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In Vitro Rescue of Helianthus Annuus Embryos by Ovule Culture

John C. Volin

IN VITRO RESCUE OF HELIANTHUS ANNUUS

EMBRYOS BY OVULE CULTURE

This thesis is approved as a creditable and
independent investigation by a candidate for the degree,
Master of Science, and is acceptable for meeting the
thesis requirements for this degree. Acceptance of this
thesis does not imply that the conclusions reached by the
candidate are necessarily the conclusions of the major
department.

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JCV

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INTRODUCTION

Interspecific hybridization between the cultivated sunflower, Helianthus annuus L., and other Helianthus species is an important aspect of sunflower improvement, because it extends the array of genes available for use in breeding programs. Sunflower species have demonstrated resistance to many pests that affect the cultivated sunflower (Rogers et al., 1982). Oil content and quality may also be improved through interspecific hybridization, as Helianthus species are sources of genetic variation for these traits (Chandler, 1976).

Attempts to cross H. annuus with sixteen other Helianthus species were only 40 % successful (Whelan, 1978). It is generally assumed that failure of interspecific hybridization in sunflowers is due to abortion of the hybrid embryos during early developmental stages (Chandler and Beard, 1983). In other genera such as Gossypium (Beasley, 1940) and Hordeum (Schooler, 1960), the solution to this problem has been to rescue these embryos prior to abortion by growing them on an artificial media. Embryo rescue in sunflower, however, has only been successful at the torpedo stage or later (Chandler, 1976). The torpedo stage normally occurs four to seven days after fertilization (Espinasse et al., 1985).

The purpose of this investigation can be summarized in two objectives. The first was to improve current in vitro techniques in sunflower by providing for the rescue of younger embryos, for example, the globular to heart stages which normally occur one to four days after pollination.

The second objective was to investigate how the embryo develops in vitro during the first eleven days after pollination, compared to its normal development in vivo.

LITERATURE REVIEW

Van Tieghems in 1873 was able to germinate excised embryos of <u>Helianthus</u> <u>annuus</u> using moist chambers. Culture of excised plant embryos <u>in</u> <u>vitro</u>, using salt solutions as well as organic nutrients, has been done since the turn of the century. However, artificial media developed for the nutritional requirements of excised embryos have only been strictly defined since 1940 (Narayanaswami and Norstog, 1964). Most species, including sunflower, follow a typical growth pattern. The first stage is round or globular, followed by heart, torpedo, and vascular stages, respectively, as defined for sunflower by Newcomb in 1973 (see also Espinasse et al., 1985). In most species, embryos at the vascular stage and older can be grown on a simple medium composed of essential macro- and micronutrients. Embryos at the torpedo stage and younger are usually much harder to grow <u>in</u> <u>vitro</u>. They require additional nutrients such as vitamins, amino acids, auxins, cytokinins, or complex extracts from nurse tissues (Raghavan, 1976).

Monnier (1978) found that young embryos are particularly sensitive to the type of salt base used in <u>in</u> <u>vitro</u> culture. The mineral salts that promote growth may be toxic, but non-toxic solutions are not able to induce

normal differentiation. Optimal physical conditions also have to be defined. It has been found that a higher osmotic pressure is often beneficial in culturing immature embryos (Paris et al., 1953). Norstog (1967) suggested plating the embryos on a low sucrose medium and transfering them after two to three days to a medium higher in sucrose. Sucrose, is the source of energy for most embryo culture media; in addition it regulates osmotic pressure. Chandler and Beard (1983) found that a high sucrose percentage decreased premature germination in sunflowers, and that a high sucrose, low auxin medium gave excellent results in allowing young excised hybrid embryos, those that were three to seven days after pollination, to develop into healthy enlarged embryos.

Chandler (1976) stated that auxin in high concentrations may be harmful to the growth of young embryos, but a low concentration of auxin may be beneficial. He found that 1.0 mg/l indoleacetic acid (IAA), a natural auxin, caused sunflower embryos to grow as undifferentiated callus instead of as normal embryos. It caused premature germination and death of very young embryos, while it caused older embryos to germinate abnormally or not at all. The callus formation was eliminated by changing the auxin component from 1.0 mg/l IAA to .05 mg/l napthaleneacetic acid (NAA), a synthetic

auxin, or even to eliminate the auxin. There was a slightly higher survival rate with low auxin compared to no auxin (Chandler and Beard, 1983).

Cytokinins are another class of compounds which are of special importance in embryo culture. The ones used most frequently are kinetin, benzyladenine (BA), and zeatin (Gamborg and Shylak, 1981). By increasing the cytokinin and decreasing the auxin, shoot growth is promoted. In contrast, root development is promoted more when the auxin is increased and the cytokinin is decreased (Murashige and Skoog, 1962).

Other factors also influence embryo growth in vitro. One of these is the pH of the media. It has been found that an initial pH of 5.5 to 5.8 is optimum for plant embryos. Another environmental factor that is important is temperature. A temperature between 26 to 28 $^{\circ}C$ is considered a good standard temperature for achieving optimum embryo growth (Gamborg and Shyluk, 1981). In general cool white fluorescent lamps and photoperiods of 12 to 18 hours give normal growth. Irradiance levels of 45 to 65 uEm^{-2}s^{-1} are adequate.

Vitamins also need to be considered. Plant embryos need vitamins for normal development. One vitamin that is absolutely essential for most plant cells is thiamine (Ohira et al., 1976). Growth is also enhanced by including

other vitamins, such as m-inositol, pyridoxine and nicotinic acid, although these are not absolutely required for in vitro growth.

The level of salt used in embryo culture is another important factor. Salt bases are generally classified as either high or low. A high-salt base refers to a medium that is higher in macronutrients as compared to a low-salt base (table 1). Studies have shown that a high-salt medium such as Murashige-Skoog (MS) was superior to low-salt bases for older ovules, but normal embryos of cotton, (Gossypium hirsutum), did not develop when younger embryos were cultured (Eid et al., 1973). Beasley and Ting (1973) found also that high-salt bases were superior to low-salt bases for cotton. Although Beasley and Ting did not study embryo development, they found that fiber growth was best when they increased KNO_3 and deleted the NH_4^+ from the MS medium. Stewart and Hsu (1977), plating Gossypium hirsutum ovules two days after pollination on the Beasley-Ting salt medium, observed that embryo growth and development was enhanced if NH_4^+ was included in the medium.

It has been found that normally abortive embryos resulting from interspecific crosses of cultivated cotton could be successfully cultured and grown to maturity through the use of ovule culture. Many times hybrid embryo failure occurs during the mid- to late stages of

Table 1. Salt base compositions for four standard *in vitro* culture nutrient solutions.

Salt	Mineral Content (mM)						Additives		Ratio	Total
Bases*	N	K	Ca	Mg	S	P	Vitamins	Amino Acids	NH4/NO3	Salts
B5	27	25	3.0	1.0	3.0	1.3	Yes	Yes	.08	Low
B5S	27	25	3.0	1.0	3.0	1.3	Yes	No	.08	Low
MS	101	27	3.0	2.0	2.1	2.0	Yes	No	.69	High
NN	27	10	1.5	0.8	2.1	0.5	Yes	No	.50	Low

*References: Gamborg et al. (1968), Murashige and Skoog (1962), and Nitsch and Nitsch (1969).

embryogenesis for many plant species (Stewart, 1981).
Frequently the failure of interspecific crosses in
sunflowers is due to incompatibility of the embryo with the
endosperm or the inactivity or death of the endosperm, and
not to the inviability of the embryo (Chandler, 1976).

The main purpose of _in_ _vitro_ embryo culture is to
provide the necessary nutrients when endosperm failure
takes place. In those incidences where endosperm death
occurs early in embryo development, removal of the small
embryo is virtually impossible. When these conditions
occur, an avenue that may be utilized is ovule culture,
whereby the entire ovule is plated on the media to support
embryogenesis long enough that one can later rescue the
embryo at a larger size (Stewart, 1981). The first
objective of this investigation is based on this concept.
Sunflower ovules are plated one to four days after
pollination, and one week after plating, the embryos are
excised from the ovules and plated on fresh media. Embryos
are ready for plating when they are globular or heart
shaped. Among 18 replications of 15 embryos per
replication, the embryos averaged only .62 mm in length at
the time of plating. Only embryos at the torpedo stage or
larger have been previously rescued _in_ _vitro_.

Ultrastructural and biochemical studies by
Bhatnagar and Sawhney (1981) showed that the endosperm was

essential for embryos not only prior to maturity but also during its germination. The embryo of sunflowers is enveloped by an endosperm that is one cell thick. The endosperm cells are characterized by thick walls and dense cytoplasm full of storage materials (lipids and proteins). Prior to radicle emergence during germination, the protoplasts of the cells facing the radicle tip underwent certain structural modifications, including vacuole formation due to storage material mobilization (Psaras, 1985). This showed that early in embryo development in sunflower, the endosperm may have a vital role in the embryos' survival.

Chandler (1976) was able to grow embryos from three new interspecific crosses in <u>Helianthus</u> species. This demonstrated that embryo abortion could be avoided by the use of embryo culture. Whelan (1976) reported that some interspecific sunflower embryos aborted too early to be successfully rescued using normal culture techniques. For most genera interspecific crosses have only been successful with those embryos that have developed past the heart stage of embryo development (Raghavan, 1976).

MATERIALS AND METHODS

General Procedures

Eight inbred lines and two hybrid lines of Helianthus annuus were used in this study: HA89, HA99, HA300, CM400, RHA273, RHA274, RHA297, RHA299, HY894 and HY903 (Table 2). Plants were planted every two weeks in the greenhouse throughout the study. The plants were grown in 152 mm pots containing 2:1:1 soil:peatmoss:perlite (v:v:v). The temperature in the greenhouse was maintained at 22 $\pm$ 5 ^{O}C and the photoperiod at 19 hours. Daily sunlight was supplemented with light from high pressure sodium lamps (100 uEm^{-2}s^{-1}, photosynthetically active radiation).

Plants were bagged one day before flowering. In the morning anthers were removed before anthesis. After emasculating for three days, the inner rings of the capitulum that had not yet gone through anthesis were removed. On the afternoon of the third day, when all the stigmata were receptive, the flowers were sib-mated.

Depending on weather conditions, embryos of the same age can differ in size and shape (Espinasse et al, 1985). Therefore, embryos were measured with an ocular micrometer in a dissecting microscope one to four days after pollination. To determine when embryos were ready to

Table 2. Genotypes used throughout the study.

Genotypes	Preliminary Test Experiment									Objectives				
	1						2	3	4	1				2
	2 DAP*				4 DAP		4 DAP	4 DAP	2-4 DAP	2-4 DAP				2-4 DAP
	MS	B5S	NN	B5	MS	B5S	MS	B5	B5	B5	B5S	MS	NN	NN
HA89 x Hy903	x**	x			x	x								
RHA299 x Hy903	x	x			x	x								
Hy894 x Hy903			x	x										
HA99 x CM400			x	x										
Hy903 x RHA297					x	x								
RHA299 x RHA297							x							
Hy894 x RHA297							x							
Hy903 x RHA299							x							
Hy894 sib-mated								x	x	x	x	x	x	x
HA300								x						
CM400								x						
Hy903									x	x	x	x	x	
RHA297									x	x	x	x	x	
RHA273									x					
RHA299										x	x	x	x	
HA99										x	x	x	x	
RHA274														x
HA89														x

* = days after pollination.
** = test included this genotype.

be plated, a sample of 15 embryos was evaluated. If embryos were between 0.1 and 1.0 mm in length, and globular or heart shaped, they were ready to be plated.

Chandler and Beard in 1983 selected dark colored ovaries to determine the age of their embryos. However, in this study the dark colored ovaries were discarded, and the white or partially darkened ovaries were used. The selected ovaries, approximately 6 to 10 mm in length, were then separated from the capitulum and put in a beaker. The ovaries were surface sterilized in a 0.5 % NaOCl solution for 7 to 10 minutes in a sterile laminar flow hood. Then the ovaries were rinsed three times, for 5 minutes each, in sterile water.

For ovule culture, ovules approximately 3 to 8 mm in length were excised from the ovaries and plated on 15 ml of 8 % agar media in 60 x 15 mm petri dishes that were then labelled and sealed with parafilm tape. The petri dishes were placed in a growth chamber with a temperature of 26 $\pm$ 2 ^{o}C, with a photoperiod of 19 hours supplied by cool white fluorescent lights of 45 uEm^{-2}s^{-2}. One week later, embryos were excised from the ovules with the use of a dissecting microscope under an aseptic hood, and plated on fresh media of the same concentration.

