

THE PHYSIOLOGY OF FLOWERING
WITH CONTRIBUTIONS BY IN VITRO TECHNIQUES

by

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PREFACE

I hereby declare that this thesis, submitted for the degree of Doctor of Philosophy in the Faculty of Science, University of Natal, Pietermaritzburg, except where the work of others is acknowledged, is the result of my own investigation.

A handwritten signature in black ink, consisting of a large, stylized loop followed by a horizontal line extending to the right.

C.W.S. DICKENS

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ABSTRACT

In vitro techniques are providing a useful tool in the investigation of the factors controlling flowering and in particular in the quest for the identity of the floral stimulus. Numerous specialized systems have been developed and have been reviewed critically in this thesis.

Initial exploratory attempts were made to establish the in vitro flowering of several species. This was done with a view to conducting in vitro grafting experiments, where the transmission of the stimulus from donor to receptor could be examined under controlled conditions. Glycine max Merrill. and Kalanchoe blossfeldiana Poellniz. were successfully induced to flower on a low nutrient hormone free medium. Flowering was also achieved on inflorescence explants of Nicotiana tabacum L. cv. Trapezond (DNP), but could not be obtained on Nicotiana tabacum cv. Maryland Mammoth (SDP) or on Nicotiana sylvestrus L. (LDP) explants.

The above plants were all used in in vitro grafting experiments, where callus was used as an intermediary tissue through which substances could pass from one plant to another. No conclusive results showing the transmission of any substance were noted. Attempts were also made to induce flowering on inflorescenc segments of Nicotiana sylvestrus by grafting onto inflorescence segments of Nicotiana tabacum cv. Trapezond which did itself flower in vitro. No flowering of the Nicotiana sylvestrus explant occurred despite prolific callus growth.

Two aspects of this initial examination were enlarged upon, namely the in vitro flowering of Glycine max and Kalanchoe blossfeldiana.

Nodal explants of Glycine with or without a leaf attached, were found to be sensitive to photoinduction by SD conditions. This stimulation was maintained on transfer to non-inductive conditions, although the extent of the flowering response was dependent on the nutrient status of the medium and of the parent plants from which explants were taken. The advantage of this system, is that as induction occurs in the explant itself while in culture, this gives an opportunity to investigate the nature of the floral stimulus. This is not always possible where explants are taken from plants which have been induced or have reached anthesis.

This system of in vitro flowering of Glycine explants, was used in an investigation of the incorporation of possible precursors into cytokinins. This was done as cytokinins are thought to be required during the induction process and thus synthesis could be taking place during flower induction.

Radiolabelled adenine and isopentenylpyrophosphate were both assimilated by the explants. No conclusive results were obtained about their incorporation into any of the tested cytokinins. Several unknown compounds were produced containing high levels of radioactivity.

Kalanchoe explants also responded to SD inductive cycles by producing flowers. Numerous factors affecting this induction were investigated. The physiological state of the parent plants was found to affect the flowering performance of the explants. The physical culture conditions, vessel size and medium volume also affected flowering. Various additions were made to the culture medium to test their effect. In other investigations, most of these substances had been shown to be inductive

or stimulatory of flowering. Included here were sucrose, phenolics, hormones and plant extracts. Gallic acid, a proposed inhibitor of flowering in Kalanchoe, was found to be inhibitory of vegetative growth in general, and not of flowering specifically. As both flowering and vegetative responses were affected by these applied substances, several parameters were studied in an attempt to construct some idea of the internal response of the plant during SD induction. In Kalanchoe, induction is manifest not only in the form of flowers, but also as leaf succulence and leaf colour.

In the last section of this investigation into the nature of the floral stimulus, an attempt was made to radiolabel the floral stimulus in Xanthium strumarium L. plants grown in vivo. Application of $^{14}\text{CO}_2$ was accompanied by manipulation procedures designed to induce the plant to translocate the labelled stimulus away from the source of application of $^{14}\text{CO}_2$, to a sink region. Soon after their arrival in the sink bud, the labelled assimilates were extracted in three different ways. Separation on paper and TLC was followed by exposure to X-ray film, to locate the radiolabelled derivatives. These investigations produced consistent and reproducible results, and indicated that several substances are no longer found after induction has begun, while several new substances are produced. None of these labelled compounds could be identified due to their low concentrations.

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CHAPTER 1.

THE INDUCTION AND EVOCATION OF FLOWERING

IN VITRO: A REVIEW

INTRODUCTION

The physiological processes involved in flowering are many and varied, and range from the genetic capability of the plant to flower, to the perception of stimuli, the production of some flower initiating signal and the control of the development of meristems to form flowers and ultimately fruits. Whether the separation of these general processes can be justified, remains to be seen, as it is possible that the regulatory mechanisms may be firmly intertwined. Yet it is tempting to try to isolate what is probably the most significant event in the process, the production of some substance or substances which brings about the change from vegetative to reproductive growth. After CHAILAKHYAN (1937) proposed the existence of a single flowering stimulus called florigen, attention was channelled towards the finding of a single flower promotor. This theory dominated research into flowering for decades, and still attracts a fair measure of support. CHAILAKHYAN (1958) himself eventually realized that some other substances, possibly gibberellin, may work alongside the flowering stimulus. Other workers provided circumstantial evidence for the existence of flower inhibitors, which were thought to work antagonistically with the promotor (EVANS, 1969; REID and MURFET, 1977; WAREING and EL-ANTABLY, 1970), whichever one being present or dominant, determining the reproductive state of the plant. This theory also met with some resistance (LANG, 1965; ZEEVAART, BREDE and CETAS, 1977) as it was thought unlikely that such a ratio could be

maintained in grafting and other manipulation experiments. As time progressed, so more evidence has accumulated to include the involvement of a variety of other substances, some specific, others common. The logical progression of all this has been the postulation of theories propounding a multi-component sequence of events which takes place as plants are induced to flower. BERNIER, KINET and SACHS (1981b) presented a credible argument in favour of such a theory. They postulate that a number of factors are involved, some common, and some unique to a particular species; some increasing in concentration and some decreasing, until some point of no return is reached after which flowering is irreversible. This theory serves to explain much of the available information, including the fact that many apparently unimportant substances can induce flowering in non-inductive conditions. They also succeeded in accommodating what has long been thought of as unequivocal evidence for "florigen". the transmission of the floral stimulus across a graft union, even if the contact time is relatively short. This model also explains why grafting between two vegetative plants does on occasion, cause flowering.

Yet, despite the fact that most researchers are providing information supporting this model, the florigen theory remains an attractive and topical one, which may not be that far removed from multi-component models. It is possible that in each species or flower response type, there may be a single substance of major importance, but this need not preclude a host of other substances, all involved, in a multi-component flower stimulus. Such substances could include a variety of compounds such as plant hormones, phenolics, nutrients and even sucrose, but these will be dealt with later in detail. Where the real mystery lies, is the chain of events, or the substance (florigen?), that is produced directly

in response to the perception of environmental stimuli, and initiates the processes leading to the production of the wide variety of substances involved in flower initiation and development.

After all the years of intensive research, which have not solved the mysteries of flowering, there is need for change. BERNIER, KINET and SACHS (1981) commented that "...no satisfactory answers will arise from the continuation and refinement of the same type of experiments". As a novel approach, in vitro techniques are proving to be useful in investigating some of these problems. They allow for very much greater control of the whole or part of the plant, more efficient application of exogenous substances, the isolation of effector or affected sites, and the avoidance of complicatory influences such as bacterial or fungal contamination of wounded surfaces and of organic substances under test. Three main systems are used for in vitro flowering with different objectives, although these are not always appreciated or recognized;

(1) Whole plant culture, where plants have all the basic organs, even though they may be reduced. Such cultures may be derived from seed sown in vitro; by subculturing whole plants reproducing vegetatively in vitro; by the growth of apical or axillary buds taken from parent plants; or by the differentiation of undifferentiated tissue to form buds and subsequently whole plants.

(2.1) The culture of isolated organs or buds or parts thereof, containing meristematic regions which develop either shoots or roots and then flower in vitro. Apical and axillary bud culture may also fit here as well as the culture of stem segments, root segments and leaf explants.

(2.2) The culture of isolated organ explants with meristematic cells which produce flowers directly without the formation of roots or shoots.

(3) The culture of non-meristematic tissues such as callus, thin epidermal cell layers and pith tissue, with direct flower bud differentiation and development without the formation of shoots or roots.

All of the above systems have advantages and disadvantages. The culture of whole plants, has the advantage that intact systems can be manipulated with a high degree of control with respect to environmental and nutritional requirements. More important, is that test substances can be applied with more accuracy and with a greater assurance that the substance is taken up by the plant. If one is to examine the flowering stimulus as a multi-component system, the chance of ever achieving a medium with the correct concentrations of all the required substances is very remote. Even more difficult, would be the simulation of concentration changes, where the level of one substance increases while another decreases. This does occur naturally, and has been shown for a variety of the known plant growth regulators, during a variety of growth processes. The advantage of whole plant investigations, is that one can test either a single, or a small number of compounds, and rely on the complete plant in culture, to provide all of the other compounds required for growth and/or flowering, in their correct concentrations.

One potential problem here, is that in those systems which are maintained in non-inductive conditions, and induction is attempted with a single test substance, the physiological conditions occurring in the plant may be directed towards vegetative growth to an extent that the

inductive properties of the substance are not realized. Nevertheless, systems could be developed using plants in a marginal flowering condition, which negates some of the problems mentioned above.

The advantage of the second type of system, isolated meristematic regions which do not always form all of the representative organs, is that the confounding influence of certain organs can be eliminated. In this way, one can establish the role of these organs in flowering. Conversely, the lack of a certain organ, may prevent either the initiation of flowering, or the manifestation of the flowering stimulus.

The advantage of those systems where a meristematic zone differentiates flowers directly without the formation of leaves or roots, is that the direction of differentiation of the initials in the sink is being controlled directly in the test system without the interfering influence of the rest of the plant. There is a possibility that the explant tissue surrounding the meristematic zone may have some influence on flowering, especially in those cases where photosynthetic tissues are present. Because of this, explant size is usually kept to a minimum, especially in the case of apical meristem culture, where it is said to be desirable to isolate the apical dome alone, by removing the leaf primordia (SCORZA, 1982). Such a system would be most suited to investigations of a single flowering stimulus.

In those systems where no meristematic tissue is present, one has first to overcome the change from ground tissue to meristematic tissue, which may be unrelated to the flower stimulus and is not a requirement in in vivo plants. Thereafter the system has similar potential to that above, with the one advantage that there is a lack of organ differentiated

tissue. It is not known if the presence of differentiated cells in callus tissue has any effect on flowering.

One major factor affects all in vitro flowering systems, and that is the physiological state of the parent plant which provides the explant for culture. If the parent plant is vegetative, then flower induction must be achieved in vitro, whereas if the parent plant is already induced or is flowering, then only the expression of that induction will be investigated. Both have their advantages, but the former is likely to be more useful in investigations of the floral stimulus.

In vitro techniques thus provide several ways in which flowering can be examined, most of which cannot be carried out in vivo. The currently available information is examined in this review, in the context of the preceding discussion. Investigations supporting a single substance regulating flowering will be dealt with first, followed by investigations relating to multi-component systems. It must be noted that various works taken from the literature have been categorized, although this may not have been the intention of the researchers.

In this review, the use of terminology follows the guidelines of EVANS (1969). These are briefly;

Induction, the processes occurring in the leaf which lead to flowering.

Evocation, the process at the shoot apex which lead to flowering but are distinct from differentiation.

Floral stimulus, any translocated substance which evokes flowering.

Florigen, the immediate product/s of leaves undergoing photoperiodic induction, which causes evocation.

Table 1. Table of species which have been cultured and have successfully produced flowers in vitro.

Species	Response Group	Parent Plant Induced	Explant Source	Culture Daylength	Hormones in Medium	Promotion By:	Inhibition By:	Reference
<u>Alium sativum</u>	-	-	flower stalk	LD	-	GA ₃	-	TIZIO, 1979;
<u>Arabidopsis thaliana</u>	LDP	no	stem segments	LD	kinetin (adenine)	-	-	NITSCH, 1972;
<u>Baeria chrysostoma</u>	-	no	seed	SD	-	-	-	L. LOO, 1946a;
<u>Begonia sp.</u>	-	yes	leaves and flower stalk	LD and SD	BA, IAA phenylacetic acid	IAA	-	RINGE and NITSCH, 1968;
<u>Bougainvillea glabra</u>	-	-	-	-	-	-	-	CHATURVEDI and SHARMA, 1977; STEFFEN, 1986;
<u>Browallia demissa</u>	SDP	yes	flower bud	LD	kinetin or SD 8339	-	IAA, GA ₃	GANAPATHY, 1969;
<u>Carthamus tinctorious</u>	-	-	cotyledon	continuous	IAA, kinetin	-	-	TEJPRATHI and ANWAR, 1984;
<u>Cestrum diurnum</u>	LSDP	no	node	LD to SD	-	light	-	CAPLIN and GRIESEL, 1967;
<u>Chenopodium rubrum</u>	SDP	no	seedling apex	SD	-	-	CCC, ABA ethrel	deFOSSARD, 1972, 1974;
<u>Cichorium intybus</u>	LDP or CRP	no ?	raceme root	SD LD	-	red light GA ₃ coumaric acid anti-auxin	hydration IAA	BADILA, LAUZAC and PAULET, 1985; BADILA and PAULET, 1986; BOUNIOLS, 1974; JOSEPH and PAULET, 1975; PAULET and NITSCH, 1964; PIERIK, 1966, 1970; MARGARA, RANCILLAC and BECK, 1965; MARGARA, RANCILLAC and BOUNIOLS, 1966;
<u>Chrysanthemum "Honeysweet"</u>	SDP	no	apex	SD	-	GA ₃	IAA, GA ₃	HARADA, 1967; HARADA and NITSCH, 1959;
<u>Crepis capillaris</u>	LDP	yes	hypocotyl callus	-	IAA, IBA, kinetin	kinetin	-	JAYAKER, 1970; BROSSARD, 1979;
<u>Cucumis sativus</u>	-	no	hypocotyl	continuous	-	-	-	RAJASEKARAN, MULLINS and MAIR, 1983;

<u>Species</u>	<u>Response Group</u>	<u>Parent Plant Induced</u>	<u>Explant Source</u>	<u>Culture Daylength</u>	<u>Hormones in Medium</u>	<u>Promotion By:</u>	<u>Inhibition By:</u>	<u>Reference</u>
<u>Cuscuta campestris</u>	-	-	seedling apex	-	-	-	-	LOO, 1946b;
<u>Cuscuta epithymum</u>	-	-	apex	-	-	haustoria	-	BERTOSSI, 1956;
<u>Cuscuta reflexa</u>	SDP	no	apex	unknown or dark	IAA	-	-	BALDEV, 1959;
<u>Dianthus caryophyllus</u>	LDP ?	yes	anthers	LD	IAA, kinetin coconut milk	-	-	VILLALOBOS, 1981;
<u>Dionea muscipula</u>	-	no	leaf segments	LD	zeatin	-	-	KING, (pers. comm);
<u>Drosera natalensis</u>	-	-	leaf	LD	BA, NAA	-	-	CROUCH, (pers. comm);
<u>Glycine max</u>	SDP	no	node+leaves	SD or LD to SD	none	-	-	DICKENS and VAN STADEN, 1985;
<u>Haworthia arachnoides</u> and <u>H. cymbiformis</u>	-	-	perianth	-	-	-	-	KONISHI, HAYASHI and IKAMI, 1982;
<u>Helianthus annuus</u>	DNP or SDP	no	apex	LD	BA or kinetin	-	-	HENRICKSON, 1954; PATERSON, 1984;
'Iris' cv. "Wedgewood" cv. "Ideal"	CRP	no	bulb or apex primordia	dark	-	low temp., GA ₃ or induced scale diffusate	IAA	RODRIGUES PEREIRA, 1963, 1965; DOSS and CHRISTIAN, 1979;
<u>Kalanchoe blossfeldiana</u>	SDP	yes	flowers	LD	BA, NAA 2,4D	-	-	MARGARA and PIOLLAT, 1981;

<u>Species</u>	<u>Response Group</u>	<u>Parent Plant Induced</u>	<u>Explant Source</u>	<u>Culture Day/length</u>	<u>Hormones in Medium</u>	<u>Promotion By:</u>	<u>Inhibition By:</u>	<u>Reference</u>
<u>Lemna aquinoctialis</u> (-paucicostata)	SDP	no	whole plant	SD cont inuous	-	zeatin, BA ABA, CCC benzoic, tannic, salicylic and nicotinic acids, and vitamin K dicourmarol	CCC, ABA GA ₃ , IAA	HIGMAN and SMITH, 1969; GUPTA and MAHESHWARI, 1970; KANDELER and HUGEL, 1973; KHURANA and MAHESHWARI, 1978, 1983b, 1986; CLELAND and TANAKA, 1979; KAIHARA and TAKIMOTO, 1985a,b; FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, TAKIMOTO and CLELAND, 1986a,b; TANAKA, HORIKAWA, NISHIMURA and NASU, 1986;
<u>Lemna gibba</u>	LDP	no	whole plant	12 hour	-	GA ₃ , cAMP EDDHA, BA salicylic, benzoic and nicotinic	chlorogenic glucose IAA, GA ₃ ABA and kinetin	FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, S TAKIMOTO and CLELAND, 1985; OOTA, 1963, 1972; PIETERSE and MULLER, 1977; UMEMOTO, 1971;
<u>Lemna minor</u>	LDP	no	whole plant	-	-	-	benzoic and salicylic acids	benzoic and KAIHARA, WATENABE and TAKIMOTO, 1981;
<u>Lemna perpusilla</u> (P146)	SDP	yes/no	whole plant	SD and LD	-	BA	allogibb. acid GA ₇	BEWINK and devRIES, 1975; PRYCE, 1973;
<u>Lunaria annua</u>	CRP, LDP	yes	petiole	-	-	low temps. adenine, GA ₃ , kinetin crude extract coconut milk	IAA, NAA, 2,4D	PIERIK, 1966, 1970;
<u>Manihot esculenta</u>	CRP/LDP	-	apex	LD	IAA, BA, GA ₃	-	-	TANG, CAPPADODIA and BYRNE, 1983;
<u>Mazus pumilus</u>	-	yes	stem internode	-	IAA, kinetin GA ₃	kinetin	-	RASTE and GANAPATHY, 1970;
<u>Mesembryanthemum floribundum</u>	-	-	-	-	-	-	-	MEHRA and MEHRA, 1972;

<u>Species</u>	<u>Response Group</u>	<u>Parent Plant Induced</u>	<u>Explant Source</u>	<u>Culture Day/length</u>	<u>Hormones in Medium</u>	<u>Promotion By:</u>	<u>Inhibition By:</u>	<u>Reference</u>
<u>Mauticocalyx lynchei</u>	-	-	leaf epidermis	-	auxin cytokinin	-	-	TRAN THANT VAN, 1973b;
<u>Nicotiana rustica</u>	-	-	leaf protoplasts	-	IAA, kinetin	-	-	GILL, RASHID and MAHESHWARI, 1979;
<u>Nicotiana tabacum cv Samsun</u>	DNP	yes	thin cell layers from inflorescence	continuous	BA, NAA, IAA, kinetin	BA	-	COUSSON and TRAN THANH VAN, 1983; CROES, CREAMERS-MOLENAAR, VAN DEN ENDE and BARENDSE, 1985; VAN DEN ENDE, CROES, KEMP, BARENDSE and KROH, 1984;
<u>Nicotiana tabacum cv. Wisconsin 38</u>	DNP	yes	thin cell layers from inflorescence stems	continuous	IAA, kinetin or none	RNA base analogues, DNA from induced plants	GA ₃	CHOUARD and AGHION, 1961; AGHION-PRAT, 1965a,b; WARDELL, 1977; HILLSON and LaMOTTE, 1977; WARDELL and SKOOG, 1969a,b; TRAN THANH VAN, DIEN and CHLYAH, 1974;
<u>Nicotiana tabacum cv. Trapezond</u>	DNP	yes	stem internodes	LD	IAA, NAA kinetin	-	-	CHAILAKHYAN, AKSENOVA, KONSTANTINOVA and BAVRINA, 1974, 1975;
<u>Oncidium varicosum</u>	-	yes	inflorescence stalk	LD	NAA	-	-	BARBANTE KERBAUY, 1984;
<u>Passiflora suberosa</u>	DNP	yes	leaf, tendril, stem segments	LD	BA	BA	-	SCORZA and JANICK, 1980;
<u>Panax ginseng</u>	-	-	embryoids and root	LD	BA, GA ₄	GA ₄	-	CHANG and HSING, 1980;
<u>Perilla crispa</u>	SDP	no	apex and leaves	continuous	IAA kinetin	sucrose, ABA and phloem exudate	-	WADA and TOTSUKA, 1982; PURSE, 1984;
<u>Perilla frutescens</u>	SDP	no	apex and leaves	SD	BA, NAA IAA	-	-	RAGHAVEN, 1961; RAGHAVEN and JACOBS, 1961; TANIMOTO and HARADA, 1979, 1980;

<u>Species</u>	<u>Response Group</u>	<u>Parent Plant Induced</u>	<u>Explant Source</u>	<u>Culture Daylength</u>	<u>Hormones in Medium</u>	<u>Promotion By:</u>	<u>Inhibition By:</u>	<u>Reference</u>
<u>Perilla nankinensis</u>	SDP	no	apex	-	-	kinetin, adenine	-	CHAILAKHYAN and BUTENKO, 1959;
<u>Pharbitis nil</u>	SDP	no	apex seedlings	SD and continuous	-	low temps., NAA, GA ₃ kinetin, BA, GA ₃ , ABA and benzoic acid	ethrel, IAA, GA ₃	TAKIMOTO, 1960; HARADA, 1967; BHAR, 1970; MATSUSHIMA, ITOYUMA, MASHIKO and MIZUKOSHI, 1974; SHINOZAKI, SHIN and TAKIMOTO, 1982a,b; SHINOZAKI and TAKIMOTO, 1983;
<u>Phlox drummondii</u>	SDP	yes	flower bud	-	IAA coconut milk	IAA coconut milk	-	KONAR and KONAR, 1966;
<u>Pisum sativum</u>	CRP,	-	apex and axillary bud	-	BA, NAA	-	-	NOVAK, LUCRENTTI, DONINI, AFZA and HERMELIN, 1985;
<u>Plumbago indica</u>	SDP	no	internode	SD	-	ethylene ABA adenine	auxin GA ₃	NITSCH, 1972; NITSCH and NITSCH, 1967a,b; NITSCH, NITSCH, ROSSINI and BUI DANG HA, 1967;
<u>Salix babylonica</u>	-	-	buds	-	GA ₃ , NAA, kinetin	-	-	ANGRISH and NANDA, 1982;
<u>Scrofularia arguta</u>	LDP	-	node	12 hour	coconut milk IAA	-	kinetin-	MIGINIAC, 1972;
<u>Silene candida (=Viscaria)</u>	LDP	yes	apex and leaf primordia	-	GA ₃ , kinetin	-	Kalanchoe extracts	BLAKE, 1966, 1969, 1972;
<u>Silene cardinalis</u>	LDP	yes	apex	-	kinetin	-	-	BLAKE, 1969;
<u>Sinapis alba</u>	LDP	no	apex	SD	-	sucrose	nitrogen	DELTOUR, 1967;
<u>Spinacia oleracea</u>	LDP	no	apex	-	GA ₃ , GA ₇	-	Sandoz9789	CULAFIC and NESKOVIC, 1980;
<u>Stellaria media</u>	-	-	apex	-	-	-	-	WHITE, 1932;
<u>Rudbeckia bicolor</u>	LDP	yes	stem and leaf	LD	GA ₃ , BA, NAA	GA ₃	-	TANIMOTO and HARADA, 1982a;

<u>Species</u>	<u>Response Group</u>	<u>Parent Plant Induced</u>	<u>Explant Source</u>	<u>Culture Day length</u>	<u>Hormones in Medium</u>	<u>Promotion By:</u>	<u>Inhibition By:</u>	<u>Reference</u>
<u>Spirodela polyrrhiza</u> SP20	-	-	-	-	-	-	-	KHURANA and MEHESHWARI, 1980;
<u>Streptocarpus nobilis</u>	SDP	no yes	leaf	SD	BAP	BAP	GA ₃ IAA, KNO ₃ sucrose	ROSSINI and NITSCH, 1966; HANDRO, 1977, 1984; SIMMONDS, 1982;
<u>Saccharum officinarum</u>	SDP	yes	apex	-	-	-	-	COLEMAN and NICKELL, 1964;
<u>Thuja</u> sp.	-	-	-	-	GA ₃ -	-	-	RITCHIE, WERKMAN and LONGMAN, 1986;
<u>Torenia fourmieri</u>	SDP	yes	leaves, internode	SD	IAA, kinetin, or none	IAA, ABA, zeatin, sucrose	NAA, BA, kinetin, NH ₄ NO ₃	BAJAJ, 1972; CHLYAH, 1973; TANIMOTO and HARADA, 1981a,b,c; TANIMOTO, MIYAZAKI and HARADA, 1985;
<u>Vitis vinifera</u>	-	-	tendrill	LD	-	BA, PBA	-	SRINIVASAN and MULLINS, 1978;
<u>Wolffia arrhiza</u>	LSDP	no	whole plant	SD	-	BA, kinetin, zeatin	-	KRAJNCIC, 1983;
<u>Wolffia microscopica</u>	SDP	no	whole plant	LD	-	salicylic acid BA, kinetin zeatin benzoic acid	ABA	KHURANA and MAHESHWARI, 1983a,b; VENKATARAMAN, SETH and MAHESHWARI, 1970;
<u>Xanthium strumarium</u>	SDP	no	apex	SD	kinetin	-	-	JACOBS and SUTHERS, 1971, 1974.

1.2. INVESTIGATIONS BASED ON A SINGLE COMPOUND/FLORIGEN MODEL:

1.2.1. BIOASSAYS

For some years, in vitro techniques have been used in an attempt to provide a bioassay system, where an extracted flower stimulus or some other substance, would bring about flowering in non-inductive conditions, or conversely would inhibit flowering in inductive conditions. It is clear that the results of the work that has been done, are not conclusive and most have not been successful as bioassay systems. They do not give an indication of whether a substance is a single compound or florigen, or an extraneous substance which happens to cause the explant to flower, either for pharmacological reasons or because it forms part of the growth requirements of the flower. The reason for this assertion, is the large variety of known, and often common substances which bring about flowering in many plants, in particular in the Lemnaceae. It is unlikely that any of these substances is a florigen acting on its own to induce flowering, and thus they will be dealt with later as part of multi-component systems.

There is still a possibility that a single substance isolated from extracts could be found to have inductive properties on a wide range of species. Such a substance could be considered to be a florigen even if it is not universally distributed. Once such a compound is identified, the bioassay of it is possible provided extensive purification of the extract is carried out. This is necessary, unless a highly specific bioassay system can be developed where the explants are only responsive to florigen and not to the wide variety of substances which are known to induce flowering. Such a bioassay system would rely on the existence of

a stimulus that is common to at least a large group of plants, or a particular response type. No such substance has yet been identified, or is even known to exist, although some preliminary work has indicated that extracts of Xanthium contain flower inducers if applied to Lemna, or to Xanthium in conjunction with GA (HODSON and HAMNER, 1970). Extracts from Chrysanthemum applied to Xanthium and Chrysanthemum (BISWAS, PAUL and HENDERSON, 1966) and diffusate from induced scales of Wedgewood Iris transmitted to Iris apices both induced flowering of these apices (RODRIGUES PERIERA, 1965). All of these examples can be explained in terms of a multi-component model as described by BERNIER, KINET and SACHS (1981b), which demonstrates the inconclusiveness of these systems as bioassays.

In the determination of the suitability of a particular system as a bioassay, a number of criteria have to be considered;

(1) The physiological state of the parent plant. Plants which had been pre-induced, or were already flowering, have often been used as the source of explants, such as in the case of Streptocarpus (SIMMONDS, 1982) Torenia (TANIMOTO and HARADA, 1981a,b,c), Kalanchoe (MARGARA and PIOLLAT, 1981), Browallia (GANAPATHY, 1969), Begonia (RINGE and NITSCH, 1968) and also in the much developed Nicotiana system, where explants are usually taken directly from the inflorescence tissue (CHOUARD and AGHION, 1961; AGHION-PRATT, 1965a,b; TRAN THANH VAN, 1973a). If these systems are used as bioassays, and flowering is brought about by the addition of some substance to the medium, it is probable that this substance simply promotes the manifestation and growth of the buds or flowers, which had already been determined by the stimulus before culture. In vitro flowering in Nicotiana has only succeeded in DNP, which suggests that the

stimulus produced by photoperiodic tobacco plants has not been reproduced. Until photoperiodically sensitive tobacco species have been successfully induced in culture, this work is not likely to be valuable as a bioassay system. CHAILAKHYAN, AKSENOVA, KONATANTINOVA and BAVRINA (1974) suggested that in photoperiodic tobacco plants, the stimulus may be the same as in DNP, but the apex does not develop the ability to synthesise its own requirements for evocation and flowering, and thus relies on a continued input from the leaves. BRIDGEN and VEILLEUX (1985) supported this by grafting DNP *Nicotiana* to photoperiodically sensitive plants in vitro, but could not obtain any flowering in the sensitive explants, or inhibition in the DNP, although this does occur in vivo (LANG, 1965).

It is desirable then, that the parent or donor plant used in a bioassay of the flowering stimulus, should be strictly vegetative at the time of culture.

The nutritional status of the parent plant also affects the ability of the explants to flower. Explants of Glycine derived from parent plants grown in vitro on a nutrient deficient medium fail to flower and fruit to the extent of those from more enriched media (DICKENS and VAN STADEN, 1985).

(2) The culture medium may play an important role in the effectiveness of a bioassay. A variety of plant growth regulators are usually added to the medium in order to bring about growth (Table 1). Some systems have nevertheless avoided these additions, such as those using Nicotiana (AGHION-PRAT, 1965b), Torenia (TANIMOTO and HARADA, 1981a,b,c), Glycine (DICKENS and VAN STADEN, 1985). It is possible that growth regulators may interfere with the expression of the stimulus, or

even simulate its effects, and thus they are not desirable in a bioassay. The composition of the nutrients in the medium may also play an important role, as was indicated by TANIMOTO and HARADA (1981b).

(3) The culture photoperiod; the most desirable culture conditions for a qualitative bioassay would be those that keep control plants in a vegetative state, and the substance under test brings about flowering. One problem associated with this, is that the plant may be dominated by vegetative conditions which actively prevent the stimulus from working. For this reason, it would be desirable to develop systems using species which are marginally floristic under certain conditions. Under these conditions, there is less likelihood of strong vegetative determination. DeFOSSARD (1974) suggested that specimens should be grown under inductive conditions in order to avoid natural inhibitor production.

(4) The choice of an explant is influenced by many factors, as was outlined in the introduction. DeFOSSARD (1974) claimed that the specimen should be defoliated to reduce its responsiveness to environmental conditions, and should be reduced to the tissues which respond to florigen. This type of bioassay is dependent on a single compound stimulus, and is unlikely to work if flower induction and evocation rely on a more complex stimulus. It is possible that some input from the leaves and other organs may be necessary to support the growth of the flower primordia to a size where they can be evaluated. A lack of these general organs may prevent the expression of the floral stimulus. Conversely to this, isolated apices of Perilla would not provide suitable bioassay material, as they flower automatically if stripped of their unfolded leaves (RAGHAVEN and JACOBS, 1961).

(5) There is a possibility that a system may be developed where the floral stimulus induces concurrent changes in the plant, which may act as indicators of the presence of the stimulus. Kalanchoe is a possibility, as there are several changes associated with the transfer to inductive cycles (SCHWABE, 1969), such as the onset of leaf succulence, cell sap viscosity and increased anthocyanin levels (NEYLAND, NG and THIMANN, 1963).

1.2.2. EVIDENCE FOR THE EXISTENCE OF FLORIGEN, PROVIDED BY IN VITRO STUDIES

The culture of small explants from plants which are already flowering, has shown that the determination to flower can be carried through from the flowering parent plant to the explant. This occurs even if the explant grows considerably before flowering takes place, such as in the case of Nicotiana inflorescence sections. This was also found in Saccharum, where the apex continued through to flower in non-inductive conditions (COLEMAN and NICKELL, 1964). Explants of Glycine taken from flowering plants, flowered in non-inductive LD (DICKENS and VAN STADEN, 1985), while SCORZA and JANICK (1980) found that reculturing of Passiflora led to the exhaustion of the stimulus. In the DNP Nicotiana, the floral stimulus was stable through three subcultures, and is thus thought to be produced in all cells (CHAILAKHYAN, AKSENOVA, KONSTANTINOVA and BAVRINA, 1974). The stimulus in Pharbitis requires at least 24 hours to reach and be stabilized at the apex before culture in vitro, while in vivo, flower saturation is achieved after only 4 hours (BHAR, 1970).

Results such as these could be used to indicate that some substance which works at a very low concentration is present in the tissue. These results also question the theory that some particular concentration or balance of substances is responsible for flowering, as this is not likely to be transferred and developed during culture. If a substance/florigen is carried over during culture and continues to stimulate flowering, it seems reasonable to assume that its multiplication must occur as the explant enlarges. This suggests some involvement at the gene level, possibly of an epigenetic nature.

The presence of a flower gradient in plants has been shown repeatedly in Nicotiana (AGHION-PRAT, 1965; CHOUARD and AGHION, 1961) and in Torenia (TANIMOTO and HARADA, 1979), where only explants taken from the upper regions of the stem will form flowers, while those from the bottom remain vegetative. The possibility has been raised repeatedly that these upper sections contain either the requisite amount of a stimulus which is produced in the upper regions of the plant and forms a gradient of concentration down the stem, or they contain the correct balance of two or more substances probably derived from different parts of the plant.

There is a strong possibility that this gradient exists as a result of epigenetic changes which have taken place, and thus no particular substance need be present in the explant to carry over the flowering stimulus besides the altered DNA. Such a situation nevertheless does not preclude the involvement of a florigen, as some signal does have to be produced by the plant which causes this genetic change, or some substance is produced as a result of this change. This was supported by work on Helianthus, where the apex was found to be determinate and various treatments with hormones and other substances, had no effect on flowering (PATERSON, 1984). It is important to bear in mind the fact that much of the "flowering gradient" work is based on DNP cultivars of Nicotiana tabacum, and thus there is no production of an environmentally initiated stimulus. SCORZA and JANICK (1980) did show the existence of some deminishing stimulus in the DNP Passiflora. It is not known what events are responsible for the change to the reproductive state in DNP, but it is possible that the same physiological conditions are produced as are found in SDP and LDP. CHAILAKHYAN (1974) claimed that all DNP cells synthesize the stimulus, while in photoperiodically sensitive plants,

only the leaves produce the stimulus, and supply a continuous supply to the apex. This makes their induction in vitro difficult. ROSS and MURFET (1985) noted that in Lathyrus, the difference between DNP and LDP is under relatively simple genetic control. That there is some similarity in the stimulus is supported by grafting experiments using Nicotiana (LANG, 1965; ZEEVAART, BREDE and CETAS, 1977), where the transfer of the stimulus was achieved from one response group to another. These grafting results do not provide conclusive evidence, if examined in the light of BERNIER, KINET AND SACHS (1981b) multi-component model, where a single substance which forms part of a multi-component system may be transmitted across a graft union and bring about flowering.

The important issue here, is the separation of a florigen from all the other factors involved in flowering. Even though a group of factors may induce flowering, this does not preclude the possible existence of a florigen. This was recognised by SACHS (1977) while describing a nutrient diversion hypothesis for the control of flowering. So in reality, it may be difficult to distinguish a florigen theory from the multi-component theory to be discussed later.

1.2.3. EVIDENCE FOR THE EXISTENCE OF A FLOWER INHIBITOR

The presence of inhibitors of flowering are beyond doubt, and have been demonstrated in several experiments. Interpretation is critical here as it was pointed out by JACOBS and SUTHERS (1974) that many of the results which show the presence of a flower stimulatory substance, can also be interpreted as showing the removal of a flower inhibitor. In support of an inhibitor, JACOBS, RAGHAVEN and KAUSHIK (1965) had earlier suggested that a florigen may not exist in Perilla, and that the apex does not need to be induced to flower, but will flower automatically when the inhibitory effect of the leaves in LD have been removed by SD. BLAKE (1972) developed a bioassay for flower inhibitors, using Silene (= Viscaria) apices and found that extracts of vegetative Kalanchoe were significantly more inhibitory than flowering extracts. One problem not dealt with, was the fact that flowering leaves of Kalanchoe are significantly more succulent than vegetative leaves (HARDER, 1948) contain more water and thus the cytoplasm would be more diluted. This may have contributed to the difference in results.

RAGHAVEN and JACOBS (1961) demonstrated that in Perilla flowering is controlled by at least two components or events, the first controlling cone formation and the second controlling flower development on the cone. The former event is controlled by the presence of an inhibitor produced by young folded leaves, while the latter is photoperiodically controlled, by the production of a stimulus by the same leaves. The inhibitor was found to diffuse through the medium, from inhibitory leaves to an isolated apex in inductive conditions.

The Lemna system has identified several inhibitors of flowering, including ammonium ions (OOTA and KONDO, 1974), sugar (OOTA, 1972), several plant growth regulators and a variety of other substances (Kandeler, 1984). These will be examined later in detail, but the important point here was made by HILLMAN and POSNER (1971) and OOTA and KONDO (1974) who said that inhibition in Lemna by such a variety of diverse factors is due to the harmful action of these substances on the plasma membrane, specifically if the cAMP level in the bud cells is controlled by membrane bound adenylyl cyclase activity. This indicates that in many cases, inhibition may not be specific to flowering, but may be pharmacological.

It is notable from the preceding discussion, that the existence or identity of a florigen or specific inhibitor has not been conclusively shown in in vitro systems, as is the case in the whole of flowering research. The major obstacle in this work, is the tendency to draw distinction between florigen and multi-component theories, a distinction which may have no justification at all.

1.3. EVIDENCE FOR A MULTI-COMPONENT STIMULUS/SYSTEM

As workers searched for a single flower promoting substance, it became apparent that even though such a substance may exist, a number of other substances and processes also affect the induction and evocation of flowering although these processes are proving difficult to distinguish. As was mentioned earlier, florigen action became related to the presence of gibberellins and then inhibitors. Several other compounds have also been implicated, and several closely related growth phenomena have indicated that more than one substance or process is involved in flowering. CHAILAKHYAN (1985), enlarging on his original theory of florigen, suggests that florigen is a bicomponent complementary system of flower hormones produced as a result of both autonomous and induced regulation mechanisms, thus supporting the currently most supported theory of flower induction.

Circumstantial evidence from in vitro work, suggests that the floral stimulus is composed of several components with different functions. In SDP Perilla, isolated apices from vegetative plants cultured in non-inductive LD could be made to produce sterile cones, while in SD, fertile flowers were formed (RAGHAVEN and JACOBS, 1961), indicating the presence of at least two different components to the stimulus. Similarly in Salix, cultured dormant buds produced sterile catkins, while non-dormant buds produced fertile flowers in the axils of the bracts (ANGRISH and NANDA, 1982). STEFFEN, SACHS and HACKETT (1986) found that Bougainvillea reproductive meristems could be initiated under any condition, but florets were only formed in inductive conditions. It is now generally recognized that many of the substances involved in flowering, may be acting on secondary growth processes after the work of

the stimulus has been completed, and are probably produced as a result of the primary stimulus.

Further evidence for a multi-component stimulus was provided by FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, TAKIMOTO and CLELAND (1986a) using Lemna species. They found that extracts of flowering plants, after extensive purification, contained three fractions of flower inducing activity. These authors conclude that flowering in Lemna is controlled by several factors, including nicotinic and benzoic acids. It is not understood how these substances bring about flowering, as neither benzoic acid (FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA and TAKIMOTO, 1983a) nor nicotinic acid (FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, TAKIMOTO and CLELAND, 1986a,b) vary in endogenous concentration in response to induction. It is possible that the effect may be pharmacological as several other substances are equally stimulatory.

Also supporting multi-component systems, is work of KANNANGARA, WIGHTMAN, APSIMON and ABRAMS (1986) who extracted and separated fractions from flowering Xanthium plants which exhibited promotive activity on the rate of development of primordia in induced plants.

SCORZA and JANICK (1980) suggested that in the DNP Passiflora, the flowering stimulus may consist of a flower promoter and/or an inhibitor, and a cytokinin, the critical components of which seem to have a short life and are subject to dissipation as they are translocated through the plant. They concluded that the flower induction component of the stimulus in Passiflora is not synthesized in vitro despite the fact that small tissue explants were able to produce flowers. This is in agreement with the situation in DNP Nicotiana sp. (AGHION-PRAT, 1965; CHOURD and

AGHION, 1961; KONSTANTINOVA, AKSENOVA, BAVRINA and CHAILAKHYAN, 1969). These results again support the distinction between induction and evocation.

Two classical theories can be interpreted as supporting the multi-component model. Firstly, the antagonism that exists between flowering and rooting. GASPAR (1980) proposed that this antagonism was due to a control mechanism where inverse variations of auxin and peroxidase enzyme are responsible for flowering and rooting. Helianthus apices also failed to produce roots once flowers had been initiated (PATERSON, 1984). Flowering of Cichorium apices was inhibited by the presence of root tissue which was in close proximity to the apical bud (JOSEPH, BILLOT, SOUDAIN and COME, 1985). SHINOZAKI and TAKIMOTO (1982b; 1983) found that in Pharbitis seedlings grown in vitro, the induction by a variety of exogenous substances was always accompanied by a suppression of root elongation, although there was no effect on the root or shoot dry weight. The size of the culture vessel also influenced flowering, smaller vessels allowing for greater flowering in non-inductive conditions. This antagonism is widely appreciated but not understood, although it suggests some relationship between the physiological control of the roots and the production of flowers.

The second classical theory that supports multi-component systems, is that a high C/N ratio can stimulate or even induce flowering. This will be examined in detail under the section on nutrients. It has recently been stressed that this theory is worthy of further investigation and has been wrongly neglected (TREWAVAS, 1983).

Naturally, no investigation of multi-component models would be complete

without a detailed look at plant growth regulators. These ubiquitous compounds are known to affect flowering as well as a variety of other growth phenomena, but the disturbing fact in all, is their apparent lack of specificity. This implies that either the cell becomes sensitive to them when required (TREWAVAS, 1981), or that they are primarily involved in routine growth and development, but not in the actual initiation of flowering itself. BERNIER and KINET (1985) claim that there is sufficient information available, indicating that plant growth regulators are primary controlling agents of flowering. This uncertainty is not likely to be resolved until there is more understanding about the mode of action of all hormones.

1.3.1. HORMONES IN IN VITRO FLOWERING

1.3.1.a. AUXIN

Auxins have had a long and varied association with flowering in vitro are often considered an obligatory part of culture media, particularly if the explant is very small.

Promotive Effects

There are not many reported cases where auxins are promotive of flowering in vitro. Pharbitis seedlings could be induced to flower in non-inductive conditions in vitro, by the application of NAA, although this was always accompanied by a suppression of root elongation. Flower induction in this case was thought to be a consequence of root suppression (SHINOZAKI and TAKIMOTO, 1983). Auxin stimulation of flowering was also found in Phlox callus derived from flower primordia (KONAR and KONAR, 1966) and in Streptocarpus if applied with cytokinins (ROSSINI and NITSCH, 1966) although this is contrary to the findings of SIMMONDS (1982) who found that IAA was strongly inhibitory. NAA was also found to promote bud development in Perilla and was essential in the medium (TANIMOTO and HARADA, 1980). Possibly the most extensive work in this area, is that work of TANIMOTO and HARADA (1981b,c) who found that in Torenia stem segments, IAA promoted the initiation and development of flower buds if applied early on on culture. It is important to note that the explants were taken from induced plants, and thus auxin may simply have been supporting the expression of the flower buds. The level of endogenous IAA in the tissue was found to remain constant regardless of the physiological state of the explant, but became undetectable after two

weeks of culture (TANIMOTO, MIYAZAKI and HARADA, 1985).

VAN DEN ENDE, KROES, KEMP and BARENDSE, (1984) found that in thin cell layers of Nicotiana, NAA inhibits the development of flowers early on, but becomes promotive later on in growth. This is also the situation in Cichorium (PAULET and NITSCH, 1964; MARGARA, 1973; MARGARA and TOURAUD, 1968). Such promotion is likely to be far removed from the floral stimulus, and is merely a cell growth promotion. VAN DEN ENDE, KROES, KEMP and BARENDSE, (1984) claimed that auxin in the medium affects the distribution or polarity of buds on the explant of Nicotiana, while BA influences the number. The same conclusion with regards to auxin was made for Streptocarpus, where auxin probably influences the transport of substances within the plant (SIMMONDS, 1985).

Auxin seems to play a role in the formation of different sexes in in vitro flowers. In Cucumis, plantlets grown produced separate sex flowers, while plantlets which received prior treatment with auxin and cytokinin, produced bisexual flowers (RAJASEKARAN, MULLINS and NAIR, 1983). In the in vivo situation, auxin is known to promote female flowers, while GA promotes male flowers (GALUN, 1959; RUDICH, HALEVY and KEDAR, 1972). The difference in the results here, is not understood.

Inhibitory effects

Auxin is widely recognized as being an inhibitor of flowering in in vitro systems, although its presence in many media may be necessary for growth. Auxin was found to be inhibitory in the SDP Plumbago (NITSCH and NITSCH, 1967b), Perilla (CHAILAKHYAN, BUTENKO and LYUBARSKAYA, 1961; RAGHAVEN, 1961); Chrysanthemum (HARADA, 1967); Streptocarpus (SIMMONDS, 1982); Lemna (FUJIOKA YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, TAKOMOTO

and CLELAND, 1985,1986; GUPTA and MAHESHWARI, 1970), in the latter case inhibiting by counteracting the inductive effects of cytokinins; Browallia (GANAPATHY, 1969) and Helianthus (PATERSON, 1984). Inhibition was also obtained in the LDP Cichorium (PAULET and NITSCH, 1964; MARGARA and TOURAUD, 1968; MARGARA, 1973); Begonia (RINGE and NITSCH 1968); Lemna sp. (FUJIOKA YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, TAKOMOTO and CLELAND, 1986b), as well as in DNP Nicotiana cultivars (AGHION-PRAT, 1965; HILLSON and LaMOTTE, 1977). Inhibition was also detected in cultures of Iris bulbs (RODRIGUES PERERIA, 1965).

In the SDP Pharbitis and Chrysanthemum, IAA retarded initiation and development of flower buds (HARADA, 1967). In Perilla, IAA inhibited flowering in SD, but allowed for the growth of sterile cones which are also produced in LD (RAGHAVEN, 1961). This inhibition by auxin is specific and is not a general growth inhibition but seems to inhibit the development of sporogenous tissue and not the formation of the calyx and corolla. No auxin inhibition resulted if the explant had two pairs of leaves, possibly as these leaves served to produce the requirements for growth.

In the LDP Cichorium, auxin was inhibitory of flowering in the first two weeks (PAULET and NITSCH, 1964; MARGARA and TOURAUD, 1968; MARGARA, 1973). Margara found auxin to be promotive during flower morphogenesis, but not during the pre-induction phase, again suggesting the involvement of auxin in tissue growth.

In DNP Nicotiana cultures, auxin is usually included in the medium (TRAN THANH VAN, DIEN and CHLYAH, 1974) and is essential for flowering (VAN DEN ENDE, CROES, KEMP, and BARENDSE, 1984), although according to CROES,

CREEMERS-MOLENAAR, VAN DEN ENDE, KEMP and BARENDSE (1985), increased auxin almost completely abolishes bud formation on older tissues. They also noted a strange situation where NAA strongly suppresses bud formation on internodes, but does not in flower stalk tissue, which is the tissue usually used in this type of culture. AGHION-PRATT (1965) found that this auxin inhibition could be partially overcome by cytokinins and that normal flowers could be produced in the absence of IAA. It was also determined that if the cytokinin level was kept constant, but the IAA level was increased, this caused a reversion of buds from flower to vegetative (WARDELL and SKOOG, 1969a,b), yet these authors also found that IAA is required for the development of normal flowers. The apparent contradiction above may be as a result of using different explant sources or parent plants at different stages of the flowering process.

In Nicotiana thin cell layers, IBA was important in bringing about flowering in liquid cultures (COUSSON and TRAN THANH VAN, 1981). HILLSON and LaMOTTE (1977) noted that IAA inhibited both flowering and vegetative bud formation if supplied with low kinetin levels, while at high kinetin levels, IAA inhibited bud formation but stimulated vegetative bud formation. This seems to be a more specific inhibition of flower induction, as IAA did not effect flower development.

The inhibition of flowering in Nicotiana by IAA, was reversed by RNA base analogues, which resulted in an increase in the number of flowers on stem segments (WARDELL and SKOOG, 1969b). These base analogues also caused the production of flowers on stem segments lower down on the plant, thus removing the floral gradient originally identified in vitro by CHOUARD

and AGHION, (1961). This gradient was also disturbed by kinetin and auxin (AGHION-PRAT, 1965a). Yet explants from vegetative plants could not be induced to flower by either of the above treatments, which suggests that they only affect the expression of flowering, but not the induction. WARDELL and SKOOG (1973) noted that the floral gradient was also reflected by DNA content, and concluded that auxin is not the only substance causing the gradient. This is supported by NOMA, KOIKE, SANO, KAWASHIMA (1984), who found no correlation between flower forming ability and endogenous IAA levels in a different cultivar. The significance of this is that the small quantity of IAA in stem segments of Nicotiana, cannot be used to explain the differing abilities of explants to flower. The only hint of a pattern of distribution, was that the concentrations of free IAA and IAA-conjugates were highest in the first and second internodes, which do normally have the ability to form flower buds, but this trend was not repeated in other tissues which also have this ability.

Auxin may inhibit flowering by inducing RNA synthesis in a way that shifts protein synthesis in favour of vegetative growth and development, rather than flowering (WARDELL and SKOOG, 1969b). Analogues may work by inhibiting the synthesis or function of IAA-induced RNA.

Endogenous IAA has been proposed to play some role in bud expression in vitro by directly suppressing the synthesis of rapidly renaturing DNA (WARDELL, 1975). Auxin is known to inhibit the synthesis of DNA and cell division (SEIDLOVA and KHATOON, 1976). Tissue capable of forming flowers is known to contain several fold more DNA than tissue that only forms vegetative buds (WARDELL and SKOOG, 1973). These authors also noted that incorporation of thymidine into the DNA of Nicotiana stem segments during

DNA synthesis, is inhibited by the same levels of IAA that inhibit flowering of these segments. Young leaves attached to these segments have the same effect on DNA synthesis, possibly due to auxins produced in them. WARDELL (1975, 1976, 1977) showed some qualitative differences between DNA extracted from the stems of flowering plants and that from vegetative plants. A purified DNA solution prepared from flowering plants could induce flowering in vegetative plants of the same cultivar.

It was shown by SILBERGER and SKOOG (1953) and KEY (1964) that auxin induced cell enlargement depends on the synthesis of RNA. VANDERHOEF and KEY (1968) also indicated that kinetin inhibits cell elongation and RNA synthesis. From this information, it is evident that increased cell enlargement and RNA synthesis in response to higher IAA levels, are closely related to the inhibition of flowering or the maintenance of vegetative growth. Kinetin counteracts IAA inhibited flowering possibly by inhibiting cell elongation and RNA synthesis. The situation in Kalanchoe seem contrary to this, as flowering is usually accompanied by cell expansion and an increase in leaf succulence (GUMMER, 1949; HARDER, 1948; SCHWABE, 1958) which suggests higher auxin levels although this has not been investigated. The accumulation and disappearance of starch in cells has been observed in regions of callus where vegetative and floral buds are differentiating (SADIK and OZBURN, 1967; THORPE and MURASHIGE, 1970). Auxin may be involved here, as IAA has been shown to reduce starch accumulation in Nicotiana callus, while kinetin enhances it (TETLEY and IKUMA, 1970). The latter authors noted that there is a complex interrelationship between starch metabolism and hormone induced growth. IAA also inhibited cytokinin induced flowering in the SDP Lemna aquinoctialis (GUPTA and MAHESHWARE, 1970). The above information

indicates that a possible regulation mechanism of the flowering process may be the balance between auxin and cytokinin.

GASPAR, PEVEL, RODUIT, MONCOUSIN and GREPPIN (1985) noted a temporary peak of auxin, associated with an inverse variation of basic peroxidase activity, which coordinates the beginning of flower development, but follows after low auxin levels associated with flower induction. As discussed earlier, auxin application during induction tends to inhibit flowering, while application during the initiation of buds tends to support growth. This is supported by Nicotiana thin cell layers, which have low peroxidase activity in the flower forming upper regions, but higher levels in the basal regions (THORPE, TRAN THANH VAN and GASPAR, 1978). There was also an increase in peroxidase in explants as floral bud differentiation began. GASPAR, PEVEL, RODUIT, MONCOUSIN and GREPPIN (1985) went on to explain the antagonism existing between flowering and rooting, as being due to opposite requirements for auxin and peroxidase for the initiation of these organs.

In their examination of auxin action on flowering, TANIMOTO and HARADA (1981c) concluded that IAA suppresses flowering if vegetative tissue explants are used, but stimulates flowering if explants are derived from flower inflorescence tissue. Similar conclusions were made by CROES, CREEMERS-MOLENAAR, VAN DEN ENDE, KEMP and BARENDSE (1985). Again this supports the auxin promotion of differentiation of flower buds, but inhibits the induction and/or evocation, possibly due to its inhibition of DNA synthesis. It thus seems unlikely that auxins form part of the floral stimulus, a conclusion also made by GASPAR, PEVEL, RODUIT, MONCOUSIN and GREPPIN (1985).

1.3.1.b. CYTOKININS

Promotive effects

Many of the media used for in vitro flowering experiments, have contained cytokinins as a constituent. In many of these systems, cytokinins were found to be essential and were able to increase the flowering response.

Those systems where cytokinins were necessary inclusions in the medium in order to bring about the growth response, included the SDP's Plumbago (NITSCH and NITSCH, 1967a,b), Streptocarpus (ROSSINI and NITSCH, 1966) and Torenia (BAJAJ, 1972; CHLYAH, 1973a, 1973b) although in the latter, TANIMOTO and HARADA (1981a,c) found that cytokinins were not necessary if explants were taken from the basal parts of old flowering plants. Cytokinins were also necessary in Begonia (RINGE and NITSCH, 1968), Arabidopsis (NITSCH, 1972), and in the DNP Nicotiana cultivars (COUSSON and TRAN THANH VAN, 1981; TRAN THANH VAN, DIEN and CHLYAH, 1974; VAN DEN ENDE, CROES, KEMP and BARENDSE, 1984; WARDELL and SKOOG, 1969c).

In some situations, cytokinins were able to induce flower bud formation in non-inductive conditions, such as in explants of the SDP's Perilla (CHAILAKHYAN and BUTENKO, 1959; CHAILAKHYAN, BUTENKO and LYUBARSKAYA, 1961; TANIMOTO and HARADA, 1980), Plumbago in conjunction with adenine (NITSCH and NITSCH, 1967b), Browallia (GANAPATHY, 1969), Lemna aequinoctialis, especially in conjunction with dicoumarol, 4-hydroxycoumarin or benzoic acid (FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA and TAKIMOTO, 1983b, FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, TAKIMOTO and CLELAND, 1986; GUPTA and MAHESHWARI, 1970; KAIHARA and TAKIMOTO, 1985b) and in Wolffia (KRAJNCIC, 1983; VENKATARAMAN, SETH and MAHESHWARI, 1970). Bud initiation was also achieved in Begonia sp. (RINGE and NITSCH, 1968), and in the DNP

Passiflora (SCORZA and JANICK, 1980) where BA was the most effective. Induction was also achieved in Vitis tendrils cultured in vitro (SRINIVASAN and MULLINS, 1978).

Cytokinins were promotive of flowering in the SDP's Streptocarpus (SIMMONDS 1982), Lemna aequinoctialis (BENNINCK and deVRIES, 1975) where cytokinins also promoted nicotinic acid induced flowering (FUJIOKA, YAMAGUCHI, MOROFUSHI, TAKAHASHI, KAIHARA, TAKIMOTO and CLELAND, 1986); in the LDP Lemna gibba if combined with salicylic acid (PIETERSE and MULLER, 1977) although cytokinins were inhibitory in photo-induced plants and promoted vegetative growth (OOTA, 1965), and in the DNP Nicotiana (TRAN THANH VAN, DIEN and CHLYAH, 1974). Promotion was also obtained in Mazus (RASTE and GANAPATHY, 1970) and in the determinate plant Helianthus (PATERSON, 1984). No effect was found in Pharbitis or Chrysanthemum (HARADA, 1967), or in Phlox (KONAR and KONAR, 1966).

Inhibitory effects

Inhibition of in vitro flowering by cytokinins has been detected in cultures of Scrofularia (MIGINIAC, 1972), and in the SDP Torenia (TANIMOTO and HARADA, 1981C) although slight promotion was obtained early on in culture. Inhibition of nicotinic and benzoic acid induced flowering was also obtained in the LDP Lemna gibba but not in the SDP Lemna aequinoctialis (FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, TAKIMOTO and CLELAND, 1985,1986b).

In the DNP Nicotiana cultivars, somewhat contradictory results have been reported, possibly due to the use of different explants taken from plants at different physiological stages. Kinetin inhibited flower bud formation and development of Nicotiana irrespective of the accompanying auxin

concentration, although vegetative bud formation was stimulated (HILLSON and LaMOTTE, 1977). Kinetin was also found to cause IAA inhibition of flower and vegetative bud formation if supplied at low concentrations. If kinetin was supplied at high concentrations, flowering was inhibited, but vegetative bud formation was stimulated. AGHION-PRAT (1965) found that low levels of kinetin could partially overcome IAA inhibition. WARDELL and SKOOG (1969a, 1969b) found that at constant low kinetin levels, increasing auxin levels cause a change in the buds from flowering to vegetative. WARDELL and SKOOG (1969a) noted that cytokinins increased the number of vegetative buds, but had no effect on the number of flowers. High concentrations of kinetin caused branching of the flower shoots to form clusters. Also in Nicotiana, cytokinins induced a greater number of vegetative buds, but inhibited flowering and root formation (TRAN THANH VAN, DIEN and CHLYAH, 1974). HILLSON and LaMOTTE (1977) noted that while flower buds increased in number in the presence of high kinetin and high auxin concentrations, vegetative buds increased in number to an even greater extent. This may be due to a stimulation of vegetative growth and consequent inhibition of flower expression, which supports the conclusions of WITTWER and AUNG (1961), who found that kinetin inhibits flowering in whole tomato plants, but stimulates vegetative growth. Similarly, in Scrofularia, kinetin inhibits flower buds in vitro, but stimulates vegetative buds (MIGINIAC, 1972). The initiation of flowering in Wolffia, by the addition of cytokinins, was also accompanied by a stimulation of leaf growth (KHURANA, TAMOT and MAHESHWARI, 1986).

The mode of action of cytokinins

From the above information, it can be seen that cytokinins have the ability to stimulate flowering in a variety of plants, but tend to be more supportive of vegetative growth. In some cases, cytokinins induce

bud formation, and in others, are promotive of growth and development, yet cytokinins could also be inhibitory of flowering. NEGRETSKII, LOZHNIKOVA and CHAILAKHYAN (1984) have shown that high cytokinin levels are associated with the upper regions of the plant, a trend which is reversed by stem girdling, which reverses the flowering gradient. This trend implies that endogenous cytokinins are stimulatory of flowering, if indeed they play any role at all. During cold flower induction of Cichorium root explants, the endogenous levels of zeatin riboside and dicafeylquinic acid increased significantly. The exogenous application of zeatin or 2iP increased the levels of dicafeylquinic acid and chlorogenic acid. The endogenous levels of both of these substances were decreased by IAA application, which was inhibitory to flowering (MIALOUNDAMA and PAULET, 1975a, 1975b; PAULET, 1979).

Adenine has been suggested as a precursor of cytokinin biosynthesis (CHEN, ERTL, LEISNER and CHANG, 1985; PETERSON and MILLER, 1976). Adenine has also been shown to be essential for the production of anthocyanins in the petals of in vitro grown Plumbago and could not be replaced by cytokinins (NITSCH and NITSCH, 1967b). Indeed, cytokinins had a depressing effect on anthocyanin synthesis which could be reversed by adenine. This antagonism between adenine and kinetin, provides possible circumstantial evidence that the role of adenine in flower development is not through its incorporation into cytokinins, and indeed, the possible role of adenine in cytokinin biosynthesis is far from clear (VAN STADEN and FORSYTH, 1984).

In Browallia, cytokinins and adenine promote flower bud development but not bud initiation (GANAPATHY, 1969). In Begonia leaf fragments, cytokinins could not induce bud formation alone, while adenine and auxin were necessary inclusions (RINGE and NITSCH, 1968). Adenine was an essential component, while cytokinins were only slightly promotive of flower bud formation. It is possible that adenine plays a dual role, as the precursor needed for the synthesis of the necessary cytokinins, and also as the purine needed for nucleic acid metabolism, and in this way is more beneficial than cytokinin alone. The synergistic relationship between adenine and cytokinin has been extensively studied (NITSCH and NITSCH, 1967a,b; NITSCH, NITSCH, ROSSINI and BUI DANG HA, 1967; SKOOG and MILLER, 1957) but is not fully understood in relation to flowering.

BERNIER, KINET, BODSON, ROUMA and JACQMARD (1974), found that in the apex of Sinapis there is an increase in mitotic activity during flower evocation, with a concomitant rise in protein, RNA and DNA synthesis. This is supported by other works and takes place possibly as a result of cytokinins (BRULFERT, GUERRIER and QUIEROZ, 1975; JACQMARD, MIKSCHE and BERNIER, 1972; JACQMARD, RAJU, KINET and BERNIER, 1976; SEIDLOVA, 1974; SEIDLOVA and CULAFIC, 1982). Adenine may initiate or promote flowering, possibly by promoting RNA synthesis at the apex or by supporting an increase in adenine-nucleotides (BODSON, 1985).

Cytokinins have been shown to stimulate the formation of meristematic zones and bud formation in Torenia stem segments (TANIMOTO and HARADA, 1982c), a proportional relationship existing for both phenomena (Figure 1.1.). This graph bears a close resemblance to the situation of flower induction in Nicotiana (Figure 1.2., VAN DEN ENDE, KROES, KEMP and

BARENDSE, 1984). It is thus possible that the prime role of cytokinins in flowering, is the development of meristematic regions, but how this relates to the different processes of induction, evocation and differentiation is not clear. There does seem to be a relationship between the accumulation of starch in the regions where buds differentiate in callus (SADIK and OZBURN, 1967; THORPE and MURASHIGE, 1970), and the occurrence of cytokinin, which is known to enhance starch accumulation (TETLEY and IKUMA, 1970).

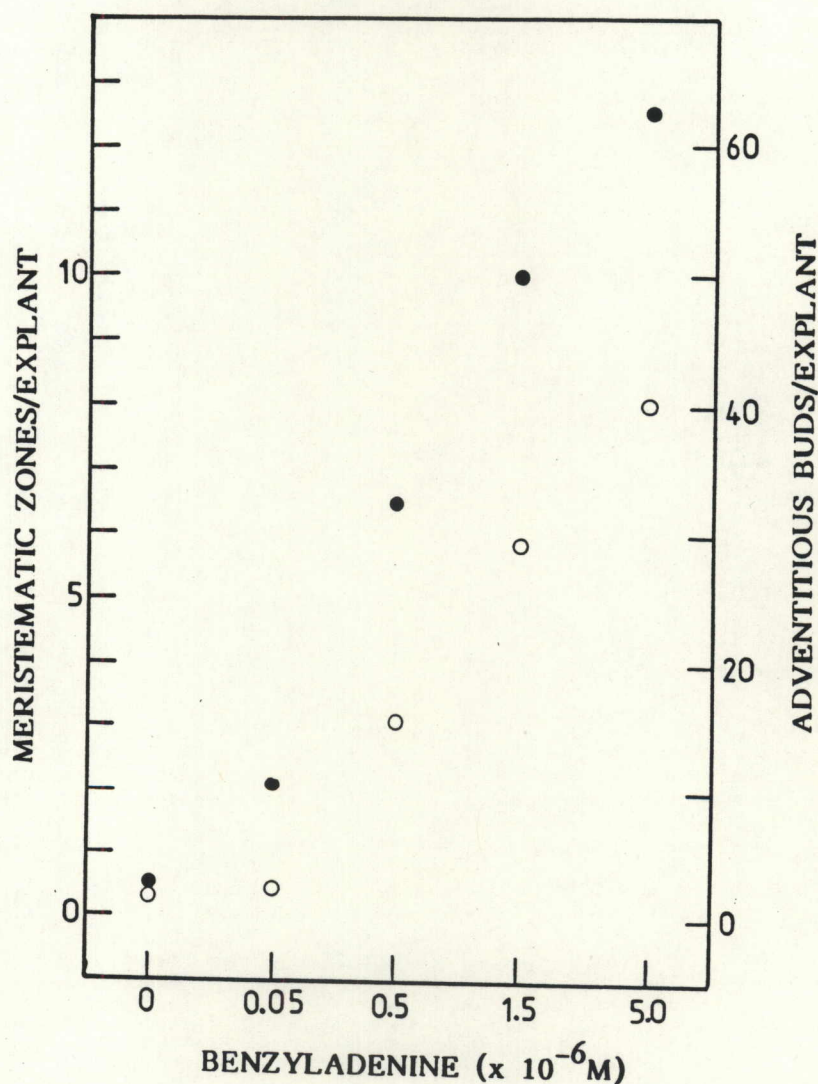


Figure 1.1. Number of meristematic zones (●) and adventitious buds (○) formed on *Torenia* epidermal explants, in response to increasing concentrations of benzyladenine. (Drawn without stated variation from data supplied by TANIMOTO and HARADA, 1982b)

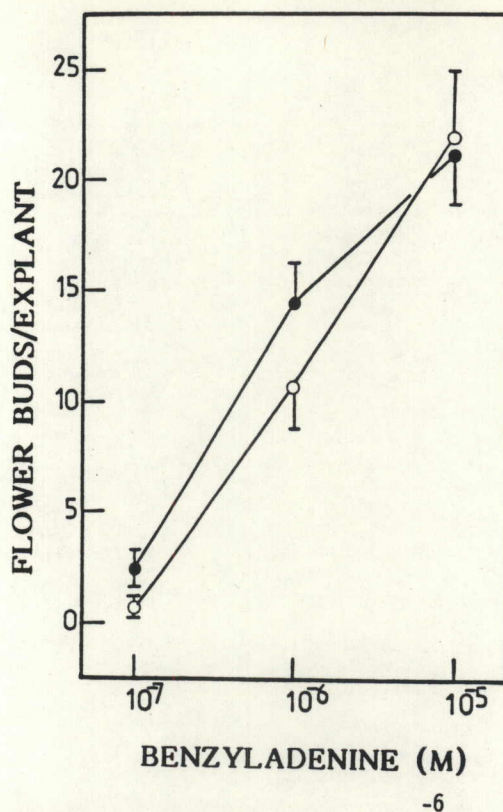


Figure 1.2. Effect of naphthaleneacetic acid at 0.1×10^{-6} M (○) and 1.0×10^{-6} M (●) and benzyladenine on *in vitro* flower bud formation on *Nicotiana* thin cell layers. (Redrawn from VAN DEN ENDE, CROES, KEMP and BARENDESE, 1984).

