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Advanced breeding in zucchini

VG002

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QLD Department of Primary Industries

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PROJECT SUMMARY

The zucchini squash industry in Queensland is valued at approximately $10M. Zucchini yellow mosaic virus (ZYMV) and or papaya ringspot virus type W (PRSV-W) often cause severe losses of marketable yield of zucchini *(Cucurbita pepo)* in all production areas with annual losses of $3.8M.

Because no cultural practice effectively controls the viruses, resistant cultivars are urgently needed. However, these are unavailable. We had previously produced partially resistant breeding lines from the resistant gourd *Cucurbita ecuadorensis* but this material required further assessment, improvement and stabilisation (by inbreeding).

In traditional plant breeding inbreeding is the procedure whereby the genetic variability within each plant is reduced by successive self-pollinations until there is essentially no difference between the parent(s) and all the progeny. This provides plants suitable for use as parents of hybrid cultivars. The process usually takes between six and eight generations of self-pollination.

However more recently ovule and anther culture methods which develop plants from unfertilised ovules and pollen have been used in breeding programmes. Production of haploid plants with the subsequent doubling of chromosome number has facilitated the rapid development of diploid inbreds from segregating populations of plants and thereby the release of cultivars.

The objective of our HRDC and QFVG (COD) funded project was therefore to develop ovule (and anther) culture in zucchini so as to hasten the production of inbred, virus-resistant lines using *C. ecuadorensis* as the source of resistance.

The programme at the Department of Primary Industries Queensland's (DPIQ) Redlands Research Station involved determining the conditions which would allow the production of large numbers of plants from ovules and subsequently determining the tissue from which these plants originated. (The programme was limited to ovule culture because the success of this technique was considered more likely than that of anther culture.) A backcrossing programme was conducted concurrently to improve resistance, quality and fertility.

A series of experiments investigated the influence on ovule germination of combinations of growth regulators (IAA, NAA, 24D, Kinetin, BAP, and 2IP at 0 to 10^{-5} M) with different durations (0-13 days) of treatment and lighting. Correlations between ovule germination and fruit, plant and ovule characters were assessed. The origin of resultant plants was also determined by evaluating progeny.

Some development occurred even in the absence of growth regulators but the best occurred when material was taken from vigorous plants at early flowering when 0.6 - 0.8 mm long ovules were extracted from fruit two days before flower opening and inoculated in darkness on growth regulators IAA, NAA (each 125 x 10^{-8}M), 24D (30 x 10^{-8}M),

Kinetin (25 x 10^{-8}M), BAP, 2IP (each 3.125 x 10^{-8}M) on MS media plus de Vaulx vitamins, 3% sucrose, 0.8% agar for 24 - 48 hours, followed by incubation on similar media without growth regulators in a 16 hr day.

One haploid plant in 26 was successfully produced using this ovule culture protocol but the efficiency needs to be increased to allow routine usage. It is postulated that the recovery of haploids can be increased by subcultures and further media modification however further investigations in such areas could require substantial resources. Other fertile plants produced from ovules, although of somatic origin and not the more desirable spontaneously doubled haploids, were virus free. The protocol therefore also provides a means of readily developing virus free breeding stock.

A semi-quantitative ELISA technique, based on biotinylated antibody and streptavidin conjugate, was developed at DPIQ's Plant Pathology Branch, to permit better evaluation of resistance to PRSV-W and ZYMV. The technique allows the quantification of the differences between plants which express only moderate levels of resistance; a situation which occurs in backcrossing.

Highly resistant but poor quality, low fertility plants were developed through a series of crosses in the backcrossing programme. This material represents a marked improvement in resistance over the original partially resistant plants which expressed increased susceptibility in the first spring after selection. With much further selection and backcrossing this highly resistant material should improve in fertility and quality. Such efforts are warranted.

Genetic transformations which may confer resistance, for example incorporation of the viral coat protein gene, should also be considered in future.

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ABSTRACT

A series of experiments based on a modified MS media investigated the effects of growth regulators (IAA, NAA, 24D, Kinetin, BAP, 2IP at 0-10^{-5} M), durations of treatment (0-13 days) and light environment on the germination of unfertilised ovules either excised or contained in fruit pieces. Correlations between ovule germination and fruit, plant and ovule characteristics were sought. The origin of 16 families from 12 distinct ovules was determined through progeny tests.

Germination of ovules commenced as early as 22 days after incubation. Some (0-2%) germination occurred, even in the absence of growth regulators. Ovule germination varied (0-20%) between samples, fruit and experiments, but there was no correlation with fruit or plant parameters. Ovules of length 0.6 mm - 0.8 mm germinated most reliably. This size was often associated with fruit two days before flower opening. Darkness during the 24 hours following initial incubation promoted the development of multiple embryos. Only about 20% of germinated ovules developed into plants.

One haploid plant was identified in a sample of 26 plants. Segregation patterns in all 16 families assessed in progeny tests indicated that germinated ovules which developed into fertile plants were derived from maternal tissue and were in fact somatic embryos rather than the desired doubled haploids from gametophytic tissue. This somatic embryogenesis produced plantlets free from zucchini yellow mosaic virus.

A sensitive semi-quantative biotin-streptavidin ELISA was developed. This made it possible to differentiate between resistant and tolerant selections.

Plants with high resistance but with low fertility and low quality were developed in the concurrent backcrossing programme.