Preliminary test

In the early phases of this investigation, four different experiments were run to determine an adequate sucrose and hormone level for embryo growth and development. The four experiments also gave a suitable plating procedure, the two-step embryo rescue procedure, for the rescue of globular and heart shaped embryos.

In experiment one, ovules were plated at two and four days after pollination on four different salt bases: B5, B5S, MS, and NN (Table 1). Four different ratios of auxins to cytokinins and two levels of osmotic pressure were used (Table 3). The osmotic pressure was determined by sucrose levels. All the media were adjusted to pH 5.8 to 5.9, and had vitamins in concentrations of 10 mg/l thiamine, 1 mg/l nicotinic acid, 1 mg/l pyridoxine and 100 mg/l m-inositol except for B5 which already has vitamins included. The media were autoclaved for 20 min at 1.0×10^5 N/m^2. Eight petri dishes for each salt base were used, 4 with 3 % sucrose and 4 with 9 % sucrose. Each petri dish within a sucrose level had a different ratio of auxin to cytokinin (Table 3). Initially six genotypes were used, Hy894, Hy903, RHA299, HA89, HA99, CM400 (Table 2). Changes in ovule length and width were measured at the time of plating and a week later. After three weeks ovules were evaluated and then dissected for determination of embryo appearance.

Table 3. Comparison of auxin to cytokinin ratios, sucrose levels, and salt bases for the preliminary tests, objective one and objective two.

		Study					
		Preliminary Test				Objective	
Factor	Level	Exp 1	Exp 2	Exp 3	Exp 4	1	2
NAA:BA	1:1			x*			
(mg/L)	1:0.5			x			
	1:0.1			x			
	1:0			x			
	0.5:1		x				
	0.5:0.5	x	x	x			
	0.5:0.1		x	x			
	0.5:0	x	x	x	x		
	0.1:1	x	x	x			
	0.1:0.5		x				
	0.1:0.1		x	x	x		
	0.1:0		x	x	x	x	x
	0:1		x				
	0:0.5		x				
	0:0.1		x				
	0:0	x	x	x	x		
Sucrose	3	x					
(%)	6					x	x
	9	x	x	x	x	x	
	12			x	x	x	
Salt	B5	x		x	x	x	
Base	B5S	x				x	
	MS	x	x			x	
	NN	x				x	x

*x = test included this factor at this level.

In experiment two, in vivo checks were added to the procedure. The auxin to cytokinin ratio was increased to 12 different ratios. Only MS and 9 % sucrose were used (Table 3). Ten ovules on each hormone level were plated four days after pollination. Genotypes Hy894, Hy903, RHA297, and RHA299 were used, and ovule length and width were measured on the day of plating and six days later.

Experiment three included 10 hormone ratios. Here the sucrose levels included 9 and 12 % sucrose, and B5 was the salt base used (Table 3). Twelve ovules from Hy894, HA300 and CM400 were plated 4-5 days after pollination on each petri dish within a sucrose and hormone level. Ovule lengths were measured at plating and a week later. Nine days after plating, embryos were excised from the ovules and plated on fresh media, this is referred to as the two-step embryo rescue procedure. Embryo lengths were measured at plating and every seventh day for two more weeks. In vivo checks of 32 to 38 ovules and 32 to 38 embryos were measured on the day of ovule and embryo excision. Embryos were scored for growth and shape on the following scale: none, round, inferior to .5 mm, .5 to 1.0 mm, or superior to 1.0 mm. All three genotypes were evaluated with respect to normal (embryos that follow the regular pattern of development) or abnormal (embryos with

misshapened cotyledons) development, presence of callus, shoots and/or roots.

The hormone ratios for experiment four were reduced to four ratios. Ovules were plated two to four days after pollination on B5 salt medium with 9 and 12 % sucrose levels (Table 3). The two-step embryo rescue procedure was again employed. Measurements were as in experiment three, and the varieties used were Hy894, Hy903, RHA273 and RHA297.

Objective 1

The preliminary test provided a base for the first objective, which was to improve on current in vitro techniques in sunflower by providing for the rescue of younger embryos. Three inbred lines and two hybrid lines HA99, RHA297, RHA299, HY894, and HY903 were used. Four basic media were used, B5, MS, B5S, and NN. Each of these four salt bases had the osmotic pressure regulated by 6, 9 and 12 % sucrose levels. The hormone concentration was 0.1 mg/l NAA for all media.

An in vivo measurement of 15 embryos, by an ocular micrometer measuring to 0.01 mm in a dissecting microscope, was performed when the ovules were first plated. After one week the embryos were excised from the ovules aseptically, and plated on fresh media of the same components. In addition, the embryos were measured under the microscope

and visual gradings were taken. The visual gradings
consisted of: shape (e.g. heart shaped), presence of
chlorophyll, callus, shoots, or roots, and whether the
embryo had survived. Measurements, both _in_ _vivo_ and _in_
vitro, of length and visual gradings were repeated every
seven days for two more weeks.

Methods chosen for statistical analysis depended on
whether the data were continuous or discontinuous in
nature. The continuous variable, length, was subjected to
an analysis of variance procedure. The visual gradings
which were discontinuous (or discrete) variables were
analyzed using a procedure for categorical data modeling.
The latter procedure determined statistical significance by
the chi-square test. Orthogonal comparisons of individual
means were performed when the chi-square tests showed
significance of main effects or interactions.

Objective 2

In the second objective of this investigation,
HY894 and its two parents RHA274 and HA89 were used to
investigate how the embryo develops _in_ _vitro_ during the
first ten days after pollination. Ovules were plated on a
NN salt base, with 0.1 mg/l of NAA and 6 % sucrose two days
after pollination. Twenty-five embryos were measured by
length, shape and presence of chlorophyll. At days 4, 6,
8, and 10, 25 embryos both _in_ _vivo_ and _in_ _vitro_ were

measured with the same grading for Hy894 and RHA274.
HA89's measurements were at days 5, 7, 9, and 11. Three
replications were used for each of the three genotypes.

Statistical analysis were performed by the same
procedures as used in objective one.

RESULTS

Preliminary Test

In experiment one, those ovules plated two days after pollination gave inconclusive results when comparing 3 and 9 % sucrose with respect to increase in ovule length (Table 4). However, ovules plated four days after pollination increased more in length on 3 % than on 9 % sucrose. Three percent sucrose had a mean percent increase in ovule length of 22 ± 1.12 %, as compared to a 19 ± 1.13 % increase for 9 % sucrose. For hormone ratios, 0.1 mg/L NAA:1 mg/L BA, 0.5:0.5, and 0.5:0 had the most increase in ovule length with an average percent of 23 ± 1.34, 23 ± 1.38, and 24 ± 1.12 %, respectively. Hormone ratio 0:0 had the least increase at 19 ± 1.11 %. At two days after pollination, B5 had the most increase in ovule length with a mean percent of 27 ± 2.19 %, followed by B5S with an average of 24 ± 1.49 % increase, and MS and NN at 22 ± 2.07, and 22 ± 1.29 %, respectively. At four days after pollination, ovules on the average grew better on B5S (21 ± 1.17 %) than on the MS (20 ± 1.11 %) salt base (Table 4). The results were not statistically different due to their standard errors.

The hormone ratio of 0.5:0.1 had the highest percentage increase in ovule length for experiment two

Table 4. Average percent increase in ovule length one week after plating for ovules plated at two and four days after pollination for experiment one.

DAP	Sucrose (%)	Medium	Genotype*	NAA:BA (mg/L)			
				0.1:1	0.5:0.5	0.5:0	0:0
2	3	MS	1	15**	19	18	13
	9			20	17	24	9
	3	B5S		27	18	27	18
	9			27	20	30	10
	3	MS	2	43	27	24	15
	9			22	34	24	24
	3	B5S		27	22	31	17
	9			24	30	30	24
	3	NN	3	23	19	27	21
	9			23	22	22	27
	3	B5		27	24	23	29
	9			38	45	44	24
	3	NN	4	20	28	20	18
	9			33	13	19	14
	3	B5		25	30	21	19
	9			19	30	22	14

Continued ...

Table 4. Continued.

DAP	Sucrose (%)	Medium	Genotype*	NAA:BA (mg/L)			
				0.1:1	0.5:0.5	0.5:0	0:0
4	3	MS	2	22	22	22	16
	9			13	22	18	23
	3	B5S		20	27	29	27
	9			16	20	27	22
	3	MS	5	15	12	17	11
	9			14	13	15	13
	3	B5S		21	18	21	14
	9			13	12	16	11
	3	MS	1	27	30	27	24
	9			24	24	23	21
	3	B5S		25	21	26	28
	9			29	22	29	17

*Genotypes 1) HA89 x Hy903; 2) RHA299 x Hy903; 3) Hy894 x Hy903;
4) HA99 x CM400; 5) Hy903 x RHA297.

**Percent of initial length; initial lengths were: 2 DAP, Genotype 1)
5.9 ± .07; 2) 4.5 ± .09; 3) 4.7 ± .09; 4) 5.0 ± .10. At 4 DAP,
Genotype 1) 7.1 ± .04; 2) 6.5 ± .05; 5) 7.2 ± .06.

(Table 5). Overall though, experiment two gave inconclusive results with respect to the best hormone ratio. The cross RHA299 x RHA297 had a higher percentage increase in ovule length over Hy894 x RHA297, which in turn had more of an increase than Hy903 x RHA299.

The two-step embryo rescue procedure was first used in experiment three. The three genotypes Hy894, HA300 and CM400 were all sib-mated. Hy894's ovules increased most in length on hormone ratios: 0.5:0.1, 1:0.5, 1:0, 0.1:0.1, and 0:0 (Table 6). Hormone ratios that had a higher increase in ovule length for HA300 were: 0:0, 1:0, and 0.1:0. CM400 had it's greatest increase in ovule length with 1:1, 0.1:0, and 0.5:0.1 hormone ratios (Table 6). Embryo length after two weeks increased more for hormone ratio 0.1:0.1 on both 9 and 12 % sucrose for Hy894. For HA300, embryo lengths increased more at hormone ratio 1:0.5, for the 12 % sucrose media and 0.1:0.1 for the 9 % sucrose media. Hormone ratio 0.1:0.1 had the most increase in embryo length on the 12 % sucrose media for CM400, while on the 9 % sucrose media 0.5:0.5 was the best for embryo growth. 0.1:0.1 and 1:0 had the greatest increase in embryo growth considering all three genotypes combined (Table 7).

In experiment three, both normal embryo development and abnormal embryo development were noted. Embryos from

Table 5. Average percent increase in length after one week of culture for ovules plated four days after pollination on a MS salt base and 9% sucrose for experiment two.

NAA:BA Ratio*	Genotype		
	RHA299 x RHA297	Hy894 x RHA297	Hy903 x RHA299
0.5:1	8**	0	0
0.5:0.5	16	7	0
0.5:0.1	24	3	4
0.5:0	13	2	0
0.1:1	19	0	0
0.1:0.5	15	0	0
0.1:0.1	13	0	0
0.1:0	7	7	0
0:1	18	5	0
0:0.5	10	7	0
0:0.1	10	0	0
0:0	17	7	2

*mg/L.

**Percent of initial length; initial lengths were: RHA299 x RHA297, 5.6 ± .12; Hy894 x RHA297, 5.8 ± .08; Hy903 x RHA299 6.2 ± .09.

Table 6. Average percent increase in length after one week of culture
for ovules plated four days after pollination on B5 salt
medium for experiment three.

NAA:BA (mg/L)	Sucrose (%)	Genotype			
		Hy894	HA300	CM400	Mean
1:1	9	10*	5	16	10
	12	12	9	20	14
	Mean	11	7	18	12
1:0.5	9	15	9	15	13
	12	13	11	12	12
	Mean	14	10	14	13
1:0.1	9	11	4	10	8
	12	12	13	17	14
	Mean	11	8	13	11
1:0	9	14	17	11	14
	12	14	13	16	14
	Mean	14	15	14	14
0.5:0.5	9	13	7	12	11
	12	13	12	10	12
	Mean	13	9	11	11
0.5:0.1	9	16	—	14	15
	12	14	8	16	13
	Mean	15	—	15	14
0.5:0	9	9	9	12	10
	12	10	12	10	11
	Mean	9	10	11	10
0.1:0.1	9	14	10	16	13
	12	14	9	10	11
	Mean	14	10	13	12
0.1:0	9	10	18	16	15
	12	13	9	14	12
	Mean	12	14	15	14
0:0	9	14	15	14	14
	12	14	17	12	14
	Mean	14	16	13	14

*Percent of initial length; initial lengths were: Hy 894, 5.7 ±
0.4; HA 300, 7.9 ± 0.6; CM 400, 8.2 ± 0.3.
— Contaminated.