According to TANIMOTO and HARADA (1986), in *Torenia* stem segments, cytokinin was responsible for inducing the meristematic divisions in the epidermis to form buds. Similar bud formation was also induced by the calcium ionophore A23187. This induction was inhibited by auxin, but not by anti-cytokinins. Bud initiation by A23187 and cytokinins was effectively inhibited by the total elimination of calcium from the medium. Various manipulations of calcium metabolism, led the authors to conclude that cytokinin induced bud initiation in *Torenia*, could be partially mediated by an increase in the level of intracellular Ca^{++} .

In *Salix*, kinetin was essential in the medium, but only to promote the

sprouting of the ready formed buds. Only buds taken from trees where bud break was beginning could produce flowers, irrespective of the hormones used (ANGRISH and NANDA, 1982).

The above work indicates that although cytokinins are involved in flowering, their role and mode of action are far from clear. It seems possible that cytokinin action is directed at nucleic acid metabolism and gene activity (ZENK, 1970), or at the transcription and/or translation level, but is unlikely to be at the level of enzyme activation (MOHR, 1972; SCHOPFER, 1977).

It has been suggested that although cytokinins do appear to be involved in flower induction and evocation, several other substances are also involved (BERNIER, KINET and SACHS, 1981b; SCORZA and JANICK, 1980). The former authors concluded that the role of cytokinins in evocation may be to control early mitotic activity and associated cell synchronization, splitting of vacuoles, precocious initiation of axillary meristems, and increased rate of appendage production by the meristem. SACHS (1977) suggested that cytokinins could be involved in nutrient mobilization towards the apex which has been induced to flower. Whatever the role of the cytokinins, it is certain that they do play an important role in flowering, although it is not clear at what physiological stage.

1.3.1.c. GIBBERELLINS

Gibberellins have been the most successful of the hormones in the induction of flowering in cold-requiring plants and rosette LDP's grown in vivo. According to BERNIER, KINET and SACHS (1981b) they earn the distinction of being the most potent florigenic compounds, although these authors do recognize that an excessive number of plants cannot be induced by these compounds. ZEEVAART and LANG (1962) went as far as saying that GA's are the physiological precursors of florigen. CHAILAKHYAN and LOZHNIKOVA (1985) recently supported the concept of the flowering stimulus being composed of gibberellins and anthesins. Yet despite their wide acclaim, this group of compounds have been neglected in in vitro investigations. A few workers have utilized them in their media, but seldom with startling results. Indeed, there seems to be little similarity between the situations in vitro and in vivo. No detailed study exists where GA stimulation in vivo has been extended to the in vitro situation.

Gibberellins were included in the culture of Manihot (TANG, CAPPADODIA and BYRNE, 1983); Mazus (RASTER and GANAPATHY, 1970); Salix, (BLAKE, 1969, 1972); Spinacea (CULAFIC, KONJEVIC and NESKOVIC, 1982); Rudbeckia (TANIMOTO and HARADA, 1982c) and Thuja (RITCHIE, WERKMAN and LONGMEN, 1986).

Promotive effects

Rudbeckia stem and leaf explants taken from flowering plants, would only flower in vitro in the presence of gibberellin (TANIMOTO and HARADA, 1982c). Explants taken from vegetative plants did not flower under any conditions, suggesting that only the expression of the flower stimulus is being affected here. The development of the calyx and corolla of Silene

(=Viscaria), and the production of pollen were all promoted by GA₃ (BLAKE 1969), which again suggests the involvement in the development of the flowers but not in the induction.

GA₃ in combination with BA supported direct flowering and the production of fertile pollen on embryoids of Panax (CHANG and HSING, 1980). Gibberellin also hastened flowering in stem tips of Chenopodium that had cotyledons attached, but was ineffectual when no cotyledons were present (DeFOSSARD, 1972). This suggested the involvement of the cotyledons, where it is possible that gibberellin catalysed the release of some substance/s necessary for flowering.

According to TANIMOTO and HARADA (1981b), gibberellin's have an indirect action on flowering in the SDP Torenia, stimulating at low concentrations but inhibiting at higher concentrations. Gibberellin's also promoted the flowering of Cichorium explants taken from vernalized plants (PAULET and NITSCH, 1964). In the SDP Pharbitis, gibberellin inhibited the initiation and development of flowers in inductive conditions (HARADA, 1967). In Cucumis, there appears to be a relationship existing between auxins and gibberellin, where auxin promotes the development of female flowers, while gibberellin promotes male flowers in vivo (RUDICH, HALEVY and KEDAR, 1972). In vitro, plantlets which had received prior treatment with auxin and cytokinin, produced both sexes separately, gibberellin not being necessary for either (RAJASEKARAN, MULLINS and NAIR, 1983). This implies some change in the requirements for gibberellin in in vitro culture, possible through an altered biosynthesis of endogenous gibberellins. Stem tips of LDP Spinacia could be induced to flower in vitro by LD, or by SD together with gibberellin (CULAFIC and NESKOVIC, 1980). gibberellin also increased LD flowering and increased the percentage of female plants.

Gibberellin was essential for the differentiation of cones on vegetative shoots of Thuja (RITCHIE, WERKMAN and LONGMEN, 1986). The involvement of gibberellin in the determinate apex of Helianthus was questioned as gibberellin inhibitors CCC and ancymidol applied in vitro had no effect of flowering, which took place despite their presence. Vegetative growth was inhibited substantially (PATERSON, 1984).

In Lemna gibba, GA₃ promoted flowering slightly under sub-optimal photoperiodic conditions, but inhibited flowering under inductive LD (OOTA, 1965). GA₃ also partially reversed CCC inhibition of photoperiodically induced flowering (CLELAND and BRIGGS, 1969). Other interactions have also been detected, in Lemna paucicostata where GA₃ and IAA nullified the inductive effects of cytokinin (GUPTA and MAHESHWARE, 1970). GA₃ also inhibits benzoic acid (FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA and TAKIMOTO, 1983b) and nicotinic acid induction (FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, TAKIMOTO and CLELAND, 1986b). The stimulatory effects of extracts from Xanthium, applied to Lemna perpusilla 6746 were reversed by the addition of GA₃ to the system (HODSON and HAMNER, 1970). Yet GA₃ was necessary in conjunction with Xanthium extract to bring about flowering of vegetative Xanthium plants. That there is possibly some endogenous contribution made by gibberellin is supported by the work of TSAO, ZHONG, TIAO and TAN (1986), where it was found that in Lemna aequinotalis 6746 gibberellin levels decreased during SD induction while ABA levels increased.

Inhibitory effects

Gibberellins have been found to be inhibitory of flowering in several plants including Plumbago (NITSCH and NITSCH, 1967b), Streptocarpus (ROSSINI and NITSCH, 1966), Torenia at higher concentrations (TANIMOTO

and HARADA, 1981c), in Mazus where flowering was delayed by two weeks, and was accompanied by stem elongation (RASTE and GANAPATHY, 1970), Browallia (GANAPATHY, 1969), Lemna gibba and L. paucicostata (CLELAND and BRIGGS, 1969; FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, TAKIMOTO and CLELAND, 1985, 1986a,b), in Pharbitis and Chrysanthemum (HARADA, 1967; CHOSHI, 1979, 1980).

In Nicotiana, gibberellin inhibited flowering of stem segments completely, although application after initial formation promoted the elongation of the flowers (AGHION-PRAT, 1965a; WARDELL and SKOOG, 1969a). The latter authors also found that this inhibition could not be reversed by the application of base analogues, as could IAA inhibition of flowering. Gibberellin also reduced starch accumulation in Nicotiana in a similar manner to auxin (TETLEY and IKUMA, 1970). As kinetin caused an increase in starch accumulation and also results in bud differentiation, gibberellin may be working to inhibit bud differentiation in a way that is somehow linked to a decrease in starch.

Vegetative bud formation on SDP Plumbago explants was inhibited by gibberellin or by LD (NITSCH and NITSCH, 1967a,b), while in SD conditions, gibberellin and auxin inhibited flower bud production.

In callus of Phlox, gibberellin was promotive of rooting at low concentrations but inhibitory at high concentrations while the best flowering was obtained with IAA and coconut milk (KONAR and KONAR, 1966). As rooting and flowering are antagonistic (GASPAR, 1980), it is possible that flowering is associated with high endogenous levels of gibberellin, resulting in poor root formation.

Cultured apical buds of Iris, flowered in the presence of GA₃ or with the diffusate from scales from large bulbs. No flowering in apices from immature bulbs occurred, showing their intrinsic juvenility (DOSS and CHRISTIAN, 1979). RODRIGUES-PEREIRA (1965) found that gibberellin did not mimic the effect of diffusate. BERNIER, KINET and SACHS (1981b) suggested that gibberellin action takes place at the shoot apex, and results in the stimulation of mitotic activity in sub-apical tissue leading to the bolting of the stem, an action which is not directly related to flowering.

There is an obvious void of information about the role of gibberellins in the flowering of cultured plants, a situation which urgently needs to be rectified.

1.3.1.d. INHIBITORS

Despite the fact that inhibitors have been implicated as playing an important role in the regulation of reproductive growth, little work has been done on their action in vitro. Of the known inhibitors, ABA has attracted most attention in this regard, but little can be concluded from the information gained. This is unfortunate since ABA is known to be partially instrumental in the creation of sinks, in particular in developing seeds (ACKERSON, 1984).

ABA applied to in vitro systems, has initiated flowering of vegetative stem segments on young stem explants of the SDP Torenia (TANIMOTO and HARADA, 1981c, 1985), but was inhibitory if applied to the apical regions of flowering plants. Stimulation of flowering also occurred in Pharbitis (HARADA, BOSE and CHERUEL, 1971), but the primary effect seemed to be due to the suppression of root elongation (SHINOZAKI and TAKIMOTO, 1983). Initiation was also obtained in the SDP Lemna aquinoctialis 6746 (HIGMAN and SMITH, 1969) but was inhibitory in LDP Lemna (KANDELER, 1985). Flower promotion by ABA, but not initiation, was also detected in the SDP Plumbago (NITSCH and NITSCH, 1967b) grown under inductive conditions, and in Perilla (PURSE, 1984), possibly as a result of the suppression of vegetative growth, a phenomenon which occurs naturally in many plants at the onset of initiation. Promotion by ABA was also obtained in Chenopodium (KREKULE and KOHLI, 1981; SEIDLOVA, KOHLI and PAVLOVA, 1981). DeFOSSARD (1972, 1973b) noted that cotyledons on excised shoot tips of induced seedlings of Chenopodium, delayed epicotyl development and flowering, although this is overcome by GA₃. Shoot tips detached from the cotyledons, were similarly delayed or inhibited by ABA and CCC but were not affected by GA₃. DeFOSSARD suggested that this inhibition

may be due to the inhibition of leaf development which in turn affects flowering.

TANIMOTO, MIYAZAKI and HARADA (1985) noted the existence of a flower gradient in Torenia plants, with the greatest flowering response occurring at the second internode. The distribution of endogenous ABA exhibited a similar pattern to this, with a decrease in concentration

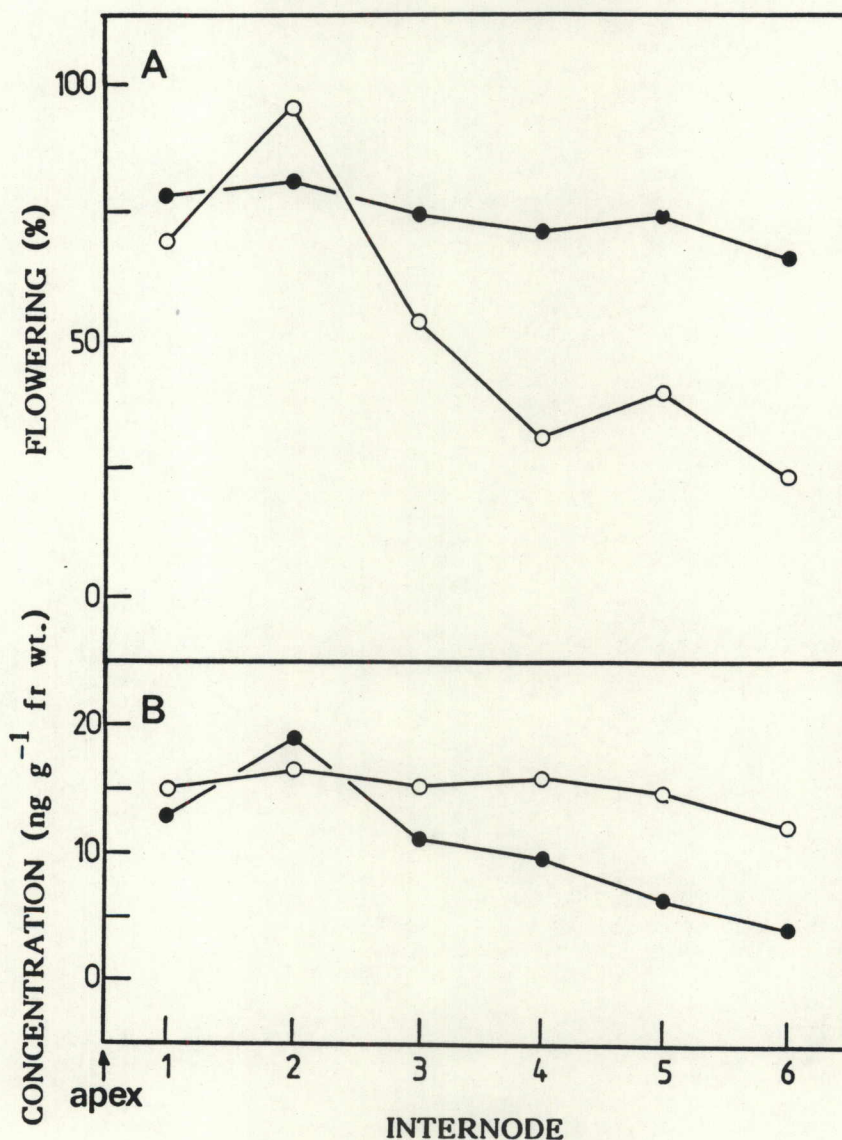


Figure 1.3. Percentage flowering and endogenous contents of ABA and IAA in Torenia stem segments excised from different parts of twelve week old plants. A. Explants cultured for four weeks with (●) or without (○) 100 ng ml⁻¹ of ABA. B. Endogenous contents of ABA (●) and IAA (○) in explants taken from twelve week old plants. (Redrawn without standard error bars from TANIMOTO, MIYAZAKI and HARADA, 1985).

towards the base and a peak at internode two. Explants taken from old plants, flowered poorly and were inhibited by exogenous ABA application, while ABA was promotive in younger plants (Figure 1.3.). These old explants also contained high levels of endogenous ABA, which was further increased by exogenous application, to become inhibitory, probably due to supra-optimal levels. The optimum endogenous levels of ABA required to regulate flower formation was between 16-20 ng/g fr.wt., which could be achieved by exogenous application. ABA was most inductive of flowering if applied to explants from vegetative plants, and in this way acted in the opposite way to auxins where stimulation was achieved in older reproductive explants (TANIMOTO and HARADA, 1981c). The endogenous levels of IAA did not reflect the flower gradient in any way.

In Lemna aequinoctialis 6746, an increase in endogenous ABA with a concurrent decrease in gibberellin occurred during SD induction (TSAO, ZHONG, TIAO and TAN, 1986). FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, TAKIMOTO and CLELAND, (1985, 1986b) and GUPTA and MAHESHWARI (1970) noted that exogenously applied ABA was inhibitory to the same plant. ABA has been implicated as a long-day canceller in Lemna (KANDELER, 1984), where ABA as well as a few other substances, cancels the effect of LD on both SDP and LDP Lemna species. KANDELER and HUGEL (1973) succeeded in obtaining flower induction in the SDP Lemna aequinoctialis 6746 with ABA, but only if accompanied by high light intensities, sucrose and a growth retardant. Lower concentrations inhibited flowering but not vegetative growth. ABA was inhibitory of flowering in Wolffia (VENKATARAMAN, SETH and MEHESHWARI, 1970).

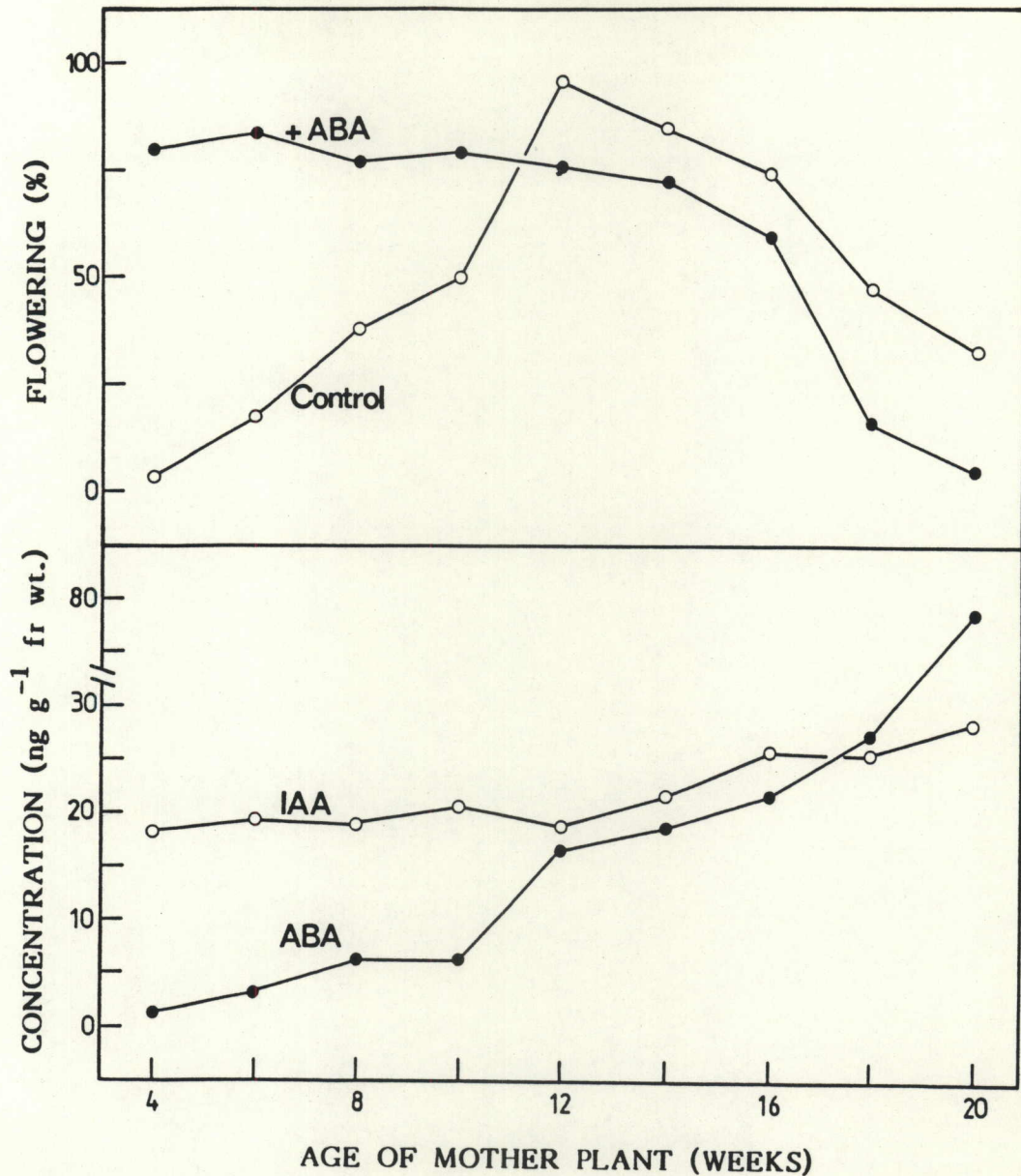


Figure 1.4. Percentage flowering and endogenous contents of ABA and IAA in *Torenia* stem segments excised from four to twenty week-old mother plants. A. The second internode cultured for four weeks with (●) or without (○) 100 ng ml⁻¹ of ABA. B. The endogenous contents of ABA (●) and IAA (○). (Redrawn without standard error bars from TANIMOTO, MIYAZAKE and HARADA, 1985).

As was discussed earlier in this review, much evidence exists to support the existence of an inhibitor of flowering in plants, although little evidence exists to place ABA in this role. In *Perilla*, an inhibitor of

flowering is produced by young leaves (RAGHAVEN and JACOBS, 1961), yet applied ABA was slightly stimulatory of flowering. Any specific flower inhibitor should ideally be inhibitory of flowering, but not of other growth processes, although some unlikely contenders fit this description such as ammonium and nitrate radicles which are inhibitory of flowering in Lemna (KANDELER, 1984).

Some proof of the existence of inhibitors of flowering have been obtained by in vitro techniques, such as the presence of inhibitors in vegetative Kalanchoe extracts applied to partially induced Silene (= Viscaria) explants (BLAKE, 1972).

It is apparent that investigations of ABA and flowering have been neglected. There are indications that ABA may be involved in the regulation of flowering, in particular in SDP, in a way that may be related to the inhibition of vegetative growth. Work is urgently needed in this field.

1.3.1.e. ETHYLENE

Little work has been done on ethylene and flowering in vitro. According to NITSCH (1972), flowering was induced by ethylene and its precursor methionine, in Plumbago. Flowering was also stimulated in in vivo Plumbago in non-inductive LD. In Helianthus apices, ACC and ethylene inhibitor AVG, both had no effect on flowering (PATTERSON, 1984) possibly due to the determinate nature of the apex. In Chenopodium (DeFOSSARD, 1973a) and in Pharbitis (SHINOZAKI and TAKIMOTO, 1983) ethrel was totally inhibitory of flowering.

The role of ethylene in flowering needs to be examined, especially in view of the interactions which occur between this substance and other hormones, in particular auxins.

1.3.2. OTHER SUBSTANCES

1.3.2.a. PHENOLICS

Numerous substances besides the recognized plant growth regulators, have been found to affect flowering in vitro. The most significant group of these substances, are the benzoic acid derivatives, which have been fairly extensively researched since it was recognized that salicylic acid may promote flowering. Much of the initial work was done by CLELAND (1974) and CLELAND and AJAMI (1974), on the role of salicylic acid extracted from aphid honeydew obtained from Xanthium plants, which had inductive properties if applied to Lemna gibba G3. These results were confused by the later findings that the endogenous levels did not fluctuate in response to the photoinductive cycles. CLELAND and KANG (1985) also noted that in Lemna gibba, salicylic and benzoic acids could reverse the inhibition of flowering caused by a lack of NH_4^+ in the medium. Conversely, GA_3 , IAA or ABA inhibited benzoic acid induced flowering in Lemna aequinoctialis 151 and 381, while zeatin supported flowering (FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA and TAKIMOTO, 1983b). BA also enhanced flowering induced by EDDHA or salicylic acid in Lemna gibba, but was ineffective on its own (PIETERSE and MULLER, 1977).

Generally in Lemna, benzoic acid (FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, TAKIMOTO and CLELAND, 1985), and salicylic acid induce flowering (TANAKA, CLELAND and HILLMAN, 1979; CLELAND, 1982, 1985). In Lemna gibba G3 though, the endogenous levels of benzoic acid did not vary with changing daylength (FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA and TAKIMOTO, 1983a), as was the case with salicylic

acid reported above. These authors concluded that benzoic acid cannot be the primary factor controlling flowering, but as it does occur in significant quantities in the plant, and can induce flowering, there is the possibility that benzoic acid may interact with other factors to influence flowering. Tannic acid has been shown to be more effective than salicylic acid at inducing flowering in Lemna aequinoctialis 6746, as it completely overrides the plants photosynthetic sensitivity (KHURANA and MAHESHWARI, 1986). It also overrides any influence of nutrient concentrations, which do affect salicylic acid induced induction.

Nicotinic acid has been extensively researched in Lemna, where it has powerful flower induction properties (FUJIOKA, YAMAGUCHI, TAKAHASHI, KAIHARA, TAKIMOTO and CLELAND, 1986b). This action is strongly daylength dependent, varying according to the response type of plant, yet the endogenous levels do not fluctuate according to daylength, which raises the possibility that other substances work in conjunction with them. Similar conclusions were made for salicylic and benzoic acids discussed above. Some relationship does exist between these substances and plant hormones, as IAA, GA₃, ABA and zeatin, all inhibited nicotinic acid induced flowering in L. gibba, although zeatin supported this flowering in L. aequinoctialis. The vitamin K antagonists dicoumarol and 4-hydroxycoumarin both induced flowering in L. aequinoctialis in a similar way to benzoic acid, as their action was also supported by BA and was daylength dependent (KAIHARA and TAKIMOTO, 1985b).

Several other phenolics and related compounds have also succeeded in stimulating flowering in Lemna, and are listed by KAIHARA and TAKIMOTO (1985b). Phenolics have also induced flowering in Wolffia (KHURANA and MAHESHWARI, 1983; KHURANA, TAMOT and MAHESHWARI, 1986).

The phenolic B-coumaric acid was also stimulatory of both vegetative and flower bud differentiation in in vitro grown cultures of Cichorium (PAULET and NITSCH, 1964). Cold was necessary for flowering, during which the endogenous levels of zeatin riboside and dicafeylquinic acid in the roots increased significantly (MIALOUNDAMA and PAULET, 1975; PAULET, 1979). BADILA and PAULET (1986) found that in Cichorium, the endogenous levels of hydroxycinnamic acid ester (especially chlorogenic acid) increased during floral induction, reaching a maximum level simultaneously with the maximum level of floral induction. Thereafter there was a rapid decrease. This occurred during induction by LD, and also during induction by 9hr white light with 15hr red light. The authors thus concluded that this increase in phenolic was not due to increased photosynthetic activity.

The involvement of the anthocyanins in flowering is not understood. NITSCH (1972) hypothesised that floral metabolism leads to the formation of organs rich in anthocyanins and possibly other phenolics, suggesting a higher activity along the pentose pathway. In Kalanchoe, an increase in the levels of anthocyanin has traditionally been associated with the onset of flowering (NEYLAND, NG and THIMANN, 1963).

An increasing variety of substances are being found with florigenic activity, although it is unlikely that any of them is the florigen. These substances could nevertheless be part of a multi-component stimulus, although there is also a distinct possibility that their effect is purely pharmacological.

1.3.2.b. NUTRIENTS

It is apparent from the literature, that most investigations of in vitro flowering, have made use of the medium developed by MURASHIGE and SKOOG (1962). This medium has a fairly high nutrient concentration, including a high level of NH_4NO_3 . It is not known whether this has been detrimental to the general investigation of the flowering process, as intensive investigations of the effects of these nutrients are seldom made. The investigations of TANIMOTO and HARADA (1981a,b) have indicated that with the correct nutrient composition, flowering can be brought about in small vegetative explants without the aid of plant hormones. They found that the dilution of the mineral salts of the MURASHIGE and SKOOG (1962) medium to one fifth of the recommended concentration, enhanced adventitious bud formation and development and in so doing enhanced flower bud formation, but did not influence the ratio of flowering to vegetative buds. Sucrose was also promotive of flowering, but the addition of the other recommended organic components of this medium, stimulated callus proliferation and vegetative bud initiation, but inhibited shoot development and flower bud differentiation. Similar work was also done by WADA and TOTSUKA (1982) who found that low nitrogen, high carbohydrate and high light intensities would induce flowering in seedlings of Perilla cultured on dilute low nutrient Whites medium.

CHLYAH (1973) had previously cultured leaf and stem fragments of Torenia on Hoaglands nutrient medium, and achieved a high rate of flower bud formation without the use of auxin or cytokinin. Both of the above investigations, found that low salts and the absence of NH_4NO_3 were supportive of flowering in vitro. The medium developed by TANIMOTO and HARADA (1981a,b) was subsequently used successfully in this thesis.

BARAN JHA, CHANDRA and CHANDRA, (1983) also employed a low salt medium to stimulate flowering of hypocotyl and leaf explants of Cuminum, as did SIMMONDS (1982) with explants of Streptocarpus. DIOMAIUTO-BONNARD (1974) found that Nicotiana glutinosa flowered in LD if provided with a plentiful supply of nutrients, but became a quantitative SDP in low nutrient levels. In Helianthus, the presence of the cotyledons on the explant, and the composition of the medium, had no effect on flowering, probably as the apex of this plant is determined to flower (PATERSON, 1984). It appears that there may be some difference between this example and that of Torenia, as in Torenia, flowering is best obtained on explants derived from flowering plants which have been induced and are thus also committed to flower. This commitment to flower, may be under a different control mechanism in the two examples. In the case of Xanthium, (JACOBS and SUTHERS, 1974), the cotyledons were present on the explant, and apparently influenced flowering only by contributing nutrients for growth. These authors had earlier (JACOBS and SUTHERS, 1971) claimed that the cotyledons of Xanthium are sensitive to daylength. It is possible that in the earlier work, the authors did not take into account the photosensitivity of leaf primordia which develop rapidly after excision. JENNINGS and ZUCK (1955) had earlier shown that Xanthium cotyledons were not sensitive to daylength and could not induce flowering.

Various salts and ions have been investigated and found to influence flowering in vitro. In Nicotiana, CoCl_2 was inhibitory of flowering of stem segments, and was eliminated from the medium (WARDELL and SKOOG, 1969). Calcium is an important component of the nutrient medium used for Torenia culture (TANIMOTO and HARADA, 1986) and supports cell division and subsequent bud formation. The elimination of calcium from the medium inhibited cytokinin and calcium ionophore A23187 induced cell division

and bud formation. This trend supported the idea that such induction of division may be partially mediated by increases in the level of intracellular Ca^{++} .

In Lemna aequinoctialis, 8-hydroxyquinoline caused a massive increase in the endogenous levels of iron and copper and yet induced flowering in non-inductive daylengths (KHURANA and MAHESHWARI, 1983; 1984), thus indicating that copper is not detrimental to flowering as has been supposed. The same phenomenon occurred in Wolffia (KHURANA, TAMOT and MAHESHWARI, 1986).

The state of the nutrient medium has also been noted to have an effect on flowering, where the solidification of the medium with agar, resulted in flower buds on explants of Cichorium, while liquid medium produced only vegetative buds (BOUNIOLS, 1974). This was thought to be as a result of the hydration of the explants, and not due to the constituents of the agar.

Nitrogen

For years, nitrogen in various forms has been recognized as having some influence on flowering. As early as the turn of the century, KRAUS and KRAYBILL (1918) noted that increasing nitrate concentrations caused increased flowering. In agreement, LOO (1946a) investigated carbohydrates and nitrogen in Baeria, and concluded that nitrate allows for the most prolific flowering, while all other nitrogen sources depress flowering. This is supported by TANIMOTO and HARADA (1981a,b) who found it necessary to eliminate NH_4NO_3 from the MS medium in use, but not KNO_3 .

Ammonium ions were also inhibitory of flowering in Lemna, where the rate of flowering decreased, and the induction time increased (OOTA and KONDO, 1974). These ions slow down the development of initiated flower primordia, possibly due to a lowering of the ATP level (KANDELER, 1984). It was concluded that sucrose acts as an "end of day" far red effect, while NH_4^+ and NO_3^- are flower inhibitors.

MOHANTY and FLETCHER (1976) stated that the presence of NH_4^+ in the medium increases the activity of nitrate reductase and promotes the growth of suspension cultures of Paul's scarlet rose. It is possible that nitrogen metabolism may be modified to affect flowering in a similar way (TANIMOTO and HARADA, 1981a,b). Recently, TANAKA, HORIKAWA, NISHIMURA and NASU (1986) noted that flowering can be induced in Lemna by the suppression of nitrate assimilation using various inhibitors, probably as a result of the suppression of nitrate reductase activity. TANAKA (1986) went on to show that Lemna *aequinoctialis* 6746 was induced to flower in CL on a nitrogen deficient medium, but flowers would develop only if the plants were transferred to a nitrogen rich medium. There was also a difference in the capabilities of NH_4^+ and NO_3^- , the latter being supportive of flowering with continuous exposure. TANAKA and TAKIMOTO (1975), found that NH_4^+ causes an increase in the free and total amount of free amino acids to a greater extent than NO_3^- . TANAKA (1986b) has suggested that NH_4^+ enhances nitrogen metabolism in a way that is unfavourable for flower induction.

1.3.2.d. CARBON/NITROGEN

Possibly the most widely researched and controversial of the nutrient effects in relation to flowering, is the ratio of carbon and nitrogen. In 1913, KLEBS proposed that a high C/N ratio brings about flowering in plants. Many workers have subsequently supported this theory, and some evidence exists in in vitro investigations to support it.

Sucrose supplied to defoliated plants of Chenopodium (LONA, 1948) and to Perilla (LONA 1949; 1950; WADA and TOTSUKA, 1982) promoted flowering in all daylengths, especially if accompanied by high light intensities and low nitrogen levels. CHAILAKHYAN (1945) noted that although sucrose promoted flowering in Perilla, as did high light intensities, the supply of nitrogen also stimulated flowering, but only in inductive SD. It is possible that in the latter case, as photoperiodic induction was taking place, the higher nitrogen levels were simply supporting the growth of the flowers. CHAILAKHYAN (1968) said that the C/N ratio does not condition the start of flowering, but is of certain importance, and indeed he recommends the use of up to 7% sucrose for flowering of Nicotiana (CHAILAKHYAN, AKSENOVA, KONSTANTINOVA and BAVRINA, 1975). HINNAWY (1956) supported this.

SIMMONDS (1982) using Streptocarpus leaf explants, found that a high KNO_3 , high sucrose medium supported vegetative growth, while a low KNO_3 and low sucrose caused flower bud development. The parent plants were pre-induced, so the stimulus itself has not been reproduced. This low sucrose level is unusual, and SIMMONDS concluded that flowering occurred in response to a decreased availability of nutrients rather than to a particular hexose/nitrate ratio.

CARBOHYDRATES

In most cases, the availability of carbohydrate does seem to be essential for flowering, as in Xanthium, where a high light intensity is required for induction, but could be partly replaced by sucrose (LIVERMAN and BONNER 1953). In vitro, this was supported by COUSSON and TRAN THANH VAN (1983) where the quantity of light supplied to Nicotiana thin cell layers, resulted in different organ production, an effect which was copied by glucose, but not in the role of an osmoregulator. A ratio of glucose and sucrose was almost able to substitute for the light requirement for the development of anthers and a style. These authors suggested that during the sequence of events leading to flower differentiation, light acts on energy-dependent sugar uptake and metabolism and on the increase of reducing potential of the chloroplasts.

AGHION-PRAT (1962) obtained partial break down of the floral gradient in flowering Nicotiana plants, where stem sections produced occasional flowers if cultured on a high carbohydrate medium. Nicotiana thin cell layers cultured in darkness on a very high sugar level of 100 g l^{-1} , produced flowers, indicating that photosynthesis plays an important role in flower differentiation, although sucrose could not completely substitute for light (TRAN THANH VAN, DIEN and CHLYAH, 1974).

Sinapis apical buds could be induced to flower by sucrose (DELTOUR, 1967) or by high light intensity (BODSON, 1977). Similarly, in Cuscuta, for flowering to occur, a high sucrose concentration removed the necessity of having a high light intensity (BALDEV, 1962). In contrast to the above, light was essential for flowering in Passiflora, and could not be replaced by sucrose (SCORZA and JANICK, 1980). In Spinacea,

photosynthesis was not necessary for induction and flowering, provided an organic carbon source was available in the medium (CULAFIC, 1982). High light intensity was able to promote flowering, as was a high sugar concentration, but only during the first 30 days of culture after which high light intensities inhibited flowering (WADA and TOTSUKA, 1982).

A point discussed earlier, was the accumulation of starch in the areas of callus where bud differentiation takes place (THORPE and MURASHIGE, 1968, 1970) and in apical buds of cauliflower which subsequently differentiate into flower buds (SADIK and OZBORN, 1967). In Nicotiana thin cell layers, explants that flower exhibit early starch accumulation and higher mitochondrial activity in the cells (TRAN THANH VAN and CHLYAH, 1976). This accumulation of starch is supported by cytokinins (TETLEY and IKUMA, 1970) which also promote bud differentiation; and is reduced by IAA and GA₃, which tend to inhibit flower bud differentiation (THORPE and MURASHIGE, 1968). It thus seems apparent that there is a complex relationship between carbohydrate and hormone levels in cells and organs, which may in some way be responsible for flowering. This does not nevertheless detract from the possibility of a flowering hormone being involved, as these events may form part of the evocational process, but not the induction process.

COUSSON and TRAN THANH VAN (1983) stated that flower bud formation has a higher carbon energy requirement than bud formation. It is possible that the hydrolysis of starch provides a large amount of substrate for oxidative phosphorylation at the time of flower initiation. These authors also suggested that the time of hydrolysis may correspond to the period where light is most essential for flowering. They suggested that light-mediated control of flower differentiation involves energy

dependent sugar metabolism, and therefore the modification of sugar supply is more dramatic than different light sources, because of two reasons. Firstly, light energy is not sufficient to trigger flowering if sugar is deprived, and secondly, exogenous sugar enhances flower differentiation in the absence of light. In Sinapis, a specific light requirement with a role not related to photosynthesis or photoinduction has been identified at the apex (BODSON, KING, EVANS and BERNIER, 1977). In this region, there is a marked increase in soluble sugars during the transition to flowering (BODSON, 1977).

Closer analysis of the role of carbohydrates in flowering, has revealed that different responses are obtained with different sugars. STEFFEN SACHS and HACKETT (1986) noted that in Bougainvillea, floret development was promoted by fructose (83%), glucose (68%) and least of all by sucrose (24%). The latter buds grew poorly when compared to those grown on fructose, but the leaves showed no difference. In Plumbago, sucrose and maltose increased flowering, while lactose, cellobiose, and mannitol were ineffective (NITSCH and NITSCH, 1967b).

In Nicotiana thin cell layers, glucose was necessary for flowering (VAN DEN ENDE, CROES, KEMP, BARENDSE and KROH, 1984), and even a brief removal from glucose, delayed flowering but did not affect initial development. In the absence of sugar, any sign of differentiation was missing. These authors could not confirm the results of TRAN THANH VAN (1977) that vegetative buds develop on a mannitol medium, which would imply an osmotic effect. This could be due to differences in explant sources.

In Plumbago, mannitol could again not replace the effect of sucrose (NITSCH and NITSCH, 1967b), supporting the idea that this was not an

osmotic effect. Sucrose also stimulated flowering in intact seedlings of Nicotiana rustica (STEINBERG, 1950) and in Pharbitis (TAKIMOTO, 1960; KIMURA, 1963).

In the case of Lemna, sugar was inhibitory of flowering, (OOTA, 1972) possibly due to catabolite repression of the transcription of floral DNA by lowering of the cAMP level in the apex. OOTA and KONDO (1974) noted that cAMP addition could partially restore the inhibition by NH_4^+ and water treatments.

The role of nutrients, and in particular carbohydrates and nitrogen, in the induction and evocation of flowering, is far from clear as so many other growth phenomena are also regulated by these substances. Whether the effects of any one of these substances is specific, or is merely coincidental remains to be determined. There is nevertheless a role for a floral stimulus which would in turn regulate the activities of these substances. A similar conclusion was made by SACHS (1977) while addressing the nutrient diversion hypothesis.

1.4. CONCLUSIONS

It is clear that in vitro techniques provide an ideal tool for the investigation of flowering physiology, as they allow a degree of control seldom achieved in in vivo studies.

There is still little justification in separating a florigen theory of flowering, from a multi-component theory, as the former may be part of the latter. The results reviewed in this review, show that a wide variety of responses are obtained in different plants, or parts thereof, with the application of an equally wide variety of substances. In an attempt to draw some conclusions from all these results, the following tentative suggestions are made;

Many of the systems developed in this field, have obvious potential as bioassay systems for flower inducing or inhibiting substances.

No unequivocal evidence exists from in vitro investigations, to support the existence of a "florigen" or a specific flower inhibitor.

Auxin appears to promote flower bud differentiation, but inhibits induction and/or evocation.

Cytokinins play an important but unclear role in flowering. Their action may be related to nutrient availability or gene activity.

Although gibberellins have been postulated by several authors to be part of the stimulus, their mode of action is obscure but may be linked to reserve mobilization.

Inhibitors have an important role to play in flowering, possibly by reducing vegetative growth and thus supporting flowering. Their mode of action is not understood.

Many phenolic compounds have inductive capabilities, particularly in the Lemna system. Although these substances may be involved in the regulation of flowering, their role may be as part of a multi-component stimulus, or their effects may be pharmacological.

Mineral nutrient composition and concentration have a marked effect on flowering. Little evidence exists to explain this.

Carbohydrate and nitrogen salts play an important role in flowering, possibly during evocation and differentiation.

Many of these conclusions implicate a role in the evocation or differentiation and growth of flowers. No unequivocal evidence exists to place any of these known substances into the role of flower inducer. Only the extensive expansion of bioassays for flowering will allow such conclusions to be made.

CHAPTER 2.

PRELIMINARY INVESTIGATIONS: THE DEVELOPMENT OF A TECHNIQUE

2.1. INTRODUCTION

The main objectives of the work presented in this chapter, were to investigate the nature of the floral stimulus in controlled conditions in vitro. The primary motivation for having sterile conditions, was so that the transmission of the stimulus from a donor, through living callus cells to a receptor, could be attempted

Transmission through living cells was being attempted because of the widely reported lack of success at passing the stimulus through non-living materials, where it could be more readily isolated. These substances included water (GALSTON, 1949), agar blocks (CHAILAKHYAN, 1982) and oil (SIRONVAL, 1951). Some transfer was obtained between Xanthium plants, across a paper barrier (HAMNER and BONNER, 1938), but later work led to the conclusion that cellular contact may have been attained through the cellulose fibers of the paper (WITHROW and WITHROW, 1943). Attempts had also been made, using a bridge of living plant material in the form of a dodder plant (Cuscuta campestris, Yuncker) parasitising two Glycine plants. No transmission of the flowering stimulus was however, achieved (FRATIANNE, 1965).

The transmission of the stimulus, or some factor which leads to the flowering of either stock or scion in grafting experiments, has provided

much of the support for the florigen theory (CHAILAKHYAN, 1937). There are numerous cases of successful transmission, which have been extensively reviewed by LANG (1965), ZEEVAART (1976) and BERNIER, KINET and SACHS (1981a,b). It is nevertheless becoming increasingly clear that the results obtained in these experiments are far from conclusive, and can be explained using the hypothesis of BERNIER, KINET and SACHS (1981b). This hypothesis suggests that flowering is controlled by numerous factors, the transmission of only one being required to bring about flowering in some instances.

This investigation was not designed to exclusively support either the florigen theory or a multi-component theory, but both are investigated as it is possible that the two theories are firmly intertwined and are fully compatible with each other. These theories are extensively reviewed in the previous section of this thesis.

The investigations reported in this chapter were **tentative and exploratory**, as they were not based on any previous work. The following steps summarize the course of the investigation presented in this chapter;

- Selection of plant material,
- Establishment in vitro,
- Flowering in vitro,
- Establishment of callus,
- Grafting of flowering and vegetative explants onto callus.

The following species of short-day plants (SDP), day-neutral plants (DNP) and long-day plants (LDP) were selected for investigation, for reasons

described below. The common feature of all, is that they have been extensively reported on in the literature, and are all eminently suitable for research into the physiology of flowering.

1. Glycine max (L.) Merrill. (SDP),
2. Kalanchoe blossfeldiana Poellniz. (SDP),
3. Xanthium strumarium L. (SDP),
4. Nicotiana sylvestrus L. (LDP),
5. Nicotiana tabacum L. cv. Maryland Mammoth (SDP),
cv. Trapezond (DNP).

2.2. FLOWERING PHYSIOLOGY IN BRIEF

2.2.1. Glycine max.

The flowering physiology of Glycine has been extensively reviewed by HAMNER (1969) and SUMMERFIELD and ROBERTS (1985). The cultivar that has been most widely researched has been the 'Biloxi' soybean, which has a critical daylength of ± 13 hours, and will flower in response to two short-days (SD). The youngest fully expanded leaves are the primary receptors of the photoperiodic input, with a single leaf being able to produce sufficient stimulus to induce the whole plant (BORTHWICK and PARKER, 1940). Flowers can be formed in the leaf axils of all the leaves, including the terminal bud. Quantitative determination of the degree of induction can be made by measuring the increase in the number of flowers with increasing number of photoinductive cycles (BLANEY and HAMNER, 1957).

Glycine was one of the first plants used to provide evidence for the existence of a flowering hormone (KUIJPER and WIERSUM, 1936; KUIJPER and SCHUURMAN, 1938). Flowering was induced in vegetative plants by grafting with flowering plants in non-inductive conditions. It is significant to note the timing of this work was concurrent with that of CHAILAKHYAN (1937) and his florigen theory, but in this case no name was given to the proposed substance. GALSTON (1949) also performed significant work on soybean, and found that the stimulus would not pass across a water-gap, but would only pass through living cells. It was this observation that led to the attempts in this thesis to use living cells to transfer the flowering stimulus.

The cultivar used in this thesis, Impala, proved to be a SDP and was readily available.

2.2.2. Kalanchoe blossfeldiana.

The flowering physiology of this plant has been reviewed recently by SCHWABE (1969; 1985). Kalanchoe is a typical SDP with a critical daylength of \pm 11 hours. A minimum of two SD are necessary for the production of flowers. Kalanchoe responds to increasing the number of inductive cycles, by producing an increasing number of flowers, the log of which forms a linear relationship with the number of SD (SCHWABE, 1956). This fact forms the basis for a quantitative determination of flowering used extensively in this thesis.

The endogenous rhythms of this plant have been extensively reviewed by SCHWABE (1969; 1985), and were not investigated further in this thesis. Consideration was given during culture and harvesting, where an effort was made to test or harvest plants at a consistent time of day.

Extensive literature has been published dealing with the inhibitors of flowering in Kalanchoe. It was found that non-inductive cycles were not simply neutral, but were actively inhibitory of flowering in Kalanchoe (HARDER and GUMMER, 1949). This inhibition was not due to an interference with the translocation of the floral stimulus (HARDER and BUNSOW, 1956), but was more likely to be a chemical substance or physiological condition. SCHWABE (1956, 1959) suggested that inhibition occurred by the blocking of an enzyme which is catalysing the production of the flower hormone. Crude extracts of vegetative plants containing this inhibitor and injected into induced plants, caused substantial inhibition of flowering when compared to flowering plant extracts (SCHWABE, 1972).

Kalanchoe is unusual in that it exhibits several other growth phenomena linked to daylength, but seemingly unrelated to flowering. These include an increase in succulence of the leaves of up to three fold (GUMMER, 1949) . This occurs without any cell division, but takes place by cell expansion. Even isolated leaves respond in this way (SCHWABE, 1958).

In Kalanchoe, a stimulus responsible for succulence is produced by leaves and transported along a single orthostichy. This substance has been named metaplasin (HARDER and VAN WITSCH, 1940).

The total organic acid content, in particular that of malic acid, is higher in leaves grown under SD than LD (SCHWABE, 1958), but only if represented on a dry-weight basis. The reverse holds true if expressed on a fresh-weight basis. SCHWABE (1969) noted the pH of expressed sap to be 5.15 in LD leaves, and 4.35 in SD leaves. Anthocyanins also reflect the daylength condition (NEYLAND, NG and THIMANN, 1963), only a small amount occurring in LD grown plants, but considerably more in SD. Unfortunately, these authors gave no attention to the associated succulence, or to the chlorophyll content. SCHWABE and WILSON (1965) also noted an increased cytoplasm viscosity associated with increasing exposure to SD.

2.2.3. Xanthium strumarium.

Xanthium is one of the classics of flowering research, and has been investigated extensively mainly because of its extreme sensitivity to photoperiodic induction. Reviews on the flowering of this plant have been compiled by SALISBURY (1969, 1985).

The critical dark period is eight to nine hours, although flowering increased up to a 15 hour night. Only a single dark period is necessary

to induce flowering. The degree of induction has been quantified by SALISBURY (1955, 1963), where different floral stages of the apical dome can be recognised under a dissecting microscope. This is basically a measure of the rate of flowering, as the flowering response is measured at a constant time after induction. The intensity of light used for growth both before and after induction markedly affects flowering, with high intensities being essential (SALISBURY, 1969).

Extensive work has been carried out on the translocation of the stimulus, from induced leaves to the apex of Xanthium (SKOK and SKULLY, 1954; LAM, 1965). Grafting has also been successful, with a variety of experiments supporting the transmission of a translocatable flower stimulus (SALISBURY, 1985). In this thesis, attempts were made to label the floral stimulus, with ¹⁴C. More detail about this is presented in Chapter 5.

2.2.4. Nicotiana sp.

These plants provide ideal material for the investigation of flowering, as a variety of response types are found within a single genus. The most commonly utilized have been N. tabacum cv. Maryland Mammoth, a SDP; N. tabacum cv. Trapezond and cv. Wisconsin 38, both DNP; and N. sylvestrus, a LDP. These species have been used to develop grafting experiments which examine the transfer of the stimulus and of inhibitors, between compatible LDP, SDP and DNP. Successful transfer has been achieved in most cases (LANG, 1965; ZEEVAART, 1958; CHAILAKHYAN, KHAZHAKYAN and AGAMYAN, 1976). The inhibition of flowering has also been achieved when a vegetative scion of N. sylvestrus was grafted onto a stock of N. tabacum cv. Trapezond (LANG, CHAILAKHYAN and FROLOVA, 1977).

Possibly even more significant has been the system developed by CHOUARD and AGHION (1961) and AGHION-PRAT (1965), and later on continued by numerous workers as reviewed earlier in this thesis, where stem segment and even epidermal cell layers of Nicotiana have been cultured in vitro and induced to flower. Much understanding of the physiology of flowering has arisen from this work, although it is limited in that only segments of flowering DNP Nicotiana species will flower in vitro. This obviously raises doubts about the significance of these investigations, as only evocation and differentiation of flowers is studied, and not induction. The history of these works is long and complex, an examination of which is beyond the needs of this thesis. A review is currently being published in HALEVY (Handbook of Flowering, Vol. V, CRC Press).

2.3 MATERIALS AND METHODS

2.3.1 Plant Material

Seeds of Glycine max cv. Impala, were obtained from McDonalds Seed Co., Pietermaritzburg, South Africa. Plants derived from these seeds proved to be SDP's, flowering and fruiting in only 27 days from sowing in eight hour daylengths, but remaining vegetative in eighteen hour days.

Kalanchoe blossfeldiana plants used were clonal material obtained from Prof. W.W. Schwabe of Wye College, London. All propagation was via vegetative means.

Xanthium strumarium seed was collected growing wild in farmlands near New Hanover, South Africa.

Seed of Nicotiana tabacum cv. Maryland Mammoth (SDP) and cv. Trapezond (DNP), and of Nicotiana sylvestrus (LDP) were kindly provided by Dr. R.B. Taylorson of the Beltsville Agricultural Research Center, Maryland, USA.

2.3.2. In Vitro Culture

All plants to be utilized, had first to be established in vitro. This was in order to have a stock of sterile plants from which explants could be taken without suffering the detrimental effects of sterilization procedures.

2.3.2.a. Glycine max:

Dry seeds were sterilized by washing briefly in absolute alcohol, followed by a soak in two per cent NaOCl, with one drop of Tween 20 for 15 minutes. The seeds were then washed three times in sterile distilled water while under sterile conditions on a laminar flow bench.

Callus

In order to obtain callus for further investigations, cotyledons were cultured on the basal medium of MURASHIGE and SKOOG (1962) (Table 2.2.b.), to which was added kinetin and NAA (naphthaleneacetic acid) in three combinations as shown below;

Kinetin	:	NAA
1.0	:	0.5 mg l ⁻¹
5.0	:	0.5 "
5.0	:	5.0 "

The last combination produced the most callus. This combination was thus used to compare three different nutrient media, in an attempt to obtain the most suitable callus. The media used were according to MURASHIGE and SKOOG (1962), LINSMAIER and SKOOG (1965) and MILLER (1965). These were cultured in low light intensities of 35 $\mu\text{Em s}^{-2 -1}$ photosynthetically available radiation (PAR).

In an attempt to induce de novo flowering on undifferentiated Glycine callus, cotyledonary callus was cultured on a variety of media, and maintained in eight hour inductive cycles, with a light intensity of 110 $\mu\text{Em s}^{-2 -1}$. The media employed were adapted from CHANG and HSING (1980), and are represented in Table 2.1. Ten replicates were used for each treatment.

Parent Plant Culture

Whole plants of Glycine were required to provide a source of explants. Sterilized seeds were placed onto a hormone free medium adapted from TANIMOTO and HARADA (1981b), who succeeded in obtaining in vitro flowering of Torenia internode segments using this medium.

Table 2.1. Different additions to the (MS) medium of MURASHIGE and SKOOG (1962) used for the culture of *Glycine max* callus in an attempt to obtain *de novo* flowering. Cultures were maintained under SD conditions at $110 \mu\text{Em s}^{-2}$. Ten replicates were used for each treatment.

<u>Treatment No.</u>	<u>Medium</u>
1.	MS + sucrose (3%)
2.	MS + sucrose (3%) + NAA(5.0 mg l ⁻¹)
3.	MS + sucrose (3%) + GA (0.1 mg l ⁻¹) ₃ + adenine sulphate(30.0 mg l ⁻¹) + tyrosine (33.0 mg l ⁻¹) + charcoal (0.5%)
4.	MS + sucrose (3%) + kinetin (1.0 mg l ⁻¹) + GA (1.0 mg l ⁻¹) ₃
5.	MS + sucrose (3%) + kinetin (5.0 mg l ⁻¹) + NAA(5.0 mg l ⁻¹)
6.	20% MS salts - NH NO _{4 3} + sucrose (3%)
7.	20% MS salts - NH NO _{4 3} + sucrose (3%) + GA (0.1 mg l ⁻¹) ₃ + adenine sulphate (33.0 mg.l ⁻¹) + tyrosine (33.0 mg.l ⁻¹) + charcoal (0.5%)

The medium consists of one fifth of the salts of MURASHIGE and SKOOG (1962) medium but without the NH NO_{4 3}. Three per cent sucrose (w/v) was the only organic component. (Further investigations into the effect of culture conditions are presented in Chapter 3.). The medium was brought to a pH of 5.8, and gelled with 0.8 per cent agar. Seeds were germinated

under either SD (8 hour light) or LD (18 hour light) conditions. The seedlings were grown in Conviron growth chambers set to have day/night temperatures of 25/20 °C; relative humidity of 40 per cent and photosynthetically available radiation (PAR) of $110 \mu\text{Em}^{-2} \text{s}^{-1}$.

Explant Culture

After 50 days, nodal explants, two centimetres long with a single leaf, were taken from the above parent plants, and subcultured onto 40 ml of the medium of TANIMOTO and HARADA, (1981b) (Table 2.2.a.) in 100 ml Erlenmeyer flasks. These explants were maintained in SD (eight hour light) or LD (eighteen hour light) conditions in Conviron growth chambers set to have day/night temperatures of 25/20 °C, relative humidity of 40 per cent and PAR (photosynthetically available radiation) of $110 \mu\text{Em}^{-2} \text{s}^{-1}$.

Table 2.2.a. Low nutrient medium adapted from the medium developed by TANIMOTO and HARADA (1981b) as a modification from MURASHIGE and SKOOG (1962).

<u>Nutrient</u>	<u>Concentration (mg l⁻¹)</u>
CaCl ₂ .2H ₂ O	88.0
MgSO ₄ .7H ₂ O	74.0
KH ₂ PO ₄	34.0
KNO ₃	380.0
H ₃ BO ₃	1.24
CoCl ₂ .6H ₂ O	0.005
CuSO ₄ .5H ₂ O	0.005
MnSO ₄ .4H ₂ O	4.46
KI	0.166
Na ₂ MoO ₄ .2H ₂ O	0.05
ZnSO ₄ .4H ₂ O	1.72
NaFeEDTA	7.46
sucrose	30 g l ⁻¹
agar	9.0 g l ⁻¹
pH 5.8	

Table 2.2.b. MS medium as adapted from MURASHIGE and SKOOG, (1962).

<u>Nutrient</u>	<u>Concentration</u> (mg l^{-1})	<u>Mass/500 ml</u> <u>stock(g)</u>	<u>Vol. stock</u> <u>ml/litre</u> <u>final medium</u>	<u>Stock</u> <u>Solution</u>
NH_4NO_3	1650.0	82.5	10	I
KNO_3	1900.0	47.5	20	II
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440.0	22.0	10	III
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370.0	18.5	10	IV
NaFeEDTA	37.3	2.0	10	V
KH_2PO_4	170.0	8.5	10	VI
H_3BO_3	6.20	0.31		
$\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$	8.6	0.43	10	VII
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3	1.115		
KI	0.83	0.042		
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	0.013		
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025	0.0013	10	VIII
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025	0.0013		
glycine	2.0	0.1		
nicotinic acid	0.5	0.025	10	IX
pyridoxin HCl	0.5	0.025		
thiamine HCl	0.1	0.005		
m-inositol	0.1 g l^{-1}			
sucrose	30 g l^{-1}			
agar	9.0 g l^{-1}			
pH 5.8				

2.3.2.b. Kalanchoe:

Shoots of greenhouse grown plants were sterilized in a number of different ways with varying degrees of success. Stem and leaf material was first washed in running tap water, followed by a 30 second wash in absolute alcohol. Various sterilants were investigated, the results of which are presented in Table 2.4., and were followed by three washes in sterile distilled water. (Treatment 2. was used for further experimentation). Nodal explants were cultured onto the medium of TANIMOTO and HARADA, (1981b, Table 2.2.a.). Cultures were maintained in non-inductive LD (18 hour light) with an intensity of photosynthetically available radiation (PAR) of either 35 or 110 $\mu\text{Em s}^{-2} \text{ s}^{-1}$ at a temperature of 25 C. These plants were used as stock plants, from which nodal explants were taken and cultured onto TANIMOTO and HARADA (1981b) medium. These cultures were placed in either LD (18 hour light) or SD (8 hour light) conditions, with photosynthetically available radiation (PAR) of 110 $\mu\text{Em s}^{-2} \text{ s}^{-1}$, a relative humidity of 40% and a day/night temperature of 25/20 °C.

Callus of Kalanchoe was established in order to provide material for grafting experiments. Portions of stem internode taken from in vitro grown plants were cultured on MURASHIGE and SKOOG (1962) medium (Table 2.2.b.), supplemented with sucrose (3%) as well as the following combinations of growth regulators;

Kinetin	:	NAA	
0.5	:	0.5 mg l	⁻¹
0.5	:	1.0 mg l	⁻¹
5.0	:	0.5 mg l	⁻¹
5.0	:	5.0 mg l	⁻¹

The medium was adjusted to pH 5.8, gelled with 0.8 per cent agar and the plants cultured in low light intensity of 15 $\mu\text{Em s}^{-2} \text{ s}^{-1}$ at 25°C.

2.3.2.c. Xanthium strumarium:

In order to establish in vitro grown Xanthium plants, nodal explants were taken from pot grown plants, washed in water, rinsed for 30 seconds in absolute alcohol, and then sterilized using different procedures as outlined in Table 2.5. After sterilization the explants were washed three times in sterile distilled water. These explants were used in stimulus transfer experiments as described in section 2.3.2.e.

Attempts were also made to establish callus in culture for the purpose of stimulus transfer. Internode sections sterilized as above, were cultured onto MURASHIGE and SKOOG (1962) medium (Table 2.2.b.), with three per cent sucrose, supplemented with varying concentrations of kinetin and NAA, as outlined in Table 2.6. in section 2.4.c.. The medium was adjusted to a pH of 5.8, and gelled with 0.8 per cent agar. Cultures were maintained in low intensity light ($0.5 \mu\text{Em}^{-2} \text{s}^{-1}$) at a temperature of 25 °C. Ten replicates were used for each treatment.

2.3.2.d. Nicotiana:

In order to establish Nicotiana plants in vitro, seeds were sterilized by washing briefly in absolute alcohol, after which they were soaked in 3.5 per cent NaOCl for 15 minutes. This was followed by three washes in sterile distilled water. Sterile seeds were drawn up in a pipette and applied to media containing the nutrients of either full strength or half strength MURASHIGE and SKOOG (1962) medium (Table 2.2.b.), or nutrient free distilled H₂O, all without sucrose. The media were adjusted to pH 5.8 and gelled with 0.8 per cent agar. The cultures of N. tabacum cv

Maryland Mammoth and cv. Trapezond were maintained in LD (18 hour light) with an intensity of $35 \mu\text{Em s}^{-2} \text{ s}^{-1}$ (PAR) at 25 °C, while N. sylvestrus was maintained in SD (8 hour light), with all other conditions being the same. After extensive growth had occurred so that the plants filled the flasks, nodal explants of these plants were taken and cultured onto the medium of TANIMOTO and HARADA, (1981b, Table 2.2.a.). These explants were placed into inductive conditions in order to attempt flower induction. LDP N. sylvestrus and DNP cv. Trapezond were placed into LD (18 hour light) of $110 \mu\text{Em s}^{-2} \text{ s}^{-1}$ (PAR), while SDP Maryland Mammoth was placed into SD (8 hour light) of $110 \mu\text{Em s}^{-2} \text{ s}^{-1}$.

To obtain callus for further investigations, a technique was developed using the principles of the original investigations of CHOUARD and AGHION (1961) and AGHION-PRAT (1965), but the medium was adapted from TRAN THANH VAN (1973). Plants were grown up in pots in a green house. Trapezond and N. sylvestrus were induced to flower, while Maryland Mammoth failed to flower in summer conditions. When N. sylvestrus and Trapezond had reached the recommended green fruit stage, inflorescence stem segments were taken, while stem segments were taken from Maryland Mammoth. These were sterilized by washing first in water, followed by absolute alcohol for 30 seconds, and then 0.1 per cent HgCl_2 for one minute. The stem segments were then washed in three changes of sterile distilled water. Segments 1.5 cm long were split longitudinally and cultured onto a medium consisting of full strength MS, with 4.5 per cent sucrose, supplemented with kinetin (10^{-6} M or 0.215 mg/l) and NAA (10^{-6} M or 0.186 mg/l). The medium was adjusted to pH 5.8 and gelled with 0.8 per cent agar. The cultures were maintained in a LD condition (18 hour light). Callus derived using this technique was utilized in in vitro grafting experiments as described later in this chapter, in section 2.3.2.e..

2.3.2.e. In vitro grafting with callus

Callus and plants grown in vitro as described in the previous sections, were utilized to test the transmission of the flowering stimulus through living cells. Transmission had not been achieved through a water gap (GALSTON, 1949), agar (CHAILAKHYAN, 1982), or an oil gap (SIRONVAL, 1951), and it is generally thought that the stimulus can only be transmitted through living cells (GALSTON, 1949).

Initial experiments were done, where nodal explants of Glycine and Kalanchoe were taken and grafted independently onto large pieces of Glycine callus. Grafting was done by drilling down into the callus with a sharp pointed blade, and then simply inserting the base of the explant into this hole. This combination was then placed onto either TANIMOTO and HARADA (1981b) medium (Table 2.2.a.) or the callus supporting medium containing MURASHIGE and SKOOG (1962) with NAA and kinetin as described previously in section 2.3.2.d.

Further experimentation was done, where different combinations of explants were placed onto common pieces of callus. Explants of the same species but in different physiological states with regard to flowering were cultured, as well as different species on the same piece of callus. The explants were grafted with or without leaves as this influences their role as source or sink, the explants with leaves usually being the donor and the explant without leaves the receptor (LINCOLN and HAMNER, 1956). Glycine callus was used in some cases and Nicotiana tabacum cv. Trapezond callus in others. These grafted cultures were in turn cultured in either SD (8 hour light) or LD (18 hour light), in a light intensity of $110 \mu\text{Em}^{-2} \text{s}^{-1}$ (PAR) at a temperature of 25 °C. The variations in culture and the results are expressed in Tables 2.7. and 2.8. in section 2.4.e..

Further grafting experiments were done where internodes of vegetative N. tabacum cv. Maryland Mammoth, flowering Trapezond and flowering N. sylvestrus were cultured by pressing together end to end, on a MURASHIGE and SKOOG (1962) medium with 4.5 per cent sucrose supplemented with kinetin and NAA as before. These cultures were maintained in LD (18 hour light) at a temperature of 25°C in a light intensity of $110 \mu\text{Em}^{-2} \text{s}^{-1}$ (PAR).

2.4. RESULTS AND DISCUSSION

2.4.a. Glycine

Seeds of Glycine were easily established in vitro, although in the later stages of the work, new seed obtained proved to be contaminated with a pathogenic fungus. This imposed a severe restriction on the development of a flowering system for this plant in vitro.

Sterilization of the initial batches of the seed was 100 per cent successful. It was important though that the washes in water were brief as prolonged soaking resulted in extensive mechanical damage, due to rapid imbibition. Imbibed seeds were also fragile and difficult to handle during culture.

Table 2.3. The effect of culturing single cotyledons of Glycine max cv. Impala on three different media. All three media were supplemented with sucrose (3%), NAA (5 mg l^{-1}) and kinetin (5 mg l^{-1}). Each treatment was comprised of ten replicates. Mass was measured after forty days.

<u>Medium</u>	<u>Callus mass (g ± SE)</u>	<u>Quality of callus</u>
Murashige and Skoog (1962)	2.86 ± 0.12	Soft white callus 20% with small roots
Linsmaier and Skoog (1965)	2.80 ± 0.12	Extremely hard callus 90% with large roots
Millers (1965)	2.65 ± 0.24	Soft white callus 30% with small roots

Callus

The three media investigated, provided substantially different types of callus, although the ultimate masses produced did not differ (Table 2.3.). The medium of MURASHIGE and SKOOG (1962) was used in all further experiments, as it produced few roots, and was soft enough to use for grafting experiments.

De novo flowering of Glycine callus

CHANG and HSING (1980) obtained in vitro flowering of callus derived from embryoids of Panax ginseng without the formation of leaves or roots. Embryoids were produced on media containing NAA or GA₃ with adenine sulphate and tyrosine (Treatments 2 and 3, Table 2.1.). These embryoids were induced to flower on a medium with kinetin and GA₃ (similar to Treatment 4), the presence of gibberellins apparently being essential. Treatments 6 and 7 contained low nutrient TANIMOTO and HARADA (1981b) medium, the latter having GA₃, adenine sulphate and tyrosine. This was done to see if this low nutrient medium would be more supportive of flowering than the high nutrient media used in other treatments.

