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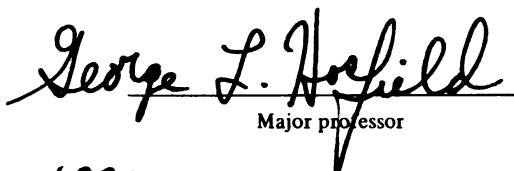
SOMATIC EMBRYOGENESIS IN COMMON BEAN (PHASEOLUS VULGARIS
L.): INFLUENCE OF MEDIA AND ENVIRONMENTAL FACTORS ON
GLOBULAR AND MATURE EMBRYOID FORMATION

presented by

Rodrigo A. Hoyos

has been accepted towards fulfillment
of the requirements for

MS degree in Crop and Soil Science


Major professor

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**SOMATIC EMBRYOGENESIS IN COMMON BEAN (PHASEOLUS VULGARIS
L.): INFLUENCE OF MEDIA AND ENVIRONMENTAL FACTORS ON
GLOBULAR AND MATURE EMBRYOID FORMATION**

By

Rodrigo A. Hoyos

A THESIS

**Submitted to
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ABSTRACT

SOMATIC EMBRYOGENESIS IN COMMON BEAN (PHASEOLUS VULGARIS L.): INFLUENCE OF MEDIA AND ENVIRONMENTAL FACTORS ON GLOBULAR AND MATURE EMBRYOID FORMATION

By

Rodrigo A. Hoyos

Opaque globules formed on the surface of primary and secondary callus of 15 bean (Phaseolus vulgaris L.) breeding lines, when primary leaf explants were cultured on B5 induction medium (IM) containing 10 to 30 mg/l 2,4-D.

Subsequently, calli with globules produced structures reminiscent of somatic embryos (embryoids) after subculture in liquid challenge medium (LCM). Calli maintained on IM for 2,3,4, and 5 weeks produced 26 to 34 embryoids/callus in LCM and calli maintained on IM for one week produced 12/callus. Well developed embryoids only occurred after primary calli were subcultured in liquid B5 medium with 0.1 to 1.0 mg/l IBA. Calli subcultured in LCM with 10 mg/l or higher IBA turned necrotic and died. Embryoids produced in LCM with 2,4-D and NAA (0.1 to 1.0 mg/l) proliferated roots and formed "frosty" appearing structures, respectively. No differences were detected in number or quality of embryoids produced in LCM from callus cultured on IM in continuous light or darkness regardless of the induction time. Even though no whole plants were regenerated, polar somatic

Rodrigo A. Hoyos.

embryoids with provascular tissues were obtained.

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TABLE OF CONTENTS

	Page
LIST OF TABLES.	vii
LIST OF FIGURES	viii
INTRODUCTION.	1
REVIEW OF LITERATURE	6
General aspects.	6
Legumes.	7
Common bean culture <u>in vitro</u>	8
Tissue type.	9
Genetic variability.	9
HORMONAL REQUIREMENTS.	10
Auxins	10
Cytokinins	10
Others	11
MORPHOGENESIS.	13
SOMATIC EMBRYOGENESIS	14
MATERIALS AND METHODS	16
Plant material	16
Experiment one	16
Induction medium (IM).	16
Response evaluation system	21
Liquid challenge medium.	21
Test of simple light effect.	23
Evaluation of somatic embryoids.	23
Experiment two	24
Benzylaminopurine (BAP) and coconut milk	24
Histology.	24
Statistical analysis	25
RESULTS	26
SCREENING OF ACCESSIONS.	26

CHARACTERIZATION OF 'JAMAPA' <u>In Vitro</u> CULTURE. . . .	27
Secondary globule formation.	27
Histology.	27
Light, culture time, and media concentration effects on 'Jamapa' callus cultured <u>in vitro</u>	33
Time on IM medium effect.	33
EMBRYO DEVELOP ON LCM.	37
Type and concentration of auxin in the liquid challenge medium (LCM)	37
Auxin type	37
Auxin concentration.	42
Time in the LCM.	46
Histology.	50
DISCUSSION	54
Further <u>in vitro</u> studies with dry beans	60
APPENDIX.	62
BIBLIOGRAPHY.	68

LIST OF TABLES

TABLE		Page
1	Bean accessions screened for globule development in B5 medium with 15 mg/l of 2,4-D and 2% sucrose	17
2	Composition of Gamborg's medium used in the experiments	20
3	Major growth regulators addenda used in the semisolid induction and liquid challenge media.	22
4	Analysis of variance table for somatic embryogenesis in liquid challenge medium (LCM)	34
5	Number of embryoids produced from leaf explants in response to time of callus culture on induction medium and time in the IBA liquid challenge medium.	35
6	Effect of time on induction medium on somatic embryoid formation	36
7	Mean number of somatic embryoids produced from callus of primary leaf explants formed in different IBA concentrations	45
8	Effect of BAP and IBA concentrations on embryoid formation in a B5 liquid medium	50

LIST OF FIGURES

FIGURE		Page
1	A. Propagation of bean seedlings under aseptic conditions. B. Leaf stage used for experiments.	18
2	Meristematic tissue. Globular structure at the surface of calli from primary leafexplants after 20 days induced on B5 medium supplemented with 15 mg/l 2,4-D.	28
3	Secondary globules (arrows) produced after transferring to fresh induction medium.	30
4	Meristematic cells with densely staining cytoplasm	31
5	Diagram of experiments realized to optimize environmental and hormonal response on somatic embryogenesis.	38
6	Morphogenesis of embryoids after 15 - 20 days in a challenge liquid medium containing 0.1 to 1.0 mg/l IBA and induced on a B5 medium.	39
7	Effect of 2,4-D and NAA in the Liquid Challenge Medium after one week in culture	43
8	Days of culture in Liquid Challenge Medium containing IBA.	47
9	Bipolar somatic embryoid	49
10	Effect of coconut milk used as a natural source of cytokinins on the development of somatic embryoids.	50

INTRODUCTION

Grain legumes are an important staple food in many countries of the world because they supply significant protein, carbohydrates and other nutrients to the diet. Since remote times humans have sought to improve the productivity of grain legumes and make them adapted to new agricultural areas. In this sense, modification through plant breeding has led to the development of new and improved genetic stocks of grain legumes. Currently, technologies such as in vitro propagation, somatic embryogenesis, and organogenesis have been used to complement plant breeding in the grain legumes.

Tissue culture techniques complement conventional plant breeding systems in several ways (Simmons, 1976). Accordingly, embryo culture has been used to make successful wide hybrids between distantly related species (CIAT, 1988). Meristem and shoot-tip propagation is useful for the development of virus-free clonal stocks, the rapid multiplication of clones and the long term maintenance of clones in vitro (Berbee et al., 1973). Lastly, unorganized cell and tissue cultures may be used for clonal propagation through somatic embryogenesis, and variability that exists in these cultures can be exploited and tested in plant improvement schemes.

Such programs might include cell line selection, mutant induction, the formation of cybrids and somatic hybrids and the stimulus of secondary product synthesis (Bajaj, 1977).

The potential offered by in vitro techniques for plant improvement is utilized to its fullest when new and fertile sexual reproducing plants are regenerated from the various culture systems. Embryogenesis and organogenesis are the two main systems by which new plants are regenerated. Moreover, genetic manipulation can be applied to these systems to alter genomic expression. The fundamental problems of plant regeneration and genetic stability are linked since both are strongly influenced by the level of organization within the culture system employed. Murashige (1974) discussed three steps necessary to successfully utilize tissue culture and the factors that in some way interact to obtain an acceptable cellular organization level. Step one is simply to attain aseptic tissue growth of the desired plant. Hence, the explant and genotype must be considered. Step two is a rapid increase of growth and development of other structures which can ultimately give rise to plants. In step three, preparation for reestablishment of the plant in soil has to be achieved. Nutrient medium characteristics and culture environment qualities need to be considered for each step.

The stages of somatic embryogenesis are similar to sexual embryogenesis in the development of a plant: globu-

lar, heart, and torpedo shape structures rapidly form after fertilization and finally the mature embryo is formed (Evans et al., 1989). In somatic embryogenesis, each stage of development has its own characteristics and requirements (Christou and Yang, 1989). The globular stage in somatic embryogenesis may be considered as an initiation process due to the fact that cellular differentiation has already begun (Williams and Maheswaran, 1986).

Various strategies have been used to sustain somatic embryogenesis in crop species past the globular stage. These strategies have required the transfer of the globular structures from callus initiated on an induction medium to a liquid maturation medium without auxin or a weak or strong auxin at various concentrations. By following such procedures, various investigators have obtained successful somatic embryogenesis in crops (Christou and Yang, 1989; Szabados et al., 1987; Kumar et al., 1988). Environmental factors such as light and temperature have also been found to be major contributous toward the initiation of embryogenesis (Seibert et al., 1975; Bhattacharya and Sen, 1980).

Light was important for somatic embryogenesis in Manihot esculenta. Crantz (cassava) by enhancing embryoid formation (Szabados et al., 1986). In Brassica campestris, callus induction and maintenance was relatively effective when cultures were kept at 25 ± 1 °C under continuous light ($18.0 \mu\text{Mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), and embryogenesis occurred at 22 ± 1 °C

under a 16 hour photoperiod of $30.0 \mu\text{Mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ illumination (Bhattacharya and Sen, 1980).

Grain legumes are recalcitrant among dicotyledons for in vitro regeneration from somatic tissues, and common bean (Phaseolus vulgaris L.), has been one of the most difficult species to regenerate (Allavena, 1983). However, globular and embryo-like structures have been observed infrequently for common bean when leaf and cotyledonary pieces were cultured on a semi-solid induction media followed by culture in a liquid media (Allavena, 1983; Martin and Sondahl, 1984). Globular structures formed more readily when high (30 mg/l) 2,4-D concentrations were a component of the induction medium (Saunders et al., 1987). The globules in common bean that formed in the presence of high auxin concentration were considered to be aberrant somatic embryos (Saunders et al., 1987). This conclusion suggested that the time callus remains on a medium with a high auxin concentration may affect embryogenesis because the explant-derived cells may express a carryover effect due to residual auxin. Auxin carryover was considered by Parrott et al. (1988) as one of the factors correlated with inefficient conversion of embryos into plants in soybean.

The present study was initiated to study the possible carry over effect that a high-auxin induction medium may have on embryogenesis after callus is transferred to a second and liquid medium.

Specific objectives of the research were to: a) Characterize the response of common bean to various 2,4-D concentrations in the induction medium on the subsequent formation of globules in the LCM and b) determine the effects of light intensity and duration and, length of time on induction medium, type and concentration of auxin and their interactions on somatic embryo development from globules in LCM, and c) ascertain genetic variability for globule and somatic embryo development stages with a stand two media sequence.

