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ANTHER CULTURE OF WINTER-HEADING CAULIFLOWER
April 1987 - March 1990

A
PROJECT REPORT FOR
THE HORTICULTURAL DEVELOPMENT COUNCIL

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SUMMARY

Anther culture of winter-heading (Roscoff) cauliflower was investigated over three seasons (1987 to 1990) using plant material supplied by three growers seed groups (Broccoli Seed Group, Codebric and Trinity Growers).

Techniques were adapted from the successful Brussel Sprout protocol developed at Wellesbourne together with media recipes from a research group from India. Plants were raised in a plastic tunnel without any supplementary heating or lighting and were therefore vernalised naturally.

Over the period of the investigation 21,000 anthers were cultured in 1987, 25,500 in 1988 and 24,000 in 1989. Nine media recipes were assessed with 5 based on the Wellesbourne media and 4 on the Indian media. A total of 236 plants were sampled.

Examination of the pollen development stages within and between buds indicated that in tunnel grown plants there is great heterogeneity in the speed of pollen development. Thus anthers of comparable sizes frequently are not comparable for pollen development stage. When the ratio of the petal length to anther length was related to pollen development stage again variability was found. However, it would appear that the criterion of the petal being about half to full length of the anther is a reasonable guide to the suitability for anther culture. The growing of plants in cool conditions (10/15 ^{o}C) may be advocated as this would slow down bud development and give a more predictable anther response rate.

Anther response was low in most plants examined. In 1987 there was no response to anther culture but in 1988 and 1989 positive response was obtained. The overall response in 1988 was 1.62% and in 1989 was 1.45% (embryoids produced per 100 anthers cultured). However, this masks an apparent genotypic effect with some plants showing a much higher response rate viz. in 1988, Trinity 22 16.3%, Codebric M 10.5%, BSG 3 13.9% and in 1989, Trinity 3001 26.7%, BSG 3$^{1}/_{2}$-63 38.4%. The total number of embryoids produced in 1988 was 152 and in 1989 was 336. Whilst this degree of response rate must still be considered to be low, it is significant that a number of responsive genotypes have been identified. These responsive genotypes have been tissue cultured or had stem/root cuttings taken from them for future use.

A protocol was refined for the sub-culturing of embryoids and their subsequent weaning. Some sub-cultured embryoids failed to respond further and went brown/black and died. When embryoids did respond many went through a disorganised phase prior to producing shoot structures. Several shoots could be obtained from the disorganised mass. At the time of writing, a total of 802 plants had been successfully weaned from 1176 shoots produced through the sub-culturing of embryoids generated during

1989. This represents a weaning success rate of 68.2%. Weaned plants were returned to the corresponding seed groups for further development work.

Ploidy determination was undertaken using the stomatal length technique on the plants weaned from the 1989 season. From this investigation it appeared that 32% were haploid, 54% were diploid and 14% polyploid. Thus it would seem that there is relatively high frequency of diploidisation in the cauliflower anther culture technique. Diploids will need to be selfed and their progeny studied before their homozygosity can be determined with certainty.

Ovule culture was attempted in 1989 and the initial stages of a protocol was developed. There was no exbryogenic response in the cultures established.

An alternative micropropagation method was refined during the investigation, that of petal culture. Proliferating cultures which subsequently produce good clonal material can be generated by using immature petals from unopened buds as the initial explant. The technique is useful as it gives an opportunity for micropropagation of genotype at a very late stage in the development of the plant. When plant selection in a breeding programme is for floral characteristics or, for example, for self incompatability then the value of this micropropagation technique is realised.

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INTRODUCTION AND BACKGROUND

The winter-heading cauliflower crop is grown largely in the frost-free regions of the UK with the largest area in the southwest but with other areas of production in Kent (Isle of Thanet), Pembrokeshire and Jersey. The crop grown in most of these areas is based on locally bred varieties maintained and multiplied by seed groups with the remainder of the area taken up by commercial varieties produced by Seed Houses (mostly Clause Ltd). The crop is also grown in the Finisterre region of Brittany particularly around Roscoff and indeed the group of varieties used for this heading period in the UK are collectively called the "Roscoff types".
All of the Roscoff types grown in the UK are of an open pollinated nature and most are based on an original selection of between 15 and 40 mother plants. In Brittany the varieties are predominantly open pollinated as in the UK but at the end of the 1980's the first products of a 20 year conventional F1 hybrid breeding programme started to become available to French growers.
The characteristics of an open pollinated variety of cauliflower are that it matures over a long period of time (4-6 weeks) and this necessitates frequent trafficking of a field to complete the harvest of the crop. Also the crop is a mixture of genotypes and this can lead to a variability of quality over the crop and a percentage may be completely unmarketable. However, such a mixture of maturity and genotypes can give the grower a buffer against adverse weather (particularly cold weather) and help in the management of the seasons production i.e. only six varieties may be necessary to cover the complete harvesting period from December to April.
The characteristics of an F1 Hybrid variety of cauliflower are that it matures over a much shorter time period (1-2 weeks) thus reducing trafficking and making more efficient use of harvesting labour. Also the crop is made up of genetically uniform individuals thus giving uniform product of high quality. Thirdly the crop may possess hybrid vigour which could offer the opportunity of closer spacing of the crop in the field and therefore increase the output per unit area. However, there are two problems associated with the growing of F1 hybrids; firstly the whole crop is at risk if there is adverse weather, and secondly there is a need to produce 10 to 20 varieties to cover the cutting period December to April.
One risk to the UK produced Roscoff cauliflower crop lies in the threat of improved French produce coming onto the market during the 1990's which is derived from F1 hybrids. Since there appear to be no Commercial Seed Houses actively involved in the production of F1 hybrid Roscoff cauliflower, it is prudent that the Seed Groups of the UK together with the Horticultural Development Council are concerned with the rapid production of F1 hybrids of this group of cauliflower. Unfortunately, F1 hybrids can only be produced by crossing two inbred lines whose genetic homozygosity is ensured. Owing to the nature of the crop and its long growing season this could take upwards of 10 to 15 years to achieve at high cost.
However, recent techniques of plant tissue culture have succeeded in

shortening this time period considerably by the in-vitro production of haploids and their subsequent induced diploidisation. This technique can lead to the production of "artificial inbreds" in only one year and therefore save an enormous amount of time and expense in a breeding programme.