None of the media investigated produced embryoids or flowers on Glycine callus. The growth of the callus was affected to a large degree, the presence of kinetin and to a lesser extent auxin being essential for callus growth. Other media produced either poor growth, or extensive browning of the tissue.

All of these Glycine callus cultures were maintained in SD conditions so that the photoperiod would be supportive of flowering, and would thus not prevent the inductive action of any of the media. No flowering resulted

from any treatment. It could thus be concluded that on the media tested, Glycine callus was not able to produce de novo flowers, although it is possible that a lack of meristematic zones prevented the manifestation of any flowering impetus. It was thus unlikely that Glycine callus would have any inductive effect on the flowering of attached explants during grafting experiments.

Glycine nodal explants

Seed of Glycine germinated well in vitro, and grew rapidly. Due to inadequate light intensities, these seedlings etiolated and thus provided only one or two nodes for later culture. This became a limiting factor in the development of studies using Glycine, as large numbers of stock plants had to be produced.

Explants taken from the above plants produced roots from the cut surface, and under suitable SD conditions, flowers or shoots from the axillary bud. On occasions, these shoots grew to have several leaves before producing flowers. The production of flowers was dependant on SD inductive cycles, showing that induction was taking place in vitro. No flowering occurred in LD conditions. It was thus successfully established that nodal explants of Glycine could be induced to flower while growing on a low nutrient, hormone free medium. This was a significant development, as most investigations in this field utilize growth regulators to assist flowering. Such works have been extensively reviewed in Chapter 1. and are tabulated in Table 1. The technique developed here allows for the investigation of the flowering stimulus without the added complicatory factors of exogenously applied hormones. The technique employs nodal explants, but these rapidly differentiate to form new stems, leaves and roots, and thus form complete plants. The merits and restrictions of whole plant culture are discussed in the literature review (Chapter 1.).

This technique of Glycine culture was developed further, to investigate the physiology and induction of the explants in vitro. This work is described in Chapter 3. Flowering and vegetative explants were also used in in vitro grafting experiments, the results of which are presented in this chapter, section 2.4.e..

2.4.b. Kalanchoe

The sterilization of Kalanchoe explants, was only possible if young rapidly growing shoots from adult plants were used. Older tissue became associated with a variety of epiphytic fungi and bacteria, some of which were impossible to eliminate. Treatment 2 (Table 2.4.) using NaOCl provided the most satisfactory decontamination, having little detrimental effect on the viability of the explants. Mercuric chloride, although an effective sterilant, inhibited or destroyed many of the explants, possibly due to its persistence after washing (YEOMAN, 1973).

Table 2.4. The percentage sterilization of Kalanchoe explants cultured on MS medium with 3% sucrose, solidified with 0.8% agar. Shoots were washed in water followed by 30 seconds in absolute alcohol before treatment with the sterilants below. Approximately 0.01% Tween 20 was added to all sterilants.

<u>Treatment</u>	<u>Sterilant</u>	<u>Time</u> (minutes)	<u>Percentage</u> <u>sterilization</u>
1.	2.0% NaOCl	15	75
2.	2.0% "	25	100
3.	3.5% "	15	95
4.	10% H ₂ O ₂	10	20
5.	0.1% HgCl ₂	2	15
6.	0.1% " ²	5	100
7.	0.1% "	10	100

Maintaining these plants under low light intensity ($35 \mu\text{Em}^{-2}\text{s}^{-1}$) produced a different growth habit if compared to cultures grown under $110 \mu\text{Em}^{-2}\text{s}^{-1}$. Cultures under low light intensity produced multiple shoots, all arising by repeated branching at the axillary meristem of the original nodal explant. The leaves tended to be smaller and of a paler colour. Under high light intensities, a single nodal explant produced one or two shoots by axillary bud extension. The leaves were several times larger, and contained more anthocyanin. SMITH and NIGHTINGALE (1979) obtained multiple-shoot proliferation of Kalanchoe in a light intensity of $20 \mu\text{Em}^{-2}\text{s}^{-1}$, but claimed that kinetin and IAA were responsible for this proliferation, although no investigation was made of the benefits of these hormones. These additions were not necessary in this investigation, light apparently playing the dominant role.

Nodal explants with two leaves taken from either of the two types of stock plants, produced roots, and shoots extending from one or both of the axillary buds. In SD conditions, these lateral shoots each produced a terminal inflorescence, branching to subtend fully formed fertile flowers. No flowering resulted in LD conditions, which indicates that induction of the explants occurs in vitro and is dependent on daylength.

As was the case with Glycine, it is significant that flowering of Kalanchoe was induced by photoperiod while growing on a low nutrient and hormone free medium. The physiology of flower induction and evocation of Kalanchoe was studied extensively in this investigation, the work being presented in Chapter 4.

Vegetative and flowering explants of Kalanchoe were also used in grafting experiments, the results of which are related in this chapter, section

2.3.2.e. For use in these experiments, maximum callus growth of Kalanchoe stem explants was obtained on a medium supplemented with kinetin (5.0 mg l^{-1}) and NAA (5.0 mg l^{-1}), yet the size of the pieces of callus obtained was not sufficient for further use. The development of techniques to produce Kalanchoe callus was thus discontinued.

2.4.c. Xanthium

The sterilization of Xanthium (Table 2.5.) was dependent on explants being taken from young rapidly growing seedlings. Older plants rapidly became infected with a variety of pathogens. These sterile explants were utilized for grafting experiments as outlined in this chapter, section 2.3.2.e.. Internode explants cultured onto a medium containing kinetin and NAA produced varying degrees of callus and roots. None of this callus growth was sufficiently abundant to use in callus grafting experiments, and was thus discontinued.

Table 2.5. Percentage decontamination of Xanthium nodal explants using various sterilants, all with 0.01% Tween 20 added. Ten replicates per treatment.

<u>Treatment</u>	<u>Sterilant</u>	<u>Time</u> (minutes)	<u>Percentage</u> <u>decontamination</u>
1.	0.1% HgCl ₂	2	0
2.	0.1% "	4	10
3.	0.1% "	8	0
4.	1.0% benomyl soln. followed by 3% NaOCl	30 + 5	80
5.	3% "	20	70

2.4.d. Nicotiana

Seeds of Nicotiana germinated well in vitro, growing rapidly to fill the flask. Nodal explants taken from these seedlings and cultured in vitro in inductive conditions, grew rapidly to fill even 500 ml flasks. No induction was achieved in any of these plants, despite long periods of culture. These plants could therefore not provide a source of explants for further culture as was done with Glycine and Kalanchoe.

Mature pot grown Nicotiana plants provided the source of tissue for callus culture. This field has been extensively researched since the technique of in vitro flowering of Nicotiana was developed by CHOUARD and AGHION (1961); AGHION-PRAT (1965); and more recently by WARDELL and SKOOG (1969); TRAN THANH VAN (1973); HILLSON and LaMOTTE (1977); CHAILAKHYAN, AKSENOVA, KONSTANTINOVA and BAVRINA (1974, 1975); BARENDSE, CROES, VAN DEN ENDE, BOSVELD and CREEMERS (1985), together with many others. It is essential that explants be taken from the inflorescence stems of flowering plants which have reached the green-fruit stage (WARDELL and SKOOG, 1969) for optimum flowering to occur. This is important because of the floral gradient which occurs in Nicotiana, where explants taken from the upper reaches of the plant produce flowers, with decreasing response down the stem until in the lower reaches, only vegetative buds are formed (CHOUARD and AGHION, 1961; WARDELL and SKOOG, 1969). This system also depends on the use of DNP Nicotiana cultivars such as cv. Trapezond or cv. Wisconsin 38.

The medium used in this investigation utilizes NAA and kinetin, neither of which are totally essential for the de novo flowering of large stem segments (CHOUARD and AGHION, 1961), but are essential for thin cell

layers (TRAN THANH VAN, 1973) possibly as these explants have very low endogenous levels of hormone. HILLSON and LaMOTTE (1977), claimed that levels of 10^{-6} M kinetin were inhibitory of the flowering of stem segments as it stimulated the growth of vegetative buds. In this thesis, kinetin and NAA at 10^{-6} M promoted both callus growth and flowering on the callus, which was an advantage for the type of work envisaged.

Using the technique as described, Trapezond explants produced callus, which differentiated flowers, many of which grew into fruits containing seeds. The flowers without exception were lacking in pigment, all being a pale green colour. Flowers were produced in 62 per cent of the Trapezond cultures. As was found by CHAILAKHYAN, AKSENOVA, KONSTANTINOVA and BAVRINA (1974, 1975) and CHAILAKHYAN (1975), none of the LDP or SDP segments produced flowers in vitro. CHAILAKHYAN (1975) claimed that this is due to the ability of all DNP cells to produce the floral stimulus, while in the case of SDP and LDP, only the leaves produce the stimulus and transmit a continual supply to the apex. The SDP Maryland Mammoth cells would produce only gibberellins in vitro, while explants of N. sylvestrus would produce only anthesins. In this way, neither of them would flower in vitro.

2.4.e. In vitro grafting of explants onto callus

Nodal explants of Glycine and Kalanchoe grafted onto callus showed few or no side effects, and continued growing rapidly. After a period of ± 30 days, the explants had usually rooted through the callus and into the medium. It is anticipated that before rooting occurred, the explants took up substances important for reproductive growth from the callus, together with the basic nutrients and water. This technique then, should provide an ideal medium for the transmission of substances from a donor explant to a receptor through living cells.

The results of these grafting experiments are presented in Table 2.6. and Plate 2.1.A. and B. No flowering of receptor plants occurred in any case. It is possible that transmission of the stimulus from the donor, through callus, to the receptor plant was not achieved. Combinations of donor and receptor, of the same and of different species, did not bring about flowering of the receptor. No further investigation using this technique was conducted.

Attempts were also made to obtain the transfer of the stimulus from a flowering donor explants to a vegetative receptor explant, both implanted in agar in a single flask. Combinations of Kalanchoe, Xanthium and Glycine produced no flowering response in any receptor (Plate 2.1.C.), and thus this technique was discontinued.

Table 2.6. In vitro grafting of donor and receptor explants of Kalanchoe, Glycine and Xanthium onto Glycine callus. Three replicates were used for each treatment.

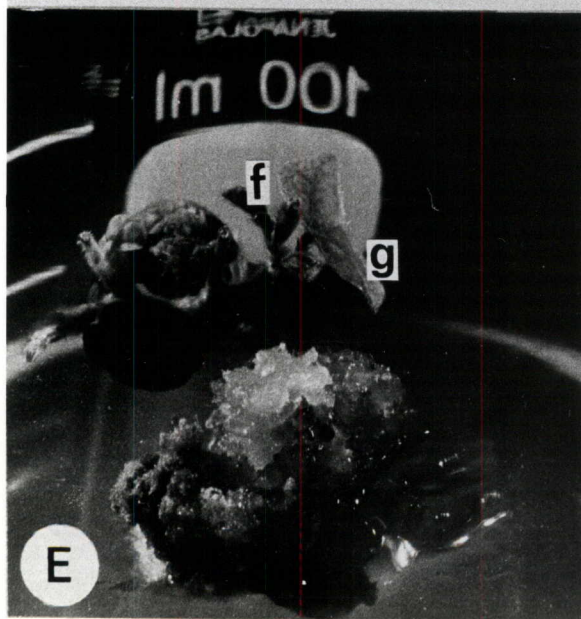
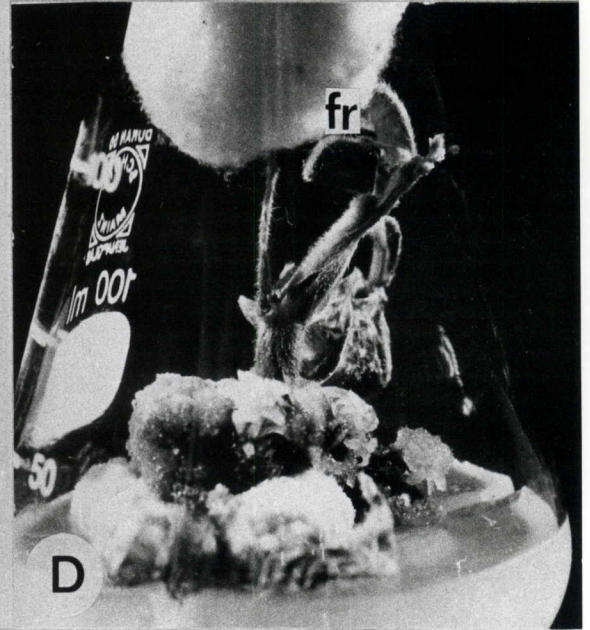
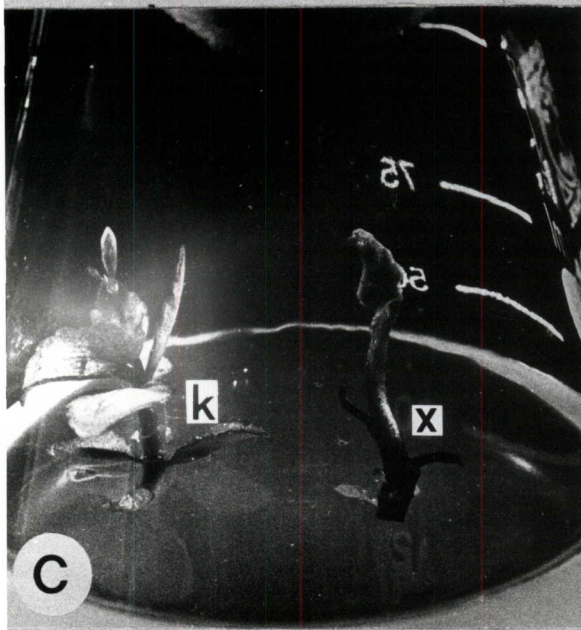
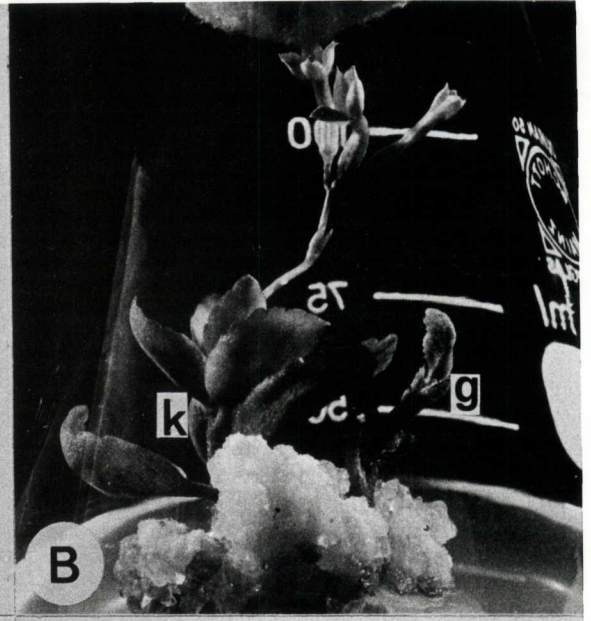
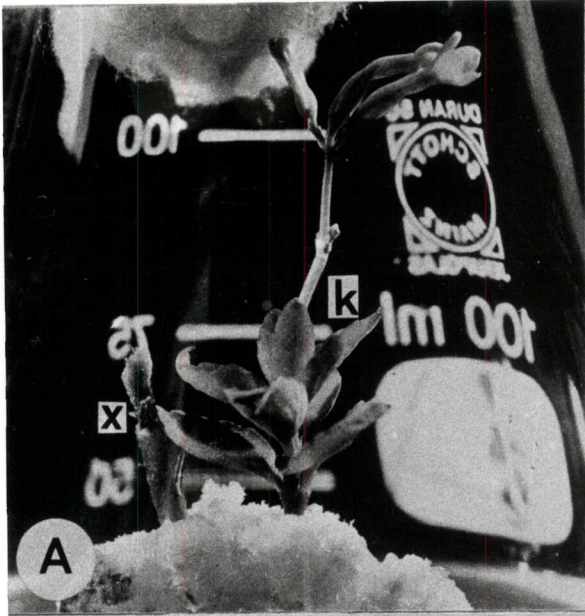
<u>No.</u>	<u>Donor</u>	<u>Receptor</u> (+leaves)	<u>Day</u> (+leaves)	<u>Response</u> <u>of Receptor</u>
1.	flowering <u>Kalanchoe</u> (+)	flowering <u>Kalanchoe</u> (-)	LD	Zero flowering Zero growth of receptor
2.	"	vegetative <u>Glycine</u> (-)	LD	nil
3.	"	" (+)	LD	nil
4.	"	vegetative <u>Xanthium</u> (-)	LD	Zero flowering poor survival of <u>Xanthium</u> explants
5.	flowering <u>Glycine</u> (+)	vegetative <u>Glycine</u> (-)	LD	nil

Flowering or vegetative stem callus of DNP Trapezond was used as a donor and a receptor to scions of Glycine or Kalanchoe grafted onto them. Half of these were placed into inductive SD and half into non-inductive LD (Table 2.7.). The aim was to examine the promotion or inhibition of flowering by the Trapezond callus on the explants, and to observe if flowering explants could bring about flowering of vegetative callus.

Table 2.7. In vitro grafting of explants with (+) or without (-) leaves onto callus of Nicotiana tabacum cv. Trapezond. This callus was in either a flowering or vegetative state. Three replicates were used for each treatment kept under either LD (18 hour day) or SD (8 hour day) conditions.

<u>No.</u>	<u>Callus</u>	<u>Explant</u>	<u>Day</u>	<u>Response</u>
6.	flowering	vegetative <u>Glycine</u> (-)	LD	10% flowering of <u>Glycine</u>
7.	flowering	"	SD	100% flowering of <u>Glycine</u> extensive secondary thickening
8.	vegetative	flowering <u>Glycine</u> (+)	LD	100% flowering of <u>Glycine</u> with fruiting
9.	vegetative	"	SD	100% flowering of <u>Glycine</u> vigorous vegetative growth and flower on callus
10.	vegetative	flowering <u>Kalanchoe</u> (+)	LD	Zero flowering of callus
11.	vegetative	"	SD	100% flowering of <u>Kalanchoe</u>
12.	flowering	vegetative <u>Kalanchoe</u> (-)	LD	fruiting of callus
13.	flowering	"	SD	Zero flowering of <u>Kalanchoe</u>

Grafting vegetative Glycine explants onto flowering Trapezond callus in LD (Treatment 6, Table 2.7.), resulted in a single flower being produced on a Glycine explant. In SD conditions (Treatment 7) flowers were produced on the Glycine explants, the stems of which became enlarged by secondary thickening (Plate 2.1.D.). The reason for this enlargement is not known. Vegetative Trapezond callus (Treatments 8 and 9) supported prolific growth and flowering of induced Glycine explants, and was itself induced to flower (Plate 2.1.F.).

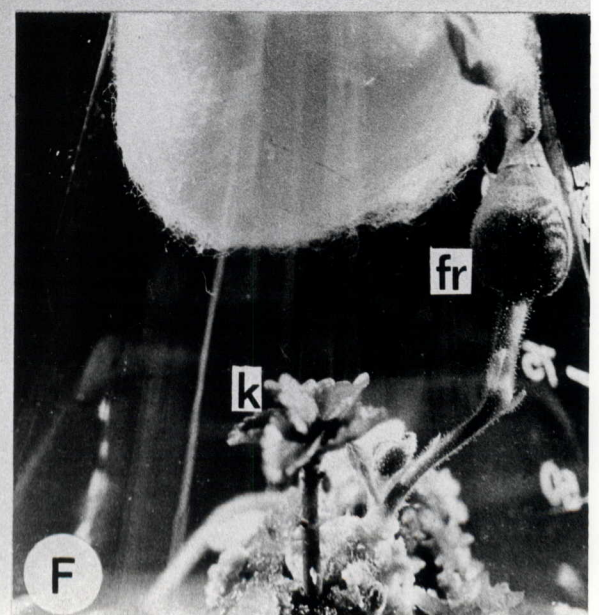
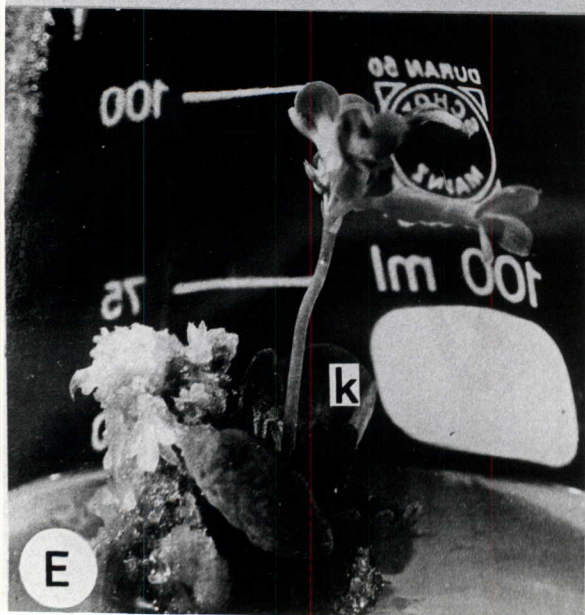
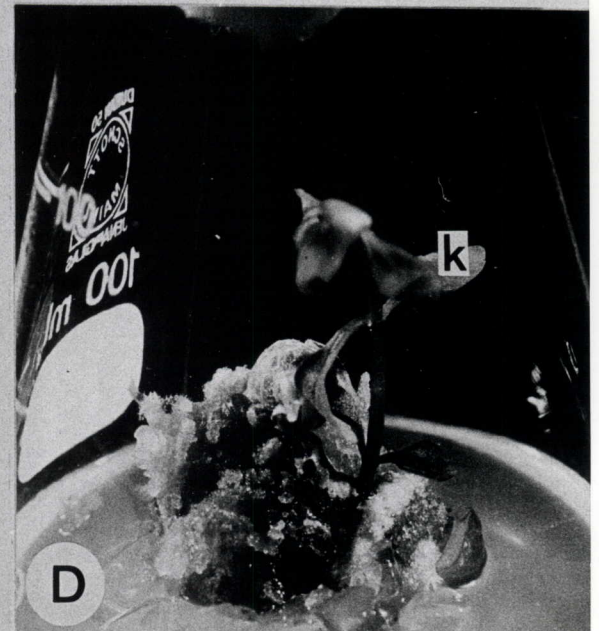
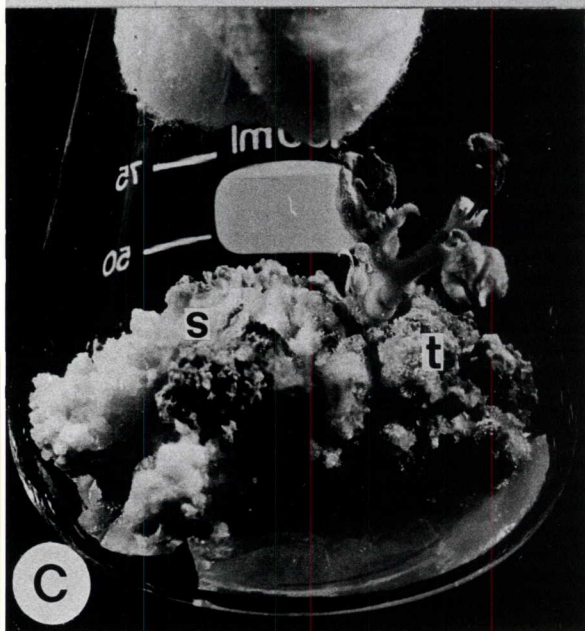
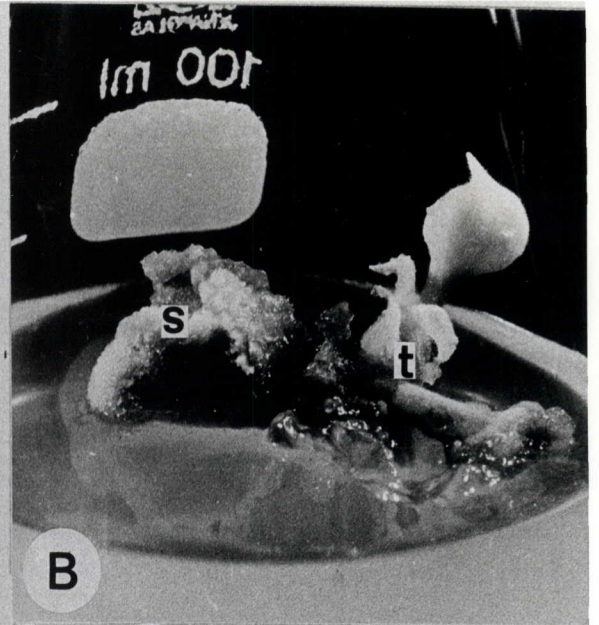
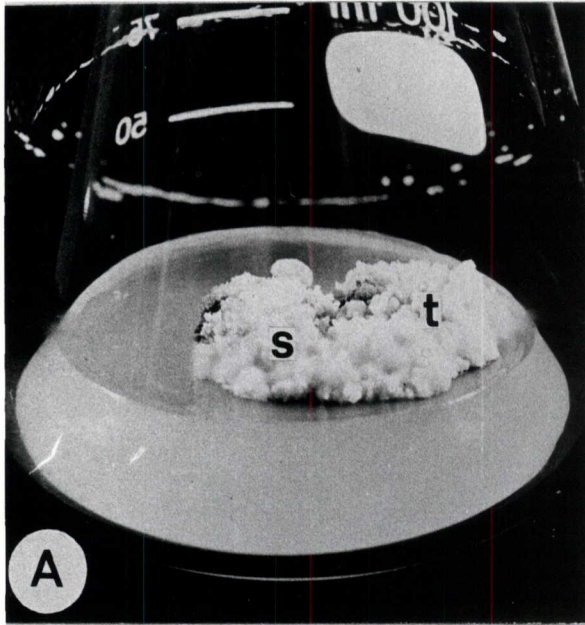


Vegetative Trapezond callus appeared to have little effect on Kalanchoe explants (Treatments 10 and 11), although flowering Trapezond callus inhibited flowering of defoliated Kalanchoe in inductive conditions (Plate 2.2.D.). Flowering callus also went on to produce fruits in association with vegetative Kalanchoe (Plate 2.2.F.)

The reasons for the above effects are not understood and need to be examined further. None of these results was of noticeable significance, which led to the discontinuation of this method as part of the investigation into flowering in this thesis. There is nevertheless a strong possibility that with further development, this technique could yield useful results.

Grafting stem explants of different Nicotiana response types together, resulted in prolific callus growth of both sections, but flowers were only produced on the Trapezond callus (Plate 2.2.A., B, and C.). Vegetative Nicotiana sylvestris has been shown to produce a substance which is graft transmissible and inhibits flowering and vegetative growth in a receptor of Trapezond (LANG, CHAILAKHYAN and FROLOVA 1977). No such inhibition occurred here on Trapezond callus, possibly as the N. sylvestris tissue was derived from reproductive plants.

This technique could prove to be most useful in the investigation of flowering, because of the controlled way in which grafting can be conducted. Grafting in vivo has already provided much knowledge to our understanding of the flowering problem, which could be supported by the unique advantages of in vitro grafting. The problems of bacterial and fungal infection at the graft union are eliminated, and very small explants could be used as scion or stock. These could include nodal



sections, buds and even thin cell layers and callus. It would be feasible to graft small areas of tissue such as the apical dome, in order to determine the exact site of action of the stimulus. Thus, although the experimental systems tentatively developed here, have much potential for further development, they were discontinued in this thesis in favour of pursuing other techniques.

2.5. CONCLUSIONS

A simple technique for the investigation of flowering in vitro has been developed using Glycine max and Kalanchoe blossfeldiana. Nodal explants of these plants, with attached leaf/leaves, can be cultured on a low nutrient hormone free medium, and can be induced to flower by photoperiodic manipulation.

As was discussed earlier in Chapter 1., the advantage of this type of culture in the investigation of flowering, is that it allows a high degree of control of all the factors externally influencing flowering, as well as of selected endogenous factors. In this way, substances can be applied to explants, without the influence of complications such as contamination of wounded surfaces and organic substances, in a way that ensures uptake of the test substance. The substance under test in this bioassay system, could be a possible "florigen", or any substance thought to play a role in a multi-component system of floral induction. The advantage of having a whole plant in this type of investigation, is that the plant itself will provide much of the support for growth if an inductive substance is applied in non-inductive conditions. The obvious disadvantage of the system, is that as a complete plant is being used, this does not allow for the isolation of the apex, or of the events taking place at the apex as it is brought to flower. Nevertheless, there is great potential in the technique, which needs to be pursued. An expansion of the technique is presented in Chapters 3. and 4. for Glycine and Kalanchoe respectively. The induction of flowering was investigated in vitro, together with the physiological responses of the plants as they were induced to flower. Numerous factors were studied in an attempt to provide clarity about the nature of the flowering stimulus.

Xanthium was not used for further in vitro investigations, although it was used extensively for other studies in Chapter 5. The three Nicotiana response types hold much promise for the investigation of flowering, but not in the way that Glycine and Kalanchoe have been used here. Their use was therefore discontinued although it must be emphasised that this was not due to a lack of potential. CHAILAKHYAN, AKSENOVA, KONSTANTINOVA and BAVRINA (1975) stated that the three Nicotiana response types are ideal for the comparison of a SDP, LDP and a DNP as all belong to the same genus, they are essentially compatible with each other with respect to grafting and other physiology.

The in vitro grafting work initiated here is an attempt to investigate the transmission of the stimulus. This system has much potential but was not developed further due to insufficient culture facilities. Little can be concluded from the results of the initial tentative experiments carried out, except that the grafts do take well, while no flowering of receptor plants occurred.

Of all the techniques and plant species investigated, the culture of Glycine and Kalanchoe nodes held the most potential for further investigation, and thus these were developed at the expense of the others. These developments are reported in the following chapters.

CHAPTER 3.

THE IN VITRO FLOWERING OF GLYCINE MAX

Plate 3.1. Nodal explants of *Glycine max* flowering and fruiting in vitro after culture on low nutrient medium (TANIMOTO and HARADA, 1981b) and exposure to SD conditions.

3.1. THE PHOTOPERIODIC CONTROL OF IN VITRO FLOWERING OF GLYCINE MAX EXPLANTS

3.1.1. INTRODUCTION

In Chapter 2., a technique was described where Glycine nodal explants will flower in response to daylength, on a low nutrient hormone free medium (section 2.3.2.a). This technique has much potential for the investigation of "florigen" or of the factors controlling flowering and forming part of a multi-component stimulus. The primary advantage of the system, is that induction takes place in the explant during culture. Much of the reported in vitro work done on flowering, makes use of explants derived from induced or flowering plants (Table 1.). This means that the inductive processes have been completed and the stimulus need not necessarily remain in the tissue. These works thus examine only the manifestation of the flower stimulus. In many of these experiments, the development of a complex medium simulating all of the requirements needed to bring about the growth of a flower bud, is suggested to constitute a copy of the flowering stimulus. It is more likely that only differentiation and possibly evocation are being studied here. In this thesis, attention is given to the induction process as well as to evocation and differentiation.

Another advantage of the system presented here, is that the medium does not induce or bring about flowering on its own, as there is an absolute requirement for SD inductive cycles. This means that the true processes of induction can be investigated, without the complication of the medium simulating the floral stimulus.

The ability of nodal explants of Glycine to perceive photoinductive cycles in vitro, and bring about flowering was investigated. The physiological state of the parent plant with regard to flowering was examined, taking into consideration the nutrient status of the medium and the resulting vigour of the plant. The ability of the explant to retain the stimulus during culture was also considered. Also examined, was the role that various organs attached to the plant play in induction and evocation.

3.1.2. MATERIALS AND METHODS

Photoperiodic induction

The basic technique used for the culture and flowering of Glycine max was as described in section 2.3.2.a., with some modifications. Seeds were germinated in vitro on both TANIMOTO and HARADA, (1981b) medium (Table 2.2.a.) as well as on a nutrient free, H₂O medium gelled with 0.8 per cent agar. The pH of both media were adjusted to 5.8 before autoclaving. These seedlings were grown under either SD (eight hour light) or LD (18 hour light) conditions as described before. Nodal explants were taken and cultured onto TANIMOTO and HARADA, (1981b) medium. Half were separated into LD and half into SD conditions. Ten explants were used per treatment and the experiment was repeated twice.

In order to test the number of days required for induction, explants were taken from LD parent plants, and cultured on TANIMOTO and HARADA, (1981b) medium (Table 2.2.a.). Batches of ten replicates were exposed to increasing numbers of SD (8 hour light) before being returned to LD (18 hour light) conditions as described above.

Manipulation of explants

One of the advantages of in vitro culture, is that various organs of the plant can be removed, or isolated to test the effect on flowering. Explants were taken from vegetative plants growing in vitro in LD. Various permutations of organ removal were carried out and the resulting explants cultured onto TANIMOTO and HARADA, (1981b) medium (Table 2.2.a.). These explants were maintained in SD (8 hour light) conditions, with the exception of treatments 9. and 10. which were in LD (18 hour light).

3.1.3. RESULTS AND DISCUSSION

Explants of Glycine flowered and set fruit under certain conditions (Plate 3.1.). From the results presented in Table 3.1., it can be seen that the nutritional status of the parent plants greatly affected the flowering and fruiting ability of the explants. Explants derived from H O₂ grown plants (Table 3.1., treatments 2. and 6.), flowered and set fruit but not as profusely as explants derived from plants grown on TANIMOTO and HARADA, (1981b) medium (Treatments 4. and 8.).

Table 3.1. Flowering and fruiting response of soybean nodal explants derived from plants grown on two different media under LD or SD. and cultured onto the medium of TANIMOTO and HARADA, (1981b, Table 2.2.a.)

No.	Germination medium	Parent plant daylength	Explant daylength	% Flowering (6 weeks)	% Flowering (10 weeks)	% Fruiting (10 weeks)
1.	H O ₂	LD	LD	0	0	0
2.	H O ₂	LD	SD	10	20	20
3.	*TH	LD	LD	0	0	0
4.	*TH	LD	SD	50	100	30
5.	H O ₂	SD	LD	20	20	0
6.	H O ₂	SD	SD	60	100	20
7.	*TH	SD	LD	40	50	0
8.	*TH	SD	SD	100	100	100

* TANIMOTO and HARADA (1981b)

The daylength to which the parent plants were exposed had an effect on the development of flowers on the explants. Explants derived from parent plants grown under SD conditions were pre-induced (Treatments 5.-8.). These explants all had the capacity to flower. Explants derived from non-induced parent plants grown under LD conditions (Treatments 1.-4.), only flowered if subjected to SD conditions after sub-culturing. This indicates that the stimulus responsible for flowering was produced within the explant in response to SD cycles. Obviously then, soybean is a SDP in vitro as is the case under natural conditions (HAMNER, 1969; THOMAS and RAPER, 1984; SUMMERFIELD and ROBERTS, 1985). It is also apparent that the explants flowered only in response to SD, and not due to the nutrient composition of the growth medium.

Explants derived from SD grown plants, and transferred to non-inductive LD cycles, exhibited a marginal flowering response, which again depended on the nutritional status of the parent plants (Treatments 5. and 7.). This indicates that some inductive stimulus is produced in parent plants under SD conditions which supports flowering after the explants are transferred to LD conditions. Many of these flowers failed to develop fully, and subsequently turned brown. None produced fruits. This indicates that continued SD cycles are essential for the maintenance of normal flower and fruit development. Similar results have been reported for whole soybean plants (HAMNER, 1969).

Explants derived from SD parent plants, and cultured under SD conditions flowered most profusely. The medium on which the parent plants were grown, had a profound effect on the rate of flower development and the ability of the explants to form fruits (Treatments 6. and 8.). All these explants were pre-induced and thus contained the floral stimulus on

subculturing. The explants were again exposed to SD conditions and thus continued to produce the stimulus. This resulted in optimal flower and fruit formation.

Increasing the number of SD inductive cycles increased the percentage of plants flowering in vitro (Figure 3.1.). Only a single explant flowered after six SD cycles, but no conclusions can be drawn about the absolute minimum daylength requirement for the induction of flowering in vitro. In Biloxi soybean grown in vivo, two SD can induce flowering (HAMNER,1969), but it was to be expected that in vitro cultures would require prolonged exposure to SD as explants are likely to undergo some shock during excission. Explants were also lacking in certain organs.

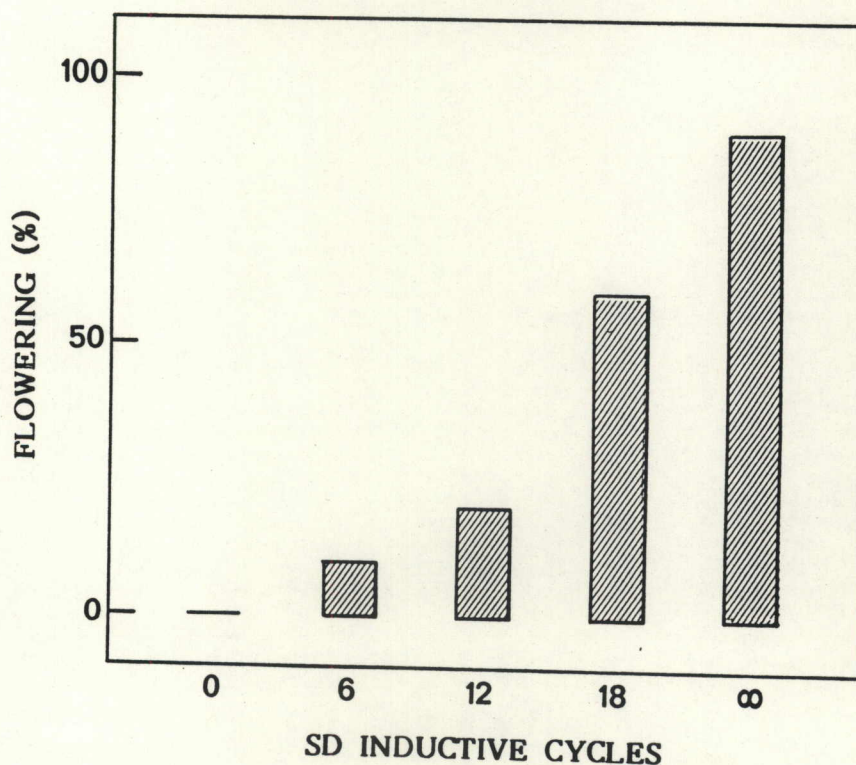


Figure 3.1. The effect of increasing numbers of SD inductive cycles on the flowering response of Glycine nodal explants cultured in vitro for ten weeks. Ten replicates were used for each treatment.

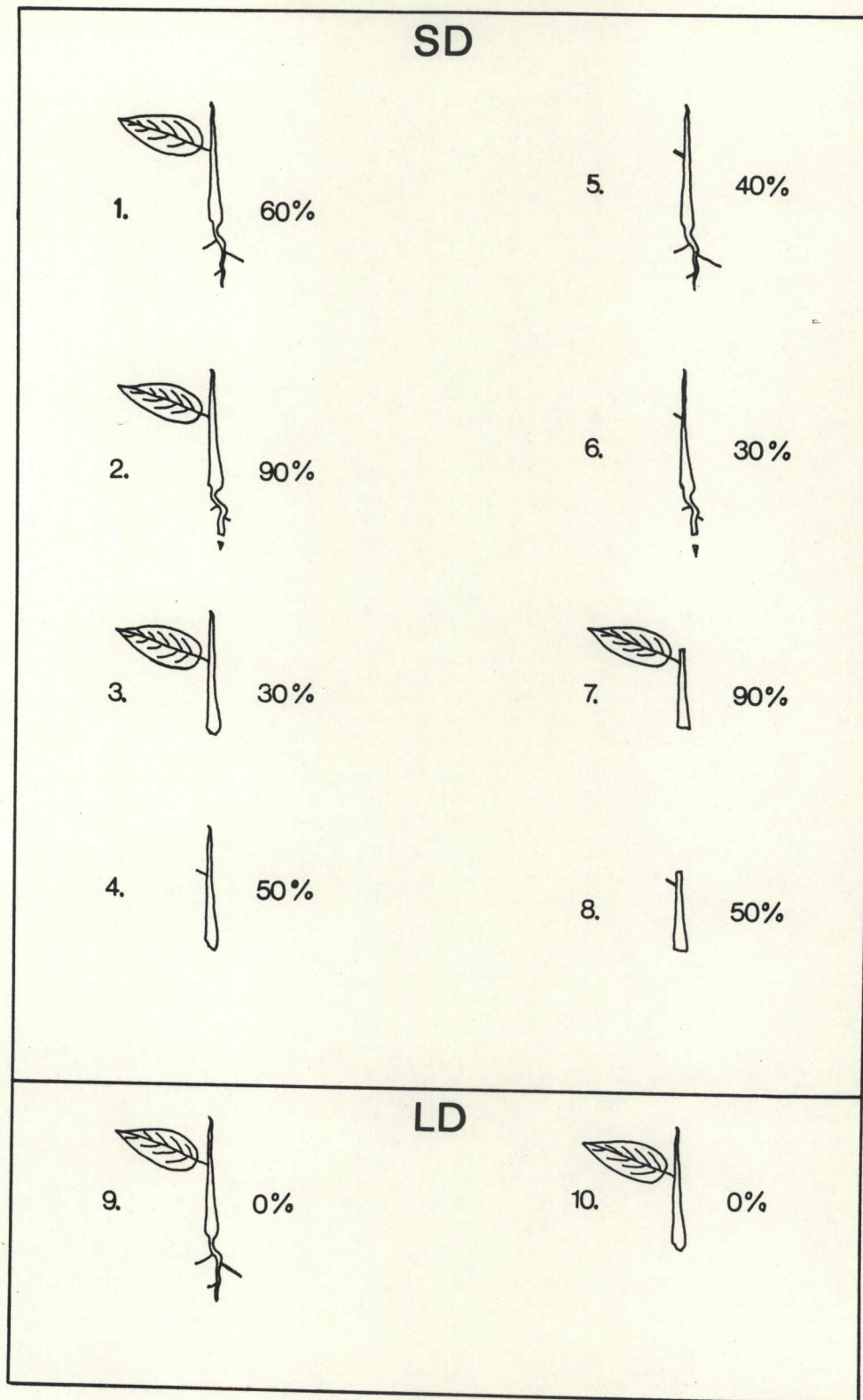


Figure 3.2. The effect of organ removal on the flowering response of *Glycine* plants after ten weeks of *in vitro* culture in SD or LD conditions.

These results indicate that increasing SD exposure resulted in an increasing flowering response of in vitro grown Glycine explants. This occurred in much the same way as in in vivo grown plants (HAMNER, 1969).

Glycine explants with different organs removed, showed different capabilities for flowering (Figure 3.2.). Whole plants (Treatment 1.) did not flower as well as the nodal explants which were used in other experiments (Treatment 7.). This trend had been noticed throughout the earlier experiments reported in Chapter 2, that seedlings generally became etiolated and flowered poorly. Nodal explants which were taken flowered rapidly.

The removal of the root apices (Treatment 2.) resulted in some increase in the flowering response. This supports the principle that roots are antagonistic to flowering (GASPAR, 1980). Removal of the entire root system caused a marked decrease in flowering associated with poor vigour and some senescence of leaves (Treatment 3.) When the leaves were removed as well, plants became more vigorous (Treatment 4.). Removal of the leaves from whole seedlings (Treatments 5. and 6.) resulted in poor flowering possibly due to the loss of these supporting organs. Approximately 30 per cent of these explants senesced. Nodal explants with a leaf attached flowered well (Treatment 7.). If the leaf was removed (Treatment 8.), 50 per cent of the explants produced flowers only, without producing any leaves. This treatment has some potential for development as a bioassay system, as a single axillary bud is cultured, and if directed towards flowering, will tend to flower without the production of any leaves. This has some advantage over apical meristem culture, where some difficulty is experienced in isolating the apical dome without the associated leaf promordia (JACOBS and SUTHERS,

1971; SCORZA, 1982). The results from this treatment also indicated that a vegetative node without leaves, can perceive photoperiodic stimuli. Induction must therefore be capable of occurring in the epidermis of the stem or in the bud itself.

3.1.4. CONCLUSIONS

A technique has been developed, where photoperiodic induction of nodal explants of Glycine max occurs, while growing in vitro on a low nutrient hormone free medium. The flowers that are produced are normal, small and cleistogamous, producing one fertile seed per pod. Most workers have found that the flowers formed in vitro are either undersized or malformed (RAGHAVEN, 1961; GANAPATHY, 1969; MEHRA and MEHRA, 1972; SCORZA, 1982), although some cases have been noted where fertile seeds are produced (CHAILAKHYAN, AKSENOVA, KONSTANTINOVA and BAVRINA, 1974, 1975)

The advantages of this type of culture as a system for the investigation of flowering, are that nodes ultimately form complete plants in vitro. The advantages and disadvantages of this are discussed extensively in Chapter 1. The main advantage, is that the explant is sufficiently developed to produce the substances necessary for flowering, which may allow the inductive properties of a substance under test to be appreciated. An added advantage of the Glycine culture system, is that no hormones are used in the medium. Nearly all of the systems developed and reported in the literature (Table 1.) have included some hormone component in the medium in order to optimise growth. This is not desirable if the floral stimulus is to be studied, as the added growth regulators may substitute for, or even influence the action of the natural flowering stimulus, particularly if the stimulus is a hormonal one. It is likely that the leaves and roots which are produced by the explants, will contribute endogenous hormones, but it is unlikely that these will be contradictory or antagonistic of the flowering stimulus, unless indeed, that is their role!

The disadvantages of the system lie in the practical sphere. Large numbers of stock or parent plants have to be produced, each providing few nodes for culture. Relatively high light intensities are needed for culture in order to avoid excessive etiolation. Light intensities of $35 \mu\text{Em s}^{-2} \text{ s}^{-1}$ (PAR) were inadequate for the production of stock plants. Intensities of three times this value were barely adequate.

The technique presented here is simple and has some scope for development. Not only can the photoperiodic induction of plants be studied, but also the contributions made by different organs on that plant. In this way, numerous parameters can be investigated, some of which may provide evidence to support a multi-component sequence of events bringing about flowering. The system can also be used to test the florigenic activity of any substance.

3.2. THE INCORPORATION OF LABELLED ¹⁴C-ADENINE AND ¹⁴C-ISOPENTENYLPYROPHOSPHATE INTO GLYCINE EXPLANTS FLOWERING IN VITRO.

3.2.1. INTRODUCTION

The work presented in the following section, is the result of an investigation where the technique of inducing Glycine to flower in vitro, was applied to an investigation of the role of precursors in the biosynthetic pathway of cytokinins as influenced by the flowering process.

Possible precursors of cytokinins

Adenine: Radioactive ¹⁴C-adenine was incorporated into the culture medium for several reasons. Adenine is the most likely precursor of the cytokinin biosynthetic pathway (PETERSON and MILLER, 1976; CHEN and PETSCHOW, 1978; STUCHBURY, PALNI, HORGAN and WAREING, 1979; NISHINARI and SYONO, 1980; CHEN, ERTL, LEISNER and CHANG, 1985), and as cytokinins have been implicated as forming part of the floral stimulus (BERNIER, KINET and SACHS, 1981b), it was anticipated that labelled adenine would be incorporated into the cytokinins forming part of the stimulus. The incorporation of adenine into cytokinins is a phenomenon that does not readily occur (VAN STADEN and CHOVEAUX, 1981; VAN STADEN and FORSYTH, 1984; 1985), so it was hoped that the production of the flowering stimulus would provide the incentive for adenine incorporation. The apparent need for cytokinins in the development of the fruit (DAVEY and VAN STADEN, 1978; VAN STADEN, 1983) is also a factor which could prompt their synthesis. Adenine was also suitable for this investigation, as

several workers have shown a role for adenine itself in the process of flowering. RINGE and NITSCH (1968) found that adenine was essential for bud formation on Begonia leaf fragments and seems to form a synergistic relationship with cytokinins (SKOOG and MILLER, 1957; NITSCH and NITSCH, 1967; NITSCH, ROSSINI and BUI DANG HA, 1967). Part of the role of adenine in flowering may be in the synthesis of nucleic acids or nucleotides, and other substances in the adenine salvage pathway (BARNES, 1961; DOREE, 1973; ASHIHARA and NOBUSAWA, 1981).

Isopentenylpyrophosphate: The isopentenyl side chain of the cytokinin molecule has been postulated to arise from isopentenylpyrophosphate (IPP) which in turn may be derived from mevalonic acid. This does appear to be the case in tRNA cytokinins (HALL, 1973; LETHAM, 1978; LETHAM and WETTENHALL, 1977), but little evidence exists to support this event in the synthesis of free cytokinins. A few cases have been reported where mevalonic acid has been incorporated into free cytokinins (BARNES, TIEN and GRAY, 1980; BURROWS and FUELL, 1981; HALBACH and KLAMBT, 1981), but in all cases the degree of incorporation was very low and requires further substantiation.

3.2.2. MATERIALS AND METHODS

¹⁴[U- C]Adenine incorporation

Soybean parent plants were cultured in vitro on the medium of TANIMOTO and HARADA, (1981b, Table 2.2.a.) as described previously in section 2.3.2.a. Nodal explants were taken and cultured in SD (8 hour light) on TANIMOTO and HARADA, (1981b) medium with [¹⁴U- C]adenine (Amersham) with a specific activity of 11.47 GBq mmol⁻¹ added at a concentration of 1.92×10^{-7} M, resulting in an activity of 8.8×10^4 Bq per flask of 40 ml.

Plants were harvested after three months by separating flowering from fruiting plants, and then by dividing into roots, shoots, flowers, pod wall and seeds. Portions were weighed individually and then frozen in liquid nitrogen. Plant tissue was ground in 80 percent ethanol and extracted for 24 hours in the cold. The extract was filtered, taken to dryness and then taken up in two millilitres of 80 per cent ethanol. The agar was frozen, thawed, taken to dryness and then taken up in 80 per cent ethanol. A precipitate was removed by filtration. The pH was adjusted to 2.5 before passing through ten grams of Dowex 50. The Dowex was eluted using 100 ml of 5 N NH₄ OH, which was taken to dryness and then eluted in two millilitres of 80 per cent ethanol.

The extracts were separated by running on Whatmans No. 1 paper with propan-1-ol : ammonium hydroxide : water (10 : 1 : 1 v/v) as the solvent. Peaks of radioactivity were located by eluting thin strips of the paper with methanol and counting in a Beckman (LS 3800) liquid scintillation counter.

The located peaks of radioactivity (Figure 3.3.) were further examined by eluting from the paper with washes of 80 per cent and then 50 per cent ethanol. This solvent was reduced to dryness and then eluted in one millilitre of 100 per cent methanol. A sample of this (100 μ l) was applied to silica gel plates (Merck Silica Gel 60 F₂₅₄) and separated two dimensionally using two solvent systems, butan-1-ol : acetic acid : water (12 : 3 : 5 v/v) and then butan-1-ol : ammonium hydroxide : water (6 : 1 : 2 v/v). Small aliquots of authentic adenine, adenosine, zeatin, ribosylzeatin, isopentenyl adenine and isopentenyl adenosine, were added to each extract to facilitate location by UV fluorescence at a wavelength of 254 nm as shown in Figure 3.4.A.. After separation, these markers were removed from the TLC plate, eluted in one millilitre of methanol and measured for radioactivity using a liquid scintillation counter. The remainder of the plate was divided up and the distribution of radioactivity also determined (Figures 3.5. - 3.7.).

Many of the extracts were enzymatically treated in order to test for the presence of conjugated forms of the adenine derivatives. Alkaline phosphatase treatment was carried out by taking 100 μ l of extract to dryness, before adding 0.5 units of enzyme dissolved in 200 μ l buffer at pH 9.5 (Tris, 0.1 M; MgCl₂, 0.01 M). This was incubated at 35 C for six hours. The enzyme was precipitated using 2 ml 100 per cent ethanol, passed through a millipore filter (0.22 μ m), taken to dryness and re-dissolved in 100 μ l of 100 per cent methanol. This extract was again separated two dimensionally on TLC plates as described above.

B-glucosidase treatment was carried out in the same way as above, but using a buffer at pH 5.39 (0.02 M Tris).

Large peaks of radioactivity which were present on the TLC plates but were not identified, were subjected to further investigation by HPLC.

[8-¹⁴C]Adenine and [1-¹⁴C]IPP incorporation

Glycine plants were cultured *in vitro* on TANIMOTO and HARADA, (1981b) medium as described in section 2.3.2.a. Nodal explants from plants cultured under long days were taken and cultured as before using 40 ml medium per flask. The precursors added to the medium prior to autoclaving were [8-¹⁴C]adenine (Amersham) and [1-¹⁴C]IPP ammonium salt in 0.005 M phosphate buffer (Amersham). Adenine with a specific activity of 1.96 GBq mmol⁻¹ was added at a concentration of 7.86×10^{-7} M resulting in activity of 6×10^4 Bq per flask. IPP with a specific activity of 2.07 GBq mmol⁻¹ was added at a concentration of 4.78×10^{-7} M resulting in activity of 4×10^4 Bq per flask. The explants were maintained under short daylengths as described previously.

After three months, the cultured plants were divided into roots, stem, flowers and fruits. These were frozen rapidly in liquid nitrogen and then extracted in cold 80 per cent ethanol for 48 hours. The extract was filtered through a Millipore filter (0.22 µm), taken to dryness and then re-dissolved in 500 µl H₂O. These extracts were separated directly by HPLC, using the technique of LEE, MOK, GRIFFIN and SHAW (1985). A Varian 5000 instrument was used and fitted with a Hypersil 5 ODS column (5 µm, C18 bonded, 250 X 4 mm i.d.). Samples were eluted with a linear gradient of methanol (5-50%) over 90 minutes in TEA buffer at a flow rate of one ml per minute. The aqueous buffer consisted of 0.2 M acetic acid adjusted to pH 3.5 with triethylamine (TEA). Fractions of one ml were collected and 0.1 ml of each was added to three ml Ready-Solve EP scintillation fluid (Beckman) and used to determine the radioactivity. A Beckman LS 3800 scintillation counter was used.

Peaks of radioactivity which had a similar retention time to authentic cytokinin standards, were further investigated by treating in one of three ways. Substances suspected of having riboside- or glucoside-substituted side-chains were acid hydrolysed by adding two ml 1N HCl to a dry sample. This was heated in a boiling water bath for one hour before being dried and taken up in 100 μ l H₂O for HPLC analysis. Radioactive peaks suspected of being cytokinins with a double bond in the side chain were oxidised using KMnO₄. The fraction was dried and re-dissolved in one ml H₂O. A few drops of dilute KMnO₄ solution were added until the pink colour persisted (MILLER, 1965).⁴ An excess of ethanol was added and the resulting precipitate removed. The filtrate was dried and the residue re-dissolved in 100 μ l of H₂O for analysis by HPLC. A final examination of the ¹⁴C-IPP extract² was made by HPLC analysis, but using a TEA buffer adjusted to pH 4.8 in place of the previously used buffer at pH 3.5. This resulted in a change of the retention times of the authentic standards.

3.2.3. RESULTS AND DISCUSSION

¹⁴[U-¹⁴C]Adenine Incorporation

The distribution of adenine-derived radioactivity in the explants is represented in Table 3.2.. The greatest concentration of radioactivity was found in the flowers, and the fruits if measured on a fresh weight basis. It is significant that there was only a two-fold increase in radioactivity found in the total fruit as compared to the flowers. This despite a much larger increase in mass.

Table 3.2. Distribution of ¹⁴C-adenine-derived radioactivity within flowering and fruiting soybean explants. Results are expressed as a percentage of total radioactivity, the mean dpm plant⁻¹ and the concentration of activity as dpm g⁻¹ fresh weight.

	<u>Flowering plants</u>			<u>Fruiting plants</u>			
	Roots	Shoots	Flowers	Roots	Shoots	Pod wall	Seed
% of total	11.5	84.2	4.3	35.2	38.8	13.5	12.5
dpm plant ⁻¹ x 10 ³	53.5	389.5	20.4	62.1	68.5	23.7	22.1
dpm g ⁻¹ fr wt. x10 ³	50.7	772.4	1251.9	42.9	152.9	313.9	406.7

These results indicate that the flowers are a powerful sink for adenine derivatives. It appears that this occurs mainly during flower development, although it is possible that as the amount of ¹⁴C-adenine in the medium diminished with time, so the fruits were not able to continue

importing from the medium. The level of ^{14}C -adenine in the medium was nevertheless reasonably high at the end of culture, so had not been completely depleted (Figure 3.3.).

There was also a greater concentration of adenine derived activity in the stem and leaves of flowering plants compared to fruiting plants. It is possible that the plants had exported much of this activity during maturation, as fruiting plants were commencing with senescence. In both cases, the roots contained the least activity.

The initial paper separation of extracts of these organs, resulted in three peaks of adenine derived radioactivity (Figure 3.3.); a large stationary peak (Peak 1.); a peak at R_f 0.3-0.4 (Peak 2.), which co-chromatographed with an authentic ^{14}C -adenine standard; and a small peak running to between R_f 0.7-1.0 (Peak 3.). The histograms are plotted as DPM per gram fresh weight, and it is apparent that they reflect the same trends of abundance as was reported in Table 3.2.. The agar medium was found to contain fairly high levels of activity associated with the authentic ^{14}C -adenine standard.

These peaks of radioactivity reported above (Peaks 1., 2. and 3.), were further separated using two dimensional TLC (Figures 3.5. to 3.7.). Only two peaks with significant amounts of radioactivity were found associated with the authentic standards. Extracts from the stems of flowering plants after treatment with B-glucosidase, produced activity associated with the ribosyl-zeatin standard (Figure 3.5.a.). Extracts from the stems of fruiting plants after treatment with alkaline phosphatase also produced activity associated with ribosyl-zeatin (Figure 3.5.b.). Confirmation of the identity of these peaks could not be obtained using HPLC. Besides these two cases, no significant peaks of radioactivity

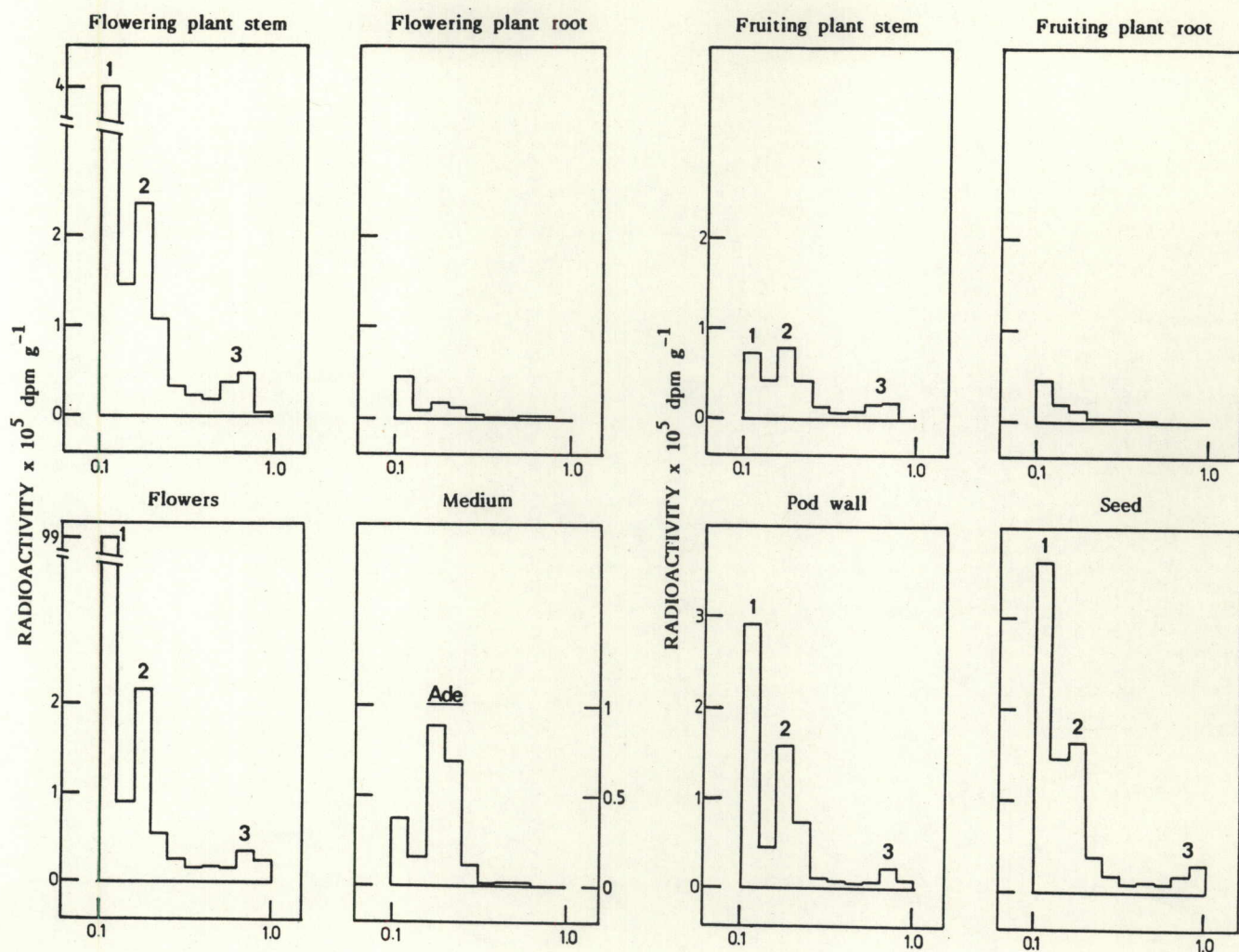


Figure 3.3. Radioactivity determined from separated extracts of different organs of *in vitro* grown *Glycine max* plants, and growth medium containing ¹⁴C-adenine derivatives. Separation was performed on Whatmans No. 1. chromatography paper using the solvent system propan-1-ol : ammonium hydroxide : water (10 : 1 : 1 v/v).

were found associated with any of the six standards used, excepting in the case of the agar medium, where high activity was associated with authentic adenine. This again indicated that ¹⁴C-adenine was present in the medium throughout the entire period of culture. Treatment of the extracts with enzymes did not always have any effect.

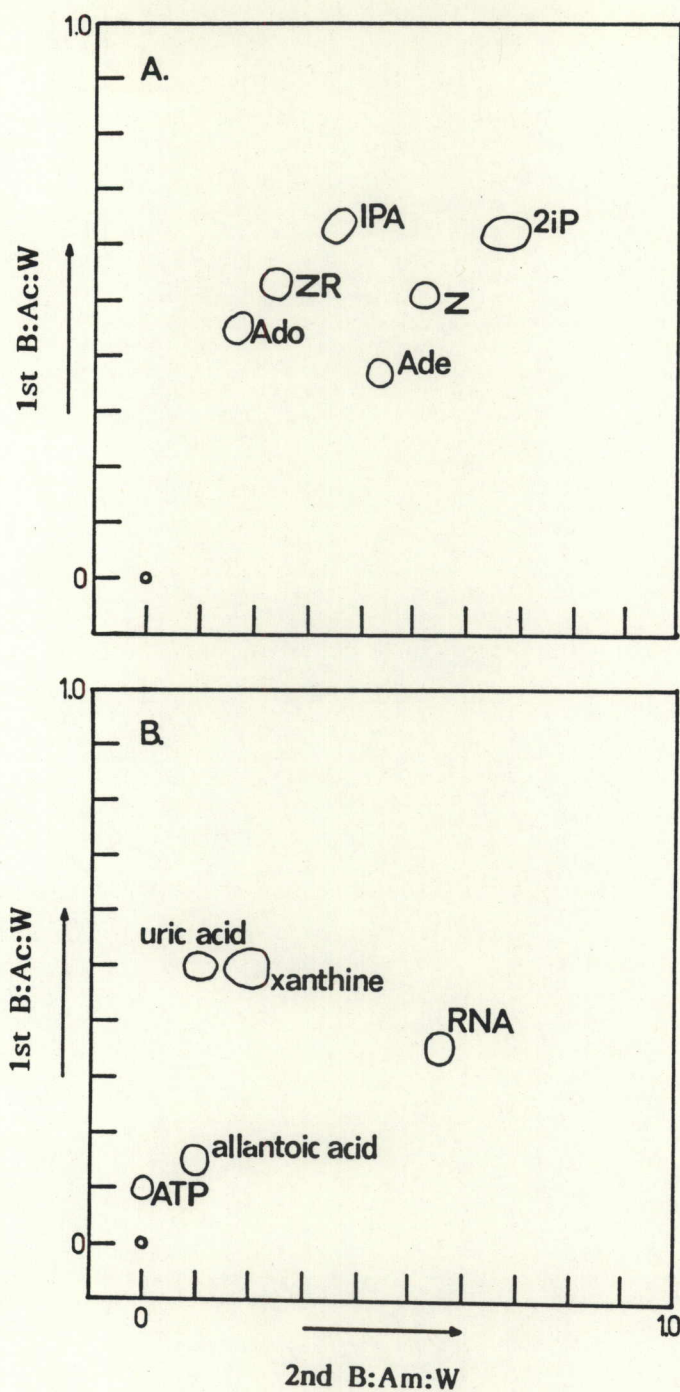


Figure 3.4. Two dimensional separation on Silica Gel F TLC plates of cytokinin standards (A.) and adenine derivatives (B.) using butan-1-ol : acetic acid : water (12 : 3 : 5 v/v) as the first solvent and butan-1-ol : ammonium hydroxide : water (6 : 1 : 2 v/v) as the second. Compounds were located under UV light at 254 nm. Cytokinin standards used were adenine (Ade); adenosine (Ado); zeatin (Z); ribosyl- zeatin (ZR); isopentenyladenine (2iP) and isopentenyladenosine (IPA).

There were nevertheless, significantly large peaks of radioactivity present at various locations on the TLC chromatograms. Certain trends were evident, depending on the origin of the extract;

Extracts derived from Peak 1. of any of the extracts as represented in Figure 3.3., again resulted in slow moving peaks after two dimensional separation on TLC. Separation of these substances was obviously poor. Their identity is not known although they are strongly polar, possibly nucleotides. Other adenine derivatives such as ATP and allantoic acid (ASHIHARA and NOBUSAWA, 1981) did remain near to the origin using these solvent systems (Figure 3.4.B.). Several of these extracts also contained substances which co-chromatographed with uric acid and xanthine standards. These results suggest that ¹⁴C-adenine was metabolised by the adenine salvage pathway (ASHIHARA and NOBUSAWA, 1981).

Peaks labelled as Peak 2. in Figure 3.3., when eluted and separated further by two dimensional TLC, yielded variable results (Figures 3.6.a. and b.). None co-chromatographed with the authentic adenine standard as was the case when separated on paper. Peaks were found, both at the origin, and at the solvent front of both solvent systems. No change was induced by enzyme treatment (C.). The identity of these substances is not known, although phenolics such as salicylic acid are known to run to the front in these solvents (HARBORNE, 1973). Salicylic acid has been implicated as being involved in flowering (CLELAND and AJAMI, 1974). The possible involvement of this substance is discussed extensively in the literature review (Chapter 1.).

