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THE INDUCTION OF FLOWERING *IN VITRO* IN STEM SEGMENTS OF *PLUMBAGO INDICA* L.

I. THE PRODUCTION OF VEGETATIVE BUDS

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Summary. Internode segments excised from vegetative stems of *Plumbago indica*, an absolute short-day plant, can be induced to form *in vitro* a callus, or roots, or vegetative shoots, or inflorescences and flowers. Callus formation was polarized when indolyl-3-acetic acid, L-tryptophane, naphthylacetamide, or 2,3-dichlorophenylacetic acid were incorporated into the medium, but not with isatin, phenylacetic acid or 2,4-dichlorophenoxyacetic acid (2,4-D). Roots without buds developed on cultures receiving 2,3-dichlorophenylacetic acid or 2,4-D in the presence of a cytokinin and adenine.

Bud formation occurred generally when no auxin was present in the medium, although low concentrations of IAA encouraged bud formation (in the presence of a cytokinin and adenine). Various cytokinins strongly promoted bud formation, but adenine had to be present in the medium in addition. Adenine could not be replaced by adenosine, guanine, guanosine, cytosine, thymine or uracil. The presence of sugar (supplied in the form of various disaccharides) was necessary for bud formation, the concentration in the medium being critical. Long days inhibited bud formation, and so did three different gibberellins applied under short days. Several amino acids, glutamine, asparagine and riboflavine also tended to reduce budding.

Introduction

A New Approach to the Physiology of Flowering. Progress in the physiology of flower induction has been hampered for years by the lack of a suitable test for assaying substances or extracts with potential, flower-inducing properties. Working with whole plants has a number of disadvantages, such as bulk, difficulty of introducing flower-inducing extracts into the right tissue, and interference of the rest of the plant with the chemical treatments given to various regions. The use of plant fragments placed in nutrient media avoids these difficulties. In addition, it may provide more precise answers as to the type of substances which are manufactured by other parts of the plant and are specifically needed for flowering. In a similar manner, *in vitro* culture of excised roots has demonstrated that certain vitamins were indispensable for the continuing growth of tomato roots, a result which could not have been gained by working with whole plants.

In an effort to simplify the problem, we have endeavored to work with stem pieces initially devoid of roots and leaves and to induce on these cultures the formation of both vegetative and floral buds.

Brief Historical Sketch. The induction of flowering *in vitro* has so far been attempted mostly by trying to grow excised shoot apices. The results thus obtained have contributed relatively little to our knowledge of the flowering process, probably because of the technical difficulties in getting isolated apices to make satisfactory growth (see review by NITSCH, 1966).

Another approach has been opened up by SKOOG's observation that a piece of tobacco stem produced five flowers while being cultured *in vitro* (1955). This phenomenon was found again by Mrs. AGHION-PRAT who studied it extensively (1965) and concluded that: (a) formation of flower buds on stem cultures could be obtained only if starting from plants which were already flowering; the technique was in fact most successful when segments from the inflorescence region were used; (b) for unknown reasons, flower buds were not produced by *Nicotiana* species which are photoperiodic, such as *Nicotiana tabacum* var. "Maryland Mammoth", a short-day plant, or *N. sylvestris*, a long-day plant.

The floral buds obtained on *Nicotiana* stem pieces were only the *expression* of the flowering status already present in the tissues at the time they were excised. In no case was there an actual *induction* of flowering *in vitro* from explants which were initially strictly vegetative. This holds also for the flowers obtained by PAULET (1965a) on stem segments of *N. suaveolens*, because the explants had been taken from plants which were flowering, and by STEWARD *et al.* (1966) on cell masses derived from PAULET's cultures.

Actual *induction* of flowering on tissues excised from vegetative plants and initially devoid of buds has been achieved with both long-day and short-days species. With *Cichorium intybus* L., a long-day plant requiring vernalisation, PAULET and NITSCH (1964a) succeeded in producing flowers on explants from vernalized roots, provided the cultures were maintained under long days. The conditions which lead to flower-bud formation have been described (PAULET and NITSCH, 1964a, b; PAULET, 1965b; NITSCH, 1965b). Working with a short-day species, *Plumbago indica*, NITSCH and NITSCH (1965) achieved the induction of flowering *in vitro* on stem segments excised from purely vegetative plants. Flowering has also been induced *in vitro* in the case of another short-day species, *Streptocarpus nobilis* (ROSSINI and NITSCH, 1966).

