Morphogenic Potentialities of Flower Buds of *Kalanchoe pinnata* Pers. grown *in vitro*

BY

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With two Plates

ABSTRACT

Excised flower buds of *Kalanchoe pinnata* Pers. representing two early stages of development (designated sets I and II), were cultured on modified White's medium (WB). They failed to attain full development on WB or on WB containing any of the following supplements: indole-3yl-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), kinetin, or coconut milk (CM). A slight stimulation of the growth of corolla was caused by kinetin (10 ppm). IAA (1 ppm) and NAA (1 ppm) induced rooting from the cut end of the pedicel and from the proliferated torus tissue situated between the sepals and petals. 2,4-D (1 ppm) either singly or in concert with CM (10 per cent) stimulated the formation of shoot buds and root growth. Addition of kinetin (1 and 10 ppm) to WB favoured shoot formation, but suppressed rooting. Flower buds of set II developed shoot buds more readily than those of set I. Thus, the primordia of floral organs present in the immature buds lose their ability for normal morphogenesis under culture conditions. Buds destined to form flowers can be made to revert to vegetative growth.

INTRODUCTION

THE phenomenon of flower initiation has been studied extensively (Lang, 1965), but the factors controlling the sequential development of different floral organs have received only meagre attention. Although some work along these lines has already been carried out using the intact plant (Plack, 1957; Cusick, 1959), the technique of organ culture offers many distinct advantages (Galun, Jung, and Lang, 1963).

Many investigators have cultured post-anthesis flowers for the study of fruit physiology (Nitsch, 1965). It is only in recent years that immature flower buds have been grown to gain an insight into the factors controlling floral morphogenesis (Galun, Jung, and Lang, 1962, 1963; Tepfer, Greyson, Craig, and Hindman, 1963; Tepfer, Karpoff, and Greyson, 1966; Blake, 1966; Porath and Galun, 1967; Brulfert and Fontaine, 1967). There are only two reports of the occurrence of microsporogenesis in cultured flower buds (Blake, 1966; Porath and Galun, 1967).

The present work was initiated to test whether an immature flower bud of *Kalanchoe pinnata* could attain its full development *in vitro* and to what extent its morphogenic pattern could be altered by the manipulation of the

[Ann. Bot. 32, 825–32, 1968.]

nutritive medium. The findings have been briefly communicated elsewhere (Mohan Ram and Wadhi, 1966).

Materials and Methods

Flower buds used in the experiments were obtained from plants of *K. pinnata* Pers. growing in the departmental garden. Buds of two different stages of development, designated sets I and II, were selected for investigation (Plate 1A and B). Flower buds of set I measured 3 mm in length and each comprised 4 green sepals (3 mm in length), 4 yellowish-white petals (1 mm in length), 8 small stamens with anthers at archesporial stage of development, and 4 carpels in which ovules had not differentiated (Plate 2A). The buds of set I were grown with or without sepals. Flower buds of set II were smaller and younger than those of set I and measured 0·5 mm in length (Plate 1A and B). The flower buds of set II consisted of well-developed sepals and primordia of other floral organs.

Portions of the inflorescence containing buds of the desired size were collected and dipped in 0·2 per cent cetrimide (trade name Cetavlon) for 5 min and were then washed in running tap water. They were surface-sterilized in freshly prepared chlorine water for 10 min followed by two changes in sterile distilled water. Buds of set II were dissected aseptically under a stereoscopic microscope.

Modified White's basal medium (WB) consisting of the following (in mg/l) was employed: $MgSO_4.7H_2O$—360; $Ca(NO_3)_2.4H_2O$—260; Na_2SO_4—200; NaH_2PO_4—165; KNO_3—80; KCl—65; $MnSO_4.4H_2O$—3; $ZnSO_4.7H_2O$—0·5; H_3BO_3—0·5; $CuSO_4.5H_2O$—0·025; $Na_2MoO_4.2H_2O$—0·025; $CoCl_2$—0·025; Ferric citrate—10; glycine—7·5; niacin—1·25; thiamine hydrochloride—0·25; calcium pantothenate—0·25; pyridoxine hydrochloride—0·25; sucrose—20,000, and agar—8000. The following supplements were added separately to the medium before autoclaving: naphthaleneacetic acid (NAA), and indole-3yl-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), coconut milk (CM), and kinetin.