REVIEW OF LITERATURE

General aspects. Embryogenesis is the formation and development of an embryo from an egg cell. In nature, embryogenesis results after fertilization of an egg by a male gamete. In sexual embryogenesis, Fossard (1976) described a lag period followed by mitotic divisions of the zygote. At first, a ball of cells forms, these quickly develop into a heart-shaped structure and finally a torpedo-shaped embryo. Final mitotic divisions give rise to well developed cotyledon(s), a minute plumule and a radicle. Embryogenesis can also arise when cells of somatic tissues follow a developmental pathway similar to sexual embryogenesis. In somatic embryogenesis, the cells go through all the stages as for sexual embryogenesis, namely globular, heart, and torpedo (Evans et al., 1984).

Plant cells have the potential to differentiate upon cell division and develop organs and complete plants (Gamborg and Shyluk, 1981). This developmental phenomenon is called totipotency. When totipotency occurs, the cells involved undergo dedifferentiation, lose their specialization in the organism, and proliferate by cell division to form a mass of undifferentiated cells (callus). Callus

formed in response to an appropriate stimulus can differentiate again to form either the same or a different cell type.

Tisserat et al. (1979) reported somatic embryogenesis in 32 families, 81 genera, and 132 plant species. In each case Murishige and Skoog (MS) medium modified with a high auxin concentration was used most frequently to induce callus. The preferred auxin was 2,4-D. Of the embryogenic crop species in which 2,4-D was used in the primary culture medium, 12.5% of the reports indicated that somatic embryogenesis on callus transferred to a secondary medium with a reduced level of 2,4-D. For the remaining species, somatic embryogenesis on callus were transferred to a secondary culture medium lacking of 2,4-D. In each species the pattern of development was similar to that reported for the Gramineae (Evans and Sharp, 1981). The secondary medium (maturation medium) contained a weak auxin (41.6%), only a cytokinin (33.3%), or no growth regulators (16.6%). Flick et al., (1983) reported that a few legume species have been regenerated in vitro, but in most cases regeneration is at low frequency or limited by the source of explant.

Legumes. Many species of the Leguminosae are particularly difficult to propagate in vitro. The notable successes have been with the forage legumes. In alfalfa, (Medicago sativa L.), Walker et al. (1979) demonstrated the quantitative con-

trol of root and shoot organogenesis by growth regulators of the auxin and cytokinin type and by basal media. Kao and Michailuk (1981) were able to regenerate plants from mesophyll protoplasts of alfalfa and red clover plants (Trifolium pratense L.). Phillips and Collins (1980) successfully regenerated soybean plants from callus derived from cell suspension cultures.

For seed legumes, plant regeneration via somatic embryogenesis in soybean (Glycine max) and pea (Pisum sativum) demonstrated that embryogenesis is not a phenomenon restricted to just a few Leguminosae taxa. In soybean, Ranch et al. (1986) used the liquid medium of Tilton and Russell (1984) supplemented with BA and activated charcoal, for maturation of somatic embryos. The embryos germinated on B5 or MS medium supplemented with 0.6 μ M IBA. Hammat and Davey (1987) induced germination by desiccating soybean somatic embryos in sterile Petri dishes until they had reached 40-50% of their original size. Prior to desiccation, the somatic embryos had been matured for one month on B5 medium with 0.1 mg/l IBA.

Common bean culture in vitro. Attempts to develop an effective, in vitro system for dedifferentiation of bean somatic tissues, are reported in the literature which dates back about 30 years beginning with the investigation of Jeffs and Northcote on cell suspensions (1967). Since then, studies

have been published about callus culture and cell suspension (Liau and Boll, 1970; Allavena and Rossetti, 1983; Martins and Sondahl, 1984), effects of different hormone types and concentration on callus and somatic embryoid development (Crocomo et al., 1976; Mok and Mok, 1977; Saunders et al., 1987), and the use of organic compounds such as coconut milk and 'aguamiel' (Agave sap) (Lopez et al., 1978). Along with the various investigations mentioned above, researchers have also found some influence of explant source and genotype on the success of bean tissue culture in vitro (Haddon and Northcote, 1976; Mok and Mok, 1977; Martins and Sondahl, 1984)

Tissue type. Various types of tissue, namely, primary leaves, immature embryos (Allavena and Rossetti, 1983), specific areas of mature cotyledons and primary leaves, the distal one-third portion of primary leaves without the midvein, (Saunders et al., 1987), shoot apex, leaf, petiole, epicotyl, cotyledon, hypocotyl pith, inner hypocotyl, whole hypocotyl, outer hypocotyl, root stele, whole root, root cortex, and anthers (Haddon and Northcote, 1976) have all been used in attempts to regenerate plants of common bean.

Genetic variability. Genetic variability of common bean has been observed in response to callus formation (Mok and Mok, 1977), and proembryo formation (Martins and Sondahl, 1984).

Hormonal requirements

Auxins. Responses of common bean genotypes to auxin effects were examined by Mok and Mok (1977), and were useful in defining genotypic variation. Since the differential responses of bean callus tissues to different types of auxin such as picloram, 2,4-D, NAA, IAA as determined by optimal growth, apparent inhibition of callus formation seemed large enough to characterize individual genotypes. The concentration of 2,4-D required for the development and further growth of callus tissue from bean hypocotyl tissue depended upon genotype of plant from which the explants were taken. The growth regulator, 2,4-D or picloram, was required for the callus growth of the bean varieties examined. In lima beans (*P. lunatus* L. cv. Kinston) callus near optimum growth occurred at 4.4 mg/l 2,4-D, whereas, Lima bean callus, PI. 194314, was inhibited at this concentration. There was also an eight-fold difference in the auxin requirement for callus growth of common bean Romana and two other bean genotypes (Mok and Mok, 1977).

Cytokinins. Mok et al., (1982) reported similarities among some bean genotypes in respect to their response to cytokinin. The activity of N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (thidiazuron), a synthetic plant growth regulator, was approximately equal to that of zeatin in promoting the growth

of callus. In lima bean 'Jackson Wonder' callus showed cytokinin autonomy after treatment with either substance, whereas, lima bean, PI. 260415, remained cytokinin dependent under all conditions tested.

Others. GA_3 and ABA were tested in bean morphogenesis (root initiation and vascular nodules) studies in vitro on callus obtained from hypocotyl of P. vulgaris var. Canadian Wonder (Haddon and Northcote, 1976). ABA at concentrations greater than 1 μM inhibited not only tissue differentiation (root initiation) but also cell differentiation (vascular nodules). The inhibition was not overcome by the addition of GA_3 . In contrast, root initiation was stimulated by GA_3 (0.1 - 45 μM) at a 5:1 auxin : kinetin ratio (6 μM of NAA and 1 μM kinetin). Concentrations at this ratio did not normally induce differentiation. ABA at concentrations of 0.1 - 30 μM also stimulated root formation in the absence of kinetin. The ABA inhibition was effective even in the presence of gibberellic acid at concentrations of 30 or 45 μM .

In addition to hormones, vitamins and other compounds have been used to induce morphogenesis in common bean.

A number of different amino acids and amides have been found advantageous in some tissue cultures (Murashige, 1974). Gamborg et al. (1968) found that the addition of 3 to 8 Mm glutamine was beneficial for the growth of soybean

suspension cultures. This compound also has been useful in the culture of immature embryos from interspecific hybrids between Phaseolus vulgaris and P. acutifolius (Mok, et al., 1978)

Tonin et al. (1981) studied the effect of amino acids or amino acid mixtures on the stimulation or inhibition of root growth and morphogenesis in tissue cultures of common bean leaf explants. The amino acids studied were arginine, aspartic acid, cysteine, glutamic acid, histidine, isoleucine, methionine, phenylalanine, proline, threonine, tyrosine, and serine. Root morphogenesis was promoted by a modified MS supplemented with 1.0 mg/l KIN, 5.0 mg/L IAA, and three amino acids: arginine (60.0 mg/l), aspartic acid (50.0 mg/l), and cysteine (10 mg/l). However, when the same medium was complemented with another group of amino acids: glutamic acid (65 mg/l), glycine (25 mg/l), and histidine (10 mg/L), an inhibitory effect on both growth and morphogenesis was observed. On the other hand, a low concentration of glutamic acid (0.5 mg/) was promotive of growth, while higher concentrations were inhibitory.

The effect of caffeine and nicotine on callus growth and root morphogenesis of the dry bean, Bico de Ouro, was studied by Peters et al., 1976. Along with nicotine (1-200 mg/l), a modified BM (Veliky and Martin, (1970) plus IAA 11.5 μ M and KIN 1.0 μ M; BM minus KIN; BM minus KIN and IAA were tested. Distinct morphogenetic responses were observed

on the different media used. On BM, the nicotine effect inhibited root induction. On BM minus KIN, nicotine did not produce callus growth, but was associated with root induction. In the absence of both IAA and KIN, nicotine did not promote callus growth or root formation.

Jeffs and Northcote (1967) explored the biological role of various monosaccharides, disaccharides, trisaccharides and sugar derivatives instead of sucrose on the induction process. They only found areas of differentiation in calli induced on 2,4-D carrying wedges containing IAA, sucrose or other glucosyl disaccharides such as trehalose and maltose.

Morphogenesis

Root and vascular nodule formation has been induced on bean callus obtained on a defined B5 medium with 2,4-D and 20 g/l sucrose after transferring to an induction medium without 2,4-D, but with NAA, kinetin and 30 mg/l sucrose (Haddon and Northcote, 1975).

Root morphogenesis in common bean has also been noted by Liao and Boll, 1970 and Olieman-van der Mer et al., 1971 who studied adventitious root formation from cultivated epicotyls and found sugar, auxin and light to be important limiting factors. Liao and Boll (1970) inhibited root morphogenesis by changing the inorganic fraction of the nutrients, but found no inhibition by manipulation of the organic

fraction.

Saunders et al. (1987) reported control of root morphogenesis in common bean using a wide range of 2,4-D concentrations. Explants rooted more frequently at lower 2,4-D concentrations and a type of globular structure was produced on primary leaf and distal cotyledon explants of pinto bean cv UI 114 using high 2,4-D concentrations.

Shoot morphogenesis in *P. vulgaris* was reported by Crocomo et al., (1976). These authors grew sections of bean leaves on Veliky and Martin's, 1970 salts and vitamins medium plus 10 g/l agar, 2 mg/l IAA, 1 mg/l NAA. and 0.2 mg/l KIN. Extract from bean seeds soaked for two hours in tap water prior to homogenation was added to the medium at a concentration of one-fourth seed/ml. Two plantlets, which developed a root system, were induced in a group of 9 bean cultures.