The present series of articles will describe the experimental conditions leading to the induction of inflorescence and flower formation on excised stem segments of *Plumbago indica*. In order to obtain a flower bud, it is first necessary to stimulate the production of buds in general. The first article of this series will deal therefore with the factors which are necessary for the formation of vegetative buds *in vitro* on cultured stem segments of this species.

Materials and Methods

Plant Material. The plant used was *Plumbago indica* L., clone "Angkor", a strict short-day plant (NITSCH, 1965c). The stock plants were derived from a single specimen by means of cuttings. They were grown on glass fiber covered with "Vermiculite" and watered with the mineral solution used routinely in the Gif Phytotron (NITSCH, 1965c). The pots were placed in a Superglasshouse (see NITSCH, 1965a) at 28° C during the light period and at 20° during the dark period. Day-length was extended to 16 hours by means of a combination of fluorescent and incandescent lamps. Under this regime, the stock plants have been maintained in completely vegetative conditions for over 3 years.

Preparation of the Explants. The first internode below the first, completely unfolded leaf of vigorously-growing shoots was excised with a razor blade, and the two cut ends were sealed by molten paraffin. The internodes were then dipped into 70% ethanol and sterilized for 3—5 min in a filtered suspension of 7% calcium hypochlorite in water. They were rinsed twice with sterile water and cut into cylinders of 7 mm in length by means of parallel razor blades. The average diameter was approximately 4 mm. The stem pieces were then laid horizontally on the nutrient media, about one half of each cylinder being immersed into the agar.

Media. Of the two formulas tested initially (NITSCH and NITSCH, 1965) only formula K was used in the present experiments. The basal medium contained (in mg/l):

a) the following salts: $Ca(NO_3)_2 \cdot 4\,H_2O$ (500), KNO_3 (125), $MgSO_4 \cdot 7\,H_2O$ (125), KH_2PO_4 (125), $MnSO_4 \cdot 4\,H_2O$ (25), H_3BO_3 (10), $ZnSO_4 \cdot 4\,H_2O$ (10), $Na_2MoO_4 \cdot 2\,H_2O$ (0.25), $CuSO_4 \cdot 5\,H_2O$ (0.025), $CoCl_2 \cdot 6\,H_2O$ (0.025), plus 5 ml of a solution containing 7.45 mg/l of the sodium salt of ethylenediaminotetraacetic acid (EDTA) and 5.57 mg/l of $FeSO_4 \cdot 7\,H_2O$;

b) glycine (2), *myo*-inositol (100), nicotinic acid (5), pyridoxin hydrochloride (0.5), thiamin hydrochloride (0.5), folic acid (0.5) and biotin (0.05);

c) agarose[1] (10 gm/l).

Various ingredients, specified in the text, were added to this basal medium.

The media were poured into 25 mm-wide test tubes; these were usually capped with stainless-steel caps[2]. Sterilization was obtained either by autoclaving for 15—20 min at 115 lbs pressure or by filtration through "Millipore" membranes according to a technique described by NITSCH and NITSCH (1957).

Environment of the Cultures. The cultures were grown under two light regimes: short days of 10 h and long days of 16 h. The combination of fluorescent and incandescent lamps used gave an illumination of about 700 ft. c. outside the culture tubes. The temperature was about 30° C during the light period and 20° during the dark period.

Scoring of the Results. The presence or absence of vegetative or flowering buds on the cultures was recorded at various time intervals, the final results being generally evaluated after 3 months of culture. The results are expressed either as the *percentage of cultures* producing a particular type of buds or as the number of buds produced per 10 cultures.

Experimental Results

Potentialities of the Plumbago *Stem Sections.* As shown in Fig. 1, we have been able to induce 4 types of growth and differentiation in the

[1] Agarose is a neutral component of agar, isolated by ARAKI (1956).

[2] Made by Bellco Glass Inc., Vineland, N.J., USA.

stem explants of *Plumbago indica*, namely, the formation of (a) a callus, (b) roots, (c) vegetative buds, and (d) inflorescences and complete flowers. The conditions leading to each type of growth will now be described.

a) Conditions Leading to Callus Formation. Polarity. Stem segments of *Plumbago indica* behave as similar explants of many other Dicotyledons in that they need an auxin in order to form a callus. We have tested the effects of several auxins, both natural and artificial, at various concentrations.

Natural Auxins and Related Compounds. Indolyl-3-acetic acid (IAA), L-tryptophane, isatin and phenylacetic acid all stimulated callus formation, in the presence of benzyladenine (10^{-6} *M*) and adenine (10^{-4} *M*), IAA from 10^{-5} *M* on, L-tryptophane from 10^{-4} *M* on, isatin from 10^{-3} *M* on, and phenylacetic acid from 10^{-4} *M* on.