The number of flower buds used for each treatment is mentioned in Tables 1 and 2. The conditions of the room in which the cultures were stored were as follows: temperature, 25±2 °C; light intensity, 10–100 f.c.; and relative humidity, 65 per cent. The cultured flower buds were fixed, at various periods after inoculation, in formalin–acetic–alcohol for histological studies. Following the customary methods of dehydration and paraffin embedding, microtome sections were cut at a thickness of 8–15 μ and they were stained with haematoxylin–erythrosin or safranin-fast green.

Results

Flower Buds of Set I

Table 1 summarizes the effects of various growth-regulating substances on flower buds of sets I and II. The flower buds of set I cultured on WB

remained closed and invariably dried up. In those grown on WB+NAA (5 ppm) the region of the torus between the calyx and corolla showed initiation of meristematic activity 1 week after planting. The enlarged torus lost its usual green colour and became chalky white. Several root primordia emerged

TABLE 1

Influence of growth-regulating substances on the morphogenic potentialities of flower buds of Kalanchoe pinnata *after 12 weeks of culture*

Treatment	No. of flower buds cultured	Responses: Shoot bud formation No.	(%)	Rooting only No.	(%)	Callus formation No.	(%)
Set I							
WB	24	0	0	0	0	0	0
WB+NAA (5 ppm)	21	0	0	15	71·4	0	0
WB+CM (10%)+2,4-D (1 ppm)	24	**4	16·6	16	66·2	4	16·6
WB+kinetin (10 ppm)	22	2	9	0	0	0	0
Set II							
WB	24	0	0	0	0	0	0
WB+NAA (1 ppm)	23	1	4·7	14	60·8	0	0
WB+NAA (5 ppm)	24	0	0	14	58·3	0	0
WB+CM (10%)	12	0	0	5	41·6	0	0
WB+2,4-D (1 ppm)	23	10	43·5	*13	56·5	0	0
WB+CM+2,4-D (1 ppm)	23	13	56·5	*10	43·4	0	0
WB+kinetin (1 ppm)	12	1	8	0	0	0	0
WB+IAA (1 ppm)	24	0	0	12	50·0	0	0
WB+IAA+kinetin	24	0	0	0	0	0	0

* Callus also. ** Roots also.

TABLE 2

Effect of different concentrations of kinetin on the formation of shoot buds from flower buds of set II after 9 weeks of culture

Concentration of kinetin (ppm)	Number of flower buds cultured	Shoot bud formation No.	(%)
0·1	24	0	0
1·0	24	0	0
10·0	24	7	29·1

from the torus (Plate 1D) in 71·4 per cent of cultures. They developed into short, thick, white roots (Plate 2C) covered profusely with hairs. In flower buds with intact sepals, the roots either pierced through the sepals or grew along their inner surface and emerged at the apex of the bud. The petals and stamens shrivelled up and turned brown but the carpels remained fresh (Plate 2B). Transference of the rooted flower buds to WB stimulated the development of numerous fresh, thin and elongated roots.

On WM+CM (10 per cent)+2,4-D (1 ppm) rooting was stimulated in 66·2 per cent of the cultures. In another 16·6 per cent of the cultures a pale-yellow, friable callus appeared first which organized roots ultimately. Callus formation occurred in the torus at the bases of petals and stamens. Although sepals became callused occasionally, carpels never did so. In the remaining 16·6 per cent of cultures shoot buds and roots differentiated from the enlarged torus without the intervention of callus formation. As many as 10–15 shoots developed in each flower bud. While some shoots had long internodes and several pairs of leaves with crenate margins, others were fasciated with atypical leaves. The shoots remained vegetative throughout.