Somatic embryogenesis

Most somatic embryogenesis studies in beans conducted in the past 10 years used a two step culture approach. First, a semisolid induction medium supplemented with high auxin concentration is used. The second step is a liquid or semisolid medium with no or very low auxin concentration. Only a small number of reports in bean are available on embryoid formation from somatic tissue cultured in vitro

(Allavena and Rossetti, 1983; Schieren and Jacobsen, 1986; Saunders et al. 1987; Martins and Sondahl, 1984)

Allavena and Rossetti (1983) observed somatic embryogenesis when young primary leaves from 8-day-old seedlings were used in the experiments. Explants were placed on B5 basal medium plus 18 μM 2,4-D. After 20 days, calli were transferred to 10 ml of liquid medium and placed on a gyratory shaker. The liquid medium consisted of B5 salts, sugar, and vitamins plus the following growth regulators: 0.53 μM NAA, 2.3 μM KIN, 0.29 μM GA₃, and 0.11 μM ABA. Cultures were maintained at 16/8 hour photoperiod with low light intensity at 25 ± 0.5 °C. After 10 to 12 days of culture on the liquid medium a few embryos developed, but no mature plants were obtained.

Martins and Sondahl (1984) reported formation of the early stages of somatic embryogenesis in liquid media from callus induced on shoot apices.

Schieren and Jacobsen (1986) reported the formation of some embryo-like structures in a liquid RB medium (Breuer, 1985) supplemented with 4 mg/l BA and 0.1 mg/l IBA. The initial explants (surface sterilized leaf segments) were ached on RB solid medium with different concentrations of Picloram (0.1, 0.19, 0.38, 1.0 mg/L) instead of 2,4-D.

MATERIALS AND METHODS

Plant material. Initially, several hundred *P. vulgaris* ($2n = 24$) accessions, maintained by the USDA dry bean genetics program, were evaluated to ascertain their ability to produce meristematic tissue from primary leaf explants in vitro. Fifteen of the best responders were chosen for this study (Table 1). Plants to provide explants were grown from seeds that were surface-sterilized in 70% EtOH for 15 to 30 s, and immediately soaked (15 to 20 min) in a 15% Clorox solution. Seeds were rinsed three to four times in sterile water before germinating on sterile cotton in 0.25 l jars (Fig. 1A) under continuous light from cool white fluorescent bulbs ($19 \mu\text{En}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 28 ± 2 °C. Leaf discs 5 mm in diameter were cut with a cork borer from the leaf lamina of primary leaves before they were fully expanded and used as the explants (Fig. 1B). Explants were usually taken from seven day-old plants.

Experiment one

Induction medium (IM). The five millimeter explants, selected at random, were placed in 100 x 20 mm sterile Petri dishes containing approximately 30 ml of B5 induction medium

Table 1. Bean accessions screened for globule development in B5 medium with 15 mg/l 2,4-D and 2% sucrose.

Accession	Source*	Testa color**	Callus fresh weight (g)	Explants that developed globules (%)
Ruddy	Cornell	Lt Rd	0.42	43.8
Mexico 12-1	CIAT	Yw	0.40	12.5
Carioca	Brazil	Bg-Mottled	0.44	25.0
Black magic	MSU	Bl	0.26	18.8
Jalpatagua-72	Guatemala	Bl	0.33	50.0
San Fernando	Costa Rica	Bl	0.15	18.8
Jamapa	CIAT	Bl	0.38	47.0
Othello	USDA-WA	Bg-Mottled	0.42	46.5
Aurora	Cornell	Wh	0.34	18.8
Calima	CIAT	Rd-Mottled	0.31	25.0
Bat 38	CIAT	Rd	0.43	31.3
Bat 1507	CIAT	Rd	0.19	12.5
Bat 1654	CIAT	Rd	0.26	12.5
Bac-95	CIAT	Rd	0.35	6.3
A-5	CIAT	Rd	0.32	31.3

* CIAT, MSU, USDA-WA = Centro Internacional de Agricultura Tropical, Cali, Colombia; Michigan State University; USDA, Prosser, Wash., respectively.
 ** Bg, Rd, Yw, Bl, Wh, Lt Rd = beige, red, yellow, black, white, and light red, resp actively

Figure 1. A) Propagation of bean seedlings under aseptic conditions in a 0.25 l jar

B) Leaf stage used for explants, note that leaves are still unfolded

A



B



Table 2. Composition of Gamborg's medium used in the experiments.

Compound	Concentration	
	(mg/l)	Molecular equivalents
NaH_2PO_4	150.0	1.05 mmol
KNO_3	2500.0	24.7 "
$(\text{NH}_4)_2\text{SO}_4$	134.0	1.01 "
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	250.0	1.01 "
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	150.0	1.02 "
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	10.0	59.2 μmol
H_3BO_3	3.0	48.5 "
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	2.0	6.96 "
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	1.03 "
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025	0.108 "
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025	1.05 "
KI	0.75	4.52 "
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8	100.0 "
$\text{Na}_2 \cdot \text{EDTA} \cdot 2\text{H}_2\text{O}$	37.3	100.0 "
Sucrose	20.0*	58.4 mmol
Myo-Inositol	100.0	555.0 μmol
Nicotinic acid	1.0	8.12 "
Thiamine·HCL	10.0	29.6 "
Pyridoxine·HCL	1.0	4.86 "
Agar	8.0*	8.0

* Values are grams

(IM) described by Gamborg et al. (1968) to form callus (Table 2). The induction medium contained 0.8% Difco Bacto-agar, 2% sucrose, and was supplemented with 15 mg/l 2,4-D (Table 3). The pH was adjusted to 5.8 with 1.0 to 5.0 N KOH or 0.1 to 1.0 N HCl and the media sterilized for 20 min at 15 p.s.i. (121 C).

Response evaluation system. Responses were evaluated for the stimulation of embryogenic tissue on IM following the features described by Vasil, et al. (1984); and Buchheim, et al. (1989). The description of embryogenic callus consisted of compact, organized appearing tissue, opaque or white to pale yellow in color.

'Jamapa' was selected from the 15 accessions to continue experiments on embryoid development. Intact single callus (200-400 mg) from 'Jamapa' explants cultured on the semisolid IM were taken at random and transferred sequentially each week for six weeks to 125 ml Erlenmeyer flasks containing 50 ml of LCM. The LCM cultures were placed on a reciprocating incubator at 120 rpm.

Liquid challenge medium. Embryogenic callus was challenged to produce somatic embryos in a liquid medium (LCM) containing B5 salts (Gamborg et al., 1968) with 3% sucrose and selected growth regulators. The somatic embryoid was defined by presenting two defined ends; root and cotyledon

Table 3. Major growth regulators addenda used in the semi-solid induction and liquid challenge media.

Medium and component	Concentrations tested (mg/l)
<u>Induction Medium (IM)</u>	
2,4 Dichlorophenoxyacetic acid (2,4-D)	15
<u>Challenge Liquid Medium (CLM)</u>	
2,4 Dichlorophenoxyacetic acid (2,4-D)	0.01 - 100.0
Naphthaleneacetic acid (NAA)	0.01 - 100.0
Indolebutyric acid (IBA)	0.01 - 100.0
Benzylaminopurine (BAP)	0.01 - 1.0
Coconut milk	0.02 - 20.0 *

* % v/v

like end.

Growth regulators NAA, 2,4-D, or IBA were added to the LCM in concentrations of 0.0, 0.01, 0.1, 1.0, 10.0, 100.0 mg/L (Table 3). The pH of the LCM was adjusted to 5.8 with 1.0 to 5.0 N KOH or 0.1 to 1.0 N HCl and sterilized for 20 min at 15 p.s.i. (121 °C).

Test of simple light effect. Leaf explants were maintained two different controlled environments: constant light and complete darkness. A culture room was set-up in continuous light at $10-66 \mu\text{M m}^{-2}\cdot\text{s}^{-1}$ at 28 ± 2 °C. The continuous dark environment was maintained in a incubator with a temperature of 28 ± 2 °C.

Evaluation of somatic embryoids. Evaluations were made on the small, green globular protuberances that formed on the calli surface from the 'Jamapa' explants. The number and type of differentiated structures that developed from globules was determined visually. Evaluations were made every 5 days for 20 days over the same samples counting them looking through the bottom of Erlenmeyer divided in equal sectors (nondestructive evaluation). Differentiated structures with a green apex and yellow color in the radicle area were examined with a light microscope.

Experiment two

Benzylaminopurine (BAP) and coconut milk. Based on preliminary results of time on IM, type and auxin concentration and the time in the LCM, the following standard time sequence was selected: 3 weeks in the IM with continuous light, and 15 days in LCM to study the effect of cytokinins on embryoid formation. IBA, along with the two sources of cytokin was used in the LCM in concentrations of 0.01, 0.1, 1.0 and 10.0 mg/l. The 100.0 mg/l concentration had a deleterious effect on the callus and prevented embryoid formation. This IBA concentration was not used further in experiments. Concentrations of 0.0, 0.1, 0.3, 1.0 mg/l BAP and 0.02, 0.2, 2.0 and 20% v/v coconut milk were used (Table 3).

Histology. The differentiated structures produced from bean leaf callus were fixed for at least 20 hours in FAA formalin, acetic acid, and 70% ethanol (90:5:5 v/v/v). The structures were dehydrated by passage through a tertiary butyl alcohol (TBA, 2-methylpropan-2-ol) series (Sass, 1958) and embedded in paraffin (Paraplast). Sections of the wax-embedded specimens 8 μ m thick were cut with a microtome. The sections were fixed to a glass slide with Haupt's adhesive (appendix II), where they were again encouraged to expand by the addition of a 3% v/v formalin solution. Sections were stained in Harris' Hematoxylin. Slides were

made permanent using Canada Balsam and viewed under a light microscope (Zeiss, Germany).

Statistical analysis:

For the first experiment, a split plot arrangement of treatments was arranged in a completely randomized design. A factorial arrangement of the three factors (time on IM, light duration on IM, and auxin concentration in LCM) comprised the whole plot and the sub-plot (split) was the time globules remained in the LCM.

For the second experiment, only the description of cultures and tables of means are presented.

RESULTS

Screening of Accessions

Callus production. Leaf disks from the 15 dry bean accessions produced translucent or friable callus after 30-35 days in culture on IM containing 15 mg/l 2,4-D. Callus arose from the adaxial side of the leaf explants. The abaxial side was touching the medium. Many calli had several smooth green or translucent globular structures up to 4 mm in diameter that resembled the globules reported by Saunders et al (1987). There was variability among the accessions for the number of explants that produced calli containing the globules; the range was from 6.3% for Bac-95 to 50% for 'Jalpatagua 72' (Table 1). 'Ruddy' and 'Jamapa' were similar (44 and 47% respectively) to 'Jalpatagua' for the percentage of explants that produced callus with globules. On the other hand, 'Mexico-12', Bat 1507, and Bat 1654 had similar but low percentages of explants producing callus containing globules; 12.5, 12.5, 12.5 %, respectively.