Haploid plant production

A haploid plant is best described as a plant whose cells contain a single set of complete chromosomes.

Haploids have great value as intermediates in the production of homozygous plants (inbreds) which are much sought after in breeding programmes (Dunwell 1986). Haploid plants may be produced by the in-vitro culture of the haploid cells in the flower:

Androgenesis: culture of the male haploid cells i.e. the microspores/pollen through isolated microspore or pollen culture or through the entire culture of the anthers (pollen bearing sacs). One potential problem of using anther culture is that the anther itself is made of diploid mother plant cells and if it responds itself to the culture conditions then diploid clones of the mother plant may be produced. In most anther culture protocols the removal of the filament from the anther is recommended as this structure is very responsive to in-vitro culture.

Gynogenesis: culture of the female haploid cells i.e. the ovules or the ovaries. As with anther culture, female haploid cells are frequently surrounded by diploid mother cells which can respond to culture. A second problem of gynogenesis is the relatively low number of ovules in a flower compared to the number of pollen grains in an anther.

What is actually happening when a haploid cell in the flower is forced to grow into a haploid plant in-vitro is an induction of embryogenesis without the normally necessary act of fertilisation. Thus the pollen or ovary is stimulated to "switch-on" its embryo development genes and show totipotency by then developing into a complete plant. This "switching-on" of the embryo development gene sequences is artificially induced by the culturing technique together with certain treatments such as heat or cold shock.

The first products of haploid induction are termed embryoids. These are usually swellings emanating from a ruptured anther wall or ovule. In some plant species these embryoids appear structurally very similar to zygotic embryos and can go through a very similar developmental sequence; proembryo to globular to heart shaped to torpedo to plantlet. In other species the embryoids may increase in size without an initial increase in complexity and resemble a callus growth. This in turn will undergo embryogenesis around its periphery and produce embryoids - this may produce several clones of the original embryoid but may also lead to different genotypes being produced since callus is prone to somaclonal variation. The production of one plant per embryoid can be considered as the more straight-forward method.

The products of in-vitro culture of anthers or ovules can never be guaranteed to be haploid and require screening after induction and usually after weaning to determine their genetic make-up. Embryoids could be diploid or triploid or tetraploid through spontaneous chromosome doubling at some stage in their development. Also, haploid nuclei could fuse with other haploid nuclei (e.g. generative and vegetative nuclei) and produce a non-homozygous embryoid. Also, as has already been mentioned, apparent embryoids can be derived from somatic mother cells in which case they would be diploid and very heterozygous.
The most promising protocol for the use of these techniques in a breeding programme is therefore one which produces a high frequency of haploid plants.

Factors affecting embryoid production
Many factors can affect the degree of responsiveness of a plant to embryoid production.
There is considerable evidence in some species that individual genotypes vary enormously in responsiveness and that this is a heritable trait carried by a limited number of nuclear genes (Uhrig 1985, Chen 1986, Henry and de Boyser 1985). In a species (like Roscoff cauliflower) where the degree of heterozygosity is high then this may not be important since even from a single responsive individual genotype a large number of genetically different haploids are obtainable for potential use as inbreds. In Brassica species such genotypic variability is not uncommon (Ockendon 1985) and appears to exist in the cauliflower species also (Ockendon 1989).
The growth conditions of the donor plants can affect plant responsiveness. In particular, the plants should normally be grown under stress-free conditions in terms of nutrients and water. Also, agrochemicals (fungicide) can influence culture response and should not be used on the flowering spikes. In some breeding programmes the culture conditions for the donor plants have been optimised by the use of controlled environments to reproduce ideal conditions. Thus for Brassicas a growing environment with a high light intensity appears necessary but the optimum growing temperature appears very species dependent (Keller et al 1983).
Many species flower over a long time period and this is especially the case in Brassicas and there is some evidence to suggest that more success is obtained in culturing from flowers in the early part of the flowering cycle (Keller et al 1983).
Other factors fall into the remit of the culture technique and include:

Development stage - of pollen or ovule

Shock treatment - pretreatment of material prior to culture incubation i.e. heat/cold shock for a few hours or many days

Sterilisation technique

Media composition - solid or liquid, osmotic potential, mineral salt

base, vitamin supplement, hormone mixture and concentration, organic supplements, presence/absence activated charcoal, presence of silver nitrate.

Incubation - temperature, light/dark, culture vessel ventilation..

Anther culture

Anther culture was first refined in the mid-sixties in Datura innoxia (Guha & Maheshwari 1966) and in the 25 years since then has been applied successfully to over 200 species with many of these species in the families Solanaceae and Graminae. Active research in this field is today limited to only a few species but is used in a more routine manner in several important agricultural crops e.g. oil-seed rape.

Anther culture involves the in-vitro culture of the entire anther (minus the filament) and is more commonly used than pollen culture owing to its easier implementation and also because in several species it has been shown that the anther wall itself help stimulate sporophytic development of the microspores/pollen. Pollen culture is usually applied to species where anther culture has failed.

Measuring the success of anther culture is not straightforward but can be divided into several stages:

1. maximising the production of embryoids per anther cultured.

Obviously the higher the response of the anthers then the better the technique is considered to be working. Also, highly responsive and predictively responsive individuals or species allow for easier experimentation and refinement of optimal conditions. In reality, it is common to achieve a wide range of responsiveness between anthers even within a responsive individual. A response of over 100 embryoids produced per 100 anthers cultured would be considered to be good. However where the production of inbred lines in a species is very time-consuming lower rates of response can be considered acceptable.

2. maximising the production of haploids from embryoids.

As explained earlier, the production of haploids via anther culture gives a greater degree of confidence to the technique since haploid embryoids can only have been derived from microspores/pollen whereas diploid embryoids need to be checked for homozygosity before they are deemed useful to a breeding programme.

3. maximising the number of weaned plants from culture.

