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thesis entitled
A Non-Destructive Assay for
Gibberellin Production in vitro
and its Application to Callus
Cultures of Phaseolus and Nicotiana

presented by

Mrs. Barbara J. Thompson

has been accepted towards fulfillment
of the requirements for

M.S. degree in Horticulture

Frank G. Dennis, Jr.
Major professor

Date December 22, 1980

A NON-DESTRUCTIVE ASSAY FOR GIBBERELLIN PRODUCTION *IN VITRO* AND ITS APPLICATION TO CALLUS CULTURES OF *PHASEOLUS* AND *NICOTIANA*

By

Barbara Lake Thompson

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Horticulture

1980

ABSTRACT

A NON-DESTRUCTIVE ASSAY FOR GIBBERELLIN PRODUCTION IN VITRO AND ITS APPLICATION TO CALLUS CULTURES OF PHASEOLUS AND NICOTIANA

By

Barbara Lake Thompson

The barley half-seed halo assay was adapted for use as a non-destructive test of gibberellin (GA) content of both callus tissues and culture media. Sterile barley half-seeds were placed in direct contact with callus cultures or media for 24 hours. Gibberellins in the callus or media stimulated synthesis of α-amylase in the half-seeds. Seeds were transferred aseptically to starch plates for 72 hours. The plates were flooded with KI/I_2 solution, whereupon clear halos appeared around the half-seeds where the starch had been digested by the α-amylase. Factors affecting the halo diameters after exposure to GA_3 standards were determined. Using these methods a study was made of the gibberellin content of Phaseolus vulgaris and Nicotiana forgetiana tissue cultured in vitro to determine the feasibility of using callus as a source of gibberellin production. Callus cultures were tested during initiation and subsequent subcultures. Both calli produced only small quantities of gibberellins in vitro.

ACKNOWLEDGMENTS

Special thanks to my husband, Joel, for his love and support, to Dr. Ken Sink and Dr. Peter Carlson for the use of laboratory facilities, and to Dr. Frank Dennis for his guidance during the course of this research.

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INTRODUCTION

Research on the physiological roles and effects of gibberellins, other than GA_3 and GA_{4+7}, is limited by the small quantities available. An adequate supply of these gibberellins would permit extensive studies in areas of plant hormone research thus far untouched. The current interest in tissue cultures that produce pharmaceuticals and other secondary metabolites raises the possibility that gibberellins may be produced in a similar manner. In particular, do they accumulate at some point in culture or would altering environmental conditions induce gibberellins to accumulate *in vitro*?

The major objective of this research was to determine (a) the gibberellin content of callus and (b) effect of subculturing, medium composition, and light exposure on gibberellin concentration. Callus cultures were initiated on explants of immature bean cotyledons, *Phaseolus vulgaris* L. 'Montcalm'. Beans were chosen for this work because immature bean seeds have high concentrations of gibberellins and thus might retain or attain the capacity to produce them *in vitro*.

The gibberellin content of callus was determined using a bioassay. Most bioassays for gibberellins involve

extraction of the tissue; however, to determine the gibberellin content of normally growing callus at various time intervals, the bioassay would have to be non-destructive. The barley half-seed halo assay offered the potential for directly testing callus in vitro and was successfully applied to monitor GA-like content of callus cultures in these studies.

LITERATURE

I. Production of Secondary Metabolites *In Vitro*

The potential for the industrial production of secondary metabolites through tissue cultures has yet to be fully realized (Staba, et al., 1965; Street, 1973; Butcher, 1977; Alfermann and Reinhard, 1978). The production of these compounds *in vitro* involves developing large scale callus or cell suspension cultures that yield an economically significant quantity of the chemical of interest. These chemicals are generally either secreted into the liquid phase of the medium or compartmentalized within the cell; in either case appropriate extraction methods must be developed. To make extraction profitable the cultures must remain productive over long periods of time.

Thus far, plant physiologists have concentrated their efforts on the production of medicinal compounds by tissue culture techniques. To date no published references are known on the possibility of producing plant hormones such as gibberellins (GAs), auxins, or cytokinins on an industrial scale. Commercially available gibberellins are presently limited to only GA_3, GA_4, and GA_7. These GAs are extracted from cultures of *Fusarium moniliforme* in its reproductive form, *Gibberella fujikuroi*. The other known gibberellins

are obtained in microgram quantities from plant tissues. Thus, comparisons of physiological activities and commercial use are limited by difficulties in obtaining sufficient quantities.

There are many possible approaches to finding an alternative system which yields high quantities of gibberellins. However, a living plant system seems most reasonable since gibberellins are endogenous growth hormones. Because extractions of plant tissues yield low quantities of gibberellins per unit weight, culturing specific tissues which contain high levels of gibberellins would appear to be a promising approach.

The many advantages of a tissue culture system for the production of secondary compounds have been discussed (Butcher, 1977). Tissue cultures of most plants are easy to establish and maintain either as callus or as cell suspensions. An added advantage in such systems is that environmental conditions are readily controlled. Nutrient and hormone needs can be easily met and precursors of the desired compound can be incorporated into the medium.

Recognition of the biosynthetic potential of plant tissue cultures began with the production of rubber from guayule cultures (Arreguin and Bonner, 1950). A patent issued in 1956 to Routien and Nickell defined ten cultures with the potential for large scale production of secondary compounds. Since then the possibilities of using tissue cultures for the synthesis of economically valuable compounds have been discussed in several articles (Staba, et al., 1965; Street, 1973; Butcher, 1977; Tabata, 1977; Alfermann and Reinhard, 1978; Dougall, 1979a, 1979b).

The use of tissue culture for the production of secondary metabolites is based on the assumption that cells will maintain their chemical as well as morphological totipotency *in vitro*. Scientific evidence is available to both support and refute this assumption. With the methods commonly used for callus and cell culture, metabolites normally produced by the intact tissue may or may not be found in the cells cultured, in callus tissue produced by them, or in the culture medium. For example, *Digitalis lantana*, which produces cardiac glycosides *in vivo*, ceases to produce them *in vitro*. In contrast, *Ruta graveolens* produces coumarins and alkaloids and *Andographis paniculata* produces paniculides *in vitro* which are not found in the respective whole plants. When secondary metabolites are produced, the concentrations are often very low. However, some secondary compounds are produced in much greater quantities *in vitro* than *in vivo*. Dougall (1979a, 1979b) gives several examples; cell cultures of *Morinda* and *Galium* produce 20 times more anthraquinones than do their intact roots.

Numerous secondary metabolites have been identified in plant tissue cultures (Staba, 1963; Carew and Staba, 1965; Staba, et al., 1965; Puhan and Martin, 1971; Tabata, 1977; Alfermann and Reinhard, 1978; Kemp and Stoltz, 1979; Salem and Reinhart, 1979). Some of these compounds, a few of which are produced at substantially higher levels than in

intact plant tissue(s), are listed in Table 1. In some cases a major problem involves the detection of the compound produced. Specific tests for gibberellins, which are colorless, generally require extraction of the tissue, followed by bioassay. Nickell (1958) detected gibberellin-like substances in extracts of tissue cultures using the dwarf pea bioassay (Table 2).

Alfermann and Reinhard (1978) suggested a basic strategy for the production of secondary compounds in vitro. First, cultures of a species that produces the compound of interest are established. Second, cultures of this species are screened for productivity and appropriate strains selected. Third (sometimes included with step two), the factors which influence the growth and productivity of the culture are varied to determine the optimal cultural conditions for production. Fourth, methods for extracting and concentrating the desired chemical are developed. Just how each of these steps is to be carried out must be determined for the specific species and compounds being investigated.

Analytical techniques for selecting and maintaining productive strains of cells are currently being developed for a wide range of compounds. Freeze-preservation methods permit the storage of viable callus tissues of certain species over long periods of time. Chemostat or turbidostat methods allow the maintenance of cultures in a steady state over extended periods. Radioimmunoassay systems are being

Table 1. Some secondary compounds produced by plant tissue cultures.

Compound	% dry wt.	Conc. relative to conc. in vivo	Species	References
ajmalicine	-	>1x	Catharanthus roseus (C^1,S^2)	Zenk, et al. 1977 Arens, et al. 1978 Deus 1978
α-ketoglutaric acid	-	-	Helianthus annuus and Vinca	Scott, et al. 1955
anthocyanins	-	>1x	Haplopappus (C,S)	Reinert, et al. 1964 Constable, et al. 1971 Stickland and Sunderland 1972
	-	-	Daucus (C,S)	Alfermann and Reinhard 1971
anthraquinones	10	20x	Morinda citrifolia (S)	Zenk, et al. 1975
	6	10x	Cassia tora	Tabata, et al. 1976
	-	20x	Galium sp. (S)	Bauch and Leistner 1978 Wilson 1978 Zenk 1978
ascorbic acid	-	-	Nicotiana tabacum, crown gall	Routien and Nickell 1956

[1]C=callus culture [2]S=suspension culture

Table 1. (Cont'd.)

Compound	% dry wt.	Conc. relative to conc. *in vivo*	Species	References
atropine	-	-	*Atropa belladona* (root C)	West and Mika 1957
betalains	-	-	*Beta* (C)	Constable 1967
camptothecin	-	-	*Camptotheca acuminata*	
citric acid	-	-	*Agave*, *Ginkgo* and *Rosa*	Staba 1963
coumarin	-	-	*Melilotus officinalis*	Weygand and Wendt 1959
diosgenin	1.5	-	*Dioscorea deltoidea* (S)	Kaul, et al. 1969
glutathione	-	-	*Nicotiana tabacum* (S)	Bergmann and Rennenberg 1978
glycyrrhizan	4	-	*Glycyrrhiza glabra*	Tamaki, et al. 1973
fumaric acid	-	-	*Tagetes sp.* and *Helianthus annuus*	Scott, et al. 1955
L-DOPA	1 w/v medium	-	*Mucuna pruriens* (S)	Brian 1974

Table 1. (Cont'd.)

Compound	% dry wt.	Conc. relative to conc. in vivo	Species	Reference
nicotine	0.291	-	Nicotiana rustica	Tabata, et al. 1976
rosmaric acid	-	-	Coleus blumei (S)	Zenk, et al. 1977
oxalic acid	-	-	Rumex tumor	Routien and Nickell 1956
saponins	21	1x	Panax ginseng (S)	Jhang 1977
scopolamine	-	-	Hyoscyamus niger (root)	Metz and Lang 1966
			Datura stromonium (S)	Strohs 1969
serpentine	-	>1x	Catharanthus roseus (C,S)	Döller, et al. 1976 Roller 1978 Döller 1978
ubiquinone-10	-	-	Nicotiana tabacum (S)	Ikeda, et al. 1976

Table 2. Relative activities of gibberellin-like substances in tissue cultures of various plant species (Nickell, 1958).

Species	Culture	Relative activity in dwarf pea assay
Vinca rosea	stem, crown gall	+
Helianthus annuus	petiole, crown gall	+++
Melilotus officinalis	stem, crown gall	+
" "	root, virus tumor	+
" "	stem, virus tumor	+
" "	root callus	+
Agave toumeyana	leaf callus	++
Ilex aquifolium	stem callus	+
Phaseolus vulgaris	cotyledon callus	+

developed to screen cells for high-yielding strains (Kostenbauder, et al., 1974; Weiler, 1978).

Continuous culture systems may permit cultures to be maintained at a stage of maximum metabolite production. The continuous culture systems make it possible to maintain a constant density, growth rate, chemical composition, and metabolic activity (Butcher and Ingram, 1976). Chemostat cultures maintain the density of the culture by constant addition and removal of nutrient medium in the vessel. In turbidostat systems, the suspension is maintained at a specific optical density automatically by the addition and removal of medium (Wilson, et al. 1971).

Wilson (1978) investigated the use of chemostat cultures for the production of anthraquinones by Galium mollugo. Production was from 7 to 30 times less than that of batch cultures, but was increased to similar levels by

manipulation of limiting factors. One of the distinct advantages of the chemostat system is that the effects of various components of the medium on metabolite production can be easily verified independently by their addition or removal.

Production of secondary compounds on an industrial scale awaits the solution of several major problems. The most prominent of these is the instability of cell strains. While some cells can synthesize the product of interest, other cells do not. Often cells unpredictably lose or regain this ability, especially when subcultured. Thus, loss of productivity can never be totally prevented under present cultural methods (Alfermann and Reinhard, 1978). Cultures must be checked continually for production levels--an impractical procedure in large scale batch cultures.

The production of secondary compounds by tissue culture has received considerable attention in recent years. The emphasis has been on products of pharmaceutical value while the possible production of plant hormones such as gibberellins has been neglected. Considering the limited supplies of various gibberellins, a system that would yield useful quantities would be of great value to plant research. Such investigation may provide additional sources of the known gibberellins.