Two fast moving peaks from extracts of the flowers and pod walls and referred to as Peak 3. in figure 3.3., when separated further on TLC, showed contradictory results (Figure 3.7.). Only the extract from the pod walls contained substances which moved to the solvent front.

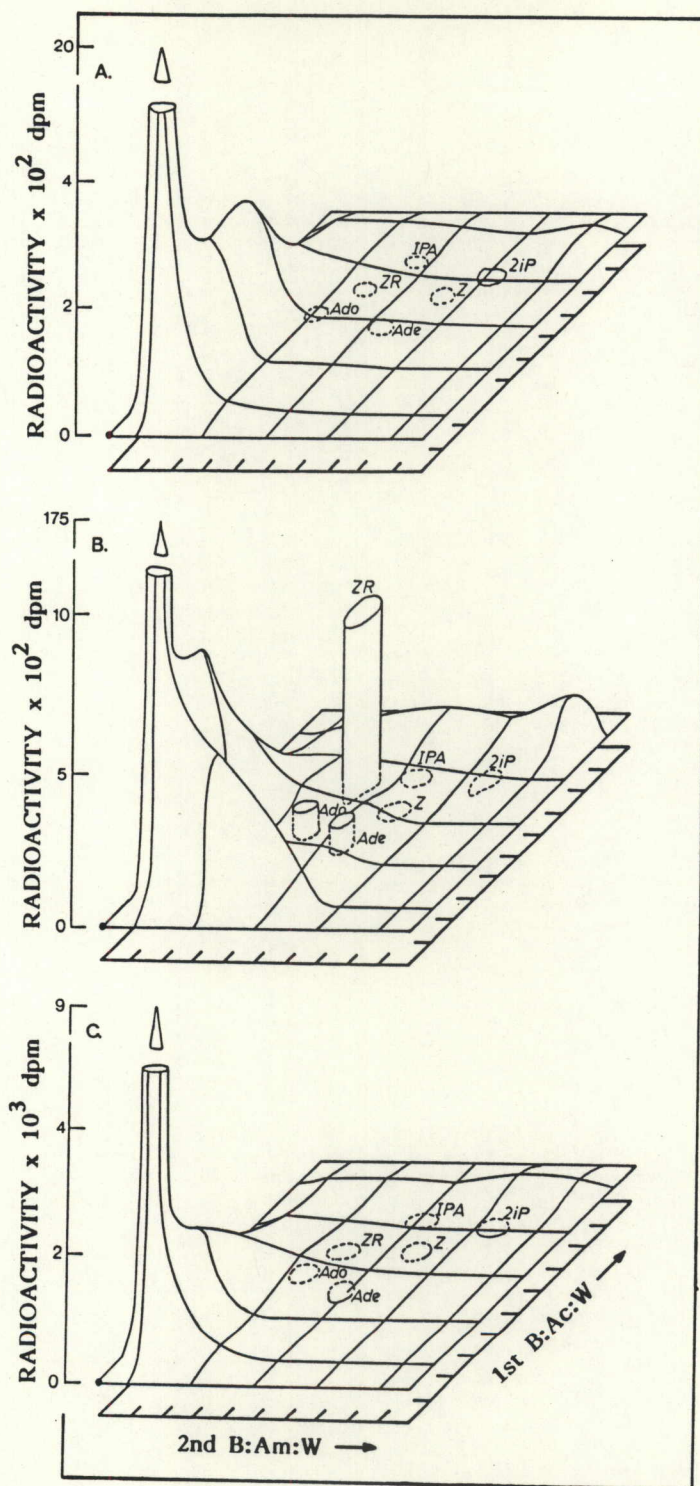


Figure 3.5.a. Radioactivity determined after separation on Silica Gel TLC plates of the slow moving **Peak 1**. (Figure 3.3.) extracted from the **stems of flowering *Glycine* plants grown *in vitro***. Solvent systems were as shown in Figure 3.4. A., untreated extract; B., after treatment with B-glucosidase; C., after treatment with alkaline phosphatase.

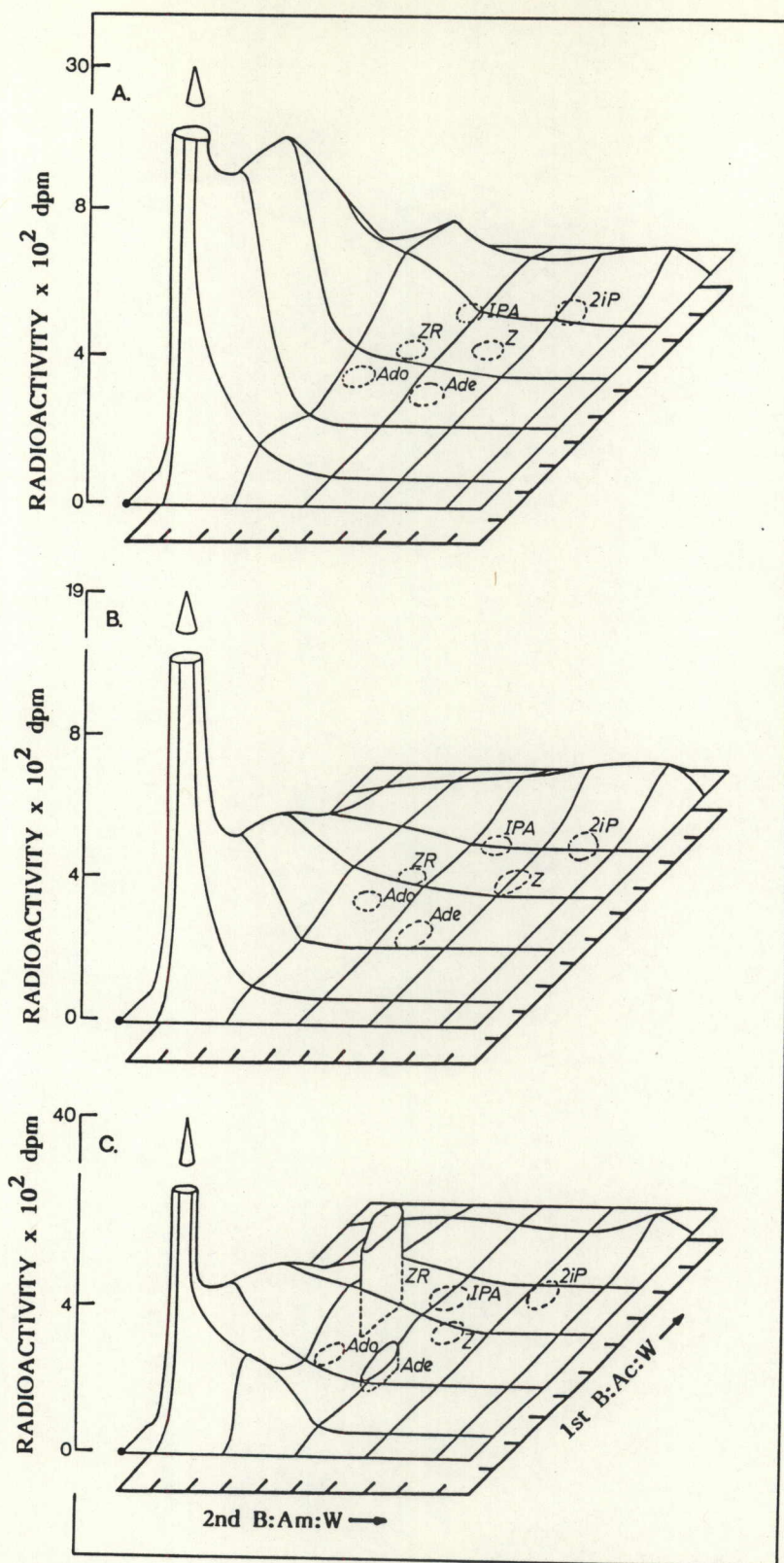


Figure 3.5.b. Radioactivity determined after separation on Silica Gel TLC plates of the slow moving **Peak 1**. (Figure 3.3.) extracted from the **stems of fruiting *Glycine*** plants grown **in vitro**. Solvent systems were as shown in Figure 3.4. A., untreated extract; B., after treatment with B-glucosidase; C., after treatment with alkaline phosphatase.

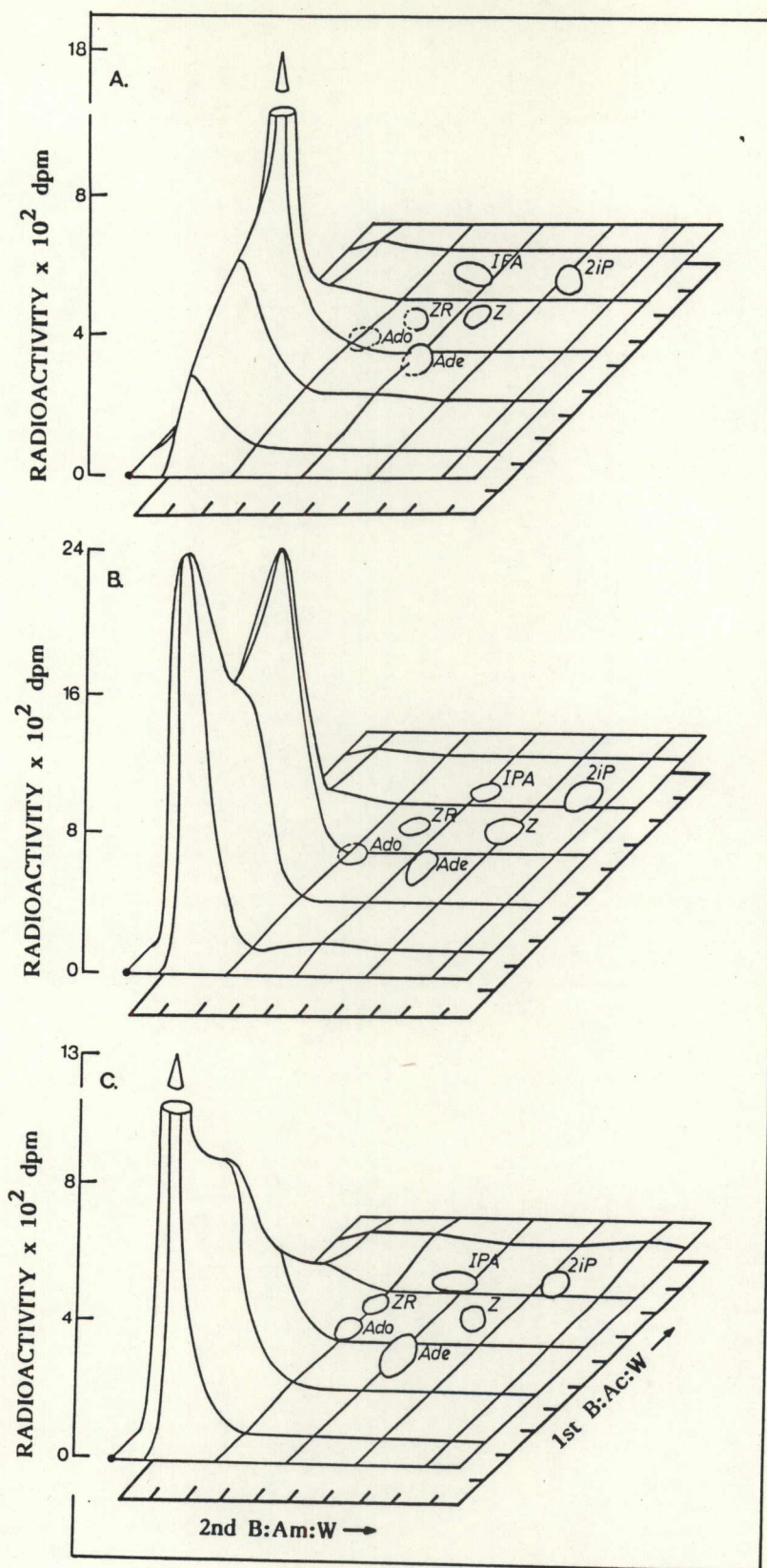


Figure 3.5.c. Radioactivity determined after separation on Silica Gel TLC plates of the slow moving Peak 1. (Figure 3.3.) extracted from the **flowers (A.)**, **pod wall (B.)**, and **seeds (C.)** of *Glycine* plants grown *in vitro*. Solvent systems were as shown in Figure 3.4.

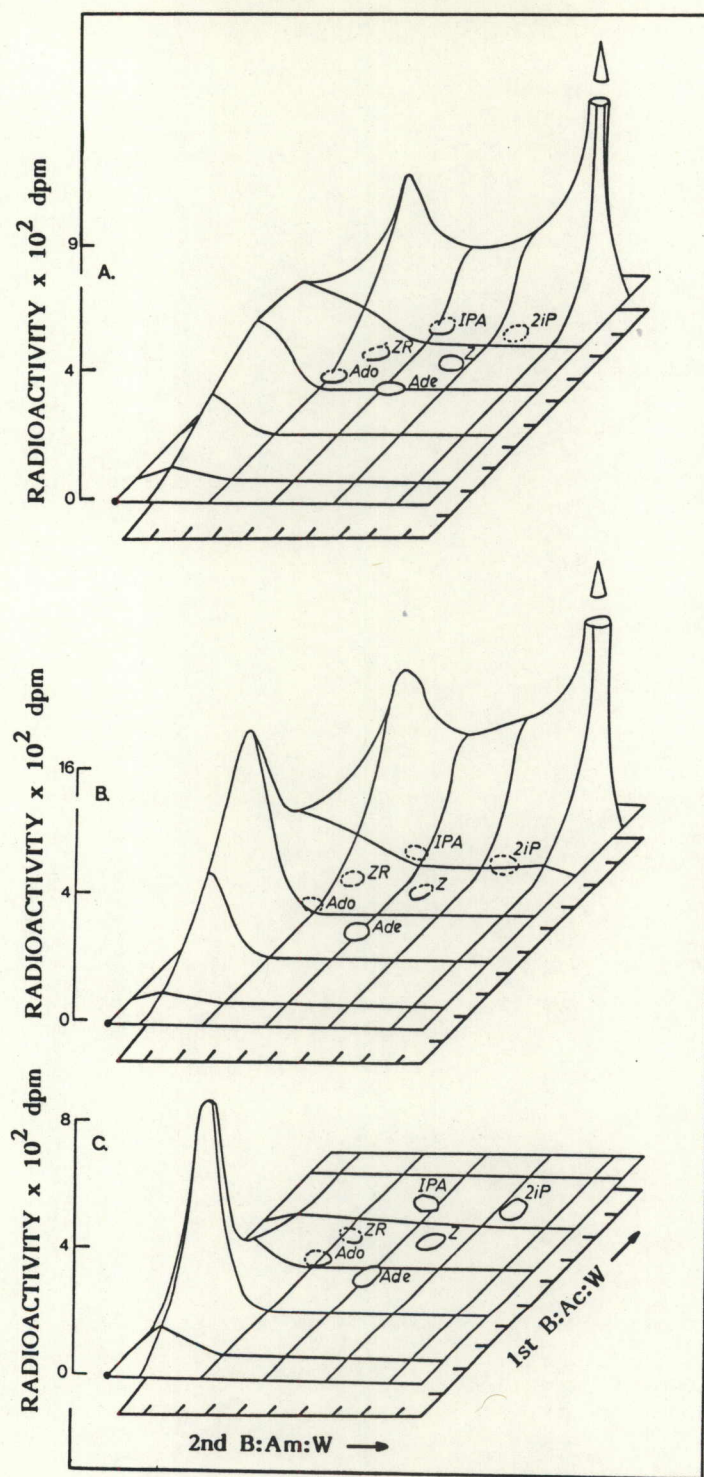


Figure 3.6.a. Radioactivity determined after separation on Silica Gel TLC plates of **Peak 2**. (Figure 3.3.) extracted from the **stems of fruiting (A.) and flowering (B.) plants and the flowers (C.)** of *Glycine* plants grown *in vitro*. Solvent systems were as shown in Figure 3.4.

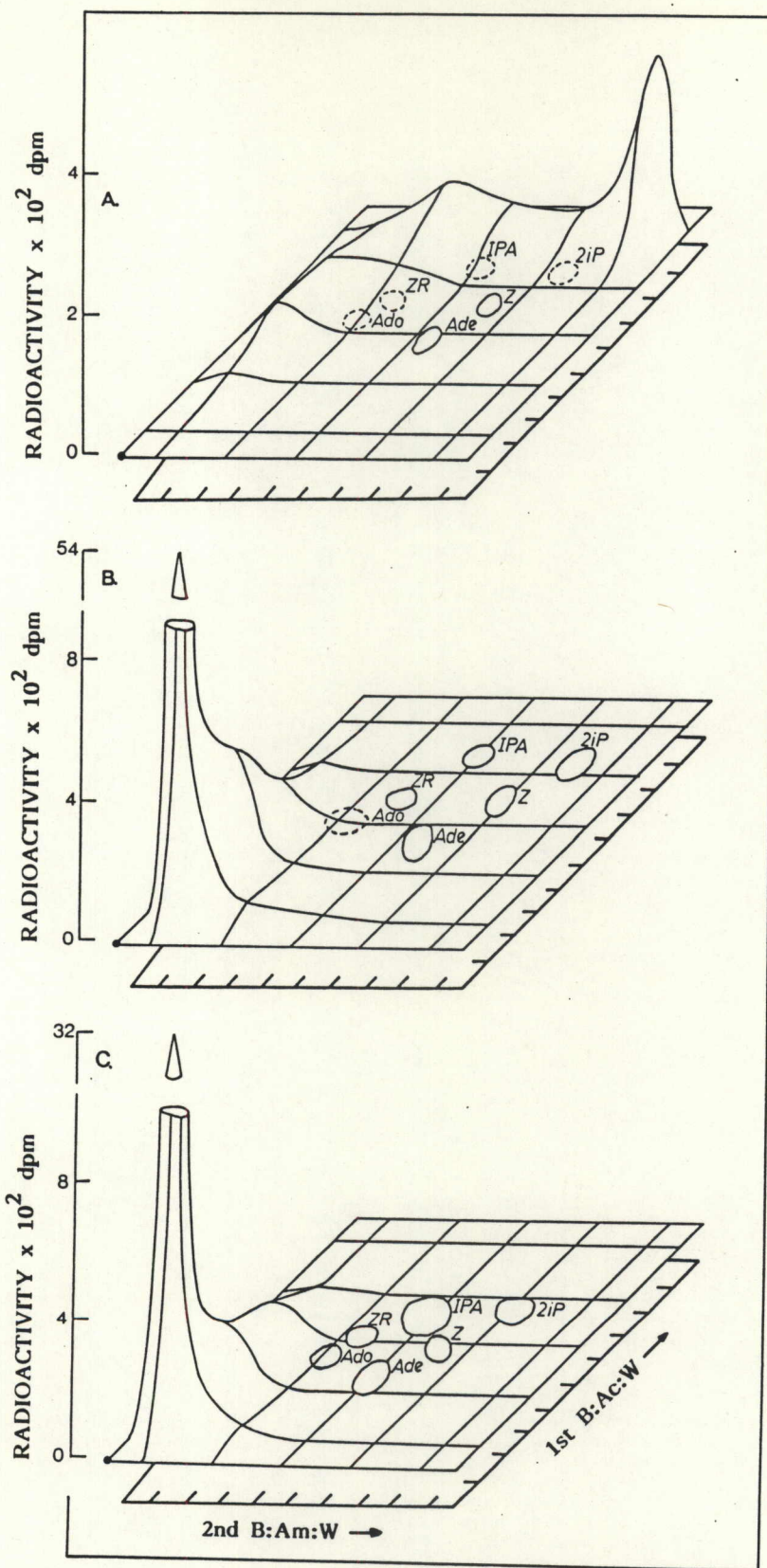


Figure 3.6.b. Radioactivity determined after separation on Silica Gel TLC plates of **Peak 2**. (Figure 3.3.) extracted from the **seeds (A.)** and **pod wall (B.)**. The pod wall extract was also treated with B-glucosidase (C.). Solvent systems were as shown in Figure 3.4.

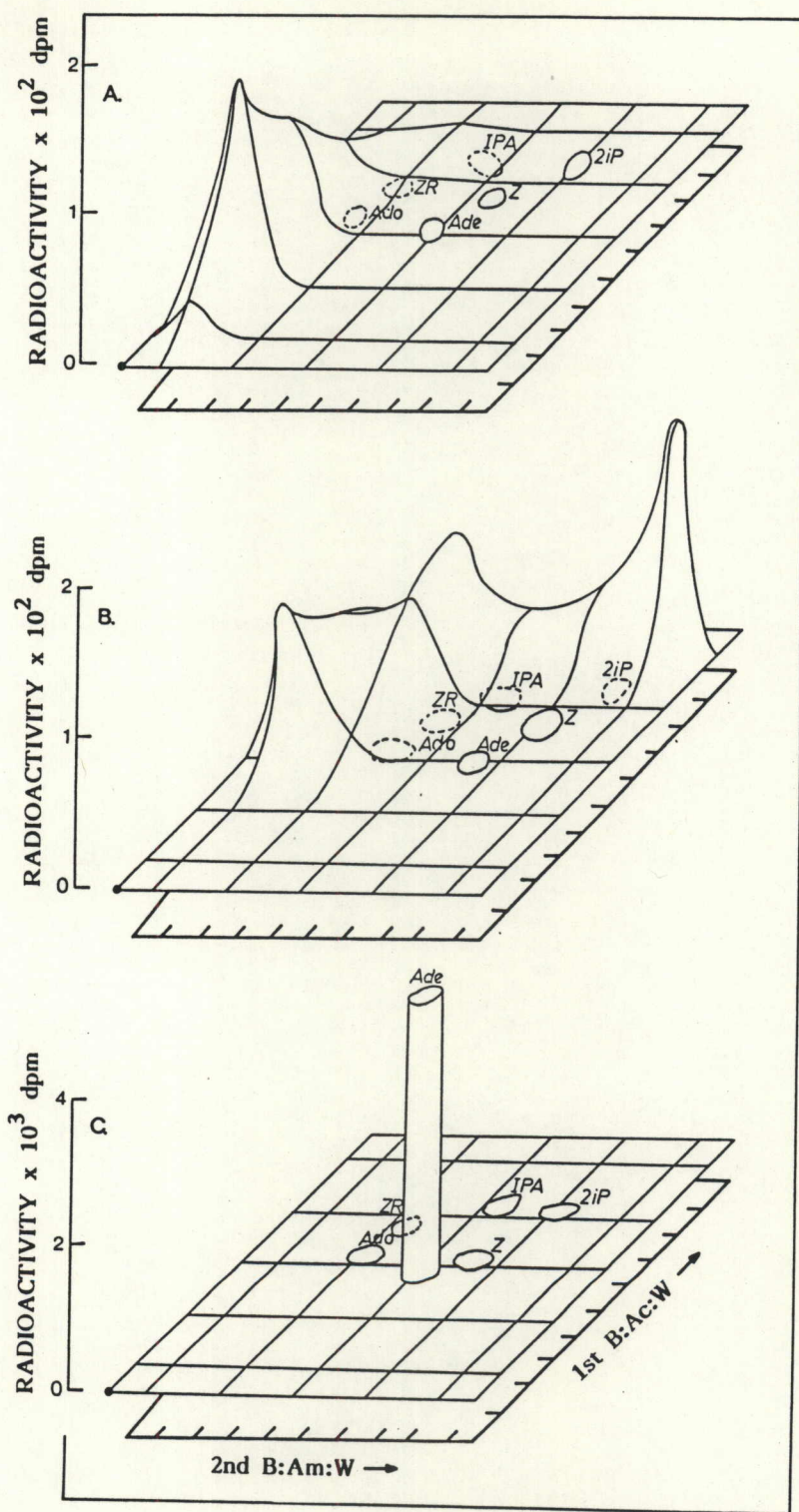


Figure 3.7. Radioactivity determined after separation on Silica Gel TLC plates of the fast moving **Peak 3**. (Figure 3.3.) extracted from the **flowers (A.)** and **pod wall (B.)** of *Glycine* plants. The **agar medium (C.)** was also separated. Solvent systems were as shown in Figure 3.4.

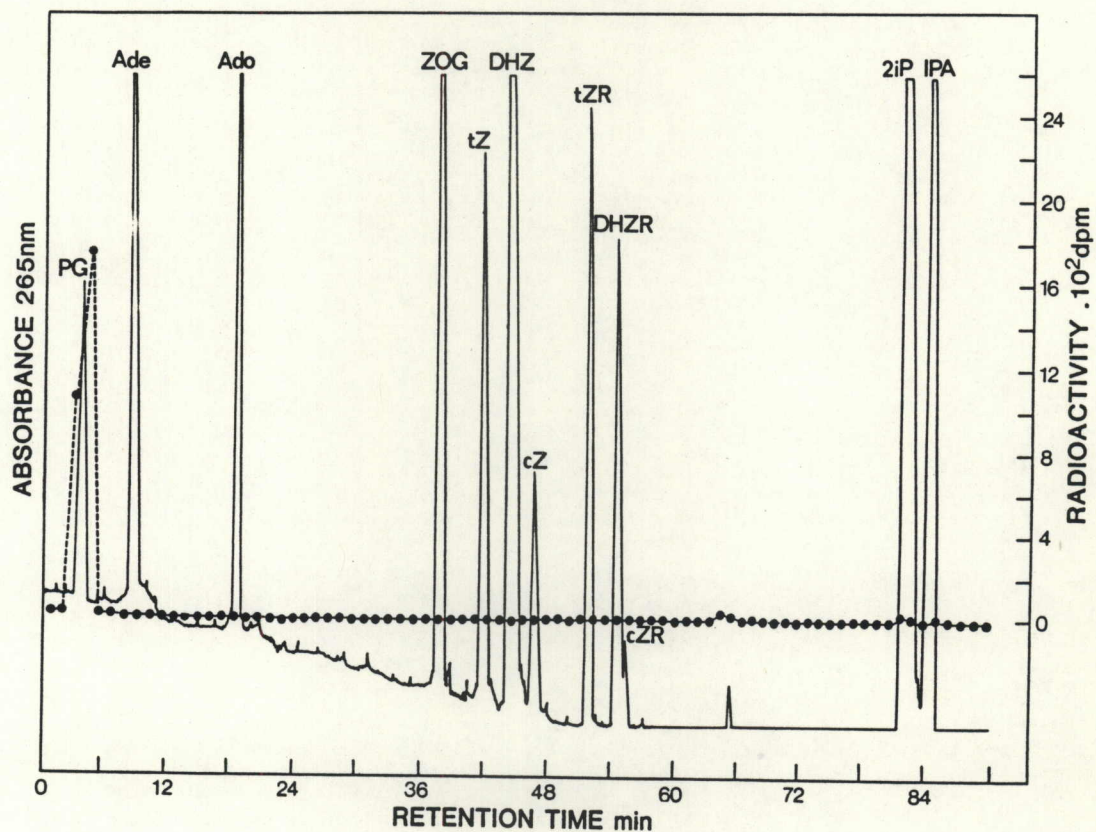
All of these peaks from the extracts as represented in Figure 3.3., were again separated, but using the greater sensitivity and resolution of the HPLC. In all cases, radioactivity was eluted from the sample with a low retention time. No activity was found associated with any of the standards used, with the one exception of the agar medium, which again was found to contain radioactivity associated with authentic adenine.

Due to insufficient material for further investigation, this experiment was repeated with some modifications. A timeously published paper by LEE, MOK, MOK, GRIFFIN and SHAW (1985) allowed for the direct analysis of crude extracts for radiolabelled cytokinins. This provided a distinct advantage, in that extracts could be analysed without the several purification steps as outlined in the preceding work. Not only did this save on time, but it also avoided the possibility of chemical decomposition during successive handling procedures. These results are reported in the following section.

[8-¹⁴C]Adenine and [1-¹⁴C]IPP incorporation

At the termination of the experiment, the medium containing ¹⁴C adenine was found to contain on average 3.2×10^4 Bq per flask, indicating that nearly half the available adenine was taken up. In the previous section, this radioactivity was tentatively identified as being adenine.

Extracts from flowers and fruits contained low levels of radioactivity, with only one peak associated with puranyl glycine (Figure 3.8.). As no peaks were associated with any of the other cytokinin standards, and due to the low radioactivity levels, this extract was not investigated further.



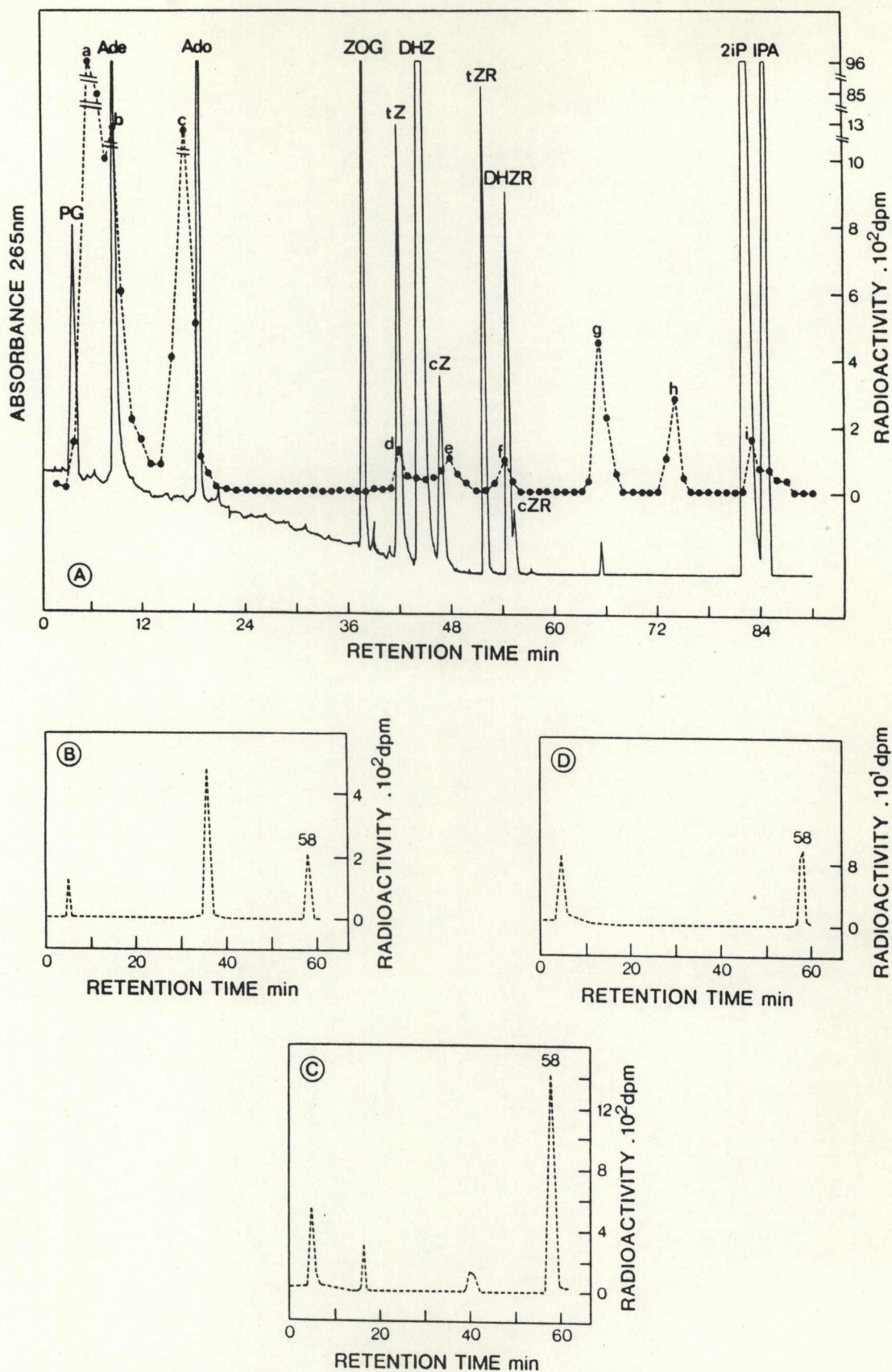
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Figure 3.8. The distribution of C-adenine derived radioactivity from a **flower and fruit extract** (●●●) and UV trace of authentic cytokinin standards (—) after separation by HPLC. Cytokinin standards used were adenine (Ade); adenosine (Ado); dihydrozeatin (DHZ); dihydro-ribosylzeatin (DHZR); isopentenyladenosine (IPA); purinyl glycine (PG); trans-zeatin (tZ); trans-ribosylzeatin (ZOG) and isopentenyladeine (2iP).

Extracts from the shoots of ^{14}C -adenine treated plants yielded a number of peaks of radioactivity (Figure 3.9.), several of which had similar retention times to the authentic standards ade, ado, tZ, cZ, DHZR and 2iP. In addition, three radioactive peaks, (a), (g) and (h) were detected, but did not co-chromatograph with any of the available standards.

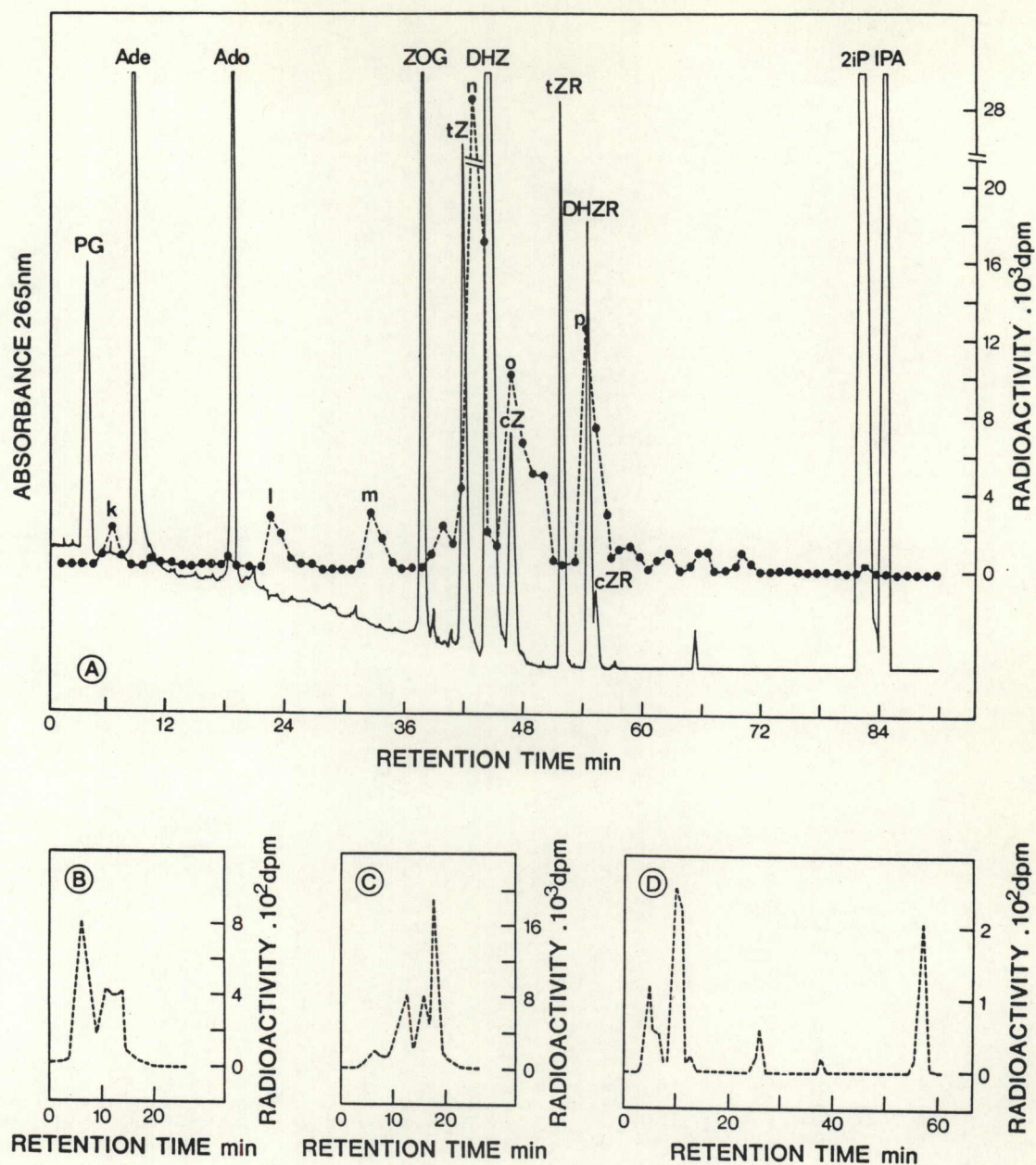
The oxidation by KMnO_4 of the substance associated with peak (d) resulted in the detection of two major peaks of radioactivity, one of which had a retention time of 58 minutes, and a minor peak which co-chromatographed with PG (Figure 3.9B). Acid hydrolysis of substances associated with peak (g) resulted in a shift of radioactivity, one of the peaks subsequently detected having a retention time of 58 minutes. Three smaller peaks were also found with lower retention times (Figure 3.9C). The oxidation by KMnO_4 of the substances associated with peak (i) which had the same retention time as 2iP, yielded two peaks of radioactivity, one at 58 minutes and another at 4.5 minutes (Figure 3.9D).

The above results suggest that the substances associated with both peaks (d) and (i) had saturated bonds which were affected by KMnO_4 treatment. The substances represented by peaks (d), (g) and (i) all yielded a radioactive peak with a retention time of 58 minutes after chemical treatment, suggesting that all three substances had a common component. The identity of this substance is presently not known. Due to the low levels of radioactivity associated with cytokinin-like compounds, insufficient material was available for further investigation.



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Figure 3.9. The distribution of ¹⁴C-adenine derived radioactivity from **shoot extract** (●●●) and UV trace of authentic cytokinin standards (—) after separation by HPLC. A, total extract; B, peak (d) after oxidation by KMnO₄; C, peak (g) after hydrolysis by HCl; and D, peak (i) after oxidation by KMnO₄. Cytokinin standards used were adenine (Ade); adenosine (Ado); dihydrozeatin (DHZ); dihydro-ribosylzeatin (DHZR); isopentenyladenosine (IPA); purinyl glycine (PG); trans-zeatin (tZ); trans-ribosylzeatin (ZOG) and isopentenylnadeine (2iP).

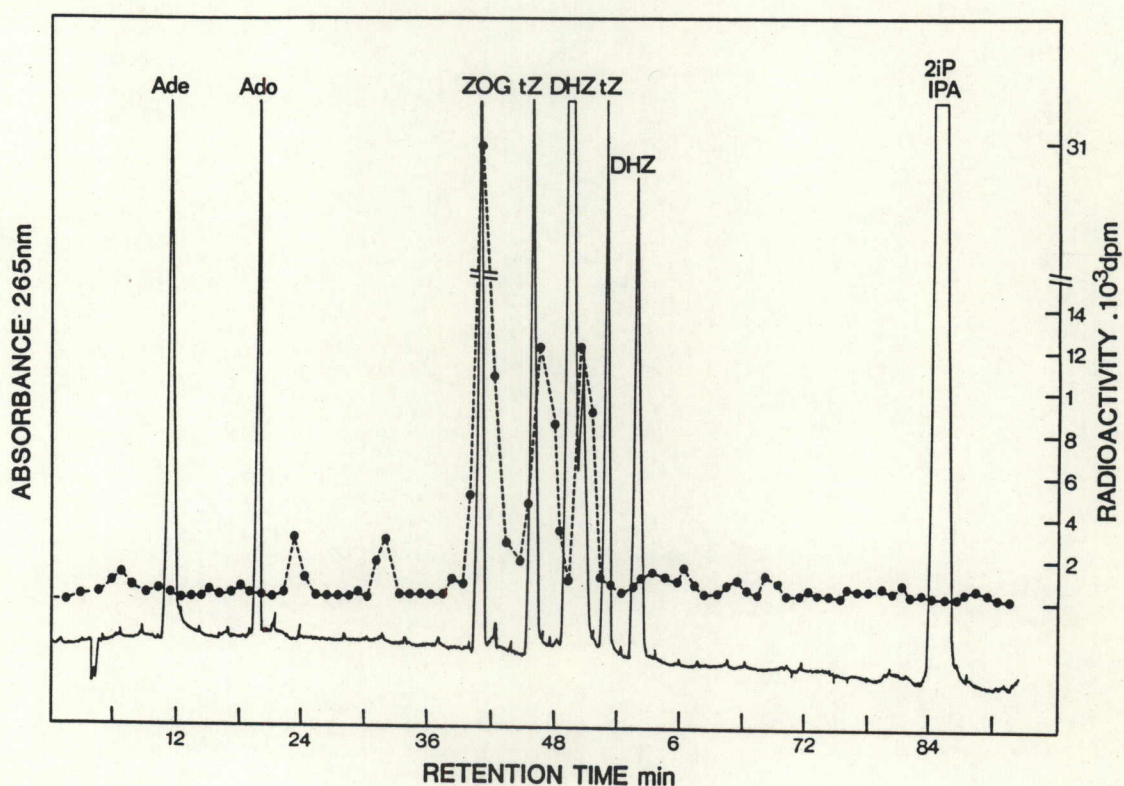


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Figure 3.10. The distribution of C-IPP derived radioactivity from **shoot extract** (●●●) and UV trace of authentic cytokinin standards (—) after separation by HPLC using a buffer adjusted to **pH 3.5**. A, total extract; B, peak (n) after oxidation by KMnO₄; C, peak (o) after oxidation by KMnO₄; and D, peak (p) after hydrolysis by HCl. Cytokinin standards used were adenine (Ade); adenosine (Ado); dihydrozeatin (DHZ); dihydro-ribosylzeatin (DHZR); isopentenyladenosine (IPA); purinyl glycine (PG); trans-zeatin (tZ); trans-ribosylzeatin (ZOG) and isopentyladeine (2iP).

Shoots (Figure 3.10A), and flowers and fruits (Figure 3.11) of ^{14}C -IPP treated plants were found to have similar peaks of radioactivity after HPLC separation. Several of these peaks had similar retention times to authentic cytokinins. Only the shoot extract was examined further in detail.

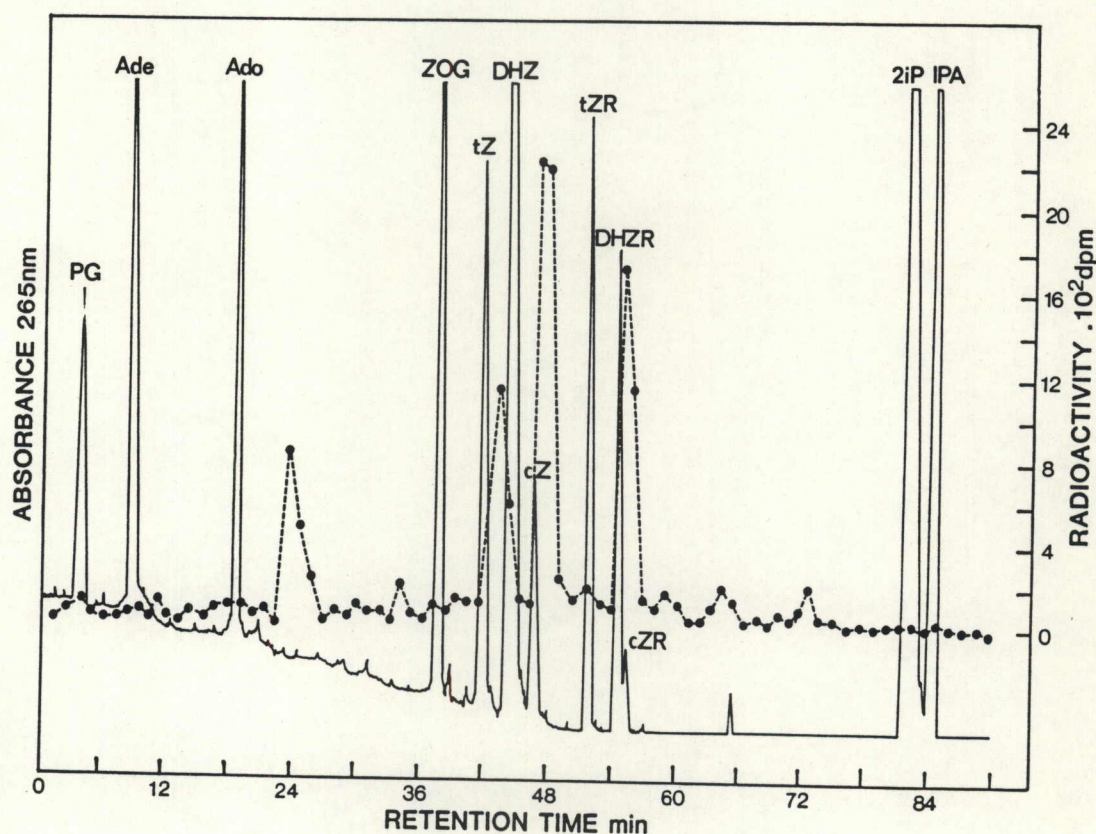
The oxidation by KMnO_4 of the substances associated with peak (n) resulted in the subsequent detection of two peaks of radioactivity (Figure 3.10B). The oxidation of the substances associated with peak (l)



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Figure 3.11. The distribution of ^{14}C -IPP derived radioactivity from **flower and fruit extract** (●●●) and UV trace of authentic cytokinin standards (—) after separation by HPLC using a buffer adjusted to pH 3.5. Cytokinin standards used were adenine (Ade); adenosine (Ado); dihydrozeatin (DHZ); dihydro-ribosylzeatin (DHZR); isopentenyladenosine (IPA); purinyl glycine (PG); trans-zeatin (tZ); trans-ribosylzeatin (ZOG) and isopentenyladeine (2iP).

produced four peaks of activity (Figure 3.10D). The hydrolysis of the substances associated with peak (p) by HCl resulted in four peaks, one of which had a similar retention time to adenine (Figure 3.10D). HPLC separation of the total extract after ¹⁴C-IPP treatment, but using an aqueous buffer adjusted to pH 4.8 with TEA (Figure 3.12) caused the peaks of radioactivity to shift so that they no longer co-chromatographed with the same standards as in Figure 3.10. This result effectively shows that the radioactivity was not associated with any of the cytokinins used as standards. There was therefore no detectable incorporation of IPP into the cytokinins investigated. Radioactivity remaining in the medium amounted to on average 2.8×10^4 Bq per flask.



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Figure 3.12. The distribution of ¹⁴C-IPP derived radioactivity from **shoot extract** (●●●) and UV trace of authentic cytokinin standards (—) after separation by HPLC using a buffer adjusted to **pH 4.8**. Cytokinin standards used were adenine (Ade); adenosine (Ado); dihydrozeatin (DHZ); dihydro-ribosylzeatin (DHZR); isopentenyladenosine (IPA); purinyl glycine (PG); trans-zeatin (tZ); trans-ribosylzeatin (ZOG) and isopentenyladeine (2iP).

3.2.4. CONCLUSION

The presence of adenine in the medium at the termination of the experiment, indicates that adenine, and probably also IPP were available to the explants for the entire period of culture during which time flowering and fruit set took place. In some other investigations, the levels of adenine were found to become rapidly depleted (BARNES, 1961; DOREE, 1973). The level of radioactivity found in the substances associated with each of the above peaks was very low compared to the initial amount of ¹⁴C-adenine incorporated into the medium. This was to be expected, as most adenine is known to be metabolised to nucleotides, nucleic acids and the other products of the adenine salvage pathway (BARANKIEWICZ and PASZKOWSKI, 1980; ASHIHARA and NOBUSAWA, 1981).

The concentration of adenine derivatives in the flowers of soybean explants suggests that flowers act as a strong sink for adenine and/or its derivatives early in their development. Adenine may be important here as a component of either the cytokinins or of nucleotides and nucleic acids, both of which are known to stimulate flower initiation. Cytokinins have been postulated to form part of the flowering stimulus (WAREING, HORGAN, HENSON and DAVIS, 1977; BERNIER, KINET, JACQHARD, HAVELANGE and BODSON, 1977); have been able to stimulate flowering in several plants; and are often essential in the medium for *in vitro* flowering experiments (Table 1.).

Adenine and some other nucleic acid constituents have been shown to initiate and promote flowering, possibly by promoting RNA synthesis at the apex (BERNIER, KINET and SACHS, 1981b) or by supporting an increase

in adenine nucleotides (BODSON ,1985). In Plumbago indica, adenine was essential for the formation of petal colour (NITSCH, NITSCH, ROSSINI and BUI DANG HA, 1967), while cytokinins were not necessary and could not substitute for adenine (NITSCH and NITSCH, 1967). This indicates the possibility that adenine was not being used in the form of a cytokinin. In Perilla on the other hand, adenine, adenosine and kinetin could all promote flowering in non-inductive conditions (CHAILAKHYAN and BUTENKO, 1959).

Previous investigations have indicated that adenine does play a role in flowering, possibly as part of a multi-component sequence of events accompanying flower initiation and/or growth. There is the possibility that adenine's role is partly through its incorporation into cytokinins, although this is an event which has not been conclusively proved to occur, previously or in the results presented here.

CHAPTER 4.

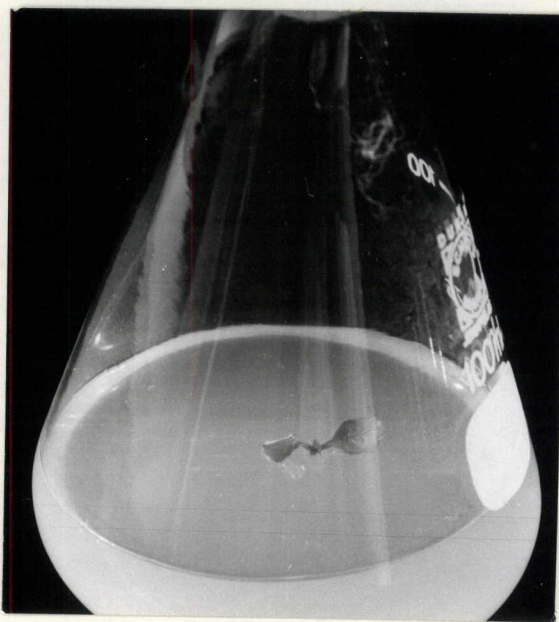
THE IN VITRO FLOWERING OF
KALANCHOE BLOSSFELDIANA

Plate 4.1. Nodal explants of Kalanchoe blossfeldiana with leaves, vegetative in LD conditions but flowering in SD conditions.

CHAPTER 4. THE IN VITRO FLOWERING OF KALANCHOE BLOSSFELDIANA

4.1. INTRODUCTION

To continue the work done on Kalanchoe and described in Chapter 2. section 2.3.2.b., an investigation was made of the factors affecting the flowering of Kalanchoe explants in vitro. This was initially done to optimise the flowering response in vitro, but was rapidly expanded to include studies on the physiological state of the tissue before and after culture. This system was tested extensively as a bioassay for factors affecting flowering, both endogenously and exogenously.

Many of the in vitro flowering investigations reported in the literature (Table 1.), have utilized parent plants which were either induced or flowering prior to excision of the explants. This includes the well investigated Nicotiana system developed by CHOUARD and AGHION (1961). As was reported in Chapter 1., the culture of previously induced material must be treated with some reservation if the stimulus of flowering is to be investigated, the stimulus for flowering will already be present, or will have performed its function in the tissue at the time of culture. Indeed, several cases exist where the flowering stimulus has persisted in the tissue despite being cultured in non-inductive conditions (KONSTANTINOVA, AKSENOVA, BAVRINA and CHAILAKHYAN, 1969; WARDELL and SKOOG, 1973). The physiological condition of the Kalanchoe parent plants prior to explant excision was thus considered here, to test what effect this would have on the subsequent flowering of the explants. The flowering response of a variety of manipulation treatments was measured, as well as the concurrent increase in succulence and pigmentation of the

leaves. An anatomical investigation was also made to enlarge on the situation with regard to the increase in succulence and pigmentation.

The influence of the basic culture conditions on the flowering of Kalanchoe explants was also investigated. That the mineral components of the medium have some effect on in vitro flowering is widely accepted, and is reviewed in Chapter 1.. The most significant and thorough investigation of this type, was that of TANIMOTO and HARADA (1981a,b), who found that by manipulating the concentration of the medium, and eliminating NH_4NO_3 , flowering could be achieved on internode segments of Torenia taken from induced plants.

Other than the work of SHINOZAKI and TAKIMOTO (1982b), the effect of the volume of medium, and thus the total availability of nutrients, has not been investigated before with regard to flowering. This latter work, although referred to as in vitro, is not a 'tissue culture' system, but simply plants growing in glass containers. These authors found that by reducing the sized of the container, root elongation was suppressed and flowering increased. This supported the conclusions of GASPAR (1980) who noted the antagonism that exists between rooting and flowering. It was thus necessary to investigate the role that these factors play in the flowering of Kalanchoe.

As was discussed in Chapter 1., carbohydrates play a substantial role in the control of flowering. The most significant conclusions from the literature, are that carbohydrates can replace the light or daylength requirement for flowering in certain species (LIVERMAN and BONNER, 1953; BALDEV, 1962; COUSSON and TRAN THANH VAN, 1983). The role of carbohydrates in this stimulation of flowering is not clear, but is apparently not purely an osmotic effect (NITSCH and NITSCH, 1967b; FRIEND, BODSON and BERNIER, 1984).

Much evidence has been presented in the literature to show that the levels of endogenous sugar increased in plants during induction to flower, even if induction is achieved in shorter day-lengths which supply less light for carbon assimilation. BODSON (1977) and BODSON and OUTLAW (1985) noted an increase in the free sugars, in particular sucrose, in the apical bud of Sinapis during LD (long-day) induction and during induction by SD (short-day) displacement. The latter treatment supplies the same photosynthetic flux as non-inductive SD. MIROLO, BODSON and BERNIER (1985) suggest that increased carbon assimilation into floral buds during induction could be specifically related to floral transition, but cannot alone trigger the complete sequence of floral evocation. Alternatively, this accumulation is not related to the floral transition. BODSON and OUTLAW (1985) concluded that the role of increased sucrose at the apex, is not primarily through changing osmolarity, or through the provision of the necessary substrate for increased cell proliferation. These authors claim that sucrose hydrolysis may be rate limiting during glycolysis, and thus higher sucrose concentrations may serve to accelerate sucrose hydrolysis. FRIEND, BODSON and BERNIER (1984) noted that in Brassica, the promotion of flowering by sucrose was not an osmotic effect, as neither mannitol, PEG nor sodium chloride were effective at inducing flowering. The addition of sucrose to the medium lowered the leaf number, but promoted flowering, thus indicating that promotion was not a general promotion of growth.

The increase in sugar levels in response to photoperiodic induction, is generally confined to the apical regions of the plant, and is due to both a redistribution of sugars from the base of the plant to the apex (SACHS, 1977), and to the hydrolysis of starch reserves in the apex. It is also

noteable from the literature, that sugar levels that are optimal for flowering are far higher than those optimal for vegetative growth. CHAILAKHYAN, AKSENOVA, KONSTANTINOVA and BAVRINA (1975) even recommended levels as high as seven per cent for optimum in vitro flowering of Nicotiana. Levels higher than this were however inhibitory to flowering. VAN DEN ENDE, BARENDSE, KEMP and CROES (1984) noted that in thin cell layers of Nicotiana, the presence of some metabolizable sugar is required in the medium. This requirement cannot be replaced by light.

The effect of increasing sucrose concentrations in the medium on the flowering of Kalanchoe explants grown in vitro under inductive SD conditions was thus investigated. Tentative experiments prior to this had indicated that sucrose alone would not induce flowering in LD conditions.

The effects of nitrogen on flowering form part of the oldest theories of flower induction (KLEBS, 1913; KRAUS and KRAYBILL, 1918). Nitrogen is thought to be required in low concentrations for flower induction to occur. In in vitro work, nitrogen effects were found to be significant, and are reviewed in Chapter 1. section 1.3.2.c.. The most comprehensive investigation was that of TANIMOTO and HARADA (1981a,b), who found that by lowering the $\text{NH}_4^+ \text{NO}_3^-$ level (but not necessarily that of KNO_3), flowering could be induced on Torenia stem segments. Similar work conducted on Perilla supported this conclusion (WADA and TOTSUKA, 1982; WADA and SHINOZAKI, 1985). A recent publication by TANAKA (1986) provided most of the guidelines for the research done on nitrogen in this thesis.

TANAKA (1986) reported that Lemna aequinoctialis would flower in non-inductive continuous light, if cultured on a nitrogen deficient medium for three days or more, before being transferred to a nitrogen rich medium containing either NH_4NO_3 or KNO_3 . Nitrogen was required for the manifestation of the flowering stimulus but was inhibitory of flower induction. This is supported by other work where the suppression of nitrate assimilation by nitrate reductase inhibition, also induced flowering (TANAKA, HORIKAWA, NISHIMURA and NASU, 1986).

The work of TANAKA (1986) was thus used as a model after extensive modification, to test the inductive ability of nitrogen manipulations on the flowering of Kalanchoe explants. Various permutations of nitrogen manipulation were investigated in different day-lengths. Also investigated, was the possibility that nitrogen addition would support the manifestation of the flowering stimulus in induced Kalanchoe explants transferred to non-inductive LD. Such a carry over of the stimulus had not been achieved in earlier attempts on the simple medium of TANIMOTO and HARADA, (1981b, Table 2.2.b.).

Although the roles of plant hormones and phenolics in flowering, are far from clear, there is now much evidence to indicate that they are involved in some way. The evidence derived from in vitro investigations has been extensively reviewed in Chapter one, section 1.3.. Some **general conclusions** on the role of hormones in flowering in vitro can nevertheless be made from the extensive volume of literature available .

Auxin is generally inhibitory of flowering in SDP if applied before or during induction. Once differentiation of flowers has commenced, auxin promotes flower development. Auxin is nevertheless implicated as forming part of the sequence of events accompanying the transition from vegetative to reproductive growth.

Cytokinins have been implicated as forming part of the floral stimulus in select cases, yet the results for these hormones are many and varied, ranging from total initiation, to enhancement of initiation, to inhibition. Their action may be related to nutrient availability or gene activity.

Gibberellins are the most 'florigenic' of all known hormones, having the ability to induce flowering in several plants from all photoperiodic response groups. These results are highly variable and the trends obscure. Gibberellins are least successful at inducing SDP to flower, although they may play some role in the development of the inflorescence. It is possible that their mode of action may be linked to reserve mobilization. Alternately, it has been suggested that their action is to influence the production of 'florigen' (ZEEVAART, 1978).

Abscisic acid greatly influences flowering, in both a promotive and an inhibitory way, but it is no longer regarded as being a specific flower inhibitor or promotor. In vitro work has however provided much new evidence to support the possibility of endogenous ABA being involved in flowering. This aspect urgently requires further investigation.

Phenolics have proved highly inductive, but only in a limited number of species, in particular the members of the Lemnaceae. It is not known what the role of these substances is in flowering, as the endogenous levels of these substances seldom reflect changes that support the results of exogenous applications.

Previous hormone investigations using Kalanchoe have established very little. Auxin inhibited the number of flower buds in SD grown Kalanchoe, but after increasing numbers of SD and thus increased induction, auxin became less effective as an inhibitor (HARDER and VAN SENDEN, 1949; VAN SENDEN, 1951). This supports the earlier conclusion that auxin is supportive of flower growth, but is inhibitory of induction.

TIBA (2,3,5-triiodobenzoic acid), which affects the polar transport of auxin, caused flower inhibition in Kalanchoe (VAN ZEIST and KOEVOETS, 1951), while ethylene caused leaf abscission and closure of flowers (MAROUSKY and HARBAUGH, 1979). Gibberellic acid was inhibitory of flowering in Kalanchoe (HARDER and BUNSOW, 1956; 1957; 1958) if applied before or during induction. Previously induced plants exhibited extensive peduncle elongation (SCHMALZ, 1960). ABA and xanthoxin, natural plant inhibitors, both inhibited flowering if injected into Kalanchoe leaves (SCHWABE, 1972).

Numerous attempts have been made over the years to extract the endogenous floral stimulus, and latterly, the floral inhibitor from plant tissues. Extractions by various workers, have produced little evidence of the existence of either a floral stimulus or of an inhibitor, (BONNER and BONNER, 1948; ROBERTS, 1951; BONNER and LIVERMAN, 1953; ROBERTS and STUCKMEYER, 1960; LINCOLN, MAYFIELD and CUNNINGHAM, 1961; MAYFIELD, LINCOLN, HUTCHINS and CUNNINGHAM, 1963; LINCOLN, CUNNINGHAM and HAMNER, 1964; BISWAS, PAUL and HENDERSON, 1966; CARR, 1967; TOMITA, 1968; HODSON and HAMNER, 1969; BLAKE, 1972; PRYCE, 1972; CLELAND, 1974; CHAILAKHYAN, GRIGORIEVA and LOZHNIKOVA, 1977; WARDELL, 1977; CHAILAKHYAN, 1978; PURSE, 1984; SUZUKI, YAMAGUCHI and TAKAHASHI, 1985; CHAILAKHYAN and LOZHNIKOVA, 1985). Extracts made by these workers, have usually been tested in some bioassay system, by foliar spray, stem flap application, injection into the plant, or incorporation into culture media. Where successful flowering has been induced by an extract, the percentage flowering of assayed plants has been very low. The results of these experiments have not been confirmed, by the authors themselves or by other workers. In no case have the results been conclusive. Unfortunately techniques have often been inadequately described, rendering them unrepeatable.

A variety of extraction systems and solvents have been used for these extractions, and have tentatively indicated that flower regulating substances are water soluble (MAYFIELD, LINCOLN, HUTCHINS and CUNNINGHAM, 1963; LINCOLN, CUNNINGHAM and HAMNER, 1964; PRYCE, 1972; SCHWABE, 1972; PURSE, 1984). There is also evidence that the compound/s is organic and is soluble in polar solvents, but is altered in the presence of organic solvents (PRYCE, 1972).

In most of the bioassay systems described, consideration was given to the effects of the substance on flowering, but little or no consideration was given to the effects on growth in general. This is to the detriment of these investigations, as is evidenced by the well known flower stimulation of LDP rosette plants by gibberellins, an action which may occur as a result of the stimulation of stem elongation. This lack of information is even more disquieting in investigations of inhibitors, as inhibitors may retard not only flowering, but growth in general, and yet be indicated as floral inhibitors. Some indication of such inhibition was given by BLAKE (1972), where Viscaria (= Silene) apices supplied with vegetative Kalanchoe extracts, produced fewer leaf pairs concurrently with the inhibition of flowering. This implies that the inhibitor was not specific to flowering. Subsequently PRYCE (1972), using the same system as BLAKE, noted that flowering as well as vegetative leaves contained floral inhibitors if organic solvents were used. This same inhibitor was further isolated and identified as gallic acid, although this could not be verified by ZEEVAART, (1976).

It is clear that there is little to be gained by reviewing all the historical details of the various extractions and results, as none to these has stood the test of time and /or critical examination.

Kalanchoe explants cultured in vitro were used in this thesis, as a bioassay to test for the presence of promoters or inhibitors of flowering. The advantages and disadvantages of such a system are discussed in Chapter 1., section 1.2.1.. The most pertinent conclusions from the literature, which were used in the development of extraction techniques in this investigation, are that the stimulus (and/or the inhibitor) seems to be soluble in water, is possibly heat stable, but suffers from a loss of activity if fractionated or purified too extensively. These factors determined the guidelines for the crude extracts investigated here.

4.2. MATERIALS AND METHODS

The basic technique for the culture and in vitro flowering of Kalanchoe is as described in section 2.3.2.b. Kalanchoe parent plants were grown in vitro on a medium consisting of a fifth strength of the salts according to MURASHIGE and SKOOG (1962), together with 30 g l⁻¹ sucrose, adjusted to pH 5.8 and gelled with 0.8 per cent agar (Table 2.2.a). This medium was devised by TANIMOTO and HARADA (1981b). These parent plant cultures were maintained under LD (18 hour day) conditions at a temperature of 25 °C. Light intensity was $\pm 50 \mu\text{Em s}^{-2} \text{ }^{-1}$ of photosynthetically available radiation. Nodal explants with or without leaves attached were taken and cultured onto the same medium as described above, with modifications as pertinent to the experiment. These cultures were maintained under either LD (18 hour day) or SD (8 hour day) conditions with day/night temperatures of 25/20 °C. Light intensity under both day-lengths was $110 \mu\text{Em s}^{-2} \text{ }^{-1}$ photosynthetically available radiation. Several parameters affecting flowering in this system were investigated and are documented in the following sections.

4.2.1. The effects of physiological state on the in vitro flowering of Kalanchoe explants

Preliminary investigations were made to test firstly, the role of the physiological state of the parent plants on the resulting growth and flowering of daughter explants, and secondly, the effect of the presence or absence of leaves on the explants.

Two sources of in vitro grown plants were used, half being vegetative and grown under LD (18 hour day), and the other half in the flowering condition having been grown under SD (8 hour day) for eight weeks. Nodal

explants were taken and cultured with or without attached leaves, onto TANIMOTO and HARADA (1981b,) medium (Table 2.2.a.). All of these cultures were maintained in SD (8 hour day) conditions at a light intensity of photosynthetically available radiation of $110 \mu\text{Em s}^{-2} \text{ }^{-1}$. Temperature was set at 25/20°C (day/night), and humidity at 50 per cent.

In a repeat but more elaborate experiment, cultures of all the above permutations were placed into both LD (18 hour day) and SD (8 hour day) conditions, the former testing the maintenance of the stimulus in the explants when transferred to non-inductive conditions. The parent plants of all cultures differed from the first experiment, in that they were small multiple shoots of vegetative plants. The SD plants had been induced by 10 weeks of SD, but were not flowering due to being confined in small test tubes.

This latter experiment was again repeated with some modifications, using SD parent plants which had reached anthesis. In all experiments, ten replicates were used per treatment, although some losses were experienced due to contamination. This last experiment was closely analysed, with various growth parameters being measured. The rate of flower development (per cent flowering), as well as the mass of the resulting organs, and the degree of anthocyanin and chlorophyll production were all recorded. It has already been indicated earlier in section 2.2.2. that Kalanchoe adopts a red colouration and succulence of its leaves in response to SD induction. The method of determination of these factors is described below.

Pigment determination

According to HARBORNE (1973), anthocyanins are unstable in neutral or alkaline solutions, and thus must be extracted with solvents containing acid. Extracts should then be stored in the dark at low temperatures. The recommended solvent of methanol with one per cent concentrated HCl was tested, together with DMSO (dimethyl sulphoxide) with one percent HCL. The latter solvent was examined because of its ability to extract chlorophyll from fresh tissues without the need for maceration (HISCOX and ISRAELSTAM, 1979).

In initial tests, 0.05 g of leaf material was taken from dark green vegetative Kalanchoe plants, and also from pale red coloured flowering plants. The leaf material was placed into four millilitres solvent. Those in acidified methanol were macerated and shaken for three hours at room temperature. Leaves in acidified DMSO were left intact, and were incubated at 60 °C for three hours. The absorbance spectrum of each was determined using a Varian DMS 90 UV spectrophotometer, set with a range from 350 nm to 700 nm. The dilution of the extract for measurement was kept constant, and was made using the same solvent. Instrument settings and plotter sensitivity were also kept constant.