Obviously the technique is only of use in a breeding programme if the products of in-vitro culture can be weaned successfully and raised to mature flowering plants. Thus an established and successful tissue culture protocol for the species is desirable.

Anther culture of Brassicae

Anther and microspore culture of various species of Brassicae has provided reliable methods for the production of haploid plants and subsequent homozygous lines:

B. campestris (Keller & Armstrong 1979)

B. hirta (Klimaszewska & Keller 1983)
B. juncea (George & Rao 1983)
B. napus (Keller & Armstrong 1977)
B. oleracea var. italica (Keller & Armstrong 1981)
B. oleracea var. capitata (Kuo et al 1983)
B. oleracea var. gemmifera (Ockendon 1984, 1985).

Reviewing these reports it evident that the conditions necessary for successful anther culture varies greatly between species and frequently genotype variability to culture was high. For most Brassica species it appears that high light intensities and low temperatures during the growth of donor plants favour culture responsiveness.

At the beginning of this project there was very little evidence in the literature concerning B. oleracea var. botrytis (cauliflower). Bagga et al (1982) working in India reported some work on this species but species responsiveness and plant ploidy were not reported.

EXPERIMENTAL WORK 1987-1990

AIMS

The main aim of the sponsored project was to investigate the possibility of developing an anther culture system for use in winter-heading cauliflower. It was necessary at the outset to impose certain restrictions on the investigations which brought the project into the reality of the situation that exists with the grower sponsors who will be the main benefactors of the experimental results. The main restriction was to attempt to produce a technique that would be attainable by growers with limited facilities of controlled growth chambers and with little sophisticated laboratory equipment.
The second aim was to characterise the products of the in-vitro work (if any) and provide the sponsoring growers with potential diploidised haploids for use in their breeding programmes.

Outline plan of experimentation

The experimentation was based largely around the three potential flowering seasons. Flowering of this group of plants usually occurs from April to August and there is a 12 month growing period i.e. sown or cultured (tissue culture or root/shoot cuttings) in July-August and flowering the following spring/summer. With the sponsorship commencing on April 1st 1987 this posed some problems with the first flowering season.
A summary diagram of the plan of experimentation is given in Figure 1. The experimentation followed a logical sequence of events dictated by the flowering season. Maximum technical effort was put into anther culture to maximise the numbers of anthers cultured. In this way, very low response rates to anther culture would be detected and help lead to the refinement of technique.

Aside from the main investigation of anther culture two other avenues were investigated - petal culture (noted in some of the anther culture plates) and ovary culture.

Personnel

The following Personnel have worked on the project over the 3 years:
Dr Brian Grout (original Project Leader - resigned from the Polytechnic in Dec 1988)
Dr Michael Fuller (took over as Project Leader from Dr Grout)
Mrs Susan Turton (in a part-time capacity for the duration of the project)
Mr David Hawkins(Sandwich Student April 88 - Sept 88)
Miss Karen Pulford (Sandwich Student April 89 - Sept 89)
Dr Julie Strange (part-time March 89 - July 89)
Mrs Norma Simkin (part-time Jan 90 - March 90)

Figure 1 Plan of experimentation.

A 87	Order and set-up Plastic Tunnel - Growing environment
M	
J	Start anther culture ; Examine pollen development stages
J	Maximise the number of anthers cultured
A	ditto
S	Examine anthers cultured ; Investigate Petal Culture
O	Set-up Second Cycle of plants in tunnel
N	
D	Report to Growers
J 88	
F	
M	
A	Start Anther Culture ; Investigate Pollen:bud size ratios
M	Rationalise Media - start using Silver Nitrate
J	Maximise numbers of Anthers Cultured.
J	ditto
A	ditto
S	Characterise embryoids produced
O	Attempt to wean embryoids
N	Set-up Third Cycle of plants in tunnel
D	Report to Growers
J 89	
F	
M	
A	Start Anther Culture
M	Investigate Ovary culture
J	Maximise numbers of anthers cultured
J	ditto
A	ditto
S	Score and characterise embryoids produced
O	Raise embryoids to plantlets
N	ditto
D	ditto
J 90	Report to Growers
F	Wean plantlets and characterise ploidy
M	ditto

1987 SEASON

OBJECTIVES

The main objective of the first flowering season was to maximise the number of anthers put into culture on the maximum number of media types with potential for successful anther response. Since the plants that were supplied to the project were already flowering at the start of the contract then anther culture had to commence prior to any testing of techniques. Throughout the season routine examination of a number of buds for pollen development stage was undertaken to try and predict the optimum bud size for culture.

MATERIALS AND METHODS

Examination of Pollen Development Stage

Many investigations of anther culture have revealed that the pollen is only responsive to embryogenesis for a short period of time. This begins just after the release of the microspores from the tetrad after the second meiotic division but before the first mitotic division of the microspore. This stage is referred to as the uninucleate stage. The general developmental sequence is as follows:-

Pollen mother cells
V
Diads (1st meiotic division)
V
Tetrads (2nd meiotic division)
V
Tetrad breakdown release of uninucleates
V
Mitotic division, giving pollen tube nucleus and generative nucleus
V
Vacuolisation and appearance of exine
V
Lobed pollen grain with thick exine

The determination of the pollen development stage requires the examination of several anthers on each occasion and many workers have sought to find indicators of pollen development stage in easy recognisable floral structures e.g. bud size, colour, petal size, anther size and colour and ratios such as petal size to anther size. This investigation looked at these characters in order to try and determine the optimum bud size for sampling of winter-heading cauliflower.

Procedure

On several occasions through the flowering season buds were sampled from

plants and their anthers prepared for microscopic examination. This involved the dissection of buds under a dissecting microscope (x20), measurement of floral parts, removal of anthers, removal of filaments, squashing of anthers in Aceto-orcein stain on microscope slides, incubation at room temperature for 10 to 15 minutes and then examination under a Zeiss transmission light microscope (x400).