Callus with embryogenic potential from the accession 'Jamapa' was selected for further study.

Characterization of 'Jamapa' in vitro Culture

Secondary globule formation. When pieces of callus from 'Jamapa' containing globules (Fig. 2) were transferred to fresh IM, secondary globules emanated from the primary globules. (Fig. 3). However, when the secondary and primary globules were transferred to a secondary solid maturation media (MM) selected because of its efficacy to regenerate soybean (Christou and Yang, 1989; Hammatt and Davey, 1987; Buchheim et al. 1989), peas (Rubluo et al. 1984) and tepary beans (Kumar et al. 1988), no shoots or buds developed.

Histology. Histological analysis of thin sections of the globules produced on callus derived from 'Jamapa' were examined under a light microscope (100-400x) and revealed that the green area at the distal part of the globule consisted of a group of tightly aggregated cells with little organization (Figs. 4B and C). In addition, calli had some green areas not only at the distal part of the globules, but also in the callus. Meristematic areas also were observed. (Fig. 4A).

Figure 2. Meristematic tissue. Globular structures at the surface of calli from primary leaf explants after 20 days induced on B5 medium supplemented with 15 mg/l 2,4-D.

A



B

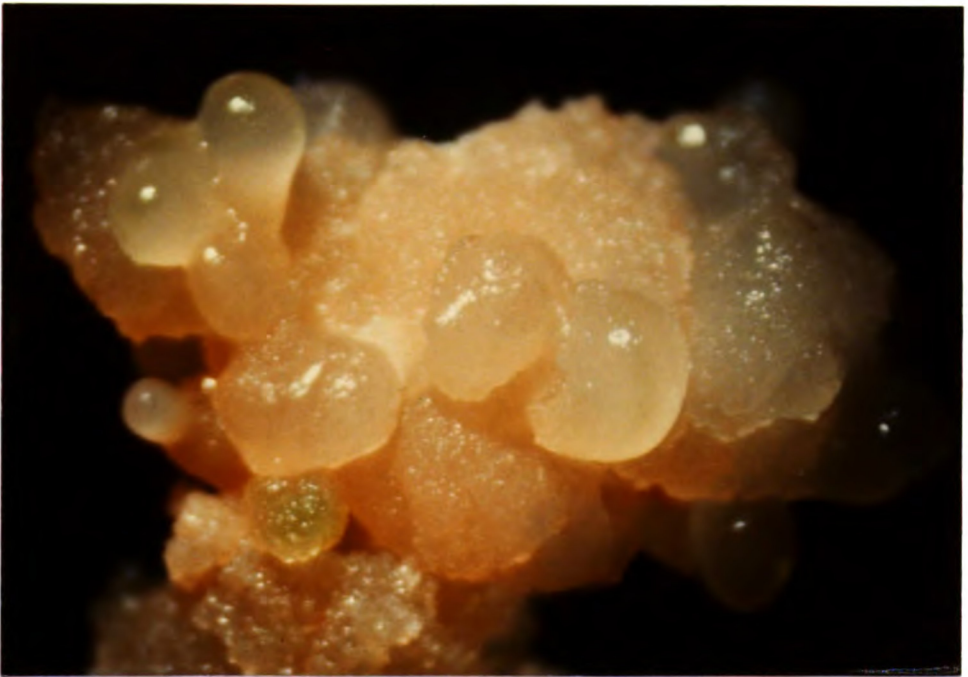


Figure 3. Secondary globules (arrows) produced after transferring to fresh induction medium

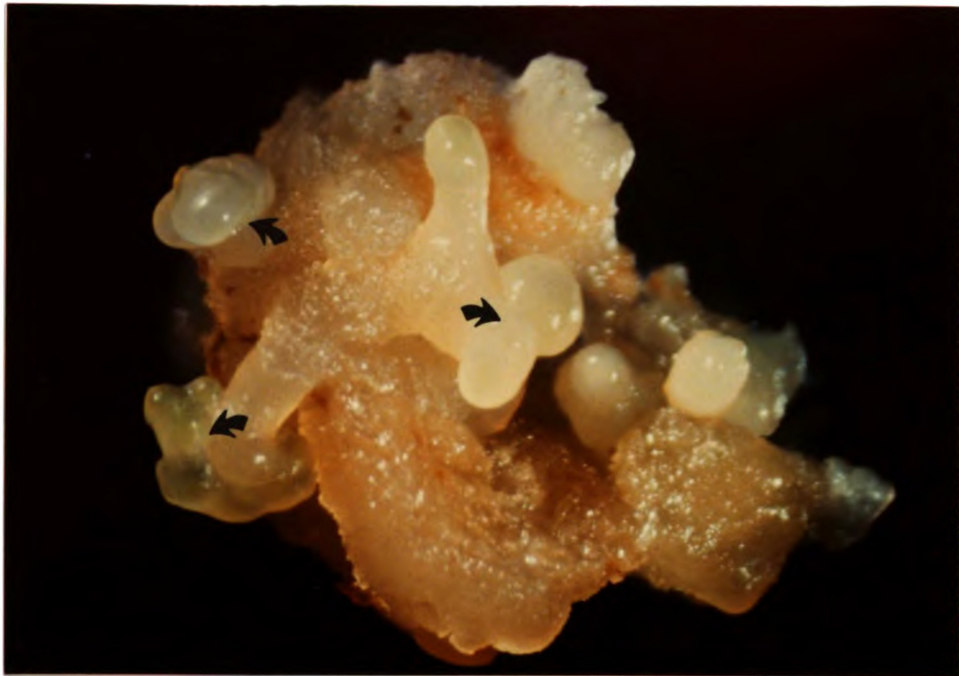
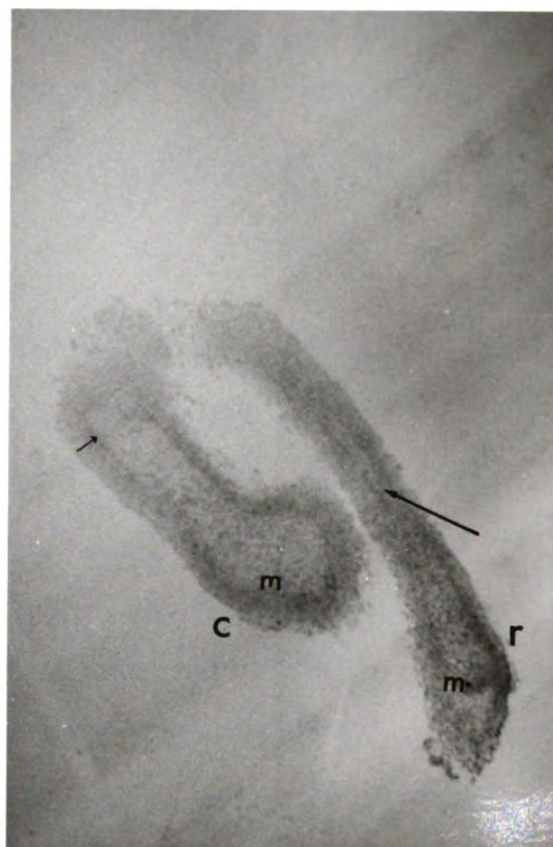
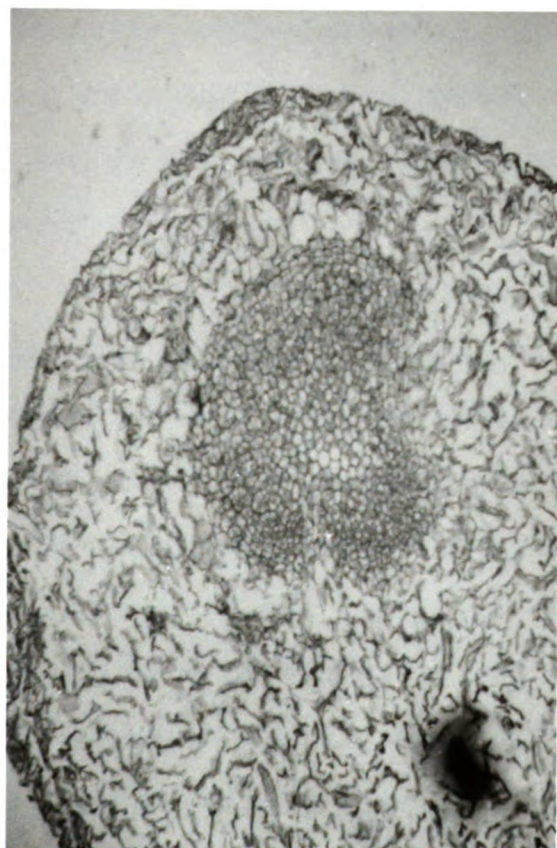
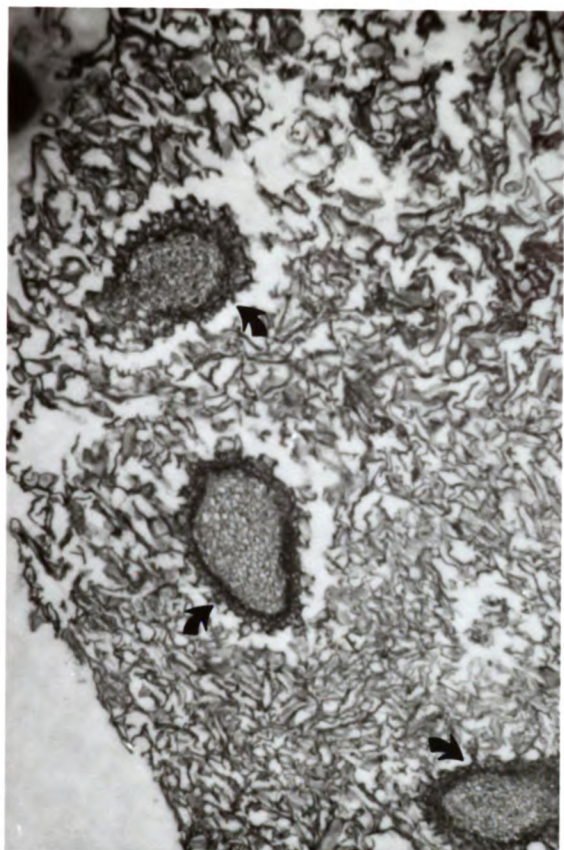


Figure 4. Meristematic cells with densely staining cytoplasm.