From the absorbance spectra (Figure 4.1.) it was apparent that DMSO with HCl extracts more anthocyanin at 535 nm than does methanol and HCl. This peak of anthocyanin at 535 nm was more evident in extracts from red leaved plants in both solvent systems. In all subsequent work, anthocyanin levels were measured only at 506, 535 and 547 nm.

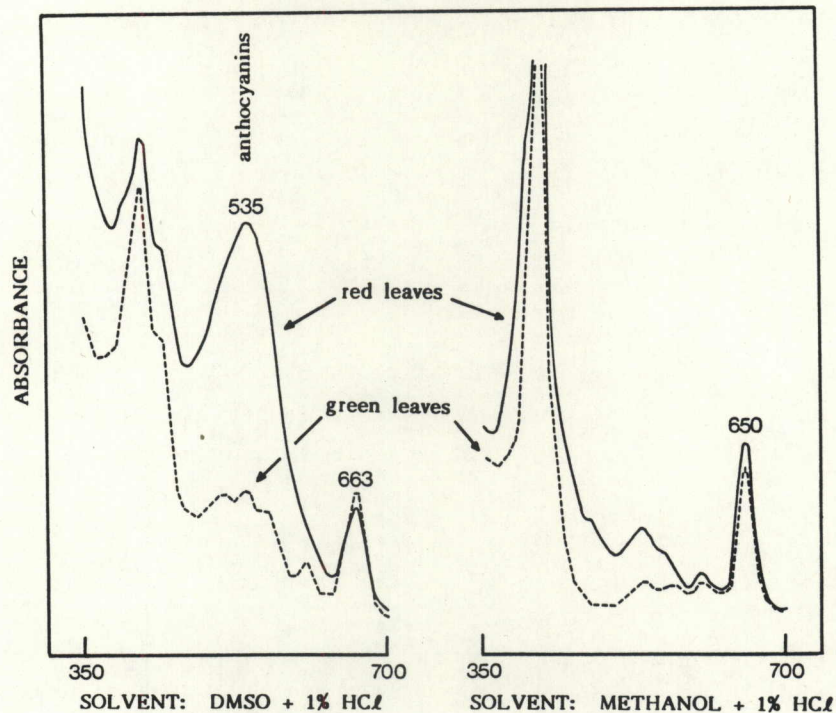


Figure 4.1. Absorption spectra of 0.05 g *Kalanchoe* leaf material taken from green vegetative plants (----) and red pigmented plants (—) and extracted using different acidified solvents.

A comparison was made of the chlorophyll extraction techniques of HARBORNE (1973) using 80 per cent acetone with a trace of Na_2CO_3 to prevent pheophytin formation, and that of HISCOX and ISRAELSTAM (1979) using absolute DMSO (dimethyl sulphoxide). In the former, 0.05 g leaf tissue was macerated and the mixture shaken for three hours at room temperature. In the case of DMSO, whole tissue was incubated in 4 ml DMSO at 60 C for three hours. Absorption spectra were obtained over a range of 300 nm - 700 nm. The absorption spectra for extracts using these two solvents, (Figure 4.2.) indicate that acetone is more efficient at extracting chlorophyll a. at 663 nm. Ethanol and methanol extractions

produced peak values similar to DMSO, the results of which are not presented here. This is contrary to the work of HISCOX and ISRAELSTAM (1979) who found that DMSO was most efficient. It was nevertheless advantageous to use DMSO, as large numbers of samples could be investigated relatively easily. Results obtained later, also indicated that DMSO provided consistent values between similar samples. There was also little loss of absorbance over storage times of several months. In all subsequent work, values of absorbance were only determined at wavelengths of 663 nm and 645 nm, the peaks associated with chlorophylls a. and b.. The absolute concentrations of chlorophyll were not determined, as it was the relative amounts which were of interest. In later experiments, freeze dried tissue was used to avoid the possible influence of varying leaf succulence (section 4.2.3.).

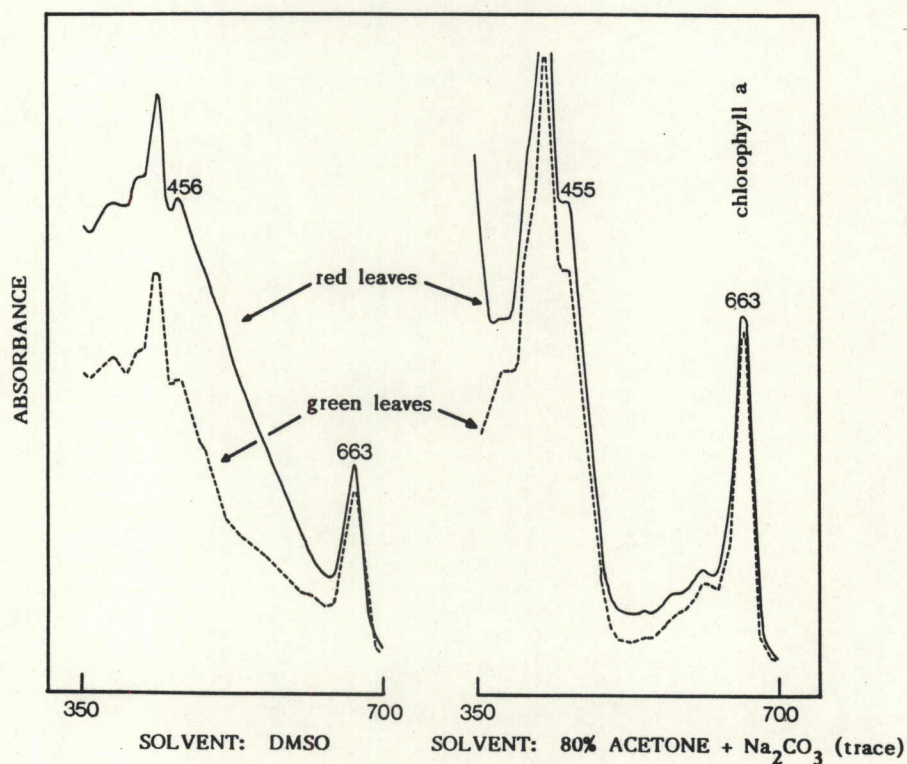
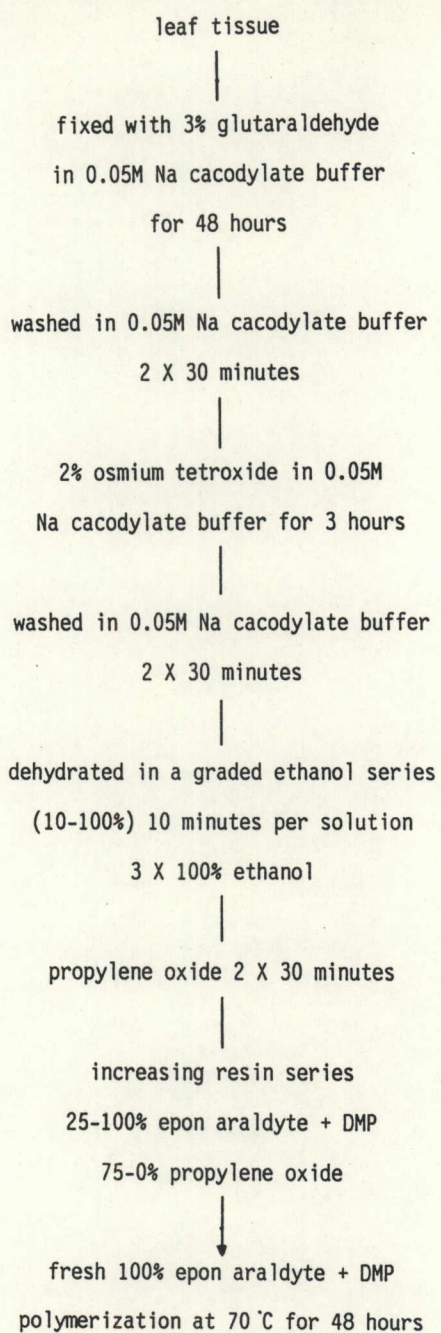


Figure 4.2. Absorption spectra of 0.05 g *Kalanchoe* leaf material taken from green vegetative plants (---) and red pigmented plants (—) and extracted using different acidified solvents.

Anatomical investigation

Leaves from flowering and vegetative plants were selected with a cross-lamina width of approximately one centimetre. The leaf tissue was fixed and embedded in resin according to the procedure outlined below. Thick sections for light microscope investigation were cut on an ultramicrotome with a glass knife. These were mounted on glass slides, stained with Ladd's Multiple Stain, and photographed using an Olympus BH2 microscope. Thin sections for transmission electron microscopy were cut using a glass knife. These were mounted on copper grids and stained with uranyl acetate and lead citrate for fifteen minutes each (REYNOLDS, 1963). These sections were examined with a Joel 100 CX electron microscope with an acceleration voltage of 80 kV.

Technique for resin embedding of Kalanchoe leaf material.



4.2.2. The effect of culture conditions and nutrients on the flowering of Kalanchoe explants

Kalanchoe nodal explants derived from vegetative parent plants grown in vitro under a light intensity of $35 \mu\text{Em s}^{-2} \text{ s}^{-1}$ at 25°C were used for all treatments. The basal medium employed, was low nutrient TANIMOTO and HARADA, (1981b) medium (Table 2.2.a.). Container size and medium volume were varied as indicated in Table 4.1.. The flasks of treatment A. were sealed with commercial PVC cling-wrap, to restrict gaseous exchange, while all other culture vessels were closed with non-absorbant cotton wool stoppers. Cultures were maintained under $110 \mu\text{Em s}^{-2} \text{ s}^{-1}$ (photosynthetically available radiation) in SD conditions at 25°C .

Table 4.1. Altered physical culture conditions for the culture of Kalanchoe explants under inductive SD (8 hour day). All cultures were grown on TANIMOTO and HARADA (1981b) medium (Table 2.2.a.) with ten replicates for each treatment.

<u>Treatment</u>	<u>Physical condition</u>
Control	40 ml medium in 100 ml Erlenmeyer flasks
A.	as above, sealed with PVC film
B.	20 ml medium in 100 ml Erlenmeyer flasks
C.	20 ml medium in 50 ml Erlenmeyer flasks
D.	10 ml medium in 40 ml test-tubes

Nutrient effects

Explants were cultured on altered media as indicated in Table 4.2.. Temperature was set at 25 °C under SD (8 hour day) conditions at 110 $\mu\text{Em s}^{-2}$ (PAR).

Table 4.2. Medium alterations employed to test the effect on the flowering performance of *Kalanchoe* nodal explants cultured in inductive SD (8 hour day). Forty millilitres of medium were used in 100 ml Erlenmeyer flasks stoppered with non-absorbant cotton wool. Ten replicates were cultured for each treatment.

<u>Treatment</u>	<u>Medium</u>
E.	Full MS (MURASHIGE and SKOOG, 1962) medium
F.	Full MS salts without NH NO
G.	50% MS salts without NH NO
H.	20% MS salts without NH NO (= Control TANIMOTO and HARADA, 1981b medium)
I.	20% MS salts with 50% NH NO
J.	20% MS salts with full NH NO
K.	20% MS salts without NH NO + Full MS vitamins

* (Full NH NO refers to 1650 mg l^{-1}).

Flower pigmentation

The flowers of Treatments A. - K. were tested for anthocyanin and chlorophyll content. Fresh flowers were extracted for pigment as in section 4.2.1. A sample of 0.1 g fresh mass from each treatment was extracted and the absorbance determined at 535 nm for anthocyanins and 663 nm for chlorophyll (Table 4.9.).

Ethylene determination

Samples of the atmosphere inside the flasks of treatment A. and the control (Table 4.1.) were taken by inserting a micro-syringe needle through the cotton wool stopper and withdrawing 0.1 ml of gas for ethylene determination. This gas was injected into a Varian Gas Chromatograph Series 3700 fitted with a Porapak S/S column (length 2 metres; I.D., 2 mm; mesh range 100/120; maximum temperature 250 °C). The machine was set with the Ion Detector at 300 °C; the Injection Port at 80 °C; and the column at 50 °C for maximum separation. Ethylene was detected using a flame ionization detector (FID), and a record was made using a Linear plotter.

4.2.3. The effect of carbohydrate and nitrogen on the in vitro flowering of Kalanchoe

Vegetative Kalanchoe nodal explants were cultured on TANIMOTO and HARADA (1981b) medium with varying concentrations of sucrose from zero per cent to six per cent. These cultures were maintained in SD (8 hour day) conditions as described in section 4.2.. After all likely plants had reached anthesis, the experiment was terminated and various vegetative and flowering parameters measured.

Leaf area

Leaf area of all plants was measured while still fresh with a LI-COR, inc. LI-3100 Area Meter.

Pigment levels

Anthocyanin and chlorophyll were extracted from freeze dried tissue and the levels of these pigments determined as described in section 4.2.1..

Endogenous carbohydrate levels

Endogenous free sugars and starch in leaf tissues were extracted as shown in Figure 4.3. and the endogenous concentrations determined as in Figures 4.4. and 4.5. using techniques adapted from EBELL (1969a,b). The standard for free sugar determination was D-glucose at concentrations of $0-100 \mu\text{g ml}^{-1}$ in a saturated benzoic acid solution. The standard for starch determination was D-glucose at concentrations of $0-90 \mu\text{g ml}^{-1}$ in a 0.25 per cent benzoic acid solution.

Figure 4.3. Method used for soluble free sugar and starch extraction from leaves of *Kalanchoe* (adapted with modifications from EBELL, 1969a,b).

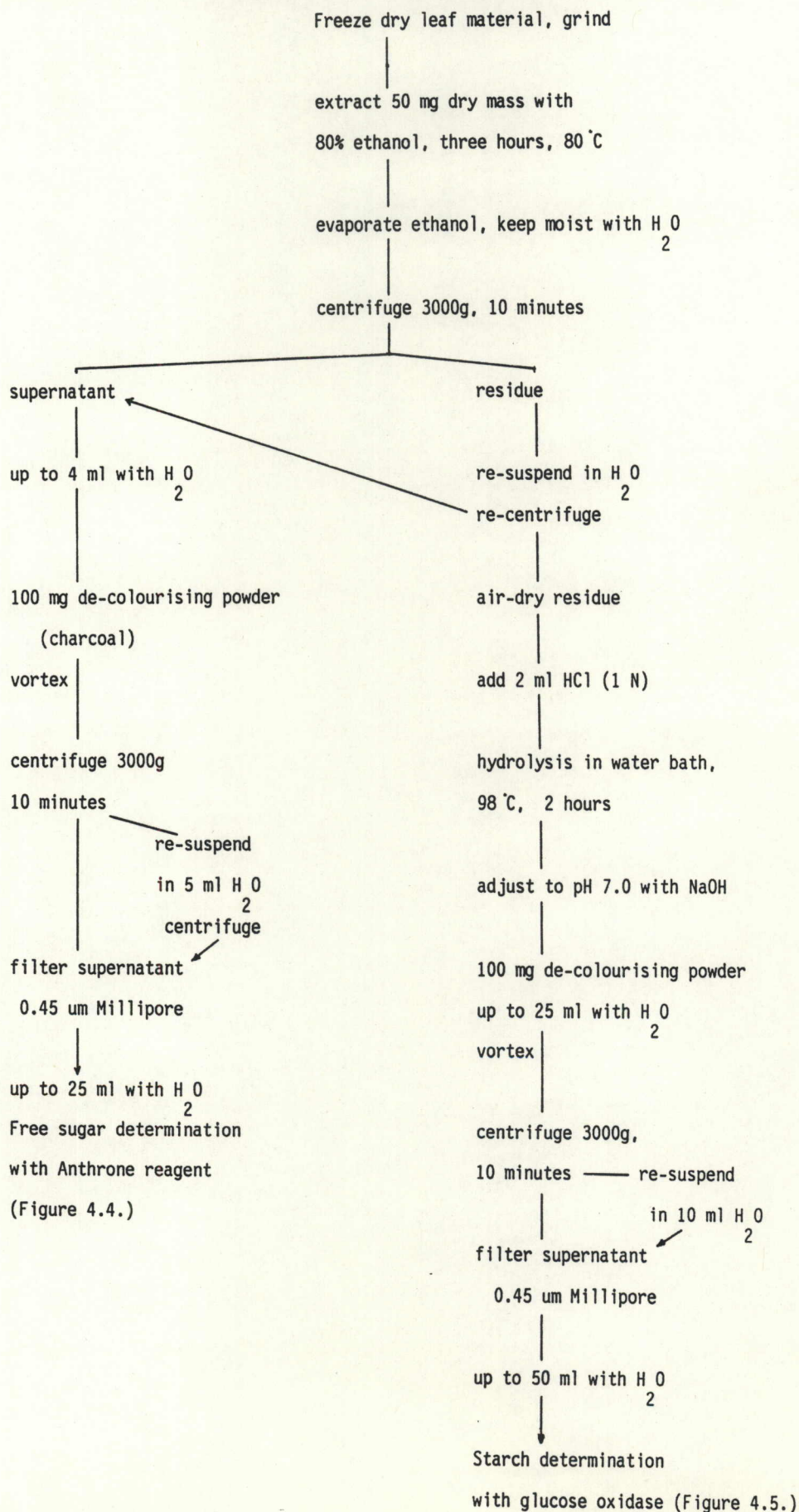
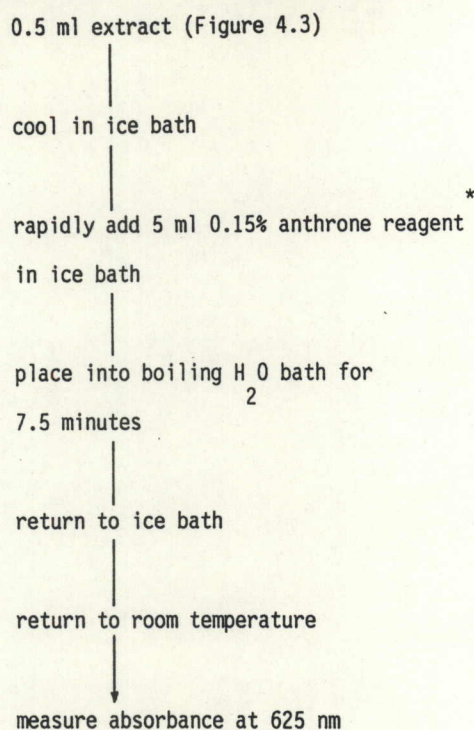


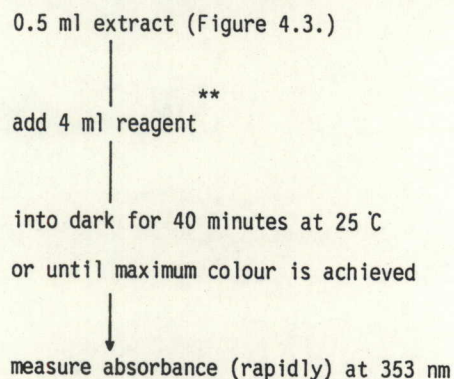
Figure 4.4 Determination of soluble free sugars using anthrone reagent (adapted from EBELL, 1969a).



* Anthrone reagent: 1.5g anthrone in 1000 ml solvent

solvent = 20% ethanol : H₂SO₄ (1:3)

Figure 4.5. Determination of starch content using glucose-oxidase (adapted from EBELL, 1969b).



** Reagent: Dissolve C in 100 ml each of A and B. Dilute to 250 ml with H₂O, adjust to pH 6.0; keep cold in the dark.

A. 41g KH₂PO₄ ; 21g K₂HPO₄ ; 6.5g (NH₄)₂MoO₇ · 4H₂O; up to 1 litre

B. 2g KI in 100 ml H₂O (use fresh)

C. 1000 units glucose-oxidase (use fresh).

Anatomical investigation

Leaves of equal size (lateral width) from each of the treatments, were embedded and sectioned as described in section 4.2.1.. One variation was introduced, in that caffeine (1.0 per cent) was added to the fixatives during fixation. This was done in order to stabilize and prevent the re-distribution of anthocyanin during embedding (DUKE and VAUGHN, 1982). This also results in greater contrast in the sections, as the caffeine penetrates the membranes, rendering them more electron dense (CHAREST, BRISSON and IBRAHIM, 1986).

Nitrogen effects

Kalanchoe nodal explants with leaves were taken from vegetative plants grown in LD (18 hour day) and cultured on modified TANIMOTO and HARADA (1981b) medium (Table 2.2.a.). Nitrogen (both NO_3^- and NH_4^+) was initially eliminated from the medium and later added in different ways as indicated in Table 4.3.. Additions to the medium of some treatments were made after 28 days, by injecting small volumes of concentrated filter sterilized solutions of the salts NH_4NO_3 and/or KNO_3 through the cotton wool plugs in the top of the flasks. The salts added at "Full strength" were according to the concentrations recommended by MURASHIGE and SKOOG (1962). This added liquid was rapidly absorbed by the agar gelled medium. Day-lengths were varied as shown in Table 4.3..

Table 4.3. Permutations of NH_4^+ and NO_3^- added to a modified nitrogen free TANIMOTO and HARADA (1981b) medium (Table 2.2.a.). Cultures with *Kalanchoe* nodal explants were maintained under either LD (18 hour day) or SD (8 hour day) conditions. Ten replicates were cultured for each treatment.

<u>Treatment</u>	<u>Initial medium</u> +(Daylength)	<u>Additive after 28 days</u> +(Daylength)
L.	Zero N (LD)	Zero N (LD)
M.	Zero N (LD)	Full strength $\text{NH}_4^+ \text{NO}_3^-$ (LD)
N.	Zero N (LD)	Full strength KNO_3 (LD)
O.	Full strength $\text{NH}_4^+ \text{NO}_3^-$ + KNO_3 (LD)	-
P.	Zero N (SD)	Zero N (SD)
Q.	Zero N (SD)	Full strength $\text{NH}_4^+ \text{NO}_3^-$ (SD)
R.	Zero N (SD)	Full strength KNO_3 (SD)
S.	Full strength $\text{NH}_4^+ \text{NO}_3^-$ + KNO_3 (SD)	-
T.	Zero NH_4^+ (SD)	(LD)
U.	Full strength $\text{NH}_4^+ \text{NO}_3^-$ in (SD)	(LD)
V.	Zero NH_4^+ (SD)	Full strength $\text{NH}_4^+ \text{NO}_3^-$ (LD)

-1

*Full strength $\text{NH}_4^+ \text{NO}_3^- = 1650 \text{ mg l}^{-1}$
 $\text{KNO}_3 = 1900 \text{ mg l}^{-1}$

4.2.4. The effect of phenolics and hormones on the in vitro flowering of Kalanchoe

Phenolic investigation

Gallic acid was filter sterilized (Millipore 0.22 μm) and added to the autoclaved medium, resulting in gallic acid concentrations of 10^{-5} M to 10^{-7} M. Ten replicates were used for each treatment, cultured with Kalanchoe nodal explants and maintained in both LD and SD conditions. This experiment was repeated using the higher concentrations of gallic acid (100 - 500 mg l^{-1}) as utilized by PRYCE (1972). Ten replicates of each treatment were subjected to LD and ten to SD.

Tannic and nicotinic acids were added to the basal medium prior to autoclaving, at concentrations of 10^{-4} M and 10^{-6} M. Ten replicates were used for each treatment, maintained in SD (8 hour day) conditions.

Hormone investigation

Hormones were added to the medium at concentrations of 10^{-6} M and 10^{-7} M. Benzyladenine (BA) and naphthaleneacetic acid (NAA) were added prior to autoclaving, while abscisic acid (ABA), gibberellic acid (GA) and BA with salicylic acid were filter sterilized (Millipore 0.22 μm). Kalanchoe nodal explants with leaves were cultured onto these media. Ten replicates of each treatment were used, for both LD and SD culture. Flowering and vegetative characteristics were measured after anthesis, using techniques as described in section 4.2.3..

4.2.5. The effect of endogenous inhibitors and promoters on the in vitro flowering of Kalanchoe

Crude leaf extracts

According to SCHWABE (1972), the expressed sap of a single Kalanchoe leaf from LD culture, inhibited flowering if injected into a plant in SD conditions. Thus, in **Experiment 1.**, single, sterile leaves of similar size, were taken from either flowering or vegetative plants grown in vitro and crushed into the basal TANIMOTO and HARADA (1981b) medium (Table 2.2.a.) in each flask. These were crushed into the medium after autoclaving, but before the agar gelled. In another treatment, vegetative leaves were included before autoclaving to test the effect of the heating process on the properties of the extract. Kalanchoe nodes with leaves were cultured onto these media and maintained in SD (8 hour day) conditions.

This experiment was repeated twice with some modifications (**Experiments 2. and 3.**). Crushed leaves from both flowering and vegetative plants were added to the medium both before and after autoclaving. Two leaves were added to each flask in these later experiments. Five replicates of each treatment were maintained in SD conditions and five in LD conditions. In all three experiments, a control was employed where no crude extract was incorporated into the basal medium.

Separated leaf extracts

A basic technique modified from PRYCE (1972) was used for the extraction of inhibitors (and promoters) from Kalanchoe. The quantity of leaf material extracted from both flowering and vegetative plants was measured at a rate of two leaves of equal size per flask to be cultured. These

were frozen in liquid nitrogen, crushed and mascerated in distilled water, filtered twice, and the volume reduced to three millilitres at 35 °C under vacuum. These extracts were applied to Whatmans No.1 chromatography paper, and separated decendingly using a solvent system comprised of butan-1-ol : acetic acid : water (4 : 1 : 5 v/v), which is suitable for polar substances. The paper was divided into five Rf. values, each of which was divided into ten equisized portions. These portions were included in the medium of each flask before autoclaving. Five replicates of each Rf. were cultured with Kalanchoe nodal explants and maintained in both SD (8 hour day) and LD (18 hour day) conditions at $110 \mu\text{Em s}^{-2} \text{ }^{-1}$.

4.3. RESULTS AND DISCUSSION

4.3.1. The effects of physiological state on the in vitro flowering of Kalanchoe explants

It is evident from the results presented in Table 4.4. and Figure 4.6., that the physiological state of the parent plant influenced the flowering performance of the explants, yet the manner in which this was occurring was not clear.

Table 4.4. The effect of culture conditions and the presence or absence of leaves, on the flowering response (%) of Kalanchoe explants. Figures in parentheses are values for repeat experiments.

<u>Treatment No.</u>	<u>Day regime</u>	<u>Experiment 1.</u> (%)	<u>Experiment 2.</u> (%)	<u>Experiment 3.</u> (%)
+LEAVES	a. LD-LD	-	0	0
	b. SD-LD	-	0	0
	c. LD-SD	60	85	100
	d. SD-SD	60	75(60)	100(100)
-LEAVES	e. LD-LD	-	0	0
	f. SD-LD	-	0	10
	g. LD-SD	0	100	70
	h. SD-SD	0	50(0)	16(66)

The explants had an absolute requirement for SD inductive cycles during their culture and would not flower under LD, with the exception of one SD derived explant in the third experiment. Besides this example, the stimulus was not carried over in SD derived explants during culture, as had happened in Glycine (Chapter 3.). The well documented inhibitor of flowering in Kalanchoe, which is produced in LD (SCHWABE, 1956) may have actively overridden the presence of any stimulus carried over during culture. Carry over of the stimulus was achieved in later experiments, by modifying the medium, but only after extensive induction had taken place. This work is reported in section 4.3.2..

Explants derived from LD conditions and cultured in SD (LD-SD), seemed to have a greater capacity to flower than explants derived from SD's (SD-SD). (This was also the case in Glycine as was reported in Chapter 3.) This was particularly evident in explants without leaves. This latter treatment (SD-SD) without leaves produced highly inconsistent results, and was repeated five times. All that could be concluded from these results, was that the plants did not flower readily, and were stunted vegetatively. The likely explanation is that plants taken from vegetative plants were more robust and more able to flower, while SD derived explants were either depleted of reserves due to having already produced flowers, or were committed to flowering and could not readily revert back to vegetative growth. It usually occurred that some vegetative growth, even just leaf expansion, took place before flowers were produced.

The rate at which explants began to flower (Figure 4.6.) is a measure of the physiological state or inclination of the explants to produce flowers. It is noticeable that explants with leaves transferred into SD conditions (Treatments c. and d.) flowered most rapidly. Flowering in explants without leaves transferred to SD conditions (Treatments g. and h.) was delayed, as they produced some leaves before flowering. These leaves were generally small in size.

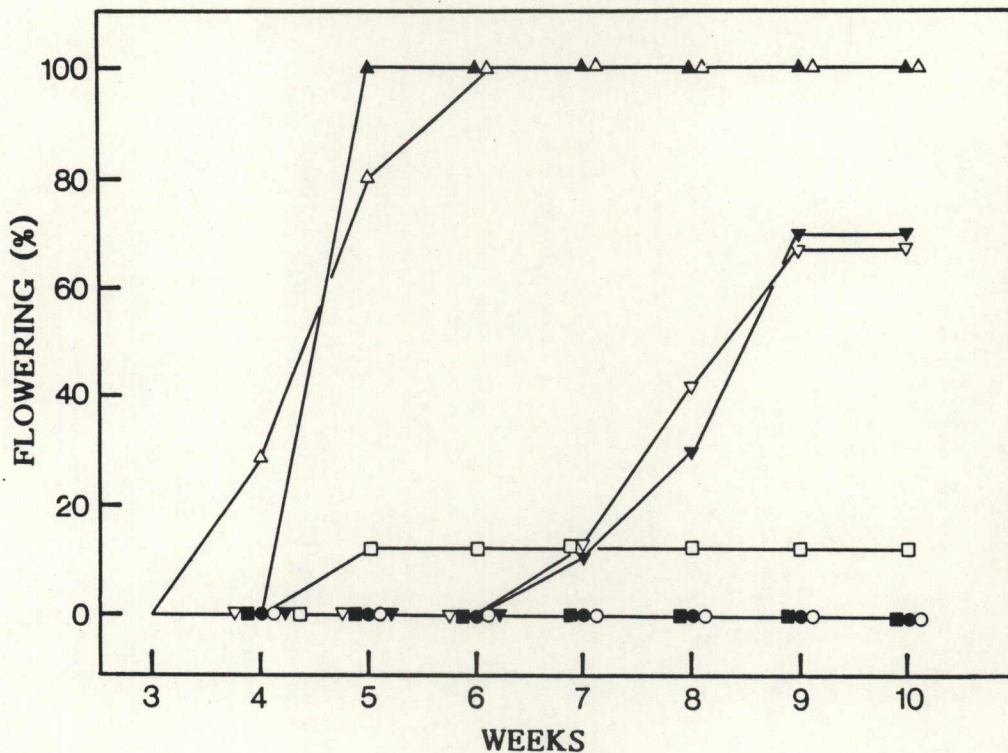


Figure 4.6. Rate of flowering of *Kalanchoe* explants with (a. - d.) or without (e. - h.) leaves after exposure to different day-lengths. Treatment a.- ● (LD-LD); b.- ○ (SD-LD); c.- ▲ (LD-SD); d.- △ (SD-SD); e.- ■ (LD-LD); f.- □ (SD-LD); g.- ▼ (LD-SD); and h.- ▽ (SD-SD). Ten replicates were used for each treatment.

Measurement of the shoot, root and flower masses of all these explants after the termination of the experiment, provided some interesting results (Figure 4.7.).

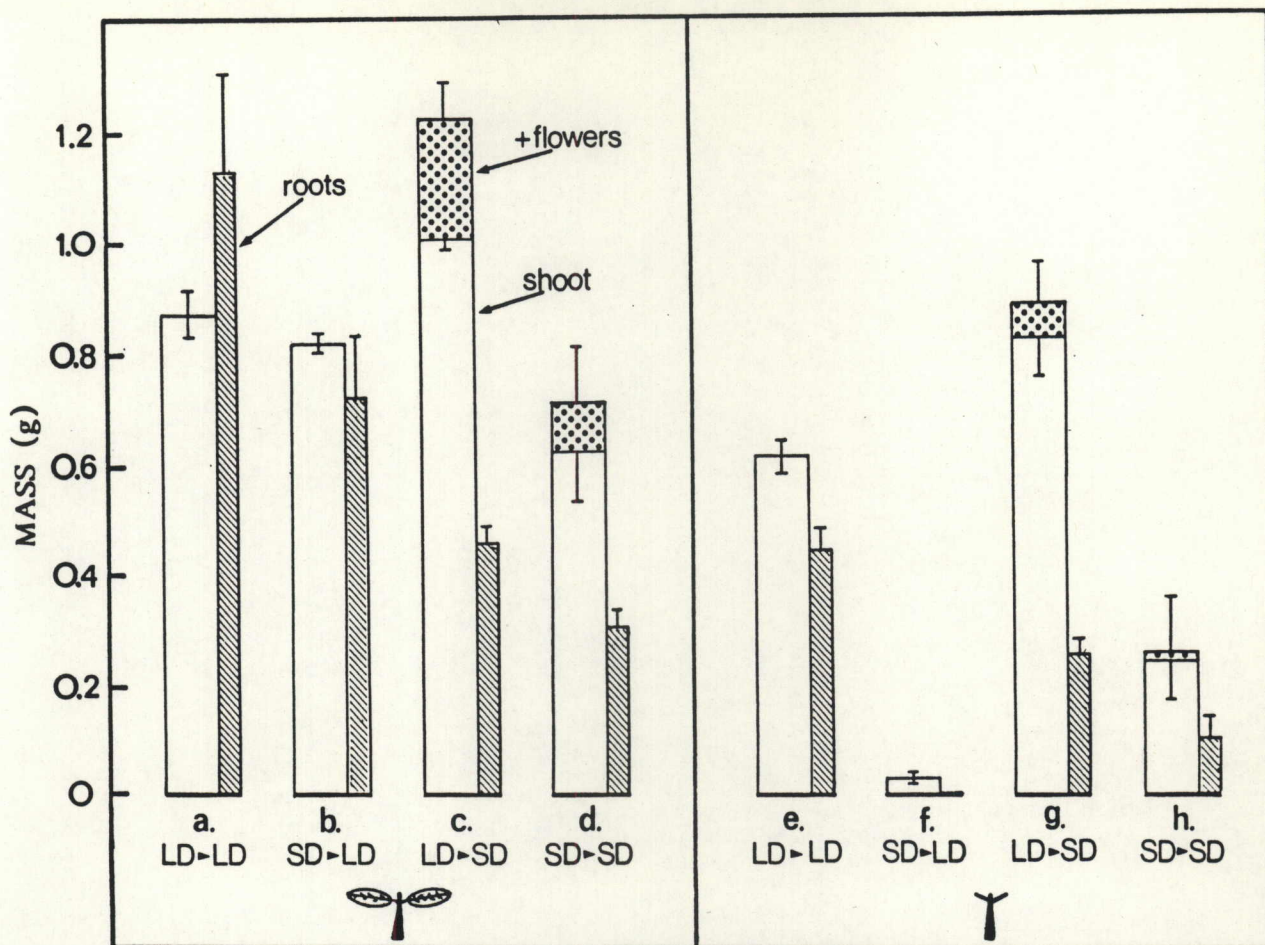


Figure 4.7. Mass of shoots, flowers and roots produced by *Kalanchoe* explants with (a. to d.) or without (e. to h.) leaves. Explants were taken from plants grown under either LD or SD and cultured under either LD or SD as indicated. Bars represent the standard error on the mean.

It was noticeable that with the increasing involvement of SD inductive cycles, there was a decline in the mass of roots produced. This supports the theory that flowering and rooting are antagonistic to one another (GASPAR, 1980). It is also clear that explants subjected to LD-SD treatments (c. and g.) produced the greatest vegetative growth. This was unexpected, as these explants were exposed to half of the total photon flux compared to LD-LD (a. and e.) plants, as a result of the shorter day-lengths. Much of this increased mass was probably due to differences in succulence, as SD plants accumulate much more liquid than LD plants (GUMMER, 1948; ZABKA AND McMAHON, 1965).

Unfortunately, dry masses were not determined for all samples, as the tissue was used for pigment determination. The exceptions were the explants from Treatments b. and d., which were measured for dry mass (Table 4.5.). Leaves from Treatment d. (SD-SD with leaves) were 3.15 fold more succulent than those from Treatment b. (SD-LD with leaves), which indicates that although explants from Treatment b. (SD-LD) were of similar mass to those from Treatment d. (SD-SD), they had assimilated more carbon.

Table 4.5. The succulence of leaves taken from explants exposed to Treatments b. (SD-LD with leaves) and d. (SD-SD with leaves). Equisized fresh mass samples were taken from each treatment.

	<u>Fresh mass (g)</u>	<u>Dry mass (g)</u>	<u>Succulence</u> <u>(Fresh/Dry mass)</u>
Treatment b.	2.645	0.359	7.37
Treatment d.	2.651	0.114	23.25

A noticeable feature of these manipulation treatments, was that the explants adopted vastly different leaf colours during culture, depending on both the daylength that the parent plant was exposed to, as well as the daylength that the explant was cultured in (Figure 4.8.). The most outstanding red colour was visible where explants with leaves were taken from SD parent plants and cultured in SD conditions (Treatment d.). These took on an intense red colour after two weeks. This colour persisted for several weeks, but faded as the explants grew. This decrease is represented below in Table 4.6. and in Figure 4.8.

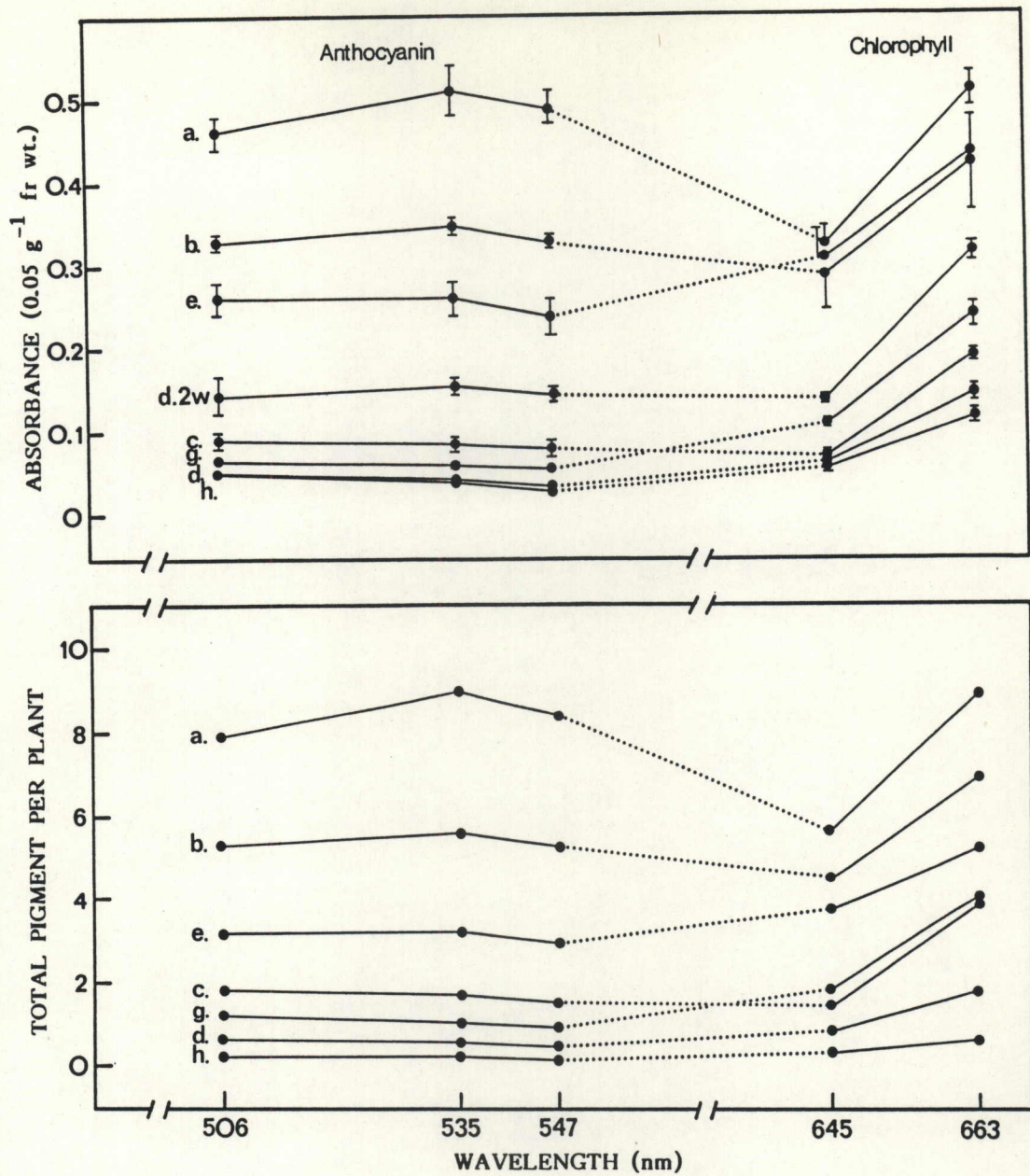


Figure 4.8. Anthocyanin and chlorophyll levels in the leaves of *Kalanchoe* explants after ten weeks of culture. Explants were taken with (a. - d.) or without (e. - h.) leaves after exposure to different photoperiods. Treatment a. (LD-LD); b. (SD-LD); c. (LD-SD); d. (SD-SD); e. (LD-LD); f. (SD-LD); g. (LD-SD); and h. (SD-SD). Pigment levels are also shown for treatment d. after two weeks of culture (d.2w). Ten replicates were used for each treatment.

Table 4.6. The change of leaf pigment levels in explants taken from parent plants grown in SD, and cultured in SD (Treatment d.). The results are means of three replicates \pm standard error.

	<u>Absorbance</u>				
	<u>Anthocyanin</u>	<u>Anthocyanin</u>			<u>Chlorophyll</u>
	506nm	535nm	547nm	645nm	663nm
Two weeks after culture	0.145 \pm 0.002	0.154 \pm 0.001	0.146 \pm 0.001	0.137 \pm 0.002	0.314 \pm 0.01
Eight weeks after culture	0.050 \pm 0.002	0.043 \pm 0.002	0.034 \pm 0.002	0.067 \pm 0.006	0.148 \pm 0.01

The lower absorbance values after eight weeks for extracts from leaves derived from SD and cultured in SD (Treatment d.) were used in the graphs of Figure 4.8. despite the fact that pigment levels were higher after two weeks of culture (d.2w Figure 4.8.). This was necessary, as the explants could only be sacrificed for pigment determination after anthesis. Thus, the differences indicated between the pigment levels of leaves from all treatments in Figure 4.8. do not represent the situation when colour differences were most noticeable. It is interesting to note though, that while the anthocyanin levels of visibly red leaved explants exposed to

SD-SD conditions (Treatment d.) were expected to be extremely high, they were still lower than the levels of pigments in leaves from Treatments a., b. and e. which were associated with LD and were more green in colour. If represented as total pigment per plant, (Figure 4.8.) the situation did not change markedly. These apparently misleading results may have been different if pigments had been determined on a dry mass basis. It was found that leaves from plants exposed to SD-SD conditions (Treatment d.) were more succulent than those exposed to SD-LD conditions (Treatment b.) (Table 4.5.), indicating a possible reason for some of the discrepancy in the levels of pigments. Multiplying the anthocyanin and chlorophyll absorbance values for the more succulent leaves from Treatment d. by the resulting conversion factor of 3.15, still does not bring the pigment levels up to a comparable level with leaves from Treatment b.. It does not therefore seem likely that the low anthocyanin levels found in the red coloured succulent leaves, are exclusively a result of dilution of the cytoplasm, but are a real difference. This does not explain though, the red colour of the leaves in Treatment d. (SD-SD with leaves). It is possible that the low anthocyanin levels in red coloured leaves may have been due to the unmasking of the red pigment, either by the lower chlorophyll levels, or by the swelling of the cells. The absorbance spectra of representative samples of each treatment are presented in Figure 4.9..

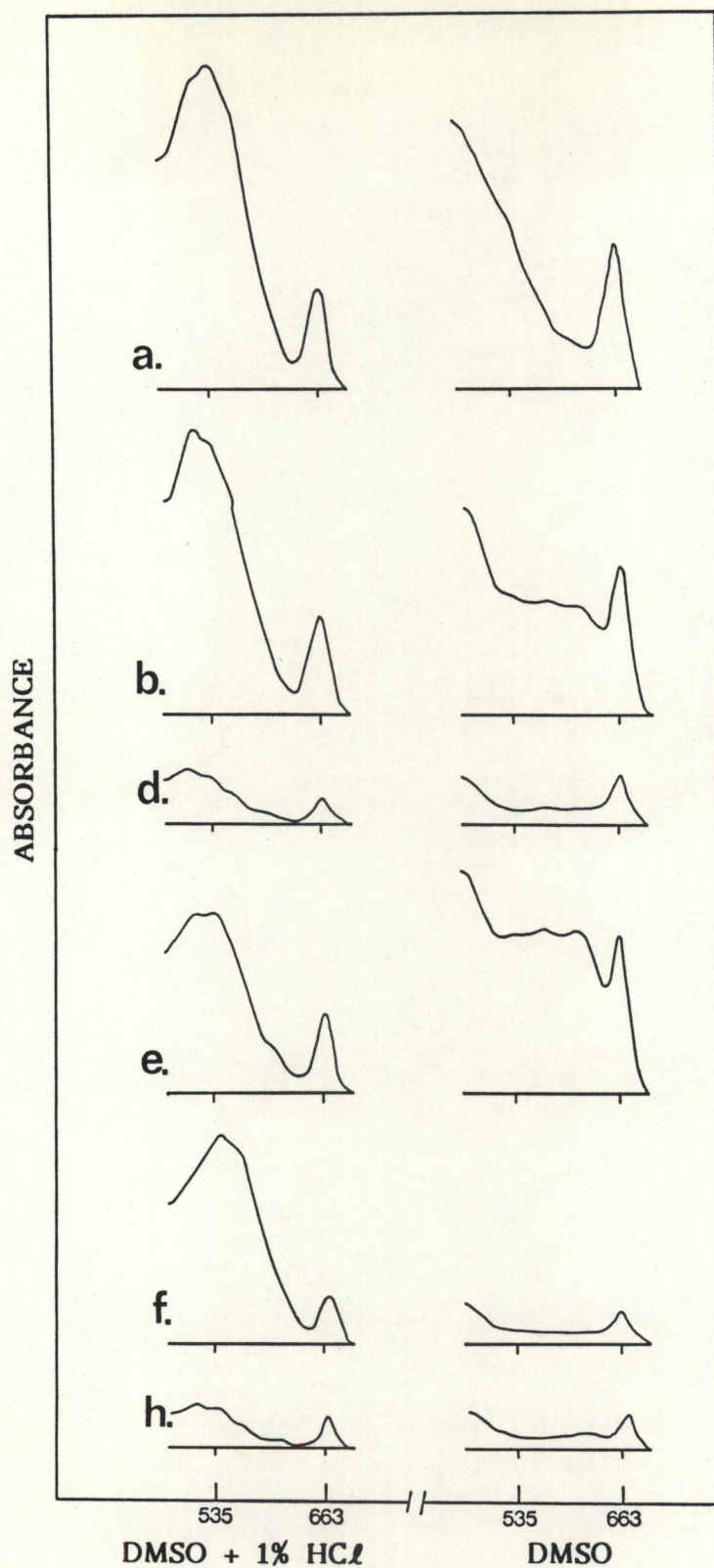


Figure 4.9. Absorption spectra of pigments from extracts from *Kalanchoe* leaves taken from Treatments a, (LD-LD); b, (SD-LD); and d, (SD-SD), all which had leaves at the time of culture, and from Treatments e, (LD-LD); f, (SD-LD); and h, (SD-SD), which had no leaves at the time of culture. DMSO + HCl was used to extract the anthocyanins, and DMSO for chlorophyll.

Anatomical sections of leaves exposed to SD-LD conditions (Treatment b.) and those exposed only to SD (Treatment d.) indicated that the leaf from the LD derived plants of Treatment b. had a dense layer of pigment, probably anthocyanin, in the sub-epidermal layer (Plate 4.2.). In the more succulent and apparently red leaf of SD derived Treatment d., the cells containing this pigment were separated by pigment-free cells. It is possible that the red appearance of leaves from Treatment d., is a result of the lower chlorophyll levels, which are masking the presence of the anthocyanins in leaves exposed to LD (Treatment b.). The thinner leaves from LD plants do have a darker, more densely coloured appearance, which does support this possibility. The masking of the anthocyanins by chlorophyll was also noted in a later experiment, where pigment levels were determined on a dry mass basis (section 4.3.3.).

The present results are contrary to those presented by NEYLAND, NG and THIMANN (1963), who reported that levels of anthocyanin increased in leaves of Kalanchoe in response to SD inductive cycles. They also noted that the levels of leuco-anthocyanidin, a colourless precursor of anthocyanin, decrease in response to SD. No consideration was given to the succulence of the leaves during experimentation, and it is assumed that fresh tissue was used for the extraction. Leuco-anthocyanidin levels were determined by measuring the absorbance of extracts using vanillin and HCl in 30 per cent ethanol. An acidic aqueous extract of the leaves was used to determine anthocyanin levels. This extraction procedure was not recommended by HARBORNE (1973), because although anthocyanins are soluble in water, extraction is likely to be incomplete.

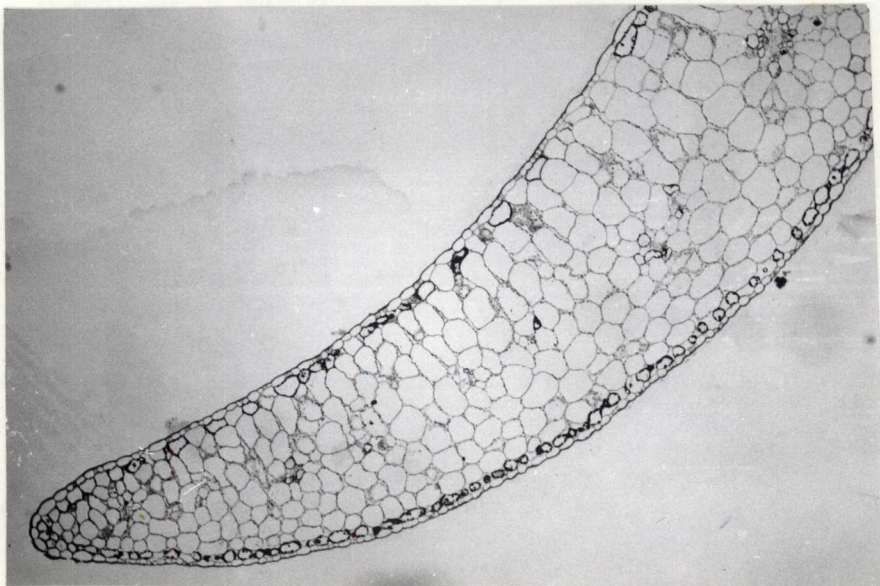
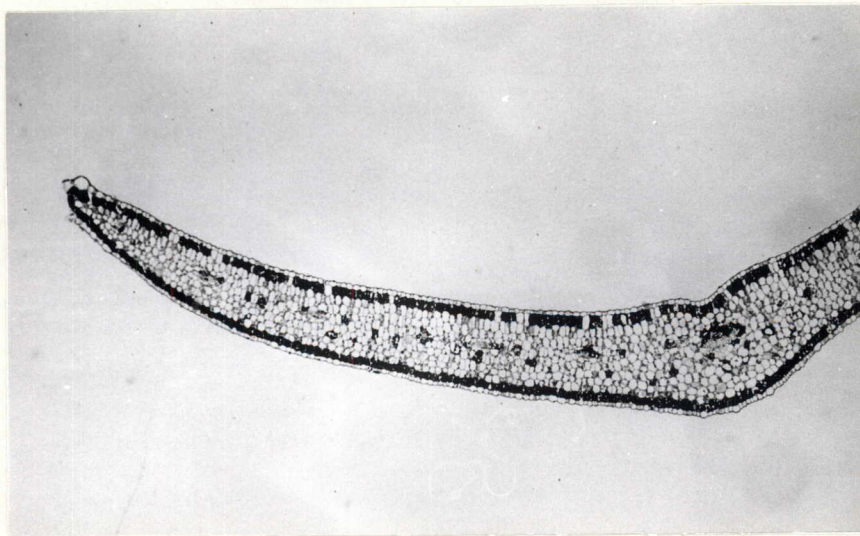


Plate 4.2. Cross sections of leaves taken from Kalanchoe explants grown under LD conditions (top) and SD conditions (bottom). Sections were cut from leaves of equal width. Magnification X 20.

According to HARBORNE (1973), the anthocyanins are aglycones formed when anthocyanidins are hydrolysed with acid. The extraction of tissues for anthocyanins by hydrolysing with strong acids, may result in the conversion of colourless leuco-anthocyanidins to form coloured delphinidin, cyanidin and pelargonidin amongst others. This may give false values for the amount of coloured pigment in the tissue, and may be the reason for the discrepancy in the results presented here, and those of NEYLAND, NG and THIMANN (1963). It is possible that the documented change in the acidity of Kalanchoe leaves (SCHWABE, 1958) in response to photoperiod, may have some effect on the colour of the leaves. Kalanchoe leaves were found to contain more acid, especially malic acid, in SD than in LD if expressed on a fresh mass basis. Culturing explants on a TANIMOTO and HARADA (1981b) medium acidified to pH 4.5 with HCl, did not result in any visible difference in the colour of the leaves.

The succulence of the leaves, or the degree of cell expansion is most apparent (Plate 4.2.), leaves of the same width being thicker in SD than in LD. This is in agreement with the work of GUMMER (1949) and SCHWABE (1958). This increase in thickness is due to an increase in cell expansion and not cell division, as both leaves were approximately ten cells thick. According to ESAU (1965), the number of cells at right angles to the plane of the leaf, is constant. Yet it is clear that the LD derived vegetative leaf had many more cells laterally from margin to margin than the SD derived leaf. This could be due to the SD leaf of the same size being physiologically and morphologically younger than the LD leaf, the marginal meristem (ESAU, 1965) having produced fewer cells. This means that leaves of the same age as determined by number of cells, would be larger in both thickness and surface area if derived from SD conditions. This is contrary to the conclusions of GUMMER (1949) and

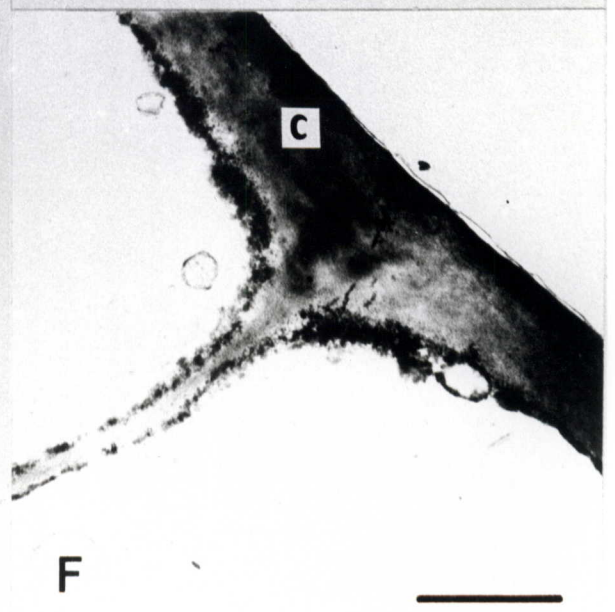
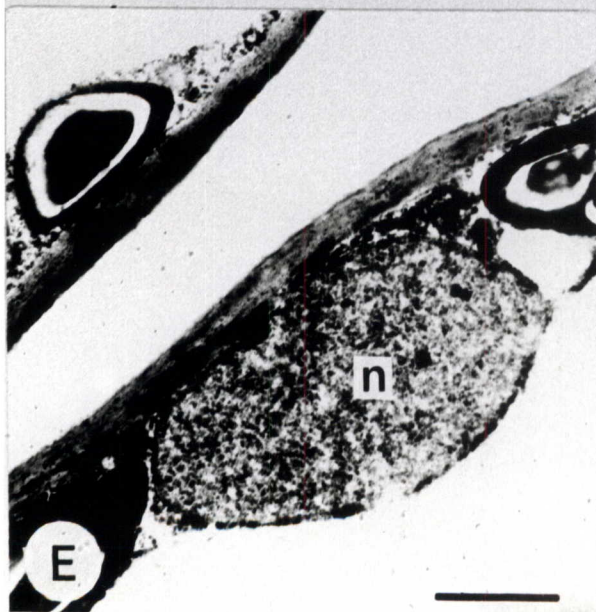
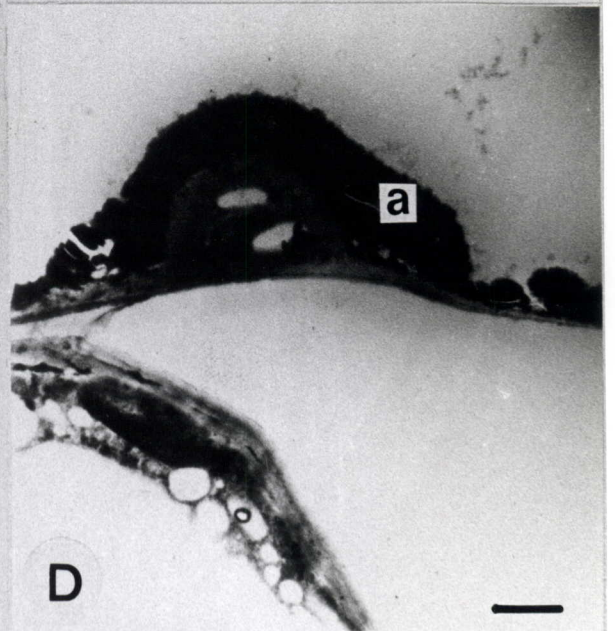
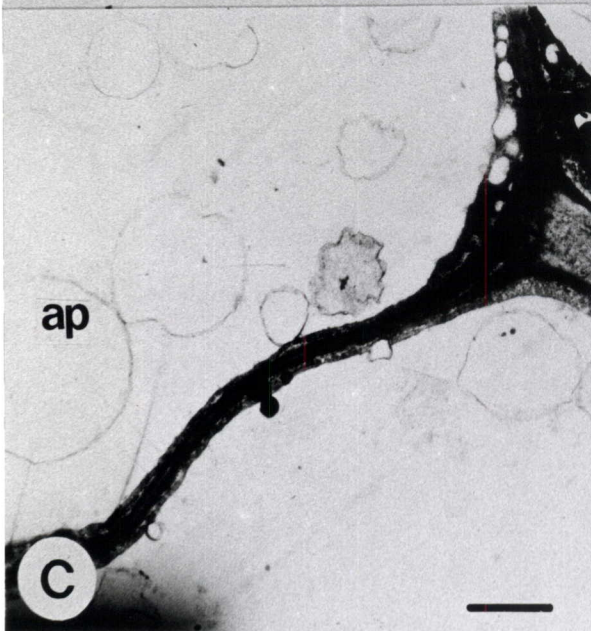
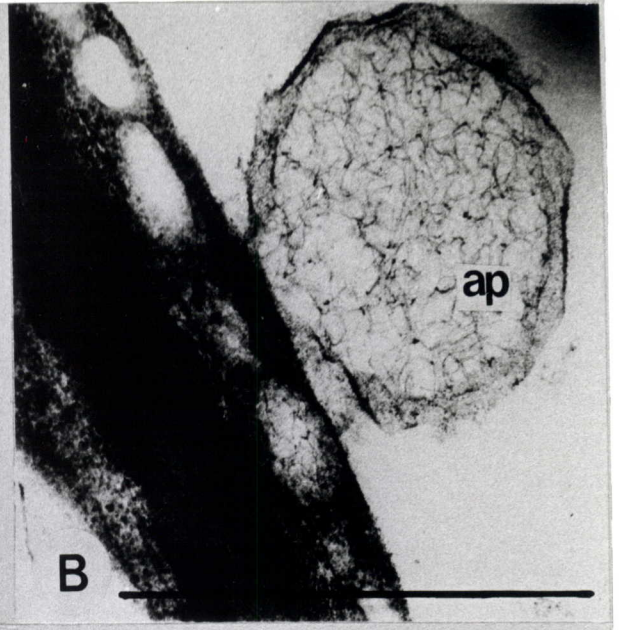
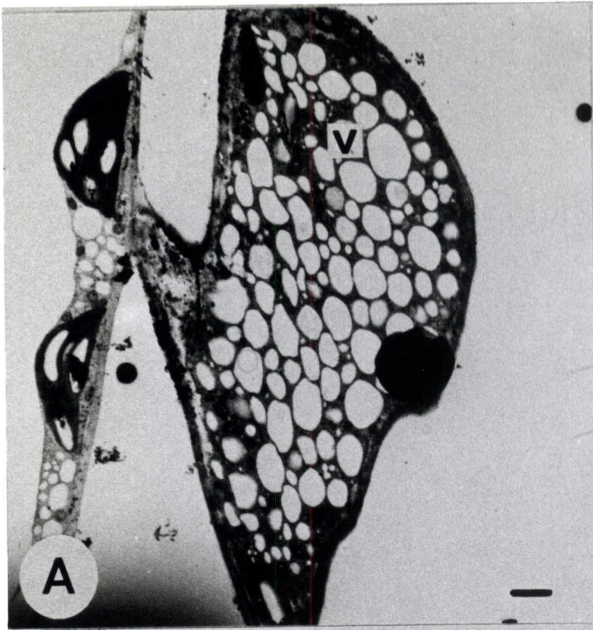
SCHWABE (1958) who stated that expansion only occurred at right angles to the surface of the leaf. It is clear from the light micrographs in Plate 4.2., that the cells had expanded in all directions.

The most outstanding difference between in vitro grown LD vegetative and SD flowering derived leaf tissue (Treatments b. and d.) at the ultrastructural level, was the presence of large numbers of small globular vacuoles which were found in the leaves of flowering plants (Plate 4.3.A.). These vacuoles were often associated with chloroplasts in the thin layer of dense cytoplasm around the perimeter of the cell.

Also found in leaves derived from both vegetative and flowering plants, were large organelles filled with fibrillar material (Plate 4.3.B. and C.), which according to NOZZILILLO (1972), PECKET and SMALL (1980), and DUKE and VAUGHN (1982) are anthocyanoplasts. These organelles are probably responsible for the production of anthocyanins and have been observed spilling their contents into the cell vacuole (DUKE and VAUGHN, 1982).

Cells containing anthocyanin were difficult to section (Plate 4.3.D.), but where successful, showed large masses of electron-dense material lying against the cell wall and over the chloroplasts. In the leaves of vegetative plants, (Plate 4.3.E. and F.), no secondary vacuoles were present, while anthocyanoplasts were present in the epidermal cells (Plate 4.3.F.).

The presence of anthocyanoplasts in plant tissue has only been demonstrated on three previous occasions in the literature (DUKE AND VAUGHN, 1982). The function of the numerous secondary vacuoles found in cells from flowering plants, is not known.



4.3.2. The effect of culture conditions and nutrients on the flowering of Kalanchoe explants

The results of this investigation are presented in Figure 4.10. and Table 4.7.. Sealing the top of the culture vessels with PVC film (Treatment A.) had a most significant effect on flowering, which was totally inhibited. This despite the fact that the shoot fresh mass was only slightly reduced, and the dry mass was unaffected. This implies that the plant was utilizing the sucrose carbohydrate source in the medium for its growth requirements, but this was not available for flowering.

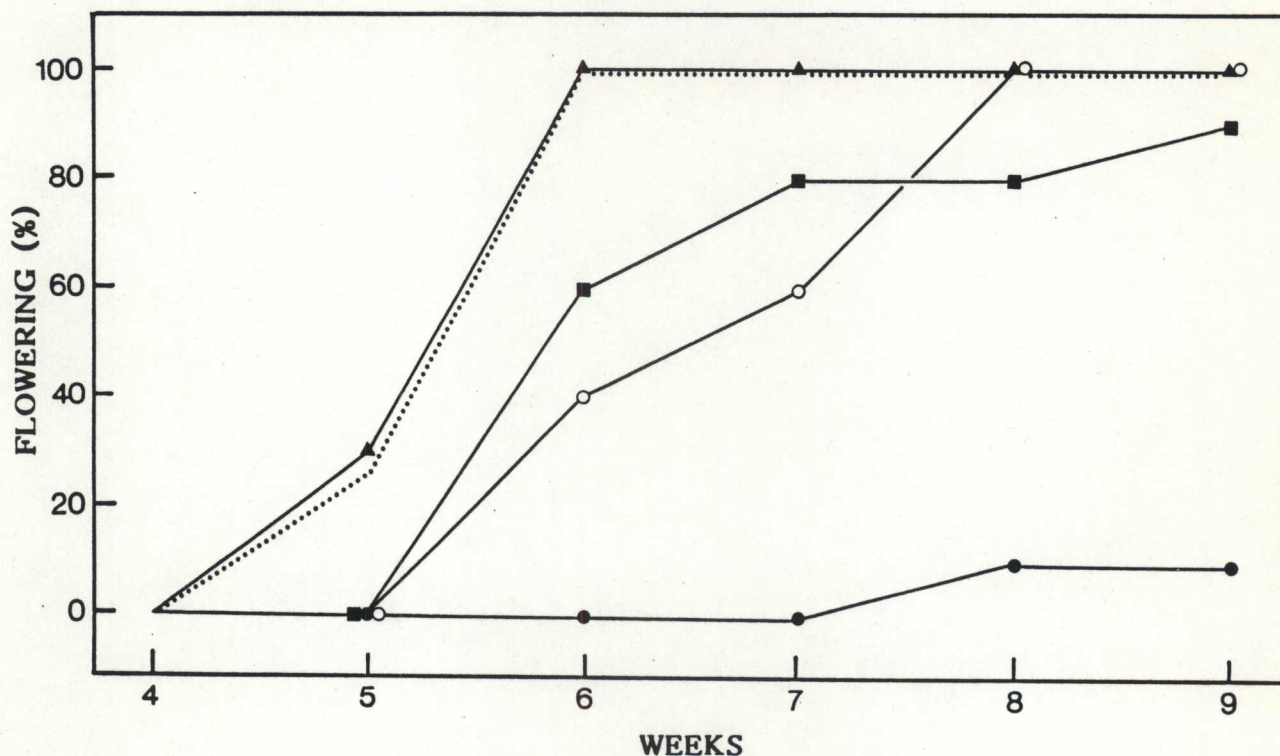


Figure 4.10. Rate of flowering of Kalanchoe explants restricted by medium and flask volume and induced to flower by SD exposure. Control (.....), 40 ml medium in 100 ml Erlenmeyer flasks; Treatment A (●) as control but sealed with PVC film; Treatment B (■) 20 ml medium in 100 ml flasks; Treatment C (△) 20 ml medium in 50 ml flasks; Treatment D (○) 10 ml medium in 40 ml test-tubes. Ten replicates were used for each treatment.