Since kinetin is known to induce shoot buds from various parts of the plant (Miller, 1961), it was thought desirable to test its effects on excised flower buds also. Some of the buds grown on WB+kinetin (10 ppm) remained green for as long as 20 weeks. In 12 out of 22 buds the corolla tube showed a little enlargement. Shoot buds made their appearance in only 2 out of 22 buds; rooting remained completely suppressed (see Table 1).

Flower Buds of Set II

White's basal medium by itself or in combination with the various supplements could not support the development of buds of set II into open flowers. In the presence of NAA (1 and 5 ppm) the pedicel became enlarged and a few swollen roots appeared from it as well as from the torus in 60·8 and 58·3 per cent of cultures, respectively. However, rooting was less profuse than that in the buds of set I. In only one out of 23 buds cultured on a medium containing NAA (1 ppm) did a leafy shoot develop from within the bud (Plate 1E). The green sepals opened out to allow the emergence of the growing shoot bud, which originated from the torus tissue and pushed the primordia of floral whorls to a lateral position. A few roots also developed from the torus (Plate 2D).

With the addition of IAA (1 ppm) to WB, 50 per cent of flower buds developed roots but not shoot buds at the cut end of the pedicel.

In the buds planted on WB+CM (10 per cent)+2,4-D (1 ppm) the pedicel became hypertrophied and numerous roots developed from it (Plate 1F). The tissue of the torus situated between the sepals and petals underwent profuse meristematic activity and the resulting mass of cells surrounded the primordia of petals, stamens, and carpels. On this proliferated torus, shoot meristems were organized at certain loci after a period of 7 weeks (Plate 2E and F). Leafy shoots appeared in 56·5 per cent of cultures. The number of shoots arising from a single flower bud was variable; some were normal (Plate 1F) and others fasciated (Plate 1G). In another 43·4 per cent of cultures profuse rooting and a little callus formation were observed.

Since the buds of set II developed shoot buds more readily than those of set I on CM (10 per cent)+2,4-D (1 ppm), it was of interest to test the influence of these substances when added singly to WB. When only CM was included in the medium the cultures failed to develop shoot buds; alone roots

appeared from the cut end of the pedicel in 41·6 per cent cultures. With the addition of 2,4-D alone shoot-bud formation was noted in 43·5 per cent cultures; rooting and callus formation were observed in the remaining 56·5 per cent cultures.

On WB+kinetin (1 ppm) the buds remained green for 10 weeks. At the end of 6 weeks one of the 12 buds had produced two leafy shoots (Plate 1C). Each shoot had two pairs of leaves with crenate margins and condensed internodes. Microtome sections showed that the shoot buds had their origin in the torus. In this flower bud, as well as in the remaining 11 buds, roots failed to develop.

With a view to studying the effects of kinetin in detail, experiments were set up using three concentrations of kinetin (0·1, 1, and 10 ppm). The growth responses of flower buds to kinetin are given in Table 2. With 0·1 ppm of kinetin the buds merely turned brown. With 1 ppm kinetin the buds remained green for as long as 32 weeks, but their morphogenic potentialities were completely suppressed. This response was rather disappointing since at least one out of 12 flower buds had formed shoot buds at the same concentration of kinetin in the previous experiment. However, out of 24 buds cultured on 10 ppm of kinetin, 7 developed highly stunted shoots after the lapse of 8 weeks. In the other 10 buds, which failed to form shoot buds, the petal primordia had expanded a little.

Inclusion of IAA (1 ppm) in the medium containing kinetin (1 ppm) proved ineffective for the induction of either roots or shoot buds.