II. Gibberellins in Developing Seeds of Phaseolus vulgaris

Immature seeds of Phaseolus vulgaris contain high concentrations of gibberellins (Corcoran and Phinney; 1962; Carr and Skene, 1961; Skene and Carr, 1961; Skene, 1970). The free gibberellin content of immature bean seeds is highest between 10 and 15 days after anthesis and declines with maturity. Although the concentration of free gibberellins is higher in immature than in mature beans, the concentration of bound gibberellins increases as the seeds mature (Hiraga, et al., 1974a; Yamane, et al., 1975).

Corcoran and Phinney (1962) measured the gibberellin content in developing seeds of Phaseolus vulgaris 'Black Valentine' using the dwarf corn bioassay. The highest concentration of gibberellin detected was 0.05 µg/g of tissue 10 days after anthesis.

Two peaks of gibberellin-like activity were detected in developing seeds of Phaseolus vulgaris 'Hawkesbury Wonder' using the dwarf pea bioassy (Carr and Skene, 1961; Skene and Carr, 1961; Skene, 1970). The highest concentrations were observed at 15 days (0.7 µg/g) and 26 days (0.275 µg/g) after anthesis. The average seed weight was 50 mg for the former and 600 mg for the latter. The youngest beans in each case contained the highest gibberellin-like activity.

A number of free gibberellins have been identified in immature bean seeds. Durley, et al. (1971) identified GA_1, $GA_{5/20}$, GA_6, and GA_8. Hiraga, et al. (1974b) isolated GA_1, GA_8, and GA_{38} and identified GA_4, GA_5, GA_6, and GA_{37}.

Yamane, et al. (1977) added GA_{17}, GA_{20}, GA_{29}, and GA_{44} to the list of free gibberellins known to occur in immature bean seeds.

MATERIALS AND METHODS

I. Effect of Stage of Development of Bean Seeds on Content of GA-Like Substances in Methanol Extracts

Phaseolus vulgaris was chosen for this work since the immature seed contains high concentrations of gibberellins (Corcoran and Phinney, 1962). The gibberellin content of methanol extracts of 'Montcalm' seeds was determined using the lettuce hypocotyl assay (Frankland and Wareing, 1960).

A. Greenhouse Experiment

Four bean seeds were planted in each of 20 pots (2 gallon volume) in the greenhouse under 12 to 14 hours cool white fluorescent light per day. The temperature was maintained at $20^{\circ} \pm 3^{\circ}$ C and the plants watered and fertilized as needed. Flowers were tagged at anthesis and the pods on all plants were harvested 35 days after the first flowers opened.

Seeds were divided into groups according to age, their length was measured, and they were frozen (-10° C) until extracted. For extraction, each group of seeds was macerated in a blender with approximately 10 ml cold methanol per gram of tissue. The macerates were filtered under vacuum through Whatman No. 1 filter paper and the filtrate refiltered by gravity. The methanol was evaporated under vacuum and the remaining aqueous extract frozen at -10° C.

Acidic, neutral, and butanol fractions of the extracts were separated by acid-base fractionation. The pH of the aqueous extract was adjusted to 8.0 with 3 N NH_4OH, and the extract partitioned 3 times using 10 ml distilled ethyl acetate. The combined ethyl acetate fraction was washed once with 10 ml distilled water and the water added to the aqueous phase. The pH of the aqueous fraction was adjusted to 3.0 with 3 N formic acid, and the water was partitioned 3 times against ethyl acetate. After washing the combined ethyl acetate fraction (acidic fraction) with 10 ml water and adding the wash to the aqueous fraction, the latter was washed 3 times with n-butanol as described above for ethyl acetate, and the water phase was discarded.

The acidic, neutral, and butanol fractions were left overnight at -10° C, then filtered through glass wool in cold Buchner funnels to remove ice particles. The filtrates were evaporated and the residues dissolved in 2 to 4 ml of ethanol and stored at -10° C until assayed with the lettuce hypocotyl assay.

B. Field Experiment

'Montcalm' bean seeds were planted in the field at the Horticultural Research Center in late June, July, and August. The pods were harvested at varying stages of development and separated by age into 6 groups. A random sample of seeds from each group was dissected and morphological characteristics of each group were recorded so that similar stages could be identified when selecting samples

for culture.

Each group of seeds was extracted and the extracts fractionated as previously described. The residue was taken up in ethanol and assayed with the lettuce hypocotyl assay.

II. Culture of Tissues

A. Establishing Cultures of Bean, Phaseolus vulgaris L. 'Montcalm'

Callus cultures were established of immature tissues of bean seeds of Phaseolus vulgaris 'Montcalm'. Pods were harvested from field grown bean plants when the seeds were 0.6 to 1.0 cm in length and washed to remove soil. Under aseptic conditions, the pods were dipped in ethanol, flamed to sterilized the outer surface, cut open, and the seeds removed. The seed coat was cut away and the cotyledons separated from the embryonic axis. After removal of the proximal tissue, the abaxial surface of the cotyledon was placed in contact with the agar medium in a 2 ounce screw-capped jar. The cultures were grown under cool white lights, 1800 to 2100 $\mu Em^{-2}s^{-1}$, or in the dark. Callus was subcultured about every 4 weeks unless otherwise indicated.

1. Comparison of media

Fifteen test media were prepared by adding various hormones to Murashige and Skoog (1962) basal salt and vitamins medium (see Tables 3 and 4). Embryonic axes and cotyledons from greenhouse grown beans were prepared as described above, implanted on the media, and

cultured in the dark for 5 weeks. Cultures were evaluated weekly for callus texture, color, and size. Root formation and any unusual growth of the explants were also noted.

2. Effect of 2,4-D on callus formation

Arnison and Boll (1978) reported that 2,4-D was essential for the growth and maintenance of cell suspension cultures of bush bean cotyledons. Results of the media comparison experiment above suggested that callus growth and friability increased with 2,4-D concentration. No growth occurred on most media which lacked 2,4-D. The medium MS-35 was therefore used with varying concentrations of 2,4-D (0.0, 0.01, 0.5, 1.0, and 3.0 mg/l) added to test the rate of callus initiation and subsequent growth. The pH was adjusted in all cases to 5.8. Embryonic axes and cotyledons were prepared as described above, and the cultures kept in the dark. Four cultures were used for each observation. Fresh weight was recorded after 19 days.

B. Establishing Cultures of Nicotiana forgetiana Hort. ex. Hemsley.

L. Rapport (personal communication) reported that tobacco callus contained slightly higher concentrations of GA-like substances than did other tissues tested. Nicotiana cultures were obtained from J. Passiatore (personal communication) approximately 3 months after initiation from leaf discs cultured on MS-21 and transfer to SH (Table 3), followed by subculture 4 weeks later on the same medium. They were then transferred to UM and subcultured once

Table 3. Components of Murashige and Skoog (1962) basal salts and vitamins medium (MS) and Schenk and Hildebrandt (1972) (SH). Final concentrations in mg/l unless otherwise specified.

Component	MS	SH
NH_4NO_3	1650.0	-
$NH_4H_2PO_4$	-	300.0
KNO_3	1900.0	2500.0
$MgSO_4.7H_2O$	370.0	400.0
$MnSO_4.H_2O$	-	10.0
$MnSO_4.4H_2O$	22.3	-
$ZnSO_4.4H_2O$	8.6	-
$ZnSO_4.7H_2O$	-	1.0
$CuSO_4.5H_2O$	0.025	0.2
$CaCl_2.2H_2O$	440.0	200.0
KI	0.83	1.0
$CoCl_2.6H_2O$	0.025	0.1
KH_2PO_4	170.0	-
H_3BO_3	6.2	5.0
$Na_2MoO_4.2H_2O$	0.25	0.1
$FeSO_4.7H_2O$	27.85	15.0
Na_2EDTA	37.25	20.0
Glycine	2.0	-
Inositol	-	1000.0
Myo-inositol	100.0	-
Nicotinic acid	0.5	5.0
Pyridoxine-HCl	0.5	0.5
Thiamine-HCl	0.1	5.0
2,4-D	-	0.5
p-CPA	-	2.0
Kinetin	-	0.1
Sucrose	30.0 g/l	30.0 g/l
Agar	8.0 g/l	6.0 g/l
pH	5.8	5.8-5.9

Table 4. Components added to MS basal salts and vitamins medium to prepare test media. Final concentration in mg/l and pH adjusted to 5.8 unless otherwise specified.

Components	Medium				
	MS-35	MS-36	MS-0.01	MS-0.1	MS-1.0
6-BAP	0.5	2.5	-	-	-
2,4-D	5.0	5.0	0.01	0.1	1.0
	$MS\text{-}P_1$	R3B	MS-21	MS-0	
6-BAP	0.5	1.0	0.01	-	
NAA	2.0	2.0	5.0	-	
	R2A	R3	MS-D3	R305	
IAA	2.0	5.0	2.0	5.0	
2,4-D	2.0	0.5	-	-	
6-BAP	-	-	1.0	-	
NAA	-	-	-	10.0	
Kinetin	0.3	0.3	-	0.3	
pH	6.0	6.0	5.8	6.0	
		UM (Uchimiya and Murashige, 1974)		MS-S	
Nicotinic acid		4.4		4.5	
Pyridoxine-HCl		9.5		-	
Thiamine-HCl		9.9		-	
Folic acid		-		0.5	
Biotin		-		0.05	
2,4-D		2.0		3.0	
Kinetin		0.25		-	
Casein hydrolysate		2.0 g/l		-	
				Decrease sucrose to 20.0 g/l	
				Increase agar to 9.0 g/l	
pH		5.8		5.6	

4 weeks later for these experiments.

C. Culture Media Used

The components of the media used in these experiments are listed in Tables 3 and 4.

III. Assay for Gibberellin-Like Substances

A. Barley Half-Seed Halo Assay

1. Direct assay of callus cultures

The following procedure based on the half-seed assay by Machi Fukuyama Dilworth (Fukuyama, 1971) was developed to test callus directly in vitro for gibberellin content.

Sterile barley half-seeds were placed in direct contact with callus cultures for 24 hours. The gibberellin in the callus stimulated de novo synthesis of α-amylase in the aleurone layer of the seed. The seed was transferred aseptically to starch plates where the α-amylase diffused into the plates and digested the starch. When flooded with KI/I_2 solution, the background turned blue as the starch and iodine reacted. Clear rings or halos appeared around the seeds where the starch had been digested in the agar. Since all procedures were performed under aseptic conditions, the cultures tested continued to grow.

Preparation of GA_3 standard agars. Stock solutions of GA_3 were prepared in 95% ethanol and diluted with ethanol (95%) to obtain concentrations of 1.0, 0.1, 0.01, 0.001, 0.0001, and 0.0 μg GA_3/10 μl ethanol. These solutions were filter-sterilized through Millipore filters (0.45 μm) and

stored at 0° C in sterile jars. One ml of 0.8% Difco Bacto agar solution was placed in each of 36 pathology vials, 25 ml volume, the caps were screwed on loosely, and the vials were autoclaved at 15 lbs. pressure for 20 minutes. After autoclaving, the vials were cooled to touch in a 45° C water bath. Ten µl of each GA_3 solution was added to each of 6 vials using an Eppendorf pipette with sterile tip. The final GA_3 concentrations were 1.0, 0.1, 0.01, 0.001, and 0.0001 µg GA_3/ml. The control contained 10 µl of sterile ethanol per ml of agar.

Preparation of barley seeds. Barley seeds, Hordeum vulgare 'Himalaya', diameter 0.3 cm or larger, were cut in half with a razor blade and the embryo half discarded. The remaining half-seeds were stored at 10° C. Before use they were soaked for 8 minutes in a solution of 0.7% Tween 20 and double distilled water and sterilized in a 250 ml flask containing 100 ml of full-strength 'Clorox' (5.25% sodium hypochlorite) and 2 drops of Tween 20. The seeds were sterilized for 1 hour with one change of the 'Clorox' solution and occasional swirling to release air trapped by the seeds. The half-seeds were then rinsed 10 times with 100 ml aliquots of sterile water.

Transfer to callus cultures. The callus culture jars were opened aseptically and the cut surface of each sterile half-seed was placed on the callus surface. The same procedure was used to place the half-seeds on the GA_3 standard agars. In most cases, 4 half-seeds were placed on each of

8 callus cultures for a total of 32 observations per treatment. Two half-seeds were placed on each of 6 replicate GA_3 controls for a total of 12 observations per concentration. The half-seeds were left on the callus or agar surface for 24 hours before transfer to starch/agar plates.

Preparation of starch/agar plates. Stock solutions of KH_2PO_4 (60 mg/ml) and $CaCl_2.2H_2O$ (29.4 mg/ml) were prepared in double distilled water and stored at 10° C until used.