1. INTRODUCTION

In Queensland each year the viral diseases papaya ringspot virus type W (PRSV-W) and zucchini yellow mosaic virus (ZYMV) cause losses in zucchini *(Cucurbita pepo* L.) estimated at $3.8 M. These viruses of cucurbits are also of major economic importance world wide. They are not effectively controlled by cultural practices. Resistant cultivars, though urgently required and the subject of breeding programmes, are unavailable (Provvidenti *et al.*, 1984).

Resistance to both viruses has been transferred from *Cucurbita ecuadorensis* Cutler and Whitaker into pumpkin *(C. maxima* Duch.) and a breeding line released as cv. Redlands Trailblazer (Herrington *et al.*, 1991a, 1991b). Work to transfer the resistances from *C. ecuadorensis* into *C. pepo* was also initiated and improved material partially developed.

It was expected that the release of a resistant zucchini cultivar from this material would be assisted by the development and application of ovule and or anther culture techniques for zucchini. These techniques have been successfully applied to a number of crops including tobacco, wheat, barley, rice and sugar beet (Bollon and Raquin, 1988). While some preliminary information on ovule culture of *C. pepo* was available (Chambonnet and de Vaulx, 1985) a specific protocol was lacking. It was decided to concentrate of ovule culture as the method most likely to be successful.

Yang and Zhou (1982) reviewed the induction of haploid plants from unpollinated ovaries and ovules. The conditions required varied with species but the genotype and the type and concentration of exogenous growth regulators were the most important factors. Other factors included embryo sac stage, media, sugar, substrate (solid or liquid), mode of inoculation and cultural conditions.

The experimental programme therefore sought to develop ovule culture techniques for zucchini through investigations of the responses of unfertilised ovules to plant growth regulator concentrations, plant and fruit characteristics, and light.

Doubled haploid plants, either induced or spontaneously doubled (Yang and Zhou, 1982), are expected to be homozygous. However, maternal tissue is sometimes the origin of the plantlets and unexpected variations have occurred even among plants derived from gametophytic tissue culture (Bollon and Raquin, 1988). We therefore assessed the homozygosity of a sample of progeny from ovule culture.

At the same time, since the ultimate long term objective was to produce virus resistant zucchini, a conventional breeding programme involving backcrossing was continued to improve resistance, quality and fertility.

2. GENERAL MATERIALS AND METHODS

2.1 Fruit disinfestation

Fruit were disinfested in a 1% sodium hypochlorite solution for 30-60 minutes and rinsed three times in sterile water. Stem and corolla were previously removed.

2.2 Ovule source

Ovules were dissected under a binocular microscope, using an eye scalpel and forceps, usually on the day before the flower opened, but during investigations the range of three days prior, to one day post flower opening was used. Flowers were enclosed in paper packets when it was necessary to prevent pollination. Approximately 600 ovules/person/day were dissected.

2.3 Genotypes

Early experiments used field grown plants of the commercial cultivar Regal Black, occasionally 'resistant' plants were cultured, later experiments, designed to determine the origin of ovules, involved F_1 (Regal Black x Sundance).

2.4 Media

Basic medium given below comprised macro- and micro-nutrients of Murashige and Skoog (1962) and vitamins of the 'C' medium of de Vaulx *et al.* (1981). This was amended with growth substances. Media were autoclaved for 15 minutes at 121°C.

$K NO_3$	18.8	mM
NH_4NO_3	20.6	mM
$MgSO_4.7H_2O$	1.5	mM
$CaCl_2.2H_2O$	3.0	mM
KH_2PO_4	1.25	mM
$MnSO_4.4H_2O$	100	μM
$ZnSO_4.7H_2O$	30	μM
H_3BO_3	100	μM
KI	5	μM
$Na_2MoO_4.2H_2O$	1.03	μM
$CuSO_4.5H_2O$	0.1	μM
$CoCl_2.6H_2O$	0.1	μM
$NaEDTA.2H_2O$	100	μM
$FeSO_4.7H_2O$	100	μM
Inositol	280	μM
Pyridoxine HCl	27	μM
Nicotinic Acid	5.7	μM

Thiamine HCl	1.8 μM
Calcium Pantothenate	1.0 μM
Vitamin B_{12} (Cyanocobalamin)	0.02 μM
Biotin	0.02 μM
Glycine	1.3 μM
Sucrose	87.6 mM
Agar	4 or 8 g/L or Gelrite 2.5 g/L
pH	5.75

2.5 Cultural conditions

Unless otherwise indicated, cultures were maintained at 25°C-30°C, 16 hour light (30-50 μmol.s^{-1}.m^{-2} from cool white florescent bulbs) in 250 mL polycarbonate oblique-sided pots, or 50 mm plastic petri dishes. Pots contained 30 mL and dishes 10 mL of media/container respectively. Petri dishes were sealed with Parafilm®, Nescofilm® and/or aluminium foil.

3. SPECIFIC INVESTIGATIONS

3.1 Section 1 Responses to growth regulators

3.1.1 *Objective*

To determine the optimum concentration of growth regulators necessary to produce germination of ovules.

3.1.2 *Methods*

Ovules were dissected on the day of, or one day prior to flower opening from fruit of zucchini cv. Regal Black and placed on 10 mL of Gelrite media (0.4% agar in experiment 2) amended with 16 or 20 combinations of the plant growth regulators in Table 1. Each 50 mm plastic petri dish contained five ovules with six replicates (fruit) per treatment. After 10 or 11 days ovules were transferred to similar media without growth regulators in 250 mL polycarbonate oblique-sided pots.

In experiment 1.1 (30 September 1988) the concentrations of combined auxins (IAA + NAA + 24D), Kinetin, BAP and 2IP were varied separately across coded values 1, 2, 3, 4, 5 (Table 1) with other growth regulators held at level '3'. (This involved four sub-experiments). The 'all level 3' treatment had additional duplication.

