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In vitro flowering and pod formation from cotyledons of groundnut (*Arachis hypogaea* L.)

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Summary. The response of groundnut cotyledons to the presence of various growth regulators in concentrations from 0.1 to 5 mg/l has been studied in detail using several genotypes of groundnut on two different media. Cotyledons with embryo axis, cultured on Blaydes' medium with cytokinins, produced shoots, in the axils of which 2–7 flower buds could be seen. The frequency of flower bud induction in general increased with increasing concentrations of cytokinins, the optimal levels being 3 mg/l of KN or 4 mg/l of BAP. Cotyledons without embryo axis, cultured on Blaydes' medium with BAP (0.5 mg/l), produced a cluster of flower buds directly, ranging in number from 8–28, without any vegetative growth. Excised embryo axes cultured on the same medium gave plantlets without flower buds. The growth regulators IAA, NAA, GA_3 and ABA failed to induce flower buds in independent treatments. However, lower concentrations of IAA and NAA in combination with cytokinins exerted a positive influence on flowering. The blooming of the flower buds was facilitated on media supplemented with low concentrations of cytokinins. Six percent of the induced flowers resulted in gynophore development and ultimately formed pods when cultured under complete dark conditions in modified MS medium supplemented with kinetin.

Key words: *Arachis hypogaea* L. – Cotyledons – Cytokinins – Flower induction – Pod formation

Introduction

The phenomenon of flowering, a consequence of a change in the activity of apical meristems from forming vegetative structures to forming sex organs has been a subject of intensive research (Zeevart 1976). Flowering, which ensures the sexual method of reproduction, allows recombination of gene controlled characters and permits rare favourable mutations to spread in the gene population.

The ability to control the capacity of flower formation is also of practical importance in the synchronous development of fruit, particularly in groundnut which, being asynchronous, often has considerable losses in yield (ICRISAT 1980). The earlier studies of invitro floral bud differentiation were restricted to stem segments isolated from plants in the reproductive phase such as tobacco (Aghion-Prat 1965; Wardell and Skoog 1969), *Plumbago* (Nitsch and Nitsch 1967), *Torenia* (Tanimoto and Harada 1981) and thin layers of *Nautilocalyx* (Tran Thanh Van 1973a) and tobacco (Tran Thanh Van 1973b). The existence of a physiological gradient along the main axis of the donor plant was reported for tobacco (Tran Thanh Van 1973b): segments of floral region differentiated into floral buds while segments from basal region differentiated into vegetative buds.

The present communication reports on the induction of flower buds and flowers directly from cotyledonary explants of groundnut and their further pod formation under in vitro conditions.

Materials and methods

Seeds of the 'TMV_2' groundnut cultivar were surface sterilized for 15 min in 0.1% mercuric chloride and washed thoroughly with sterile water. The seeds wee then halved into two cotyledons – one containing the embryo axis (embryonated cotyledon) and the other without the embryo axis (de-embryonated cotyledon). The cotyledons were cultured in two different media, Blaydes' (1966) and Murashige and Skoog's (MS) (1962), each containing 3.0% sucrose and gelled with 0.8% agar. Embryo axes were separately excised from the seed and inoculated onto the media. In all experiments the media were supplemented with growth regulators, BAP, KN, IAA, NAA, ABA and GA_3 in concentrations ranging from 0.1–5.0 mg/l, either alone or in combinations. Explants were also inoculated

in Blaydes' and MS basal media. Cotyledons of six other cultivars namely 'TG-19B', 'Chico', 'ICG 2875', 'CGC-5', 'ICG 4367' and 'ICG 313' were inoculated onto the flower bud inducing medium, as standardized previously, to study genotypic differences in the capacity for in vitro flowering. All cultures were grown under continuous cool white fluorescent light of 2,000 lux at 25 ± 2 °C.