The following ingredients were combined while stirring: KH_2PO_4 stock solution, 30 ml; $CaCl_2.2H_2O$ stock solution, 3 ml; double distilled water, 288 ml; and potato starch, 450 mg. The volume was adjusted to 320 ml with double distilled water and the solution boiled for 1 minute. Following centrifugation at 24 g for 20 minutes, the supernatant was stored at 10° C. Difco Bacto agar (1.5%) was added to the supernatant and the solution was sterilized by autoclaving at 15 lbs. pressure for 20 minutes. After cooling in a 45° C water bath, 25 ml aliquots were poured into each sterile Petri dish (100 x 15 mm). After solidification of the agar, the plates were stored in plastic bags at room temperature for no more than 4 days before use.

Transfer of half-seeds to starch/agar plates. After exposure to callus or standard agars, the half-seeds were transferred to agar plates. Four half-seeds were placed on a plate with the cut side contacting the agar firmly but not penetrating the agar surface. The plates were sealed with 'Parafilm' or 'Saran' and incubated for 72 hours at

20° C in the light, 400 to 500 $\mu Em^{-2}s^{-1}$.

Visualization and measurement of response. After incubation, the plates were flooded with KI/I_2 solution (300 mg KI plus 30 mg I_2 per 100 ml water). After about 3 minutes the solution was poured off and the diameter of the clear halos around the seeds were measured to the nearest mm at the widest diameter of each half-seed (Figure 1). Only circles with clearly defined edges were measured. Circles 0.5 cm (the average diameter of the half-seeds) or less in diameter were tabulated as no response. It was assumed that gibberellins diffused at the same rate through callus as through 0.8% agar. Therefore, 1 ml of 0.8% agar was equivalent to 1 ml of callus and the results were expressed in µg/unit volume. Treatment means and standard deviations were calculated using cultures as replicates.

Response to known concentrations of GA_3. GA_3 standards were prepared at concentrations ranging from 0.0 to 5.0 µg GA_3/ml. Half-seeds were incubated on GA_3 standard agars for 24 hours and on starch/agar for 72 hours.

Incubation time. The effect of incubation time, both on GA_3 standards and on starch agar, was investigated to determine the time period which would give the greatest sensitivity and the optimum dose response curve. 'Himalaya' half-seeds were sterilized and exposed to GA_3 standard agars and starch/agar plates for varying periods of time in a factorial design using 24, 48, and 72 hours of incubation on GA_3 standards and the same periods of exposure to

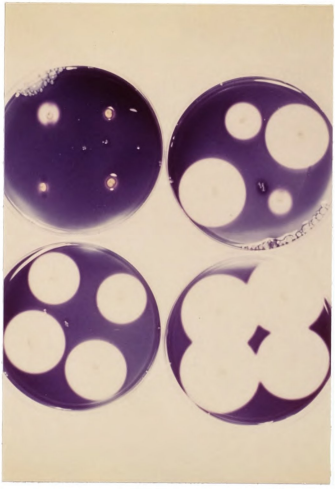

Figure 1. Halos in the barley half-seed halo assay after exposure of half-seeds to GA_3 standard agars for 24 hours and to starch/agar plates for 72 hours. Upper left, 0.0 µg/ml. Upper right, 0.001 µg/ml. Lower left, 0.01 µg/ml. Lower right, 1.0 µg/ml.

starch/agar for a total of 3 x 3 = 9 treatments. Two replicates of each concentration were used. The entire experiment was repeated. The 24/72 hour treatment was repeated three additional times with 6 replicates of each GA_3 concentration x 2 seeds per replicate making 12 observations for each concentration.

Presoaking in water. Fukuyama (1971) reported that soaking sterile half-seeds for 24 hours prior to use increased the sensitivity of the assay to low concentrations of gibberellins. To confirm this observation, barley half-seeds were sterilized, rinsed with sterile distilled water, and soaked in sterile water for 24 hours. The seeds were subsequently transferred to GA_3 standard agars for 24 hours and finally transferred to starch/agar plates for 72 hours.

Light. Fukuyama (1971) did not investigate the effect of light on the half-seed halo assay. As cultures grown both in the light and in the dark were to be assayed, the effect of light was tested. Two half-seeds were placed on twelve GA_3 standard agars of each concentration. Six replicates of each concentration were placed in the dark and 6 under cool white light, 1800 to 2100 $\mu Em^{-2}s^{-1}$, for 24 hours. All seeds were transferred to starch/agar plates and held for 72 hours at 20° C and 400 to 500 $\mu Em^{-2}s^{-1}$.

2. Direct assay of liquid culture medium

To measure gibberellin concentrations in liquid cell cultures, the assay developed by Fukuyama (1971) was modified to enable sampling of continuously growing

suspension cultures. The assay involved aseptic opening of suspension cultures 2 to 4 times a week to insert and remove half-seeds. The utmost care in flaming instruments and changing damaged foil caps had to be exercised to avoid contamination.

Preparation of GA_3 liquid standards. Since the suspension culture assay measured GA in liquid medium, standards were prepared in the same culture medium as the tissue. Media were prepared as described for solidified media except agar was omitted. One ml of the medium was placed in a culture tube, capped, and autoclaved. Ten µl of GA_3 in ethanol were added to each tube to give concentrations of 1.0, 0.1, 0.01, 0.001, and 0.0001 µg GA_3/ml of medium. Controls included both 10 µl ethanol per ml of medium and medium alone. Standard solutions were stored at 0° C between assays.

Preparation of barley seeds. Barley half-seeds were prepared as for direct assay of callus cultures previously described.

Transfer to liquid cultures. Suspension cultures were opened aseptically and 4 (2 for controls) sterile half-seeds dropped into the medium. The cultures were recapped and returned to the gyrating shaker for 24 hours. Liquid standards were rotated for 24 hours at 1 rpm in a horizontal position on a vertical wheel. The standards were tested in duplicate resulting in 4 observations for each concentration.

Preparation of starch agar plates. Starch/agar plates were prepared as for direct assay of callus cultures previously described.

Transfer of half-seeds to starch/agar plates. Half-seeds were removed from the solutions assayed, blotted on sterile filter paper, and placed cut side down in firm contact with the agar surface. The plates were covered and sealed with 'Parafilm' or 'Saran' for 24 hours.

Visualization and measurement of response. The direct assay of liquid cultures was visualized and measured as described above for the direct assay of callus cultures.

Effect of ethanol solvent and temperature of medium during addition of GA_3 to liquid standards. The method outlined above for preparing liquid GA_3 standards was based on the assumption that the ethanol solvent evaporated and thus did not interfere with the assay. To test this premise, GA_3 standards were prepared in duplicate as in Table 5, using 10 µl GA_3 solution per 1 ml of medium. Two liquid media were tested, UM and R3B. Sterile half-seeds were incubated in the solutions for 24 hours, then transferred to starch/agar plates for 72 hours.

Incubation time with starch. The effect of time of barley half-seed incubation on starch gel was tested. Liquid standards were prepared by adding 10 µl of ethanolic GA_3 solutions to 1 ml aliquots of culture medium. Half-seeds were incubated in these solutions for 24 hours and placed on starch/agar plates for 24, 32, 48, or 72 hours

before adding KI/I_2 solution.

3. Assay of extracts in starch/agar plates

The method presented here was adapted from that used by Fukuyama (1971) and involved the addition of an extract of cultured cells or media dissolved in ethanol to the starch/agar plates as they hardened. The half-seeds were placed directly on these plates, allowing both the diffusion of GA from the plates into the seed, and of the induced amylase from the seed into the plates.

Table 5. Treatments used to test effect of adding ethanol with GA_3 on halo diameter in barley half-seed halo assay.

Solvent for GA_3	Medium temperature (^oC)
Ethanol	ca. 0^o
Ethanol	ca. 70^o
Water	ca. 25^o

Preparation of starch/agar plates with extract.

Starch/agar solution was prepared as for the direct assay of callus cultures. Five ml of the autoclaved solution was pipetted into sterile Petri dishes (60 x 15 mm). Before the agar solidified, filter-sterilized extracts of GA_3 standards in ethanol were added and the plates swirled to mix thoroughly. Plates containing extracts and GA_3 standards were prepared in duplicate.

Preparation of barley seeds. Barley half-seeds were prepared as for direct assay of callus tissue. After rinsing, the cut surfaces of two half-seeds were placed on the surface of the starch/agar plate. Dishes were sealed with 'Parafilm' or 'Saran' for 60 hours.

Visualization and measurement of response. Visualization and measurement of the halo diameter was performed as for direct assay of callus cultures.

Incubation time. Starch/agar plates containing GA_3 standards were assayed for 48, 60, and 72 hours to determine optimum time for response.

B. Lettuce Hypocotyl Assay

The lettuce hypocotyl assay (Frankland and Wareing, 1960) was used to measure the concentration of endogenous GA-like compounds in extracts of fresh bean seeds. High concentrations of 2,4-D in the callus inhibited lettuce hypocotyl elongation making determination of gibberellin content of callus with the lettuce hypocotyl assay impossible without purification of the extract beyond acid-base fractionation.

One cm^2 filter paper sections (Whatman No. 1) were placed in 16 x 65 mm shell vials. Extracts dissolved in ethanol were added to each vial and allowed to dry overnight. An aqueous solution of Tween 80 (0.1%), 0.3 ml, was added. Standards were prepared by adding 0.3 ml of GA_3 dissolved in the same solution at concentrations of

0.1, 0.03, 0.01, and 0.003 μg GA_3/0.3 ml. Extracts and GA_3 standards were prepared in duplicate. Ten lettuce seeds cv. Parris Island, were added to each vial and the vials placed on moist paper towel in a clear plastic box on a lab bench for 24 hours. The container was moved to a growth chamber at 23 to 25° C under fluorescent lights (46.5 $\mu Em^{-2}s^{-1}$) for 72 hours. The length of the five longest hypoctyls in each vial was recorded.

IV. Gibberellin Content of Established Callus Cultures of Bean and Nicotiana

Callus cultures of bean and Nicotiana which had been subcultured for the 5th time (Subculture 5) were assayed biweekly for gibberellin content with the half-seed halo assay. Bean cotyledon cultures were grown on R3B and UM and Nicotiana cultures on UM. Cultures were grown in cool white light (1800 to 2100 $\mu Em^{-2}s^{-1}$). Half of the cultures were subcultured after 18 days (Subculture 6). Others were assayed until they turned brown and ceased to grow. The fresh weight of the callus was measured to relate gibberellin concentration to changes in growth rate.

V. Gibberellin Content of Callus Initiated on Bean Cotyledons and in Subsequent Subculture

If the loss of gibberellin production in established callus was due to subculturing or habituation, freshly derived callus cultures should show a pattern of production similar to that of established cultures. To test this,

callus cultures were initiated on bean seed cotyledons 0.6 to 1.0 cm in length. Embryo axes were removed and one cotyledon was placed in each culture jar. Three media were tested: R3B, MS-D3, and UM. Cultures were grown in both light (1800 to 2100 $\mu Em^{-2}s^{-1}$) and dark. Gibberellin concentration was measured with the barley half-seed halo assay once a week from the time the callus was large enough to support 4 half-seeds until it browned and ceased to grow, approximately 27 days.

VI. Gibberellin Content of *Nicotiana* Callus Following Subculture on Root and Shoot Regeneration Media

As gibberellins did not accumulate in meristematic callus, the question arose as to whether or not they might increase in callus as it regenerated plantlets. A system has not yet been devised for regenerating plantlets from bean callus, but has been for *Nicotiana*. *Nicotiana* was transferred to MS-D3 for shoot initiation, to MS-0 for root initiation, and to UM for maintaining unorganized callus. The gibberellin content was measured by direct assay with the half-seed halo assay.

RESULTS AND CONCLUSIONS

I. Effect of Stage of Development of Bean Seeds on Content of GA-Like Substances in Methanol Extracts

A. Greenhouse Experiment

Bean seeds 0.6 to 1.1 cm long (Table 6) had the highest concentration of active gibberellins when grown in the greenhouse. No GA activity was detected in the neutral fraction except in extracts of the smallest size seeds. Activity in the acidic ethyl acetate fraction was higher than in the neutral ethyl acetate fraction or the butanol fraction. Activity in the acidic ethyl acetate fraction was very low in seeds larger than 1.5 cm.

Table 6. GA-like activity (μg GA_3-eq./g f.w.) in methanol extracts of greenhouse grown bean seeds using the lettuce hypocotyl assay.

Days after anthesis	Length of bean seed (cm)	Fraction		
		Neutral EtAc	Acidic EtAc	Acidic butanol
10-15	0.6-1.1	0.03	1.50	0.00
15-20	1.1-1.3	0.00	0.20	0.03
25	1.5	0.00	0.30	-
30	1.5	0.00	0.03	-
35	2.0	0.00	0.00	-

B. Field Experiment

The youngest bean seeds tested contained the highest GA-like activity (Figure 2). Activity declined with decreasing seed size. No activity was detected in the neutral EtAc fraction. The activity of the acidic EtAc fraction was highest in seeds less than 0.7 cm in length. Seeds 0.3 to 0.5 cm in length were too small to provide viable explants for tissue culture. Seeds from 0.5 to 1.0 cm long were therefore used as a source of explants.