Table 7. Average percent increase in length after one week of culture for embryos plated four days after pollination on B5 salt medium for experiment three.

NAA:BA (mg/L)	Sucrose (%)	Genotype			
		Hy894	HA300	CM400	Mean
1:1	9	104*	76	89	90
	12	117	130	—	124
	Mean	109	99	—	104
1:0.5	9	65	—	78	72
	12	64	174	47	95
	Mean	64	—	64	84
1:0.1	9	148	126	55	110
	12	94	126	106	109
	Mean	125	126	76	110
1:0	9	150	—	—	150
	12	155	136	—	146
	Mean	152	—	—	148
0.5:0.5	9	150	63	134	116
	12	87	126	84	99
	Mean	116	95	106	108
0.5:0.1	9	135	—	56	96
	12	78	83	45	69
	Mean	111	—	50	83
0.5:0	9	180	88	61	110
	12	85	61	8	51
	Mean	124	75	30	81
0.1:0.1	9	271	150	45	155
	12	200	121	117	146
	Mean	239	138	71	151
0.1:0	9	—	89	81	85
	12	65	100	14	60
	Mean	—	94	49	73
0:0	9	163	84	18	88
	12	83	—	56	70
	Mean	124	—	36	79

*Percent of initial length; initial lengths were: Hy 894, 2.1 ± .06; HA 300, 3.9 ± .15; CM 400, 4.0 ± .16.
— Contaminated.

the lower cytokinin and auxin levels had the highest
percentage of normal embryo development (Table 8). The 9 %
sucrose media promoted a higher increase in embryo growth
than did the 12 % sucrose media. Although 9 and 12 %
sucrose were within one percentage point of each other for
normal embryo development (Table 8).

Greco et al. in 1984 induced abundant callus growth
from different sunflower explants (leaf and cotyledon
pieces, shoot apices and hypocotyl segments) using the
cytokinin 6-benzylaminopurine (6-BAP) alone. However,
using the auxin 2,4-dichlorophenoxyacetic acid (2,4-D)
alone produced poorly developed, nodular callus. In
experiment three, substantially more callus developed from
the embryos that were either higher in cytokinin (BA) or in
auxin (NAA) (Table 9). However, when comparing the same
ratio of cytokinin to auxin, the cytokinin always had a
higher percentage of callus development compared to the
auxin. The lower sucrose level (9 %) had an increase in
callus formation compared to the 12 % sucrose level (Table
9).

Experiment four had much missing data due to
contamination, and genotype RHA297 had the only complete
data. For this genotype, increase in embryo lengths and
percentages of normally developed embryos were not
statistically different because of their standard errors.

Table 8. Percent of embryos that developed normally when plated four days after (DAP) pollination on a B5 salt base for experiment three.

NAA:BA (mg/L)	Sucrose (%)	Genotype			
		Hy894	HA300	CM400	Mean
1:1	9	0*	40	17	18
	12	0	0	10	3
	Mean	0	20	14	11
1:0.5	9	0	38	0	10
	12	0	29	18	17
	Mean	0	33	9	13
1:0.1	9	20	56	8	26
	12	22	40	0	20
	Mean	21	47	4	23
1:0	9	11	---	---	11
	12	25	40	---	32
	Mean	19	---	---	26
0.5:0.5	9	0	30	0	10
	12	18	40	0	18
	Mean	10	35	0	15
0.5:0.1	9	17	---	9	13
	12	22	80	8	35
	Mean	19	---	9	26
0.5:0	9	27	80	25	42
	12	42	89	50	58
	Mean	35	84	38	50
0.1:0.1	9	50	75	25	47
	12	30	83	17	36
	Mean	41	79	21	42
0.1:0	9	---	60	8	32
	12	42	70	17	41
	Mean	---	65	13	38
0:0	9	33	100	36	56
	12	30	---	25	27
	Mean	32	---	30	44

Mean percent of normally developed embryos/sucrose level:
 9% = 28% ± 5.32.
12% = 29% ± 4.82.
*Percent of normally developed embryos.

Table 9. Percent of embryos that had callus growth one week after
          embryo excision when plated four days after pollination
          (DAP) on a B5 salt base for experiment three.

NAA:BA (mg/L)	Sucrose (%)	Genotype			
		Hy894	HA300	CM400	Mean
1:1	9	33*	89	100	72
	12	25	80	75	59
	Mean	29	84	87	65
1:0.5	9	33	30	92	53
	12	33	70	67	58
	Mean	33	50	79	55
1:0.1	9	55	50	92	68
	12	8	50	67	41
	Mean	30	50	79	54
1:0	9	36	——	——	36
	12	75	60	25	53
	Mean	57	——	——	49
0.5:0.5	9	75	70	83	76
	12	25	80	92	65
	Mean	50	75	88	71
0.5:0.1	9	67	——	75	71
	12	25	40	92	53
	Mean	46	——	83	60
0.5:0	9	17	25	67	38
	12	17	40	25	26
	Mean	17	33	46	32
0.1:0.1	9	25	10	92	44
	12	8	33	75	39
	Mean	17	21	83	42
0.1:0	9	0	40	67	35
	12	0	20	17	12
	Mean	0	30	42	24
0:0	9	8	30	0	8
	12	8	——	8	12
	Mean	8	——	4	11

*Percent of embryos with callus growth.

Although the lowest auxin concentration was the most promising in both embryo length and percentage of normally developing embryos. The 9 % sucrose media enhanced embryo growth more than the 12 % sucrose at the 0.1:0 and 0:0 hormone level (Table 10). Although the 12 % was superior over 9 % sucrose in embryo growth at the 0.5:0 and 0.1:0.1 hormone ratio. The 9 % also had a higher percentage of normal embryos compared to the 12 % sucrose media. In experiment four the percent of callus formation was insignificant in all treatments (Table 10).

Objective One

Results of the preliminary test showed that use of the two-step embryo rescue procedure made survival of very young (globular and heart shaped) embryos possible. From the in vivo checks of 18 replications of 15 embryos per replication, the embryos averaged only 0.62 mm in length. According to observations of this investigator and from the scale set by Espinasse et al, the embryos were in the beginning stages of the heart shaped stage when the ovules were first plated. Of the 18 replications of 180 embryos per replication, the embryos averaged 1.94 mm in length when excised from the ovules. This coincides with the torpedo or early vascular stage of embryo development. Detailed analysis of requirements for successful use of

Table 10. Average increase in length of RHA297 embryos two weeks after excision and percent of normal embryo development and callus growth for ovules plated four days after pollination on a B5 salt base, for experiment four.

NAA:BA (mg/L)	Sucrose (%)	Percent embryo increase		Normal embryo development	Callus growth
		Initial size (mm)	%	%	%
0.5:0	9	3.5	103	83	0
	12	3.3	148	58	17
0.1:0.1	9	2.9	114	83	0
	12	2.1	143	50	0
0.1:0	9	2.3	196	92	0
	12	3.0	83	67	0
0:0	9	2.7	163	75	0
	12	3.3	73	75	0
Mean	9	$2.9\pm.25$	144 ± 21.69	83 ± 3.47	0
	12	$2.9\pm.28$	112 ± 19.62	63 ± 5.42	4 ± 4.25
	Mean	$2.9\pm.18$	128 ± 14.85	73 ± 4.93	2 ± 2.13

this procedure was accomplished in the study of objective one.

Results of the objective one study showed that differences in growth of the five genotypes were statistically significant. For two of the genotypes, Hy894 and HA99, significant medium effects for percent survival and embryo development were observed (Table 11). It was found that Hy894's embryo development was slower on the B5 medium compared to the other three media. For HA99 it was observed that embryos did not develop as quickly on either MS or NN as on B5 or B5S (Figure 1; see appendices A, B, C, & D for detailed data). Percent survival on B5S was significantly better than the other three media for Hy894. Another varietal difference was in extent of callus formation. Callus formation was significantly increased on MS and NN on days 18 and 25 for genotype RHA297, compared to B5 and B5S. Hy894 also had a significant increase in callus formation on day 25, but just on NN and not the other three salt bases (Figure 2). Results for RHA299 showed that callus formation at 6 and 9 % sucrose was significantly higher than for 12 % on day 18, although on day 25 only 6 % sucrose was significantly higher for callus formation compared to 9 and 12 % sucrose. Callus formation for HA99 at 6 % sucrose was significantly higher on both day 18 and day 25 compared to 9 and 12 %. The other three

Table 11. A chi-square analysis of main effects and interactions for five genotypes at 11, 18, and 25 days after pollination (DAP) for objective one.

Analysis	Genotype	DAP	Factor	Chlorophyll Rating	% Survival	Ending Stage of Development				% Callus	% Shoots	% Roots	% Shoots & Roots
						Globular	Heart	Torpedo	Vascular				
Main Effects			Medium		*	*	**			**			**
			Sucrose	*						**	**	**	**
			Genotype	**	**	**	**	**	**	**	**	**	**
Interaction Effects	Hy894	11	Medium				*						
			Sucrose				*						
		18	Medium			*	*						
			Sucrose								**	**	**
		25	Medium			*				**			
			Sucrose								**	**	**
	Hy903	11	Medium										
			Sucrose	*									
		18	Medium										
			Sucrose								**	**	**
		25	Medium										
			Sucrose								**	**	**
	RHA299	11	Medium										
			Sucrose										
		18	Medium										
			Sucrose							**			
		25	Medium										
			Sucrose							*			
	RHA297	11	Medium										
			Sucrose					*					
		18	Medium							**			
			Sucrose								**	**	**
		25	Medium							**			
			Sucrose		*						**	**	**
	HA99	11	Medium				*	**	*				
			Sucrose										
		18	Medium										
			Sucrose							*	**	**	**
		25	Medium										
			Sucrose							**	**	**	**

*,** Chi-square analysis found main effects and interactions statistically significant at 0.05 and 0.01 levels of probability, respectively.

Figure 1. Percent Embryo Development for the Genotype HA99 on Four Media
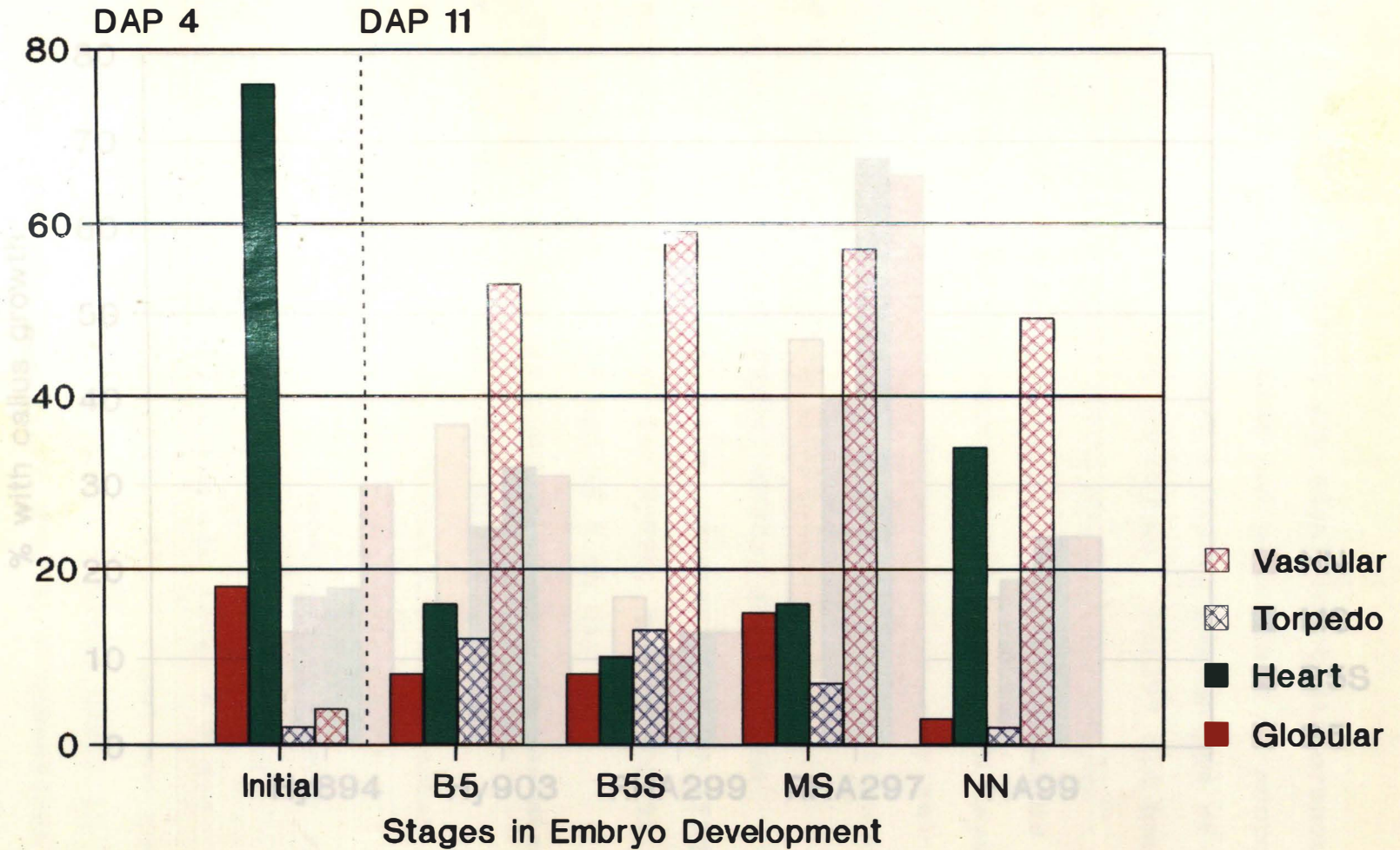
* MS Globular; ** NN Heart