For pod formation MS medium substituted with Blaydes' nitrates and incorporated with 0.5 mg/l KN was used. Cultures with elongated gynophores, those penetrating into medium, were transferred from flower inducing medium to this pod forming medium and cultured in a complete dark chamber. The cultures were examined systematically at intervals of 15 days for possible embryo growth.

Results and discussion

Cotyledons with an embryo axis, cultured on Blaydes' basal medium, produced one to two flower buds in the axils of developing shoots with a 5.2% frequency (Table 1) within 4–6 weeks of culture. The same cotyledons cultured on MS basal medium did not respond. Cotyledons without an embryo axis and excised embryo axes failed to produce flower buds when cultured on either Blaydes' or MS basal media.

Embryonated cotyledons of 'TMV_2', cultured on Blaydes' medium supplemented with cytokinins, developed 2–7 flower buds in the axils of developing shoots with a high frequency within 3 weeks of culture (Fig. 1 a). The percentage of flower bud induction increased with increasing concentrations of cytokinins, the optimal levels being 4 mg/l BAP or 3 mg/l KN (Table 1). The other growth regulators IAA, NAA, GA_3 and ABA had no effect on flower bud induction in independent treatments. However, flower bud development was not observed on MS medium containing either KN or BAP, both of which only resulted in the browning of the medium. The response of seven different genotypes (Table 2) of embryonated cotyledons cultured in 3 mg/l KN supplemented Blaydes' medium suggested that the frequency of flower bud induction was maximum (47.8%) in 'ICG 2875' while the percentage of cultures with flower buds were lowest (20.8%) in 'Chico'. However, the number of flower buds/cotyledon was least in 'ICG 2875' and maximum in 'ICG 4367'.

De-embryonated cotyledons cultured on Blaydes' medium with BAP (0.5 mg/l) produced flower buds with a 20.2% frequency from the 'TMV_2' cultivar. The flower bud formation in this case was not accompanied by vegetative growth (Fig. 1 c), unlike that of the embryonated cotyledons. In contrast to the fewer number of flower buds (two to seven) emerging from shoot axil of embryonated cotyledons, the number of flower buds from de-embryonated cotyledons was as high as 8–28. Prior to flower bud formation, the cotyledons became considerably swollen and turned extensively green within a week of culture. Cytokinin-induced chloroplast development has already been well documented (Sugiura 1963; Wozny and Szaweykowska 1975). The initiation of the flower bud was preceeded by an extended growth of a hypocotyl-like structure, occassionally ending as a blunt root. The cluster of flower buds formed away from the cotyledonary node on this extended growth (Fig. 1 c). The culture of de-embryonated cotyledons in media containing an excess of 0.5 mg/l BAP in supplemented Blaydes' medium, showed the sporadic formation of flower buds. When the level of BAP exceeded 2 mg/l, the same cotyledons produced vegetative structures without reproductive buds. The response of seven different genotypes of de-embryonated cotyledons cultured in 0.5 mg/l BAP

Table 1. The effect of varying concentrations of BAP and KN on flower bud formation from embryonated cotyledons of TMV_2 cultivar cultured on Blaydes' medium. An average of 70–80 cotyledons were cultured per treatment

Hormone (mg/l)	Cultures showing flowering response (%)		Flower buds/ cotyledon		Flower bloom (%)	
	BAP	KN	BAP	KN	BAP	KN
0.5	21.0	9.3	2.9	3.2	62.1	68.7
1.0	25.2	15.2	3.7	4.7	51.3	70.2
2.0	28.2	33.6	4.1	5.8	39.0	53.4
3.0	30.0	41.2	4.4	4.6	25.0	36.9
4.0	37.2	12.2	3.3	3.8	9.1	23.7
5.0	19.0	6.3	2.8	2.6	–	7.6
Basal medium	5.2		1.4		71.4	

Table 2. Comparative data on the frequency of flower bud formation from cotyledons of different genotypes of *Arachis hypogaea* L. cultured on Blaydes' medium. EC = Embryonated cotyledons cultured in 3.0 mg/l KN; DC = De-embryonated cotyledons in 0.5mg/l BAP. An average of 70–80 cotyledons were cultured per treatment