Discussion

The experiments demonstrated that excised immature flower buds of *K. pinnata* failed to develop into open flowers. The cultured buds, however, organized shoot buds and roots in response to certain treatments. Leafy shoots arise from potential flower buds *in vivo* in *Agave*, *Anagallis*, *Ananas*, *Anthophytum*, *Furcraea*, *Gonatanthus*, *Impatiens*, and certain Liliaceae. These may arise spontaneously (LaRue, 1942), as a result of incomplete photoinduction (Brulfert and Chouard, 1961; Nanda and Krishnamoorthy, 1967), due to infection by plant pathogens (Horsfall and Dimond, 1959), by surgical treatments (Wardlaw, 1963) or by the application of growth regulators (Dostal and Hošek, 1937; Dostal, 1964, 1965). The reversion of flowers to vegetative shoots by incomplete photoinduction or as a result of invasion by micro-organisms may arise from changes in the level of hormones, such as auxins, gibberellins, and cytokinins, which occur in the higher plants as well as in micro-organisms (Sequeira, 1963; Knapp, 1963; Letham, 1967). Dostal and Hošek (1937) demonstrated the reversion from flowering to vegetative state in higher plants by treatment with IAA. Sparrow and Gunckel (Heslop-Harrison, 1963) observed partial reversion of flowers (permitting only floral bracts) in snapdragon by irradiation. This effect was believed to be due to the initial effect of radiation on the synthesis of auxin. The potential flower buds of *Syringa vulgaris*, *Aesculus hippocastanum*

(Dostal, 1964, 1965), and *Anagallis arvensis* (Brulfert, 1961) reverted to vegetative growth by treatment with gibberellic acid. Cytokinins are not known to produce similar effects. In the present work leafy shoots developed from flower buds of *K. pinnata* when they were grown on WB+2,4-D (1 ppm) or WB+CM+2,4-D (1 ppm) or WB+kinetin (1 and 10 ppm). The morphogenic effects of gibberellins were not investigated in the present study. Hence it is surmised that the transition from flowering to vegetative state is regulated by an interplay of auxins, gibberellins, and cytokinins.

The development of leafy shoots from excised flower buds is extremely rare. LaRue (1942) observed the formation of vegetative shoots in the cultured flowers of only two plants, viz. *Nemesia strumosa* and *K. globulifera* out of a total of 92 species investigated by him. But he suspected that in *N. strumosa* the shoot buds might have already been formed prior to culture. In *K. globulifera* shoots originated from the cut end of the pedicel and not from the flower bud *per se*. In the present work shoot buds were organized on the enlarged but non-callused torus. This is in contrast to those reports in which a callus is first produced from the flower primordia or flower buds and ultimately differentiates shoot buds (Konar and Nataraja, 1964; Johri and Ganapathy, 1967).

In the cultured buds of *K. pinnata* (present work) 2,4-D either alone or in combination with CM was found to favour the formation of both shoot buds and roots. The younger the bud at culture, the greater was the capacity to form shoots. For example, the percentage of cultures of set II which regenerated shoot buds was 56·5 in contrast to 16·6 in set I. Kinetin, on the other hand, proved favourable only for shoot formation. NAA promoted only rooting except in a single instance.

LaRue (1942) observed rooting of open flowers in 3 out of 12 species of monocots and 25 out of 80 species of dicots on a simple basal medium. He further observed that the inclusion of IAA hastened rooting and that younger buds failed to root. In *K. pinnata*, roots were initiated from flower buds of both sets I and II when the medium contained IAA, NAA, or 2,4-D. The requirement of an exogenous supply of auxin in the medium for rooting of immature flower buds as observed in the present work and the easy development of roots in mature flowers on a medium devoid of auxin (LaRue, 1942) are in agreement with the existing evidence that auxin level increases during flower development (Lang, 1961).

Thus early excision and cultivation of flower buds of *K. pinnata* impedes their normal morphogenesis, but certainly invokes their totipotency for organizing the entire plant.

Acknowledgements

We are grateful to Professor B. M. Johri for his interest in the work, and to the Council of Scientific and Industrial Research and the University Grants Commission for awarding a Research Fellowship to one of us (M. W.).

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EXPLANATION OF PLATES

PLATE 1

A and B. Flower buds of sets I and II respectively at planting. A. × 10; B. × 26.