Anther culture

Plant raising

Plants provided by the sponsoring growers were delivered to Seale-Hayne at the beginning of April 1987. Plants from Trinity Growers were in large polythene plant pots, those from Broccoli Seed Group in 12 inch clay pots and those from Codebric in 10 inch plastic pots. The plastic tunnel was not erected until 29th May, and until this time the plants were kept either in a glasshouse with overhead irrigation or outside with hand watering. In June all plants were moved into the Tunnel and watered by drip feed. All pots were surface treated with Temic (aldicarb) insecticide. At this stage the B.S.G. plants and the Trinity plants were well advanced with many having reached flowering. Plant condition was not good and many plants abscised leaves as they flowered.

Anther sampling procedure

Bud clusters with a range of bud sizes but without open flowers were cut from the donor plants and the cluster placed into a labelled petri dish or culture tube containing dampened filter paper and transported to the laboratory. The procedure was standardised throughout the investigation with all procedures being carried out in a laminar flow cabinet (Table 1)

In this manner a total of between 6 and 12 petri dishes could be completed by one operator in a day i.e. 250 to 500 anthers cultured per day. As operators became skilled at this technique then 500 per day was normally achieved with a maximum of 1000 per day being the uppermost limit by a single operator.

Seven media types were used and the Media recipes are given in Table 2.

After plating the anthers onto petri dishes different pretreatments were applied before culturing at 23^{o}C in the dark. Pretreatments included varying times of incubation from 8 to 72 hours at either 35^{o}C or 4^{o}C and these were compared to no pretreatments (such pretreatment is for convenience hereto referred to as "shock").

Cultured anthers were periodically examined for any signs of culture activity and recorded.

Table 1. Generalised procedures used for the culture of anthers

bud clusters surface sterilised - immersion in 5% domestic bleach solution for 3 to 4 minutes agitated every 20 seconds or so.

V

bud clusters rinsed three times in a step-wise fashion - by immersion in sterile distilled water and shaken.

V

buds of the desired size selected and excised from the cluster.

V

sepals removed using a sterile scalpel/needle

V

anthers removed - without filament where possible (by flicking the anther outwards). If filament remained attached then this was physically removed.

V

anthers placed lengthwise onto 9cm petri dishes containing agar solidified media. Each petri dish contained 36 to 42 anthers from 7 buds.

V

petri dishes sealed with nescofilm.

Table 2. Media Recipes used in 1987

OCK 1*	Gamborgs B5 + 600 mg/l Calcium chloride dihydrate + 800mg/l Glutamine + 0.6% activated charcoal + 10% Sucrose (as domestic cane sugar) + 0.1 mg/l NAA + 0.1 mg/l 2.4-D + 0.8% Agar.
OCK 2	As OCK 1 without activated charcoal.
OCK 3	As OCK 1 with 0.4% activated charcoal
OCK 4	As OCK 2 with 5% sucrose
BA 1"	Gamborgs B5 + 10% sucrose + 0.5 mg/l BAP + 0.8% Agar
BA 2	As BA 1 with 0.6% activated charcoal
BA 3	As BA 1 with 5% sucrose

All media adjusted to a pH of 5.8 and autoclaved at 15 psi, 121 oC for 20 minutes with all ingredients added. Media poured into plates whilst warm following agitation.

* after Ockendon (1984), " after Bagga et al (1982).

RESULTS

Pollen Development Study

The results of the study are given in Table 3. Early in the study the apparent bud size to use appeared to be in the region of 2.25 to 2.5 mm with an anther to petal ratio of 1.3 to 1.6. Larger buds seemed to have pollen that was too far developed to expect it respond. However, later in the study, the desirable stage of development (uninucleate) appeared to be associated with a larger bud size 2.75 to 5.0 mm with an anther to petal ratio of 0.75 to 1.75. Despite the disparity of bud size with these two samplings the anther to petal ratio was of the same order. This highlights the importance of a development stage rather than a size stage. However, there does appear to be some overlap between the plants assessed and this could be due to genotypic differences or environmental differences. Observations of plants indicates that if temperatures are high and therefore the development rate of buds is quick, then smaller buds are produced and bud opening can occur at a small bud size. Also, it can be inferred that under high temperatures the pollen developmental sequence is quick and as a result the uninucleate stage will not last for long. Under such conditions it would be easy to "miss" the optimum stage for culturing. Thus it would seem to be good practise when culturing to culture a range of bud sizes (within the range of anther:petal ratio 0.75 to 1.75) to be sure of obtaining some anthers at the correct stage. We have examined the degree of variation within the anthers contained within a single bud and found that this can be considerable further emphasising the need to culture a range of bud sizes.

Anther culture

Tables of anther numbers cultured are given in Tables 4 to 6. These show that between 6,000 and 8,500 anthers were cultured from the plants supplied by each of the sponsoring growers groups. The majority of these were cultured without using a shock treatment.

Examination of anthers revealed that anthers cultured with their dehiscence lines down and in contact with the medium appeared healthier. Anthers cultured the other way up tended to shrivel and go brown more quickly. Once this observation had been made, all subsequent anthers were cultured, where possible, with their dehiscence lines down. Other important observations included : 1. the ability of the filament fragments to respond to culture. In the main this response was to produce roots which grew along the surface of the medium; 2. the ability of immature petal fragments to proliferate shoot structures which turned green when plates were transferred to the light.