Genotypes	Cultures showing flowering response (%)		Flower buds/ cotyledon		Flower bloom (%)	
	EC	DC	EC	DC	EC	DC
TMV_2	41.2	20.2	4.6	17.4	36.9	68.7
TG-19B	43.0	18.6	4.6	14.4	34.7	52.1
Chico	20.8	16.4	3.2	12.4	40.6	58.1
ICG 2875	47.8	11.8	2.2	9.8	31.8	54.1
CGC-5	26.6	0.0	3.0	–	33.3	–
ICG 4367	34.1	9.8	5.2	13.7	38.6	64.1
ICG 313	29.6	12.7	4.8	12.0	42.6	57.6

supplemented Blaydes' medium suggested that the frequencies of flower bud induction (20.2%) and flower buds/cotyledon (17.4) were maximum from the de-embryonated cotyledons of 'TMV$_2$' and minimum in the genotype 'CGC-5' (Table 2).

Excised embryo axes separated from cotyledons and cultured on Blaydes' medium with cytokinins produced plantlets without flower buds.

The mineral formulation of the medium seems to play an important role in the expression of flower bud primordia. In Blaydes' medium, the three major inorganic salts, ammonium (1,000 mg/l), potassium (1,000 mg/l) and calcium (499 mg/l) nitrates were present in the proportion of 2:2:1. Keeping the total amount of nitrates present in Blaydes' medium at a constant level, different combinations were tested by varying levels of these three sources of nitrogen using de-embryonated cotyledons. When nitrates were supplied only as calcium nitrate, the frequency of flower bud induction increased by 6.8% compared to Blaydes' medium with normal nitrates and 0.5 mg/l BAP. When calcium and ammonium nitrates were supplied in the proportion of 3:2, deleting potassium nitrate, the frequency of flower bud induction was maximum (32.4%). However, the frequency (22.5%) was less than the above two combinations when ammonium nitrate was deleted and calcium and potassium nitrates were present in the proportion of 3:2. In contrast to *Torenia* (Tanimoto and Harada 1981), where the removal of ammonium nitrate from the medium increased the number of flower bud primordia, in the present case ammonium is found to have a promotive influence.

The MS medium failed to promote flower buds from either of the two cotyledons when supplemented with cytokinins. However, upon substitution of the nitrates of the MS medium with that of Blaydes' resulted in flowering. Embryonated cotyledons of 'TMV$_2$' cultured in 3 mg/l KN supplemented and nitrate-modified MS medium produced flower buds with a 28% frequency, while de-embryonated cotyledons of 'TMV$_2$' cultured on the same medium with 0.5 mg/l BAP developed flower buds in 17% of the cultures.

The role of sucrose (3–12%) on flower bud induction was tested using de-embryonated cotyledons. Sucrose concentration exceeding 30 g/l did not result in flowering, possibly because of osmotic involvement. However, a high concentration of sucrose promoted in vitro flowering in *Cichorium* (Harada 1966) and *Plumbago* (Nitsch and Nitsch 1967). The low frequency occurrence (3.8%) of flower buds in Blaydes' medium without sucrose suggests that the natural reserve material within the cotyledons could supply the necessary organic constituents required for floral morphogenesis.

Experiments were also made to study the effect of segmentation of cotyledons on flower bud induction by dividing both cotyledons of the 'TMV$_2$' cultivar into three parts horizontally. When these segments of embryonated and de-embryonated cotyledons were cultured on Blaydes' medium with cytokinins, flower bud formation was observed from the first-one-third segment of embryonated cotyledons with 13.6% frequency in Blaydes' medium with KN (3 mg/l). Only a few flower buds were induced in de-embryonated segments in 5.8% of the cultures on Blaydes' medium with 0.5 mg/l of BAP.