Figure 2. Embryos with Callus Growth Effected by Media and Genotype
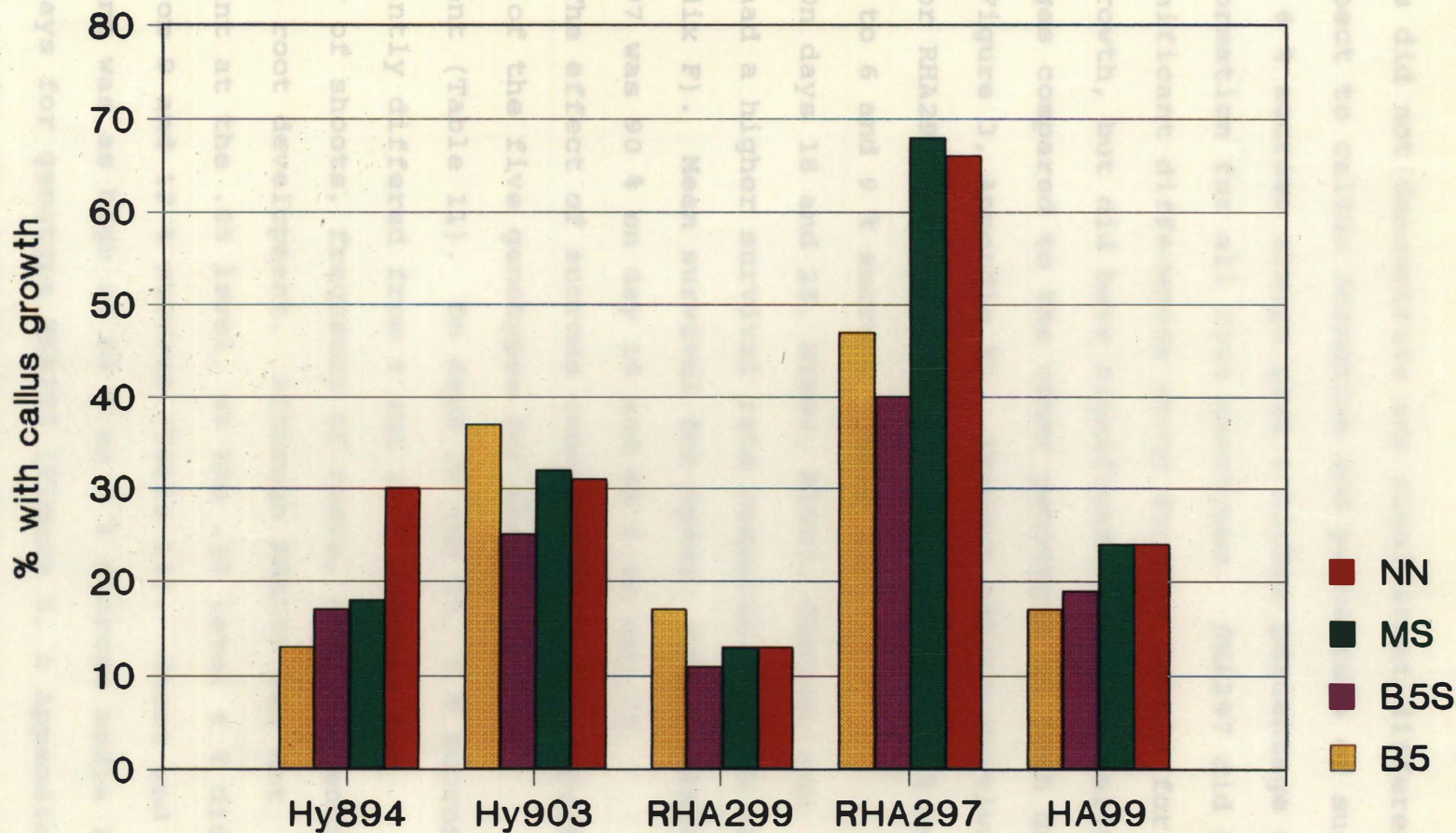
genotypes did not demonstrate any significant differences with respect to callus formation and percentage of sucrose. However, 6 % sucrose always gave a higher percentage of callus formation for all five genotypes. RHA297 did not have significant differences among sucrose levels for callus growth, but did have significantly higher callus percentages compared to the other genotypes on both days 18 and 25 (Figure 3, Appendix E). Percent embryo survival on day 25 for RHA297 was significantly better for 12 % sucrose compared to 6 and 9 % sucrose (Table 11).

On days 18 and 25, HY894, HY903, RHA299, and RHA297, had a higher survival rate compared to HA99 (Figure 4, Appendix F). Mean survival for Hy894, Hy903, RHA299, and RHA297 was 90 % on day 18 and 88 % on day 25.

The effect of sucrose concentration was consistent for four of the five genotypes for shoot and root development (Table 11). On days 18 and 25, 6 % sucrose significantly differed from 9 and 12 % sucrose for frequency of shoots, frequency of roots, and frequency of shoot and root development. Although RHA299 was not significant at the .05 level, at the .10 level 6 % did differ from 9 and 12 % sucrose (Table 11). Shoot and root development was as high as 64 % on 6 % sucrose media in only 18 days for genotype RHA297 (Figure 5, & Appendices G, H, & I). On day 11, 6 % sucrose was significantly better

Figure 3. Embryos With Callus Growth Effected by % Sucrose and Genotype
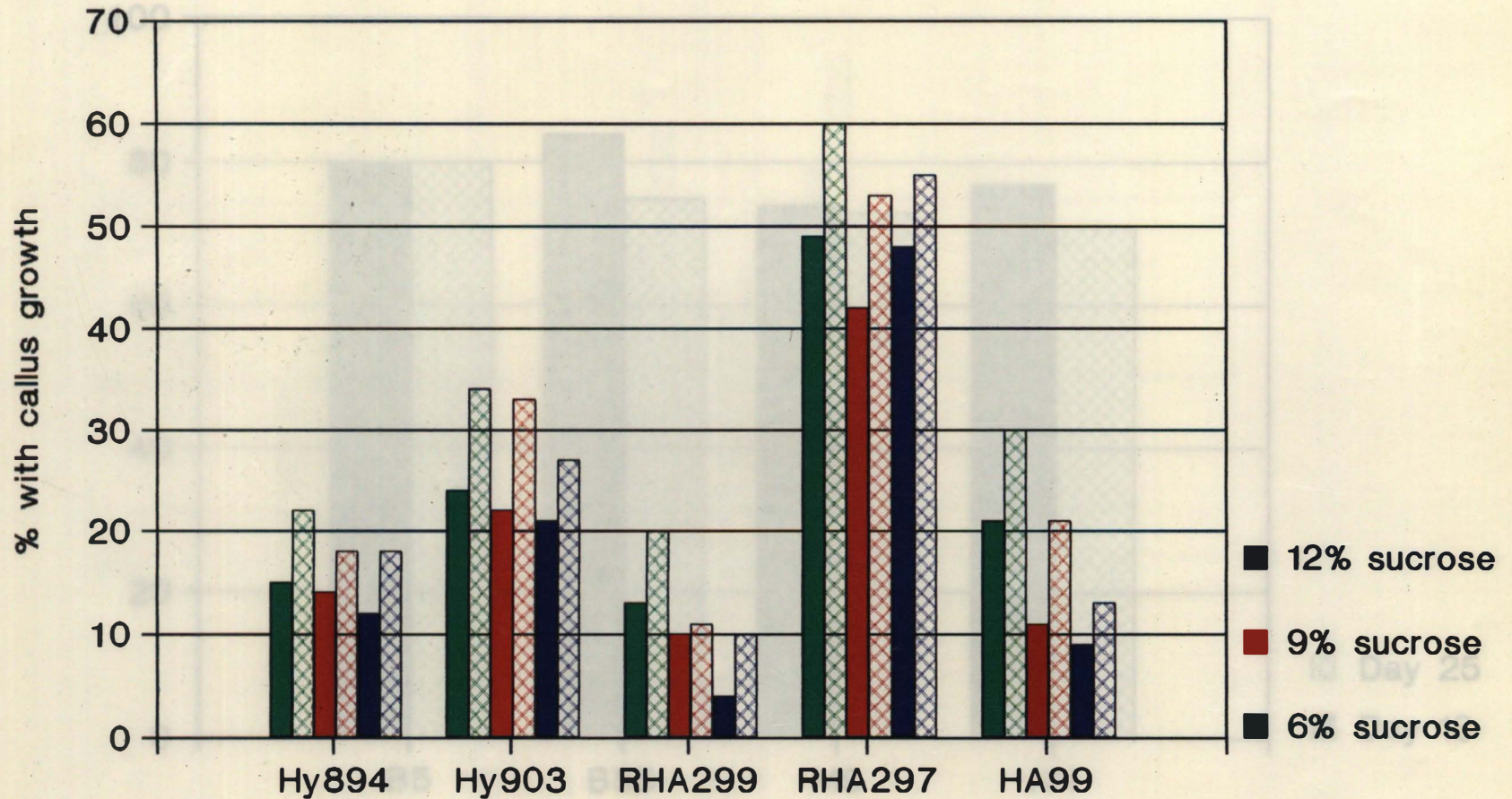
solid = day 18; pattern = day 25