Table 3. Results of Pollen Development Survey

Plant code & date	Bud length mm	Petal length mm	Anther length mm	Anther to Petal ratio	Pollen Development Stage
0830	1.5	0.5	1.1	2.2	pollen mothers
21 Apr	2.0	0.67	1.5	2.24	tetrads
	2.5	1.0	1.75	1.75	true pollen
	2.5	1.25	1.67	1.34	true pollen
	2.5	1.5	2.0	1.33	true pollen
	2.5	1.25	1.67	1.34	tetrads
	2.5	1.25	1.67	1.34	tetrads
	2.5	1.25	1.75	1.4	true pollen
	3.0	1.75	2.33	1.33	lobed pollen
	3.0	2.0	2.33	1.17	lobed pollen
	3.5	2.25	2.5	1.11	lobed pollen
	4.0	3.0	2.5	0.83	lobed, thick exine
	5.0	4.0	2.67	0.67	lobed, thick exine
	6.0	7.0	3.0	0.43	lobed, thick exine
0850	2.0	0.8	1.5	1.88	tetrads
21 Apr	2.25	1.1	1.67	1.52	uninucleate
	2.25	1.1	1.75	1.59	uninucleate
	2.5	1.5	1.67	1.11	true pollen
	2.5	1.5	2.0	1.33	true pollen
	3.0	2.33	2.25	0.97	lobed pollen
	4.25	2.67	2.5	0.94	lobed pollen
5 C/F	2.0	0.75	1.5	2.0	tetrads
24 May	2.25	0.75	1.5	2.0	tetr. + uninucl.
	2.5	1.5	1.75	1.17	true pollen
	2.75	1.5	2.0	1.33	true pollen
2423A	-	-	1.5	-	pollen mothers
13 Jun	-	-	1.5	-	pollen mothers
	-	-	1.75	-	pollen mothers
	-	-	1.75	-	pollen mothers
	-	-	2.25	-	tetrads
	-	-	2.25	-	tetrads
	-	-	2.5	-	tetrads
	-	-	2.5	-	tetr. + few uninucl.
	-	-	2.5	-	uninuc. + few tetr.
1435	2.25	1.5	0.75	2.0	early tetrads
3 Aug	2.5	0.75	1.5	2.0	tetrads
	2.5	0.75	1.5	2.0	tetrads
	2.75	1.0	1.75	1.75	early uninucleate
	3.0	1.25	2.0	1.67	early uninucleate
	3.25	2.0	2.5	1.25	early/mid uninuc.
	3.75	2.0	2.25	1.125	mid uninucleate
	4.0	2.25	2.5	1.10	mid/late uninuc.
2423A	3.25	2.5	2.5	1.0	uninucleate
3 Aug	4.25	2.5	2.75	1.1	uninucleate
	4.25	3.0	2.75	0.91	uninucleate
	5.0	3.75	2.75	0.75	binucleate + late uninucleate

TABLE 4 - Nº of Anthers cultured - Trinity Growers Plants 1987

MEDIUM	NUMBER OF PLANTS				NUMBER OF ANTHERS NO. SHOCK				NUMBER OF ANTHERS SHOCK				SUB TOTAL				
	6	7	7	9													
	10	20	21	22	10	20	21	22	10	20	21	22	10	20	21	22	TOTAL
BAI	3	3	7	7	573	259	777	959	-	-	-	-	573	259	777	959	2568
BAII	1	3	1	1	41	371	123	84	-	-	-	-	41	371	123	84	619
BAIII	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-
OCKI	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-
OCKII	3	3	5	6	375	248	546	487	-	-	-	32	375	248	546	519	1688
OCKIII	3	5	0	3	331	490	-	454	-	-	-	-	331	490	-	454	1275
OCKIV	0	0	1	1	-	-	-	-	-	-	34	65	-	-	34	65	99
					320	368	1446	1984	-	-	34	97	1320	1368	1480	2081	6249

TABLE 5 - N° of Anthers cultured - Broccoli Seed Group Plants 1987

MEDIUM	NUMBER OF PLANTS			NUMBER OF ANTHERS NO. SHOCK			NUMBER OF ANTHERS SHOCK			SUB TOTAL			
	8	9	8										
	BS6 1½	BS6 4	BS6 5	BS6 1½	BS6 4	BS6 5	BS6 1½	BS6 4	BS6 5	BS6 1½	BS6 4	BS6 5	TOTAL
BAI	8	7	5	610	846	865	-	-	-	610	846	865	2321
BAII	0	0	1	-	-	122	-	-	-	-	-	122	122
BAIII	0	0	0	-	-	-	-	-	-	-	-	-	-
OCKI	8	4	1	727	350	80	-	-	-	727	350	80	1157
OCKII	0	3	2	-	329	374	-	-	-	-	329	374	703
OCKIII	1	4	3	239	240	453	-	564	170	239	804	623	1666
OCKIV	0	1	0	-	-	-	-	26	-	-	26	-	26
				1576	1765	1894	-	590	170	1576	2355	2064	5995

TABLE 6 - Nº of Anthers cultured - Codebric Plants 1987

MEDIUM	NUMBER OF PLANTS			NUMBER OF ANTHERS NO. SHOCK			NUMBER OF ANTHERS SHOCK			SUB TOTAL			
	10	9	8										
	M	A	AM	M	A	AM	M	A	AM	M	A	AM	TOTAL
BAI	5	3	7	778	82	535	-	362	1025	778	444	1560	2782
BAII	3	1	0	288	81	-	-	-	-	288	81	-	369
BAIII	0	2	3	-	489	286	-	-	90	-	489	376	865
OCKI	0	0	0	-	-	-	-	-	-	-	-	-	-
OCKII	4	3	1	124	-	-	515	469	123	639	469	123	1231
OCKIII	6	5	1	1330	1124	157	-	43	-	1330	1167	157	2654
OCKIV	0	1	3	-	251	416	-	-	166	-	251	582	833
				2520	2027	1394	515	874	1404	3035	2901	2798	8734

DISCUSSION

Despite culturing a total of nearly 21,000 anthers from a total of 81 plants of 10 genotypes (open pollinated varieties), no embryoids were found. This finding was obviously disappointing but not discouraging. Plants were acknowledged to be in a poor condition and stressed and it is well reported that the quality of the mother plant plays a key role in determining culture response. Procedures were therefore invoked to relieve the stress conditions for the second culturing season.

The response of immature petals to tissue culture was an important accidental discovery and was to be investigated further alongside the anther culture programme in the following season.

1988 SEASON

OBJECTIVES

The main objective of the 1988 season was to repeat the mass anther culture approach attempted in the 1987 season using a fresh set of plants that were less stressed in their growing. Furthermore, a different bud size was to be assessed. Also during this season an evaluation of plants derived from petal culture was to be instigated (see Appendix 1).

MATERIALS AND METHODS

Plant raising

Plants provided by the sponsoring growers were delivered to Seale-Hayne and potted into 10 inch plastic pots containing peat based compost and treated with the pesticide Temic (aldicarb). The pots were placed into the plastic tunnel in the arrangement shown in Figure 2. Pots were watered with a drip feed irrigation system and fed at intervals with Liquinure and Maxicrop.