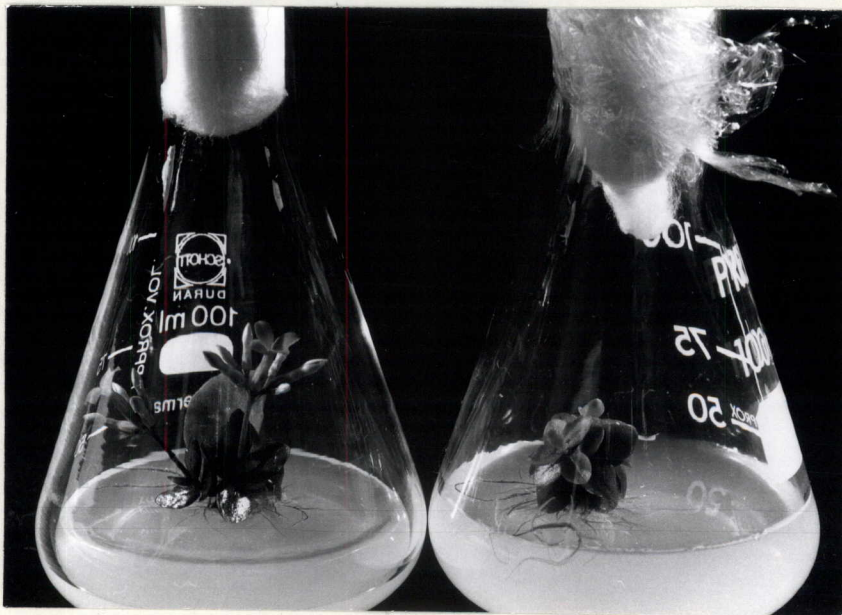
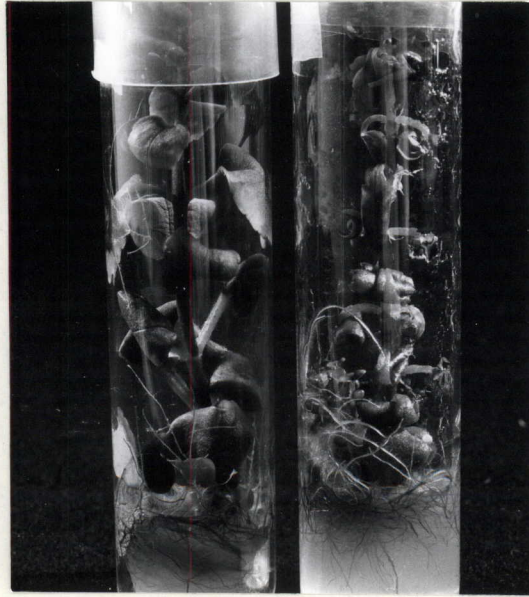


Plate 4.4. Explants of Kalanchoe after culture in test-tubes or flasks sealed with PVC wrap (right) or allowing free ventilation (left).

HARDER, BODE and VON WITSCH (1944) and IRELAND and SCHWABE (1982a) noted that CO_2 was essential for flowering of Kalanchoe in a way that could not be substituted for by the addition of carbohydrates, yet ZABKA and McMAHON (1965) noted that flowering increased in a CO_2 free atmosphere under SD. The results presented here tend to support the former conclusions, that by sealing the flask, CO_2 was excluded, inhibiting flowering in a way that could not be countered by freely available sucrose. An alternative possibility, was that ethylene build-up in the atmosphere of the flask inhibited flowering. The determination of the ethylene content in the atmosphere of these inhibited plants, when compared to flowering controls which were not sealed with plastic, revealed that there were higher ethylene levels in the flasks with inhibited explants (Figure 4.11.). Results are presented as peak height. Peak areas were 1.57 ± 0.17 for inhibited flasks, and 0.71 ± 0.32 for flowering flasks. It is not known whether these concentrations are high enough to inhibit flowering. It was also significant that the growth form of the plants differed

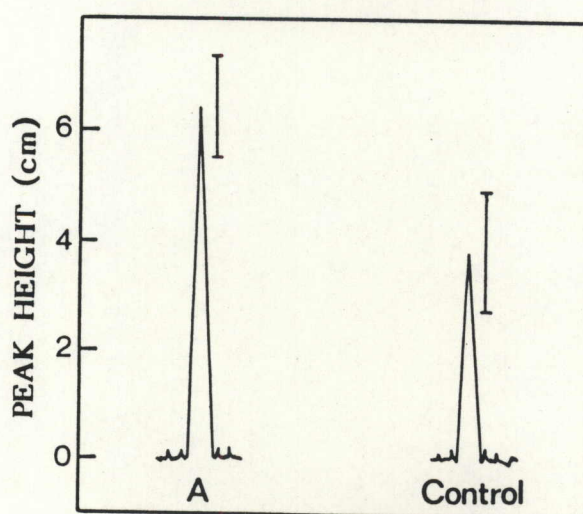


Figure 4.11. Ethylene content in the atmosphere of flasks containing Kalanchoe explants exposed to SD conditions, and sealed with PVC wrap (A.), or with cotton-wool (Control). A. did not flower, while the control flowered profusely. Values are the mean of three treatments \pm standard error.

markedly, with those in sealed flasks (Treatment A.) having small downward curved pale green leaves, with no red pigmentation (Plate 4.4.). This was also noticeable in plants grown in test-tubes.

Explants treated according to Treatments B., C. and D. (Table 4.7.) were designed to confine and restrict the growth of the roots. This was done in view of the widely recognised phenomenon of antagonism which exists between rooting and flowering (GASPAR, 1980). SHINOZAKI and TAKIMOTO (1982b) found that

Table 4.7. Vegetative and flowering characteristics of *in vitro* grown *Kalanchoe* explants cultured in SD conditions on the medium of TANIMOTO and HARADA (1981) under different culture conditions. Control, 40 ml medium in 100 ml Erlenmeyer flasks; Treatment A, as control but sealed with PVC film; Treatment B, 20 ml medium in 100 ml flasks; Treatment C, 20 ml medium in 50 ml flasks; Treatment D, 10 ml medium in 40 ml test-tubes. Ten replicates were used for each treatment. Results are presented as mean \pm standard error.

<u>Characteristic</u> <u>measured</u>	<u>Treatment</u>				
	Control	A.	B.	C.	D.
Shoot fresh mass (g)	1.06 \pm 0.13	0.78 \pm 0.10	0.64 \pm 0.06	0.69 \pm 0.02	0.38 \pm 0.02
Shoot dry mass (g)	0.025 \pm 0.009	0.026 \pm 0.003	0.027 \pm 0.008	0.03 \pm 0.00	0.02 \pm 0.01
Succulence (g fr/dry)	27.7 \pm 2.7	29.2 \pm 1.9	22.6 \pm 0.4	22.9 \pm 0.5	16.4 \pm 0.6
Root mass (g)	0.064 \pm 0.01	0.03 \pm 0.01	0.035 \pm 0.006	0.05 \pm 0.00	0.06 \pm 0.00
Flower number	13.50 \pm 1.45	-	9.66 \pm 1.20	9.9 \pm 0.6	4.0 \pm 0.3
Inflorescence mass (g)	0.14 \pm 0.01	-	0.12 \pm 0.18	0.16 \pm 0.01	0.03 \pm 0.00

Pharbitis seedlings grown in vitro in smaller containers, flowered more prolifically in non-inductive conditions than those in larger containers. These authors also found that the induction by exogenously applied substances was always accompanied by a suppression of root elongation, although there was no effect on the root dry mass. Earlier in this thesis (section 4.3.1.) results were presented which indicated that induction of Kalanchoe by SD was accompanied by a reduced mass of roots.

Reduced medium volume in the same sized container (Treatment B.), resulted in reduced root and shoot fresh mass. Flowering was slightly reduced. Similar results were found if the container size was reduced together with the medium volume (Treatment C.), although the rate of flowering was greater than explants treated according to Treatments B. and D. (Figure 4.10.). Drastic reduction of both medium and container size (Treatment D.) resulted in a greater inhibition of flowering and vegetative characteristics. It is anticipated that the principles of this reduction of flowering with a reduction in medium volume and container size, would apply in reverse if the container size was increased. Even the optimum flower numbers obtained in vitro are nowhere near the numbers obtained in pot grown plants, where flower numbers reach several hundred per plant.

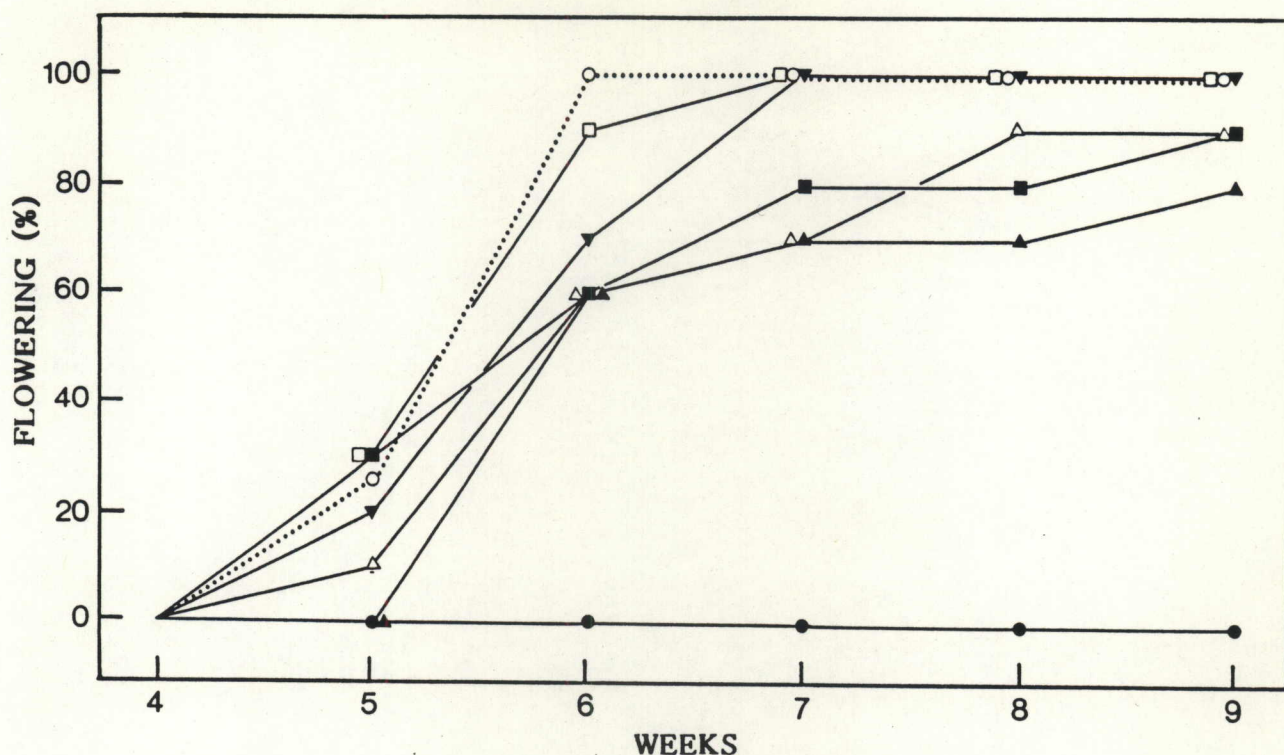


Figure 4.12. Rate of flowering of *Kalanchoe* explants cultured on different media and exposed to SD conditions. Treatment E (●) full strength MS (MURASHIGE and SKOOG, 1962) medium; Treatment F (■) salts of MS medium without NH_4NO_3 ; Treatment G (▲) 50% of the salts of MS medium without NH_4NO_3 ; Treatment H (○) 20% of the salts of MS medium without NH_4NO_3 ; Treatment I (□) 20% of the salts of MS medium with half of the recommended NH_4NO_3 ; Treatment J (△) 20% of the salts of MS medium with the full amount of NH_4NO_3 recommended; Treatment K (▼) 20% of the salts of MS medium without NH_4NO_3 but including the full vitamin component. The salt NH_4NO_3 is full strength at 1650 mg l⁻¹. Ten replicates were used for each treatment

The culture of explants onto full strength MURASHIGE and SKOOG (1962) medium (Treatment E.) resulted in the total inhibition of flowering (Figure 4.12. and Table 4.8.) with an associated inhibition of vegetative growth when compared to the control plants (Treatment H.) grown on the standard TANIMOTO and HARADA (1981b) medium (Table 2.2.a.).

Table 4.8. Vegetative and flowering characteristics of in vitro grown Kalanchoe explants cultured in SD conditions on various media. Treatment E, full strength MS (MURASHIGE and SKOOG, 1962) medium; Treatment F, salts of MS medium without NH_4NO_3 ; Treatment G, 50% of the salts of MS medium without NH_4NO_3 ; Treatment H, 20% of the salts of MS medium without NH_4NO_3 ; Treatment I, 20% of the salts of MS medium with half of the recommended NH_4NO_3 ; Treatment J, 20% of the salts of MS medium with the full amount of NH_4NO_3 recommended; Treatment K, 20% of the salts of MS medium without NH_4NO_3 but including the full vitamin component.

<u>Characteristic</u> <u>measured</u>	<u>Treatment</u>											
	E.	F.	G.	H.	I.	J.	K.					
Shoot fresh mass (g)	0.35±0.07	0.71±0.13	0.49±0.12	1.06±0.13	0.85±0.08	0.69±0.10	0.03±0.06	?				
Shoot dry mass (g)	0.02±0.01	0.03±0.01	0.02±0.01	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00					
Succulence (g fr/dry)	20.3±1.0	23.2±1.7	22.1±1.7	27.7±2.8	27.8±0.4	25.8±1.4	28.2±1.0					
Root mass (g)	0.11±0.00	0.02±0.00	0.01±0.00	0.06±0.01	0.02±0.01	0.04±0.01	0.09±0.11					
Flower number	-	7.5±1.6	7.5±1.2	13.5±1.5	9.7±1.0	8.0±1.0	13.4±0.9					
Inflorescence mass (g)	-	0.11±0.02	0.09±0.02	0.14±0.01	0.16±0.01	0.10±0.01	0.15±0.02					

MURASHIGE and SKOOG (1962) medium has high salt concentrations when compared to other nutrient media, and it is apparent that these levels are inhibitory to this system. The removal of NH_4NO_3 from MURASHIGE and SKOOG (1962) medium at full strength (Treatment F.) allowed flowering to take place, but still at a level below the control (Treatment H.). Halving the remaining salts (Treatment G.) did not make any significant difference to flowering (Figure 4.12.).

Using the low salt levels as in the control (Treatment H.), but increasing the concentration of NH_4NO_3 (Treatments I. and J.) lowered the rate of flowering (Figure 4.12.) and also the number of flowers and the shoot mass (Table 4.8.). Adding the vitamin component of MURASHIGE and SKOOG (1962) medium to the low nutrient medium, increased root mass, but otherwise had little effect on flowering (Treatment K.).

It can be concluded from these results, that full strength MURASHIGE and SKOOG (1962) medium was inhibitory of flowering in the presence of NH_4NO_3 , but not markedly if NH_4NO_3 was removed. Yet if NH_4NO_3 was added to low nutrient TANIMOTO and HARADA (1981b) medium which was normally most supportive of flowering, flowering was partially inhibited but not to the extent that inhibition occurred when NH_4NO_3 was added to full strength MURASHIGE and SKOOG (1962) medium. It thus appears that although NH_4NO_3 was slightly inhibitory of both the rate of flowering and the flower number, it is possible that this effect was partly an osmotic one. Later in this Chapter (section 4.3.3.) evidence is presented to show that NH_4NO_3 was detrimental to flower induction, but was supportive of flower growth.

Table 4.9. Absorbance readings to show anthocyanins and chlorophyll a in extracts of flowers taken from Treatment A, 40 ml medium in 100 ml flasks sealed with PVC film; Treatment B, 20 ml medium in 100 ml flasks; Treatment C, 20 ml medium in 50 ml flasks; Treatment D, 10 ml medium in 40 ml test-tubes; Treatment E, full strength MS (MURASHIGE and SKOOG, 1962) medium; Treatment F, salts of MS medium without NH NO_4^3 ; Treatment G, 50% of the salts of MS medium without NH NO_4^3 ; Treatment H, 20% of the salts of MS medium without NH NO_4^3 (= control); Treatment I, 20% of the salts of MS medium with half of the recommended NH NO_4^3 ; Treatment J, 20% of the salts of MS medium with the full amount of NH NO_4^3 recommended; Treatment K, 20% of the salts of MS medium without NH NO_4^3 but including the full vitamin component.

<u>Treatment</u>	<u>Anthocyanin</u> (absorbance 535 nm)	<u>Chlorophyll</u> (absorbance 663 nm)
A.	-	-
B.	0.236	0.082
C.	0.221	0.105
D.	0.310	0.117
E.	-	-
F.	0.250	0.109
G.	0.195	0.130
H.	0.219	0.098
I.	0.205	0.185
J.	0.176	0.176
K.	0.208	0.136

SCHWABE (1982a) also examined the effect of NH_4NO_3 on flowering of Kalanchoe, and found that at high concentrations, the salt increased the O_2 output, but had no effect on the number of flowers. The elegant work of TANIMOTO and HARADA (1981a,b) indicated that mineral salt concentrations had a marked effect on the in vitro flowering of Torenia stem segments. These authors found that by diluting the MURASHIGE and SKOOG (1962) salts to one fifth strength, they achieved enhanced adventitious and flower bud formation. By adding the other organic components of the medium, flowering was also inhibited. Several other examples, reviewed in Chapter one, also point to low nutrients, and low NH_4NO_3 being beneficial to flower induction.

4 3

Further investigations of the role of nitrogen and carbohydrates on the flowering of Kalanchoe, are made in Section 4.2.3. and 4.3.3. in view of the classical theory of a high C/N ration being responsible for flowering. An investigation was also made of the effects of nitrogen on the succulence of the leaves.

It was apparent that the colouration of the flowers of all of the preceding trials, varied according to treatment (Table 4.9.). The most noticeable colour intensity was in the leaves of Kalanchoe explants cultured on ten ml medium in test-tubes (Treatment D.). Conversely, Treatment J. had very low anthocyanin, but high chlorophyll levels. These latter explants were cultured on TANIMOTO and HARADA (1981b) medium with full strength NH_4NO_3 , the nitrogen source being responsible for the greening of the flowers.

4 3

4.3.3. The effect of carbohydrate and nitrogen on the flowering of Kalanchoe explants

The rate at which explants flowered, was markedly affected by sucrose concentration (Figure 4.13.). Concentrations of two and four per cent were stimulatory of flowering over cultures without sucrose, while concentrations above four per cent became inhibitory, almost totally so at eight per cent.

The rate of flowering has seldom been used as a measure of flowering in plants, and thus no comparable results are available in the literature. Much evidence does nevertheless exist to support the theory of flower promotion by higher carbohydrate levels. This has been extensively discussed in Chapter 1. section 1.3.2.e.

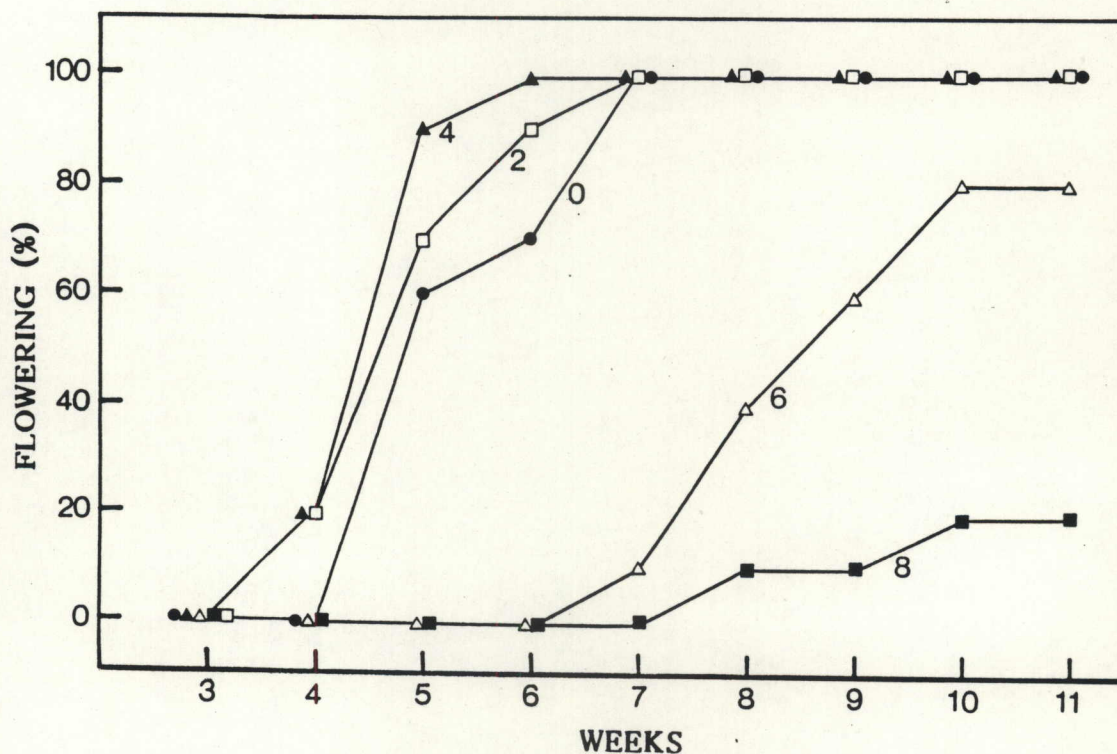


Figure 4.13. Rate of flowering of Kalanchoe explants grown in vitro under SD as affected by increasing (0% - 8%) concentrations of sucrose in the medium. Ten replicates were used for each treatment.

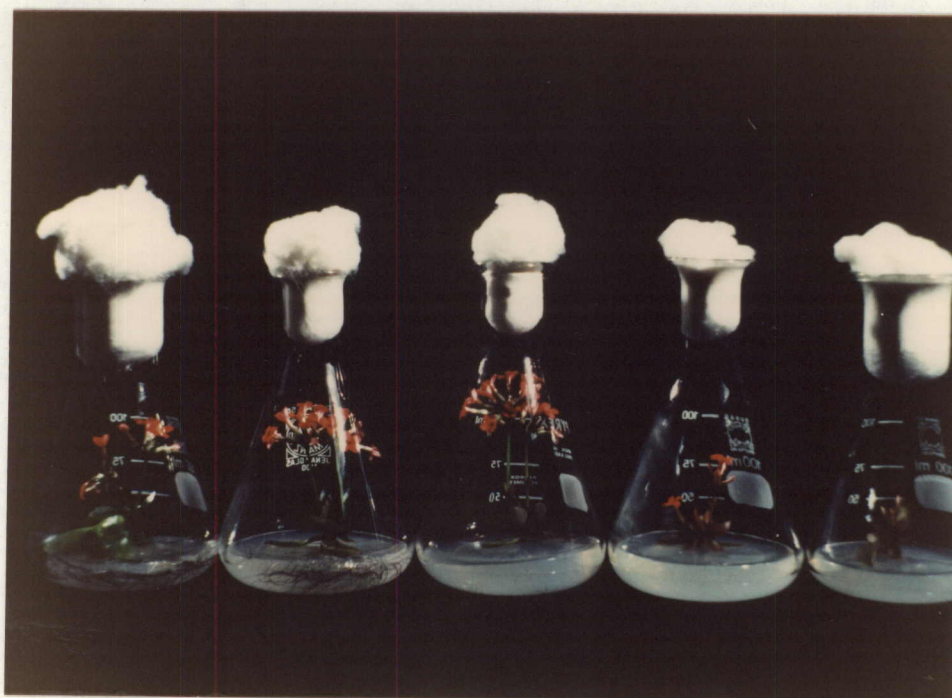


Plate 4.5. Kalanchoe nodal explants after eleven weeks of culture on TANIMOTO and HARADA (1981b) medium supplemented with increasing levels of sucrose. From left to right, 0%, 2%, 4%, 6% and 8% sucrose.

After anthesis had occurred in all likely plants (representatives of which are shown in Plate 4.5.), the plants were removed and examined in detail to observe if any of the characteristics normally affected by daylength, were affected by sucrose. The characteristics measured were shoot fresh and dry mass, succulence, root mass, leaf area and number, flower number, inflorescence mass and peduncle length. The results are represented in Figure 4.14. and Table 4.10.. Many of these vegetative and flowering characteristics became inhibited with increasing sucrose concentration. These included shoot and root fresh mass, succulence, inflorescence mass and leaf area. Those that increased or remained constant, were shoot dry mass (constant to 4%), leaf number (constant to 8%), inflorescence length (increasing to 4%) and flower number (constant to 4%).

In Brassica, leaf number was found to decrease with increasing sugar concentrations and with the associated increased flowering (FRIEND, BODSON and BERNIER, 1984). This did not occur in Kalanchoe, even at supra-optimal levels. The only characteristic that increased substantially besides the rate of flowering, was the peduncle length. It is possible that sucrose was providing resources for growth, thus manifesting the inflorescence both faster (resulting in increased flowering rate) and increasing its size. This would agree with the conclusions of TANIMOTO and HARADA (1981a), who stated that sugar promotes the development of flowers, but not their initiation. Thus there was no increase in flower initiation of Kalanchoe as represented by the number of flowers (Figure 4.14.).

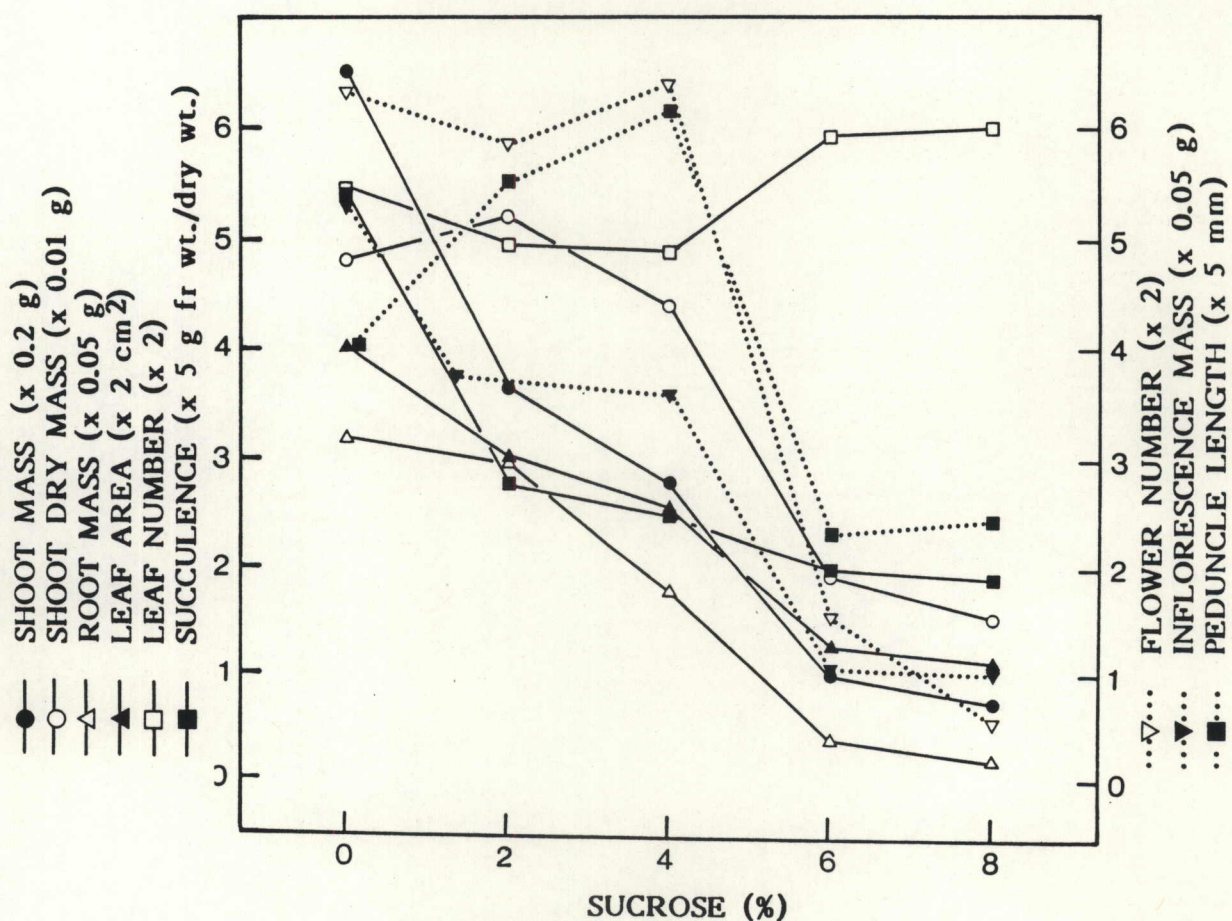


Figure 4.14. Vegetative and flowering characteristics of Kalanchoe explants after culture and induction to flower on medium containing increasing sucrose concentrations. Ten replicates were used for each treatment. Standard errors are given in Table 4.10.

Table 4.10. Vegetative and flowering characteristics of *in vitro* grown *Kalanchoe* explants cultured in SD conditions on TANIMOTO and HARADA (1981b) medium with increasing sucrose concentrations. These same results are represented diagrammatically in Figure 4.14.. Ten replicates were used for each treatment. Results are presented as mean \pm standard error.

Characteristic <u>measured</u>	Sucrose concentration (%)				
	0%	2%	4%	6%	8%
Shoot fresh mass (g)	1.30 \pm 0.13	0.73 \pm 0.04	0.56 \pm 0.07	0.20 \pm 0.02	0.14 \pm 0.03
Shoot dry mass (g)	0.048 \pm 0.005	0.052 \pm 0.003	0.044 \pm 0.002	0.020 \pm 0.002	0.015 \pm 0.002
Succulence (fr/dry mass)	27.0	14.0	12.7	9.95	9.5
Root mass (g)	0.158 \pm 0.03	0.149 \pm 0.01	0.080 \pm 0.007	0.019 \pm 0.003	0.010 \pm 0.004
Leaf area (cm ²)	8.13 \pm 0.97	5.86 \pm 0.61	5.05 \pm 0.63	2.6 \pm 0.2	2.16 \pm 0.4
Leaf number	10.8 \pm 0.9	9.9 \pm 0.9	9.8 \pm 1.3	11.9 \pm 1.4	12.1 \pm 0.9
Flower number	12.70 \pm 1.09	11.70 \pm 1.25	12.75 \pm 1.88	3.11 \pm 0.58	1.17 \pm 0.74
Inflorescence mass (g)	0.270 \pm 0.016	0.187 \pm 0.016	0.180 \pm 0.28	0.57 \pm 0.007	0.053 \pm 0.007
Peduncle length (mm)	21.3 \pm 1.8	27.5 \pm 2.3	30.8 \pm 4.0	11.8 \pm 2.2	12.3 \pm 1.84

Chlorophyll levels in the leaves were noted to decrease with increasing sucrose concentration in the medium (Figure 4.15.), while anthocyanin levels increased. The combination of these two factors resulted in an increasing red appearance of the leaves (Plate 4.5.). It is well known that anthocyanins are produced in plants as sugar accumulates in the tissues in amounts excessive for the immediate requirements for growth (GOODWIN, 1965). In *Kalanchoe*, it has been reported that increasing

anthocyanin levels are associated with increasing SD induction (NEYLAND, NG and THIMANN, 1963). Yet flower number, which normally increases with SD induction (SCHWABE, 1956), was found to remain constant in explants cultured on medium with up to four per cent sucrose under *in vitro* conditions (Figure 4.14.). At higher concentrations of sucrose, there was a marked decrease in flower number. This decrease in flower number occurred despite the fact that the anthocyanin levels increased, thus suggesting that the increase in both anthocyanins and flower number with increasing SD induction under natural conditions, is not under the same control.

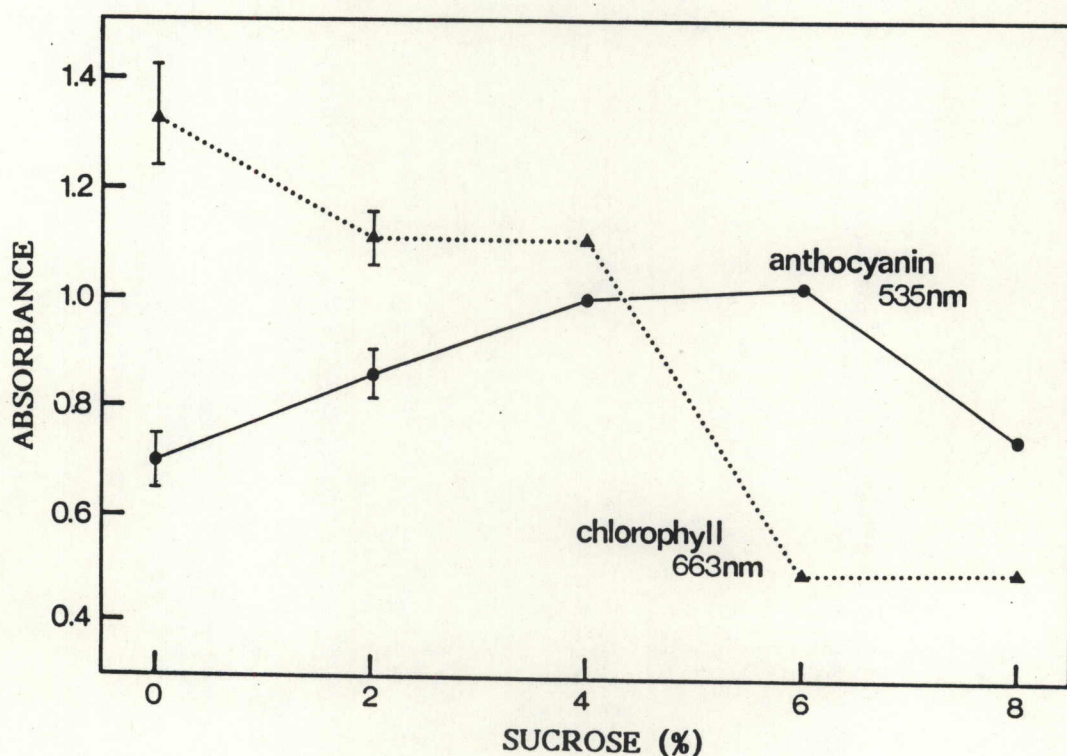


Figure 4.15. Pigment levels in DMSO (\blacktriangle) and DMSO + 1% HCl (\bullet) extracts taken from *Kalanchoe* explants grown on medium containing increasing sucrose concentrations. Ten replicates were used at each concentration. Results are mean \pm standard error.

No other evidence is available in the literature with regard to the situation of chlorophyll and SD flower induction. The decrease in chlorophyll indicated here, was not related to increasing succulence of the leaves, as chlorophyll determination was done on a dry mass basis. It is possible that the decrease in chlorophyll with increasing sucrose concentration was due to a negative feedback mechanism on the synthesis of chlorophyll, the plant having little need for chlorophyll when excess sucrose was present.

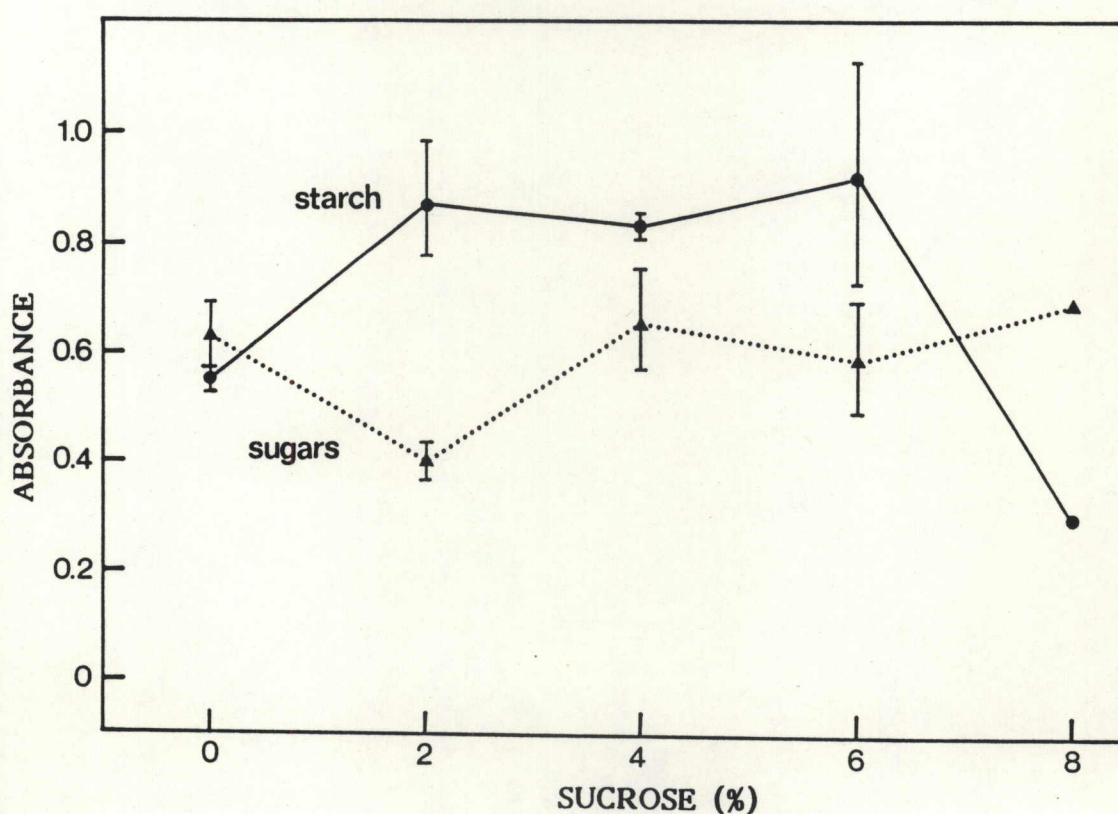


Figure 4.16. Starch and free sugar concentration in extracts taken from *Kalanchoe* explants grown on medium containing increasing sucrose concentrations. Four extracts were made from each treatment. Results are mean \pm standard error.

The determination of endogenous free sugars and starch using the techniques described in section 4.2.3., produced levels as indicated in Figure 4.16..

Standard concentrations of D-glucose produced the graphs presented in Figure 4.17. Straight lines were achieved showing accuracy in the quantification techniques employed. Absolute values were not required and thus were not calculated, as a comparison between treatments was possible using absorbance values.

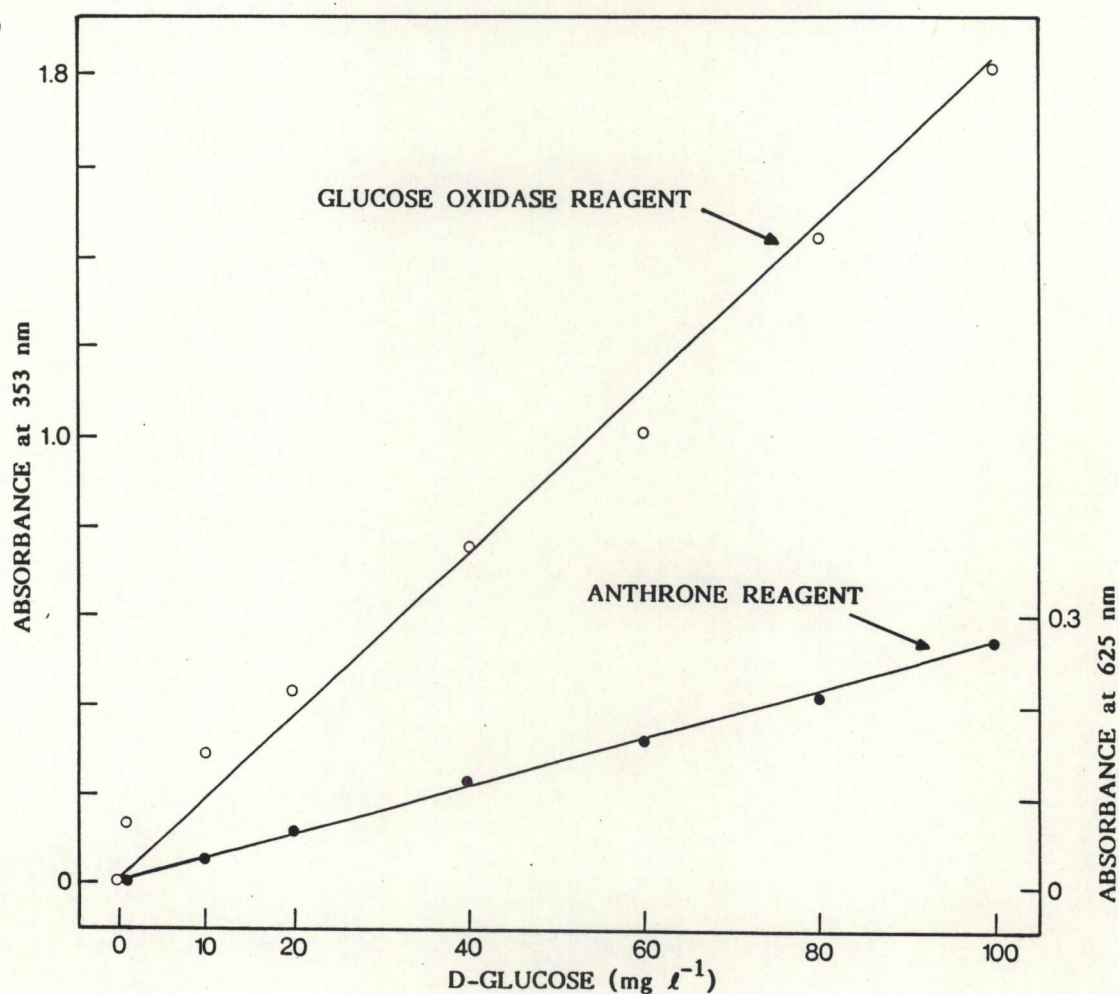
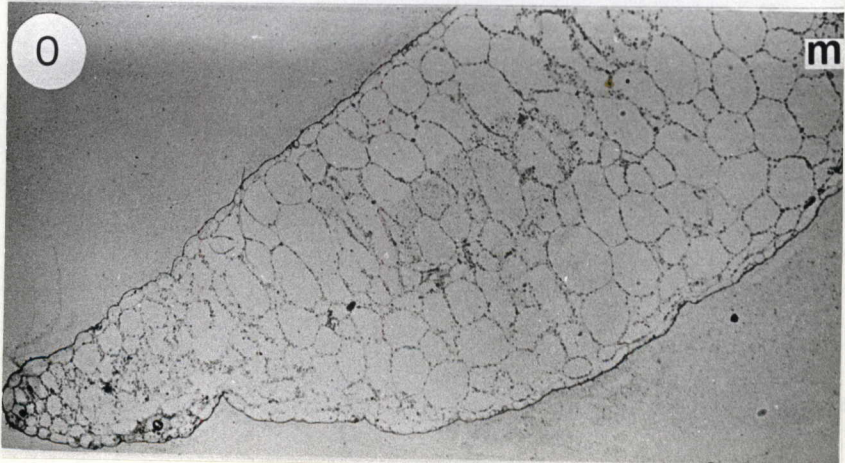
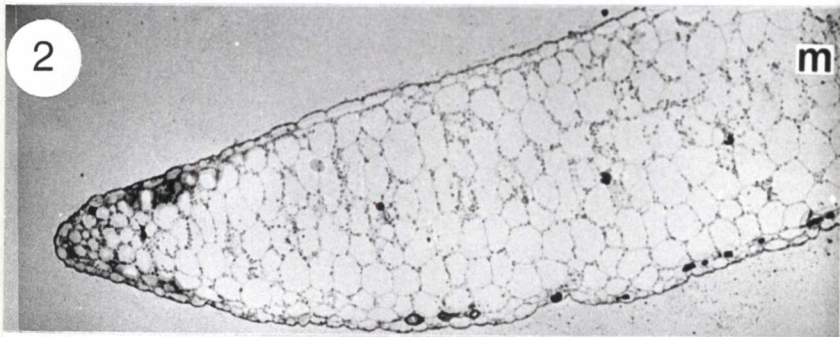
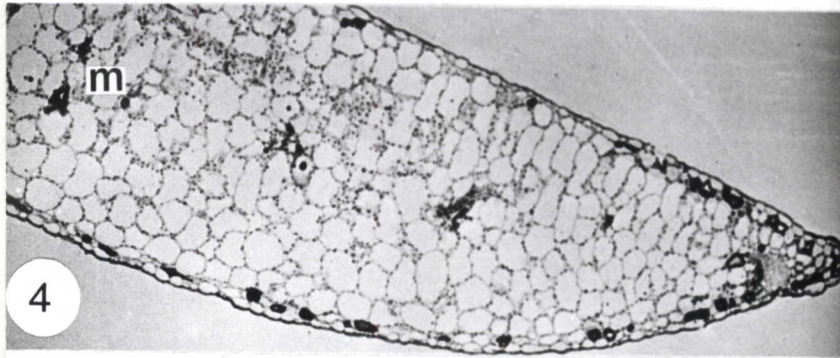
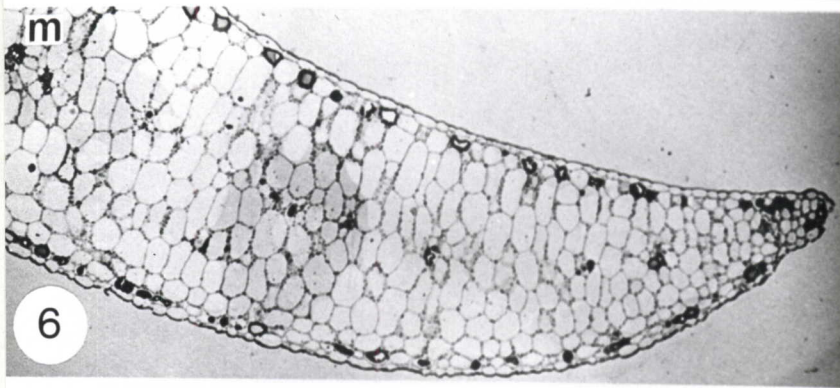
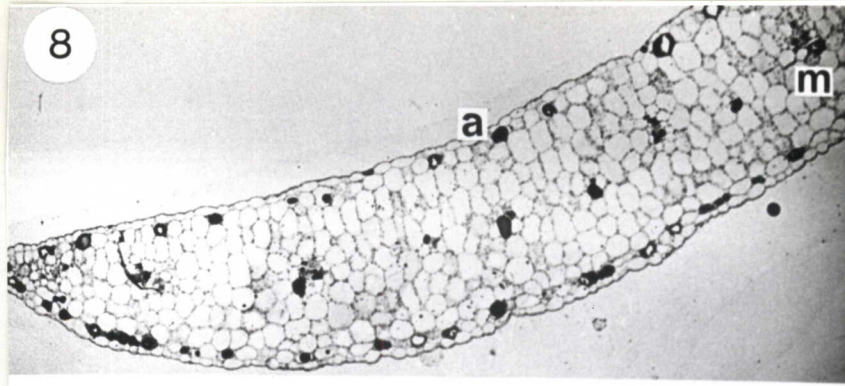


Figure 4.17. Standard curves for starch (○) as determined using glucose oxidase reagent, and for free sugars (●) using anthrone reagent. Three replicates were used at each concentration of D-glucose.

The changes in endogenous starch and sugar concentration were minimal and insignificant, and do not reflect the anticipated differences. It was anticipated that culture on increasing sucrose concentrations would result in higher endogenous concentrations of either sugars or starch or

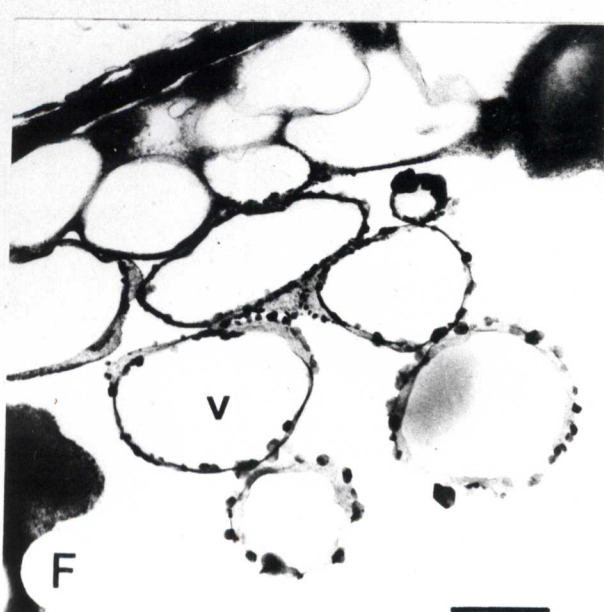
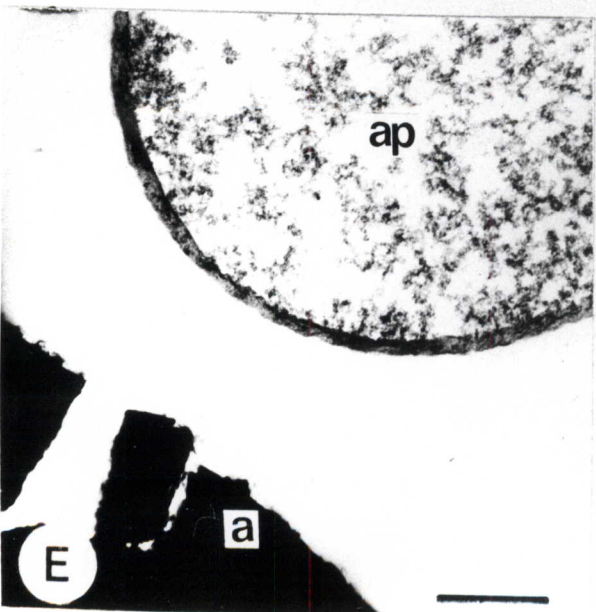
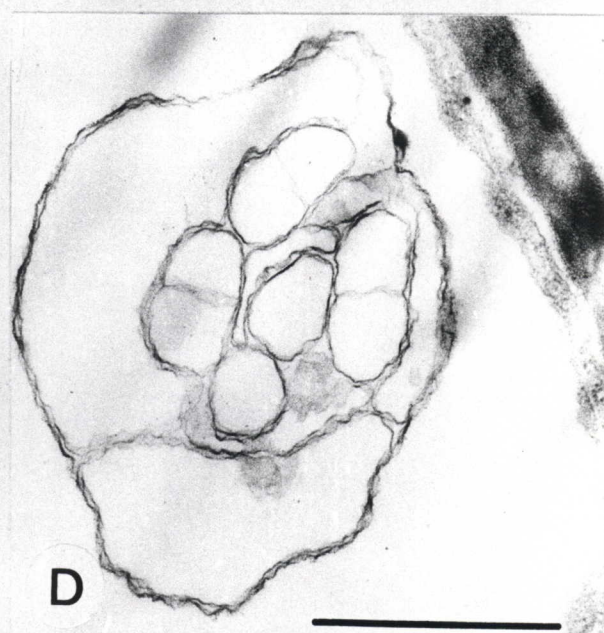
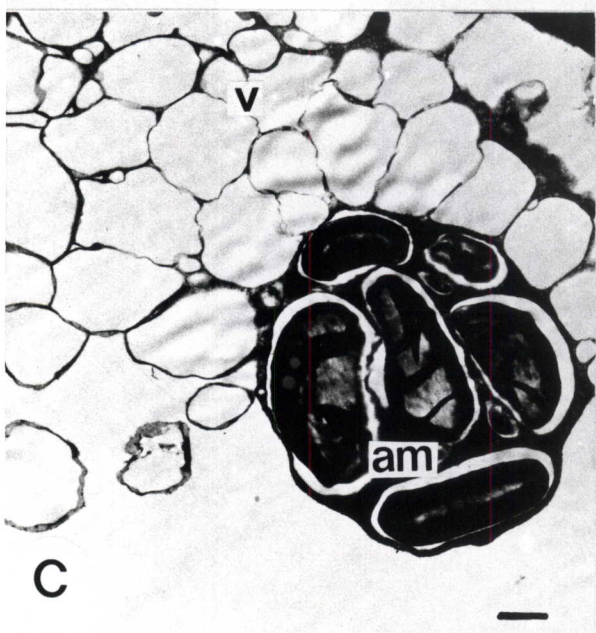
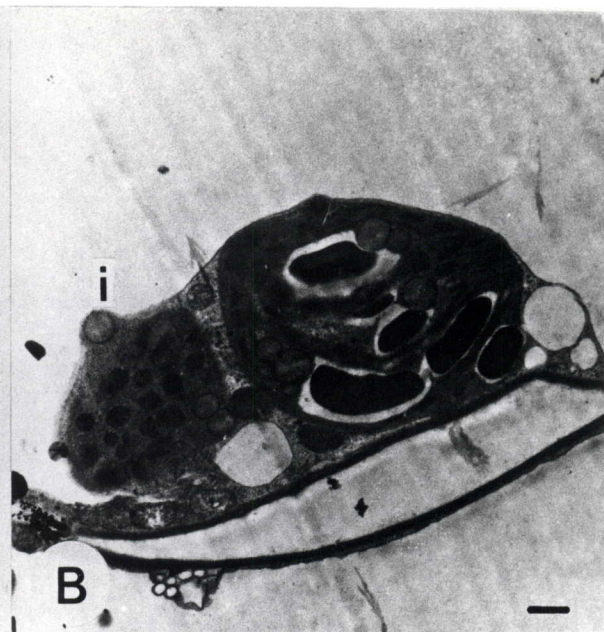
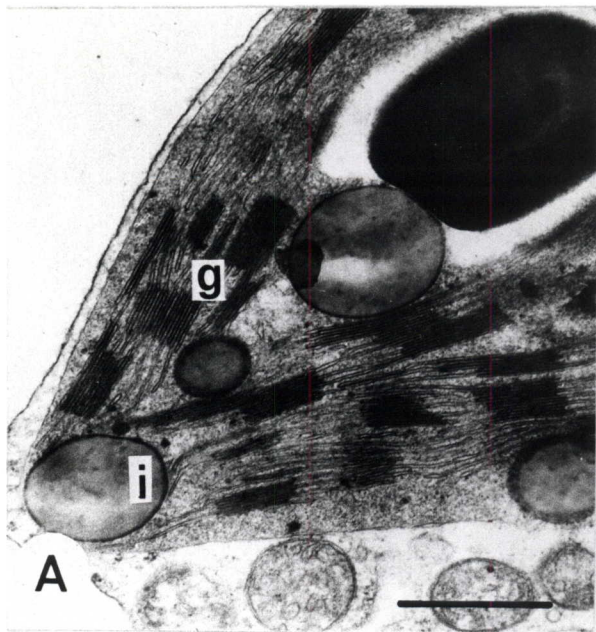
both. Due to the long culture period of eleven weeks, it is possible that high levels of sucrose in treatments at the time of culture, had been completely utilized by the explants, possibly to produce anthocyanins (GOODWIN, 1965). It is possible that higher sucrose concentrations may have resulted in higher respiratory rates, which could have accounted for carbon loss from the treatments.

Marked differences are apparent in the anatomy of Kalanchoe leaves which were cultured on increasing concentrations of sucrose (Plate 4.6.). There was a notable decrease in the size of the cells with increasing sucrose concentration which suggests a decreasing succulence. Mass measurements had determined that this was the case, (Figure 4.14.) where it was found that succulence decreased with increasing sucrose concentration. The cell walls also appear less distinct in leaves taken from plants cultured without sucrose, as opposed to those cultured on eight per cent sucrose, probably as the excess sucrose has been diverted into cellulose. The most outstanding feature of the sequence, is the increase in number of anthocyanin containing cells with increasing sucrose concentration. At the two per cent level, these anthocyanin containing cells were confined to the lower surface of the leaf, while at higher sucrose concentrations, they were found on both surfaces.



At the ultrastructural level, the most noteworthy difference between the leaves of plants cultured without sucrose and those of plants cultured on six per cent sucrose, was in the appearance of the chloroplasts (Plate 4.7.). In plants cultured on six per cent sucrose, the chloroplasts have accumulated starch to the extent that they no longer have much capacity for photosynthesis, as evidenced by the lack of grana, and could be termed amyloplasts (Plate 4.7. C.). This is in contrast to the chloroplasts from cultures without sucrose (Plate 4.7. A.), where the chloroplasts have clearly defined grana. These chloroplasts also have numerous inclusions, possibly of lipid. One of these bodies appears to be extruding from the chloroplast membrane (Plate 4.7 B.). Cultures without sucrose also contained several multivascular bodies (Plate 4.7. D.), some of which appeared to be dividing. These had a different appearance to the massed vacuoles in the cells of plants cultured on six per cent sucrose (Plate 4.7. C.). Leaves from cultures on six per cent sucrose, had numerous anthocyanoplasts (Plate 4.7. E.), seen here associated with a dense mass of what is probably anthocyanin. This substance proved extremely difficult to section, and thus could not be thoroughly investigated. Anthocyanoplasts and their possible role were discussed in section 4.3.1.. Several vacuolar bodies were also found (Plate 4.7. F.) which appeared to be associated with small electron dense bodies. The nature of these vacuoles is not understood.

(6) The dearth of functional chloroplasts in leaves cultured on six per cent sucrose, helps to explain earlier results where a marked decrease in the chlorophyll content was observed (Figure 4.15.). The higher anthocyanin content is also apparent. It is not understood why the apparent abundance of starch containing amyloplasts in cultures on six per cent sucrose, was not reflected by the starch extraction (Figure 4.16.), where



no real difference was detected between the endogenous starch contents of leaves cultured on zero and six per cent sucrose. The most likely sinks for the excess sucrose in plants cultured on high sucrose concentrations, thus appears to be through the production of anthocyanins and cell walls, and possibly a higher respiration rate.

Nitrogen

Significant promotion of the rate of flowering of *Kalanchoe* explants with nitrogen treatment, over untreated controls cultured on the medium of TANIMOTO and HARADA (1981b) was obtained and is shown in Figure 4.18..

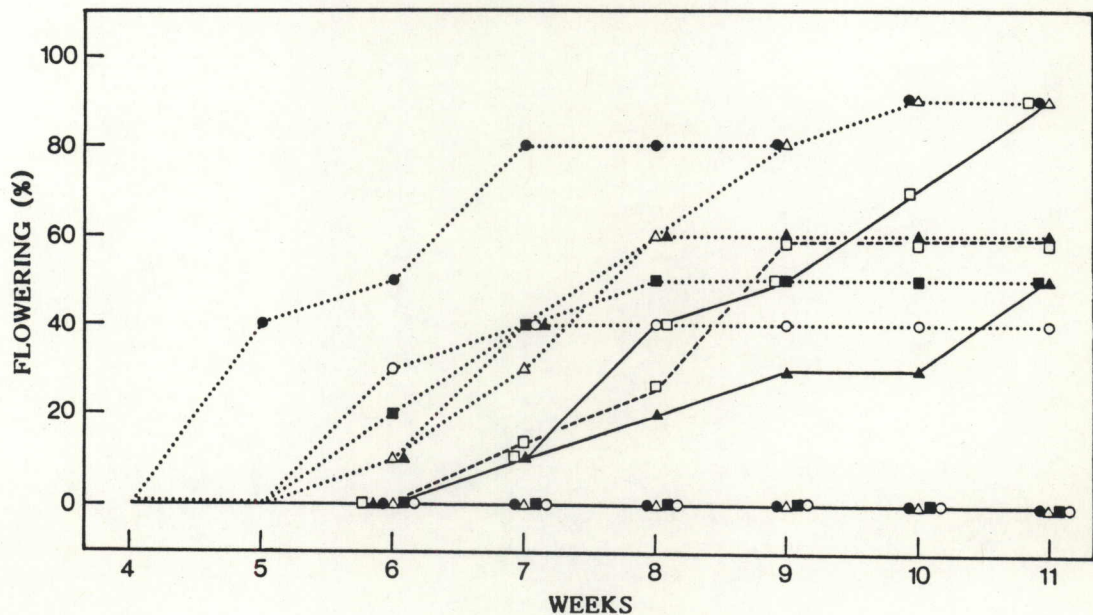


Figure 4.18. Rate of flowering of *Kalanchoe* explants subjected to variations in NH₄⁺ and NO₃⁻ concentration and daylength regimes. Changes were made after 28 days. Treatment L (—△—) zero nitrogen in LD conditions; M (—●—) zero nitrogen in LD changing to full strength NH₄⁺NO₃⁻ in LD conditions; N (—■—) zero nitrogen in LD changing to full strength KNO₃ in LD conditions; O (—○—) full strength NH₄⁺NO₃⁻ and KNO₃ from the time of culture in LD conditions; P (—▲—) zero nitrogen in SD conditions; Q (—□—) zero nitrogen in SD changing to full strength NH₄⁺NO₃⁻ in SD conditions; R (•△•) zero nitrogen in SD changing to full strength KNO₃ in SD conditions; S (•●•) full strength NH₄⁺NO₃⁻ and KNO₃ from the time of culture in SD conditions; T (•■•) zero NH₄⁺NO₃⁻ in SD changing to LD conditions; U (•○•) full strength NH₄⁺NO₃⁻ in SD changing to LD; V (•▲•) zero NH₄⁺NO₃⁻ in SD changing to full strength NH₄⁺NO₃⁻ in LD conditions. The control (—□—) was the medium of TANIMOTO and HARADA, (1981b) in SD conditions. Ten replicates were used for each treatment.

The most rapid flowering was obtained on the basal medium supplemented with full strength NH_4NO_3 and KNO_3 in SD conditions (Treatment S.), if applied at the time of initial explant culture. Explants which were deprived of a nitrogen source for four weeks, and then had full strength KNO_3 added to the medium in SD (Treatment R.) also exhibited rapid flowering. The transfer of the explants to NH_4NO_3 as opposed to KNO_3 , caused substantially slower flowering (Treatment Q.).

The addition of NH_4NO_3 after 28 days was also able to manifest the flowering stimulus in explants transferred from inductive SD to non-inductive LD (Treatment V.). This rate was only marginally faster than in explants cultured on medium without NH_4NO_3 , and transferred from SD to LD (Treatment T.). It had not been possible in earlier experiments, to obtain such a manifestation of the stimulus on transfer to LD conditions (section 4.3.1.). Explants cultured on full strength NH_4NO_3 from the time of culture in SD and then transferred to LD after 28 days, (Treatment U.), produced a slower rate of flowering than either of the previous two treatments. This suggests that NH_4NO_3 was detrimental to flowering during induction, but was supportive during growth. This is supported by the work of TANIMOTO and HARADA (1986). A total lack of nitrogen from the medium (Treatment P.) delayed flowering of the explants, probably due to a lack of growth in general. No permutation was able to bring about flowering in continual LD conditions (Treatments L. to O.) as was found by TANAKA (1986) with Lemna.

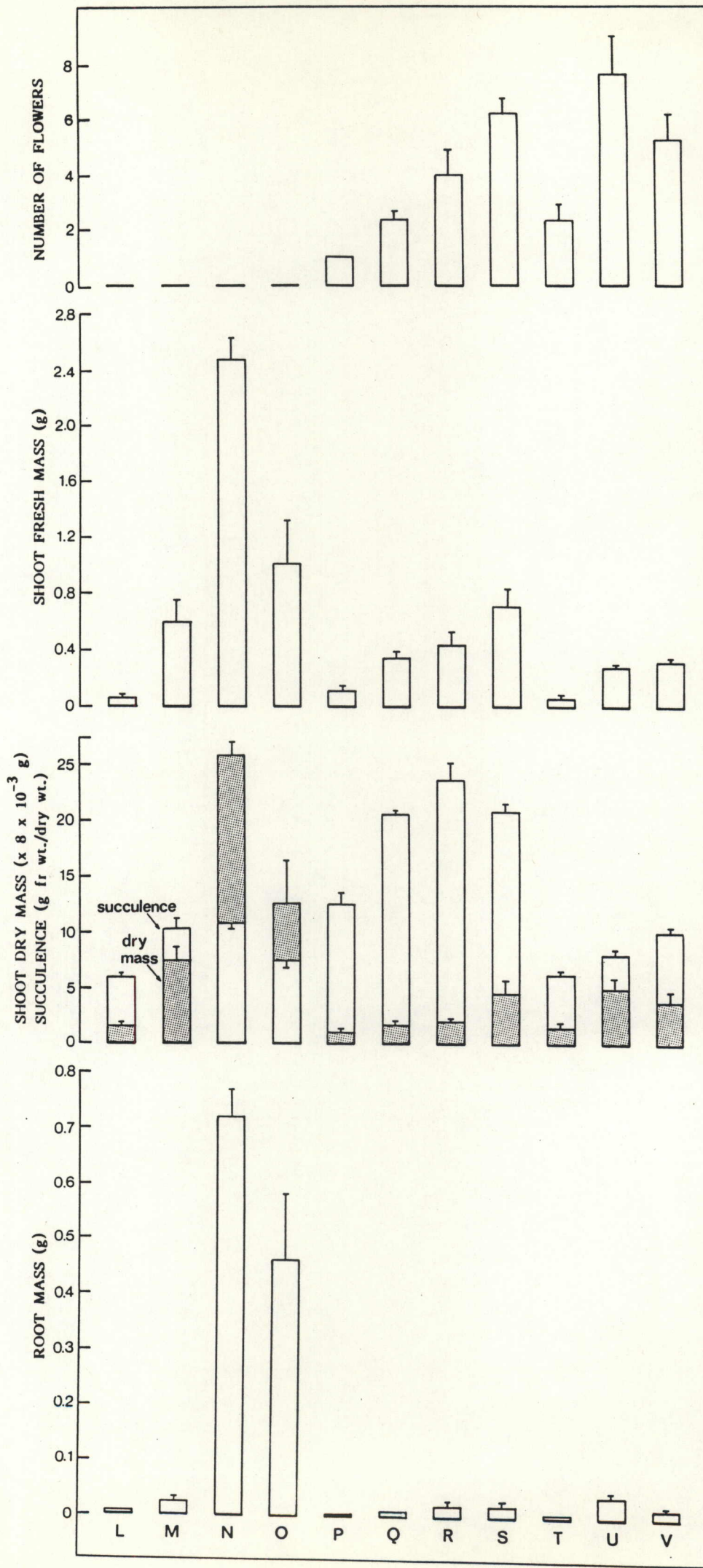
Treatments producing the greatest number of flowers, were not always those that flowered at the fastest rate. The greatest number of flowers were obtained with Treatments U. and S. (Figure 4.19.). Both of these treatments had high levels of NH_4NO_3 as opposed to KNO_3 . A lack of NH_4NO_3 (Treatment T.) resulted in low flower numbers when transferred to

LD conditions. Plants from all treatments which produced high flower numbers also produced high shoot dry mass (Figure 4.19) but not necessarily fresh mass, although fresh mass was high in explants cultured on full strength NH NO_4 (Treatment S.).