C. Set II flower bud grown for 6 weeks on WB+kinetin (1 ppm). Note the swelling of the cut end, absence of roots, and the development of leafy shoots. × 7.

D. Top view of a flower bud of set I (without sepals) after 2 weeks' growth on WB+NAA (5 ppm). Several roots have emerged from the torus. × 4·5.

E. A flower bud of set II grown on WB+NAA (1 ppm) for 20 weeks. Note profuse rooting of the pedicel and the development of a shoot and roots from within the bud. × 3.

F. Sixteen weeks' growth of a flower bud of set II on WB+CM+2,4-D (1 ppm). The pedicel has rooted profusely and a shoot has developed from within the bud. × 5.

G. Twenty-eight weeks' growth of a set II flower bud on WB+CM (10 per cent)+2,4-D (1 ppm) medium. A fasciated shoot and several roots have arisen from within the flower bud. Roots can be seen at the cut end. Remnants of the four sepals are also visible. × 2.
(r, root; s, sepal; sh, shoot.)

PLATE 2

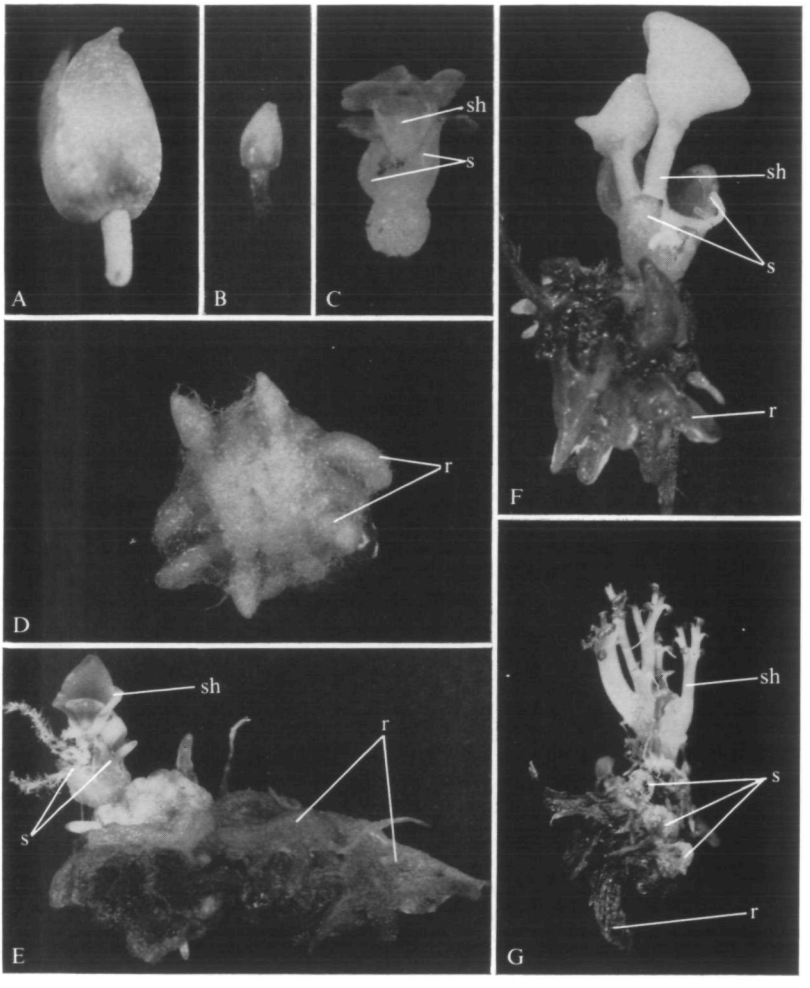
A. L.S. of a set I flower bud at planting. × 17.

B. L.S. of a set I flower bud grown for 8 weeks on WB+NAA (5 ppm). Sepals were removed at the time of fixation. Note the shrivelled stamens and petals and the emergence of roots from the torus. × 44.