Figure 4. Percent Embryo Survival for the Genotype HA99 on Four Media

Figure 5. Percent Embryos Developing Both Shoots and Roots
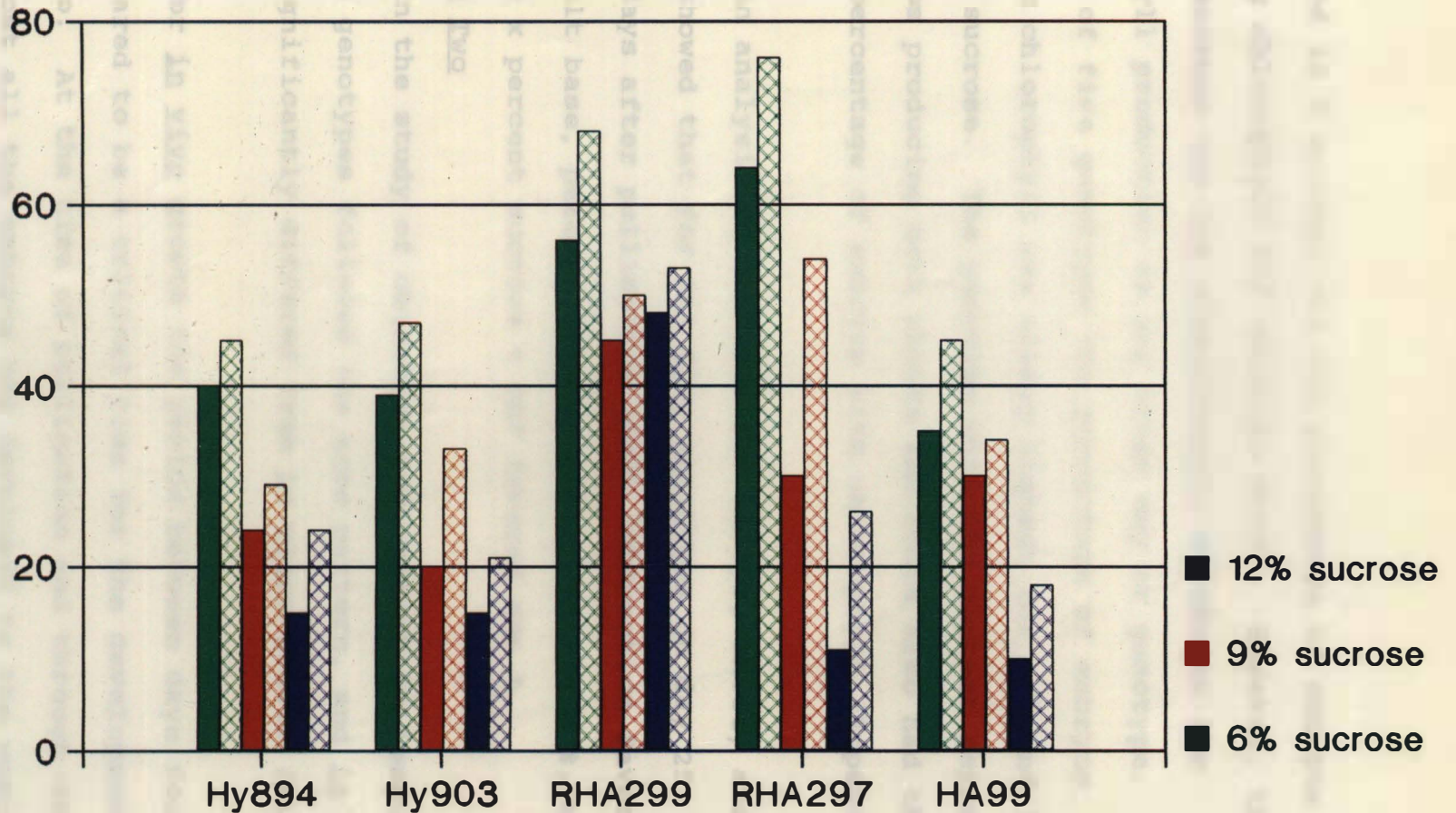
solid = day 18; pattern = day 25

than 9 and 12 % sucrose for the percentage of embryos
producing chlorophyll for genotype Hy903. However, the
percent sucrose was not significantly different for
chlorophyll production on any other day or genotype. In
four out of five genotypes the percentage of embryos
producing chlorophyll was always highest for 6 % and lowest
for 12 % sucrose. The genotype with the highest percentage
of embryos producing both shoots and roots also had the
highest percentage of embryos with chlorophyll (Appendix
J).

An analysis of variance for embryo, shoot, and root
length, showed that for all five genotypes on day 25; the
media x days after pollination (DAP) interaction favored
the NN salt base, percent sucrose x DAP favored 6 %, and
the media x percent sucrose x DAP favored NN6 %.

Objective Two

In the study of objective two, growth in length of
all three genotypes followed the same pattern, and in vivo
growth significantly differed from in vitro growth (Figures
6,7 & 8).

For in vivo growth the period between days four and
five appeared to be a critical time for the development of
the embryo. At the time of pollination and through day
five, almost all the embryos had developed to the vascular
stage (Figure 9). This critical time in embryo development

Figure 6. In vitro vs. in vivo embryo growth for genotype Hy894
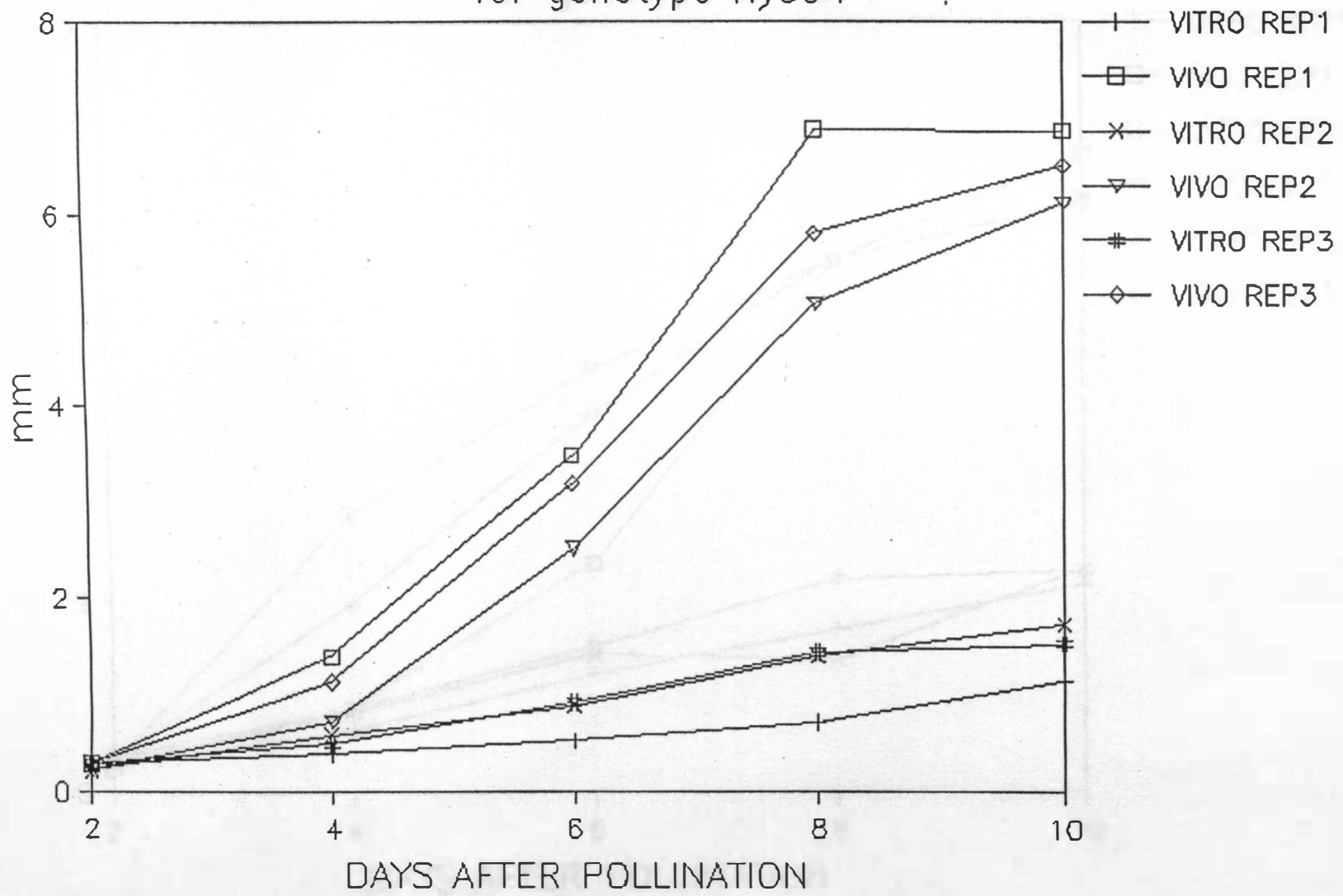
Figure 7. In vitro vs. in vivo embryo growth for genotype RHA274
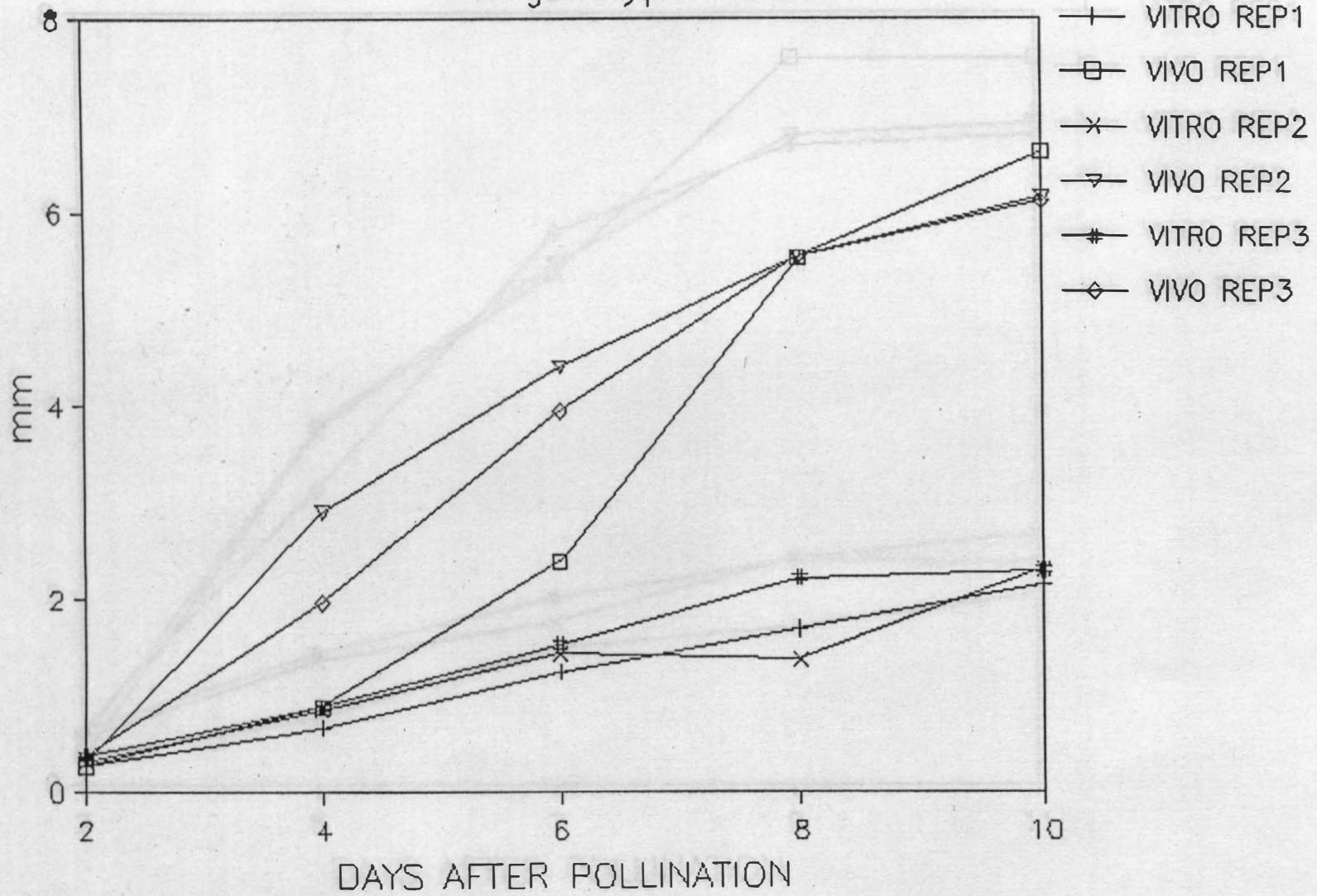
Figure 8. In vitro vs. in vivo embryo growth for genotype HA89
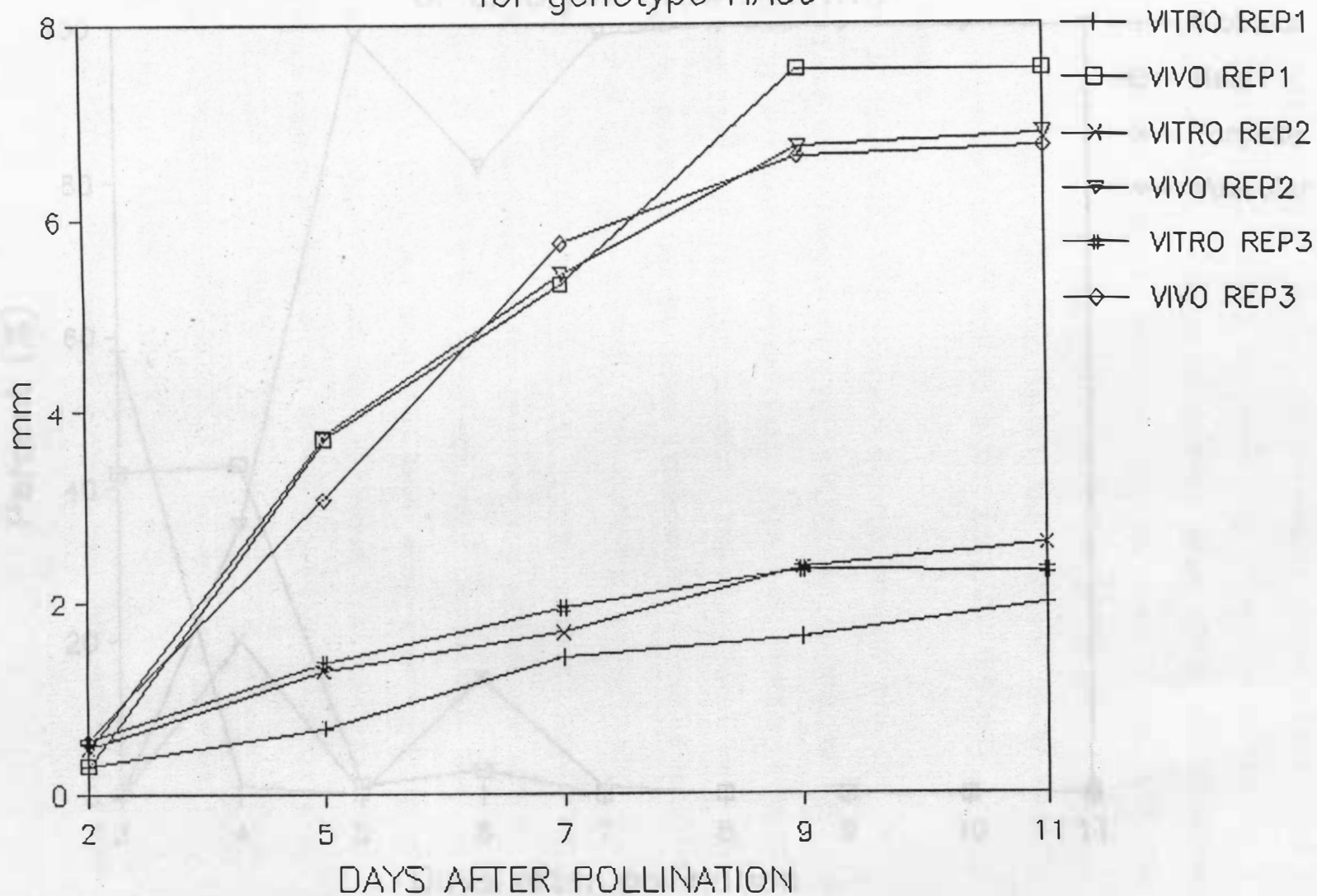
Figure 9. Change in developmental stage of embryos grown in vivo
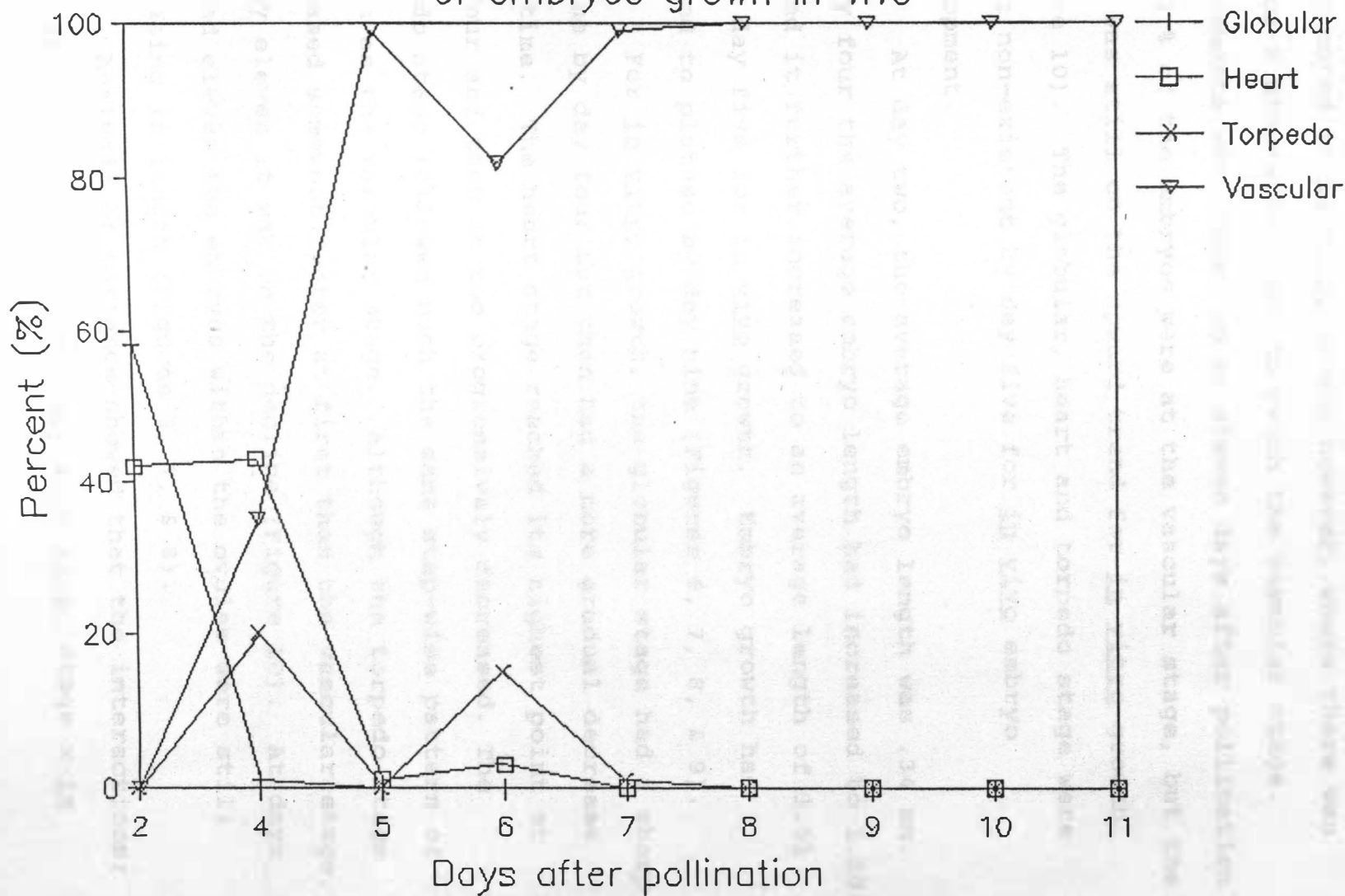
is not noted in <u>in</u> <u>vitro</u> growth however, where there was
more of a step-wise climb to reach the vascular stage.
Measurements were taken up to eleven days after pollination
and 57 % of the embryos were at the vascular stage, but the
curve was still on the upward trend for <u>in</u> <u>vitro</u> growth
(Figure 10). The globular, heart and torpedo stage were
almost non-existent by day five for <u>in</u> <u>vivo</u> embryo
development.