II. Establishing Cultures of Bean; Phaseolus vulgaris L. 'Montcalm'

A. Comparison of Media

Two types of callus were initiated on the embryonic axes. Clear, yellow, friable callus formed at the base of the primary leaves, while white, firm callus formed just above the tip of the radicle. Callus formed mostly around the edges of the cotyledon where it contacted the surface of the medium. The character of the callus depended upon the medium. Media which produced proliferating callus without roots or unusual growth of the explant for both cotyledons and embryonic axes were UM, MS-35, MS-36, MS-P_1, R3B, and R2A (see Materials and Methods page 18-20). Callus which formed on UM was the fastest growing and the most friable.

B. Effect of 2,4-D on Callus Formation

The initiation and growth rate of callus were directly related to 2,4-D concentration (Figure 3). Media

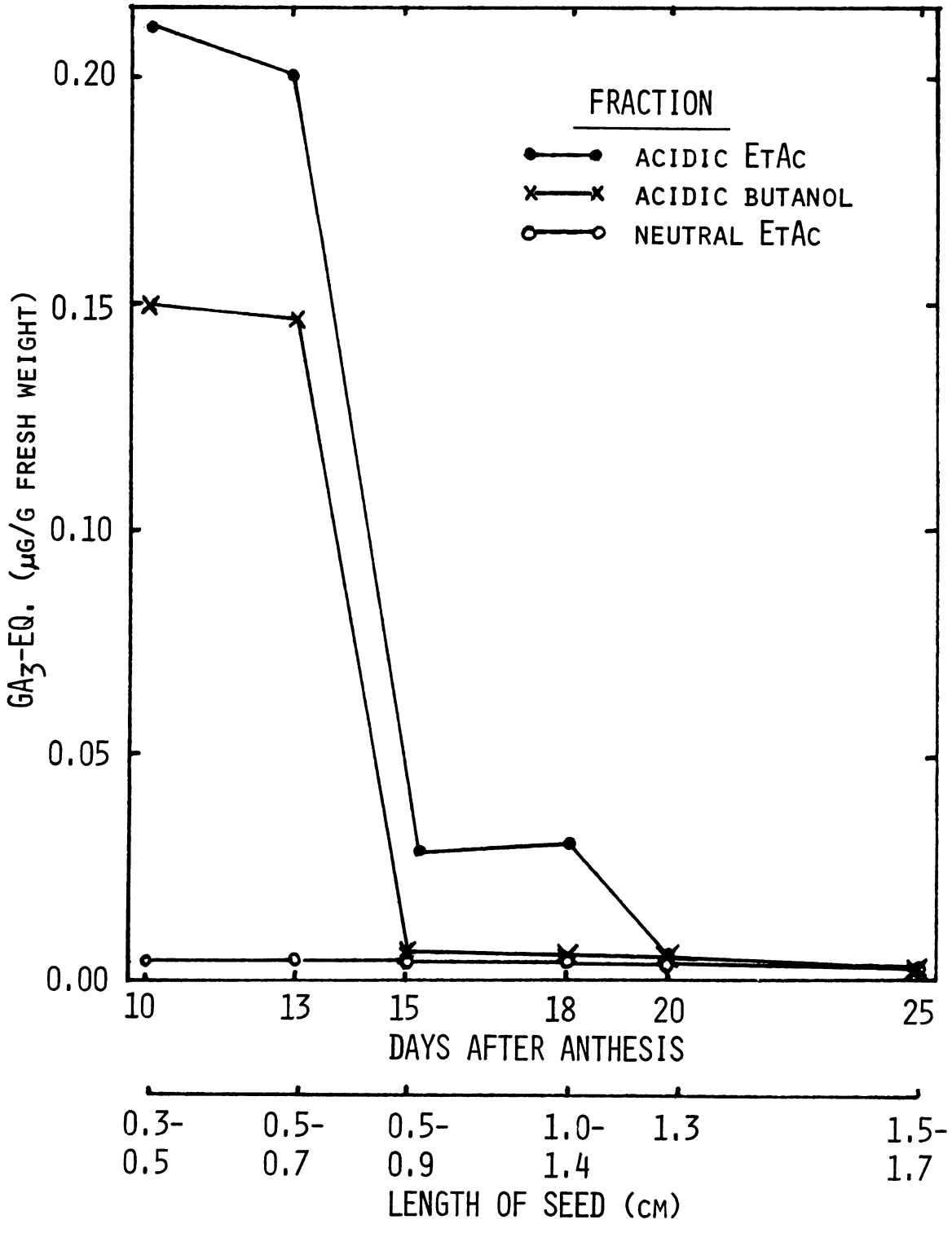

Figure 2. GA-like activity in methanol extracts of field grown bean seeds as determined by lettuce hypocotyl assay.

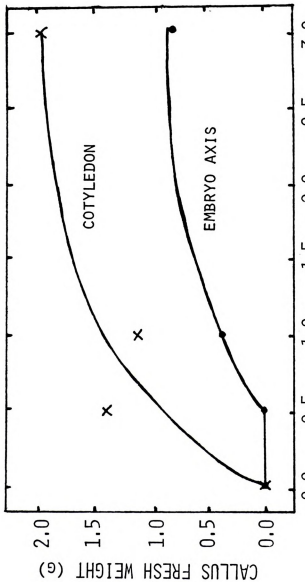

Figure 3. Effect of 2,4-D concentrations in MS-35 medium on callus formation on bean cotyledon and embryonic axis explants after 19 days of culture.

without 2,4-D did not initiate callus. Embryonic axes required higher concentrations of 2,4-D for growth than did cotyledons. Callus tissue on media containing 2,4-D remained actively dividing and light in color for 4 to 5 weeks. Fresh weight of callus increased with concentration of 2,4-D up to approximately 2.5 mg/l for cotyledons and 2.0 mg/l for embryo axes.

Of the 15 media tested previously, all those which induced callus formation contained at least 2.0 mg/l auxin. On media which contained NAA, IAA, or no auxin, callus developed that was brown, slow-growing, and ceased growth after 2 weeks.

III. Barley Half-Seed Halo Assay

A. Factors Affecting Halo Diameters After Exposure to GA_3 Standards in Agar

1. Halo diameters after exposure to known quantities of GA_3

Halo diameters of barley half-seeds exposed to increasing GA_3 concentrations in agar increased at an increasing rate to 10^{-2} µg/ml GA_3 and thereafter increased at a decreasing rate to 10^{1} µg/ml GA_3 (Figure 4). Increases in halo diameter declined sharply above 10^{-1} µg/ml with only slight additional increases at higher concentrations. The controls (ethanol in agar) consistently resulted in diameters of 0.5 cm. Therefore, the maximum level of GA_3 which could be accurately estimated in unknown samples was 10^{-1} µg/ml.

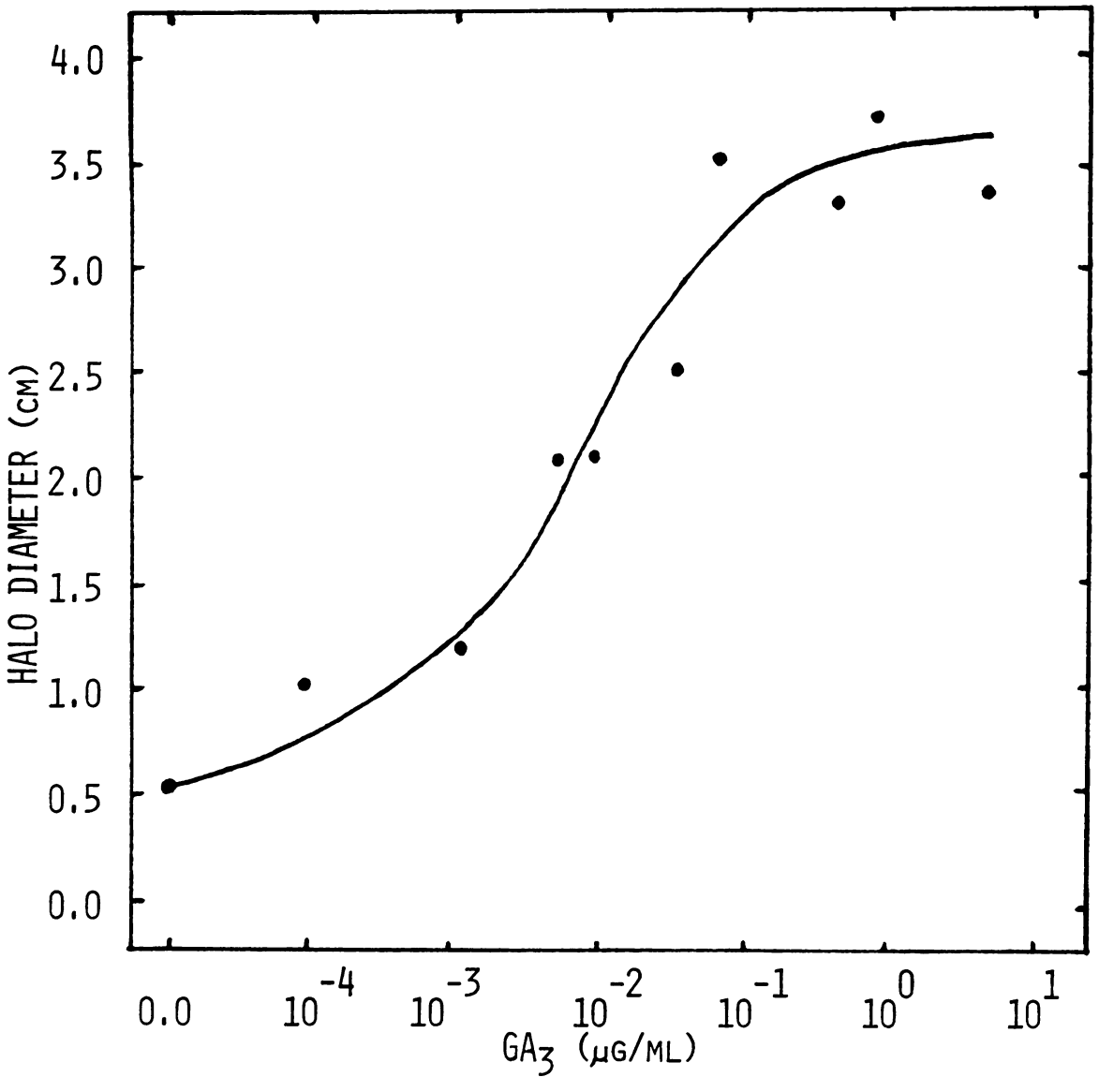

Figure 4. Halo diameters in half-seed halo assay after exposure to known concentrations of GA_3 in 1 ml aliquots of agar.

2. Incubation time

Halo diameter increased with time of exposure of half-seeds to both GA_3 and starch/agar (Figure 5). However, the slope of the response curve was maximum with the 24/72 hour treatment. Incubation of half-seeds for 72 hours prior to placement on starch/agar plates essentially negated the response to GA_3, since controls produced halos as large as those induced by GA_3. These half-seeds were very soft and white exudate remained on the agar surface after they were removed. Almost all of the endosperm had been digested. Similar results were obtained on repeating the experiment (data not shown). On three additional trials, using only the 24/72 hour treatment, the response curve was fairly consistent (Figure 6).

3. Presoak in water

The baseline of the dose:response curve was increased by soaking barley half-seeds in water for 24 hours prior to incubation on GA_3 standards. However, since the slope of the response curve was reduced (Figure 7), the seeds were used without a presoak.

Figure 5. Halo diameters of barley half-seeds incubated for various times on GA_3 standard agars and on starch/agar plates. (First figure=hours on GA_3 standards; second figure=hours on starch/agar plates.)