In experiment 1.2 (7 October 1988) the concentrations of combined cytokinins (Kinetin + BAP + 2IP), IAA, NAA and 24D were varied as in experiment 1.1.

Experiments 1.3 (14 October 1988) and 1.4 (25 October 1988) used a factorial design of growth regulators at concentration levels '2' and '4' in combinations as in experiments 1.1 and 1.2 respectively.

Experiment 2 (11 January 1989) compared the germination of ovules on media with the level '3' concentration of growth regulators with germination on media without growth regulators on 0.4% agar.

Germinated ovules were noted once to three times per week.

Table 1. Growth regulators used in Section 1

	Growth regulator concentration (x 10^{-8}M)[a]				
Coded Value	1	2	3	4	5
Auxins					
IAA	31.25	62.5	125	250	500
NAA	31.25	62.5	125	250	500
24D	7.5	15	30	60	120
Cytokinins					
Kinetin	1.56	6.25	25	100	400
BAP	0.195	0.7813	3.125	12.5	50
2IP	0.195	0.7813	3.125	12.5	50

[a] IAA = Indole acetic acid
NAA = α - Naphthalene acetic acid
24D = 2, 4-Dichlorophenoxy acetic acid
Kinetin = 6-Furfuryl amino purine
BAP = N^6-benzyl amino purine
2IP = N^6-iso pentenyl amino purine

3.1.3 *Results and discussion*

Most germination occurred in the first 42 days after dissection. Some occurred after only 22 days (Table 2).

Table 2. Rate of germination of ovules

Germinated ovules	Days from dissection					
	22-28	29-35	36-42	43-49	50-56	57-64
Number/50 incubated	1	17	14	16	1	1
Percentage	2	34	28	32	2	2

The frequency of germination of ovules ranged from 0 to 13% (Tables 3 and 4). This was better than the best result (4.3%) of Chambonnet and de Vaulx (1985). Only about 20% of germinated ovules regenerated into plants (Plate 1). Germination of ovules in similar treatments of differing experiments varied from 0 to 9% (this was significant only at P = 0.055 in F test) and indicated fruit from young plants were more productive of germinated ovules than those from old plants.

The percentage of ovules which germinated varied substantially between fruit as indicated by significant replicate effects (Table 3).

Surprisingly there was no significant effect of growth regulators (Tables 3 and 4) except for lowest levels of BAP (Table 4). In fact, the germination without growth regulators 2% (7/275) was in one experiment similar 1% (2/285) to that on the level '3' concentration of growth substances. This concentration was chosen as the base for future experimentation.

Table 3. Frequency of germination of ovules of cv. Regal Black at concentrations of growth regulators

Coded concentration of growth regulator*	Growth regulator							
	Experiment 1.1				Experiment 1.2			
	Combined Auxin	Kinetin	BAP	2IP	Combined Cytokinin	IAA	NAA	24D
1	0.03	0.07	0	0.10	0	0	0	0.03
2	0.10	0.03	0.03	0	0	0	0	0
3	0.08	0.08	0.08	0.08	0	0	0	0
4	0.13	0.07	0.10	0.13	0	0	0	0
5	0.06	0.03	0	0.03	0	0	0	0.07
Overall mean	0.08	0.06	0.05	0.07	0	0	0	0.02
LSD (P=0.05)								
n = 6, 6	0.14	0.13	0.10	0.13	-	-	-	0.06
n = 12, 6	0.12	0.11	0.09	0.12	-	-	-	0.06
F test (P=0.05)								
Concentration	NS	NS	NS	NS	-	-	-	NS
Replicates	*	*	*	NS	-	-	-	-

* Concentration of nominated growth regulator was varied from coded value 1 to 5 (see Table 1) while the others were held constant at the mid level '3'. 'Combined auxin' is IAA + NAA + 24D, 'combined cytokinin' is Kinetin + BAP + 2IP.

Table 4. Frequency of germination of ovules of cv. Regal Black at concentrations of growth regulators in factorial combinations

Coded concentration of growth regulator[a]	Growth regulator							
	Experiment 1.3[b]				Experiment 1.4[b]			
	Combined Auxin	Kinetin	BAP	2IP	Combined Cytokinin	IAA	NAA	24D
2	0.02	0.02	0.04	0.02	0.06	0.05	0.04	0.04
4	0.02	0.01	0	0.02	0.03	0.04	0.04	0.05
Overall mean		0.02				0.05		
F test (P=0.05)	NS	NS	*	NS	NS	NS	NS	NS
LSD (P=0.05)	0.3	0.03	0.03	0.03	0.03	0.03	0.03	0.03

[a]Growth regulators 'combined auxin' is IAA + NAA + 24D, 'combined cytokinin' is Kinetin + BAP + 2IP; 2 is Kinetin 6.25 x 10^{-8}M, BAP 0.7813 x 10^{-8}M, 2IP 0.7813 x 10^{-8}M, IAA 62.5 x 10^{-8}M, NAA 62.5 x 10^{-8}M, 24D 15 x 10^{-8}M; 4 is Kinetin 100 x 10^{-8}M, BAP 12.5 x $^{-8}$M, 2IP 12.5 x 10^{-8}M, IAA 250 x 10^{-8}M, NAA 250 x 10^{-8}M, 24D 60 x 10^{-8}M.

[b]Main effects only shown because there was no significant interaction.