Synthetic Auxins. Two strong auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,3-dichlorophenylacetic acid, as well as the amide of 1-naphtylacetic acid were also tested in the presence of a cytokinin and adenine. The first two compounds were active at concentrations of 10^{-8} *M* and above, 1-naphthylacetamide at 10^{-4} *M* and above. Fig. 2 shows the aspect of the cultures grown on media containing various levels of 2,4-D, in this case without a cytokinin.

Polarity of Callus Formation. Depending on the type of auxin used, proliferation developed at both ends or at one end only of the explants. At 10^{-7} *M* or above, 2,4-D stimulated callus development over the whole surface of the explants (Fig. 2). With isatin and phenylacetic acid calli developed at both extremities of the segments. With IAA, tryptophane, naphthylacetamide or 2,3-dichlorophenylacetic acid, on the other hand, the development of the calli occurred in a polar fashion. Fig. 3 gives the aspect of the types of calli produced.

b) Conditions Leading to Root Formation. Cultures developed roots when first grown on 2,4-D (10^{-6} *M*) and then transferred to a new medium containing kinetin and adenine, but lacking auxin. Roots developed on all cultures grown on 2,3-dichlorophenylacetic acid (10^{-7} *M* and above) in the presence of benzyladenine (10^{-6} *M*) and adenine sulfate (10^{-4} *M*). In all these instances no buds were formed.

There were other instances, however, in which both roots and shoots developed. This was, for example, the case, with phenylacetic acid at 10^{-5} *M* in the presence of benzyladenine (10^{-6} *M*) and adenine sulfate (10^{-4} *M*).

c) Conditions Leading to Shoot Formation. The initiation of buds on the internodal segments cultured *in vitro* was found to depend on both physical and chemical factors.

Effect of Daylength. The facility of bud formation was dependent on the photo- and thermoperiodic regimes to which the explants were

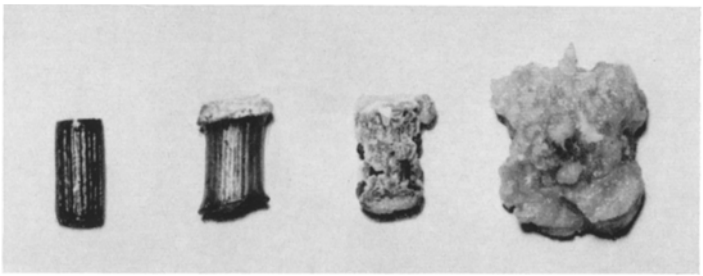

Fig. 2

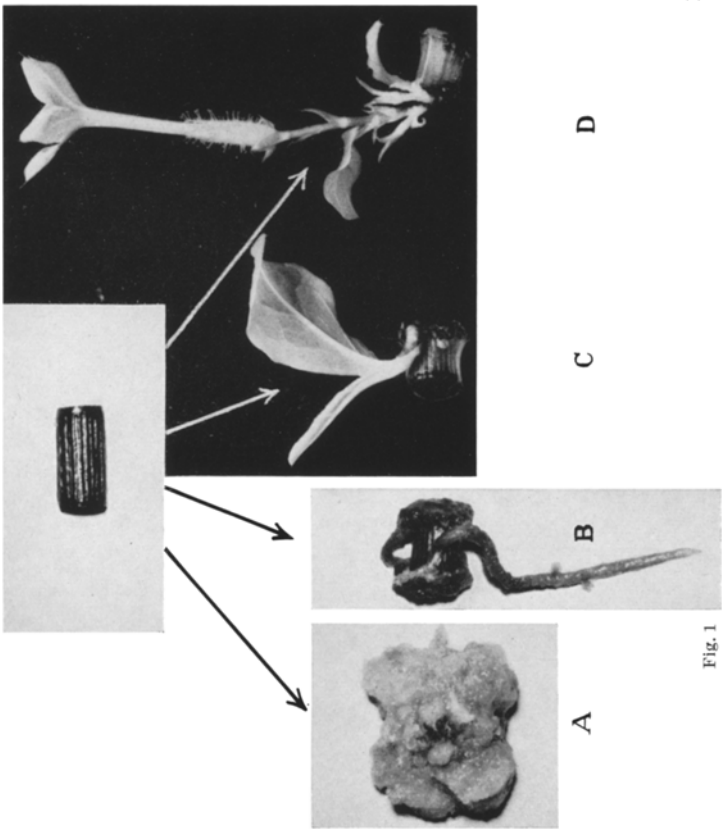

Fig. 1

Fig. 1. Morphogenetic potentialities of the internode of vegetative *Plumbago indica*. Depending on the conditions, the same explant (*top*) can produce *in vitro*: a callus (A), roots (*B*), a vegetative bud (*C*), or a flower (*D*)

Fig. 2. Production of unpolarized calli with 2,4-D on internode sections in the presence of adenine (10^{-4} *M*) and 2% sucrose, but without a cytokinin. Molar concentrations of 2,4-D: 0 (*A*), 10^{-5} (*B*), 10^{-7} (*C*), 10^{-6} (*D*). Photographed after 11 weeks of culture under 10-hour days

subjected. Thus, as Table 1 shows, more buds are formed under short days than under long ones.

