneratie van knoppen vóór of tijdens de vernalisatie bepaalde in sterke mate of effectieve vernalisatie mogelijk was. Wanneer door toediening van groeiregulatoren de knopregeneratie van cotylstekken vóór of tijdens de vernalisatie werd vertraagd of bevorderd, werd de bloei hierdoor op dezelfde wijze beïnvloed.

6. Bloei-inductie in vitro. – Vernalisatie van scheutstekken van jonge planten was niet mogelijk als gevolg van juveniliteit. Omdat pogingen om spruiten te doen ontstaan mislukten, kon de vraag niet beantwoord worden of vernalisatie van callus- of mergweefsels mogelijk is. Bloei-inductie in bladsteelstukjes van vegetatieve planten was alleen effectief, indien vóór of tijdens de vernalisatie knoppen werden gevormd en indien de vernalisatie relatief lang duurde.

7. Aanleg van wortels. – Door lage temperatuur en IAA werd de aanleg van wortels in bladsteelstukjes van oude vegetatieve planten zeer sterk bevorderd.

8. De aanleg van bloemknoppen in vitro in explantaten van generatieve planten. – De aanleg van bloemknoppen in geïsoleerde explantaten uit de hoofdassen van bloeiende planten werd beïnvloed door het bloeistadium van deze planten en door vrijwel alle milieufactoren, die over het algemeen de groei beïnvloeden zoals: grootte van het explantaat, licht, temperatuur, minerale voeding, koolhydraten. De aanleg van bloemknoppen werd over het algemeen geremd door auxinen, iets bevorderd door phytokininen en bevorderd door GA_3. De aanleg van bloemknoppen werd sterk bevorderd door extracten van vegetatieve en generatieve *Lunaria* planten en door cocosnoot melk van onvolwassen noten. In de gestrekte hoofdassen werd een wortelvormings- en een spruitvormingsgradiënt gevonden, maar geen bloeigradiënt. De physiologische heterogeniteit van het gebruikte plantmateriaal moet als een complicatie worden beschouwd.

9. Algemeen. – In de algemene discussie werden de volgende hypothesen naar voren gebracht:

1. ‚Rejuvenilisatie' als gevolg van regeneratie is identiek met juveniliteit.
2. Celdelingen worden beschouwd noodzakelijk te zijn voor het bereiken van een bepaald stadium in het regeneratieproces, dat noodzakelijk is voor de inwerking van koude.
3. Het verdwijnen van de bloeitoestand wordt veroorzaakt door het ontstaan van een nieuwe plant direct na de bevruchting.

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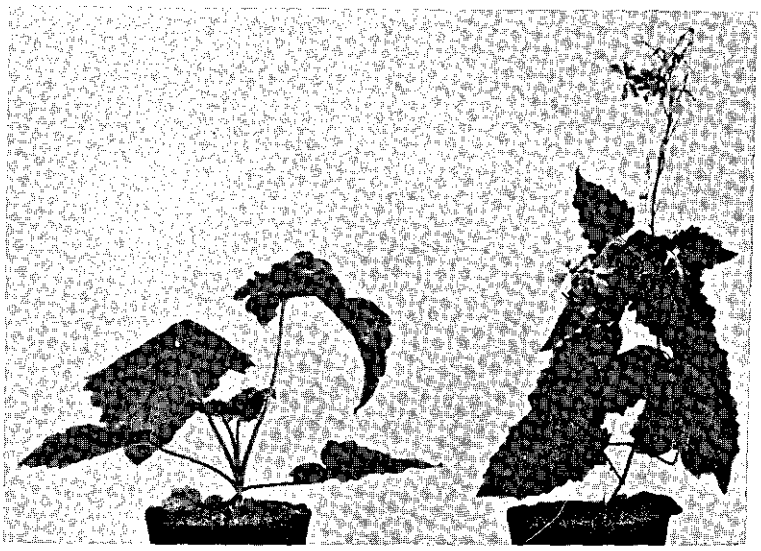


PHOTO 1. Cuttings of 2 weeks old plants, regenerated during 3 weeks before vernalization during 15 weeks, photographed 6 w. after the vernalization.
LEFT: regenerated shoot cutting without cotyledons.
RIGHT: regenerated cotyledon cutting.