4 3

Those explants that flowered at the greatest rate (Figure 4.18.) in particular those from Treatments S., R. and Q. (in descending order), were all cultured in SD conditions and all exhibited one common morphological characteristic, that of having a high leaf succulence (Figure 4.19). The succulence of plants treated in these three ways was more than double that of the other treatments that flowered. This was due primarily to a high fresh mass, as the dry masses did not vary much. It is probable that this increased succulence was as a result of culture in SD conditions, as this is well known to increase succulence, and is discussed in Section 4.3.1.. Explants from these three treatments (S., R. and Q.) also showed decreasing shoot fresh mass, dry mass and flower number corresponding to the decreasing rate of flowering. It is significant to note, that explants from Treatments U. and V., both of which were cultured on NH NO_4 and were brought to anthesis in LD, had low succulent levels, but nevertheless produced high numbers of flowers. This fact suggests that succulence is not a prerequisite for flowering, but probably occurs concurrently for unrelated reasons. This possibility has previously been suggested by SCHWABE (1969; 1985).

No flowering was induced by nitrogen manipulation in non-inductive LD, as was achieved in Lemna by TANAKA (1986). Yet it is significant to note the variation in vegetative growth between identical treatments, one kept in LD and the other in SD (Figure 4.19.).



Most outstanding were the explants from Treatment N., which had KNO_3 added after four weeks of culture. Both shoot fresh mass and dry mass, as well as root mass were exceptionally high. These were higher than explants cultured with NH_4NO_3 (Treatment M.) and also higher than explants from Treatment O. which had full strength NH_4NO_3 and KNO_3 . Due to the greater mass of tissue produced, it is apparent that the nitrogen in KNO_3 was more available for growth than that in NH_4NO_3 . It is also apparent that the presence of NH_4NO_3 in conjunction with KNO_3 (Treatment O.) suppressed the efficiency of the KNO_3 , rendering it unavailable. Similar conclusions were made by JACKSON (1973) who stated that NH_4^+ inhibits NO_3^- uptake and action. Jackson also claimed that the inhibition of NO_3^- assimilation in the roots by NH_4^+ , is most severe when the root sugar levels are low, or when the rate of transport of photosynthate is low. In this system, where the sugar levels in the medium are relatively high, this should mean that the NH_4^+ inhibition of NO_3^- should be less effective. It is thus possible that this inhibition would have been more severe on a medium containing lower sugar levels. It is also noteworthy, that the high root mass produced by explants grown without nitrogen, and then transferred to KNO_3 in LD (Treatment N.), was not achieved in explants grown on the same medium but under SD conditions. This indicates the degree of antagonism between flowering and rooting (GASPAR, 1980), as rooting was virtually totally suppressed where flowering occurred.

Succulence in LD conditions was also not elevated by increasing the nitrogen source (Treatments L. to O.) as had been proposed by TISDALE, NELSON and BEATON (1985). These authors suggested that a high nitrogen supply, together with carbohydrates (from photosynthesis or in this case from the medium), would form large amounts of protein in the protoplasm.

This protein would be hydrated, resulting in succulence. Alternatively, if nitrogen levels are low, carbohydrates would be deposited in the cell walls, resulting in firmer, less succulent cells. Protein levels do increase in response to inductive cycles in Sinapis (JACQARD, MIKSCHE and BERNIER, 1972), this increase occurring during the later stages of floral transition.

4.3.4. The effect of phenolics and hormones on the in vitro flowering of Kalanchoe explants

Phenolics

At low concentrations, gallic acid had no effect on the number of flowers produced by Kalanchoe explants (Figure 4.20.). There was also no effect on any of the vegetative characteristics measured, in either LD grown or SD grown plants (Figure 4.21.).

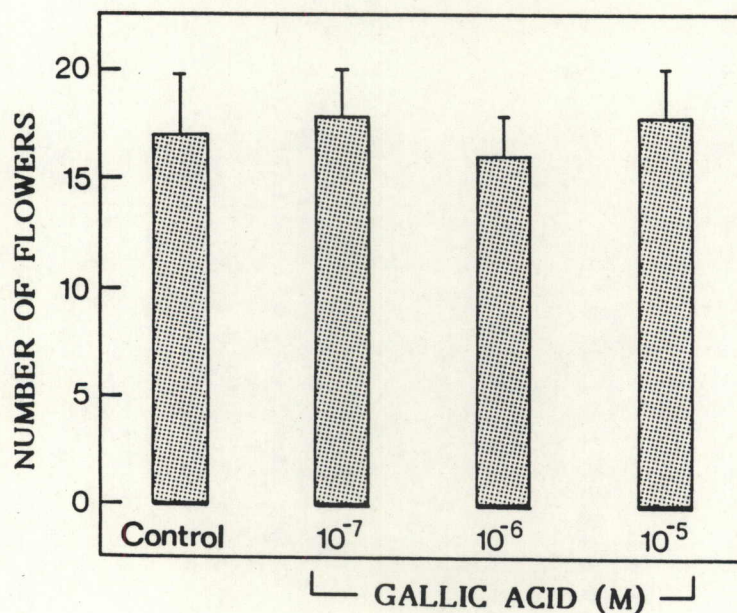


Figure 4.20. The effect of gallic acid on the number of flowers produced by Kalanchoe explants cultured in vitro under SD conditions. Ten replicates were used for each treatment. The results are expressed as mean ± standard error.

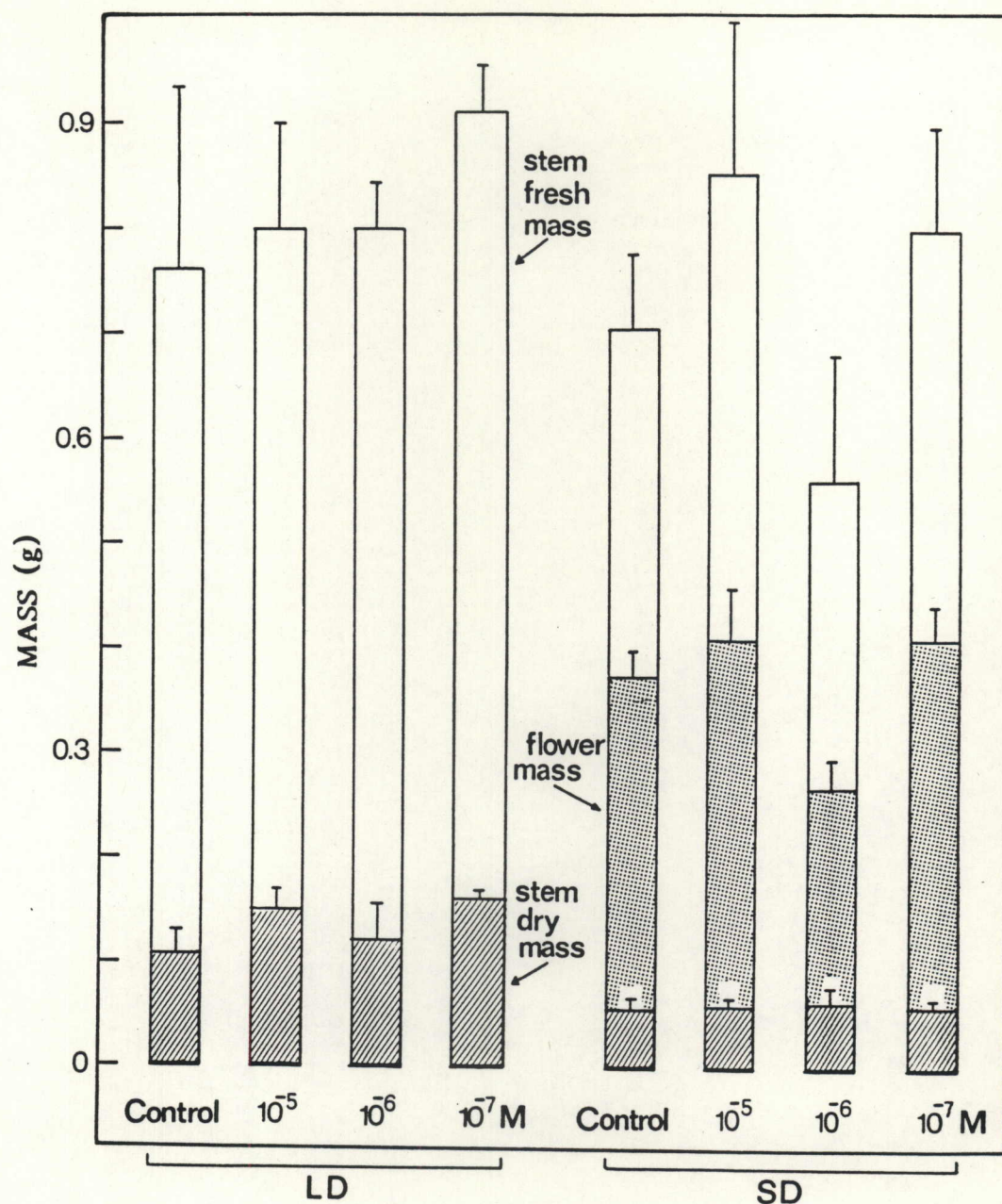


Figure 4.21. Vegetative and flowering response of *Kalanchoe* explants cultured on TANIMOTO and HARADA (1981b) medium containing increasing concentrations of gallic acid, and cultured under either LD or SD conditions. Ten replicates were used for each treatment. The results are expressed as mean \pm standard error.

In a repeat experiment using the higher levels suggested by PRYCE (1972), severe inhibition of both flowering and vegetative growth was observed (Figure 4.22.). Only those explants cultured on low concentrations of 100 mg l^{-1} succeeded in flowering at all.

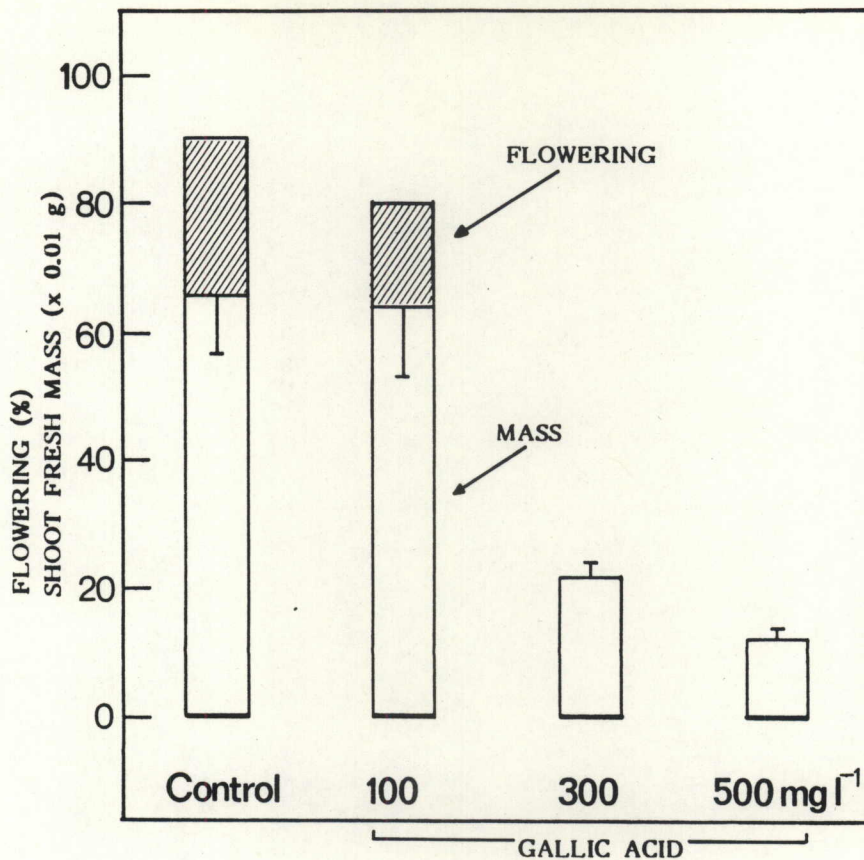


Figure 4.22. Flowering percentage and mass of vegetative growth of explants cultured on TANIMOTO and HARADA (1981b) medium supplemented with gallic acid at higher concentrations than in Figure 4.21. Ten replicates were used for each treatment. The results are expressed as mean \pm standard error.

This work is contrary to the proposals made by PRYCE (1972), who claimed that gallic acid was a specific flowering inhibitor, present in the leaves of vegetative *Kalanchoe* plants, and in flowering plants in an inactive form. That some inhibitor of flowering does exist, has been shown by SCHWABE (1972) and BLAKE (1972), the latter employing an *in vitro* bioassay system using *Viscaria* (= *Silene*) apices. The former author noted that the sap of one leaf was capable of inhibiting flowering in an entire *in vivo* grown plant, yet PRYCE (1972) used between one and three grams of leaf tissue per culture flask per apex, and double this in

extracted fractions. This author also used gallic acid at approximately 500 mg l^{-1} ($\pm 3 \times 10^{-3} \text{ M}$), and stated that this is a normal concentration for phenolic inhibitors. In this thesis, using the Kalanchoe bioassay, lower concentrations were not inhibitory to flowering, while higher concentrations were inhibitory of both flowering and vegetative growth.. The role of gallic acid in the inhibition of flowering in Kalanchoe could not be verified by ZEEVAART (1976), and was not referred to by SCHWABE (1985). The latter author recognises that the inhibitor in Kalanchoe may be a small molecule phenolic, but states that its identity is not known.

Both nicotinic and tannic acids were inhibitory of the flowering rate (Figure 4.23.) as well as of flower numbers (Figure 4.24.). Yet there was also strong inhibition of vegetative growth, so this inhibition of flowering seems to be through general growth inhibition. No induction of flowering was achieved in non-inductive LD conditions, as had been

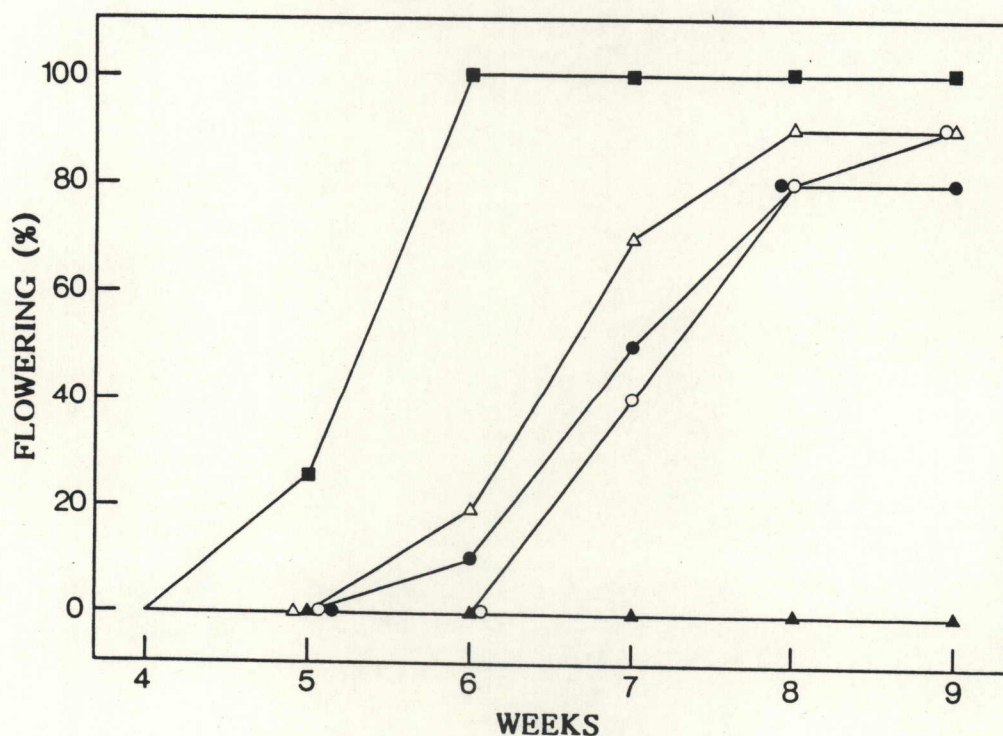


Figure 4.23. Rate of flowering of Kalanchoe explants cultured on TANIMOTO and HARADA (1981b) medium containing phenolic additives. Nicotinic acid at 10^{-6} M (○) and at 10^{-4} M (●); and tannic acid at 10^{-6} M (△) and 10^{-4} M (▲). Ten replicates were used for each treatment.

achieved in Lemna aequinoctialis with nicotinic acid (FUJIOKA, YAMAGUCHI, MUROFUSHI, TAKAHASHI, KAIHARA, TAKIMOTO and CLELAND, 1986). Although nicotinic acid had been isolated from Lemna, these authors found that there was no fluctuation in its level in response to daylength. This means that either the response was pharmacological, or that nicotinic acid operates in combination with some other substance.

No induction or promotion of flowering in Kalanchoe was achieved with tannic acid either, although this substance had proved to be a powerful flower inducer in Lemna aequinoctialis 6746 (KHURANA and MAHESHWARI, 1986). In Lemna, tannic acid was especially effective in that it completely overrode the plants photosynthetic sensitivity and the influence of nutrient concentrations.

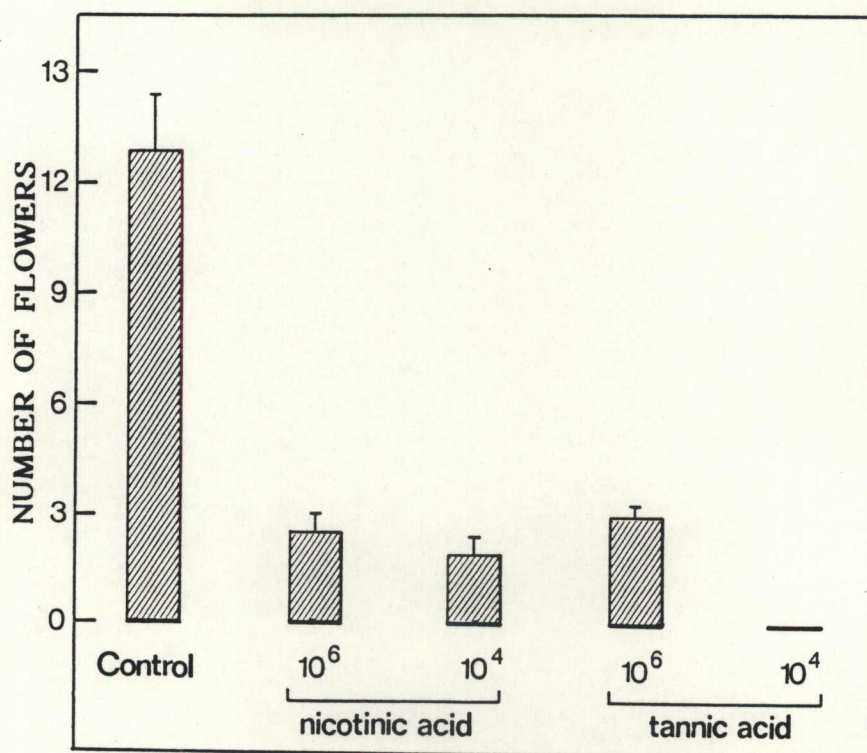


Figure 4.24. Number of flowers produced by Kalanchoe explants cultured on TANIMOTO and HARADA (1981b) medium containing phenolic additives. Ten replicates were used for each treatment. The results are expressed as mean \pm standard error.

It was also found in this work, that benzoic and salicylic acids had no promotive effect on flowering and were detrimental to vegetative growth. Again this is contrary to work with Lemna and Wolffia where these phenolics induce flowering (CLELAND and AJAMI, 1974; KHURANA and MAHESHWARI, 1983; CLELAND and KANG, 1985). No promotion was achieved in Pharbitis using these compounds, although some benzoic acid derivatives, in particular 3,4-dichlorobenzoic acid, did induce total flowering (SHINOZAKI and TAKIMOTO, 1983).

Hormones

Previous investigations of hormones and flowering in vitro have been discussed in detail in Chapter 1. and in Section 4.2.4.. There have also been many reviews on the subject (ZEEVAART, 1978; BERNIER, KINET and SACHS, 1981a,b; CLELAND and BEN-TAL, 1983), and thus the subject will not be examined exhaustively unless where directly applicable to the results.

None of the hormones investigated promoted flowering at a faster rate than the hormone-free control (Figure 4.25.), while several were severely inhibitory. NAA and GA_{-7} at 10^{-6} M had no effect on the rate of flowering, while GA_3 at 10^{-3} M was slightly inhibitory. All other treatments were inhibitory, with BA and BA with salicylic acid being totally so. BA with salicylic acid was promotive of flowering in Lemna at the same concentrations used here (PIETERSE and MULLER, 1977).

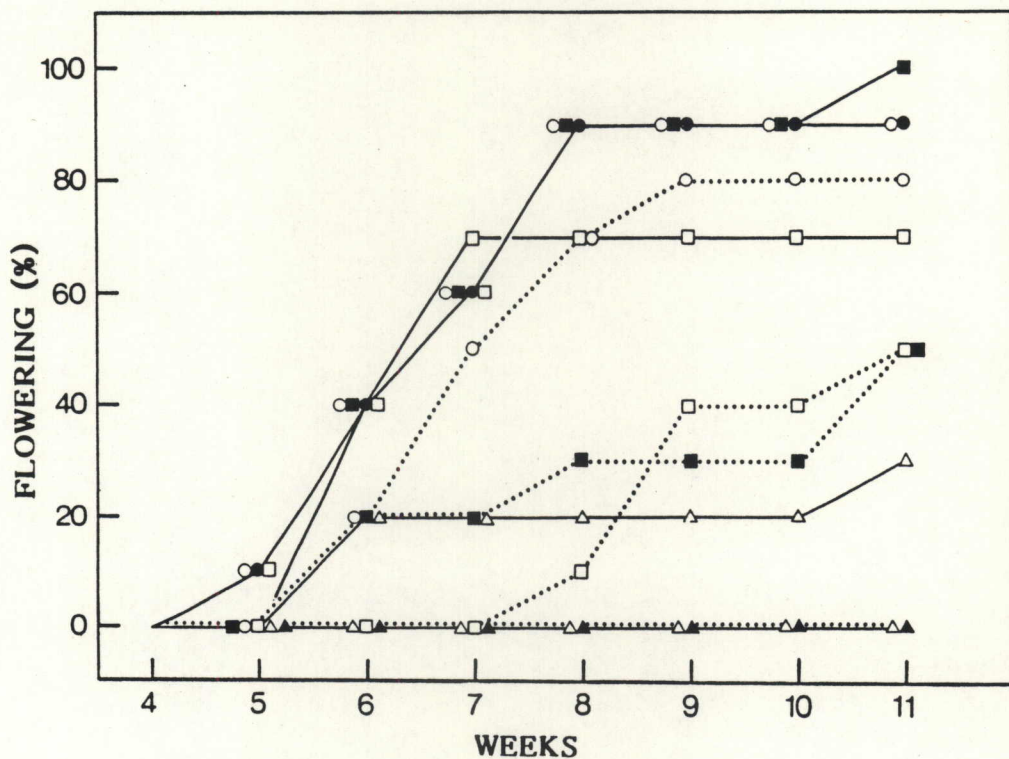


Figure 4.25. The rate of flowering of *Kalanchoe* explants grown *in vitro* on TANIMOTO and HARADA (1981b) medium supplemented with GA (○); BA (△); NAA (■); ABA (□) or BA with salicylic acid (▲) at concentrations of 10^{-7} M (—) and 10^{-6} M (.....). Ten replicates were used for each treatment.

Each of the hormones investigated, was examined for the effects it may have had on the growth and flowering characteristics of the *Kalanchoe* explants. The results for each hormone are outlined below.

Auxin

Under SD conditions, auxin caused a significant increase in the succulence of the explants (Table 4.11.), although this was not evident to the same magnitude in LD (Table 4.12.). Auxin is well known to be responsible for cell expansion, and so may play a role in the increased succulence of *Kalanchoe* leaves in response to SD which has been well

documented by GUMMER (1948) and SCHWABE (1958). Yet auxin was not able to increase succulence in LD conditions to the levels achieved in SD conditions, thus indicating that auxin is only partly responsible for the control of succulence. Under LD conditions, auxin promoted vegetative growth to a greater extent, increasing shoot fresh and dry mass, as well as root mass. The roots were thin, fibrous and abundant. Such an increase of mass was not evident in SD conditions, possibly as flowering is antagonistic of both vegetative growth and root growth (GASPAR, 1980).

Table 4.11. The effect of NAA on the flowering of *Kalanchoe* explants cultured *in vitro* on the medium of TANIMOTO and HARADA (1981) under SD (8 hour day) conditions. Ten replicates were used for each treatment. Values represented are means \pm standard error. Significant difference from the control, $P = 0.05$ (*); $P = 0.01$ (**).

<u>Characteristic</u> <u>measured</u>	<u>Control</u>	<u>NAA</u> (10 ⁻⁷ M)	<u>NAA</u> (10 ⁻⁶ M)
Percentage flowering (%)	90	90	70
Number of flowers	7.7 \pm 1.9	5.8 \pm 1.07	8.8 \pm 1.7
Flower mass (g)	0.17 \pm 0.04	0.14 \pm 0.03	0.15 \pm 0.04
Shoot mass (g)	0.45 \pm 0.13	0.56 \pm 0.1	0.54 \pm 0.15
Shoot dry mass (g)	0.03 \pm 0.008	0.028 \pm 0.005	0.033 \pm 0.009
Succulence (g fr/g dry)	15.02 \pm 1.06	18.8 \pm 1.1*	18.8 \pm 1.6*
Root mass (g)	0.056 \pm 0.01	0.057 \pm 0.02	0.15 \pm 0.04
Number of leaves	13.6 \pm 3.1	16.5 \pm 2.2	16.6 \pm 3.9
Leaf area (cm ²)	4.56 \pm 1.1	4.18 \pm 0.6	4.5 \pm 1.3
Stem length (mm)	5.9 \pm 0.88	4.9 \pm 0.7	7.5 \pm 1.6
Peduncle length (mm)	19.5 \pm 3.4	16.9 \pm 1.2	25.4 \pm 2.7

Auxin is generally inhibitory of flowering in SDP (SALISBURY, 1955), and *Kalanchoe* is no exception (HARDER and VAN SENDEN, 1949; VAN SENDEN, 1951).

The auxin antagonist TIBA was thus anticipated to promote flowering in Kalanchoe, and indeed did so (ZAWAWI and IRVING, 1968), although this was not supported by the work of HARDER and OPPERMANN (1952) and VAN ZEIST and KOEVOETS (1951), who found TIBA inhibitory of flowering. In this thesis, the higher concentration of 10^{-6} M NAA used for Kalanchoe culture, was indeed inhibitory of the rate of flowering (Figure 4.25.), but was not inhibitory of any vegetative or flowering parameters (Tables 4.11. and 4.12.). These were generally all stimulated. This indicated that NAA may have acted as a specific inhibitor of flowering, a trend which was also reported by SALISBURY (1955), or may simply have acted by diverting nutrients away from the flowering process.

In in vitro flowering cultures, auxins are often essential components of the medium, but mainly to support the growth of the flowers (Chapter 1.). It is generally thought that although auxins may play some role in flowering, they are unlikely to form part of the flowering stimulus (GASPER, 1980), and are more likely to be involved in the differentiation of flower buds (TANIMOTO and HARADA, 1981b; CROES, CREEMERS-MOLENAAR, VAN DEN ENDE, KEMP and BARENDSE, 1985).

Table 4.12. The effect of NAA on the flowering of Kalanchoe explants cultured in vitro on the medium of TANIMOTO and HARADA (1981) under LD (18 hour day) conditions. Ten replicates were used for each treatment. Values represent means \pm standard error. Significant difference from the control, $P = 0.05$ (*); $P = 0.01$ (**).

Characteristic	Control	NAA 10^{-7} M	NAA 10^{-6} M
Shoot mass (g)	0.747 \pm 0.012	0.973 \pm 0.08**	0.936 \pm 0.048**
Shoot dry mass (g)	0.15 \pm 0.003	0.164 \pm 0.006**	0.172 \pm 0.006**
Root mass (g)	0.314 \pm 0.015	0.406 \pm 0.028**	0.56 \pm 0.045**
Succulence (g fr/dry)	4.98 \pm 0.13	5.85 \pm 0.29**	5.34 \pm 0.11**

Cytokinins

From the literature, it is clear (Chapter 1.) that cytokinins play an important role in flowering, although whether at the induction, evocation or differentiation stage is not known. In Lycopersicon (WITTWER and AUNG, 1961), and in Scrofularia (MIGINIAC, 1972), cytokinins inhibited flowering, but stimulated vegetative growth which led to the conclusion that inhibition was via the support of vegetative growth. The inhibition by benzyladenine (BA) of all growth in Kalanchoe grown in SD conditions (Table 4.13.), suggests that the levels used were supra-optimal, and that lower concentrations should have been incorporated. No promotion of flowering was obtained in any instance, which is contrary to many of the investigations reviewed in Chapter 1. (section 1.3.1.2.).

Different effects were observed for BA in LD and SD conditions (Tables 4.13. and 4.14.). In SD conditions, BA was markedly inhibitory of all growth with the exception of leaf number. This inhibition was most evident at higher concentrations of BA. Rather unexpectedly, in LD conditions, BA supported increased vegetative growth. These results are not conclusive though, as 30 per cent of the explants in LD conditions did not grow at all, indicating the possibility of some variation due to explant source, a factor not encountered at any other stage in this investigation with Kalanchoe. These plants with zero growth were not included in the results in Table 4.14. .

Table 4.13. The effect of benzyladenine (BA) on the flowering of *Kalanchoe* explants cultured *in vitro* on the medium of TANIMOTO and HARADA (1981) under SD (8 hour day) conditions. Ten replicates were used for each treatment. Values represent the mean \pm standard error. Significant difference from the control, P = 0.05 (*); P = 0.01 (**).

<u>Characteristic</u> <u>measured</u>	<u>Control</u>	<u>BA</u> (10 ⁻⁷ M)	<u>BA</u> (10 ⁻⁶ M)
Percentage flowering (%)	90	40	0
Number of flowers	7.7 \pm 1.9	2.4 \pm 1.5	0**
Flower mass (g)	0.17 \pm 0.04	0.15 \pm 0.04	-
Shoot mass (g)	0.45 \pm 0.13	0.15 \pm 0.05**	0.028 \pm 0.006**
Shoot dry mass (g)	0.03 \pm 0.008	0.01 \pm 0.003**	0.005 \pm 0.001**
Succulence (g fr/g dry)	15.02 \pm 1.06	13.8 \pm 1.3	-
Root mass (g)	0.056 \pm 0.01	0.028 \pm 0.01	0**
Number of leaves	13.6 \pm 3.1	10.1 \pm 2.0	5.3 \pm 0.7**
Leaf area (cm ²)	4.56 \pm 1.1	1.61 \pm 0.5*	0.5 \pm 0.06**
Stem length (mm)	5.9 \pm 0.88	3.6 \pm 1.2	1.6 \pm 0.19*
Peduncle length (mm)	19.5 \pm 3.4	9.3 \pm 5.0*	-

Table 4.14. The effect of benzyladenine (BA) on the flowering of *Kalanchoe* explants cultured *in vitro* on the medium of TANIMOTO and HARADA (1981) under LD (18 hour day) conditions. Ten replicates were used for each treatment. Values represent the mean \pm standard error. Significant difference from the control, P = 0.05 (*); P = 0.01 (**).

<u>Characteristic</u> <u>measured</u>	<u>Control</u>	<u>BA</u> (10 ⁻⁷ M)	<u>BA</u> (10 ⁻⁶ M)
Shoot mass (g)	0.747 \pm 0.012	0.7 \pm 0.02	-
Shoot dry mass (g)	0.15 \pm 0.003	0.13 \pm 0.19	-
Root mass (g)	0.314 \pm 0.015	0.252 \pm 0.02	-
Succulence (g fr/dry)	4.98 \pm 0.13	5.38 \pm 0.2	-

Gibberellic acid

Gibberellins are regarded as being the most florigenic of the hormones (ZEEVAART and LANG, 1962; CHAILAKHYAN and LOZHNIKOVA, 1985), although their promotive effects are mainly confined to cold requiring plants and LDP's with a rosette growth habit. Nevertheless there is a substantial list of SDP's which are induced to flower by gibberellins (BERNIER, KINET and SACHS, 1981b). In Kalanchoe, gibberellic acid (GA3) is reported to be inhibitory of flowering (HARDER and BUNSOW, 1956). In this thesis, there was little or no effect by GA3 on the rate of flowering (Figure 4.25.), while vegetative characteristics were stimulated at higher concentrations in LD (Tables 4.15. and 4.16.). Leaf succulence, which has been linked to SD induction (GUMMER, 1948; SCHWABE, 1958) was promoted to a greater extent with GA3 than any other hormone tested (Table 4.15.), the leaves taking on an exceptionally watery appearance in SD with 10^{-7} M GA3. From work presented earlier in this Chapter, it does not seem likely that succulence is in any way linked to flowering, although both are affected by short day lengths.

Stem elongation has been considered to be one of the earliest signs of reproductive transition, not only of rosette plants, but also of caulescent plants such as the SDP Glycine (PARKER and BORTHWICK, 1939), Xanthium (ERICKSON and MEICENHEIMER, 1977) and even in Kalanchoe (STEIN and STEIN, 1960). This increase in internode length begins before any sign of reproductive structures have been produced. Yet, stem elongation does not always result in flowering, as can be seen where gibberellin applications result in stem elongation but not flowering (BERNIER, KINET and SACHS, 1981b). This was also the case with Kalanchoe in this study. Although GA3 stimulated both stem and peduncle elongation in SD, the rate or degree of flowering was not affected. In LD conditions (Figure

4.16.), general growth promotion was achieved by GA_3 , but no flowering resulted, despite a three fold elongation of the stem. HARDER and BUNSOW (1956; 1957; 1958) obtained inhibition of Kalanchoe flowering with gibberellins, despite the fact that internodes elongated markedly. If gibberellins were applied to Kalanchoe plants already induced, then elongation of the peduncles took place (SCHMALZ, 1960). Such elongation was also found in this study under SD conditions (Table 4.14.), but was not accompanied by inhibition.

The results presented above do not indicate the unusual nature of the roots as affected by GA_3 , as these were unbranched, elongated and very fibrous.

Table 4.15. The effect of gibberellic acid (GA_3) on the flowering of *Kalanchoe* explants cultured *in vitro* on the medium of TANIMOTO and HARADA (1981) under SD (8 hour day) conditions. Ten replicates were used for each treatment. Values represent the mean \pm standard error. Significant difference from the control, $P = 0.05$ (*); $P = 0.01$ (**).

<u>Characteristic</u> <u>measured</u>	<u>Control</u>	GA_3 (10^{-7} M)	GA_3 (10^{-6} M)
Percentage flowering (%)	90	100	90
Number of flowers	7.7 \pm 1.9	5.5 \pm 1.1	5 \pm 0.9
Flower mass (g)	0.17 \pm 0.04	0.104 \pm 0.02	0.115 \pm 0.02
Shoot mass (g)	0.45 \pm 0.13	0.42 \pm 0.08	0.35 \pm 0.08
Shoot dry mass (g)	0.03 \pm 0.008	0.02 \pm 0.003	0.02 \pm 0.003
Succulence (g fr/dry)	15.02 \pm 1.06	19.1 \pm 1.5*	16.5 \pm 1.6
Root mass (g)	0.056 \pm 0.01	0.046 \pm 0.02	0.05 \pm 0.01
Number of leaves	13.6 \pm 3.1	14.2 \pm 4.3	10.9 \pm 1.9
Leaf area (cm ²)	4.56 \pm 1.1	3.59 \pm 0.67	3.62 \pm 1.14
Stem length (mm)	5.9 \pm 0.88	5.1 \pm 0.99	11.4 \pm 1.8**
Peduncle length (mm)	19.5 \pm 3.4	23.3 \pm 2.7	31.2 \pm 2.57**

Table 4.16. The effect of gibberellic acid (GA_3) on the flowering of *Kalanchoe* explants cultured *in vitro* on the medium of TANIMOTO and HARADA (1981b) under LD (18 hour day) conditions. Ten replicates were used for each treatment. Values represent the mean \pm standard error. Significant difference from the control, $P = 0.05$ (*); $P = 0.01$ (**).

<u>Characteristic</u> <u>measured</u>	<u>Control</u>	GA_3 (10^{-7} M)	GA_3 (10^{-6} M)
Shoot mass (g)	0.747 \pm 0.012	0.78 \pm 0.02	0.804 \pm 0.017**
Shoot dry mass (g)	0.15 \pm 0.003	0.14 \pm 0.003*	0.14 \pm 0.007*
Root mass (g)	0.314 \pm 0.015	0.363 \pm 0.01	0.375 \pm 0.017*
Succulence (g fr/dry)	4.98 \pm 0.13	5.6 \pm 0.25	5.81 \pm 0.3**
Stem length (mm)	12.4 \pm 0.9	15.7 \pm 1.6	32.1 \pm 3.2**

Abscisic acid

The number of flowers per flowering Kalanchoe explant cultured under SD conditions was significantly increased by abscisic acid (ABA) treatment at 10^{-7} M (Table 4.17.). Higher concentrations were supra-optimal, resulting in inhibition of flower number. The peduncle length was also significantly greater than the control, as was the flower mass at 10^{-7} M. The rate of flowering was only slightly reduced (Figure 4.25.). This promotion of flower characteristics by ABA was unexpected, as earlier workers had shown that ABA injected into the induced leaves of Kalanchoe was inhibitory of flowering in SD conditions (SCHWABE, 1972). All other vegetative and flowering characteristics did not vary significantly at 10^{-7} M, while at 10^{-6} M most growth was suppressed.

Table 4.17. The effect of abscisic acid (ABA) on the flowering of Kalanchoe explants cultured *in vitro* on the medium of TANIMOTO and HARADA (1981) under SD (8 hour day) conditions. Ten replicates were used for each treatment. Values represent the mean \pm standard error. Significant difference from the control, $P = 0.05$ (*); $P = 0.01$ (**).

Characteristic measured	Control	ABA (10^{-7} M)	ABA (10^{-6} M)
Percentage flowering (%)	90	70	50
Number of flowers	7.7 \pm 1.9	14.5 \pm 2.2*	2.8 \pm 0.6**
Flower mass (g)	0.17 \pm 0.04	0.27 \pm 0.04	0.04 \pm 0.01*
Shoot mass (g)	0.45 \pm 0.13	0.55 \pm 0.13	0.16 \pm 0.03**
Shoot dry mass (g)	0.03 \pm 0.008	0.03 \pm 0.006	0.01 \pm 0.001*
Succulence (g fr/dry)	15.02 \pm 1.06	16.19 \pm 1.7	15.4 \pm 1.4
Root mass (g)	0.056 \pm 0.01	0.047 \pm 0.02	0.008 \pm 0.003**
Number of leaves	13.6 \pm 3.1	13.1 \pm 1.7	12.6 \pm 1.77
Leaf area (cm ²)	4.56 \pm 1.1	4.25 \pm 0.9	1.87 \pm 0.33**
Stem length (mm)	5.9 \pm 0.88	6.2 \pm 1.2	2.1 \pm 0.33*
Peduncle length (mm)	19.5 \pm 3.4	31.1 \pm 3.2*	5.1 \pm 1.12**

Although several cases of flower inhibition by ABA have been reported in the literature, there are also several cases of promotion of flowering in plants which were marginally induced (BERNIER, KINET and SACHS, 1981b). It is possible that this flower promotion was as a result of the inhibition of vegetative growth by ABA, a theory which is rejected by the latter authors as being a minor part of flower induction. Yet unexpectedly, ABA was significantly stimulatory of the vegetative growth of Kalanchoe explants in LD conditions (Table 4.18.). Succulence was also increased by ABA in LD conditions, but not to levels achieved under SD conditions.

In vitro investigations have provided the strongest support for the promotion of flowering by ABA, where this has been achieved in Plumbago (NITSCH and NITSCH, 1967b), Perilla (PURSE, 1984) and Chenopodium (KREKULE and KOHLI, 1981, SEIDLOVA, KOHLI and PAVLOVA, 1981). The strongest support for a role for ABA in flowering comes from TANIMOTO, MIYAZAKI and HARADA (1985), who first noted that the flower gradient in Torenia is reflected by the endogenous levels of ABA. Further investigation by these authors, as reported in Chapter 1., section 1.3.1.d., indicated that an optimum level of endogenous ABA was required for flower induction of stem internodes to occur. Levels below or above this were not able to initiate flowering.

Table 4.18. The effect of abscisic acid (ABA) on the flowering of Kalanchoe explants cultured in vitro on the medium of TANIMOTO and HARADA (1981) under LD (18 hour day) conditions. Ten replicates were used for each treatment. Values represent the mean \pm standard error. Significant difference from the control, P = 0.05 (*); P = 0.01 (**).

<u>Characteristic</u>	<u>Control</u>	<u>ABA</u> (10^{-7} M)	<u>ABA</u> (10^{-6} M)
Shoot mass (g)	0.747 \pm 0.012	0.688 \pm 0.019*	0.957 \pm 0.03**
Shoot dry mass (g)	0.15 \pm 0.003	0.14 \pm 0.002*	0.149 \pm 0.008
Root mass (g)	0.314 \pm 0.015	0.187 \pm 0.009**	0.119 \pm 0.005**
Succulence (g fr/dry)	4.98 \pm 0.13	4.98 \pm 0.12	6.55 \pm 0.35**

4.3.5. The effect of endogenous inhibitors and promoters on the flowering of Kalanchoe explants in vitro

The measurement of the rate of flowering of Kalanchoe explants of the first experiment (Experiment 1.), provided interesting results (Figure 4.26.). It may be seen that explants cultured on a medium containing crushed SD leaves, flowered faster than the control. Those containing LD leaf extracts, flowered at a slower rate than the control. This inhibition appeared to be lost due to autoclaving, as those extracts cultured on a medium containing autoclaved LD leaf extract, flowered at a rate similar to the control. Yet contrary to this, the measurement of the number of flowers produced, which in Kalanchoe is a measurement of the degree of induction (SCHWABE 1956), indicated that all cultures containing extracts from either LD treated plants, or SD treated plants, produced less flowers than the control. Those containing extracts from leaves exposed to LD, both autoclaved and non-autoclaved, displayed the greatest inhibition.

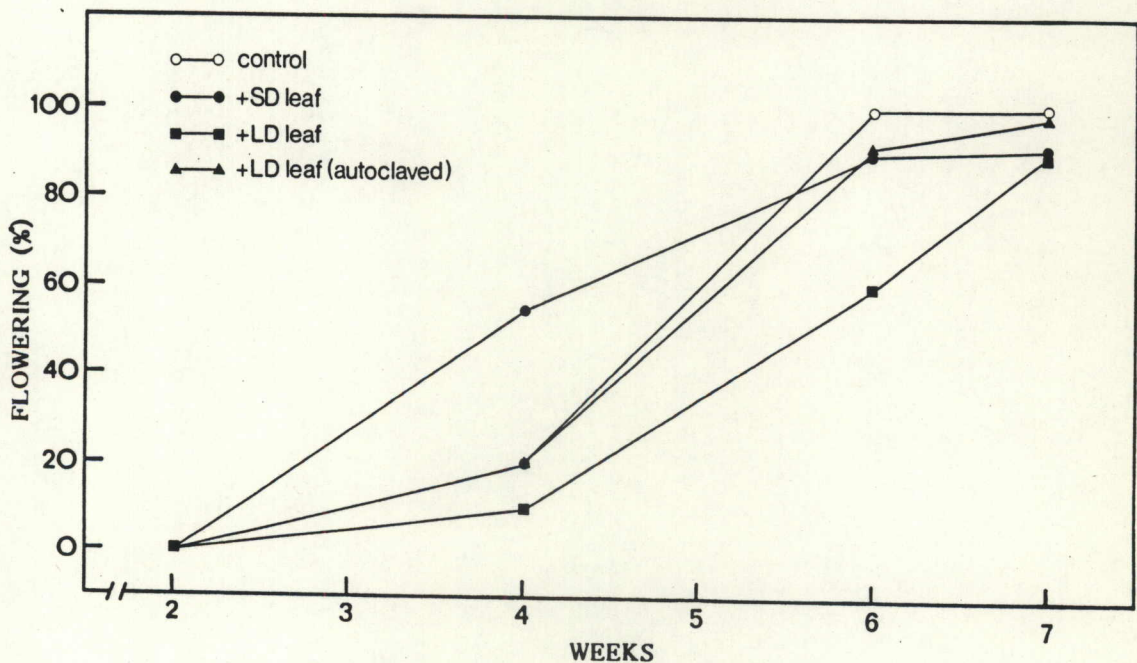


Figure 4.26. The rate of flowering of Kalanchoe explants grown in vitro on TANIMOTO and HARADA (1981b) medium supplemented with sterile crude extracts of leaves grown in SD (●) and LD (■), and by autoclaved leaves from LD (▲). Ten replicates were used for each treatment.

It is significant to note here that the promotion of the rate of flowering by SD crude extracts, was not manifest by an increased number of flowers (Table 4.19.). Similarly, an inhibitor of the number of flowers in autoclaved LD extracts, did not act to inhibit the rate of flowering. It seems thus that two different control mechanisms are present here. On the one hand, the rate of flowering, which is an indication of the inclination or ability of the plant to produce flowers rapidly. On the other hand, the number of flowers per plant, which is an indication of the degree of branching of the inflorescence. Both of these give some indication of the degree of floral induction, yet they appear to be removed from each other. This is evidenced by the results shown here, which show that at least two compounds are acting to produce a certain flowering response. This does not deny though, that "florigen" or the floral stimulus is not in overall control of both of these processes. In Lathyrus and Pisum, an investigation of the control of flowering and branching, indicated that they are controlled by different genes (ROSS and MURFET, 1985).

Table 4.19. **Experiment 1.** The effect of crude leaf extracts taken from both LD and SD grown leaves, on the number of flowers produced by Kalanchoe explants cultured on the medium of TANIMOTO and HARADA (1981) under SD conditions. Significant difference from the control, P= 0.05 (*), and P= 0.01 (**).

	<u>Untreated</u> <u>control</u>	<u>SD extract</u>	<u>LD extract</u>	<u>Autoclaved</u> <u>LD extract</u>
Number of flowers	17.6 \pm 1.65	12.6 \pm 0.9*	7.4 \pm 1.07**	7.8 \pm 0.85**

In both subsequent repeats of this experiment (Experiments 2. and 3.), no significant alteration of the rate of flowering, when compared to the control, was observed. This raises doubt about the significance of the results presented in Figure 4.26.. One possible explanation is that the leaves crushed into the medium were highly variable between experiments. Leaves within each experiment were as consistent as possible. Yet despite the above, in Experiments 2. and 3., a significant difference was found for the number of flowers produced per plant. Table 4.20.

Table 4.20. **Experiment 2.** The effect of crude leaf extracts taken from both LD and SD grown leaves, on the flowering of *Kalanchoe* explants cultured on the medium of TANIMOTO and HARADA (1981) under SD conditions. Significant difference from the control, P= 0.05 (*), and P= 0.01 (**).

<u>Characteristic measured</u>	<u>Untreated control</u>	<u>SD extract</u>	<u>Autoclaved SD extract</u>	<u>LD extract</u>	<u>Autoclaved LD extract</u>
Number of flowers	6.3 \pm 0.62	7.3 \pm 0.47	9.0 \pm 0.59*	6.0 \pm 0.46	2.6 \pm 0.7**
Peduncle length (mm)	15.5 \pm 1.72	14.75 \pm 1.51	10.5 \pm 2.77	14.2 \pm 2.25	5.5 \pm 0.5**
Inflorescence mass (g)	0.14 \pm 0.01	0.17 \pm 0.01	0.16 \pm 0.03	0.14 \pm 0.02	0.06 \pm 0.009**
Shoot fresh mass (g)	0.57 \pm 0.05	0.68 \pm 0.04	0.75 \pm 0.05*	0.59 \pm 0.05	0.33 \pm 0.04**
Shoot dry mass (g)	0.02 \pm 0.002	0.02 \pm 0.001	0.03 \pm 0.002*	0.02 \pm 0.002	0.01 \pm 0.001*
Succulence (g fr/dry)	23.18 \pm 0.4	24.39 \pm 0.74	22.55 \pm 0.95	24.45 \pm 0.202	17.64 \pm 1.60**
Root mass (g)	0.04 \pm 0.009	0.06 \pm 0.008	0.06 \pm 0.001	0.06 \pm 0.007*	0.1 \pm 0.003*

In Experiment 2. (Table 4.20.) some promotion of flower number was achieved with autoclaved LD extracts. Non-autoclaved extracts had the same tendency, but did not produce results significantly different from the control. In Experiment 3. (Table 4.21.), all extracts were inhibitory, with the exception of non-autoclaved SD extracts, which were not significantly so.

Table 4.21. **Experiment 3.** The effect of crude leaf extracts taken from both LD and SD grown leaves, on the flowering of *Kalanchoe* explants cultured on the medium of TANIMOTO and HARADA (1981) under SD conditions. Significant difference from the control, P= 0.05 (*), and P= 0.01 (**).

<u>Characteristic</u> <u>measured</u>	<u>Untreated</u> <u>control</u>	<u>SD extract</u>	<u>Autoclaved</u> <u>SD extract</u>	<u>LD extract</u>	<u>Autoclaved</u> <u>LD extract</u>
Number of flowers	13.3±0.73	12.11±0.73	11.5±0.8**	8.0±1.3**	9.62±0.53**
Peduncle length (mm)	18.2±2.12	18.46±2.6	13.85±2.0*	14.3±2.29	14.15±2.77
Inflorescence mass (g)	0.248±0.014	0.24±0.79	0.19±0.02**	0.172±0.2**	0.16±0.017
Shoot fresh mass (g)	0.96±0.042	0.94±0.06	0.88±0.084	0.83±0.08*	0.89±0.059
Shoot dry mass (g)	0.045±0.001	0.045±0.003	0.05±0.004	0.04±0.004	0.04±0.003
Succulence (g fr/dry)	21.8±0.5	20.85±0.6	21.8±0.7	20.88±0.71*	22.14±0.58
Root mass (g)	0.131±0.017	0.119±0.012	0.1±0.006**	0.109±0.012	0.08±0.008**

It thus appears that although there are some signs of the promotion of flowering by SD crude extracts, the results are not conclusive. All crude extracts appeared to be inhibitory of the number of flowers produced, LD extracts being consistently more inhibitory than SD extracts.

The analysis of various vegetative and flowering characteristics was undertaken to gauge what effect crude extracts would have on the growth of the explants. This was done in view of the fact that Kalanchoe exhibits various changes in its growth in response to SD induction, as discussed earlier, and reviewed by SCHWABE (1969; 1985).

Shoot mass, both fresh and dry, was significantly increased by the addition of autoclaved SD explants in Experiment 2., and decreased by autoclaved LD extracts (Table 4.20.), as were the flowering characteristics in the latter. This was an unexpected result, as SD induction of flowering is generally associated with a reduction in vegetative growth. Kalanchoe plants do normally gain mass through increasing succulence in response to SD induction (GUMMER, 1949; SCHWABE, 1958), but the mass gain here, was in dry mass as well. The inhibition of growth by autoclaved LD extracts, was also unexpected. The inhibitor present in LD grown plants (SCHWABE, 1956) is specific for flowering, and should have no direct influence on vegetative growth. In this experiment, inhibition by this crude extract appeared to be general to all growth.

It is important to note, that it was only autoclaved LD and SD crude extracts which achieved significant effects, while non-autoclaved extracts were unable to. This implies that either substances are produced in the extracts as a result of heating, or that the

promotive/inhibitory effects in non-autoclaved extracts are destroyed by continuing enzyme activity in the crude extract. The latter possibility is more likely. PRYCE (1972) found that the inhibitor of flowering in Kalanchoe was heat stable.

LD extracts were also significantly promotive of root mass in Experiment 2. (Table 4.20.). Flowering, and SD inductive cycles are known to be inhibitory of root growth (section 4.3.1.), but the indication here is that LD cycles have produced a promoter of root growth, which is able to operate despite the fact that the explants were maintained under SD conditions. These results were not observable in Experiment 3. (Table 4.21.). The reasons for this variation are not known.

The results of the above experiments, are highly variable and inconclusive. It appears that a large source of variation has been introduced into the different experiments. This was most likely through the leaves used for incorporation, as they were taken from plants with different origins. Any small physiological difference between these leaves, would affect the resulting performance of the bioassay, as the extract is applied whole and unpurified. It thus appeared necessary to separate extracts chromatographically to some degree before testing, although SCHWABE (1972) had successfully bioassayed the inhibitor using crude extracts of Kalanchoe leaves, injected into whole plants.

Separated extracts

Extracts from leaves that were exposed to SD or LD, which were then separated on chromatography paper, produced varying degrees of flowering. It is apparent that in LD extracts separated on paper (Figure 4.27.), there were two fractions (Rf's 0.6 and 0.8) which were promotive of the rate of flowering above that of untreated controls. Two fractions were also strongly inhibitory (Rf's 0.4 and 1.0). SD extracts also produced stimulatory effects at Rf 0.8, (Figure 4.28.) while all other Rf's contained inhibitors of flowering. The strongest inhibition from SD extracts resulted from Rf 0.4, while Rf 1.0 was less inhibitory than in the LD extract.

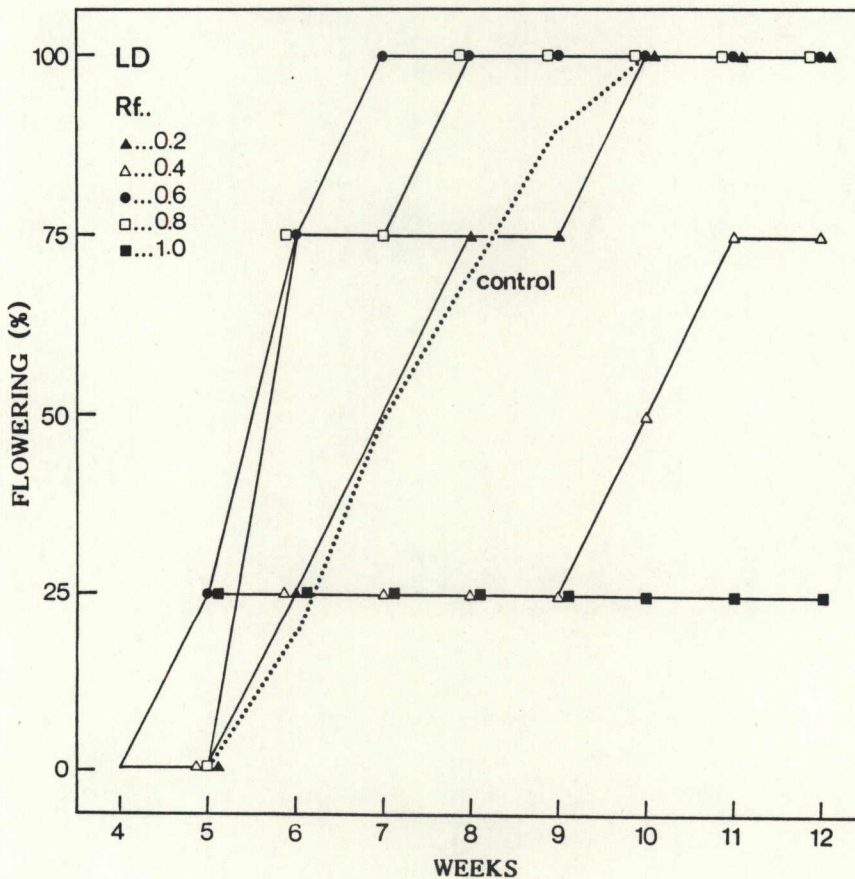


Figure 4.27. The rate of flowering of *Kalanchoe* explants grown *in vitro* on TANIMOTO and HARADA (1981b) medium supplemented with the separated extracts of leaves maintained under LD conditions. Five replicates were used for treatments which were maintained in SD conditions.

These results appear contrary to expectations, as it was anticipated that LD extracts would be more inhibitory of flowering, while SD extracts would be either neutral or promotive. The reverse appears to have occurred, with the exception of Rf 0.8, which in SD extracts was more stimulatory than in LD extracts.

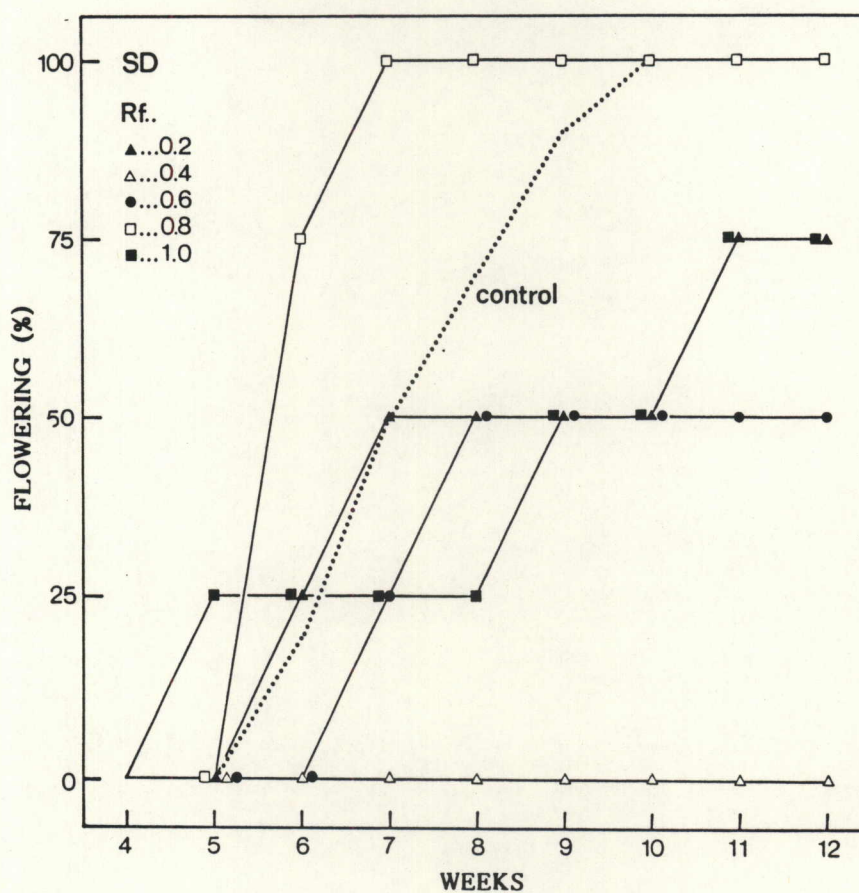


Figure 4.28. The rate of flowering of *Kalanchoe* explants grown *in vitro* on TANIMOTO and HARADA (1981b) medium supplemented with the separated extracts of leaves maintained under SD conditions. Five replicates were used for treatments which were maintained in SD conditions.

In order to determine the specificity of these effects, other growth parameters were also measured. These results are presented in Figures 4.29. and 4.30. for clarity and also in Table 4.22. and 4.23.. In LD extracts, Rf values that produced the highest rates of flowering (Rf's 0.6 and 0.8, Figure 4.27.), also produced the largest flower number, peduncle length, inflorescence mass and shoot fresh mass (Figure 4.29.). In SD extracts, the stimulation of flowering rate at Rf 0.8 (Figure 4.28.) also produced high values for vegetative and growth characteristics (Figures 4.28. and 4.30.).

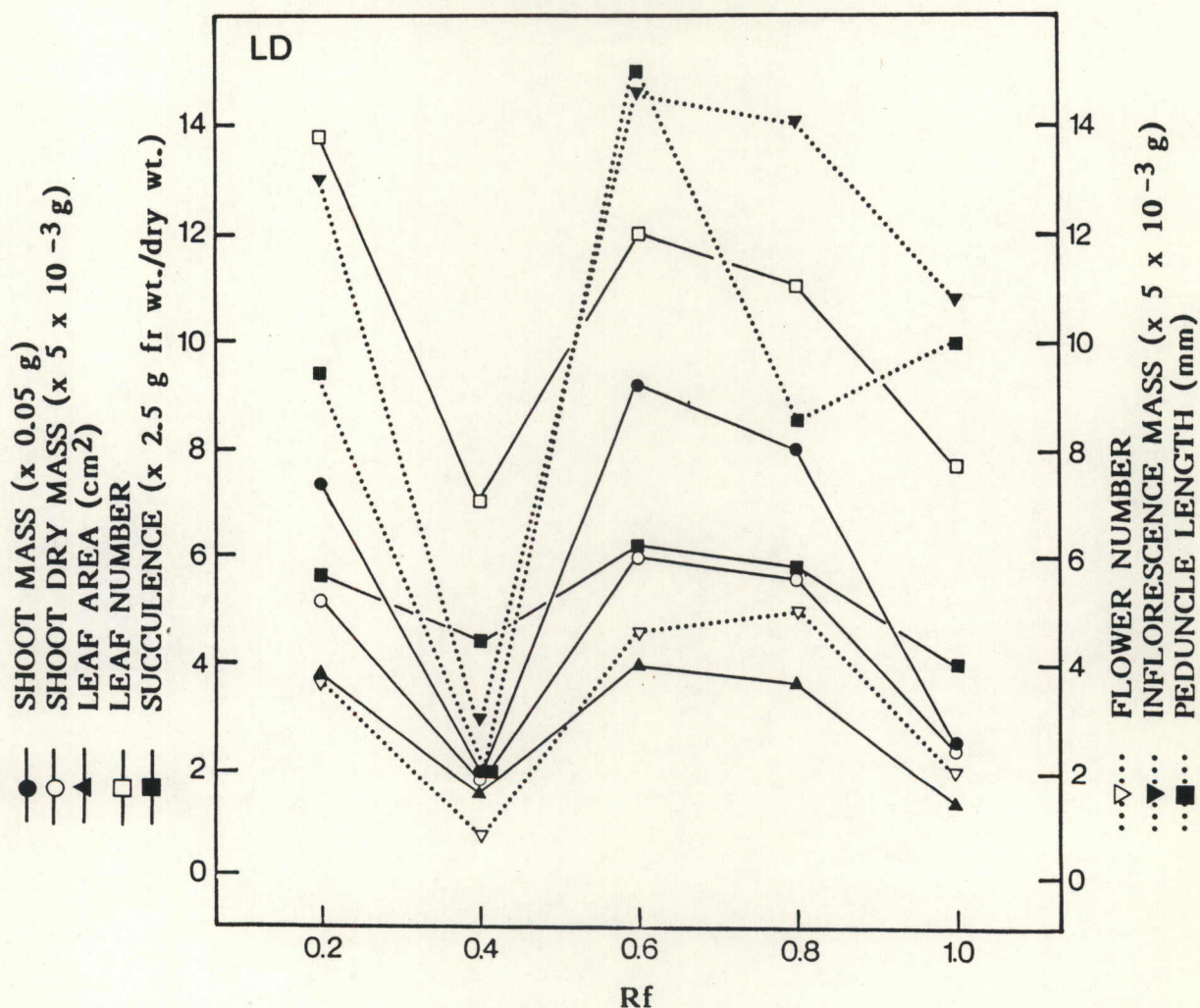


Figure 4.29. Vegetative and flowering characteristics of *Kalanchoe* explants grown on TANIMOTO and HARADA (1981b) medium supplemented with the separated extracts of plants grown under LD conditions. Five replicates were used for treatments which were grown under SD conditions.

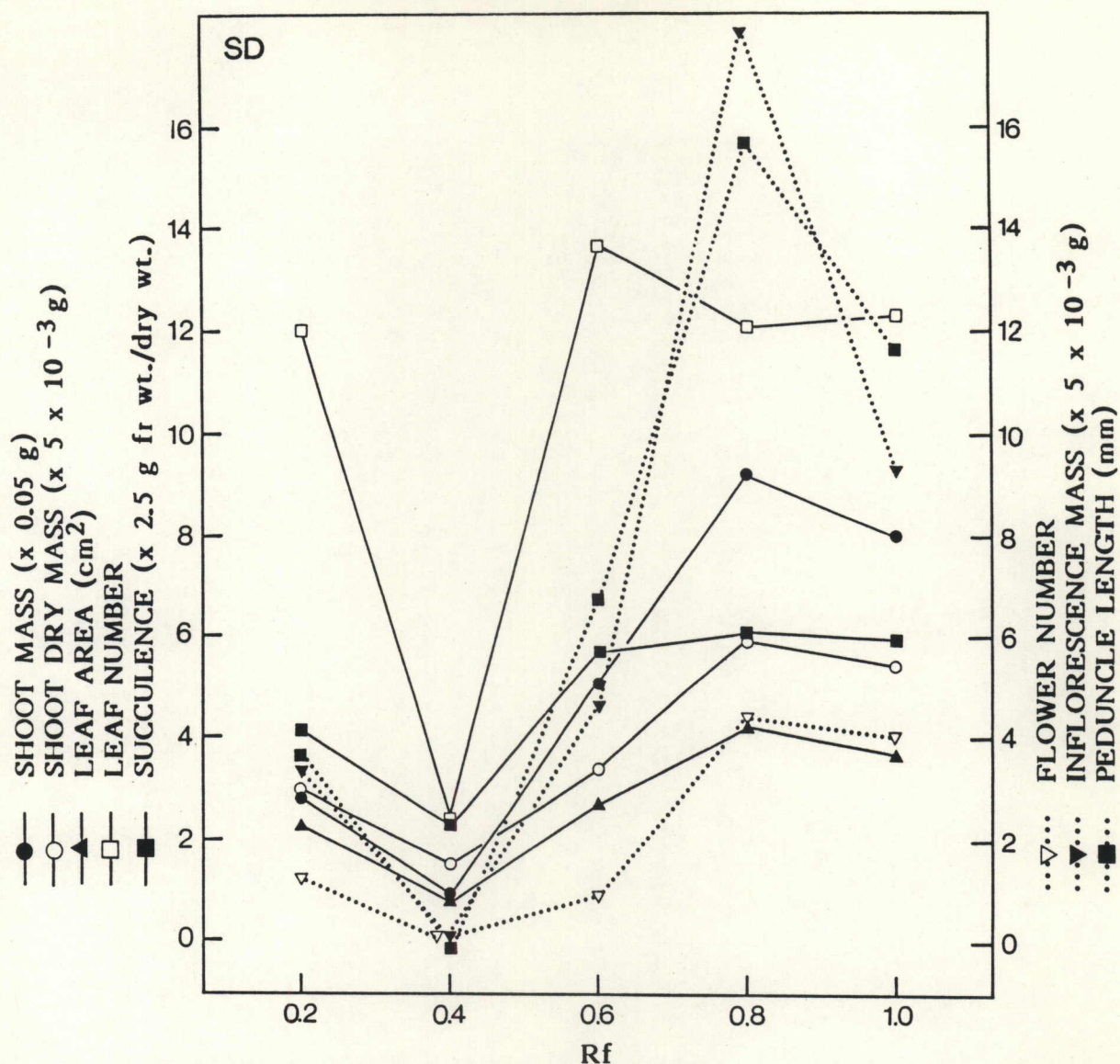


Figure 4.30. Vegetative and flowering characteristics of *Kalanchoe* explants grown on TANIMOTO and HARADA (1981b) medium supplemented with the separated extracts of plants grown under SD conditions. Five replicates were used for treatments which were grown under SD conditions.