- A. Fifteen day old-callus from induction medium supplemented with 15 mg/l 2,4-D.**
- B.C High cell division near the polar region of a globule.**
- D. Thin section of the embryoid showing tissue differentiation, R= root end; C= apex end. Note the dark staining area (m) indicating high mitotic division of meristematic cells and the provascular tissue (arrows)**



Light, culture time, and media concentration effects on 'Jamapa' callus cultured in vitro. The analysis of variance of factorials experiments revealed that the main effects of time on the induction medium (IM) and IBA concentration in LCM were highly significant. However, the effects of continuous light or continuous dark on embryoid development were not significant (Table 4). A significant interaction was noted between the factors time of callus culture on IM and the time of culture in the LCM with IBA as the auxin source (Table 4). Callus generated from explants and cultured for 5 weeks on IM before placing in LCM containing IBA followed by culturing for 20 days on this LCM gave the highest number of embryoids (50) (Table 5). A possible accumulation of 2,4-D in the callus tissue from IM may have limited the number and quality of embryoid formation in LCM when 2,4-D was used as the auxin source (carry over effect).

Time on IM medium effect. The analysis of variance indicated that the main effect (No. of embryoids produced) for time on IM was highly significant (Table 4). Based on the LSD criterion the differences were manifest between the first and second weeks and fifth and sixth weeks. No differences were detected between the mean number of embryoids produced in the LCM when callus containing globules was left on IM for three, four, five weeks (Table 6). The maximum

Table 4. Analysis of variance for somatic embryo
genesis in liquid challenge medium (LCM).

Source	Degrees of Freedom	Mean Square	F Value
Light (L)	1	2181.6	3.3
Time in IM (TIM)	5	9871.4	14.8 ***
L x (TIM)	5	1508.8	2.3
IBA conc. (I)	4	2690.7	4.0 **
L x I	4	1386.3	2.0
(TIM) x I	20	698.2	1.0
L x (TIM) x I	20	1072.7	1.6
Error	180	667.8	
Time in LM (TLM)	3	40054.6	494.2 ***
L x (TLM)	3	110.4	1.4
(TIM) x (TLM)	15	1516.6	18.8 ***
L x (TIM) x (TLM)	15	134.9	1.7
I x (TLM)	12	163.5	2.0 *
L x I x (TLM)	12	221.8	2.7 *
M) x I x (TLM)	60	120.8	1.5 *
L x (TIM) x I x (TLM)	60	145.8	1.8 **
Error	540	81.0	
Total	959	440661.9	

***, * Significant at the 1 and 5 % probability levels
respectively

Coefficient of Variation = 33.5%

Table 5. Number of embryoids produced from leaf explants in response to time of callus culture on induction medium and time in the IBA liquid challenge medium

Days in IBA LCM**	Weeks on induction medium *					
	1	2	3	4	5	6
5	23 c	8.8 c	5.9 c	13.7 d	15.6 c	3.85 c
10	13.4 b	26.6 c	42.6 a	26.5 c	34.5 b	22.89 b
15	19.0 a	32.2 a	42.1 a	35.6 b	38.15 b	41.81 a
20	13.5 b	34.1 a	35.6 b	42.3 a	49.6 a	43.7 a

Means within columns separated by LSD. P= 0.05.

**The induction medium consisted of a B5 semisolid medium complemented with 15 mg/l 2,4-D.

*The IBA liquid challenge medium consist of a B5 liquid medium complemented with different IBA concentrations (0 - 10 mg/l).

Table 6. Effect of time on induction medium on somatic embryo formation.

Callus culture time (weeks)	Embryoids formed in liquid medium (No.)
1	12 d*
2	26 c
3	31 ab
4	30 abc
5	34 a
6	28 bc

* Numbers in the same column followed by the same letter are not significantly different ($P < 0.05$). LSD value = 5.7

number of embryoids produced from callus containing globules in LCM occurred when explants were maintained on IM for 5 weeks (Table 6). For the first and the second week of callus on IM, the number of embryoids produced in LCM when callus was only maintained on IM for 1 week was low, 12, and after five weeks embryoid production began to decline.

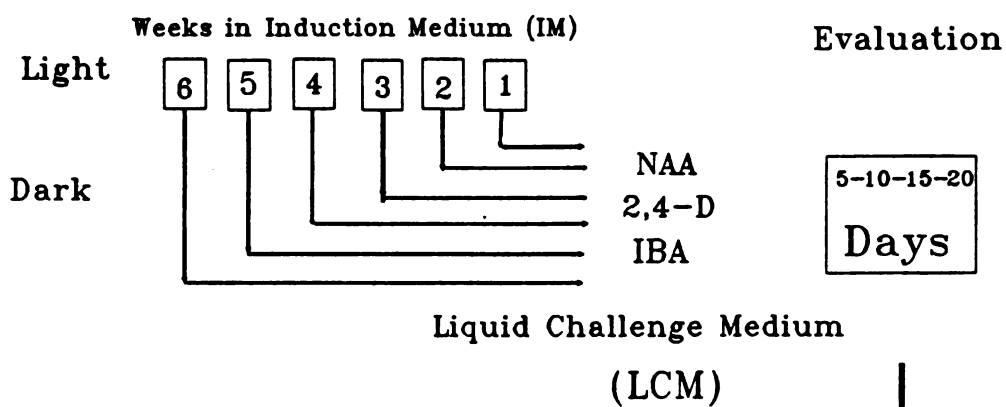
Embryoid Develop on LCM

Type and concentrations of auxin in the liquid challenge medium (LCM). Figure 5 depicts the two-step procedure followed in this study to obtain embryoids with a cotyledonary-like structure in dry beans. The first step is IM of solid medium and two characteristics are involved; light, and time in the IM. The second step is LCM and three characteristics are involved; auxin type, concentration and time in the LCM. At the bottom of Fig. 5 optimum characteristics from the first experiment were left constant and an artificial and a natural source of cytokinin were tested at different concentrations.

Auxin type. Embryoids were formed in LCM when IBA was used as the auxin. After a 2 to 3 week period callus containing globules on IM were cultured in the LCM and different stages of embryogenesis were detected (Fig. 6B). Although the

Figure 5. Diagram of experiments realized to optimize environmental and hormonal response on somatic embryogenesis

Experiment one



Experiment two

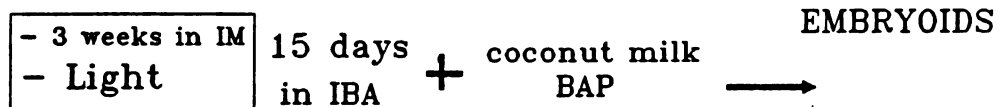
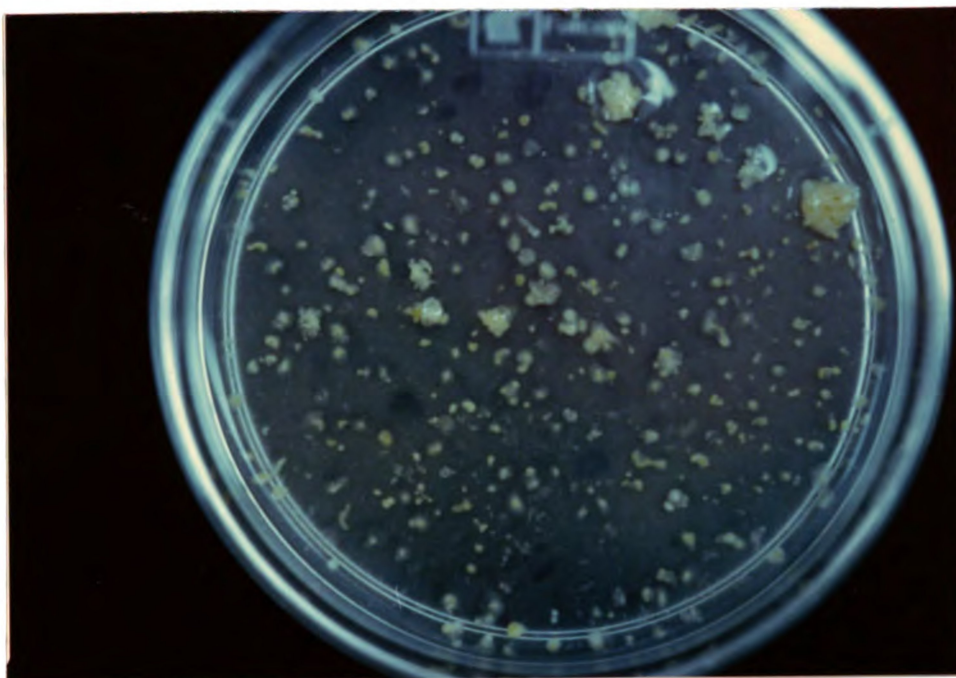


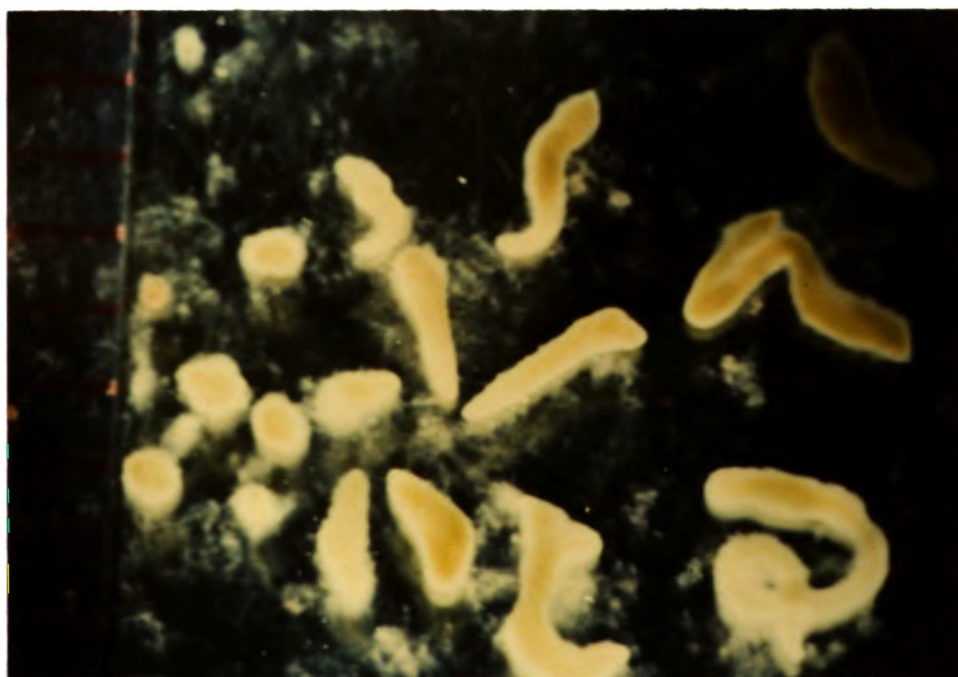
Figure 6. Morphogenesis of embryoids after 15 - 20 days in a challenge liquid medium containing 0.1 to 1.0 mg/l IBA and induced on a B5 medium.