At day two, the average embryo length was .34 mm.
By day four the average embryo length had increased to 1.50
mm, and it further increased to an average length of 3.51
mm by day five for <u>in</u> <u>vivo</u> growth. Embryo growth had
started to plateau by day nine (Figures 6, 7, 8, & 9).

For <u>in</u> <u>vitro</u> growth, the globular stage had a sharp
decline by day four but then had a more gradual decrease
over time. The heart stage reached its highest point at
day four and then it too progressively decreased. The
torpedo stage followed much the same step-wise pattern of
growth as the vascular stage. Although the torpedo stage
increased somewhat faster at first than the vascular stage,
by day eleven it was on the decline (Figure 10). At days
ten and eleven the embryos within the ovules were still
increasing in length (Figures 6, 7, & 8).

Analysis of variance showed that the interactions;
day x stage, day x <u>in</u> <u>vitro</u>, day x <u>in</u> <u>vivo</u>, stage x <u>in</u>

Figure 10. Change in developmental stage
of embryos grown in vitro
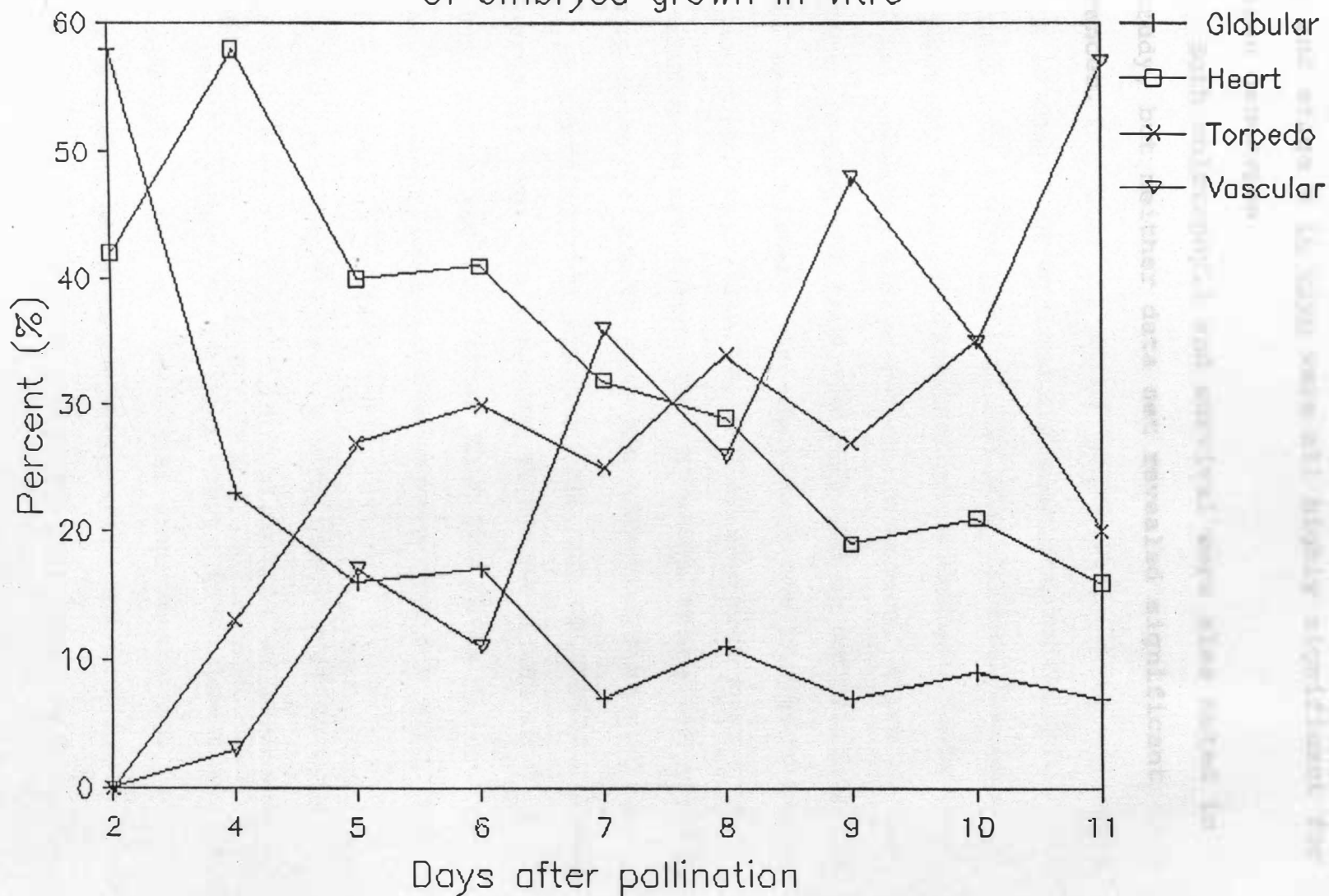
<u>vitro</u>, and stage x <u>in</u> <u>vivo</u> were all highly significant for all three genotypes.

       Both chlorophyll and survival were also noted in this study, but neither data set revealed significant differences.

DISCUSSION

Preliminary Test

From experiments one and two, it was observed that
embryos smaller than 1.0 mm in length would soon desiccate
and die if they were excised and plated directly.
Conversely, if ovules were plated and the embryos were
never excised, these embryos too would perish. This then
led to the two-step embryo rescue procedure, where ovules
were first plated and this was followed by excision of the
embryos one week later. In experiment one the best auxin
level could not be evaluated, and it appeared that
cytokinins were not essential. Although there was a slight
decrease in ovule growth when the hormones were excluded
(0:0), it appeared that hormones may not be needed. Again
in experiment two, the presence of either auxin or
cytokinin alone was not better than the check (0:0).
However, in experiment two the hormone ratio 0.5:0.1 was
best for enhancing ovule growth.