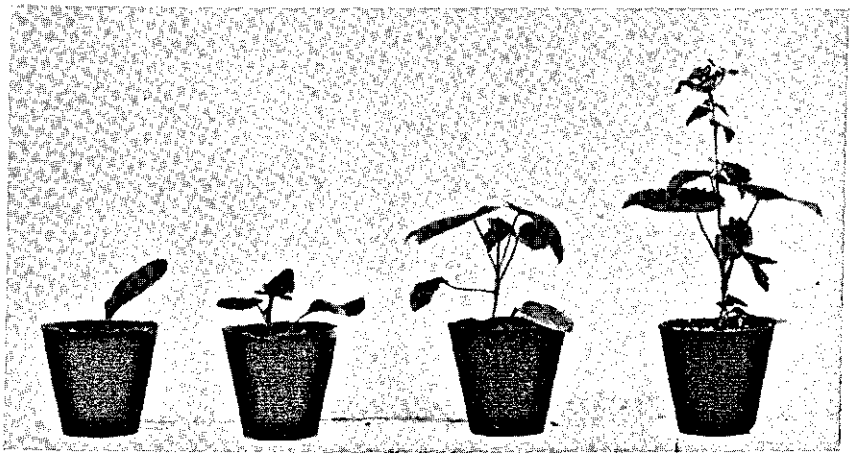


PHOTO 2. Cotyledon cuttings of three weeks old plants which regenerated during 2 weeks in the greenhouse, next were vernalized during 10 weeks and afterwards placed in the greenhouse. From left to right 0, 2, 3, 4 weeks after vernalization.

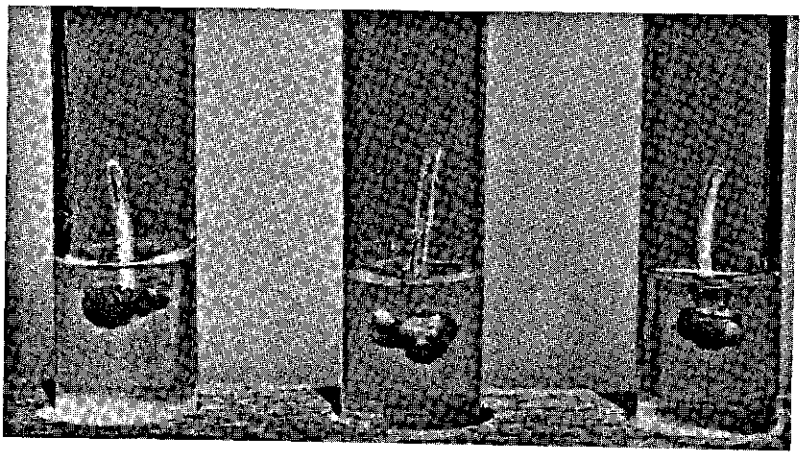


PHOTO 3. Callus induction in isolated petioles of young *Lunaria* plants.