Plate 1. Germinated ovule of zucchini

3.2a Section 2a Response of excised ovules to time on growth regulators and to lighting

3.2a.1 *Objective*

To determine the optimum duration of treatment with growth regulators and lighting conditions to produce germination of ovules.

3.2a.2 *Methods*

On the day of, or one prior to, flower opening ovules were dissected from fruit of cv. Regal Black and placed on 10 mL of 0.8% agar media amended with IAA 125 x 10^{-6}M, NAA 125 x 10^{-6}M, 24D 30 x 10^{-6}M, Kinetin 25 x 10^{-6}M, BAP 3.125 x 10^{-6}M and 2IP 3.125 x 10^{-6}M in petri dishes. Dishes were sealed with Parafilm® and half were also enclosed in aluminium foil to exclude light for 1, 3, 6, 10 and 13 days. After each time both light (16 hour light) and dark treated ovules were transferred to media without growth regulators in 16 hour light. There were five replicates (fruit) with five ovules/dish. Some source plants were infected with papaya ringspot virus type W (PRSV-W). The procedure was duplicated 7 and 9 February 1989.

3.2a.3 *Results and discussion*

The time ovules were incubated with growth regulators under different light regimes, had no significant effect on the number of ovules which germinated (Table 5). An apparent trend of decreasing germination with increasing time on growth regulators in the dark was not statistically significant.

As with other reports (de Vaulx and Chambonnet, 1985 and D'Halluin and Keimer, 1986), more than one plantlet sometimes developed from a single ovule. This was more common in the dark and in the shorter duration treatments (data not shown). Since this procedure of a short treatment time was convenient and yielded more plants, with no apparent adverse effects, it was adopted for use in further work.

There was again a significant effect of replicate (fruit) (Table 5, see also Table 3). Consequently, further experiments were conducted to determine plant, fruit or ovule characteristics which were associated with germination.

Table 5. **Frequency of germination of ovules following dark and light periods on growth regulators**

Lighting regime (L)[b]	Days (T) on growth regulators[a] 1	3	6	10	13	Mean
Light	0.08	0.04	0.08	0.08	0.12	0.08
Dark	0.24	0.12	0.12	0.04	0	0.10
Mean	0.16	0.08	0.10	0.06	0.06	0.09

Light (L)	F = NS	LSD (P = 0.05)	= 0.07
Time (T)	F = NS	LSD (P = 0.05)	= 0.11
LT	F = NS	LSD (P = 0.05)	= 0.16
Replicate	F = **		

[a]Growth regulator is IAA and NAA 125 x 10^{-8}M, 24D 30 x 10^{-8}M, Kinetin 25 x 10^{-8}M, BAP 3.125 x 10^{-8}M, 2IP 3.125 x 10^{-8}M.

[b]Light is 16 hour light.

3.2b Section 2b Response of ovules embedded in fruit pieces to orientation, time on growth regulators and lighting

3.2b.1 *Objective*

To determine the effect of orientation, placement and duration of treatment with growth regulators and lighting conditions on the germination of ovules in fruit sections.

3.2b.2 *Methods*

(a) On the day the flower opened, or day prior, the exterior of fruit of cv. Regal Black was removed. Six triangular prisms were formed by cutting down midway between placental walls and through the centre of the fruit.

The prisms were sectioned into approximately 3 mm lengths and placed on media without growth regulators. There were five replicates (fruit), three plates (samples) and five subsamples (sections) to each plate. The four treatments comprised:

(i) placental wall placed on the surface of media;

(ii) as (i) but embedded ⅔ in media,

(iii) triangular surface on media (placenta on vertical wall),

(iv) as (iii) but embedded ⅔ in media.

There was no growth regulator applied.

(b) Sections were prepared as in (a). Treatments comprised a factorial design of two light regimes (in darkness or 16 hour light), four times (1, 3, 6, 10 days) on media '3' (see Table 1), five wedges on each of two samples (petri dishes). Wedges were orientated ⅔ embedded with triangular face down. After growth regulator treatment wedges were transferred to media without growth regulators. A small sample was also grown in liquid media without growth regulators for two weeks then transferred to solid media.

3.2b.3 *Results and discussion*

(a) Best growth and ovule germination occurred when media was in good contact with the placental tissue (Table 6).

Table 6. Frequency of germinated ovules with four orientations of fruit sections

	Orientation[a]			
	1	2	3	4
				
Number of ovules germinated/75 sections	3	0	3	6

[a]P indicates location of prism face comprised of placental tissue; /// indicates media; ▵ and □ indicate location of fruit sections (triangular prism).

[b]There was no effect (P>0.05, data not shown) of time on media nor in light or dark on the germination of ovules which developed at an average frequency of 8.1% of incubated sections. Similarly plants developed from the wedges initially incubated in solution culture without growth regulators.

3.3 Section 3 Correlations with ovule germination

3.3.1 *Objective*

To determine the effect of light and correlations of ovule germination with plant, fruit and ovule parameters so as to maximise germination of ovules.

3.3.2 *Methods*

Between 18 April and 12 May, soon after flowering commenced about one month after sowing seed, 50 ovules were taken from each of 19 or 20 fruit of cv. Regal Black. Treatments comprised 24 hours in light or dark on the media described in 3.2a.2 followed by incubation in light (16 hour) on media without growth regulators. The stage of development of fruit varied between one and three days before flower opening. Each fruit had been transversely cut into four sections from blossom to stem end. These sections and a composite were treated as replicates to determine the effect of ovule position in the fruit.

Sets of five ovules were each allocated to each of five petri dishes in both light and dark treatments.