- A. Embryoids in liquid medium showing individual green embryoids.**
- B. Embryoids showing different stages of development.**

A



B



light vs dark regime had no significant effect on the number of embryoids formed in LCM, callus induced on IM in continuous dark and placed in LCM showed sparsely occurring circular green protuberances on the surface of calli after 3-4 days in culture. The number of circular protuberances increased on the callus and the number of green bodies also increased in number with the time. On the other hand, callus promoted on IM under continuous light showed numerous green patches on the callus and also on the upper polar region of the globular tissue. The green structures (meristematic centers) from the light vs dark experiment when separated from the nurse callus underwent morphogenesis to form embryoids (Figs. 6A and B). Moreover, the developmental sequence of events occurred in the same manner for calli that was promoted in continuous light as that for the dark promoted ones. Separation of the green meristematic nuclei from calli maintained in continuous light was faster than from those calli maintained in darkness (Fig. 6A).

Calli cultured in LCM with NAA or 2,4-D (0.01-1.0 mg/l), showed separation of the green meristematic centers from the callus and these structures began to differentiate.

However, the overall response of the green bodies to the two auxins was different. The green bodies in medium with NAA developed a 'frosty' appearance soon after being transferred to the LCM. The 'frosty' calli rapidly became friable and developed rudimentary structures that resembled

primordial roots (Fig. 7A). However, typical root formation occurred in the LCM only when the green bodies were cultured in the presence of 2,4-D at concentrations of 0.1-10 mg/L (Fig. 7B). Green bodies in LCM with more than 10 mg/L IBA, NAA, and 2,4-D turned necrotic and died. Because of the rapid degeneration of the callus in LCM, data were not collected when NAA and 2,4-D were used.

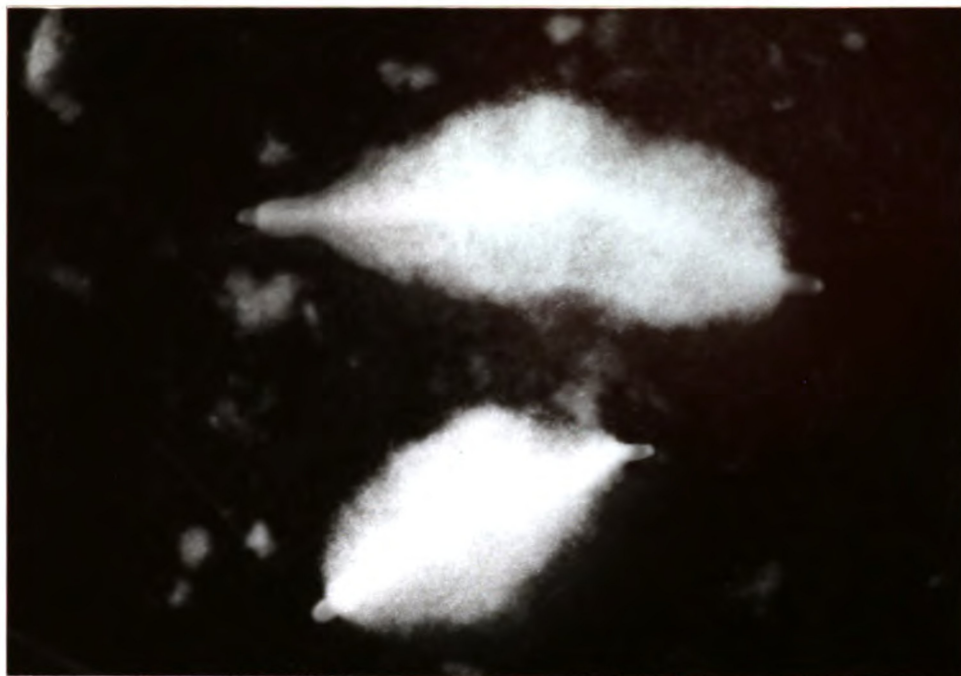
Auxin concentration. Not only the number of embryoids that were formed in the LCM, but also the quality of the embryoid was dependent on the concentration of IBA (Table 7). At no auxin treatment the number of embryoids was not significantly different. However, the type of embryoid formed did not show characteristics as well defined as with IBA treatments. Embryoid development was significantly greater at IBA concentrations of 1.0, 0.1, and 0.01 mg/l than at 10 or 100 mg/l. At the 0.01, 0.1, or 1.0 concentrations mean embryoid formation was 29, 30, and 29, respectively (Table 7). When a concentration of 10 mg/l was used, the number of embryoids decreased to an average of 21. At IBA concentration of 100 mg/l, calli turned necrotic and died and no embryoids were observed (Table 7). Similar concentrations of NAA and 2,4-D were tested without significantly improving the number of embryoids from the numbers developed in IBA. The treatment 100 mg/l IBA was excluded from the analysis because at this concentration the auxin

Figure 7. Effect of 2,4-D and NAA in the Liquid Challenge Medium after one week in culture

A. Formation of 'frosty' appearing bodies at 0.1 and 1.0 mg/l NAA

B. Root formation at 0.01 mg/l 2,4-D

A



B



Table 7. Mean number of somatic embryoids produced from callus of primary leaf explants formed in different IBA concentrations.

IBA (mg/l)	Embryos (no.)
0.0	26 ab*
0.01	29 a
0.1	30 a
1.0	29 a
10.0	21 b
100.0	0 c

* Numbers in the same column followed by the same letter are not significantly different ($P < 0.05$). LSD value = 5.2

inhibited embryoid development. The same concentration (100 mg/l) of NAA and 2,4-D had equal effects as 100 mg/l IBA on embryoid development.

Time in the LCM. Calli containing globules were left in the LCM for 20 days to ascertain time effects on the development of embryoids. The number of embryoids formed from globules increased progressively with the time they remained in the LCM (Fig. 8). The time effect was significant for globules cultured in LCM containing IBA. The greatest number of embryoids (34 and 36 embryoids) was observed after remaining in the LCM 15 and 20 days, respectively, (Appendix I). On the other hand, evaluations made at 5 days after culture in LCM showed an average of 8 embryoids produced but this number increased markedly (28) after 10 days culture in LCM. The 10 days-evaluation was significantly different from the 5 day one (Appendix I). A continued exposure of calli containing globules to auxin beyond 15 - 20 days had either no effect or a detrimental effect on the number of embryoids formed. Embryoid morphology based on subjective appeal was most desirable at 15 days compared to the 20 day at culture time in the LCM (Fig. 9). Embryoids observed at 15 days were well defined and in the apparent shoot apical region of the embryoid tissue, well defined greenish areas were observed (Fig. 9). This observation suggested the embryoid had the potential to develop a shoot. However, only the

Figure 8. Effect of the IM and IBA concentrations at 5, 10, 15, and 20 days callus cultured in LCM on the number of embryoids. W-1 to W-6 are the weeks of calli cultured in IM.

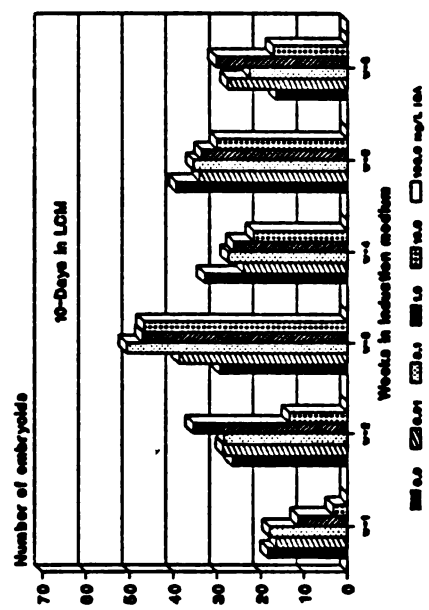
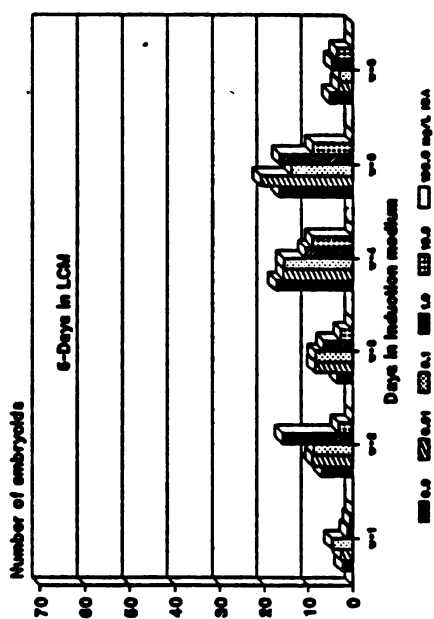
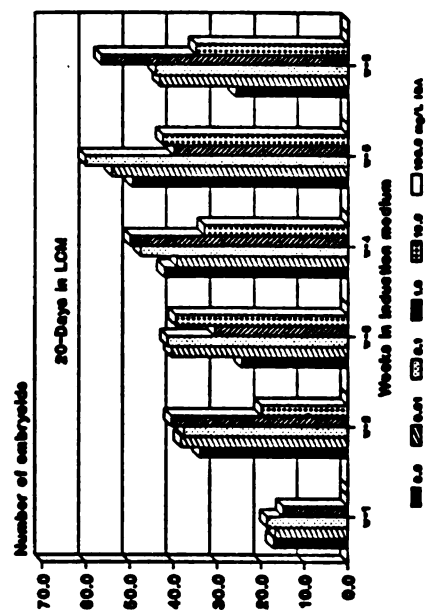
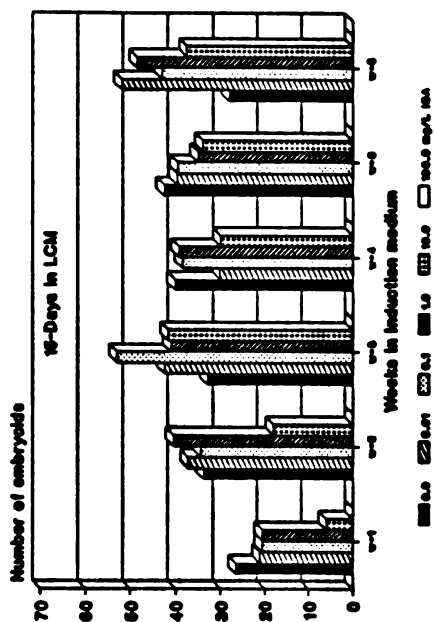
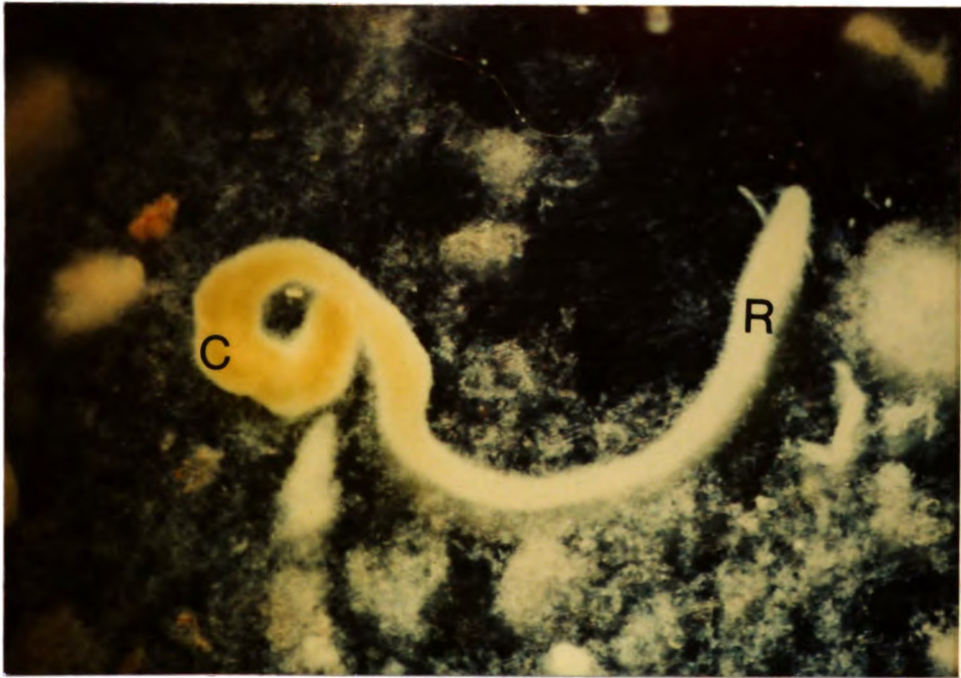