In total darkness, about 30% of the cultures produced buds, but these gave rise to erect, white stems with only scale-like structures instead of leaves (Fig. 4).

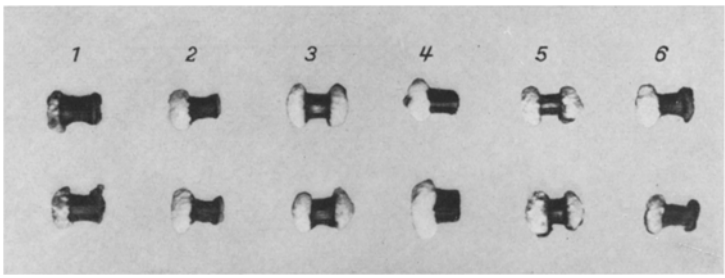

Fig. 3. Callus development on 5-mm-long explants grown in the presence of benzyladenine (10^{-6} *M*), adenine (10^{-4} M) and 2% sucrose on: *1* IAA (10^{-5} *M*), *2* L-tryptophane (10^{-3} *M*), *3* isatin (10^{-3} *M*), *4* 1-naphthylacetamide (10^{-4} *M*), *5* phenylacetic (10^{-4} *M*), *6* 2,3-dichlorophenylacetic acid (10^{-7} *M*). Photographed after 2 months of culture under 10-hour days

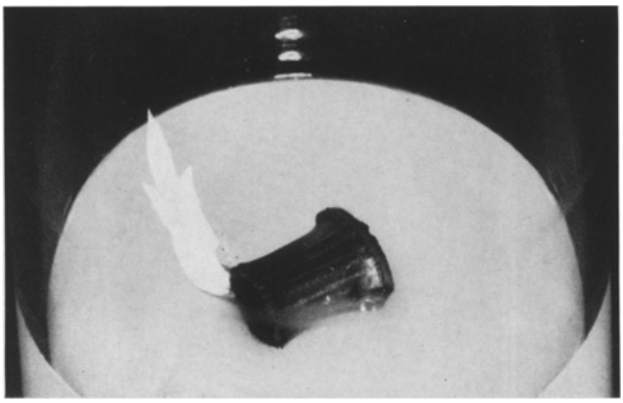

Fig. 4. Shoot developed on an internode section grown in total darkness for 3 months on a medium containing kinetin (1 mg/l), adenine (10^{-4} *M*) and 2% sucrose

Table 1. *Effect of daylength on bud formation**

	Photoperiod	Cultures with buds (%)	Number of buds per 10 cultures
1st experiment:			
Internode segments 5 mm in length.	9 hours	75	**
Medium with 10^{-4} *M* adenine and 1%	18 hours	17	**
sucrose. Results scored after 15 weeks			
2nd experiment:			
Internode segments 10 mm in length.	9 hours	92	28
Medium with 3×10^{-4} *M* adenine and	18 hours	17	4
2.5% sucrose. Results scored after 7 weeks			

* Kinetin (1 mg/l) in all cases.
** Not determined.

Effect of Various Disaccharides. Several disaccharides have been tested for their ability to support bud formation. From the results

Table 2. *Effect of several disaccharides on bud formation*
The media contained kinetin (1 mg/l) and adenine (10^{-4} *M*). The disaccharides were sterilized by filtration. Results recorded after 14 weeks of culture under 10-hour days.

Compound	Concentration (*M*)	Cultures with buds (%)	Number of buds per 10 cultures
None	0	0	—
Sucrose	3×10^{-2}	50	—
	6×10^{-2}	50	—
	9×10^{-2}	45	—
Cellobiose	3×10^{-2}	83	—
	6×10^{-2}	67	—
Sucrose	3×10^{-2}	100	50
	6×10^{-2}	91	53
	9×10^{-2}	17	27
Maltose	3×10^{-2}	100	30
	6×10^{-2}	83	20
	9×10^{-2}	73	9
Lactose	3×10^{-2}	100	27
	9×10^{-2}	91	17

Table 3. *Effect of various cytokinins on bud formation*
The media contained 2% sucrose and 10^{-4} *M* adenine. Results recorded after 3 months of culture under 10-hour days.