Plant and fruit characteristics recorded included length of fruit, breadth of fruit, length of corolla, days before flower opening, length of midrib of subtending leaf, node of flower attachment and ratio of corolla length to fruit length. Average length of ovules was calculated from measurements on a sample of five ovules taken from each fruit. Correlations were adjusted for significant treatment effects but otherwise data were pooled.

An additional experiment (31 May to 15 June) compared under the dark treatment the germination of ovules of F_1 (Sundance x Regal Black) on media enriched with growth regulators (as above), with the germination on media without growth regulators.

3.3.3 *Results*

The average number of germinated ovules was not linearly correlated ($P>0.05$) with any plant characteristic (Table 7). There was no difference between light and dark treatments nor of location of ovule within fruit.

The effect of ovule length varied between experiments as did the proportion of ovules which germinated ($X^2_{(2)} = 27.2$, $P<0.05$) but medium sized ovules, between 0.6 and 0.8 mm in length, germinated most reliably (Table 8). These commonly originated from fruit at a developmental stage of two days before flower opening. Ovules in this size range were selected for future work.

Some germination occurred even in the absence of growth regulators but application was beneficial (Table 9).

Some source plants had been infected naturally with PRSV-W, but ovule culture eliminated the infection. In only one instance was viral infection noted in regenerated plants. A similar effect occurred with partially resistant material inoculated with ZYMV.

Table 7. Correlation matrix of plant characteristics and germination of ovules

	Length of fruit	Breadth of fruit	Length of corolla	Days before flower opening	Midrib length	Node of fruit attachment	Corolla /fruit length	Ovules germinated
Length of fruit	1.0							
Breadth of fruit	0.75 **	1.0						
Length of corolla	0.56 *	0.66 **	1.0					
Days before flower opening	-0.63 *	-0.87 **	-0.84 **	1.0				
Midrib length	0.73 **	0.66 **	0.43/0.65 NS/*	-0.49/0.76 NS/*	1.0			
Node of fruit attachment	-0.29 NS	-0.43 NS	-0.71/-0.43 **/NS	0.68/0.40 **/NS	-0.21 NS	1.0		
Corolla/ fruit length	0.05 NS	0.32/0.74 NS/**	0.85 **	-0.62 *	0.06 NS	-0.71 **	1.0	
Ovules germinated	-0.35 NS	-0.25 NS	-0.46 NS	0.27 NS	-0.35 NS	0.46 NS	-0.35 NS	1.0

NS = correlation is not significant ($P \geq 0.05$)
* = significant correlation $P < 0.05$
** = significant correlation $P < 0.01$
/ indicates difference of significance between experiments

Table 8. **Germination of ovules of various average lengths, cv. Regal Black**

Ovule length (x mm)[b]	Ovules germinated in experiments[a] 1	2	3	Pooled (Experiment 1-3) Percent	Number	Total
0.5 ≤ x <0.625	9(250)[c]	53(300)	1(50)	10.5	63 (537)	600
0.625≤ x <0.75	8(100)	24(200)	22(200)	10.8	54 (446)	500
0.75 ≤ x <0.875	9(145)	16(250)	47(450)	8.5	72 (773)	845
0.875≤ x <1	5(200)	2(50)	11(200)	4.0	18 (432)	450
1 ≤ x	0(100)	10(200)	0(100)	0.3	10 (390)	400
Totals	31(795)	105(1000)	81(1000)	7.8	217 (2578)	2 795
$X^2_{(4)}$	11.7	25.5	18.7	37.8		
Pr	0.02	<0.01	<0.01	<0.01		

[a]Experiments 1, 2, 3 conducted 20 April, 12 May and 10 May 1989 respectively
[b]Mean length of five ovules
[c]Parenthesis encloses the number of ovules incubated

Table 9. **Germination of ovules of F_1 (Sundance x Regal Black) with and without applied growth regulator[a]**

Growth regulator	Ovules germinated	Ovules ungerminated	Total
Present	20	580	600
Absent	4	586	590
Total	24	1 166	1 190

$X^2_{(1)} = 9.3$ $P<0.01$

[a]Growth regulator comprised IAA 125 x 10^{-8}M, NAA 125 x 10^{-8}M, 24D 30 x 10^{-8}M, Kinetin 25 x 10^{-8}M, BAP 3.125 x 10^{-8}M, 2IP 3.125 x 10^{-8}M applied for 24 hours in dark. Ovules were then transferred to 16 hours light, 27°C with no growth regulators.

3.4 Section 4 Summary of ovule culture protocol

The protocol most favourable to ovule germination was considered to be:

- plant vigour - vigorous,
- plant stage - early flowering,
- fruit stage - two days before flower opening,
- ovule size - 0.6 - 0.8 mm long.
- environmental conditions:
- 24-48 hours on growth regulators (IAA and NAA at 125 x 10^{-8}M, 24D at 30 x 10^{-8}M, Kinetin at 25 x 10^{-8}M, BAP and 2IP at 3.125 x 10^{-8}M) in darkness,
- transfer to basic media* without growth regulators in 16 hour light of medium intensity (30-50μmol.s^{-1}.m^{-2}), preferably avoiding a long light period immediately after transfer,
- establishment of plants in pots requires rapidly growing plants in culture,
- control of fungus gnats (Family *Mycetophilidae*) for example with 0.2 mL/L Diazinon drench is essential.

Ovule size was considered more critical than fruit stage or plant stage.

*Refer Section 2 for basic media (Murashige and Skoog [1962] media with vitamins of de Vaulx *et al.* [1981], 0.8% agar and 3% sucrose).