Figure 9. Bipolar somatic embryoid. Note root-like end (R), and opposite end with green pigmentation and cotyledon-like lobes (C).



region distal to the apex developed further. This areaproduced a root; hence, establishing a polarity to the embryoids. After 20 days in LCM embryoids began to degenerate the apical (cotyledonary) part into friable callus while the root elongated to 3-5 cms.

The addition of a cytokinin (BAP) or coconut milk (a natural source of hormones and other compounds) (Tulecke et al., 1961) to the liquid medium inhibited embryoid formation. At BAP concentrations (0.03, 0.1, 0.3, and 1.0 mg/l) free cell proliferation in the medium was observed (Table 8). It was also noticed that more free cells and a thicker free cell suspension developed in all BAP treatments than in IBA treatments. As the concentration of coconut milk increased, the inhibition of embryoid number increased (Fig. 10). A cell suspension was also observed in the coconut milk medium and the medium was more dense as the proportion of coconut milk was increased. Allavena and Rossetti (1983) also found that adding cytokinin to the medium was detrimental to embryo-like structure formation.

Histology. Examination of the thin section for embryoids developed in the IBA LCM under light microscope (100x) (Figure 4C), roughly shows polarization (a connective vascular tissue from the apex to the root tissue), but it shows clear meristematic ends, one for the root portion and the other for the apical region. This includes small size, dense

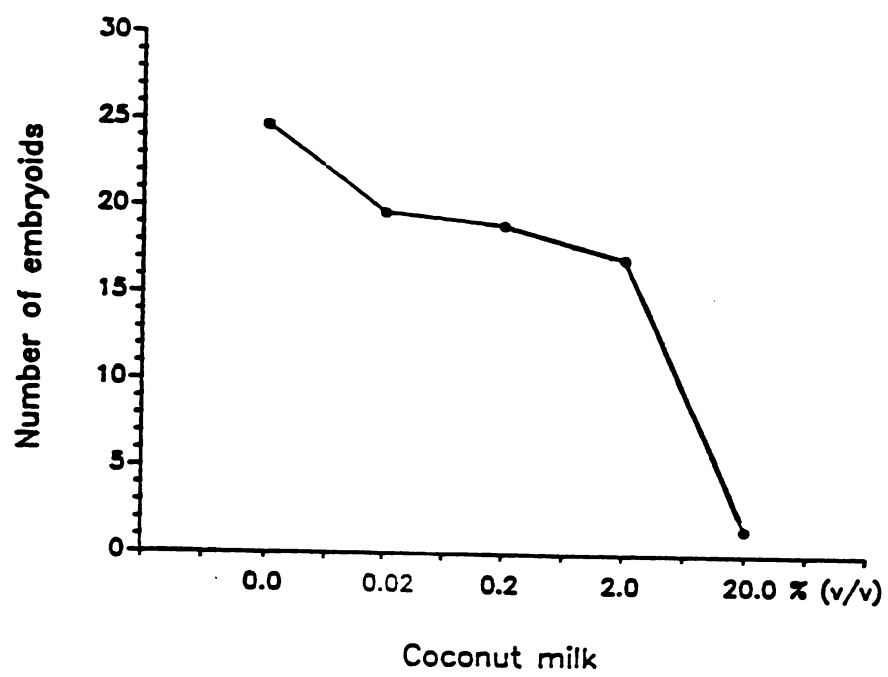
Table 8. Effect of BAP and IBA concentrations on the embryoid formation in a B5 liquid medium.

BAP mg/L	IBA mg/L	Fresh weight (mg)	E**
0.0	0.0	200.0	13.3
	0.1	400.0	19.3
	1.0	100.0	10.5
	10.0	100.0	8.8
0.03	0.0	100.0	0.0
	0.1	100.0	0.0
	1.0	90.0	0.65
	10.0	100.0	0.15
0.1	0.0	200.0	0.0
	0.1	80.0	0.0
	1.0	100.0	0.0
	10.0	100.0	0.15
0.3	0.0	200.0	0.0
	0.1	100.0	0.0
	1.0	100.0	0.0
	10.0	70.0	0.0
1.0	0.0	200.0	0.0
	0.1	80.0	0.0
	1.0	100.0	0.0
	10.0	200.0	0.0

* Calli fresh weight after 15 days in Induction medium.

**E Mean of the number of embryoids from 3 evaluations.

Figure 10. Effect of coconut milk used as a natural source of cytokinins and other compounds on the development of somatic embryoids.



cytoplasmic contents, large nucleoli, small vacuoles and a profusion of starch grains and rapidly dividing meristematic cells. These features of meristematic cells are common for all systems with embryogenic cells from which embryoids are visibly derived (Williams and Maheswaran, 1986).

DISCUSSION

The type of callus observed when 2,4-D was used at 15 mg/l in the initiation medium in B5 medium agrees with the findings of Saunders et al. (1987) in drybeans using a MS medium and in soybean using also a MS medium (Finer, 1988). Saunders et al. (1987) reported different responses of the callus tissue at 10, 30, and 40 mg/l 2,4-D using pinto beans (*P. vulgaris*) as a source of immature leaves explants. At 10 mg/l these authors obtained some roots and globules and at 30 and 40 mg/l mostly globules. However, in a preliminary study, it was found that at 30 and 40 mg/l, the 'Jamapa' callus turned necrotic after the 10-15 days in culture. Lowering the 2,4-D to 15 mg/l led to somatic tissues resembling those observed by Finer in soybean with 40 mg/l. The different responses of the two varieties to 2,4-D may be due to the endogenous auxin production or to the different organic salts used.

The globular stage reminiscent of somatic embryoids (Saunders et al., 1987) developed directly on the surface of the callus. The majority of the globular structures were opaque with an occasional globular structure manifesting a green color at the apical area. Secondary globules were

initiated when the meristematic tissue was transferred to fresh medium. Formation of secondary globules may be useful as a constant source of explants in somatic embryogenesis studies.

Growth requirements for in vitro callus promotion indicate a need for a strong auxin (Mok and Mok, 1975; Saunders et al. (1987)). For beans, 2,4-D appears to be an adequate auxin to use for promoting callus from explants on IM; however, 2,4-D probably has too strong of an effect on promoting bean embryoid development in LCM. According to Evans et al. (1981) the auxin used in secondary culture (LCM) may be the same auxin as used in the primary culture but at a reduced concentration or a weaker auxin may be used at the same or higher concentration than the strong auxin. Different responses of the callus were noticed in this work in the LCM. Possible somatic embryogenesis development was indicated by external characteristics such as the shape and size of clumps in LCM (Figs. 6B and 9) and observations, under light microscope, of embryoid thin layer sections (Fig. 4C).

The transference from a higher to a lower auxin medium for triggering the somatic embryo development is effective in crops such as Atropa belladonna, L., cotton (Gossypium hirsutum L.), eggplant and some oil plants which require an auxin for primary culture and a weak auxin during secondary culture at the same or higher concentration and the inclu-

sion of cytokinin (Evans and Sharp, 1981). The results of this research showed that transference from a strong or higher auxin concentration to a lower or weaker auxin also is applicable for dry beans as it is for soybean (Parrot et al., 1988)

Of the three auxins examined (NAA, 2,4-D, and IBA) in the LCM (secondary medium), IBA had the least inhibitory effect in producing somatic embryoids. Since IBA is the weakest auxin of the three, it may lessen the inhibition noted with no auxin in the LCM some embryoids were formed, they did not represent the desired characteristics (green cotyledon-like apex and a root part). The endogenous auxin/cytokinin ratio may have been influenced in the embryoid formation. However, with IBA the number and quality of the embryoids were enhanced. The morphogenesis of embryoids noted in this study was similar to that of other researchers. There were some green structures that were initiated from very tiny cell clumps which later developed into more differentiated structures. Similar green differentiated structures were observed with *P. acutifolius* in liquid medium before transferring them to a defined solid medium for regeneration (Kumar et al., 1988). Allavena and Rossetti (1983), and Schieren and Jacobsen (1986) described the formation of embryoids up to the torpedo stage developed for dry beans working with Tb 108 and a breeding line P263. However, clear evidence of embryoid morphology such as is

displayed in Figure 5B was lacking in previous reports. In this study one can follow a sequence in the development of embryoids. Initially embryoids were represented in a small cell clump, followed by an initiation of regions and polarization, and finally a distinguishable true root portion and a green apical region. The embryos produced roots, but no shoots development was observed.