Cytokinin	Concentration (*M*)	Cultures producing buds (%)
None	0	0
Kinetin	10^{-7}	0
	10^{-6}	30
	10^{-5}	60
Kinetin	10^{-5}	92
Benzyladenine	10^{-7}	0
	10^{-6}	83
	10^{-5}	75*
SD-8339	10^{-7}	17
	10^{-6}	100
	10^{-5}	92*
Zeatin	10^{-7}	25
	10^{-6}	100
	10^{-5}	100*
Triacanthin filtered	10^{-5}	0
Triacanthin autoclaved	10^{-5}	0

* Buds numerous, but small.

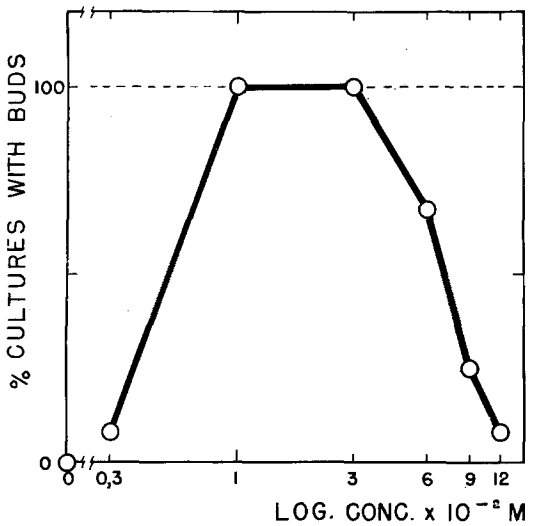

Fig. 5. Effect of sucrose concentration on the number of cultures producing buds in the presence of kinetin (1 mg/l) and adenine (10^{-4} *M*). Results after 9 weeks of culture under 10-hour days

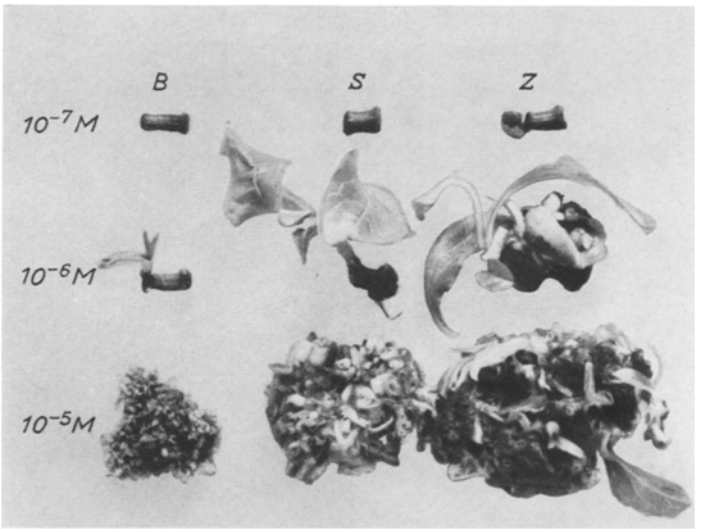

Fig. 6. Effect of cytokinin concentration upon bud formation. *B* 6-benzyl-adenine, *S* SD-8339 (6-benzyl-9-tetrahydropyrane-adenine), *Z* zeatin. All media contained 2% sucrose and adenine (10^{-4} *M*). Photographed after 10 weeks of culture under 10-hour days

of Table 2 it can be seen that sucrose, cellobiose, maltose and lactose support the formation of buds. With lactose, however, the buds remain small.

The concentration of the disaccharide added to the medium has a great influence upon the number of buds initiated and on their further