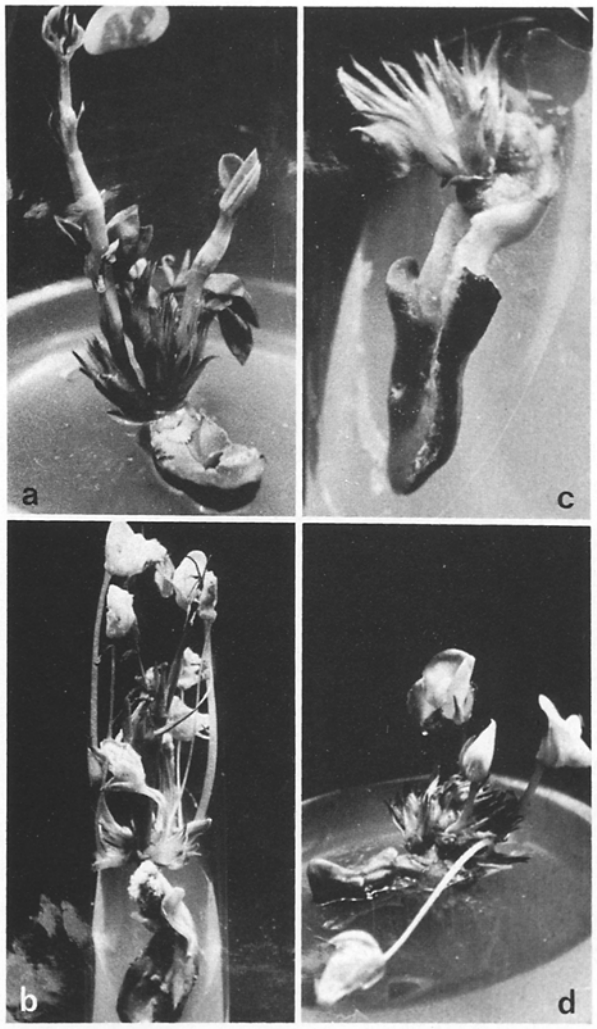

Fig. 1. **a** Cotyledon with embryo axis, cultured on Blaydes' medium with KN (3 mg/l), showing the growth of a shoot and flower buds; **b** blooming of the flower buds from cotyledons with embryo axis in the same medium; **c** cotyledons without embryo axis, cultured on Blaydes' medium with 0.5 mg/l BAP, showing direct flower bud formation without any vegetative growth; **d** blooming of the flower buds from cotyledons without embryo axis in the same flower bud inducing medium

The initiation of flowering may be explained by the presence of flower bud initials in the region of embryonic axis on the cotyledons (Fortainer 1957). The addition of cytokinins might have triggered the mechanism promoting the precocious manifestation of their expression.

The two types of cotyledons, with or without embryo axis, showed differential cytokinin requirements for flower bud induction. Embryonated cotyledons require more cytokinins, probably to neutralise the greater endogenous auxin or auxin-like substances synthesised by the growing shoot. On the other hand, the

production of flower buds from de-embryonated cotyledons was completely regulated by exogenous hormone supplementation and hence required lower levels of cytokinins. From each of the flower bud clusters two to five flower buds bloomed (reached anthesis) at a time. The frequency of flower bud bloom was 68% in Blaydes' medium with 0.5 mg/l of KN (Fig. 1b, d). However, it was marginally high in basal medium. The frequency of flower bloom decreased with increasing concentrations of hormones (Table 1). The blooming of the flower buds was facilitated by continuous transfer to fresh medium with low BAP (0.2 mg/l) at intervals of 10 days.