The plants from each sponsoring group were subdivided into groups and prefixed with the growers code as follows:

Broccoli Seed Group (BSG)	-	$1^1/_2$; 2; 3; 4;
Codebric	-	CM; CMA; CF; CFM;
Trinity	-	08; 11; 14; 17; 18; 21; 22; 24; AT.

The BSG and Trinity growers provided plants surplus to requirement and selections were made from these at sampling times.

On three dates (1 Feb, 16 March, 28 April) all plants were scored for the following: virus presence and severity of infection, vigour (rated 1-4), stage and condition of curd/flower buds.

Hot, sunny weather in May and June accelerated flower development and this necessitated a decapitation procedure in order to stimulate a second flush of flower buds for later sampling. In some cases this was done twice. The decapitation was a continuous process from 10th May to 28th July.

Anther Culture

Anther culture was carried out over the time period 21st April to 11th August. Initially, the same bud size was sampled as in 1987 that is small buds in the range 2-4 mm. The bud size was then changed to the larger size 3-6 mm following a site meeting of workers from Wellesbourne.
The anther culture procedure was the same as that described previously. All cultured anthers had their filaments removed.

All anthers were given a thermal shock of 35°C in the dark for 16-24 hours and then cultured at 23°C in the dark.

Media

The Media recipes are given in Table 7. A rationalisation of media to be used was made in an attempt to increase numbers of anthers plated onto a specific medium. In this way, if embryo formation occurs at a very low rate then there is an increased chance of detecting it. An important addition to the media recipes was the addition of Silver Nitrate. This followed discussions with colleagues at Wellesbourne who had noted increased anther culture success rates in recalcitrant varieties of Brussel Sprouts when silver nitrate was incorporated into media (Biddington et al 1989).

Table 7 Media used in the 1988 season

CODE	RECIPE
BAI	Gamborg's B5 + 10% sucrose+ 0.5 mg/l BAP + 0.8% Agar
BA II	as BAI + activated charcoal
BA IV	as BAI + 500 ppm silver nitrate
OCK I	Gamborg's B5 + 600 mg/l calcium chloride dihydrate + 800mg/l glutamine + 0.5% activated charcoal + 10% sucrose + 0.1 mg/l NAA + 0.1 mg/l 2,4-D + 0.8% Agar
OCKII	as OCKI without charcoal
OCKV	as OCKII + 500ppm silver nitrate
G2(weaning)	Gamborgs B5 + 2% sucrose (no hormones)
X26(rooting)	M & S + 3% sucrose + 2mg/l I.B.A.

all media adjusted to pH 5.8

RESULTS

Tables of anther numbers cultured produced are given in Tables 8 to 11. These tables show that for each of the six chosen media between 2,000 and 3,000 anthers were cultured in the bud size range 2-4mm and 1,000 to 2,000 in the bud size range 3-6mm. These anthers were selected from between 70 and 91 plants all of which were differing genotypes. Numbers of anthers cultured from the three different seed groups is summarised below:

Group	Anthers cultured 2-4mm	Anthers cultured 3-6mm	Total
Trinity	5279	4503	9782
Codebric	4496	2684	7180
BSG	6370	2213	8583

Results in the initial part of the season were disappointing with a total of 16,145 anthers of the size range 2-4mm cultured yielding only three embryoids. When the stage of anther development was shifted however, more response was obtained. In the size range 3-6mm a total of 9,400 anthers cultured yielded 152 embryo's (see tables 12 to 15). The overall response rate is calculated as being 1.62% (embryoids produced per 100 anthers cultured). Between plants the response rate varied from less than 1% to over 16%.
Of the cultivars tested, all but two showed some response to anther culture.

When data was pooled across media types it was found that all media gave some response to anther culture (Table 15). However there was a tendency for the OCK media to be more responsive than the BA media and for the most responsive medium to be the OCK medium without activated charcoal but with added silver nitrate.

Table 8 No. plants and anthers cultured of plants supplied by Trinity Growers 1988

	NUMBER OF PLANTS							TOT.	NUMBER OF ANTHERS BUD SIZE RANGE 2½-4MM							SUB TOT.	NUMBER OF ANTHERS BUD SIZE RANGE 3-6MM							SUB TOT.	TOTAL
	08	11	14	21	22	24	AT		08	11	14	21	22	24	AT		08	11	14	21	22	24	AT		
Medium	8	1	7	5	5	6	1	33																	
BAI	8	1	7	4	5	6	1	32	439	-	-	166	216	178	-	999	126	41	324	48	79	129	45	792	1791
BAII	6	1	5	4	4	5	1	26	436	-	-	167	167	177	-	947	44	36	213	44	46	92	42	517	1464
BAIV	5	1	7	3	5	5	1	27	132	-	-	83	215	180	-	610	87	41	328	45	97	93	42	733	1343
OCKI	5	1	6	4	4	6	1	27	296	-	-	168	168	173	-	805	47	39	270	38	40	128	42	604	1409
OCKII	8	1	7	4	5	5	1	31	367	-	-	168	169	174	-	878	239	53	409	42	86	92	46	967	1845
OCKV	8	1	7	4	5	6	1	32	441	-	-	209	223	167	-	1040	183	46	358	41	86	131	45	890	1930
									2111	0	0	961	1158	1049	0	5279	726	256	1902	258	434	665	262	4503	9782

Table 9 No. plants and anthers cultured of plants supplied by Codebric Limited 1988

	NUMBER OF PLANTS				TOT.	NUMBER OF ANTHERS BUD SIZE RANGE $2\frac{1}{2}$-4MM				SUB TOT.	NUMBER OF ANTHERS BUD SIZE RANGE 3-6MM				SUB TOT.	TOTAL
	F	FM	M	MA		F	FM	M	MA		F	FM	M	MA		
Medium	9	7	7	6	28											
BAI	7	6	6	4	23	294	170	141	36	641	44	90	174	122	430	1071
BAII	7	5	5	6	23	332	171	126	111	7407	-	51	129	126	306	1046
BAIV	7	6	5	6	22	265	295	124	118	8020	84	94	83	133	394	1196
OCKI	6	6	5	5	22	285	168	147	116	7165	-	84	130	125	339	1055
OCKII	9	7	6	5	27	335	167	137	120	7598	85	159	229	131	604	1363
OCKV	9	7	6	7	27	339	253	132	114	8380	131	127	228	125	611	1449
						1850	1224	807	615	4496	344	605	973	762	2684	7180