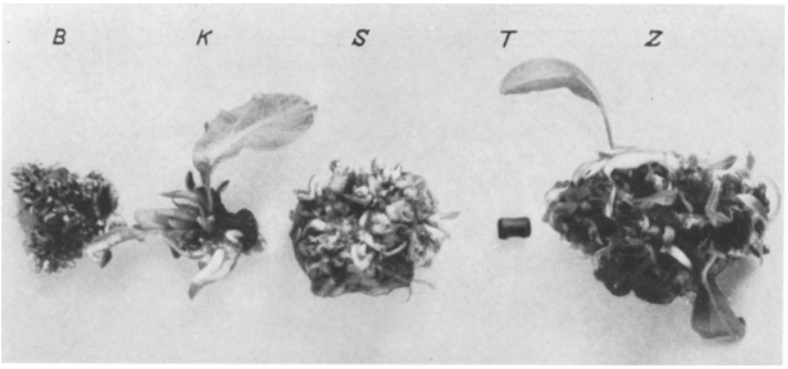

Fig. 7. Comparative effect of benzyladenine (*B*), kinetin (*K*), 6-benzyl-9-tetrahydropyrane-adenine (*S*), triacanthin (*T*) and zeatin (*Z*), all at 10^{-5} *M* on bud formation. All media contained 2% sucrose and adenine (10^{-4} *M*). Photographed after 10 weeks of culture under 10-hour days

Table 4. *Necessity of adenine for the induction of bud formation with kinetin*
Sucrose concentration: 2%. Results recorded after 3 months of culture under 10-hour days.

Kinetin concentration	Adenine concentration	Adenosine concentration	Cultures producing buds (%)
0	0	0	0
10^{-7} *M*	0	0	0
10^{-6} *M*	0	0	0
10^{-5} *M*	0	0	40**
0	10^{-4} *M*	0	0
10^{-7} *M*	10^{-4} *M*	0	0
10^{-6} *M*	10^{-4} *M*	0	30**
10^{-5} *M*	10^{-4} *M*	0	60
1 mg/l	0	0	7
1 mg/l	10^{-6} *M**	0	17
1 mg/l	10^{-5} *M**	0	8
1 mg/l	10^{-4} *M**	0	33
1 mg/l	3×10^{-4} *M**	0	100
1 mg/l	0	0	0
1 mg/l	0	10^{-6} *M*	0
1 mg/l	0	10^{-5} *M*	0
1 mg/l	0	10^{-4} *M*	0
1 mg/l	0	10^{-3} *M*	0
1 mg/l	10^{-4} *M*	0	67
1 mg/l	3×10^{-4} *M*	0	100

* Sterilized by filtration.
** Very small buds.

development. In the case of sucrose, the greatest number of buds per culture was produced at the concentration of 10^{-2} *M* (Fig. 5). At this

Table 5. *Effect of various auxins and related compounds on bud formation*
The media contained benzyladenine (10^{-6} *M*), adenine (10^{-4} *M*) and 2% sucrose. The mode of sterilization is indicated in the footnotes. Results scored after 4 months of culture under 10-hour days.

Auxin	Concentration (*M*)	Cultures producing buds (%)	Number of buds per 10 cultures
None	0	83	33
IAA*	10^{-8}	100	28
	10^{-7}	92	30
	10^{-6}	75	10
	10^{-5}	60	15
L-tryptophane*	10^{-6}	100	31
	10^{-5}	83	18
	10^{-4}	30	7
	10^{-3}	0	0
Isatine*	10^{-4}	91	18
	10^{-3}	0	0
None	0	91	18
IAA**	10^{-6}	100	25
Phenylacetic acid**	10^{-6}	83	12
	10^{-5}	50	13
	10^{-4}	0	0
Naphthaleneacetamide**	10^{-6}	91	18
	10^{-5}	91	21
	10^{-4}	0	0
2,3-Dichlorophenylacetic acid**	10^{-8}	25	3
	10^{-7}	0	0
	10^{-6}	0	0

* Filtered.
** Autoclaved.

concentration and below, however, the buds remained small; at concentrations 3—6 times higher, they developed into shoots. When the concentration was increased still further, both bud initiation and bud development became inhibited.

Effect of Cytokinins. Serial concentrations of various cytokinins were tested in the presence of 10^{-4} *M* adenine. The results, shown in Table 3, indicate that buds can be produced with kinetin, benzyladenine, 6-benzyl-9-tetrahydropyrane-adenine (SD-8339), and zeatin at the proper concentrations, but not with triacanthin, even when the latter had been autoclaved with the medium for 15 min.

As shown in Fig. 6, the number of buds per culture increased very markedly with the concentration of cytokinin, 10^{-5} *M* producing in most cases an abundance of very small buds which generally did not

develop into normal shoots. Fig. 7 gives a photographic comparison of the effect of 4 different cytokinins and of triacanthin, each at the concentration of 10^{-5} *M*.

Effect of Adenine. In the course of our experiments it was noted that the presence of adenine was necessary in order to obtain buds with cytokinins. Data from three different experiments, given in Table 4, show this effect. The results of the second and third experiments are especially clear-cut in that the series supplied with 1 mg/l of kinetin alone did not produce any bud, whereas with increasing concentrations of adenine an increasing percentage of the cultures produced buds. The average number of buds per culture also increased with the concentration of adenine.