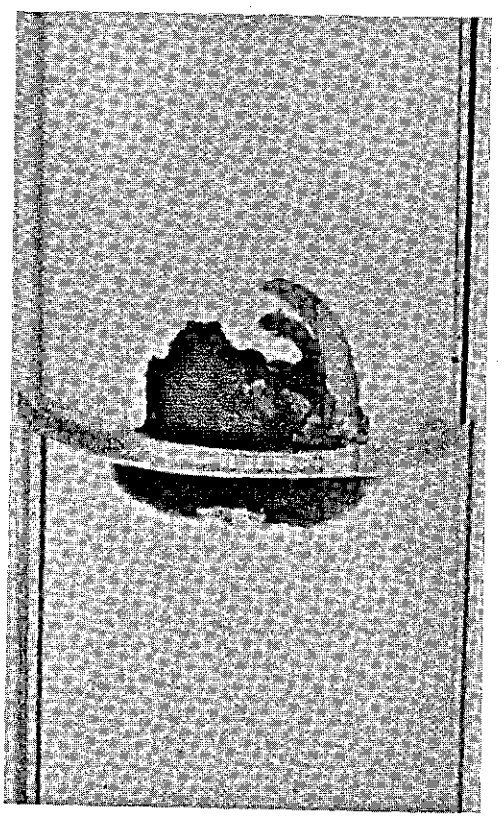


PHOTO 4.
Abnormal sprout formation with 2 completely deformed leaves in an isolated callus tissue of *Lunaria*.

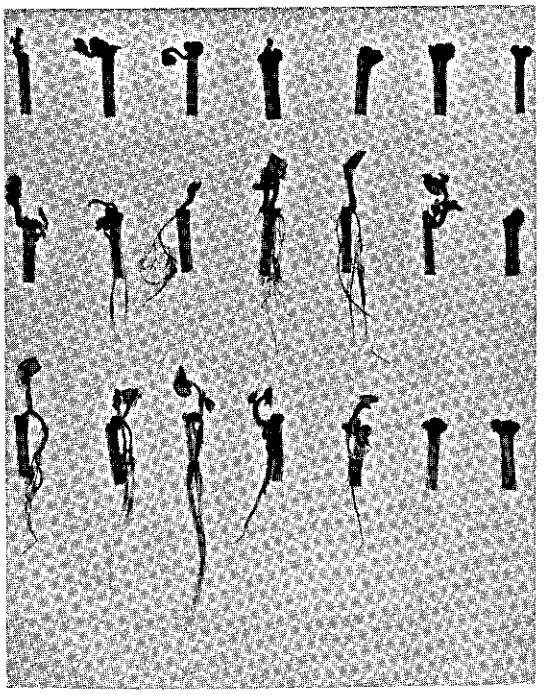


PHOTO 5.
The effect of IAA on the regeneration of petioles of young *Lunaria* plants.
Top row: control. Middle row: IAA 10^{-7}. Bottom row: IAA 4.10^{-7}.

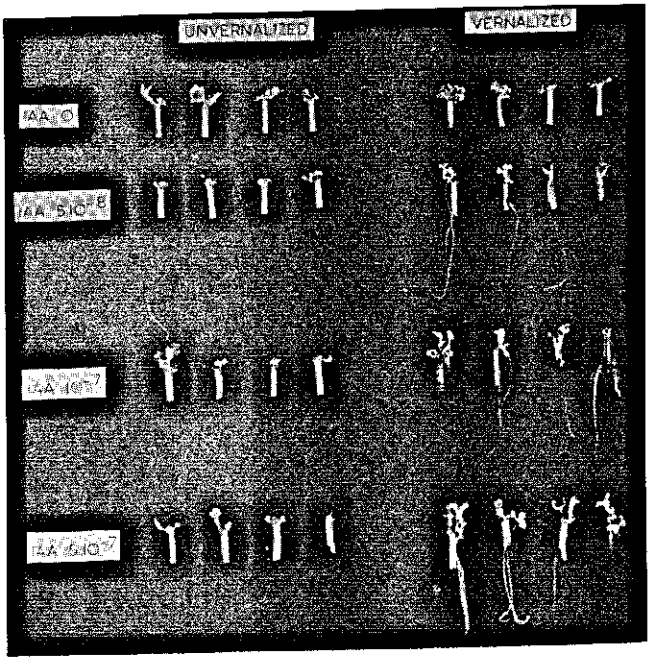


PHOTO 6.
The influence of IAA and vernalization on the regeneration of isolated petioles of *Lunaria annua.*

PHOTO 7.
Sprout formation and flower initiation at 26 °C in an isolated petiole of *Lunaria* after flower induction in vitro during 15 weeks at 5 °C without sprouts. Photo taken 16 weeks after the end of the vernalization.