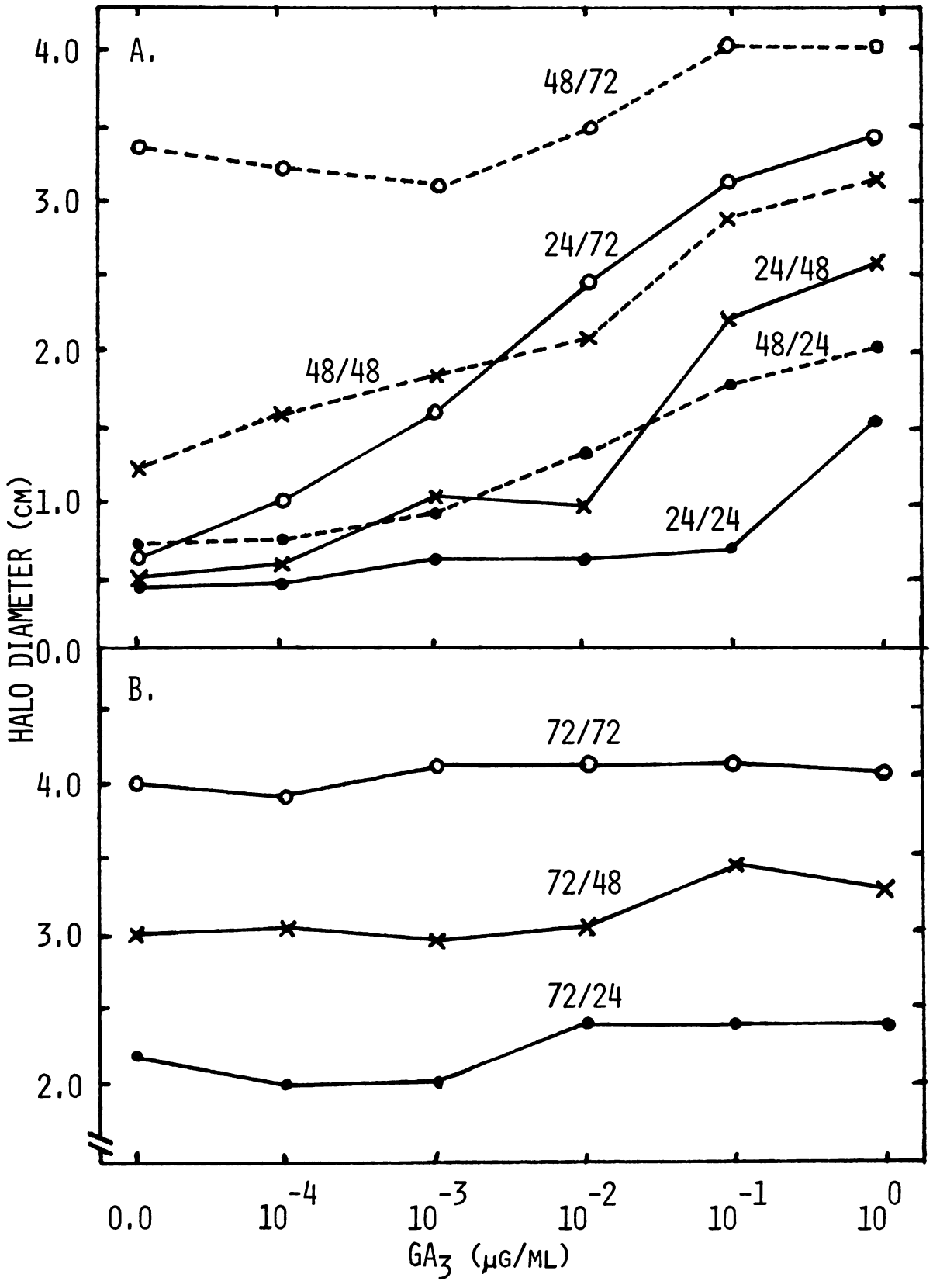
A.
48/72
24/72
24/48
48/24
48/48
24/24
B.
72/72
72/48
72/24
HALO DIAMETER (cm)
4.0
3.0
2.0
1.0
0.0
0.0
10^-4
10^-3
10^-2
10^-1
10^0
GA3 (µG/ML)

Figure 5.

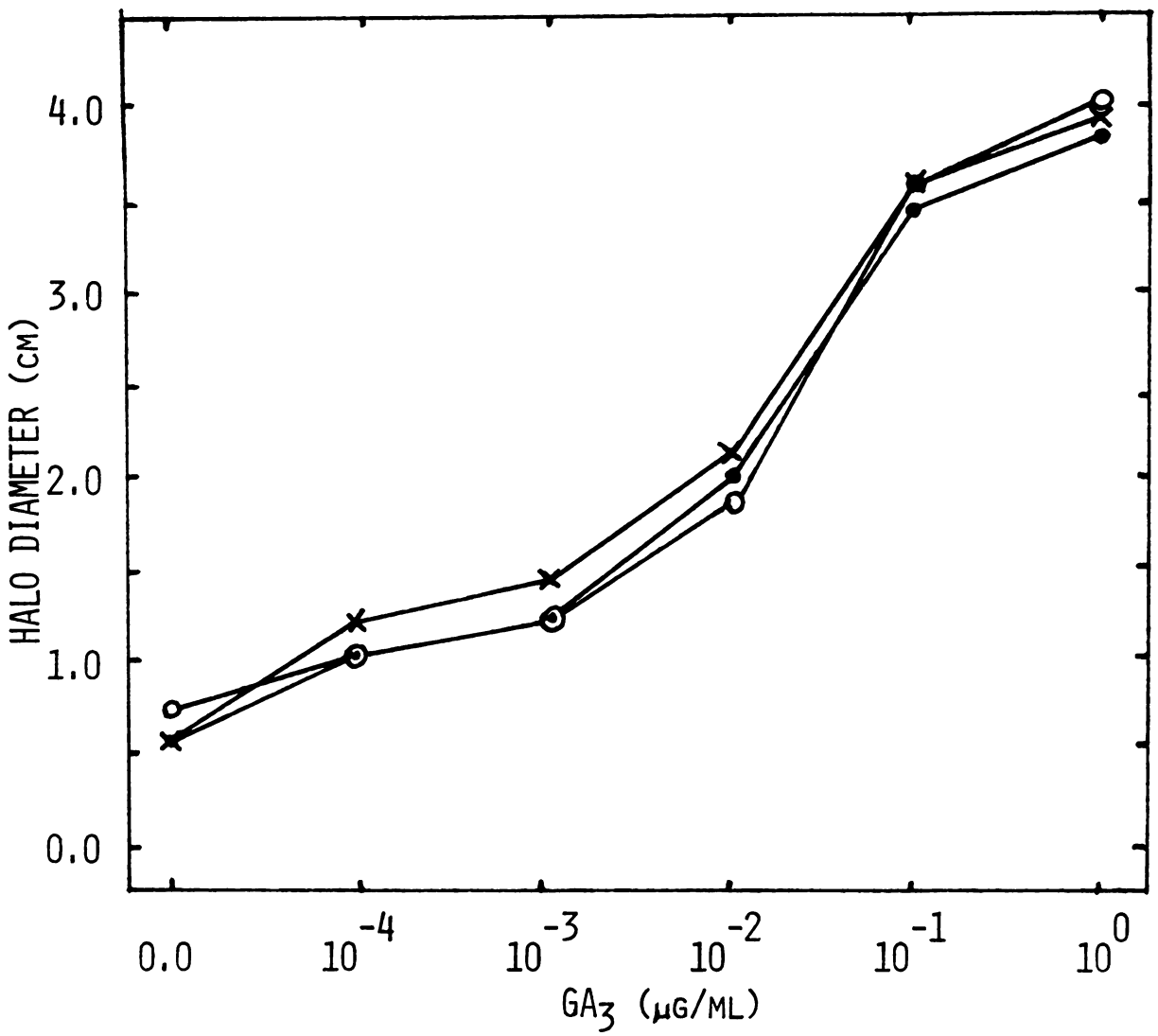

Figure 6. Halo diameters of barley half-seeds after 24 hour exposure to GA_3 standards and 72 hour exposure to starch/agar plates. Means for 3 trials.

Figure 7. Halo diameters in barley half-seed halo assay following 24 hour presoak in water. Bars represent one standard deviation above and one standard deviation below the mean.

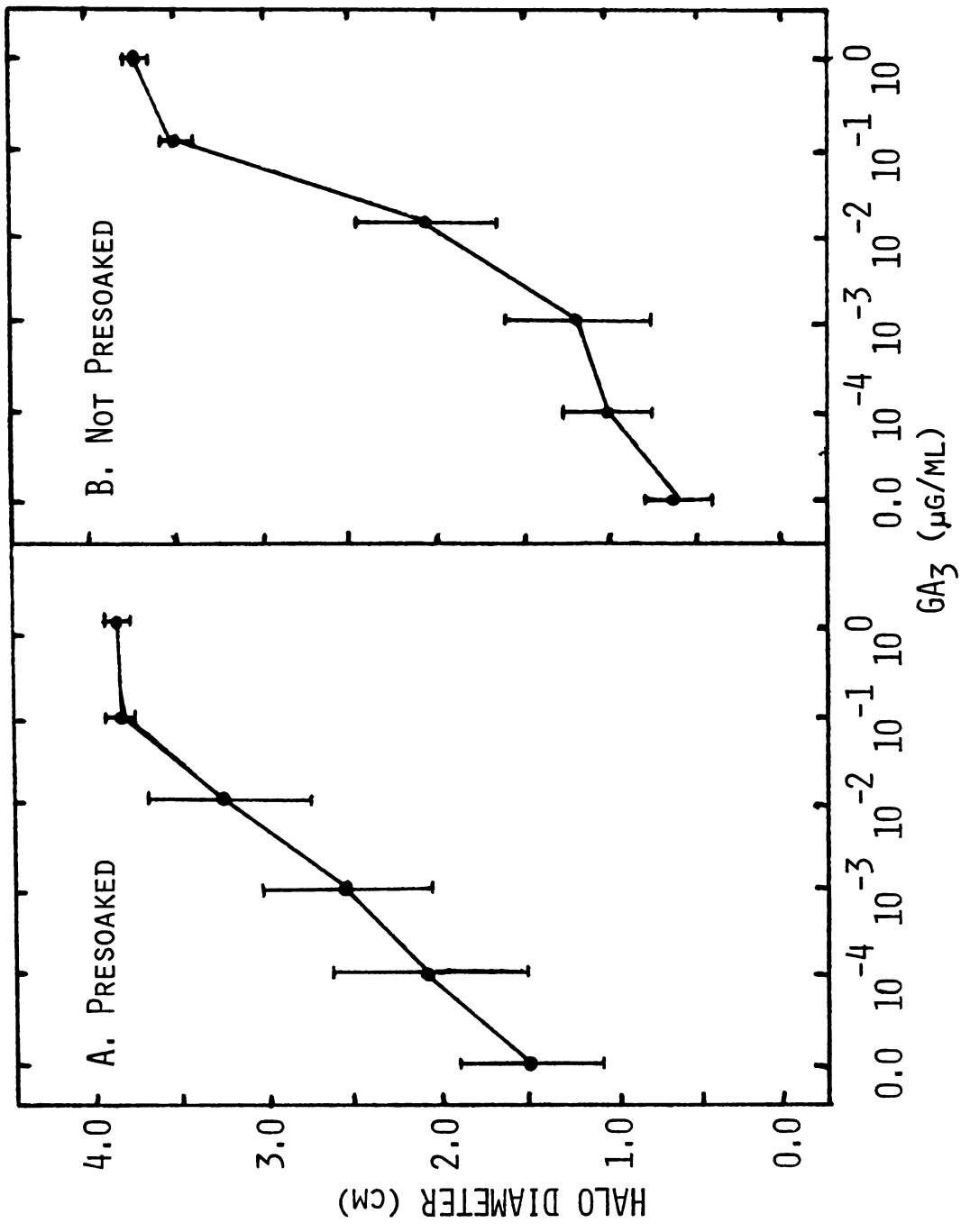
A. PRESOAKED
B. NOT PRESOAKED
HALO DIAMETER (CM)
4.0
3.0
2.0
1.0
0.0
0.0 10^-4 10^-3 10^-2 10^-1 10^0
GA3 (µG/ML)

Figure 7.

4. Light

Light had no appreciable effect on response of barley half-seeds (Figure 8).

B. Factors Affecting Halo Diameter After Exposure to GA_3 in Aqueous Solution

1. Effect of ethanol solvent and temperature of medium during addition of GA_3 to liquid standards

Method of adding GA_3 to medium had no appreciable effect on response of half-seeds (Figure 9). The poor response curve was due to the length of incubation of starch/agar plates. The time was shortened in subsequent experiments.

2. Incubation time with starch

For half-seeds incubated in aqueous solutions of GA_3, the shorter the time of exposure to the starch/agar plate the greater the slope of the halo diameter response curve (Figure 10). This differed from the results obtained with half-seeds incubated on agar containing GA_3 in which the optimum response curve was obtained with a 24/72 hour combination. The difference in optimum time of assay between solid and liquid culture may be that in the liquid standard the GA_3 diffuses throughout the seed within 24 hours, and that on transfer to the starch/agar plate the amylase begins to diffuse immediately. In seeds placed on GA_3 standard agars, on the other hand, the GA_3 may still be diffusing through the seed after 24 hours and more time on the starch/agar plate is required for equivalent response.