Inhibition of the rate of flowering in explants exposed to fractions of extracts derived from leaves exposed to LD (Rf's 0.4 and 1.0), was also reflected by the inhibition of most other flowering and vegetative characteristics measured. These results imply that for promotion and inhibition of flowering by fractions on different Rf values, the alteration of flowering was also a general alteration of growth. This

suggests that none of the promotive or inhibitory effects were specific to flowering. The results produced by extracts taken from plants exposed to SD produced the same general conclusions, as the stimulation of flowering by Rf 0.8 was a general one for all growth factors measured. The strongest inhibition of flowering by Rf 0.4, was also the strongest inhibition of growth.

If the results from extracts of leaves exposed to LD (Figure 4.29.) are compared with those exposed to SD (Figure 4.30.), support is obtained for the greatest degree of promotion of floral characteristics by Rf 0.8 derived from SD plants. No indication was obtained of the specific inhibitor of flowering which is known to occur in LD conditions (SCHWABE 1956).

Table 4.22. The effect of extracts derived from leaves exposed to SD conditions on the flowering of *Kalanchoe* explants cultured *in vitro* on the medium of TANIMOTO and HARADA (1981) under SD (8 hour day) conditions. Ten replicates were used for each treatment. Values represent the mean \pm standard error. Significant difference from the control, P = 0.05 (*); P = 0.01 (**).

Characteristic measured	Rf value				
	0.2	0.4	0.6	0.8	1.0
Number of flowers	1.20 \pm 0.58	0	0.8 \pm 0.4	4.2 \pm 1.3	4.0 \pm 1.2
Peduncle length (mm)	3.6 \pm 0.8	-	6.7 \pm 3.9	15.6 \pm 6.4	11.6 \pm 3.8
Inflorescence mass (g)	0.02 \pm 0.00	-	0.02 \pm 0.0	0.09 \pm 0.00	0.05 \pm 0.02
Shoot mass (g)	0.14 \pm 0.03	0.04 \pm 0.01	0.25 \pm 0.07	0.46 \pm 0.12	0.4 \pm 0.1
Shoot dry mass (g)	0.014	0.007	0.017	0.03	0.027
Succulence (g fr/dry)	10.0	6.0	14.7	15.3	14.8
Number of leaves	12.0 \pm 2.1	2.4 \pm 0.4	13.6 \pm 2.9	12.0 \pm 2.1	12.3 \pm 1.4
Leaf area (cm ²)	2.03 \pm 0.5	0.69 \pm 0.16	2.7 \pm 0.67	4.3 \pm 0.8	3.7 \pm 0.8

Table 4.23. The effect of extracts derived from leaves exposed to LD conditions on the flowering of *Kalanchoe* explants cultured in vitro on the medium of TANIMOTO and HARADA (1981) under SD (8 hour day) conditions. Ten replicates were used for each treatment. Values represent the mean \pm standard error. Significant difference from the control, P = 0.05 (*); P = 0.01 (**).

<u>Characteristic</u> <u>measured</u>	<u>Rf value</u>				
	0.2	0.4	0.6	0.8	1.0
Number of flowers	3.8 \pm 1.9	0.8 \pm 0.4	4.6 \pm 1.8	5.0 \pm 0.3	2.0
Peduncle length (mm)	9.6 \pm 2.3	2.0 \pm 0.41	14.9 \pm 2.9	8.5 \pm 2.6	10.0 \pm 5.9
Inflorescence mass (g)	0.07 \pm 0.03	0.2 \pm 0.0	0.07 \pm 0.02	0.07 \pm 0.03	0.054
Shoot mass (g)	0.37 \pm 0.15	0.1 \pm 0.0	0.46 \pm 0.15	0.4 \pm 0.10	0.13 \pm 0.60
Shoot dry mass (g)	0.026	0.009	0.03	0.028	0.013
Succulence (g fr/dry)	14.2	11.0	15.3	14.3	10.0
Number of leaves	13.8 \pm 1.2	7.0 \pm 1.1	12.0 \pm 2.0	11.0 \pm 1.1	7.7 \pm 1.5
Leaf area (cm ²)	3.7 \pm 0/5	1.5 \pm 0.3	4.0 \pm 0.9	3.7 \pm 0.9	1.5 \pm 0.7

4.4. CONCLUSIONS

The work presented in the first section of this chapter, indicated that factors present in the parent plant at the time of explant excision, affect the resulting ability of the explant to flower, as well as various vegetative growth parameters. It was not determined initially, whether it is possible for the stimulus to be carried over during culture, although this was achieved in a later experiment after 28 days of induction, the results of which are presented in section 4.3.2.. Generally, a reduced capacity for root growth was associated with an increasing involvement of SD, irrespective of whether the explant could be induced to flower or not. A similar reduction was found for both anthocyanin and chlorophyll contents. The leaves of plants associated with SD also became more succulent, which resulted in a higher shoot fresh mass, but lower dry mass. This raises an unusual conceptual problem; as the pigment levels are reduced on a fresh mass basis, possibly due to dilution by increasing succulence, what is the significance to the plant? Does a succulent plant operate and metabolize the same as a non-succulent plant with the same dry mass? This does not appear so, in view of the fact that Kalanchoe undergoes a conversion to crassulacean acid metabolism (CAM) after several weeks of SD cycles (DELEENS and QUEIROZ, 1984), an event that may accompany the increase in succulence of the leaves.

The viscosity of the cell cytoplasm (SCHWABE AND WILSON, 1965) as well as its acidity (SCHWABE, 1958) have been shown to change concomitantly with increasing succulence in SD conditions. It is not known if there is any causal relationship between these changes, and the induction and evocation of flowering, but until evidence is found to disprove any

correlation between them, these factors provide useful concurrent characteristics or expressions which can be studied in conjunction with flowering. It is for this reason, that many of these factors were measured in the later sections of this investigation as all of these factors may form part of a multi-component stimulus or sequence of events leading to flowering.

No significant alteration of the flowering of Kalanchoe explants cultured in SD was achieved with any of the phenolics tested. The reputed inhibitor of flowering in this plant, gallic acid had no effect at 10^{-5} M to 10^{-7} M. Other phenolics utilized, tannic and nicotinic acids, were inhibitory of flowering at both 10^{-4} M and 10^{-6} M. This inhibition included vegetative growth, and was thus not a specific inhibition of flowering. Benzoic and salicylic acids had no effect on flowering. Furthermore, no induction of flowering in non-inductive LD was achieved by any of the phenolics tested. The role of these substances in flowering is far from clear. There is no unequivocal evidence to support any of these substances as either a flower inhibitor or promoter. It is possible that their role is to work in combination with other substances or that their effects have been pharmacological.

Auxin (NAA) partially increased the succulence of explants, thus simulating the effects of SD induction. Yet this was not achieved in non-inductive LD, indicating that succulence is not exclusively under the control of auxin. The rate of flowering of Kalanchoe explants was inhibited by auxin application, while vegetative growth was generally stimulated. It is not known if this inhibition of flowering is specific, or is a result of stimulated vegetative growth.

Cytokinins (BA) proved to be inhibitory of all growth and flowering in Kalanchoe explants in SD, but did not seem to be so in LD. These results were not conclusive due to high variation in the results.

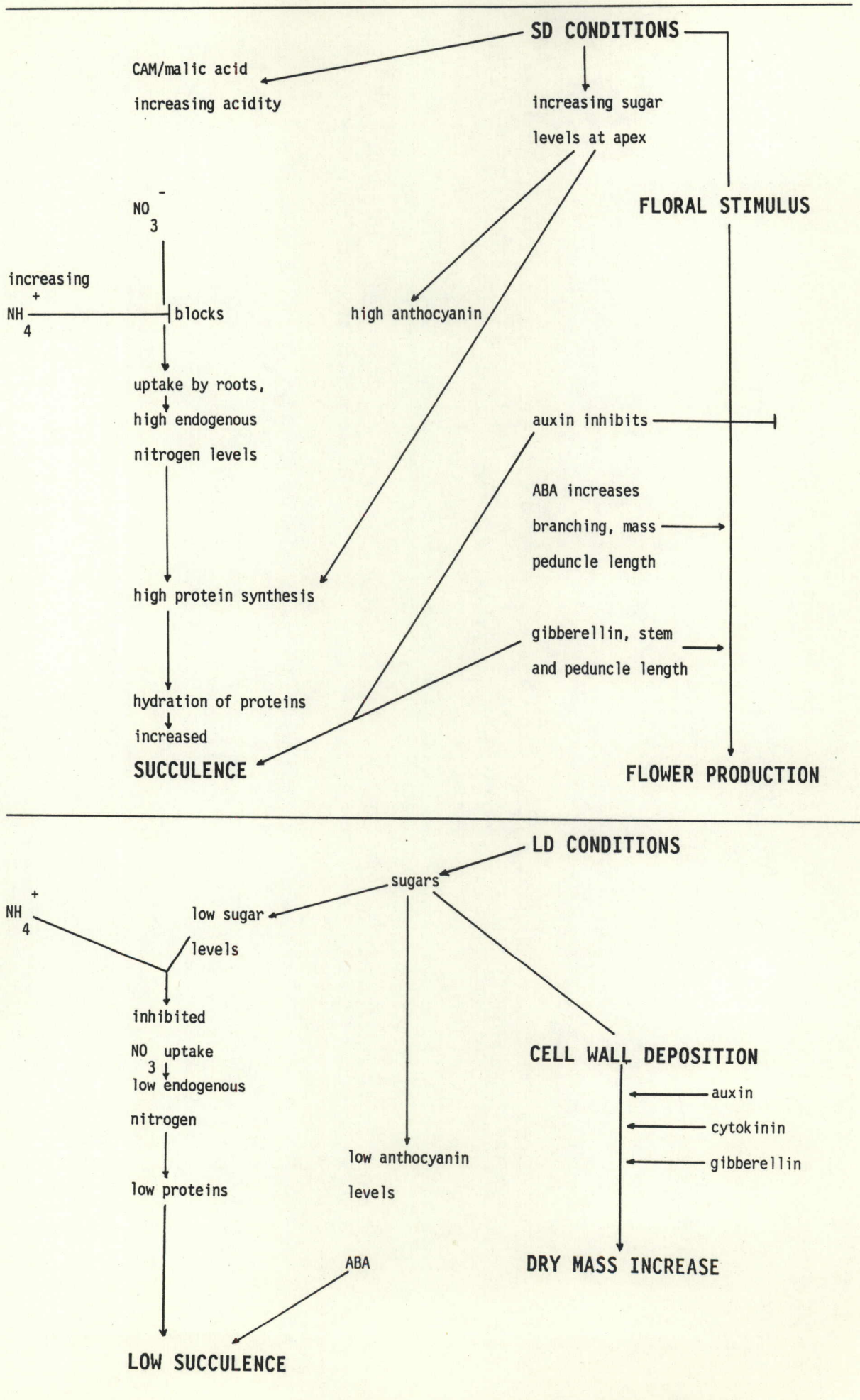
Gibberellic acid significantly promoted vegetative growth, in particular succulence, while flowering was not affected. The elongation of the stem did not influence flowering, supporting conclusions taken from the literature, and separating these two events as being unrelated.

Abscissic acid was the only hormone tested which caused an increase in the number of flowers per plant, together with other flower characteristics. This stimulation did not appear to be due to an inhibition of vegetative growth.

Attempts to use this in vitro flowering system to test for the presence of inhibitors or promoters of flowering met with little success. Initial investigations indicated that the rate of flowering and the number of flowers produced, were under different control mechanisms. These conclusions were not borne out in subsequent investigations. No unequivocal conclusions could be made from any of these results, and it is clear that the technique of extraction needs considerable refinement if it is to work. There are advantages nevertheless, in pursuing the use of crude extracts, as in this form, the stimulus could be unchanged and in its original state.

A tentative scheme for the involvement of carbohydrate and nitrogen as well as hormones in the flowering and growth of Kalanchoe plants is presented below (Figure 4.31.). This is a compilation of the results obtained, and general principles deduced by other workers, and quoted earlier in the discussion.

Figure 4.31. A tentative scheme for the involvement of carbohydrates, nitrogen and hormones in the flowering and vegetative growth of *Kalanchoe*, under SD and LD conditions.



Chapter 5.

ATTEMPTS TO USE RADIOISOTOPES TO LABEL THE PRODUCTS OF PHOTOINDUCTION IN XANTHIUM STRUMARIUM

5.1. INTRODUCTION

Radiolabelled isotopes have proved most useful in the elucidation of numerous biochemical cycles and pathways. The most reputed of these is probably that of BENSON, BASSHAM, CALVIN, HALL, HIRSCH, KAWAGUCHI, LYNCH and TOLBERT (1952) which helped elucidate the processes of photosynthesis. The basic techniques used by these authors, were to feed ^{14}C to photosynthetic algae, which were then sacrificed at decreasing time intervals and extracted for substances carrying the label. Autoradiographs were used to locate the new products formed, which contained the label. In this way, the exact sequence of biochemical change could be determined.

A similar trend in flowering research, has led to a new approach to the quest for "florigen", although this has not met with much success. The main problem here, is that the time required for the production of the flowering stimulus, is likely to be very much greater than the few seconds required for sugar synthesis during photosynthesis. This means that numerous unrelated substances would also acquire the label during any attempt to label the stimulus. AHARONI, GOLDSCHMIDT and HALEVY (1985) fed ^{14}C -acetate to Pharbitis plants under different daylengths. Two significant peaks of radioactivity resulted in vegetative plants, and disappeared during the first long night of flower induction. Light breaks, which prevented flowering, reformed these peaks, which were found

in most above ground organs. These authors to date have not identified these peaks, but noted that some modification of their chemical nature may take place during extraction.

DURZAN (1983) successfully extracted numerous substances involved with the synthesis of nitrogenous compounds during flowering, but was unable to identify many of them. The proteins produced in Xanthium during induction to flower, were examined by SHERWOOD, EVANS and ROSS (1971), but no difference in the bands after electrophoretic separation was found. NITSAN (1962) did find changes in two proteins which were only synthesised during SD induction in Xanthium. BLEDSOE and ROSS (1978) applied ¹⁴C-mevalonic acid to Xanthium, but found no difference in uptake, distribution or chromatographic pattern between induced and vegetative plant extracts.

The transport of the stimulus in Xanthium has been extensively studied, and this plant thus provides an ideal species for such an investigation, as it was envisaged that the plant could be induced to transport the stimulus to a remote sink, where it could be separated from the bulk of the unrelated substances. SKOK and SKULLY (1954) determined that in Xanthium, the stimulus would move out of an induced leaf in daylight, six hours after the close of the first long dark period. Amounts sufficient to induce flowering would move out within ten hours. These authors used single leaved plants which were prepared by removing all but the youngest fully expanded leaf. This leaf supplied stimulus to the apex. LAM (1965) showed that in Xanthium, the stimulus could be forced downwards as far as the cotyledonary buds, if the plant was decapitated and disbudded except for the sink buds. The stimulus is known to move fairly rapidly

through the phloem, in Lolium at a velocity of 2.4 cm hour^{-1} , (EVANS and WARDLAW, 1966;), and in Pharbitis at $24-37 \text{ cm hour}^{-1}$ (KING, EVANS and WARDLAW, 1968). In both cases this rate was slightly slower than the rate of carbon assimilate transport and appeared to be independent. CHAILAKHYAN and BUTENKO (1957a,b) reported that in Perilla, the two travel at the same velocity.

The objective of this experiment was to induce the transport of the stimulus, together with basic assimilates (probably mostly sucrose), to an active sink remote from the leaf where $^{14}\text{CO}_2$ was applied. This sink in induced plants could then be expected to contain basic assimilates and the floral stimulus, while the bud of vegetative non-induced plants would contain only assimilates. The difference between the two compositions could be most revealing. The timing of harvest is important though, as the time must be long enough for the stimulus in induced plants to reach the sink bud, but short enough to avoid the numerous other products formed once the labelled assimilates arrive in the bud. It was hoped that it would be possible to attach a sensitive Geiger counter to the stem above the receptor bud, to warn of the arrival of the assimilates, This instrument was unfortunately not sensitive enough. This meant that the arrival of the stimulus could not accurately be determined.

5.2. MATERIALS AND METHODS

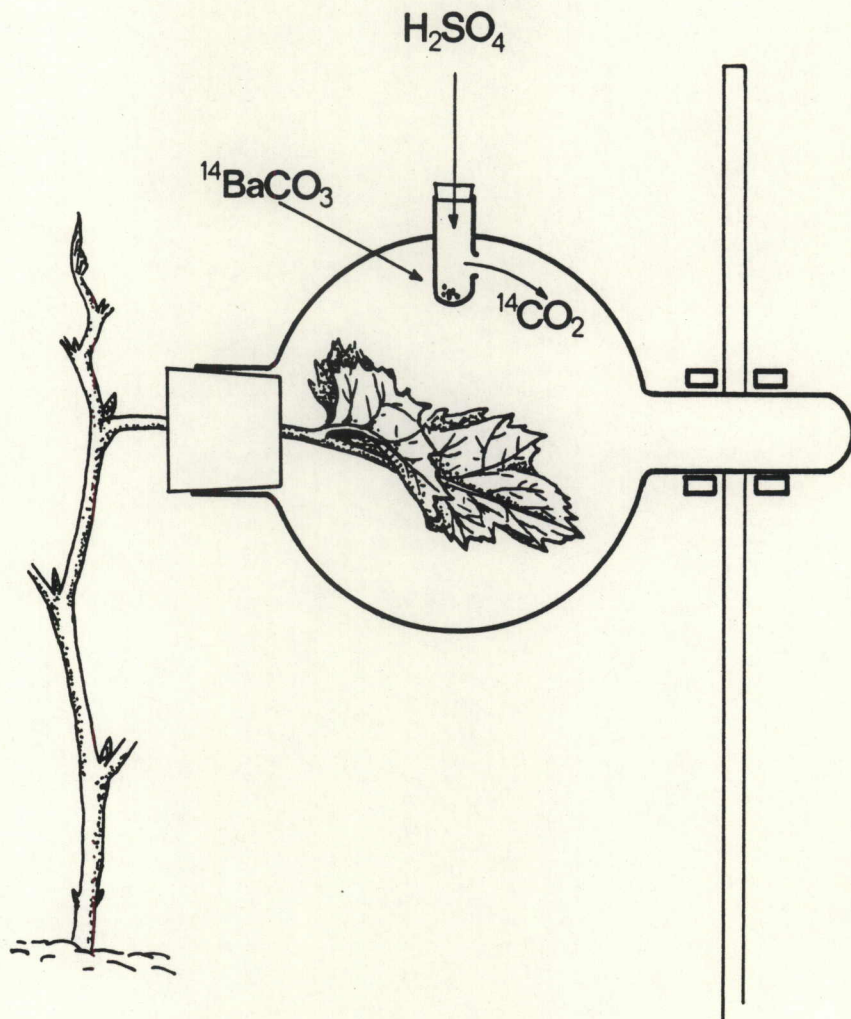


Figure 5.1. Technique for the incorporation of $^{14}\text{CO}_2$ into Xanthium strumarium.

Experiment 1.

Xanthium strumarium L. seeds were germinated and grown in pots under LD (18 hour day) conditions until they had approximately five leaves. Plants were exposed to $^{14}\text{CO}_2$ by placing the youngest fully expanded leaf, which according to KHUDARI and HAMNER (1954) is the most receptive to photoperiodic induction into a flask as shown in Figure 5.1.. Care was taken not to constrict the phloem, and gaps were plugged with petroleum jelly. Radiolabelled $^{14}\text{BaCO}_3$ was placed into the flask, and the $^{14}\text{CO}_2$ released by injecting dilute H_2SO_4 through the rubber plug. Six plants were so treated, half remaining in LD (18 hour day) conditions while exposed to $^{14}\text{CO}_2$, and the other half in SD (8 hour day) conditions. Light intensity of photosynthetically available radiation, was $150 \mu\text{Em}^{-2} \text{ s}^{-1}$. Plants were harvested after one and four days, by removing the treated leaf, and by excising the upper three axillary buds and the apical bud, which would normally form flowers in response to SD.

After exposure to $^{14}\text{CO}_2$, the apical bud, axillary bud and a small portion of the treated leaf were frozen rapidly in liquid nitrogen, and ground in a mortar and pestle while frozen. A small amount of this was scraped up with a spatula while still frozen, and applied to a small area on Whatmans No. 3MM chromatography paper. This was rapidly moved into the vapour emitted from boiling ethanol, covered with a beaker, and exposed for ten seconds, after which time, chlorophyll and other substances were beginning to diffuse into the paper. These crude extracts were separated two dimensionally, first with the single phase iso-propanol : ammonium hydroxide : water (10 : 1 : 1 v/v) or PAW; and secondly with the two phase butan-1-ol : acetic acid : water (4 : 1 : 5 v/v) or BAW. The upper phase of BAW was used as the solvent. The lower phase was used to equilibrate the tank. Partitioning must occur for twelve hours. After separation, the chromatograms were placed into close contact with X-ray film, pressed firmly in a press and kept in the dark for four weeks.

Extraction

Treated leaves were further extracted and separated into different groups of compounds. The frozen leaf was crushed and placed into boiling 95 per cent ethanol for fifteen minutes. This extract was centrifuged at 3000g for ten minutes, and the supernatant isolated. The pellet was resuspended in 80 per cent ethanol, and again centrifuged. The supernatants were combined.

The aromatic compounds in the extract were removed by shaking with 25 g deactivated charcoal for one hour at 5 °C. (Deactivated charcoal was prepared by mixing 150 g activated charcoal with two litres of five per cent acetic acid. This was filtered and thoroughly washed with ten litres distilled water until all acid had been removed). The extract with charcoal was filtered and the charcoal washed and again filtered with 150 ml of 30 per cent ethanol. The filtrates were combined, and contained the amino acids, sugars and organic acids. This was reduced in volume to 25 ml 80 per cent ethanol. The aromatic compounds were removed from the charcoal by washing with 200 ml ethanol, followed by 200 ml ethyl ether and benzene (1 : 1 v/v) for two hours. These were combined and evaporated at 60 °C. The sediment was resuspended in 50 ml 80 per cent ethanol. This extract contained the aromatic compounds. High levels of radioactivity remained adsorbed to the charcoal after removal of the aromatics. The charcoal was thus washed with 300 ml 50 per cent acetic acid. This filtrate was taken to dryness and the residue eluted in 25 ml 80 per cent ethanol.

Small amounts (30 µl) of each extract were applied to silica gel TLC plates (Silica gel 60 F Merck), and separated two dimensionally in a vertical direction. The aromatic compounds were separated using ten per

cent acetic acid, and then BAW (n-butanol : acetic acid : water) in a ratio of 63 : 10 : 27 v/v. All other fraction were separated using PAW (isopropanol : ammonium hydroxide : water) at a ratio of 10 : 1 : 1 v/v and then BAW (butan-1-ol : acetic acid : water) at a ratio of 4 : 1 : 5 v/v. Dried TLC plates were pressed firmly against X-ray film and exposed for six weeks in the dark.

Experiment 2.

Plants were treated in a similar way as those in Experiment 1, with some minor alterations. The plants were exposed to $^{14}\text{CO}_2$ and maintained in LD (18 hour day) at $250 \mu\text{Em}^{-2} \text{ s}^{-1}$ for two days. Fresh $^{14}\text{BaCO}_3$ was added. and then the plants were separated, two receiving one SD cycle, two four SD cycles, and two four LD cycles. Shoot apices, axillary buds and leaves were harvested six hours after the end of the last long dark period. All buds, and small leaf samples were crushed and applied to Whatmans No. 3MM chromatography paper as described earlier in Experiment 1. For greater separation, 80 per cent phenol w/v at pH 5.4 was used as the first solvent, and BAW (4 : 1 : 5 v/v) the second solvent.

Leaves were also extracted with solvents as before, and separated, again using 80 per cent phenol at pH 5.4 as the first solvent. This solvent was not used for the aromatics, where ten per cent acetic acid in chloroform followed by 45 per cent ethyl acetate in benzene were used. These chromatograms were exposed to X-ray film for six weeks before developing.

Experiment 3.

Xanthium plants were grown in pots under natural LD conditions (high light intensity). Six plants were debudded of all buds except for the single axillary bud above the position of initial cotyledon attachment. Bud extension took place for 30 days, after which a short branch (± 5 cm) was formed, having only small leaves. At this stage, $^{14}\text{CO}_2$ was applied to the youngest fully expanded leaf occurring on the same orthostichy as the expanding bud. Three such plants were subjected to SD (8 hour day) and three to LD (18 hour day), all in full sunlight. At the end of the first night, all other leaves, including the small leaves and axillary buds on the lateral branch, were removed, leaving only the treated leaf and the apical bud on the expanding branch. After two nights of exposure to $^{14}\text{CO}_2$ and towards the end of the following day, the apical bud (± 1 mm) of each plants was harvested. This was rapidly frozen in liquid nitrogen and crushed while solid. One millilitre of 80 per cent ethanol was added and the tissue macerated in this. This extract was applied directly of TLC plates (Merck Silica gel 60 F₂₅₄), avoiding the debris, and separated using 80 per cent phenol (w/v) at pH 5.4 as the first solvent system, and BAW, ie. butan-1-ol : acetic acid : water (4 : 1 : 5 v/v) as the second solvent. These chromatograms were exposed for three months in the dark.

Standards

Sugar and amino acid standards were separated using the same solvent systems as above. Sugars were investigated by developing chromatograms with a spray reagent consisting of 0.3 per cent amino hippuric acid and three per cent phthalic acid in ethanol. The chromatograms were then developed at 140 °C for eight minutes. Amino acid chromatograms were developed by spraying with 0.1 per cent ninhydrin in acetone.

5.3. RESULTS AND DISCUSSION

Experiment 1.

The crude separation of the components of the apical and axillary buds, as well as leaves by direct application to chromatography paper, did not result in adequate separation. The images of radioactive spots, appearing on X-ray film, were large and diffuse. No apparent differences in the radioactive spots from the different treatments was observable. These results are not presented further, as they were of little value. The technique obviously required further refinement, as according to BIDWELL (1962), the technique works to a greater efficiency for photosynthetic assimilates than by extracting compounds in solvents prior to application and separation. Yet it is apparent from the illustrations provided by Bidwell, that the radioactive spots obtained were also not very distinct.

The chromatographic separation of extracts from different treatments was accomplished with great constancy and accuracy. No apparent differences existed between chromatograms derived from different LD treatments, and nor was there any difference between SD treatments. The results are thus summarized for comparison by observing a representation of only a single chromatogram from each extract of plants exposed to one SD and one LD.

It was apparent in the extracts containing aromatic compounds (Figure 5.2.) that spots 4, 5 and 6 which were present in extracts resulting from LD conditions, disappeared in SD, and were replaced by spots a., b., c. and d.. None of these spots were identified. The bulk of the radiolabelled extract ran to the front in both solvents (spot 10).

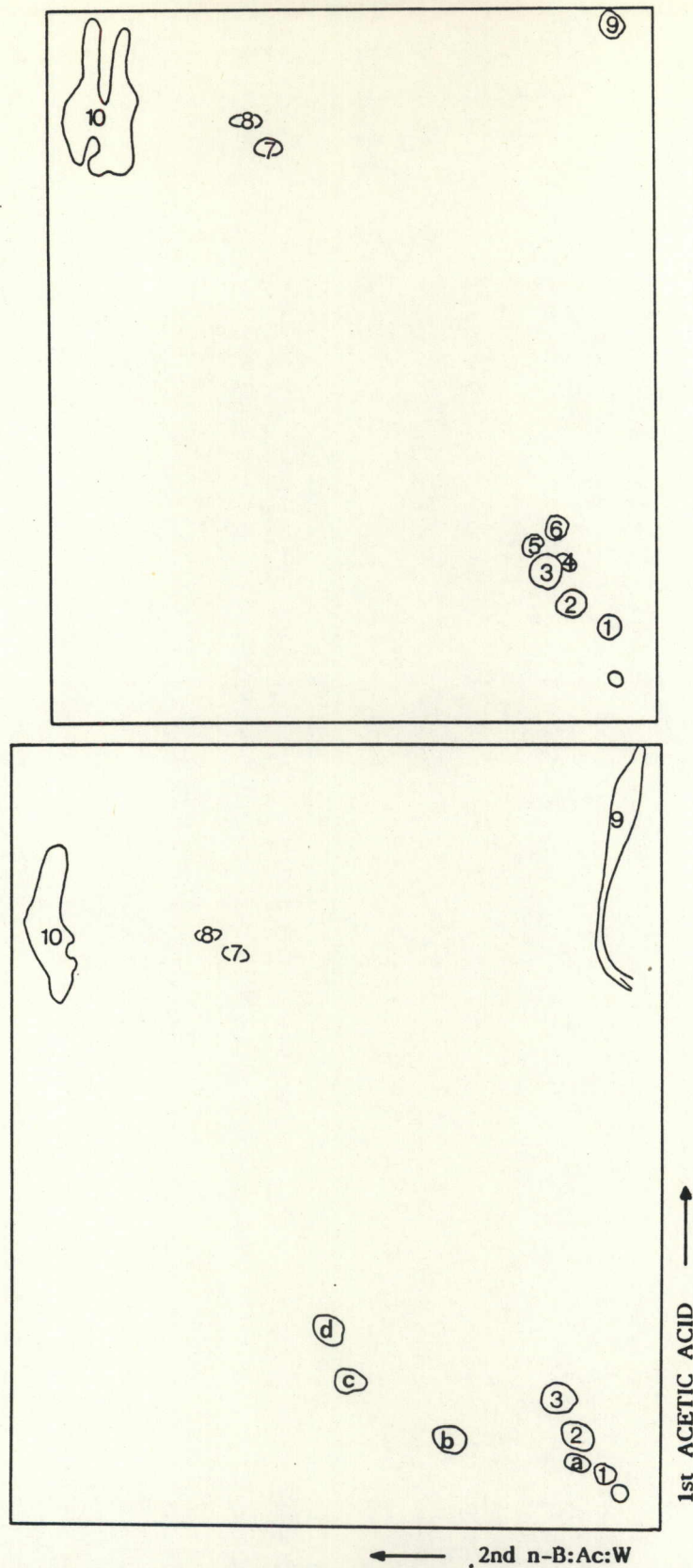


Figure 5.2. Autoradiograms of radiolabelled aromatic compounds derived from Xanthium leaves exposed to LD (top) or SD (bottom). Compounds were separated on Silica gel F254 plates using the solvents acetic acid (10%) and then n-butanol : acetic acid : water (63 : 10 : 27 v/v).

Many substances were removed from the charcoal by an acid wash (Figure 5.3.). Several faint spots in LD extracts were not visible in SD extracts, while several new ones appeared, most of which were seen at high Rf values (a. - j.). Spot 9 also increased in its radioactive intensity in SD conditions.

Extracts containing sugars, amino acids and organic acids, contained large spots of activity (Figure 5.4.), one of which is probably sucrose (spot 5 or 8). Spots 6 and 7 were darker in SD extracts than LD extracts, suggesting an increasing abundance of these substances. Several smaller spots were also not present in SD extracts, while several (a. - h.) new spots appeared.

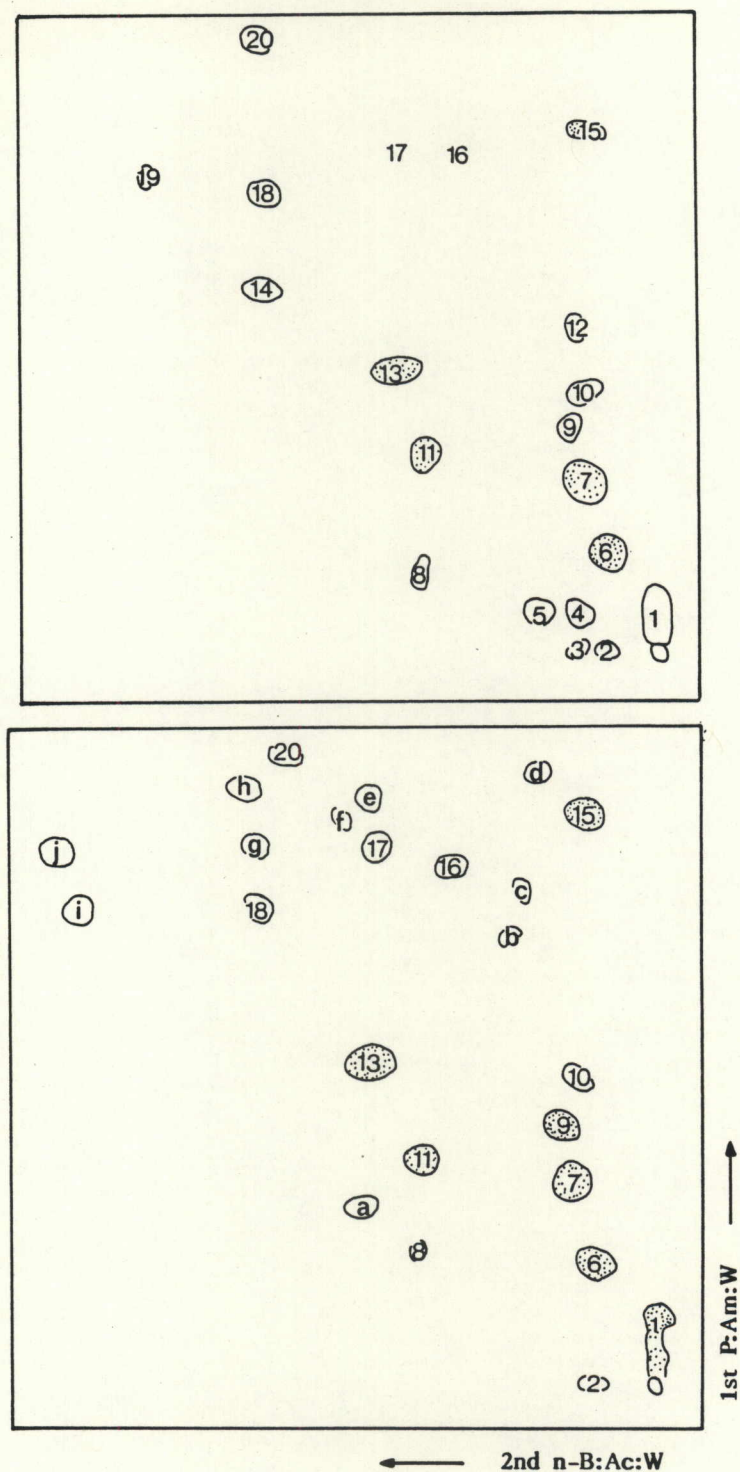


Figure 5.3. Autoradiograms of radiolabelled substances removed during extraction from the de-activated charcoal by acetic acid. Extracts were derived from *Xanthium* leaves exposed to LD (top) or SD (bottom). Compounds were separated on Silica gel F254 TLC plates using the solvents isopropanol : ammonium hydroxide : water (10 : 1 : 1 v/v) and then butan-1-ol : acetic acid : water (4 : 1 : 5 v/v).

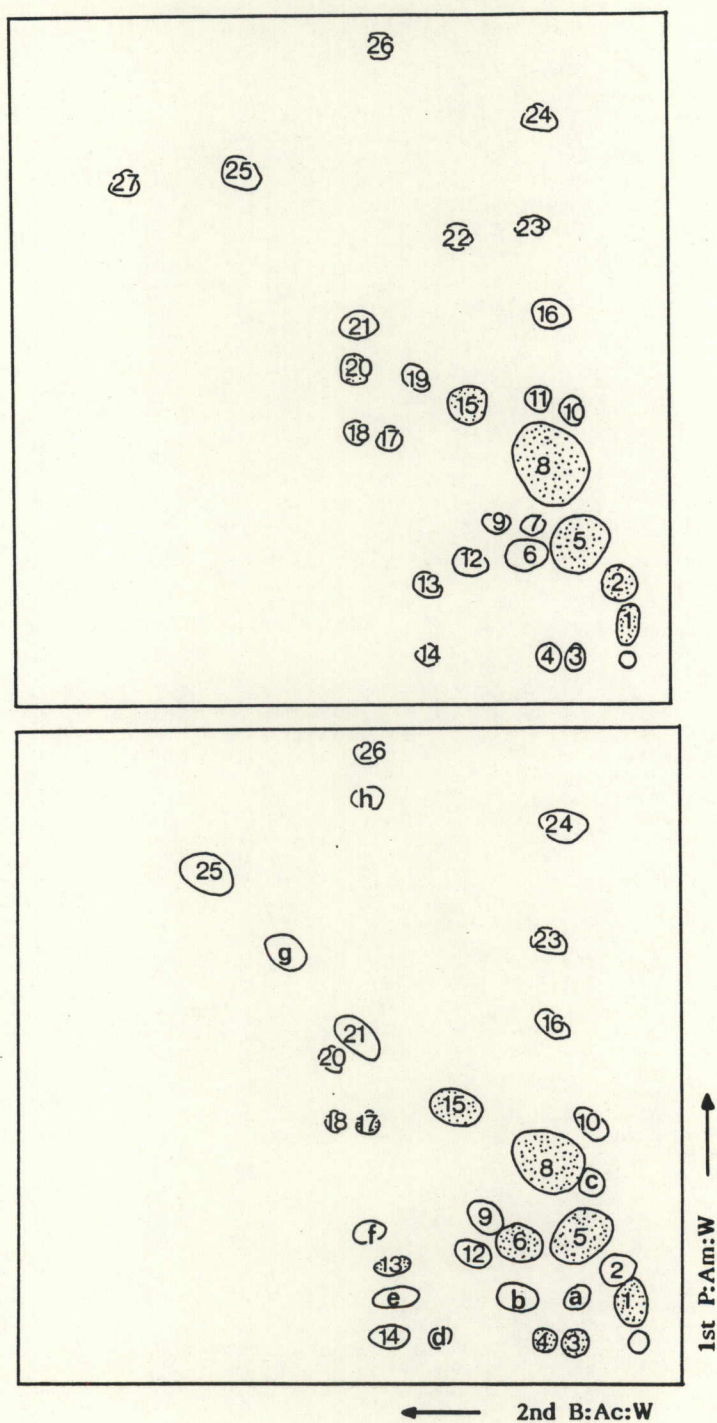


Figure 5.4. Autoradiograms of radiolabelled sugars, amino acids and organic acids. Extracts were derived from Xanthium leaves exposed to LD (top) or SD (bottom). Compounds were separated on Silica gel F254 TLC plates using the solvents isopropanol : ammonium hydroxide : water (10 : 1 : 1 v/v) and then butan-1-ol : acetic acid : water (4 : 1 : 5 v/v).

Experiment 2.

As in the previous experiment, crude tissue application directly to paper did not provide satisfactory results, and are not presented here. Although images of several separated compounds were visible on the X-ray film, these were large and diffuse.

Extracts containing aromatic compounds (Figure 5.5.) were found to contain more substances in SD (spots a. - g.) than LD extracts. The diffuse spot 15 in LD extracts was found to consist of two distinct spots in SD extracts.

The charcoal acid wash (Figure 5.6.) was found to contain a large number of common substances, but with many appearing in response of SD cycles (spots a. - q.), and many disappearing. Possibly the most noticeable was the appearance of a large dark spot i.. The identity of this substance is not known.

The most noticeable changes in the organic fraction (Figure 5.7.) were the darkening after SD exposure, of spots 2 and 26 and the fading of spot 32.

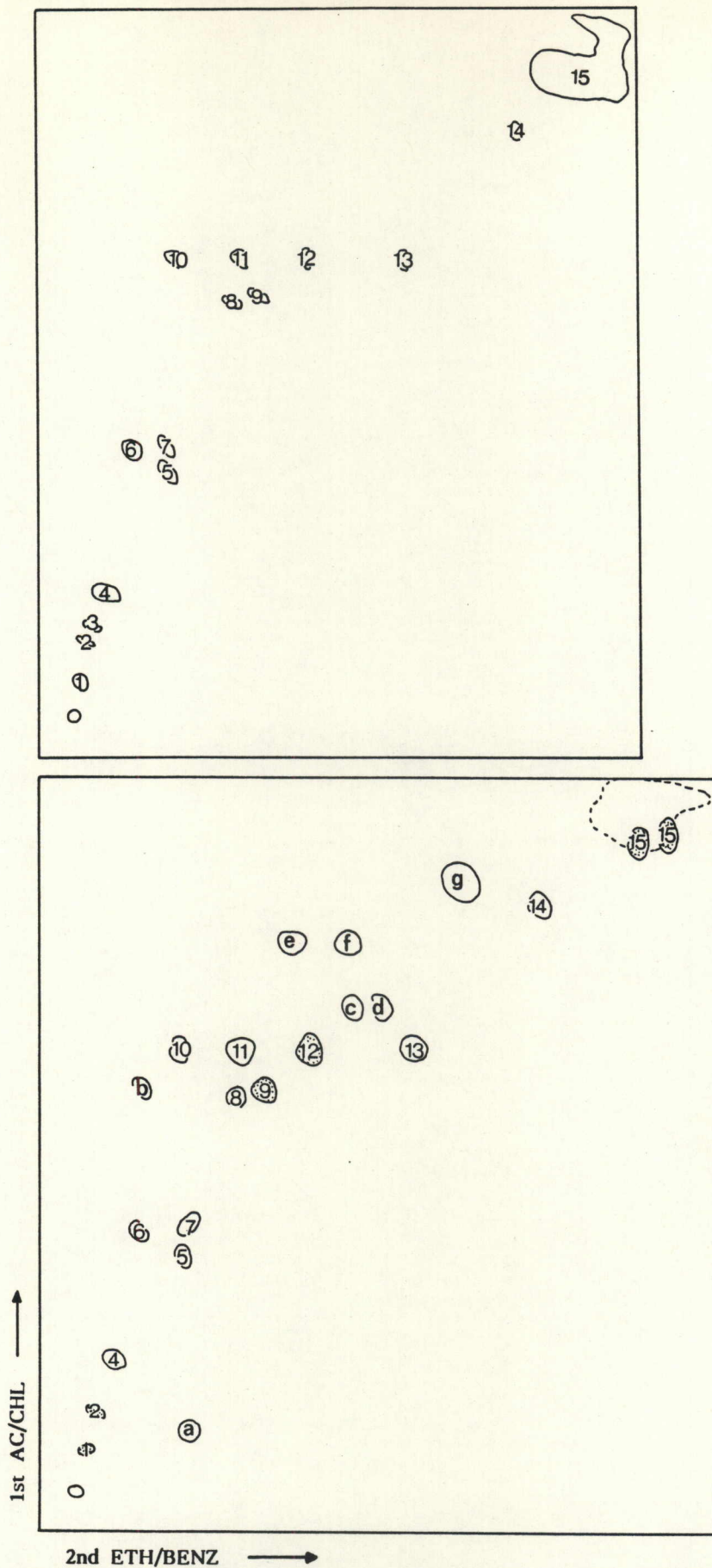


Figure 5.5. Autoradiograms of radiolabelled aromatic compounds derived from Xanthium leaves exposed to LD (top) or SD (bottom). Compounds were separated on Silica gel F₂₅₄ plates using the solvents 10% acetic acid in chloroform and then 45% ethyl acetate in benzene.

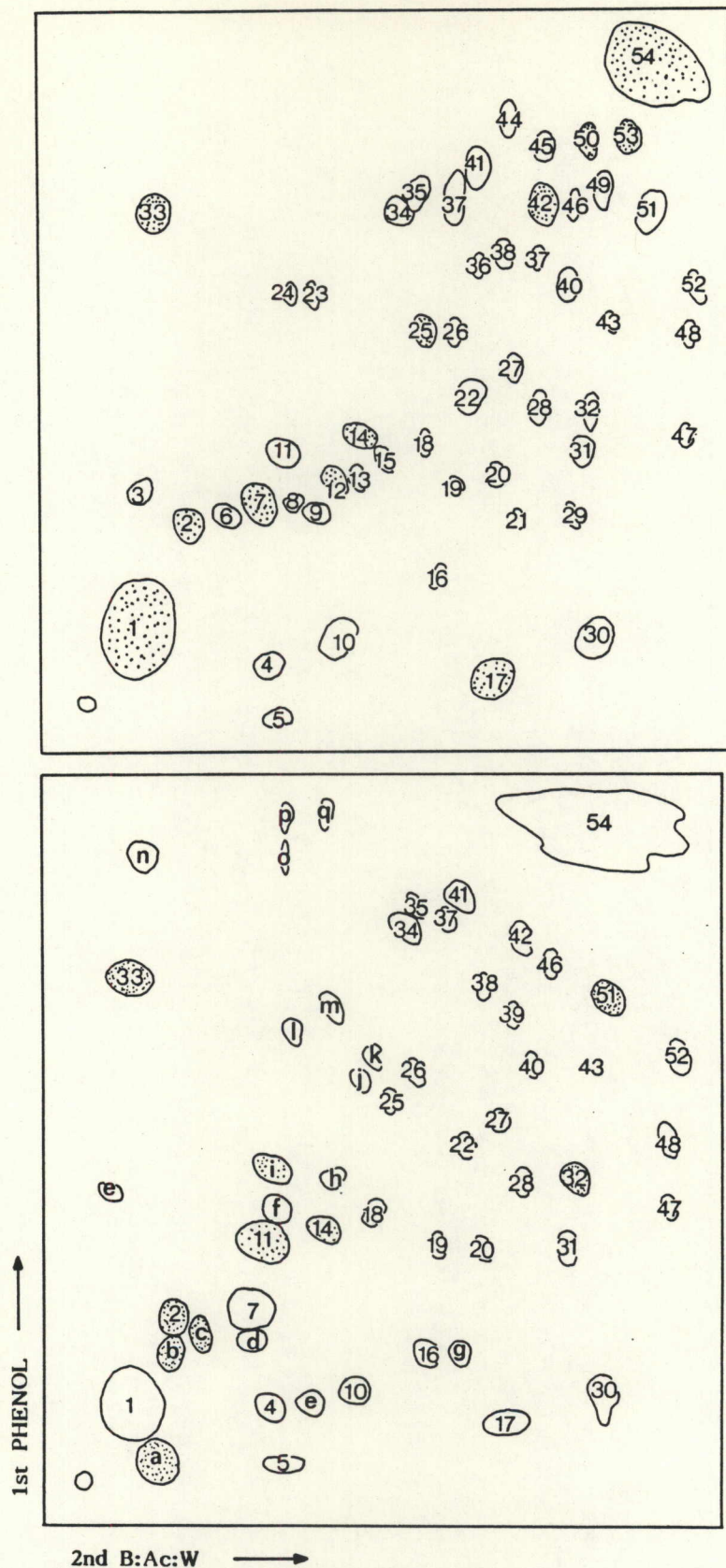


Figure 5.6. Autoradiograms of radiolabelled substances removed during extraction from the de-activated charcoal by acetic acid. Extracts were derived from *Xanthium* leaves exposed to LD (top) or SD (bottom). Compounds were separated on Silica gel F254 TLC plates using the solvents phenol (80% w/v at pH 5.4) and then butan-1-ol : acetic acid : water (4 : 1 : 5 v/v).

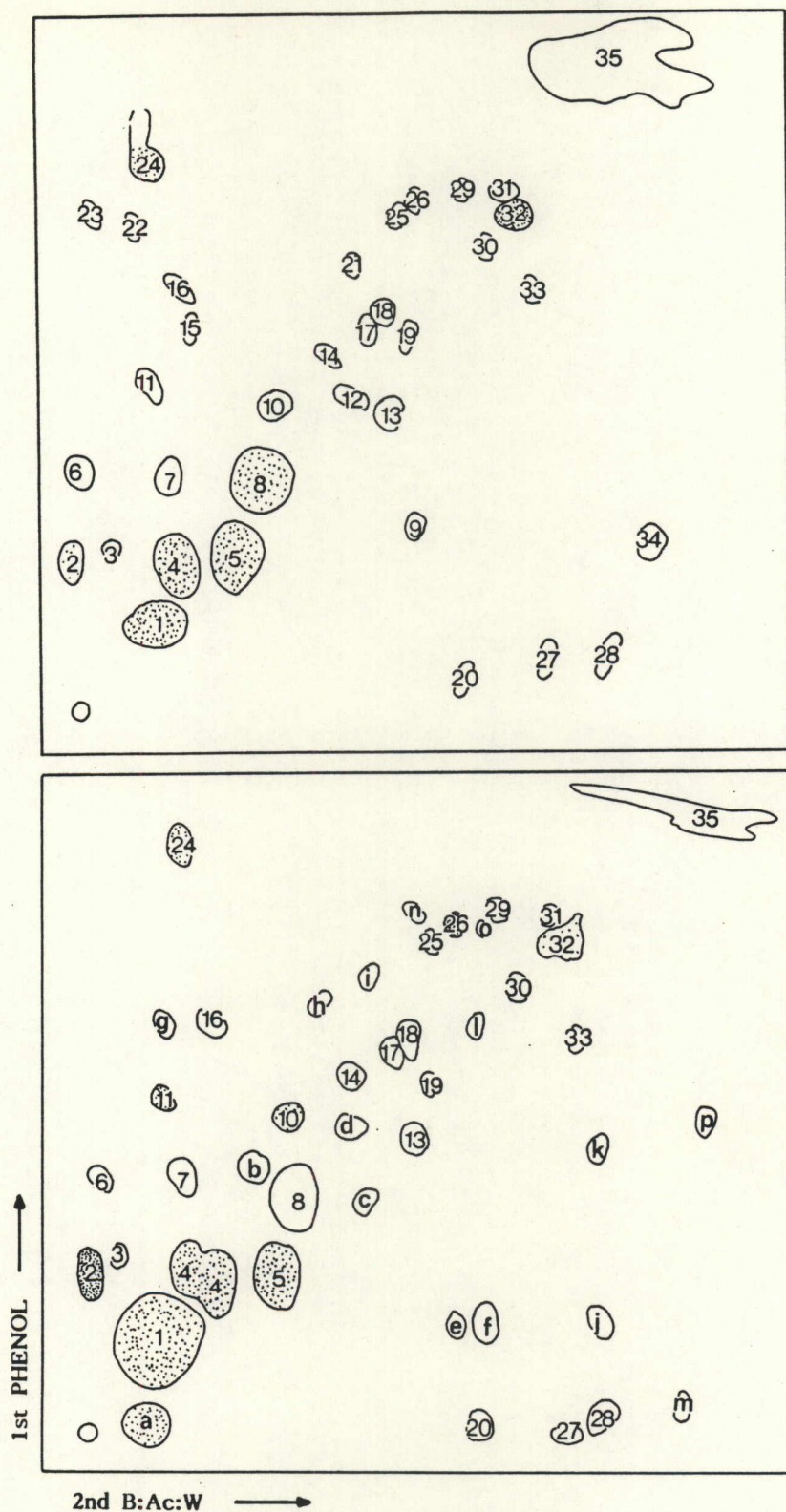


Figure 5.7. Autoradiograms of radiolabelled sugars, amino acids and organic acids. Extracts were derived from *Xanthium* leaves exposed to LD (top) or SD (bottom). Compounds were separated on Silica gel F254 TLC plates using the solvents phenol (80% w/v at pH 5.4) and then butan-1-ol : acetic acid : water (4 : 1 : 5 v/v).

Experiment 3.

The methodology of this experiment is the result of the refinement of the techniques presented in the previous two experiments. Exposure conditions were optimal, as were separation techniques. The method utilized natural high light intensities, which are essential for the induction of Xanthium (HAMNER, 1940). Earlier preliminary experiments in this investigation, had shown that SD induction with light intensities of $110 \mu\text{Em}^{-2} \text{s}^{-1}$ were not able to induce flowering.

Labelled $^{14}\text{CO}_2$ was applied to the youngest fully expanded leaf, with the other leaves remaining attached to the plant. The remaining leaves were removed only after the first night, in view of the fact that the stimulus only moves out of the leaf six to ten hours into the following day (SKOK and SKULLY, 1954). These leaves would thus serve to provide assimilates for vegetative growth to the bud until just before the stimulus moves into the system. At this time, the untreated leaves were removed so as to remove the sources of unlabelled stimulus and assimilates, thus preventing dilution of the stimulus at the bud. The reason that an axillary bud far removed from the leaf was used, was so that substances which became labelled, but were not readily transported, would not be present in bud extracts. As the stimulus is known to be transported rapidly through the plant, in close association with carbohydrates, the ideal situation would be to isolate the sink bud as soon after the first stimulus reached it.

A leaf occurring on the same orthostichy was also selected to facilitate the transport from leaf to bud. This was in view of the known restriction of the stimulus to a single orthostichy (HARDER, 1948; MURRAY, MAUK and NOODEN, 1982). This was also shown for "metaplasin", the hypothetical substance controlling leaf structure in Kalanchoe (HARDER and VAN WITSCH, 1940)

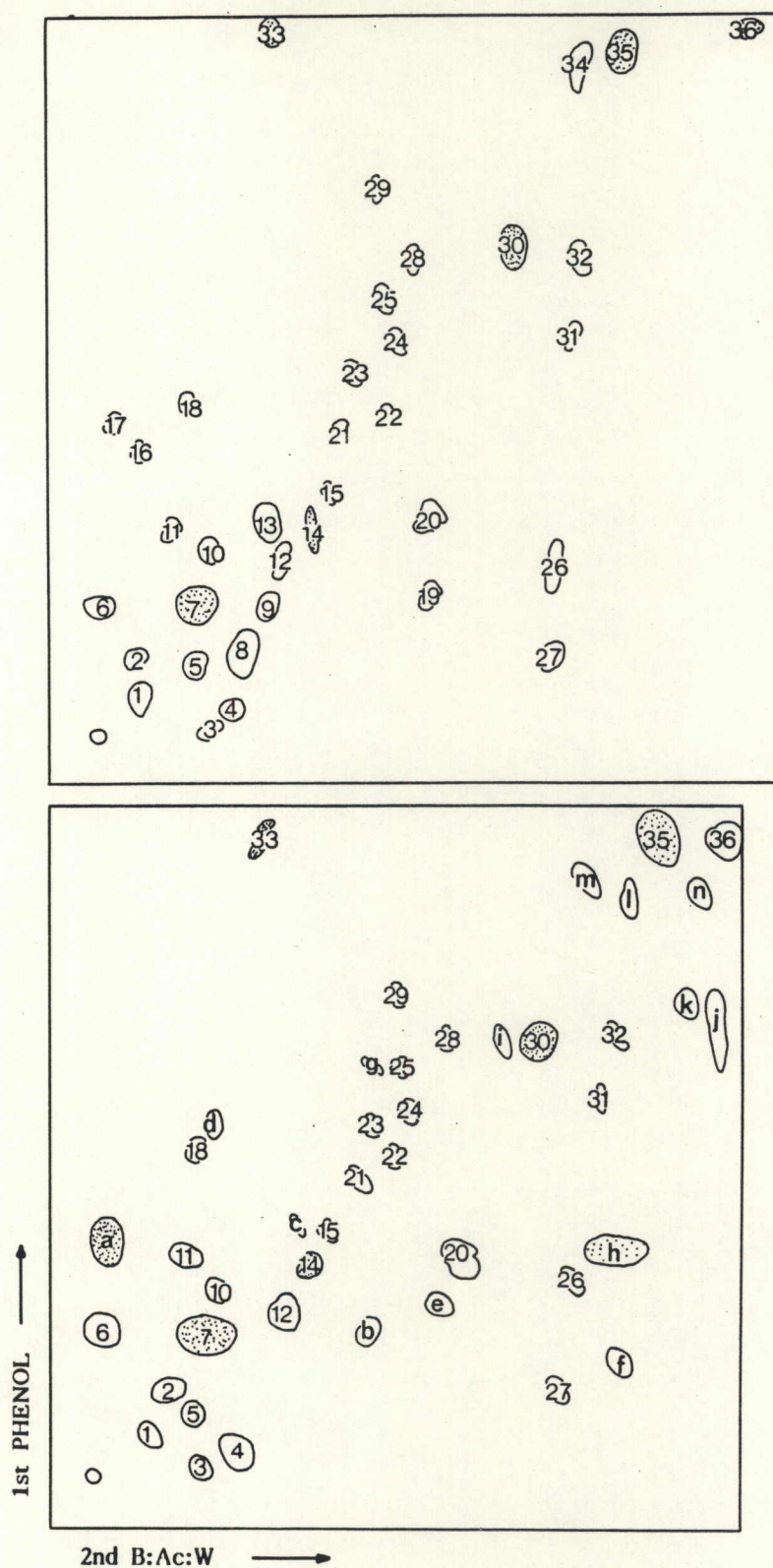


Figure 5.8. Autoradiograms of radiolabelled extracts derived from *Xanthium* leaves exposed to LD (top) or SD (bottom). Compounds were separated on Silica gel F254 TLC plates using the solvents phenol (80% w/v at pH 5.4) and then butan-1-ol : acetic acid : water (4 : 1 : 5 v/v).

The autoradiographs resulting from this Experiment 3., from crude bud extracts were near to identical for the three replicates within each treatment (Figure 5.8.). Only one of the three replicates for each treatment is presented here. The most noticeable changes, are the appearance of two large spots (a. and h.) and the greater intensity of spot 6 after SD treatment. Several other changes are also evident. Spots 8,9,13,16,17,19 and 34 were lacking in SD exposed extracts, while spots a. - n. were produced in response to these inductive cycles.

Standards

The sugar and amino-acid standards, as separated with the same solvent systems, are presented in Figures 5.9. and 5.10.. It is likely that spot 7 (Figure 5.8.) represents sucrose, due to the natural abundance of sucrose in plants systems, the intensity of the radioactivity in the spot, and the location of the sucrose standard. Besides this, no other conclusions with regard to the identity of the substances represented by the spots can be made. The most sophisticated analytical techniques are not capable of dealing with the minute amounts of substance contained in each spot. This is unfortunate, as it would be most revealing to identify spots a., h. and 6 in particular.

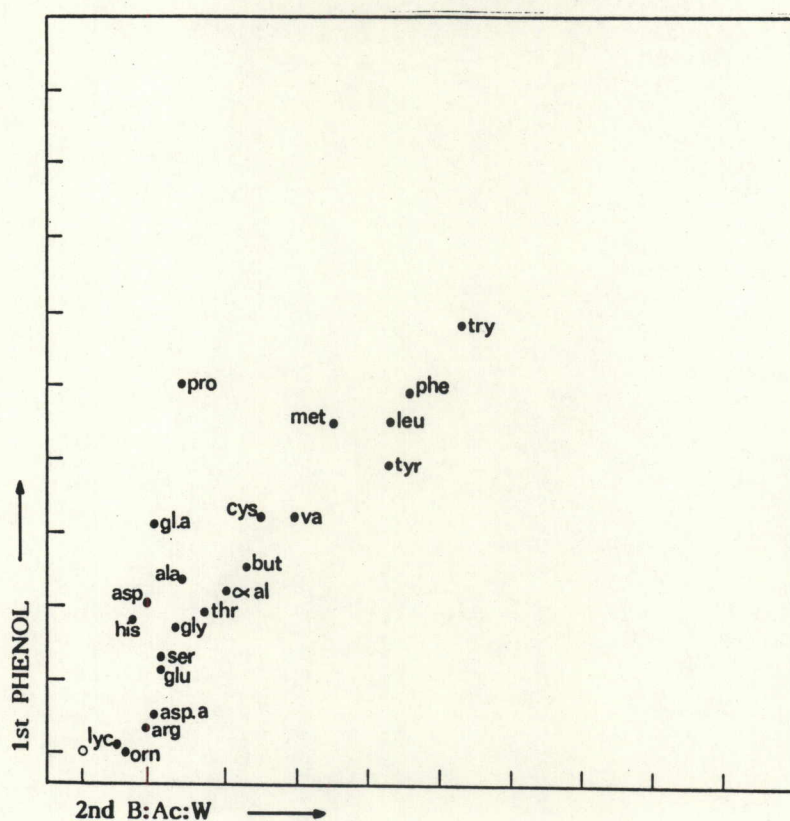


Figure 5.9. Distribution of amino acid standards after separation on Silica gel F TLC plates. Standards applied were (with abbreviations), DL- α -alanine (xal); L-alanine (²⁵⁴ala); L-arginine monohydrochloride (arg); DL-asparagine (asp); L-aspartic acid (asp.a); 4-amino-n-butyric acid (but); L-cystine (cys); DL-glutamine (glu); L-glutamic acid (gl.a); glycine (gly); DL- histidine dihydrochloride (his); DL-leucine (leu); DL-lycine monohydrochloride (lyc); L-methionine (met); L-ornithine monohydrochloride (orn); DL-B-phenylalanine (phe); L-proline (pro); L-serine (ser); L-threonine (thr); DL-tryptophan (try); L tyrosine (tyr); DL-valine (va).

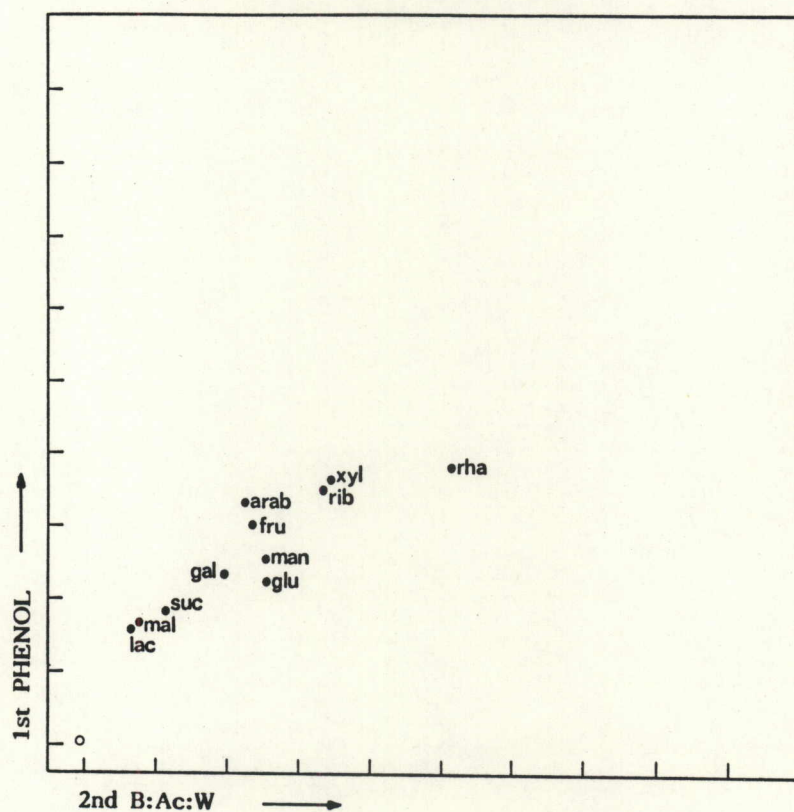


Figure 5.10. Distribution of sugar standards after separation on Silica gel F TLC plates. Standards applied were (with abbreviations), L(+)-arabinose (arab); D(-)-fructose (fru)²⁵⁴; D(+)-galactose (gal); D-glucose (glu); x-lactose (lac); D(+)-mannose (man); maltose (mal); L(+)-rhamnose (rha); D(-)-ribose (rib); sucrose (suc); D(+)-xylose (xyl).

5.4. CONCLUSIONS

It is apparent from the work presented here, that the technique of BIDWELL (1962) was not suitable for this investigation mainly due to the large number of substances produced during long exposures to $^{14}\text{CO}_2$. Paper chromatography was also not suitable for the separation of these numerous substances, and is surpassed by TLC which is reported to be a hundred fold more sensitive for autoradiograms than paper (STAHL, 1969).

In the three experiments conducted, autoradiograms made from the separated extracts of leaves and buds containing radiolabelled assimilates all produced highly reproducible results. Large numbers of common "spots" were noted on the autoradiograms. In response to SD induction, several "spots" disappeared, while others were formed. These "spots" were not identified due to their minute quantities and it is not known if any of them are promoters or inhibitors of flowering. There are known to be at least one of the former and two of the latter in Xanthium (CLELAND, 1970).

The technique as developed, has potential to help explain some of the changes which occur in a plant during induction to flower. In order for this to be successful, the investigation would have to undergo large-scale upgrading to produce sufficient amounts of the substances associated with each "spot" for identification. It is envisaged that techniques such as this will ultimately be the means to identifying the flowering stimulus or inhibitor in plants.

CONCLUSIONS

An investigation has been made into the factors controlling in vitro flowering in various species. This was done in order to gain some insight into the internal situation of the plant, as it is exposed to inductive cycles and begins to produce reproductive organs. The control of a wide variety of growth responses was investigated, and has given an indication of the complex series of events which accompany the change to reproductive growth. It was not possible to differentiate between those processes involved in induction, and those involved in evocation and differentiation of flowers. It was found though, that certain growth phenomena which accompany flowering in Kalanchoe, appeared to have no direct relationship to flowering. Thus, SD induced succulence of the leaves and increasing anthocyanin colouration in Kalanchoe leaves did not in themselves appear to have any direct link to the production of flowers, as manipulation of each was possible without affecting the other. Rooting on the other hand, appeared to have some direct link to flowering.

The techniques developed and utilized here, of inducing flowering in explants of Glycine and Kalanchoe growing on a low nutrient hormone free medium, have some potential for further development. Their greatest potential is in the development of a bioassay for the floral stimulus. The advantage of this type of system, is that nodal explants, which grow to produce whole plants, have all the organs necessary to produce the wide variety of substances needed for the manifestation of the flowering stimulus. The culture of isolated apices, on the other hand, has the serious limitation in that the explant may be induced to flower, but may not be able to produce any sign of the flower. Apical bud culture

systems also require the incorporation into the medium of a complex array of substances such as hormones, to support the growth of the bud. This poses a problem in any investigation of the flowering stimulus, especially if the stimulus is indeed hormonal in nature. It also poses problems if the stimulus is not a single compound, but a complex sequence of factors, as many of these may be interfered with by the hormones in the growth medium.

Attempts made to radiolabel assimilates of plants exposed to LD on the one hand, and induced to flower by SD on the other, met with some success. It is envisaged that techniques such as these could ultimately provide the means to understand the floral stimulus, whether it be a single substance, or a complex system of substances. A time-course study of the change in these assimilates during induction to flower, could be most revealing. Marked changes were noted between the labelled assimilates in the buds of induced plants compared to non-induced plants. These differences could give some indication of the nature of the stimulus. It is essential though, that techniques be developed with which to establish the identity of these numerous labelled assimilates.

As to the nature of the flowering stimulus. The original theory of "florigen" as a single substance promoting flowering is widely regarded as being too simplistic. As a consequence, much support is given to theories dealing with the stimulus as a complex sequence of events. It is important to appreciate here, that these two theories may yet prove to be compatible. Some initial, individual substance must be produced in the leaves in response to photoinduction, from where it or its products are transported to the apex. At some stage in this sequence, there is likely to be a substance which fits the description of "florigen". Until this substance is found or disproved, the quest will continue.

Whereto in the future? It is by no means envisaged that the systems utilized here are the only or the best for the elucidation of the flowering process. There are numerous other techniques with much potential, but it is important to quote from BERNIER, KINET and SACHS (1981a,b), " no satisfactory answer will arise from the continuation and refinement of the same type of experiments". These authors also stated "...that a reconsideration of accepted notions of the physiology of flowering is absolutely and urgently required.". These are the notions that are providing the incentive to devise novel systems to investigate the physiology of flowering. It is hoped that some of the systems developed in this thesis, would fit into this category.

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