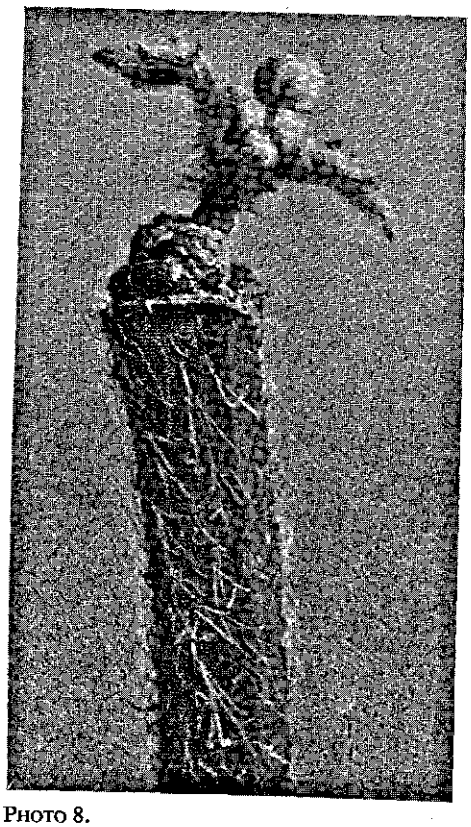


PHOTO 8.
Sprout and flower bud formation at 20 °C in an isolated petiole of *Lunaria* after 20 weeks vernalization. The basic medium contained kinetin 5.10^{-8}. Sprout formation took place during the vernalization whereas no roots were formed.

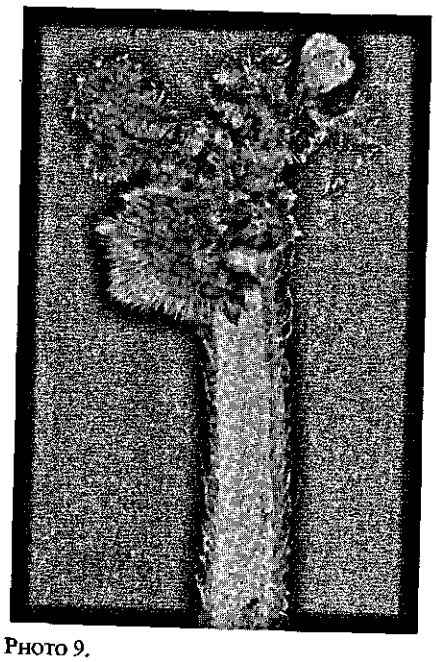


PHOTO 9.
Sprout and flower bud formation at 20 °C in an isolated petiole of *Lunaria* without any regeneration before the vernalization. Duration of vernalization: 16 weeks. For explanation see 8.5.5.3.
Medium: control.

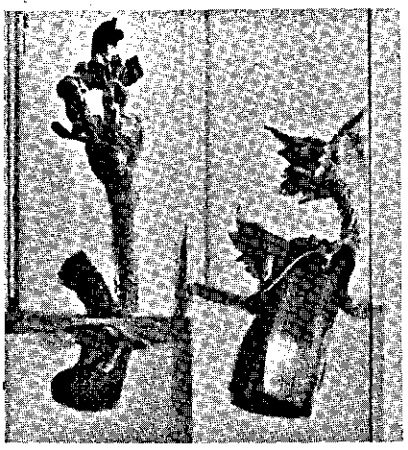


PHOTO 10.
Regenerating explants of the main axis of a flowering *Lunaria* plant, left: flowering; right: vegetative.