Table 10 No. plants and anthers cultured of plants supplied by Broccoli Seed Group 1988

	NUMBER OF PLANTS				TOT.	NUMBER OF ANTHERS BUD SIZE RANGE 2½-4MM				SUB TOT.	NUMBER OF ANTHERS BUD SIZE RANGE 3-6MM				SUB TOT.	TOTAL
	1½	2	3	4		1½	2	3	4		1½	2	3	4		
Medium	9	8	9	8	34											
BAI	7	6	6	8	27	291	248	212	293	1044	40	82	86	94	302	1346
BAII	6	4	5	7	22	259	250	210	353	1072	42	-	37	55	134	1206
BAIV	6	4	5	6	21	214	83	165	209	671	40	91	40	98	269	940
OCKI	8	4	7	6	25	312	285	263	288	1148	83	43	89	48	263	1411
OCKII	8	8	9	8	33	297	252	255	334	1138	74	165	250	134	623	1761
OCKV	8	8	9	8	33	306	330	243	418	1297	88	173	209	152	622	1919
						1679	1448	1348	1895	6370	367	554	711	581	2213	8583

Table II - Overall Totals of Plants and Anthers Cultured 1988

Medium	Total No. Plants	Total No. Anthers bud size 2½-4mm	Total No. Anthers bud size 3-6mm
BAI	82	2684	1524
BAII	71	2759	957
BAIV	70	2083	1396
OCKI	74	2669	1206
OCKII	91	2775	2194
OCKV	82	3175	2123

Table 12 Embryoid Data Sheet for Trinity Growers Plants 1988

	NUMBER OF RESPONSIVE PLANTS							TOT.	NUMBER OF EMBRYOIDS							TOT.	# % RESPONSE RATE OF ANTHERS						
Medium	08	11	14	21	22	24	AT		08	11	14	21	22	24	AT		08	11	14	21	22	24	AT
BAI	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	1.26	0	0
BAII	0	1	0	0	0	1	1	3	0	4	0	0	0	9	1	14	0	11.11	0	0	0	9.78	2.38
BAIV	0	0	0	1	1	0	0	2	0	0	0	1	4	0	0	5	0	0	0	2.22	4.12	0	0
OCKI	0	1	0	0	1	0	0	2	0	3	0	0	4	0	0	7	0	7.69	0	0	10.0	0	0
OCKII	0	0	1	0	1	0	0	2	0	0	1	0	3	0	0	4	0	0	0.24	0	3.48	0	0
OCKV	2	1	1	1	1	1	0	7	2	1	13	2	14	1	0	33	1.09	2.17	3.63	4.87	16.27	0.76	0
								17	2	8	14	3	26	10	1	64							

* All embryoid data presented refers to anthers cultured from buds in the size range 3-6mm

\# % response rate calculated as $\frac{\text{No. embryoids obtained}}{\text{No. anthers cultured}} \times 100\%$

Table 13 Embryoid Data Sheet for Codebric Limited Plants 1988

	NUMBER OF RESPONSIVE PLANTS				TOT.	NUMBER OF EMBRYOIDS				TOT.	# % RESPONSE RATE OF ANTHERS			
Medium	F	FM	M	MA		F	FM	M	MA		F	FM	M	MA
BAI	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BAII	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BAIV	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OCKI	0	1	0	1	2	0	1	0	2	3	0	1.19	0	1.6
OCKII	0	0	1	0	1	0	0	24	0	24	0	0	10.48	0
OCKV	0	0	1	0	1	0	0	12	0	12	0	0	5.26	0
						0	1	36	2	39				

Table 14 Embryoid Data Sheet for BSG Plants 1988

	NUMBER OF RESPONSIVE PLANTS				TOT.	NUMBER OF EMBRYOIDS				TOT.	# % RESPONSE RATE OF ANTHERS			
Medium	1½	2	3	4		1½	2	3	4		1½	2	3	4
BAI	0	0	1	0	1	0	0	1	0	1	0	0	1.16	0
BAII	1	0	0	0	1	4	0	0	0	4	9.52	0	0	0
BAIV	1	0	0	0	1	1	0	0	0	1	2.5	0	0	0
OCKI	1	0	1	0	2	2	0	2	0	4	2.4	0	2.24	0
OCKII	1	0	2	0	3	1	0	2	0	3	1.35	0	0.8	0
OCKV	0	2	3	0	5	0	7	29	0	36	0	4.04	13.87	0
						13	8	34	0	49				

Table 15 - Overall response rate of anthers (in 3-6mm range) with respect to medium used 1988

Medium	No. Anthers Cultured Bud Size 3-6mm	No. Embryoids to date	% Response Rate
BAI	1524	2	0.13
BAII	957	18	1.88
BAIV	1396	6	0.43
OCKI	1206	14	1.16
OCKII	2194	31	1.41
OCKV	2123	81	3.82
Total	9400	152	1.62%

Embryo sub-culturing

Following the identification of embryos on the culture plates they were allowed to increase in size to 2 to 5mm before subculturing. They were picked off the plates using forceps and transferred directly to Gamborgs B5 media and incubated in the light at 23^{o}C. Several different characteristic embryoid shapes were noted as have been referred to in the literature (globular, heart-shaped, torpedo).

When embryoids were subcultured there were several courses of events which were noted:

Embryo increased in size in an unorganised manner and either remained white, turned brown and died or turned green.

Embryo increased in size in a relatively organised manner, turned green and produced one or more shoots.

Where shoots were produced these were subcultured again onto Gamborg's B5 medium and allowed to root. When sufficently rooted plants were weaned into compost in a propagator and finally into pots. Weaning was difficult with these plants and only 12 plants were weaned in the first stage of weaning and of these only 5 survived to the potted stage.