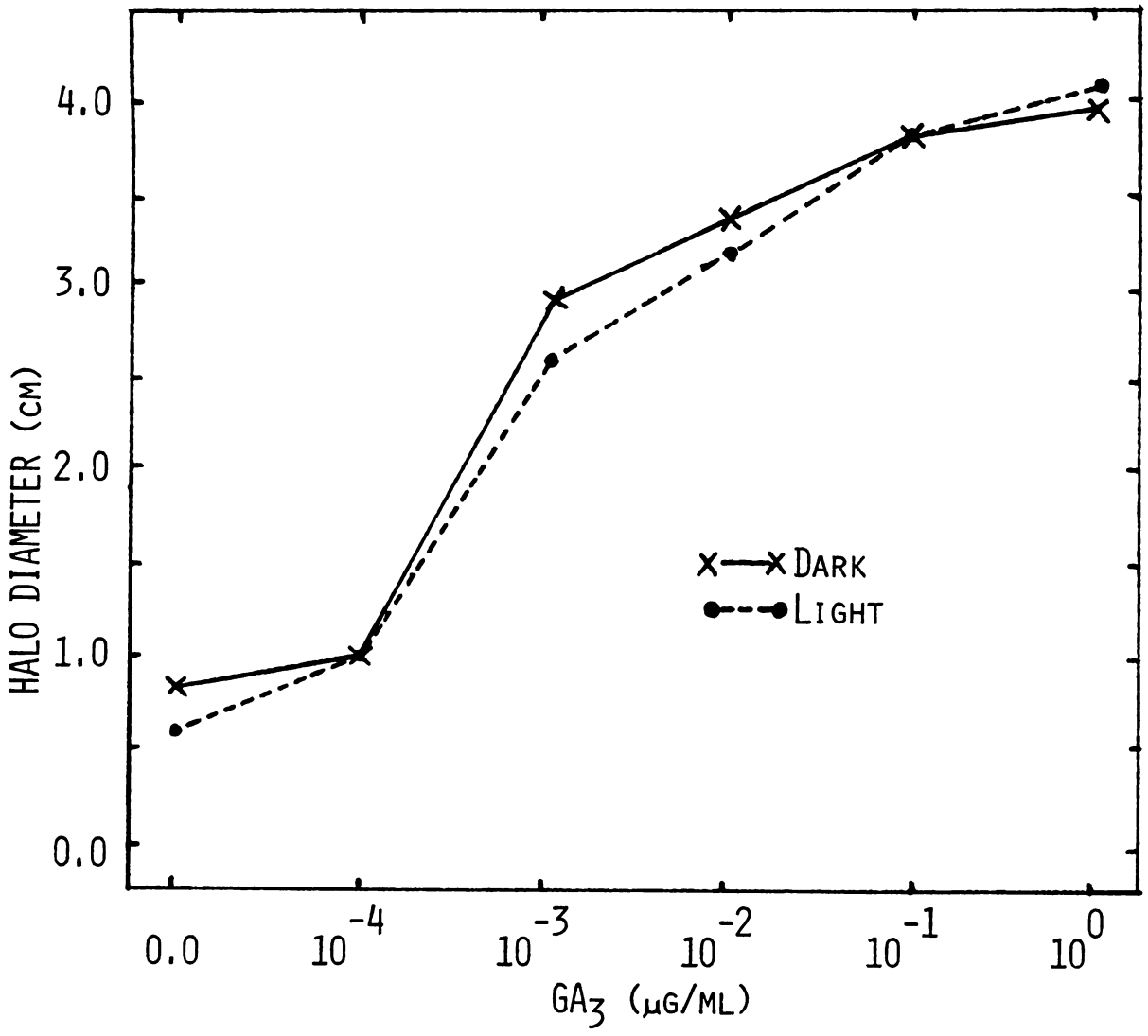

Figure 8. Effect of light during 24 hour incubation on GA_3 standard agars on halo diameters in half-seed halo assay. All half-seeds subsequently incubated for 72 hours on starch/agar plates in light.

Figure 9. Effect of method of adding GA_3 to 2 liquid media on halo diameters in half-seed halo assay. Incubation time--24/72 hours.

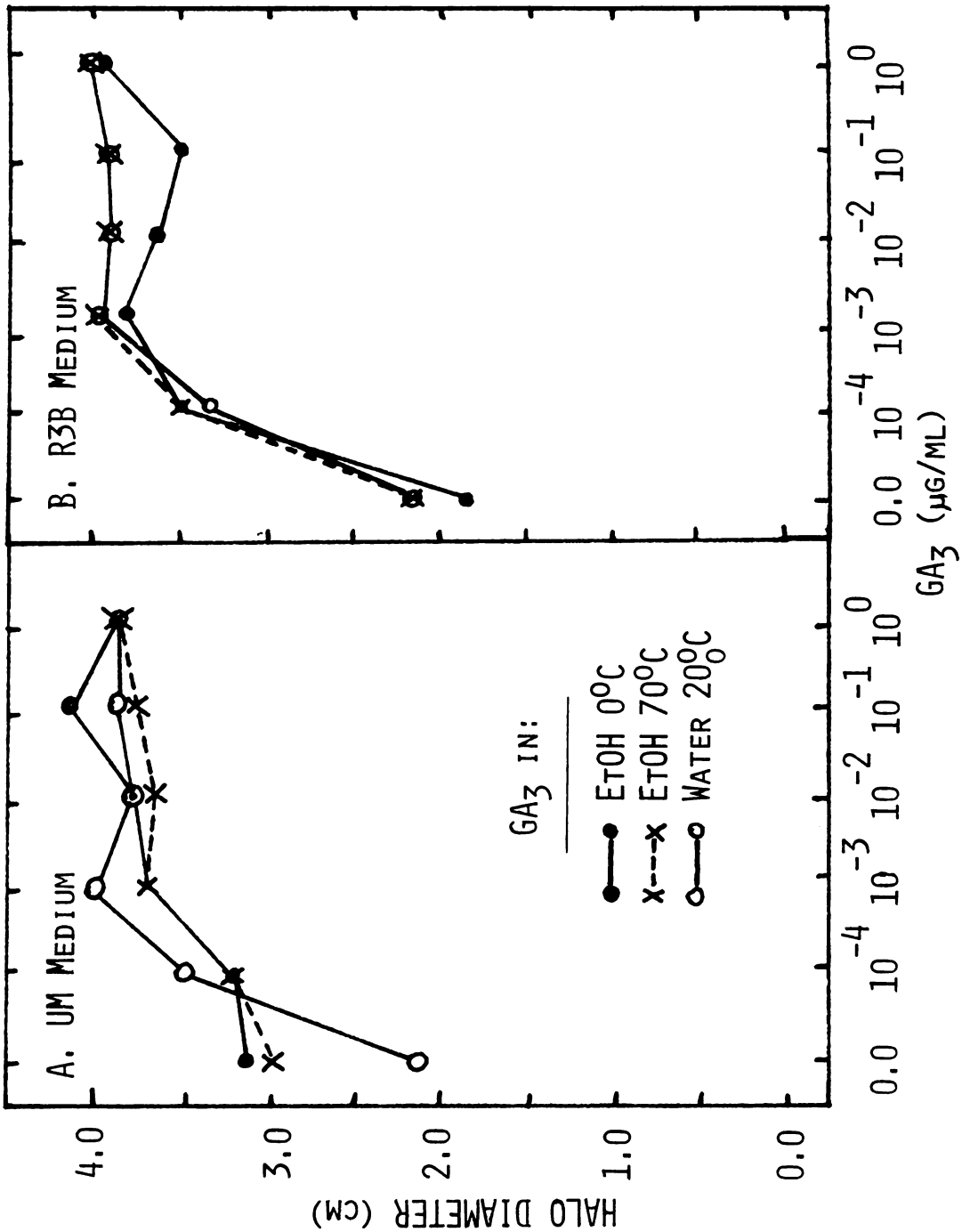
A. UM MEDIUM
B. R3B MEDIUM
HALO DIAMETER (CM)
4.0
3.0
2.0
1.0
0.0
GA_3 IN:
ETOH 0°C
ETOH 70°C
WATER 20°C
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0}
0.0
GA_3 (µG/ML)

Figure 9.

Figure 10. Effect of incubation time on starch/agar plates following 24 hour exposure to GA_3 liquid standards on halo diameters in half-seed halo assay. (First figure=hours in GA_3 liquid standards; second figure=hours on starch/agar plates.)

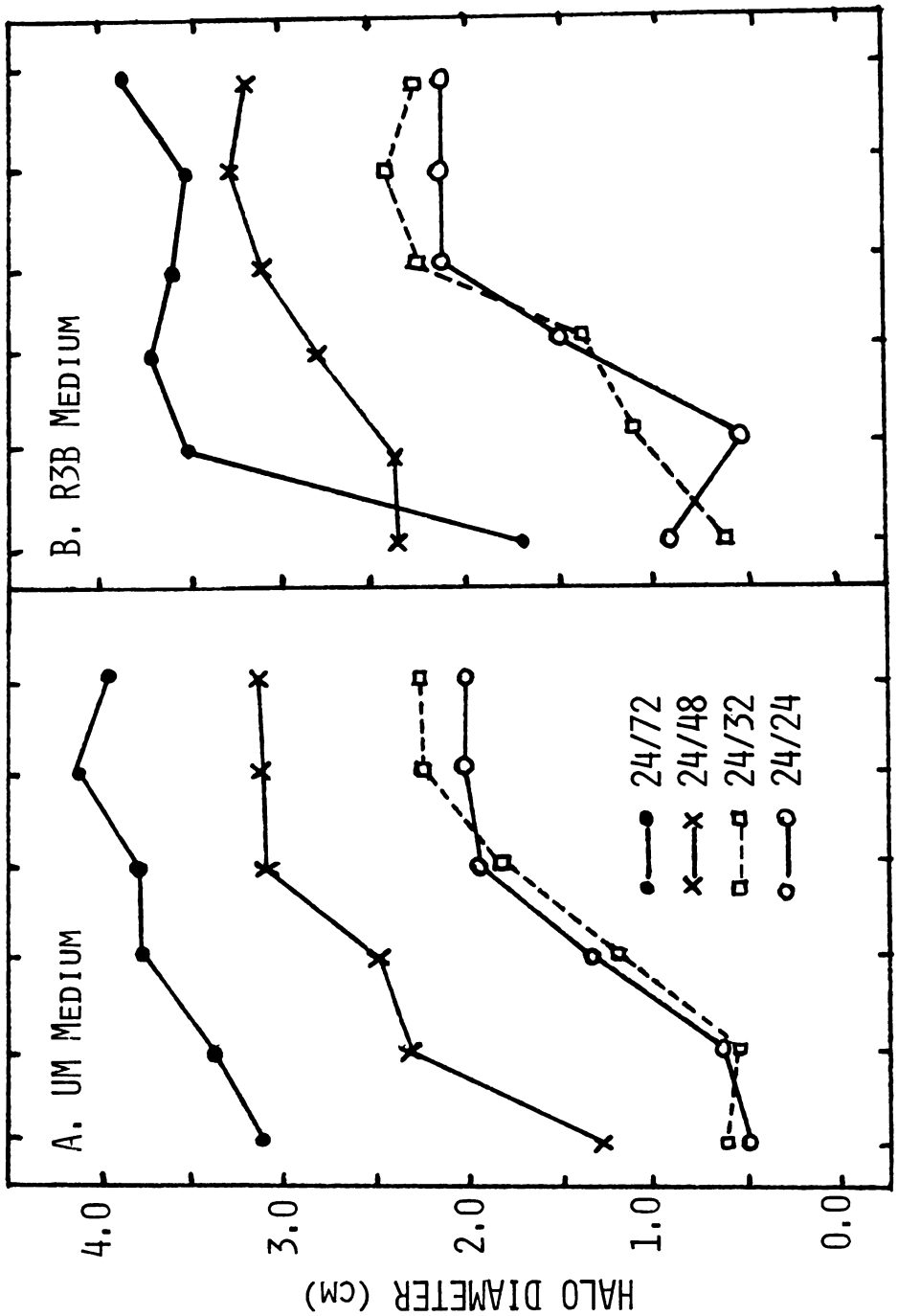
A. UM MEDIUM
B. R3B MEDIUM
HALO DIAMETER (CM)
4.0
3.0
2.0
1.0
0.0
0.0 10^-4 10^-3 10^-2 10^-1 10^0
0.0 10^-4 10^-3 10^-2 10^-1 10^0
GA3 (µG/ML)
24/72
24/48
24/32
24/24

Figure 10.

C. Incubation Time for Assay of Extracts in Starch/Agar Plates

When both GA_3 and starch were incorporated in agar, the optimum dose response curve was obtained after 60 hours (Figure 11).

IV. Gibberellin Content of Established Callus Cultures of Bean and Nicotiana

The gibberellin content of bean callus on UM (Figure 12, Subculture 5) was initially 0.02 GA_3-eq. µg/ml and attained the maximum level of 0.1 µg/ml on day 4. Subsequently, the GA content decreased to 0.0002 µg/ml on day 11 and increased to 0.004 µg/ml on day 14. After another decrease, it reached 0.01 µg/ml on day 32 before once again declining.

A similar pattern of GA_3 content was observed for bean callus on R3B (Figure 13, Subculture 5). Initially the gibberellin content was 0.1 µg/ml. On day 11 it was 0.0001 µg/ml and increased slightly on day 14. It decreased again on day 18 and increased to 0.004 µg/ml on day 32 before declining.