3.5 Section 5 Origin of plantlets

3.5.1 *Objective*

To determine the origin of plants produced from germinated ovules.

3.5.2 *Methods*

Although numerous plants were grown from ovules of cv. Regal Black, the origin of plants could not be determined readily because of the absence of easily identified variation in the parentage. While some plants set seed only poorly, pollen production appeared normal.

Hybrid zucchini cv. Sundance has an unmottled leaf, yellow skin and crook-neck fruit. Cv. Regal Black has a mottled leaf, dark green skin and straight-neck fruit. The F_1 we produced by crossing these produces mottled leaf, medium yellow-green fruit with a slight neck.

First generation after ovule culture

Plants from 22 different germinated ovules from plants of F_1 (Sundance x Regal Black), five as duplicated plants, were grown to maturity with plants of the F_1, cv. Regal Black, and Sundance (selfed). These were evaluated for leaf and fruit variation.

Fertility was assessed by counts of differentially stained pollen (Alexander, 1969) from at least two flowers of 26 plants, viz. four Regal Black-ovule-derived plants, and the 22 F_1-ovule-derived plants. Self-pollinations and cross-pollinations were also attempted where appropriate.

Ploidy of the one plant with consistently low fertility was assessed from chromosome counts of root tip squashes stained with aceto-orcein.

Second generation after ovule culture

Self-pollinated seed of 16 F_1-ovule-derived plants, parents, F_1 and three F_2 families from the original F_1 population were grown in a randomised trial to assess homozygosity and consequently whether ovule derived plants were from the desired gametophytic, or the maternal tissue. Each family from each ovule-derived plant had between 19 and 26 individuals. Of the 16 original F_1-ovule-derived plants, eight were as duplicates derived from four separate original ovules.

Plants were assessed for leaf mottle and fruit colour as indicators of homozygosity.

3.5.3 *Results*

First generation after ovule culture

One haploid plant was identified among the 26 assessed. The haploid (n=20) number of chromosomes was found in root squashes of the one F_1-ovule-derived plant which had consistently low (17%) pollen viability. This plant originated under the developed protocol but was cultured in darkness for 48 hours and was one of the earliest ovules to germinate. Unfortunately this plant had low vigour and died before chromosome doubling could be conducted.

Fertility measured as pollen staining was 94-100% for the remaining 21 of the 22 F_1-ovule-derived plants and three of the four Regal Black-ovule-derived plants assessed. The other Regal Black - ovule-derived plant varied in pollen staining and had a diploid number of chromosomes.

Despite high pollen stainability, self-pollination of four F_1-ovule-derived plants was not successful following three to seven attempts. However fruit set readily when cv. Regal Black was used as the pollen source.

Fruit on all fertile F_1-ovule-derived plants were relatively similar (Table 10) - the small variation which occurred was considered due to residual heterozygosity in their origins from different F_1 plants. Fruit on the haploid plant had immature fruit colour similar to the F_1 but fruit were slightly constricted in the centre.

Table 10. Fruit colour of parents and ovule-derived progeny

Cultivar	Colour[a] Visual	L*	a	b[b]
Regal Black	dark green	49	-12	15
Sundance	cream yellow	80	-8	45
F_1	yellow-green	60	-16	27
Ovule-derived progeny	yellow-green	58	-13	24

[a]Assessed on at least two fruit/plant on the day of flower opening.
[b]L*, a, b was with Minolta CR200 colour meter.

Second generation after ovule culture

Within each of the 16 families from the 12 separate F_1-ovule-derived plants segregation occurred for both leaf and fruit characteristics (Table 11). This indicates that the fertile ovule-derived plants were not in a homozygous condition. Except for one line, segregation of the F_2 ovule derived progeny with respect to mottled or not was similar to that in the standard F_2. However, this segregation was better described by a 1:1 ratio than the 3:1 expected from the literature (Robinson *et al.*, 1976). Segregation in the ovule derived line '7' (Table 11) which deviated ($P<0.05$) from the experimental F_2 segregation was consistent with the accepted 3:1 hypothesis.

Fertile ovule-derived lines differed ($P<0.01$) from each other with respect to immature fruit colour segregation pattern. However in one case (2 a and b in Table 11) differences ($P<0.05$) occurred even between the two lines derived from the same ovule-derived plant. All ovule-derived and F_2 populations had at least one plant which produced dark green fruit similar to that of cv. Regal Black whereas most fruit were yellow-green with some being yellow.

This indicates that the majority of the fertile ovule-derived plants were of maternal origin. Under the protocol used, there was no evidence of plants being derived from spontaneously doubled haploids as sometimes occurs in ovule culture.

Table 11. Segregation in families produced by self-pollination of ovule-derived plants

Population	Leaf mottle		Colour of immature fruit		
Self-pollinated ovule-derived families[a]	Present	Absent	Green	yellow-green	yellow
1a	12	10	7	6	8
1b	12	14	7	7	9
2a	11	14	9	6	7
2b	6	13	3	16	2
3a	7	16	6	7	7
3b	10	13	8	5	8
4a	11	11	4	13	4
4b	10	14	8	14	2
5	12	15	7	11	8
6	10	17	11	8	6
7	19	5	4	12	6
8	11	13	11	10	5
9	10	12	4	19	1
10	13	9	5	15	2
11	8	13	4	14	3
12	16	10	6	14	4
Others					
F_2	14	12	7	4	11
F_1	6	0	0	5	0
Regal Black	12	2	16	0	0
Sundance®	0	24	0	0	7

[a] Populations 1-12 were derived by self-pollination of plants derived from ovule culture of F_1 (Sundance x Regal Black); a and b indicate duplicate families produced through self-pollination of separate plants originating from the same ovule-derived plant.