In experiment three, a wide variety of hormone
ratios seemed to be suitable for promoting an increase in
embryo length. However, normal embryo development occurred
at a much higher percentage on the lower auxin and
cytokinin concentrations as compared to higher
concentrations. Comparison of the lower auxin and

cytokinin concentrations for callus induction showed that the lowest auxin concentration had the lowest percentage of callus formation. The 9 % sucrose level demonstrated a percentage of callus formation higher than the 12 % sucrose level.

Experiment four showed that auxin had a beneficial influence for embryo growth and that a lower auxin concentration will give a higher percentage of normal developing embryos. Cytokinin was the least beneficial for normal embryo growth and development. For embryo growth, a sucrose x hormone interaction was noted. At 9 % sucrose 0:0 was the best, but on the 12 % sucrose media, 0.1:0.1 hormone ratio was the best. The sucrose levels did not show any difference in callus formation.

In both experiment three and four, the test of sucrose levels showed that 9 % was superior for increasing embryo growth compared to 12 % sucrose. However, it gave inconclusive evidence in experiment three, for distinguishing between 9 and 12 % sucrose for percentage of normal embryo development. In experiment four the results indicated that 9 % sucrose had a higher percentage of normal embryo development.

From the preliminary test's results and previous documentation, one could conclude that a low auxin concentration is more beneficial than a high auxin

concentration for the normal development of the cultured embryo. Low auxin combined with low cytokinin is also beneficial for normal embryo development. However, in agreement with the literature for sunflowers (Greco et al, 1984), cytokinins promoted more callus formation than did auxins. Thus the 0.1 mg/L NAA and 0 mg/L BA proved to be a suitable hormone concentration for the survival of globular and heart shaped embryos for the two-step embryo rescue procedure.

Nine and 12 % sucrose media gave inconclusive evidence for normal embryo development, but gave the indication that 9 % may be superior. This then gave premise to add a third lower sucrose level (6 %), while continuing both the 9 and 12 % sucrose in the following objective. It was apparent in the preliminary test that there was a genotype difference for ovule and embryo growth, normality and for callus growth.

Objective One

Sunflower embryos were rescued at a younger age than had previously been accomplished, mainly due to a proper hormone and sucrose level, and the two-step embryo rescue procedure.

To help clarify the results, an explanation of the four salt bases is necessary. The four types of media were chosen for a specific purpose. B5 basal medium contains

amino acids where the other three basal media do not. B5S has the same amount of macronutrients as B5 but lacks amino acids and vitamins. B5S and NN have macronutrients that are closely related, because the amounts of nitrogen in both are equal, but the form of nitrogen is not the same. B5S has a higher ratio of NO_3 to NH_4 compared to NN, while NN and MS have a similar ratio of NO_3 to NH_4, but MS is considered a high-salt base because it has a larger amount of nitrogen compared to NN and the other salt bases. B5S, MS, and NN all have vitamins added to compensate for the vitamins in B5 (Table 1).

Significant genotype differences were observed in the study identified as objective one. For HA99, a higher ratio of NO_3 compared to NH_4 was beneficial to the development of younger embryos. However, the difference in NO_3/NH_4 ratio did not affect development of the other four genotypes. The slower development on the B5 salt base for Hy894 maybe due to the addition of amino acids to the medium, although B5 was not significantly slower in embryo development than the other four genotypes. Percent survival for Hy894 on the B5S medium was significantly better than the other three media. Again this could be due to the form of nitrogen in MS and NN, and to the presence of amino acids in B5. Since the B5 salt medium was significantly slower in embryo development and lower in

survival for Hy894, addition of amino acids to the medium is not needed.

Percentages of callus formation were highest on MS and NN for HY894 and RHA297. The trend for callus formation due to sucrose levels was that a lower sucrose level, such as 6 %, promotes an increased callus percentage compared to higher levels of sucrose.

Among the five genotypes HA99's embryo survival rate was the lowest.

It has been well documented that embryo growth and germination are affected by the sucrose concentration, that higher osmotic values obtained with sucrose prevent precocious germination of differentiated excised plant embryos, and that lower concentrations of sucrose were better for the growth of older and larger embryos (Narayanaswami and Norstog, 1964). This was substantiated for Helianthus species by Chandler and Beard in 1983, when they found that low-sucrose media could not be used to initially culture very small excised embryos. They also found that a higher concentration of sucrose, either 120 or 150 g/L, decreased the incidence of premature germination.

However, in objective one 6 % sucrose was significantly better than 9 and 12 % sucrose for shoot and root development. This does not necessarily contradict the literature, because when the embryo was first plated it was

still contained in the ovule, which already had a high osmotic value. A week later, when the embryos were excised and again plated on 6, 9 and 12 % sucrose media, they were much more developed. This is evident by the size of the embryos upon excision from the ovules. This, then, agrees with literature reports showing that for older and larger embryos the percentage of germination and percentage of embryos producing roots are improved by lowering the sucrose concentration (Chandler and Beard, 1983). In only 18 days a high percentage of embryos on the lower sucrose level (6 %) had produced both shoots and roots. As can be seen in figure 5, the 12 % sucrose media had a marginally higher frequency of shoots and roots for RHA299 compared to 9 %. However, for 12 % sucrose on days 18 and 25 it had 22 and 26 % contaminated embryos, respectively, compared to only 4 and 8 % for the embryos plated on the 9 % sucrose media. This may be why RHA299 did not follow the same pattern as the other genotypes.

There was a positive relationship among shoot and root development, chlorophyll production, and sucrose level, for four out of the five genotypes. The lowest sucrose level (6 %) had the highest percentage of embryos producing both shoots and roots and had the highest percentage of embryos with chlorophyll.

Objective Two

Embryos of all three genotypes did not grow as rapidly in vitro as in vivo during the first eleven days after pollination. In vivo growth starts to plateau by day nine, where in vitro growth is still increasing. Embryo development progressed faster in in vivo compared to in vitro conditions.

Objective two gave some important information for objective one. Because of the low percentage of death and the high percentage of vascular stage embryos for in vitro growth in objective two, it appears that one could save embryos at an even younger age than was done in objective one. The average starting size of embryos in objective one was 0.62 mm in length, and only 0.34 mm in length for objective two. Since the embryos were still growing both in length and in developmental stages by day eleven one could also leave the embryos in their ovules longer than a week after plating.

SUMMARY

It is generally assumed that failure of interspecific hybridization in sunflower is due to abortion of the hybrid embryos during early developmental stages (Chandler and Beard, 1983). Embryo rescue has been the solution to this problem, but embryos in sunflower have only been successfully rescued from the torpedo stage or later.

This research had two objectives; to improve current in vitro techniques to rescue embryos younger than the torpedo stage, and to show the comparison of both in vitro and in vivo embryo development the first eleven days after pollination. This work consisted of a preliminary test and an experiment for each objectives one and two. The preliminary test was further divided into four experiments.

Experiment one had four ratios of auxins to cytokinins, each within a sucrose level, either 3 or 9 % sucrose, and four salt bases; B5, B5S, MS & NN. Ovules were plated two to four days after pollination (DAP) and lengths and widths were measured at the time of plating and a week later. Experiment two incorporated in vivo checks. Only MS salt base and 9 % sucrose were used, although twelve hormone ratios were added. Ovules were plated four

DAP and measured at the time of plating and six days later. Hormone ratios were reduced to ten combinations for experiment three. Twelve percent sucrose was included along with the 9 % sucrose and only B5 salt base was used. In experiment three, the two-step embryo rescue procedure was employed for the first time (ovules were first plated followed by excision of the embryos a week later). Ovules were measured at plating and a week later. Embryos were measured at excision and then every seven days for two more weeks. Experiment four also used a B5 salt base and 9 and 12 % sucrose; however the hormone ratios were reduced to four.

For the first objective four basic media were used; B5, B5S, MS, and NN with three levels of sucrose; 6, 9, and 12 %. Hormone concentration was 0.1 mg/L NAA and 0 mg/L cytokinin. Ovules were plated one to four days after pollination, then they were excised and plated on fresh media. Measurements were performed by the use of a microscope for length. Other measurements were; shape, presence of chlorophyll, callus, shoots, or roots, and whether the embryo survived. Measurements were repeated every seven days for two more weeks.

For objective two, ovules were plated on a NN salt base, with 0.1 mg/L NAA and 6 % sucrose two days after pollination. Measurements at day 4, 6, 8, and 10 of

length, shape and presence of chlorophyll were performed both _in_ _vivo_ and _in_ _vitro_ for 25 embryos for genotypes Hy894 and RHA274. The same measurements were done for HA89, only at days 5, 7, 9, and 11.

Experiments one and two of the preliminary test showed that embryos had to at some time be excised from the ovules or they would perish. This led to the two-step embryo rescue procedure used in experiment three. In experiment one the most suitable auxin level could not be evaluated, but it did appear that cytokinins were not essential. For experiment two, 0.5 mg/L NAA:0.1 mg/L BA hormone ratio was the most promising. Experiment three showed that normal embryo development occured more on lower auxin and cytokinin levels. Both high auxin and cytokinin levels gave increased percentages in callus induction. It was apparent though that a lower auxin concentration had a lower percentage of callus growth compared to the low cytokinin. The lower sucrose level (9%) demonstrated a higher percentage of callus growth than the 12 %. Experiment four showed that a lower auxin level gives a higher percentage of normal embryos than does a lower cytokinin level. For callus formation, sucrose levels did not show any difference. Both experiment three and four showed that 9 % sucrose was superior for percentage increase in embryo growth compared to 12 % sucrose.

For objective one, sunflower embryos were successfully rescued at a younger age than had previously been accomplished. There were varietal differences for the form of nitrogen (NO_3 or NH_4) required for maximum growth, for the percentage of callus growth, and for embryo survival. It was found that amino acids were not necessary in the medium. The 6 % sucrose level was significantly better for shoot and root development, but this low sucrose level also gave an increase in callus growth. By only day 18 up to 64 % of the embryos in one genotype had developed both shoots and roots.

For objective two, it was found that there were significant differences between in vivo growth and in vitro growth. In vivo growth started to plateau on day nine, where as in vitro growth was still increasing at this date. Objective two showed that embryos could be saved at just two days after pollination, with an average embryo size of .34 mm. Objective two also showed that embryos were still increasing in length and development at eleven days after pollination. Thus for the two-step embryo rescue procedure one could leave the embryos in the ovules longer than a week and possibly could increase the number of embryos developing into plants.

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Appendix A. Frequency of embryos at the globular stage at 11, 18, or 25 days after pollination (DAP) that were plated on a particular salt base and sucrose level.

DAP	Genotype		Hy894	Hy903	RHA299	RHA297	HA99
11	B5	6%	2*	4	2	2	9
		9	7	0	0	2	7
		12	7	7	0	0	9
	B5S	6	4	7	2	0	7
		9	2	13	2	5	13
		12	2	11	0	0	4
	MS	6	7	7	2	0	2
		9	7	9	0	2	18
		12	4	4	2	0	7
	NN	6	4	2	0	0	2
		9	7	2	2	2	4
		12	4	16	0	0	2
18	B5	6	0	2	3	0	0
		9	4	0	0	0	0
		12	0	0	0	0	0
	B5S	6	2	0	0	0	10
		9	0	3	4	0	8
		12	0	0	0	0	0
	MS	6	0	2	0	0	3
		9	0	0	0	0	5
		12	2	0	0	0	3
	NN	6	2	0	0	0	10
		9	0	0	0	0	5
		12	0	0	0	0	7
25	B5	6	0	2	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
	B5S	6	2	0	0	0	13
		9	0	3	0	0	3
		12	0	0	0	0	0
	MS	6	0	0	0	0	3
		9	0	0	0	0	3
		12	0	0	0	0	3
	NN	6	2	0	0	0	3
		9	0	0	0	0	3
		12	0	0	0	0	0

*Percent of globular shaped embryos; n = 45.

Appendix B. Frequency of embryos at the heart stage at 11, 18,
or 25 days after pollination (DAP) that were plated on a
particular salt base and sucrose level.