Nicotiana callus on UM (Figure 14, Subculture 5) contained 0.1 GA_3-eq. µg/ml and gradually declined to 0.0004 µg/ml on day 14. Gibberellin content increased slightly on day 18 and decreased on day 21. It then increased through day 32 when it peaked at 0.01 µg/ml before declining again.

Established callus cultures oscillated in gibberellin content in µg/ml of tissue (Figures 12, 13, 14, Subculture 5).

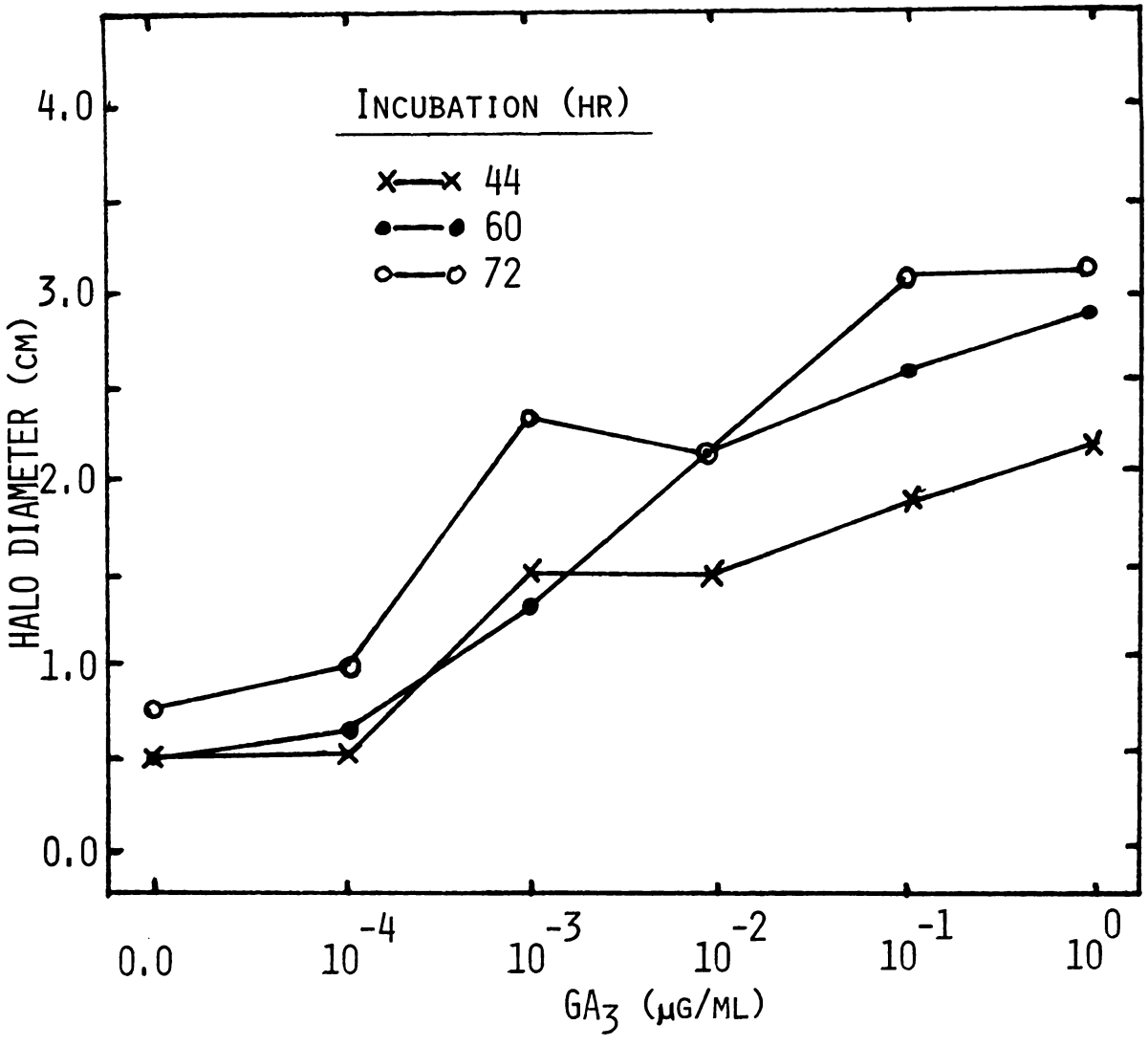

Figure 11. Effect of incubation time on halo diameters of barley half-seeds on agar plates containing both GA_3 standards and starch.

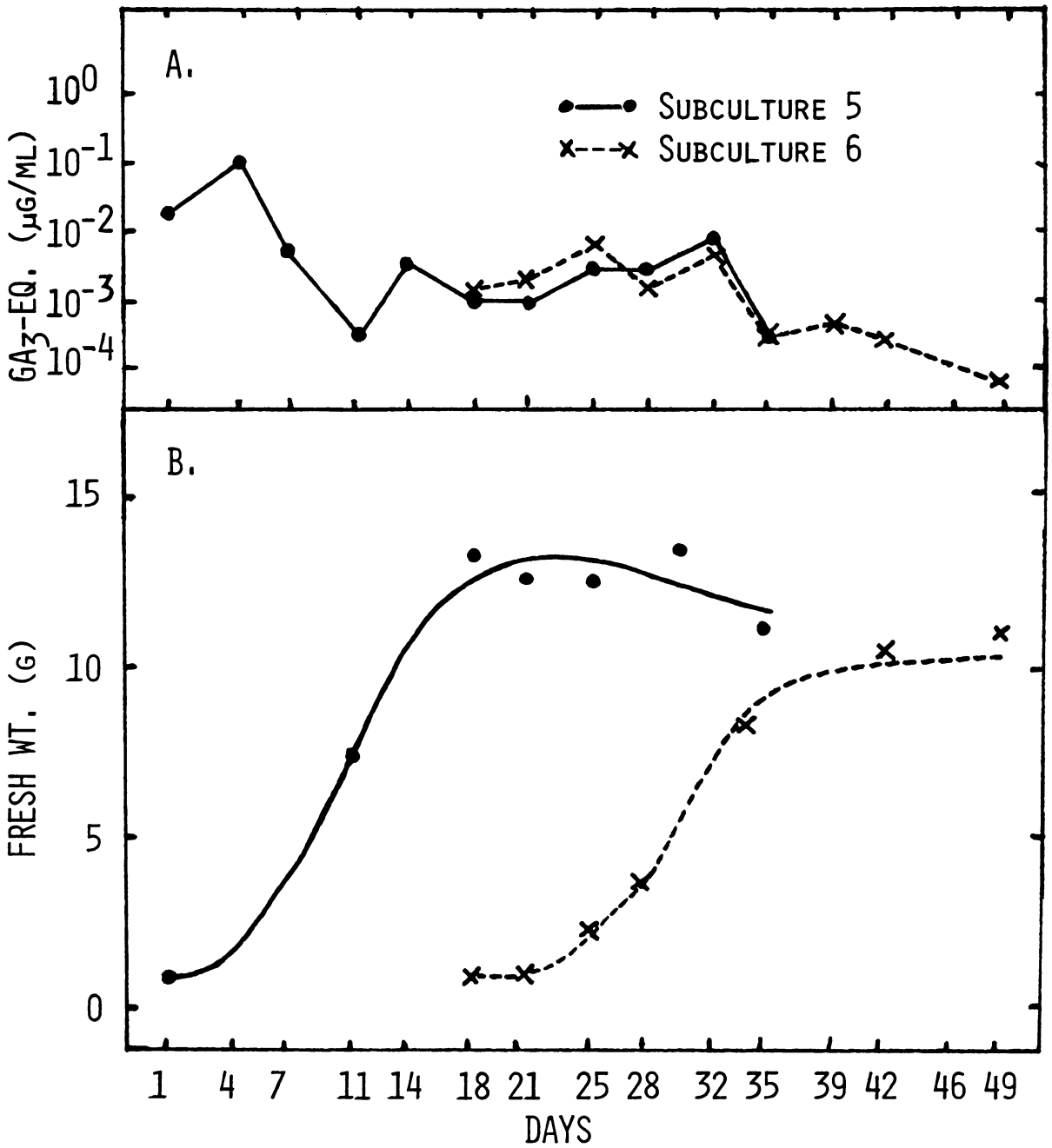

Figure 12. (A) Gibberellin content and (B) fresh weight of established bean callus during subculture on UM. Subculture 6 initiated from subculture 5 on day 18.

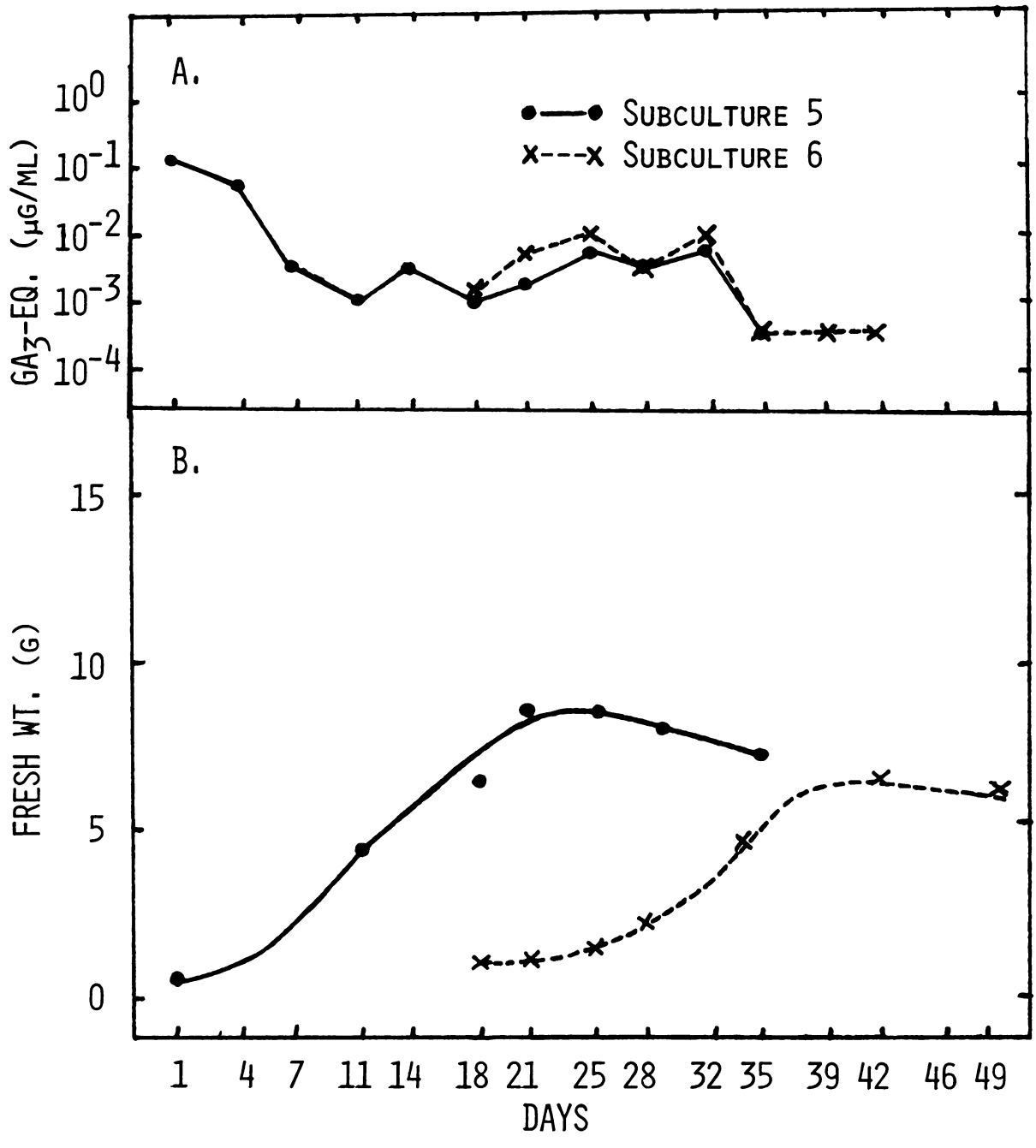

Figure 13. (A) Gibberellin content and (B) fresh weight of established bean callus during subculture on R3B. Subculture 6 initiated from subculture 5 on day 18.