X^2 within ovule-derived lines; mottle $X^2_{(15)} = 20.7$, $P = 0.15$; fruit colour $X^2_{(30)} = 56$, $P < 0.01$.

3.6 Section 6 Selection for resistance

3.6.1 *Objective*

To produce and select backcross populations for improved resistance.

3.6.2 *Methods*

Twice each year from September 1988 to June 1991 a series of field crossing blocks was established to evaluate and or produce progeny of *Cucurbita* (zucchini like) plants with improved resistance, fertility and or agronomic characters. This procedure usually involved tissue culture (embryo rescue), establishment in pots in glasshouse and transplantation to field, mechanical inoculation with PRSV-W, subsequent evaluation and hand-pollination.

3.6.3 *Results*

The line 1487-04 had been previously derived from crosses involving *C. pepo* (zucchini), *C. moschata* (butternut pumpkin) and *C. ecuadorensis* (resistant to PRSV-W and ZYMV) and initially expressed high tolerance, but showed severe symptoms following transplantation and inoculation (18 October 1988) with PRSV-W. Later 1487-04 recovered slightly. Most open-pollinated progeny from this line expressed moderate to severe symptoms following mechanical inoculation, but two of the 96 plants produced through embryo rescue tissue culture appeared to have high tolerance. Nevertheless, PRSV-W virus was recovered from all plants. Fertility of pollen ranged from 0 to 20%. Self-pollinated seed and crosses to zucchini were produced with approximately 45 and 20 seedlings respectively being established. These were severely affected by PRSV-W and discarded. Further crosses of the tolerant line (1590 N3), derived from (1487-04), to *C. ecuadorensis* and *C. pepo* (zucchini) were made to improve resistance and seed yield.

The backcross to zucchini had high fertility (61% stainable pollen) but poor tolerance to PRSV-W. The cross back to *C. ecuadorensis* segregated 7 'bush':11 'vine' for bush type and initially showed no symptoms. Only one plant subsequently showed symptoms. Plants were male sterile (no stainable pollen). However a few female flowers would set when zucchini pollen was used and at least 120 plants were produced over three seasons. Combined infection of PRSV-W (inoculated) and ZYMV (natural spread) severely affected much of the latest population. However approximately 20 embryos from four resistant plants were rescued. Fertility, based on embryo numbers, varied between two and 40 embryos/fruit.

These and residual material of earlier generations could form the nucleus for continued development of resistant cultivars through conventional breeding.

A resistant selection 'Nigerian Local' of *C. moschata* was obtained. Resistance judged on leaf symptoms was high but virus (PRSV-W) was recovered from uninoculated leaves (Table 12). ZYMV resistance was also high. No progeny was developed from this line.

Table 12. Response of *C. moschata* lines to inoculation with ZYMV and PRV-W

Cultivar	ZYMV-K	ZYMV-G4	PRSV-W[a]	Recovery of PRSV-W from sixth leaf[b]
Butternut	wilt, necrosis	wilt, necrosis	50 chlorosis	9/9
Kens Special	vein clear, epinasty	chlorosis	70 vein clear	31/31
Nigerian Local	resistant hyper-sensitive[c]	resistant hyper-sensitive[c]	0	22/24

[a]Proportion of leaf area showing symptoms and predominant symptom type.

[b]Number of zucchini plants infected/number inoculated with bulk sap extract from sixth leaf above inoculated cotyledon.

[c]Hypersensitive small white spots on cotyledons.

3.7 Section 7 ELISA to quantify resistance

3.7.1 *Objective*

To develop an ELISA evaluation technique which allows the quantification of intermediate levels of resistance.

3.7.2 *Methods*

Methods are described in Dietzgen and Herrington (1991).

3.7.3 *Results*

The biotin-streptavidin (BA)-ELISA system detected the three potyviruses infecting cucurbits, the watermelon strain of papaya ringspot virus (PRSV-W), zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus 2. BA-ELISA was four to eight times more sensitive than double antibody sandwich ELISA and detected all three viruses in the nanogram range. BA-ELISA was virus-specific but did not differentiate between different pathotypes of ZYMV. The use of egg-white avidin-enzyme conjugate or the simultaneous incubation of biotinylated antibody and streptavidin-enzyme conjugate decreased the sensitivity of BA-ELISA. The concentrations of PRSV-W and ZYMV in field-infected cucurbit specimens and in resistant breeding lines were estimated from calibration curves derived from a dilution series of purified virus in extracts of uninfected plants on each test plate. Thus it was possible to determine semi-quantitatively the degree and uniformity of resistance in plants from these lines and to differentiate resistant from tolerant selections. (See also Dietzgen and Herrington 1991).

4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 General discussion

4.1.1 *Ovule culture media*

The type and concentration of plant growth regulators are generally considered to be major factors in determining the success of haploid culture (Yang and Zhou, 1982). However, we found virtually no differences (Tables 3 and 4) across the type and concentration range (0 - 500 x 10^{-6}M) studied. Chambonnet and de Vaulx (1985) and van Geyt *et al.* (1987) used a similar range of concentrations (1 - 100 x 10^{-6}M, 0 - 300 x 10^{-6}M) to induce the production of plants from ovules of zucchini and sugarbeet respectively, while cereals required higher concentrations (approximately 10^{-5}M) (Yang and Zhou, 1982). We used the lower concentrations of plant growth regulators because in general, higher auxins are associated with regeneration of plants from somatic tissue rather than from the haploid gametophytic cells (Yang and Zhou, 1982). There was no obvious effect of auxin: cytokinin ratio. The response to this ratio varies with species (Yang and Zhou 1982, van Geyt *et al.*, 1987).