Thus, adenine is essential for the production of buds, even in the presence of a cytokinin such as kinetin. In fact, in certain series, adenine at 10^{-4} *M* was able to induce the formation of a few buds in the absence of any cytokinin in the medium.

The promotive action of adenine on bud formation seems to be very specific since none of the other substances tested alone, not even adenosine, had a similar effect. Guanine (from 10^{-6} *M* to 3×10^{-4} *M*), cytosine (from 10^{-7} to 10^{-4} *M*), uracil (10^{-7} to 10^{-4} *M*) or thymine (10^{-7} to 10^{-5} *M*), all sterilized by filtration, did not give an appreciable increase in bud formation in the presence of 1 mg/l of kinetin and 2% sucrose.

Table 6. *Effect of three gibberellins on bud formation*

All media contained kinetin (1 mg/l), adenine (10^{-4} *M*) and 2% sucrose. The gibberellins were sterilized by filtration. Results assessed after 15 weeks of culture under 10-hour days.

Gibberellin	Concentration (*M*)	Cultures producing buds (%)
None	0	60
GA_1	10^{-7}	50
	10^{-6}	0
	10^{-5}	8
GA_3	10^{-7}	30
	10^{-6}	17
	10^{-5}	8
GA_7	10^{-7}	9
	10^{-6}	33
	10^{-5}	8

However, when adenine was already present in the medium at an optimal concentration (3×10^{-4} *M*), bud formation was enhanced further by the addition of thymine.

Effect of Auxins, Gibberellins and Other Substances (Amino Acids, Amides, Urea, Riboflavin). Different concentrations of both natural and synthetic auxins have been added to media containing a cytokinin (such as kinetin or benzyladenine) and adenine. As expected, auxins tended generally to decrease bud formation, sometimes in a drastic manner. This is shown by the results in Table 5. IAA, however, when used at concentrations of 10^{-8} *M* to 10^{-6} *M*, often increased slightly the production of buds in the presence of a cytokinin and adenine.

Table 7. *Effect of amino acids, amides and urea on bud formation*

All media contained kinetin (1 mg/l), adenine (10^{-4} *M*) and 1.5% sucrose. All the nitrogenous compounds were sterilized by filtration. Results recorded after 15 weeks of culture under 10-hour days.

Compound	Concentration (*M*)	Cultures (%) producing	
		embryonic buds*	developed buds
None	0	25	50
L-alanine	3×10^{-4}	17	25
	10^{-3}	17	25
	3×10^{-3}	8	17
L-arginine	3×10^{-4}	8	17
	10^{-3}	42	33
	3×10^{-3}	42	17
L-serine	10^{-3}	36	17
	3×10^{-3}	8	0
Urea	3×10^{-4}	0	75
	10^{-3}	8	67
	3×10^{-3}	0	58
None	0	8	42
L-aspartic acid	3×10^{-4}	27	36
	10^{-3}	8	17
	3×10^{-3}	9	9
L-glutamic acid	3×10^{-4}	44	22
	10^{-3}	30	10
	3×10^{-3}	33	0
L-asparagine	3×10^{-4}	45	27
	10^{-3}	36	36
	3×10^{-3}	8	0
L-glutamine	10^{-3}	18	27
	3×10^{-3}	45	9

* Buds which did not develop beyond a size of 1—2 mm.

Three different gibberellins, GA_1, GA_3 and GA_7 (sterilized by filtration), were tried, each at three different concentrations. As shown in Table 6, bud formation was reduced in all cases.

The effect of a number of organic, nitrogenous compounds was also tested. As shown by the data of Table 7, amino acids and amides at the concentrations used, tended to reduce bud formation or, at least, bud development. The latter effect is indicated by the percentage of cultures producing embryonic buds which did not develop further to any great extent.

Urea, in contrast, did not inhibit bud development, but rather stimulated it to some extent.

Riboflavine (sterilized by filtration and then added to the media) inhibited bud formation at 10^{-6} *M* and above.

Discussion

Before studying how flowers could be produced *in vitro*, it was necessary to investigate the conditions which lead to bud formation in general. Two aspects of budding have to be distinguished, namely, bud *initiation* and bud *development*. These two processes can be separated experimentally. For example, lactose allows the formation of buds, but these remain very small, whereas the same molar concentration of sucrose leads to good bud development. Also, relatively low concentrations of sucrose (below 10^{-2} *M*) or high concentrations of cytokinins (above 10^{-6} *M*) stimulate the formation of numerous buds (see Fig. 6), but these do not develop to any great extent; other concentrations may lead to the initiation of a smaller number, which however develop into leafy shoots.