DAP	Genotype		Hy894	Hy903	RHA299	RHA297	HA99
11	B5	6%	20*	20	9	7	13
		9	33	22	9	13	16
		12	20	24	9	2	20
	B5S	6	2	11	27	2	11
		9	16	16	11	9	11
		12	13	25	17	16	7
	MS	6	18	22	16	7	16
		9	18	16	11	4	16
		12	9	13	18	4	16
	NN	6	7	11	9	4	36
		9	22	20	16	16	42
		12	9	20	20	4	24
18	B5	6	4	4	0	0	4
		9	13	7	0	2	0
		12	9	4	5	0	16
	B5S	6	2	2	5	0	7
		9	0	10	2	0	8
		12	0	16	4	7	0
	MS	6	13	2	2	0	3
		9	7	2	2	0	5
		12	0	4	3	0	0
	NN	6	2	2	0	0	8
		9	2	9	7	2	13
		12	4	9	6	2	9
25	B5	6	4	2	0	0	2
		9	11	7	0	2	0
		12	4	2	2	0	12
	B5S	6	2	0	3	0	10
		9	0	0	2	0	5
		12	0	0	0	7	0
	MS	6	9	0	0	0	3
		9	4	0	2	0	0
		12	0	2	3	0	0
	NN	6	2	0	0	0	5
		9	0	2	3	2	3
		12	4	2	3	2	4

*Percent of heart-shaped embryos; n = 45.

Appendix C. Frequency of embryos at the torpedo stage at 11, 18, or 25 days after pollination (DAP) that were plated on a particular salt base and sucrose level.

DAP	Genotype		Hy894	Hy903	RHA299	RHA297	HA99
11	B5	6%	7*	4	9	13	11
		9	7	4	5	2	13
		12	7	0	23	0	11
	B5S	6	0	4	11	10	13
		9	7	0	7	5	11
		12	11	0	5	0	16
	MS	6	4	4	16	9	4
		9	2	7	7	10	11
		12	4	2	4	2	7
	NN	6	9	0	16	7	2
		9	7	0	11	0	2
		12	9	0	9	11	2
18	B5	6	0	0	0	0	0
		9	0	0	0	0	5
		12	4	0	0	0	0
	B5S	6	0	0	3	0	0
		9	2	0	0	0	3
		12	0	0	0	0	2
	MS	6	0	0	0	0	0
		9	0	0	0	0	0
		12	2	0	0	0	5
	NN	6	0	0	0	0	0
		9	2	0	0	0	0
		12	0	0	0	0	0
25	B5	6	0	0	0	0	0
		9	0	0	0	0	0
		12	2	0	0	0	0
	B5S	6	0	0	0	0	0
		9	0	0	0	0	0
		12	0	0	4	0	0
	MS	6	0	0	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
	NN	6	0	0	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0

*Percent of torpedo shaped embryos; n = 45.

Appendix D. Frequency of embryos at the vascular stage at 11, 18, or 25 days after pollination (DAP) that were plated on a particular salt base and sucrose level.

DAP	Genotype		Hy894	Hy903	RHA299	RHA297	HA99
11	B5	6%	67*	69	80	76	58
		9	44	69	80	82	56
		12	60	69	63	98	44
	B5S	6	87	76	56	83	58
		9	76	64	80	77	56
		12	71	59	76	82	62
	MS	6	58	67	64	80	53
		9	60	60	82	82	56
		12	80	76	71	93	62
	NN	6	76	82	69	84	52
		9	62	69	67	82	36
		12	73	58	69	82	60
18	B5	6	89	84	93	93	78
		9	64	87	92	93	80
		12	78	84	79	100	53
	B5S	6	89	89	84	92	73
		9	96	75	89	91	70
		12	93	69	89	89	76
	MS	6	67	78	88	93	70
		9	69	76	93	93	63
		12	89	84	94	100	68
	NN	6	89	84	83	93	60
		9	80	80	80	91	46
		12	78	71	91	96	73
25	B5	6	78	84	94	93	78
		9	67	87	92	93	83
		12	80	84	81	100	60
	B5S	6	87	87	81	92	70
		9	98	79	88	91	68
		12	93	75	87	89	78
	MS	6	67	78	92	93	70
		9	69	78	89	93	70
		12	91	84	94	100	70
	NN	6	87	84	85	93	65
		9	84	84	85	89	50
		12	76	76	91	96	78

*Percent of vascular shaped embryos; n = 45.

Appendix E. Frequency of embryos with callus growth at 11, 18,
             or 25 days after pollination (DAP) that were plated on a
             particular salt base and sucrose level.

DAP	Genotype		Hy894	Hy903	RHA299	RHA297	HA99
11	B5	6%	0*	0	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
	B5S	6	0	0	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
	MS	6	0	0	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
	NN	6	0	2	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
18	B5	6	11	33	18	38	22
		9	9	24	16	51	5
		12	9	16	3	31	7
	B5S	6	13	24	8	31	17
		9	9	15	16	27	8
		12	16	16	7	27	2
	MS	6	11	20	17	62	18
		9	20	18	9	44	18
		12	7	33	3	69	15
	NN	6	24	18	10	53	23
		9	20	27	5	44	13
		12	18	18	0	71	11
25	B5	6	13	42	26	49	29
		9	11	44	15	53	15
		12	13	24	7	40	10
	B5S	6	16	29	10	44	23
		9	11	24	14	30	23
		12	24	21	4	38	8
	MS	6	16	36	21	78	30
		9	22	22	9	62	28
		12	16	38	11	71	15
	NN	6	42	30	20	60	35
		9	27	38	10	69	18
		12	20	24	11	73	20

*Percent of embryos with callus growth; n = 45.

Appendix F. Frequency of embryos that did not survive at 11, 18,
or 25 days after pollination (DAP) that were plated on a
particular salt base and sucrose level.

DAP	Genotype		Hy894	Hy903	RHA299	RHA297	HA99
11	B5	6%	4*	2	0	2	9
		9	9	4	7	0	9
		12	7	0	5	0	16
	B5S	6	7	2	4	5	11
		9	0	7	0	5	9
		12	2	5	2	2	11
	MS	6	13	0	2	4	7
		9	13	9	0	2	0
		12	2	4	4	0	9
	NN	6	4	4	7	4	9
		9	2	9	4	0	16
		12	4	7	2	2	11
18	B5	6	7	9	5	7	18
		9	18	7	8	4	15
		12	9	11	17	0	31
	B5S	6	7	9	8	3	10
		9	2	13	4	9	13
		12	7	16	7	4	22
	MS	6	20	18	10	7	25
		9	24	22	4	7	28
		12	7	11	3	0	25
	NN	6	7	13	18	7	23
		9	16	11	14	7	36
		12	18	20	3	2	11
25	B5	6	18	11	6	7	20
		9	22	7	8	4	18
		12	13	13	17	0	29
	B5S	6	9	13	16	8	23
		9	2	18	10	9	23
		12	7	23	9	4	23
	MS	6	24	22	8	7	25
		9	24	22	9	7	28
		12	9	13	3	0	28
	NN	6	9	16	15	7	28
		9	16	13	13	9	45
		12	20	22	6	2	18

*Percent of non-surviving embryos; n = 45.

Appendix G. Frequency of embryos with shoot development at 11, 18,
             or 25 days after pollination (DAP) that were plated on a
             particular salt base and sucrose level.

DAP	Genotype		Hy894	Hy903	RHA299	RHA297	HA99
11	B5	6%	0*	2	0	0	2
		9	0	0	0	0	0
		12	0	0	0	0	0
	B5S	6	0	0	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
	MS	6	0	0	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
	NN	6	0	2	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
18	B5	6	53	36	70	71	31
		9	29	22	49	24	23
		12	16	13	24	16	2
	B5S	6	38	40	63	56	33
		9	29	20	60	36	35
		12	11	20	61	0	24
	MS	6	33	40	48	67	38
		9	13	20	44	33	35
		12	18	18	34	24	5
	NN	6	53	56	53	71	40
		9	33	27	46	36	26
		12	16	20	54	20	16
25	B5	6	64	44	77	82	56
		9	38	49	62	60	48
		12	33	24	42	40	19
	B5S	6	49	53	65	75	53
		9	40	47	64	66	35
		12	24	27	52	24	28
	MS	6	40	49	68	78	40
		9	22	29	64	76	43
		12	27	31	43	60	20
	NN	6	56	70	65	87	43
		9	36	36	53	58	26
		12	20	29	63	50	29

*Percent of embryos producing shoots; n = 45.

Appendix H. Frequency of embryos with root development at 11, 18, or 25 days after pollination (DAP) that were plated on a particular salt base and sucrose level.

DAP	Genotype		Hy894	Hy903	RHA299	RHA297	HA99
11	B5	6%	0*	2	3	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
	B5S	6	0	0	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
	MS	6	0	0	0	0	0
		9	0	0	3	0	0
		12	0	2	0	0	0
	NN	6	0	0	0	0	0
		9	0	0	0	0	0
		12					
18	B5	6	47	38	68	69	31
		9	27	22	46	24	23
		12	18	16	21	13	2
	B5S	6	36	42	61	53	40
		9	27	23	60	36	35
		12	11	18	61	0	22
	MS	6	31	42	45	60	38
		9	11	18	44	24	33
		12	18	20	34	13	5
	NN	6	49	49	53	69	33
		9	33	20	46	33	26
		12	16	13	54	16	16
25	B5	6	56	40	77	76	51
		9	33	44	56	49	33
		12	31	16	34	27	7
	B5S	6	44	53	61	69	50
		9	29	37	62	59	35
		12	18	23	52	16	25
	MS	6	33	49	64	67	35
		9	20	24	53	51	40
		12	24	27	43	36	15
	NN	6	51	56	63	84	40
		9	33	29	50	56	26
		12	22	22	63	25	27

*Percent of embryos producing roots; n = 45.

Appendix I. Frequency of embryos with both shoot and root development at 11, 18, or 25 days after pollination (DAP) that were plated on a particular salt base and sucrose level.

DAP	Genotype		Hy894	Hy903	RHA299	RHA297	HA99
11	B5	6*	0	2	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
	B5S	6	0	0	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
	MS	6	0	0	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
	NN	6	0	2	0	0	0
		9	0	0	0	0	0
		12	0	0	0	0	0
18	B5	6	44	33	68	69	31
		9	27	22	46	24	23
		12	16	13	21	13	2
	B5S	6	36	36	61	53	33
		9	27	20	60	36	35
		12	11	18	61	0	22
	MS	6	31	38	45	60	38
		9	11	18	44	24	33
		12	18	18	34	13	5
	NN	6	49	49	53	69	33
		9	33	18	46	33	26
		12	16	11	54	16	16
25	B5	6	51	36	77	76	51
		9	33	44	56	49	33
		12	31	16	34	27	7
	B5S	6	44	49	61	69	50
		9	29	34	62	59	35
		12	18	23	52	16	25
	MS	6	33	47	64	67	35
		9	20	24	53	51	40
		12	24	24	43	36	15
	NN	6	51	56	63	84	40
		9	33	24	50	56	26
		12	22	20	63	25	27

*Percent of embryos producing both shoots and roots; n = 45.

Appendix J. Frequency of embryos that developed chlorophyll at 11, 18, or 25 days after pollination (DAP) that were plated on a particular salt base and sucrose level.

DAP	Genotype		Hy894	Hy903	RHA299	RHA297	HA99
11	B5	6%	0*	4	9	0	7
		9	0	0	2	0	0
		12	0	0	0	0	0
	B5S	6	7	4	4	0	0
		9	2	2	0	0	0
		12	4	0	0	0	0
	MS	6	2	4	7	0	0
		9	2	2	0	0	0
		12	0	0	0	0	0
	NN	6	2	9	0	0	4
		9	0	2	0	0	2
		12	0	2	0	0	0
18	B5	6	73	69	90	91	17
		9	49	80	90	91	80
		12	58	71	67	41	62
	B5S	6	72	84	84	32	67
		9	82	75	76	84	65
		12	78	49	86	82	67
	MS	6	67	73	79	91	68
		9	64	67	80	91	60
		12	67	62	80	96	63
	NN	6	82	80	78	91	60
		9	69	62	75	84	46
		12	53	56	74	96	58
25	B5	6	76	80	94	93	78
		9	62	87	92	93	83
		12	60	80	71	96	67
	B5S	6	76	87	81	89	73
		9	82	76	79	91	73
		12	76	59	87	87	70
	MS	6	71	76	87	93	73
		9	67	73	84	91	70
		12	67	78	83	96	65
	NN	6	84	79	85	93	58
		9	69	69	75	87	50
		12	53	71	77	98	64

*Percent of embryos develping chlorophyll; n = 45.