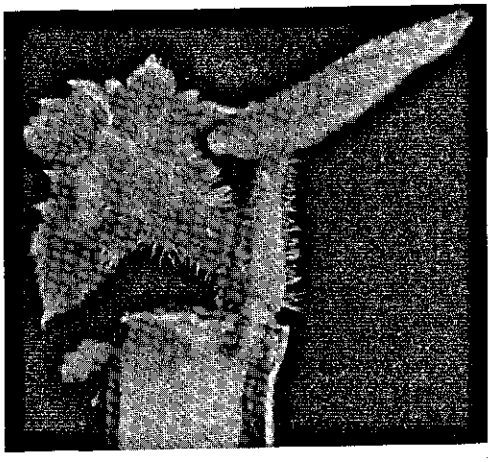


PHOTO 11.
Formation of a vegetative sprout in an explant derived from the main axis of a *Lunaria* plant without opened flower buds.

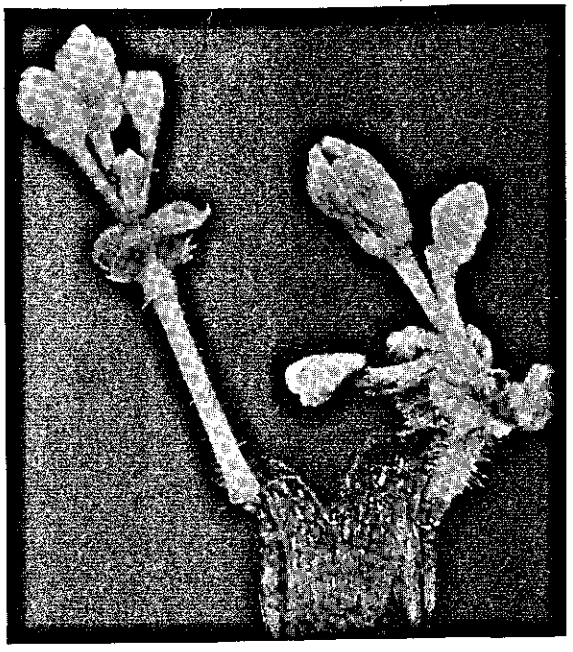
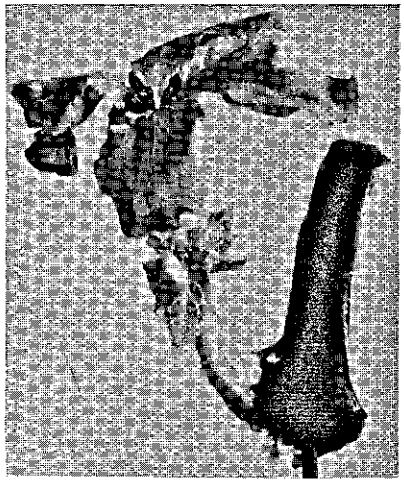


PHOTO 12.
Formation of generative sprouts in an explant derived from the main axis of a *Lunaria* plant in full bloom.

PHOTO 13.
Bud formation at the base of the explant in the culture medium at 20 °C, followed by quite normal flower bud development.



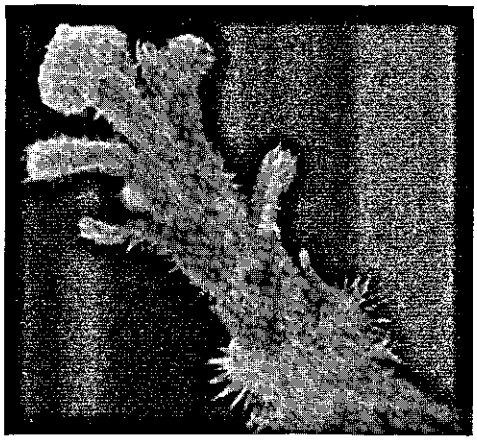


PHOTO 14.
Formation of an underdeveloped inflorescence at a low glucose concentration of 0.5 per cent.

PHOTO 15.
Part of the elongated axis of a flowering *Lunaria* plant isolated in vitro, which regenerated a vegetative sprout (left) and an inflorescence (right) without bracts and leaves.