Among the factors which have been investigated, some have been found to favor bud initiation and development, others to inhibit these processes.

Factors Promoting Bud Formation. Conditions which favor bud formation on internodal segments of *Plumbago indica* are: (a) an adequate sugar supply, (b) a low level of auxin, (c) cytokinins, (d) adenine.

Various disaccharides were capable of stimulating bud formation. With lactose, however, as just mentioned, the buds tended to remain small, whereas they developed well with sucrose, cellobiose or maltose. The concentration of sugars in the medium appeared to be critical, as was shown by PAULET (1965a) in the case of *Nicotiana glauca*. Relatively low concentrations (such as 3×10^{-3} *M*) allowed the formation of numerous buds, but these remained very small. They developed well when the sucrose level in the medium was 10 times higher. As shown by NITSCH and NITSCH (1965), the sucrose level has a determining influence upon the orientation of these buds towards the vegetative or the flowering state. This effect will be described in detail in the second article of this series.

As is well known from the literature (see review by NITSCH, 1965b), auxins tend to inhibit bud formation. There are however reports on promotion of bud formation by low concentrations of auxins (see, for example, STICHEL, 1959). The incorporation of IAA (10^{-8} to 10^{-6} *M*) into the medium speeded up bud formation in *Plumbago indica* and caused nearly 100% of the cultures to produce buds. It should be emphasized, however, that this effect occurred when a cytokinin and adenine were present in the medium. In their absence, IAA caused only the production of roots, not of shoots.

The stimulatory effect of cytokinins upon bud formation is well-known (SKOOG and MILLER, 1957). In the case of *Plumbago* cultures, kinetin, benzyladenine, 6-benzyl-9-tetrahydropyrane-adenine and zeatin all were effective in promoting the differentiation of buds. Triacanthin has been claimed to be active as a cytokinin by BEAUCHESNE (see CAVE *et al.*, 1962). However, ROGOZINSKA *et al.* (1964) demonstrated that triacanthin is not active *per se*, but can be transformed during autoclaving into its isomer 6-(γ, γ-dimethylallylamino)-purine which is a very active cytokinin. In the case of *Plumbago*, triacanthin was totally inactive, even after being autoclaved for 15 min with the medium (Fig. 7).

Adenine has long been known to promote bud formation in tobacco stem segments (SKOOG and TSUI, 1948). Since then the discovery of kinetin and other cytokinins has overshadowed its role, and the idea has been put forward that kinetin and similar cytokinins were merely more efficient adenine molecules. It came therefore as a surprise that adenine was required for bud formation in addition to a cytokinin (Table 4). This effect seems to be very specific of adenine, since neither adenosine nor guanine, cytosine, uracil or thymine could substitute for it. Similar results have been obtained with cultures of tobacco stem (SKOOG and MILLER, 1965) and of tobacco pith (NITSCH *et al.*, 1966).

Factors Inhibiting Bud Formation. Conditions which inhibit bud formation include: (a) long photoperiods, (b) gibberellins, (c) auxins at relatively high concentrations, (d) amino-acids, and (e) riboflavin.

The effect of long days (Table 1) resembles the one described by HEIDE (1965) with *Begonia* leaf cuttings. The inhibitory effect produced by long days may be attributed in part to the stimulation of gibberellin production by long photoperiods (see NITSCH, 1963). In fact, as shown in Table 6, gibberellins reduce budding in *Plumbago* stem segments, as they do in tobacco callus cultures (MURASHIGE, 1964).

Amino acids such as alanine, arginine, serine, aspartic and glutamic acids and their corresponding amides, depressed budding and tended to inhibit the development of buds (Table 7). L-proline and hydroxy-L-proline did not seem to be inhibitory at concentrations of 10^{-4} *M* or below, although they reduced bud development at 10^{-3} *M*.

Finally riboflavin inhibited bud formation, whereas the mixture of glycine, *myo*-inositol, nicotinic acid, thiamin, folic acid and biotin routinely added to all media promoted budding, as shown by parallel series of cultures which were or were not supplied with these addenda.

Advantages of Plumbago indica *as an Experimental Plant.* An important feature of the plant material used in the present investigations resides in the fact that it constitutes a clone, derived from a single plant (NITSCH, 1965c). The results presented above show how versatile internodal tissues of this clone can be: depending on the cultural conditions,

they can produce a callus, or roots, or vegetative shoots, or inflorescences and flowers. The development of flowers *in vitro* will be dealt with in the second article of this series.

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