In this study when NAA was used as the auxin source, cellular clumps developed initially and quickly (3-4 days) became frosty and died. This observation is contrary to the findings of Parrott et al., 1988, in soybean who reported that after a 12-14 days exposure of cotyledonary tissue to NAA there was a good balance between embryo number and embryo quality. In response to the use of 2,4-D in the IM followed by NAA in LCM cell clumps produced single roots. This cell differentiation (rhizogenesis) may be formed from single cells or very small clumps of cells. The differences noted between the number and quality of soybean embryos and bean embryoids may have been due to a carryover of the 2,4-D present in the previous medium in which leaf explants were cultured for different periods of time. Another explanation might be due to a synergistic activity of the meristematic tissue inducer and the concentration of the growth regulator used in the secondary medium. However, with IBA, a significant interaction was observed between the time explants were in the IM culture and the time that the callus was

maintained in the liquid challenge medium on the number of embryoids formed was observed. It could be seen that the callus in the IM needs a particular endogenous and exogenous auxin ratio to respond favorably in terms of embryoid formation in the LCM.

The embryoids obtained in LCM with IBA as the auxin failed to develop further. A diversity of conditions were tested to develop a strategy for producing mature plants. When somatic embryoids of carrots (Daucus carota L.), soybean and bromegrass (Bromis inermis) were transferred to hormone free (Gamborg 1975; Gamborg et al., 1970), or a low auxin media (Lazzeri et al., 1985) or a desiccation process, either into an empty Petri dish (Hammat and Davey, 1986) or medium with a high osmotic potential (Buchheim et al., 1989), complete development of the embryoid was obtained. Under these conditions the embryoids from dry beans failed to germinate.

There are some effects that might influence such development of the embryoid formation that have to be considered. Those effects are genotype, explant, light, temperature, medium etc. Martins and Sondahl (1984) suggested that varieties respond differently in the amount and quality of callus formation. In this investigation and using leaf and cotyledonary explants it was possible to observe differences among accessions tested. Saunders et al (1972) suggested that genotypic response is one of the main factors to

consider in somatic embryogenesis. Another important factor in embryogenesis is the type of explant. Kartha (1982) suggested that tissues from young and actively growing plants give the overall responses in in vitro culture. In this research immature (5-7 days old) primary leaves were used. In addition, immature cotyledonary tissue (10 - 14 days after anthesis) developed numerous globular structures (data not shown).

The non-significant light effect on callus produced on IM agrees with results obtained in soybean by Hammatt and Davey (1987). However, the opposite effect was observed in cassava by Szabados et al., 1987, who they reported that embryogenic callus maintained in complete darkness enhanced somatic embryo development.

The use of a synthetic cytokinin (BAP) and a natural source of cytokinins (coconut milk) did not enhance embryoid formation in the LCM, but it had an inhibitory effect. It may be that, responses to overall embryogenesis is family dependent. Lippmann and Lippmann, (1984) found similar results in soybean. The addition of cytokinins (BA, Kinetin, Zeatin at 0.5 and 1.0 μMol) to the culture medium containing 5 μMol of 2,4-D inhibited the induction of embryogenic tissue and of embryoids. In addition, Hammatt and Davey (1986) reported that the auxin present in the coconut milk used may increase the total auxin concentration in the medium to levels exceeding endogenous levels found in the

somatic embryos of the soybean cultivars screened.

In regards to the meristematic areas noted upon microscopic examination of callus, Henshaw et al. (1982) suggested that meristematic centers provide the means by which an intimate arrangement of cells might arise so that organogenesis can be initiated. Street (1976) hypothesized that the ultimate fate of a meristematic center is dependent on the development of polarity. Hicks (1980) referred to that specialized cells with the feature observed (Figure 4A) as "meristemoids". However, the meristematic centers reported in this work were surrounded by a layer of friable tissue (non-meristematic cells with small nuclei and large vacuoles). This external non-meristematic layer of cells might impede the growth of the inner and meristematic cells.

When calli with virescent areas, "meristemoids" or meristematic centers were placed in liquid medium, they separated from the nurse tissue and initiated a process of differentiation leading to a clear-cut polarization of the embryoid.

Since the development of zygotic embryogenesis has been found quite similar to somatic embryogenesis (Christou and Yang, 1989), characterization of the development of somatic embryogenesis in liquid medium might contribute to recognizing the developmental stage to which additional treatments may be applied. This study provided information for the development of a model to characterize the biochemical,

molecular and morphological aspects of development in dry bean somatic embryogenesis.

FURTHER in vitro STUDIES WITH DRY BEAN

Characterization of the morphological process of somatic embryogenesis and determination of similarities between somatical and sexual embryo formation are of great importance for further in vitro studies in dry bean. These studies may be achieved through isozyme, other molecular and anatomical studies.

If one can confirm that some stages of somatic embryogenesis are physiologically identical to the sexual stages, it may be useful to determine 1.) the appropriate stage of embryoid to transfer to a maturation medium and 2.) a series of media to be evaluated. Studies have been reported in beans which relate nutritional requirements during sexual embryogenesis with embryo morphology in early stages of development (Smith, 1973). No studies have been reported for the relationship of nutrition with morphology in somatic embryogenesis.

Ethylene accumulation has been detected in Petri dishes containing calli produced from bean explants. An enhanced formation of globules on the 'Jamapa' and 'Jalpatagua-72' callus was observed by using 10 mg/l of Silver nitrate (AgNO_3) an ethylene inhibitor (data not shown). Since

ethylene accumulation had an inhibitory effect on somatic embryogenesis of corn (Vain et al., 1989), carrots (Roustan et al., 1989), and denovo shoot morphogenesis in Brassica campestris ssp (Chi and Pua, 1989) it may prove fruitful in trying to develop shoots from callus to evaluate the effect of ethylene on somatic embryogenesis in dry bean.

APPENDIX

APPENDIX I

Mean number of somatic embryoids of callus from seven-day-old primary leaves explants in liquid medium. Data represent the mean of four replications collected every 5 days for 20 days.

Time in liquid medium (TLM) (Days)	Number of embryoids (Mean)
5	8 c*
10	28 b
15	35 a
20	36 a

* Numbers in the same column followed by the same letter are not significantly different ($P < 0.05$). LSD value = 1.614

APPENDIX II

Details of procedure for fixing, embedding, sectioning and staining small specimens.

1. Fix specimens in FAA for at least twenty hours at 5 °C.
2. Pass specimens through TBA series (see below). One hour in each solution except TBA 2, at room temperature. The tertiary butyl alcohol series for dehydrating specimens before embedding in wax.

TBA	Water	:	95% Ethanol	:	TBA	:	100% Ethanol
1	50	:	40	:	10	:	0
2	30	:	50	:	20	:	0
3	15	:	50	:	35	:	0
4	0	:	50	:	50	:	0
5	0	:	0	:	75	:	25

3. Change to pure TBA and leave overnight on oven top.
4. Add wax ships to specimens in TBA. Put specimens in vials inside oven at 70 - 80 °C and let TBA evaporate. Leave for 6 - 12 hours.
5. Remove wax and replace with fresh molten wax three times. Leave for 6 - 12 hours per wax change.
6. Pour specimens and molten wax into prepared mould on hot plate. Orientate specimens at with forceps. Allow

wax blocks to cool down.

7. Section specimens at 8 μm with a microtome.
8. Float sections on warm water to allow expansion and place on slide smeared with Haupt's adhesive (see below). Add a few drops of 3% formalin to allow further expansion.

Haupt's Adhesive: Dissolve 1 g gelatin in 100 ml distilled water at 60 °C. Add 2 g Phenol crystals and 15 ml of glycerol. Stir and filter.

9. Immerse slide in xylene baths (5 and 3 min. each) and then Xylene-Ethanol (50:50) for 3 minutes.
10. Rehydrate sections in ethanol series (100%, 95%, 70%, 50%, 30%) for 2 mi each and then distilled water (1 min).
11. Stain in Filtered Hematoxillin dye. 20 minutes.
12. Dehydrate sections - pass quickly trough distilled water and ethanol series, Xylene and ethanol (50:50), 2 washes of xylene.
13. Attach cover slip to specimens on slide with Canada Balsam.

APPENDIX III

Interaction effect of Light, time in liquid medium (TLM) (day of evaluation), and IBA concentration on the number of somatic embryoids.

Light	Time in LM days of eval.	IBA con. mg/L	No of embryoids
Light	20	0.01	46.00 a
Darkness	20	1.0	44.17 ab
Light	20	0.1	42.88 ab
Darkness	15	0.1	41.29 ab
Light	15	0.01	41.29 ab
Darkness	20	0.1	40.42 bc
Darkness	15	1.0	40.21 bc
Light	15	0.1	35.67 cd
Light	20	10.0	5.03 d
Darkness	20	0.01	34.79 d
Light	15	1.0	34.72 d
Darkness	15	0.0	34.08 d
Light	15	0.0	33.90 de
Light	20	1.0	33.88 de
Light	10	1.0	33.03 def
Darkness	15	0.01	32.88 def
Light	15	10.0	32.72 def
Light	20	0.0	32.70 def
Light	10	0.01	32.58 def
Darkness	10	0.1	32.17 defg
Darkness	20	0.0	31.83 defg
Light	10	0.0	28.90 efgh
Light	10	0.1	28.63 fgh
Darkness	10	1.0	28.46 fgh
Light	10	10.0	27.13 ghi
Darkness	10	0.0	25.58 hi
Darkness	10	0.01	23.88 hi
Darkness	15	10.0	23.00 i
Darkness	20	10.0	22.63 i
Darkness	10	10.0	17.00 j
Light	5	0.01	12.46 jk
Light	5	10.0	10.63 kl
Light	5	0.0	9.300 kl
Light	5	0.1	9.292 kl
Darkness	5	0.1	8.750 kl
Darkness	5	0.0	8.083 kl
Darkness	5	1.0	7.917 kl
Darkness	5	0.01	7.583 klm
Light	5	10.0	6.917 lm
Darkness	5	10.0	2.625 m

* Numbers in the e same column followed by the same letter are not significantly different ($P < 0.05$). LSD value = 5.105

APPENDIX IV

Interaction effect of Time on induction medium (TIM) (weeks), and Time in liquid medium (days of evaluation) on the number of somatic embryoids.

Time in IM (weeks)	Time in LM (day of evaluation)	Mean	
1	20	49.60	a
6	20	43.70	b
3	10	42.55	b
4	20	42.35	b
3	15	42.15	b
6	15	41.81	bc
5	15	38.15	cd
4	15	35.60	de
3	20	35.35	de
5	10	34.50	de
2	20	34.12	e
2	15	33.17	e
2	10	26.57	f
4	10	26.45	f
6	10	22.89	fg
1	15	18.98	gh
5		15.57	hi
4	5	13.73	i
1	20	13.48	i
1	10	13.45	i
2	5	8.780	j
3	5	.900	jk
6	5	3.850	k
1	5	2.300	k

* Numbers in the same column followed by the same letter are not significantly different ($P < 0.05$). LSD value = 3.9

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