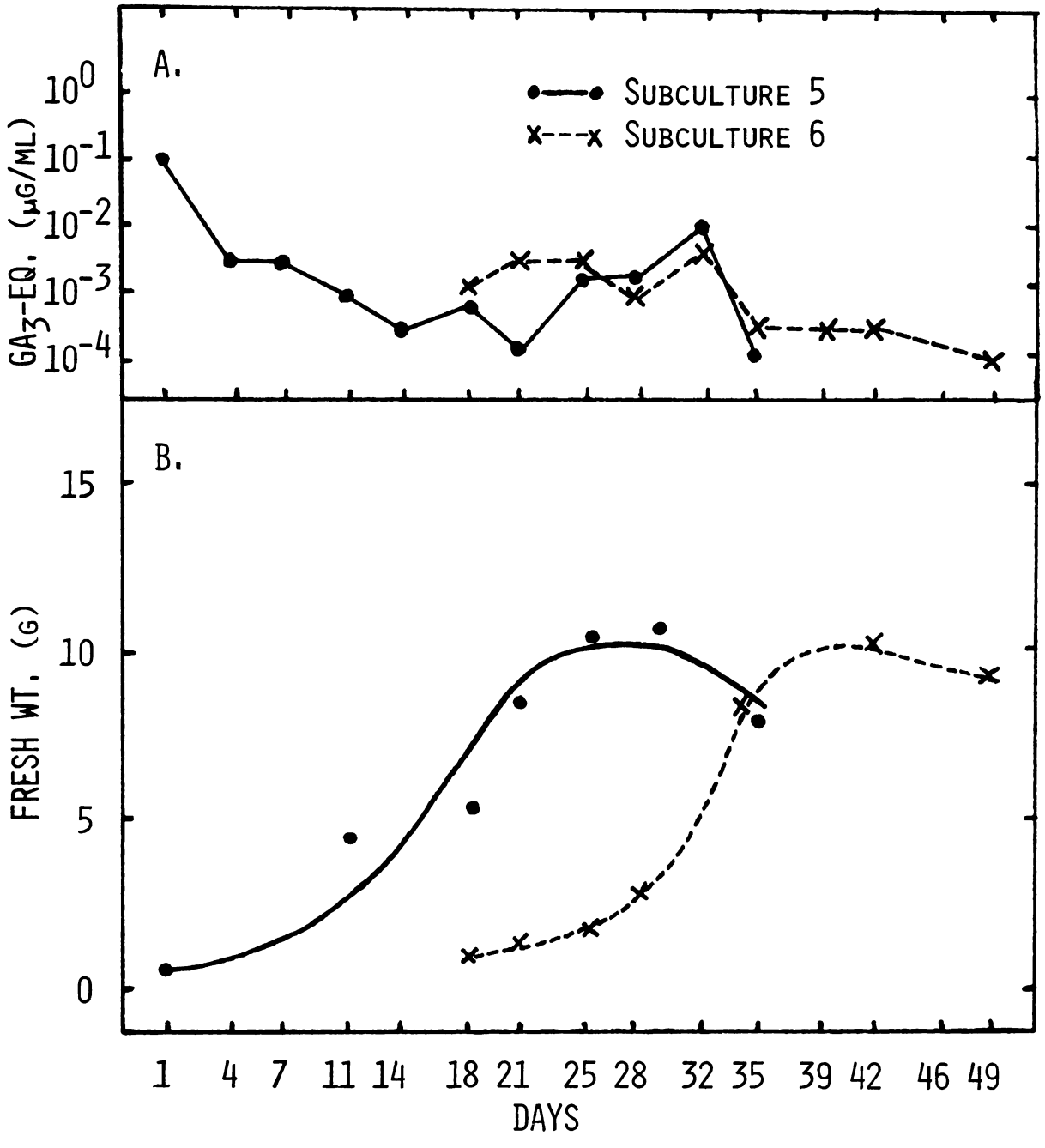

Figure 14. (A) Gibberellin content and (B) fresh weight of established Nicotiana callus during subculture on UM. Subculture 6 initiated from subculture 5 on day 18.

The highest concentration in subculture 5 occurred on the first or fourth day of culture. The gibberellin content decreased through the rest of the logarithmic phase of growth and increased throughout the linear phase. It declined again as callus neared maximum size. Gibberellin level increased to the second highest concentration at the end of the senescence phase.

When subcultured a different pattern was observed (Figures 12, 13, 14, Subculture 6). The gibberellin content increased in the logarithmic growth phase, decreased in the linear phase, increased again at the start of the senescence phase, and declined as the callus senesced.

This may have been due to physical changes in the callus at subculturing, or to habituation of the callus to hormones. The gibberellin content in the initial stages of subculture 6 paralleled that in the latter stages of subculture 5 in most cases suggesting that environmental conditions may have been responsible for the observed patterns.

Gibberellin content in µg/culture of subculture 5 increased as the callus senesced (Figure 15); however, for subculture 6 this pattern was not repeated. In subculture 5, it appears that gibberellin synthesis and degradation was occurring in the callus.

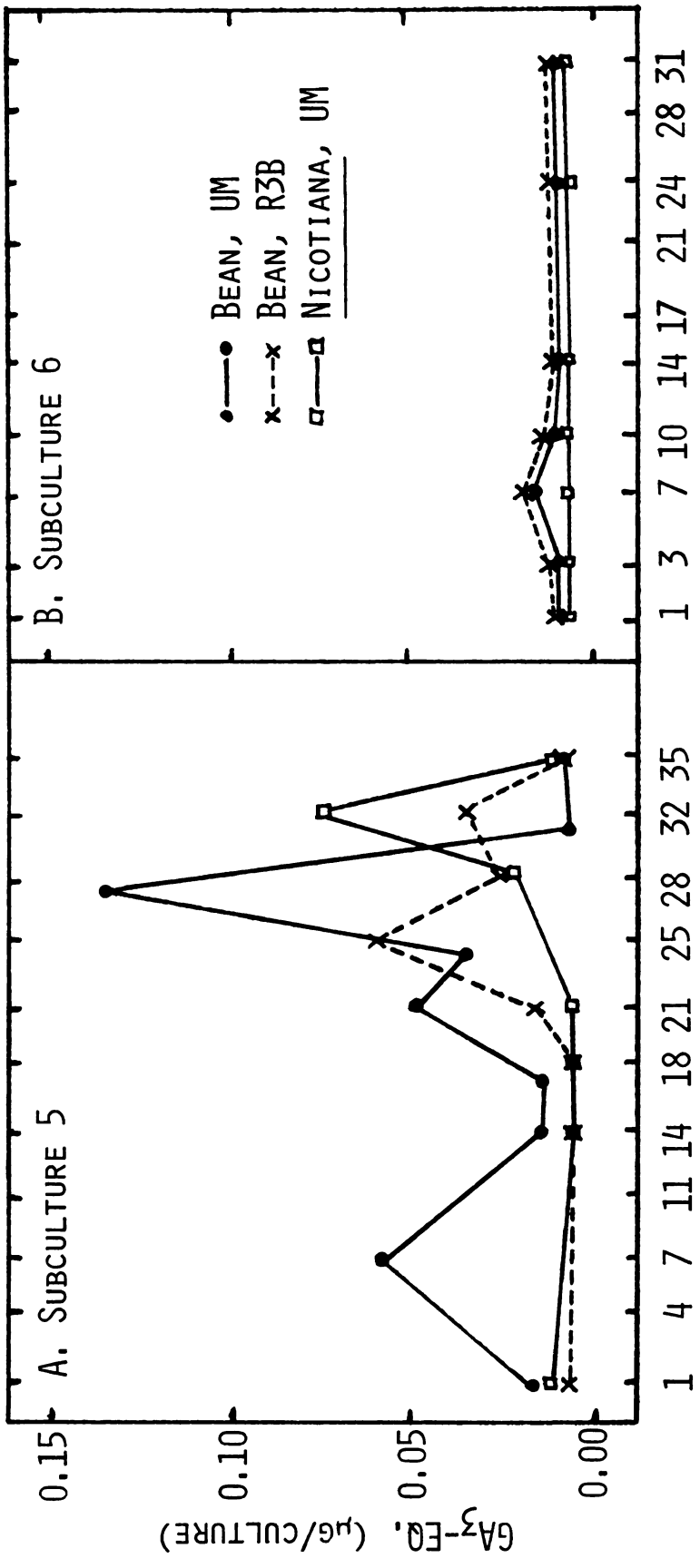

Figure 15. Gibberellin content per culture of established callus subcultures. Subculture 6 initiated from subculture 5 on day 18. GA content per culture was calculated by multiplying GA content in Figures 12, 13, 14, A. x weight of callus in B. (One ml of 0.8% agar was assumed to be equivalent to 1 g of callus.)

V. Gibberellin Content of Callus Initiated on Bean Cotyledons and in Subsequent Subculture

During initiation and first subculture callus did not reflect the increases and decreases in gibberellin content found in subculture 5 (Tables 7, 8). Light did not affect gibberellin content. Tissues grew somewhat faster in the light than in the dark, but the decline in growth began at about the same time. Thus cultures did not grow as large in the dark as in the light.

Table 7. Effect of light and medium on gibberellin content (ng/ml) of callus initiated on bean cotyledons. Cultures initiated July 29.

Assayed on (day)	Medium					
	UM		R3B		MS-D3	
	Light	Dark	Light	Dark	Light	Dark
13	5.0	4.0				
20	0.4	2.1	0.0	0.0		
25	0.1	0.52	0.0	0.0	0.0	0.0
33	*	0.3	*	0.0	*	0.1
40		0.8		0.0		0.1

*Cultures lost due to accident.

Table 8. Gibberellin content (ng/ml) of first callus subculture. Tissues subcultured September 2 or 3.

Assayed on (day)	Medium				
	UM	R3B		MS-D3	
	Dark	Light	Dark	Light	Dark
2 or 3	0.22	0.18	0.1	0..0	0.1
6 or 7	0.3	0.17	0.1	0.1	0.2
10 or 11	0.1	0.1	0.1	0.0	0.1
13 or 14	0.0	0.0	0.0	0.0	0.0
21 or 22	0.0	0.0	0.0	0.0	0.0

VI. Gibberellin Content of *Nicotiana* Callus Following Subculture on Root and Shoot Regeneration Media

Regeneration of shoots from *Nicotiana* callus did not occur during the first transfer. However, the callus changed morphologically in response to different media. Callus on shoot regeneration medium became nodular and developed isolated bright green spots. Callus on root regeneration medium became white, opaque, and firm. Callus on UM remained grayish to clear, soft, and friable. No significant change in gibberellin content accompanied these visible morphological changes in the callus (Table 9). GA activity was greater than control in only 2 cases, and did not exceed 0.2 ng/ml of tissue at any time.

Table 9. Gibberellin content (ng/ml) of *Nicotiana* callus during culture on MS-0, MS-D3, and UM media.

Assayed on (day)	Medium		
	MS-D3	MS-0	UM
3	0.0	0.1	0.0
10	0.2	0.0	0.0
17	0.0	0.0	0.0
24	0.0	0.0	0.0

DISCUSSION

In the majority of cases, gibberellins are not required components of plant tissue culture media. The addition of GA_3 generally has little or no effect on the growth of cultures. For example, bean cultures do not require gibberellins in the medium; hence if required for growth, they must be produced endogenously. In this study, both bean and Nicotiana produced very low levels of gibberellins in vitro.

The barley half-seed halo assay was adapted for measuring gibberellin levels in vitro. For callus cultures, the optimum dose:response curve was achieved when the half-seeds were incubated on callus for 24 hours, then on starch/agar plates for 72 hours. The assay was unaffected by light versus dark or a presoak in water.

Although whole bean seeds in vivo contained 0.21 µg GA_3-eq./g fresh weight, callus initiated in vitro on cotyledons contained less than 0.1 µg GA_3-eq./g. Nicotiana leaf callus also contained less than 0.1 µg GA_3-eq./g of tissue during subculture and transfer to regeneration media.

The gibberellins produced may not have been detected by the barley half-seed halo assay. Fukuyama (1971) investigated the specifity of this assay and reported that

half-seeds are primarily sensitive to C-19 gibberellins, such as are present in immature bean seeds. If the callus produced the same C-19 gibberellins, they would have been detected. The callus could have produced C-20 gibberellins or inactive gibberellins not detectable with the barley half-seed halo assay. The assay did detect GA_3 in both the medium and callus when various concentrations of GA_3 were added to UM media. The halo diameters were similar to those of standard concentrations.

The cellular site of gibberellin production in bean seeds is not fully established. Alpi, et al. (1975) suggested that the suspensor was the site of gibberellin synthesis in embryos of Phaseolus coccineus. In the experiments described here, immature cotyledons with the proximal end removed were used as explants. Thus, although the cells of immature cotyledons contained gibberellins in vivo, they may not have had the ability to produce them in vitro.

The fluctuation in gibberellin content observed in callus may indicate that synthesis and degradation both occur in vitro. If this were the case, gibberellin content as measured at any one time might not indicate the total produced by the callus.

The bean callus cultures in these experiments did not contain enough gibberellins to serve as an alternative source of these compounds. However, further investigation of plant tissues in vitro may provide such a source.

Callus cultures may be induced to synthesize high quantities of gibberellins when subcultured on media containing gibberellin precursors. Cell suspension cultures may exude high levels of gibberellins into the liquid culture medium. Investigation of these and other systems *in vitro* may yield an economically feasible system for producing large quantities of "rare" gibberellins.

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