Each of the growth regulators, NAA, IAA, GA_3 and ABA, had no influence on flower induction from both cotyledon types when applied alone, although GA_3 promoted considerable growth of the shoot in embryonated cotyledons. Auxin inhibited flower formation in *Plumbago* (Nitsch and Nitsch 1967), while tobacco stem segments required the presence of lower levels of IAA for floral bud differentiation (Wardell and Skoog 1969). The interaction of various hormones on flower bud induction was tested using de-embryonated cotyledons (Table 3). Low concentrations (0.1 mg/l) of IAA or NAA in combination with BAP (0.5 mg/l) have a promotive influence on flowering. GA_3 exerted an antagonistic effect on flower bud induction as is evident from the low frequency of flowering response in combination with BAP. The culture of cotyledons in ABA along with BAP did not even result in chloroplast development. Lower levels of KN or MAP exerted a promotive influence on flowering in combination with BAP as is evident from the increased frequency of flowering response compared to higher concentrations of KN or MAP.

The fertility of pollen in induced flowers, as tested by acetocarmine staining, was found to be 80–88%. In field grown plants, one, or at the most two, flowers of an inflorescence reaches anthesis in a given day (Smith 1950, 1954), while two to five flowers per day were typical of in vitro grown cotyledons. Six percent of the induced flowers resulted in gynophore development, indicating successful in vitro fertilization.

Table 3. The effect of various combinations of hormones on flowering from de-embryonated cotyledons of TMV_2 cultured in 0.5 mg/l BAP containing Blaydes' medium

Hormone (mg/l)	No. of cotyledons cultured	% of flowerbud formation	Flower buds/ cotyledon	Flower bloom (%)
BAP	72	20.2	17.4	68.7
0.1 IAA + BAP	64	22.4	10.2	4.6
0.1 NAA + BAP	48	37.1	12.8	6.8
0.5 KN + BAP	52	24.1	5.4	39.4
1.0 KN + BAP	48	16.2	4.3	33.33
0.5 MAP + BAP	56	30.2	11.4	27.6
1.0 MAP + BAP	61	27.8	10.4	16.5
0.5 GA_3 + BAP	49	7.5	5.2	20.8
1.0 GA_3 + BAP	56	–	–	–

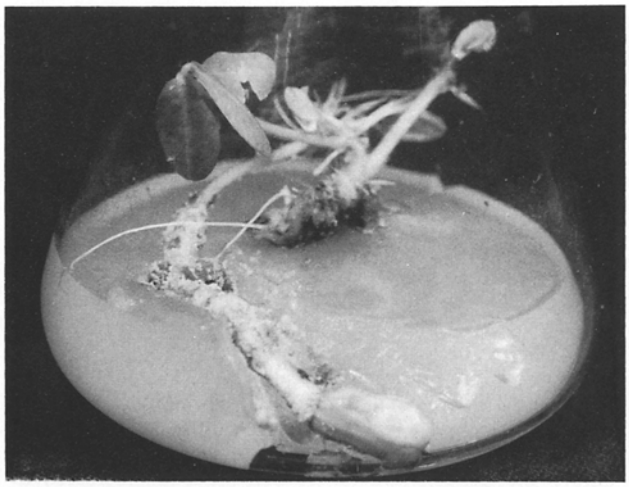

Fig. 2. Pod formation from the gynophore of induced flower in modified MS medium containing 0.5 mg/l KN when cultured in dark

Pod formation from excised gynophore tips of groundnut in chemically defined medium has already been reported (Ziv and Zamski 1975). In the present communication we report the formation of pods from the gynophores of induced flowers. Cotyledon cultures transferred to MS medium substituted with Blaydes' nitrates and incorporated with 0.5 mg/l KN showed various stages of growth in pod formation when observed at intervals of fifteen days. The growth of the pod was complete within 60–75 days. However, interruption of dark-grown cultures with light leads to chlorophyll development in the pod, followed by complete cessation in the growth of the pod. Twenty-six percent of the gynophore developed cultures transferred to dark showed pod formation (Fig. 2). The successful formation of pods under in vitro conditions provide a potentially useful system for generating variability for this crop.

The successful induction of in vitro flowering in groundnut offers a unique system with which to study the molecular basis of flowering and the physiological factors controlling meristems from making leaves to floral parts. The findings also have an implication for possible improvement in the reproductive efficiency of groundnut by harmonal manipulation.

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