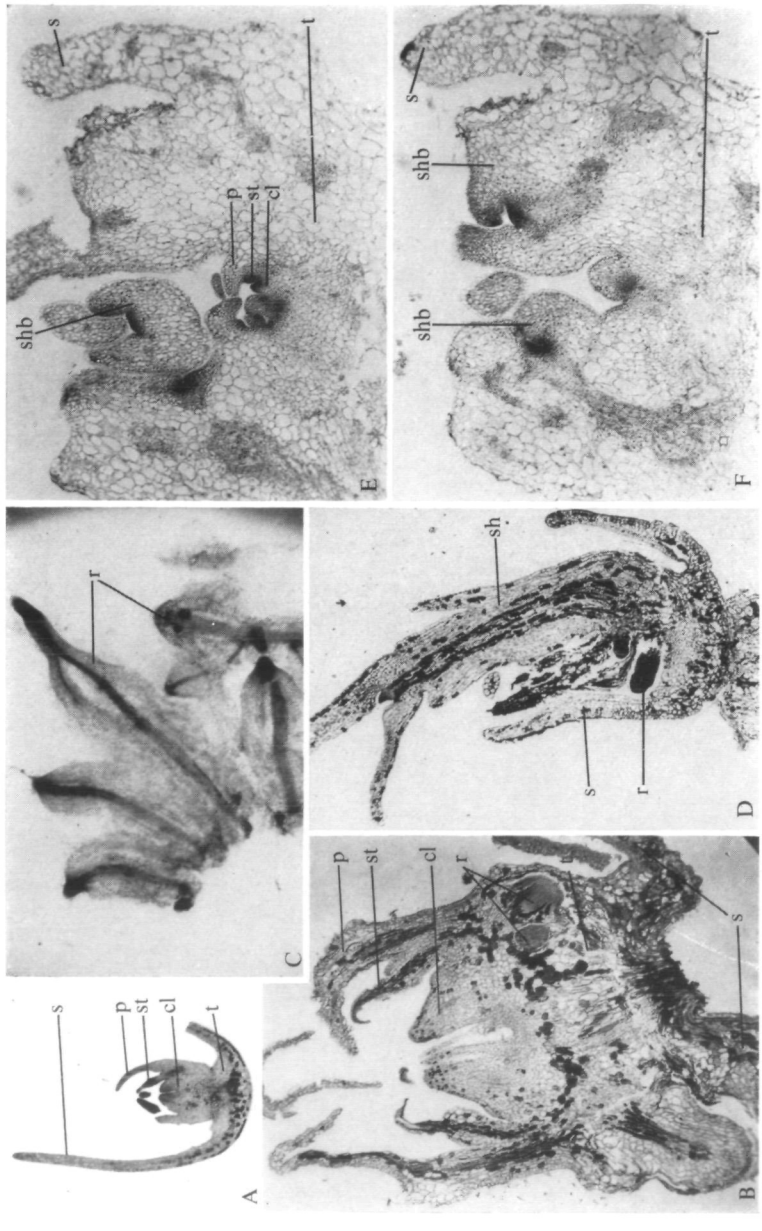
C. Whole mount of a few fasciated roots from a 6-week-old culture of set I flower bud on NAA (5 ppm). Note the development of lateral roots. × 23.

D. L.S. of a flower bud of set II grown for 20 weeks on WB+1 ppm of NAA (same as shown in Plate 1E). Roots and shoot have originated from the torus. × 30.

E and F. Portions of longitudinal sections of a flower bud of set II which was grown for 7 weeks on WB+CM+2,4-D (1 ppm). The torus tissue situated between the sepal and petal primordia has become hypertrophied and shoot buds have differentiated from it. × 46.
(cl, carpel; p, petal; r, root; s, sepal; sh, shoot; shb, shoot bud; st, stamen; t, torus.)



H. Y. MOHAN RAM AND MRIDUL WADHI



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