4.1.2 *Ovule culture correlations*

The lack of linear correlations between ovule growth and plant or fruit characteristics (Table 7) is consistent with results for sugar beet (D'Halliun and Keimer, 1986). However in our work the largest (>0.875 mm) and sometimes the smallest (<0.625 mm) ovules germinated less than intermediate sizes (Table 8), indicating that there may be a preferred developmental stage for plant regeneration. Other observations have consolidated this view. Yang and Zhou (1982) indicated that in general, a broad range of developmental stages of embryo sac development (uninucleate to mature) was acceptable for plant development, but that there are variations between crops. Observations suggest that the stage of flower opening and embryo size may be poorly correlated over diverse environments and in the absence of data on stage of embryo sac development, ovule size may be the most reasonable guide to potential responsiveness of ovules.

4.1.3 *Origin of plantlets*

Doubling of haploids is induced to produce homozygosity but this commonly spontaneously occurs in tissue culture. Doubled haploid plants are expected to be homozygous. However in ovule culture, diploid plants may arise from either the gametic cells or from somatic cells of the embryo sac (Bollon and Raquin, 1988). Consequently

homozygosity of regenerates is difficult to control (Bollon and Raquin, 1988). Despite the relatively small size of our sample, the variation observed in progeny of fertile regenerates in our experiments (Table 11), is best described as originating from normal segregation following self-pollination of maternal plants, that is the fertile plants are of somatic origin.

The reason for this stimulated development of maternal tissue is uncertain, though a major difficulty in ovule cultures generally (Yang and Zhou, 1982). Development of maternal tissue is unlikely to be due to excessive exogenous growth regulators, a common cause (Yang and Zhou, 1982), because our levels were low and plants were also produced in the absence of exogenous growth regulators. Microscopic observation indicated that the volume of gametophytic tissue in ovules was quite small in proportion to the maternal tissue. Random stimulation of cell division and embryogenesis would give a commensurately much higher frequency of diploid plants of somatic origin. Some genotypes used were, in part, selected to be 'responsive' and there may have been inadvertent selection for somatic embryogenesis.

However only about 20% of germinated ovules were successfully established as plants. The ploidy of the non-established ovule-derived tissue was not determined. This should be assessed in future. Subsequently methods which increase the frequency of plant regeneration (for example from callus) and establishment may lead to higher yields of haploid plants. Reincubation using the ovule culture media and conditions may be useful. However substantial resources could be required to improve on the haploid production protocol presently described.

4.1.4 *Quantification of resistance - ELISA*

The ELISA system developed using biotin and streptavidin (Dietzgen and Herrington 1991) allows semi-quantitative evaluation of resistance. However, because sap of different species may produce differing calibration curves from which to estimate virus content (Scott *et al.* 1989, Dietzgen and Herrington, 1991), caution is still required in assessing the relative resistance of plants in segregating populations. This may be of special importance when the populations involved are derived from divergent species, as occurs in our programme.

4.1.5 *Backcross breeding*

Maintaining resistance to PRSV-W and ZYMV in a backcrossing programme is dependent upon producing plants in sufficient numbers

to have a high probability that at least one of the progeny carries high levels of resistance to both viruses, and then being able to identify this resistance.

Large populations would most easily be obtained with the resistance derived from *C. moschata* cv. Nigerian Local but this plant becomes systemically infected with PRSV-W (Table 12) and the *C. ecuadorensis* in our programme has a higher level of resistance. Also although fertility in our progeny of *C. ecuadorensis* is low, some recombination (for example bush type) has occurred and the prospect of transferring resistance to zucchini is favourable.

4.1.6 *Future direction and breeding*

At present the most appropriate strategy to develop virus resistant zucchini cultivars would seem to be to continue with a *C. ecuadorensis* based backcrossing programme aimed at producing large populations. Selection for resistance within each two or three consecutive generations should occur, so as to improve average resistance and fertility. Some backcrosses to *C. moschata* are warranted but other workers are also using this material. Genetic transformation to incorporate viral coat protein genes into plants, has been shown to be a promising means of producing virus resistance in a number of crops (Beachy *et al.*, 1990). This technique may provide another method of transferring novel resistance genes and is being explored elsewhere using PRSV and ZYMV. While development costs are expensive this technique ought to be considered in future as a possible viable alternative if interspecific crossing is unduly slow or difficult.

4.2 Conclusions

The ovule culture technique developed has offered limited immediate gains in the production of homozygous (and virus resistant) plants suitable for use as parents but it would be useful for eliminating virus from selected and desirable plants. The recovery of haploids may be improved by developing systems which stimulate the production of large numbers of plants from zucchini leaves, cotyledons, hypocotyls or callus. This area of research warrants further investigation; commencing in the case of ovule culture after determining the ploidy of the germinated ovules which subsequently fail to develop properly. Reincubation using ovule culture media and conditions may be appropriate. While this area of research may require substantial resources the results produced would also be applicable to the technology required in genetic transformation protocols.

In the absence of protocols for producing haploids consistently and in large numbers continued backcrossing of selected progeny is likely to be the most

economical procedure to develop virus resistance in zucchini in the short or medium term. Populations suitable for this procedure have been developed and should be developed further. Virus elimination through ovule culure is a useful technique able to provide a clean, selected population for crossing. In the longer term, other procedures (for example genetic transformations) should also be considered in developing virus resistant zucchinis.

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