DISCUSSION

The results of the second season of culturing were an improvement on the first season with some positive responses. The turning point of the experimentation appeared to be the adoption of the larger bud size for culturing. This does not appear to reconcile with the theoretical results of the pollen development stage study explored in the 1987 season. However, as was mentioned earlier, the lack of synchrony of pollen development stage at any one set bud size together with the confinement of most of the culturing of the bud size range 3-6mm on buds from the secondary flower flush could contribute to the lack of predictability of the technique. Even where response was obtained to anther culture, the very low response rate hindered a rigorous analysis of either technique or media type. Thus although some response was encouraging, further investigation in the 3rd season will need to retest the more successful protocol now developed and attempt to increase the response rate of anthers so that "sensible" scientific experimentation can be carried out on the donor material.

1989 SEASON

OBJECTIVES

The main objective of the 1989 culturing season was to apply the improved culture system developed in 1988 to the 1989 plants and attempt to increase response rate to anther culture. Greater numbers of anthers per individual plant were to be assessed in order to highlight responsive plants quickly.
A second objective was to investigate Ovule culture as an alternative means of haploid culture. This investigation is written up in Appendix . During the 1989/90 period of investigation Ploidy levels were to be investigated in plants derived from embryoids.

MATERIALS AND METHODS

Plant raising
Plants were raised in the same manner as reported above for 1988. Fresh plants were used for this season and fewer plants than before were used. Growers supplied 20 plants of their own choice from each group as follows:

BSG	1; $1^1/_2$; $3^1/_2$; 5. (5 plants per variety)
Codebric	CND; CM. (10 plants per variety)
Trinity	20 individually coded plants.

Hot sunny weather again accelerated flower development in the plants leading to a need to decapitate flowering heads and work on the secondary flush of flower buds.

Anther culture

Anther culture was carried over the time period April 4th to August 31st. The procedures used were those refined in 1988. Buds in the size range 3-6mm were used throughout the culturing period. All anthers had their filaments removed and were given a heat shock of 35^{o}C for 16-24 hours once plated. All plates were subsequently incubated at 23^{o}C in the dark and visually inspected every 2 weeks.

Media
Only four media were used in this season namely BAII, BAIV, OCKII and OCKV (see table 3 for media recipes). The majority of anthers were cultured on the medium OCKV since this gave the best response in the 1988 season.

Ploidy determination

The determination of the ploidy of anther derived Brassica plants is not straightforward. Cauliflower chromosomes are small and difficult to see in root tip squashes. Cell size determination (normally stomata guard cell length) is considered to be the quickest method of determination and there is often a quantitative distribution of sizes within a population of plants of similar ploidy. Haploids are easiest to detect at the fowering stage by virtue of bearing sterile small flowers and no pollen. Ockendon (1989) reported work on ploidy determination in anther culture derived cauliflowers and concluded that it was virtually identical to the situation found in Brussel Sprouts. The technique of determining the guard cell length was considered to be reliable in distinguishing haploids from diploids but some overlap between diploids and tetraploids was suggested. The range of size determination was given as:-

	Guard cell length (um)		
	Mean	Range	No. plants
Haploid	16.9	15.6 - 18.4	4
Diploid	24.3	21.2 - 26.8	18
Triploid	30.5	28.2 - 33.8	18
Tetraploid	32.0	28.2 - 37.0	56
Octoploid	36.0	-	1

In the current investigation only the technique of guard cell length was used to give an indication of ploidy level. This is due mainly to the availability of weaned plants at the correct timing in relation to the timing of the grant monies i.e. a large number of plants were only weaned over the period March - June 1990 and the grant monies terminated at the end of March 1990.

The plants selected for ploidy determination represented one plant from each sub-group where a sub-group is a group of plants from a single embryo or cluster which could not be separated at first sub-culture. All plants had been weaned into 10 cm pots and bore 4 to 6 fully expanded leaves. Samples (1 cm leaf discs) were taken from the third leaf back from the youngest visible leaf. Lower epidermal strips were peeled off the leaf discs and mounted in water on a microscope slide, covered with a cover slip and then examined under a micropscope at x400. Guard cell length was recorded by use of an eyepiece graticule which was calibrated using a standard graduated slide. Fifteen measurements were recorded per slide. In addition to the plants from anther culture, control diploid plants from seed derived material were also examined.

RESULTS

Tables 16 to 19 give the results of the number of anthers cultured per plant and per media type. It can be seen that numbers per plant ranged from 240 to 980 and that a total of nearly 24,000 anthers were cultured in

Table 16 - Nº of anthers sampled - B.S.G. 1989

Plant Number	Line 1	Plant Number	Line 1½	Plant Number	Line 3½	Plant Number	Line 5	
10	339	9	414	47	238	69	775	
13	283	18	329	54	402	74	286	
15	340	21	335	63	315	86	327	
18	580	24	331	75	651	90	326	
21	576			80	978	97	247	
TOTAL	2118		1409		2584		1961	8,072

Table 17 - Nº of anthers sampled - Codebric 1989

Plant Number	Line C/ND	Line C/M
1	288	884
2	364	289
3	325	338
4	437	476
5	482	-
6	379	746
7	337	843
8	485	393
9	367	334
10	821	241
Total	4285	4544
		TOTAL 8829

Table 18 - Nº of anthers sampled - Trinity Growers 1989

Plant Number		Plant Number	
0822	270	2603	-
1810A	236	2618	240
1810B	327	2620	-
1907	64	3001	318
1921	1690	5001	322
1922	330	5005	311
2155	276	5011	331
2507	717	5026	280
2508	-	2509	318
2510	310		
			TOTAL 6890

Table 19. Media x No. Anthers Sampled

	KENT	BSG	CODEBRIC	TOTAL
BAII	3081	2618	2909	8608
OCKV	3809	4203	5632	13644
BAIV	-	873	288	1161
OCKII	-	378	-	378
	6890	8072	8829	23791

this season.

The overall response rate to anther culture in terms of embryoids produced was similar to the 1988 season with an overall response of 1.45% when calculated over all plants but this rises to 3.88% when calculted over the responsive plants only (see Table 20). However the pattern of response was different. In 1988, most of the lines tested produced a low level of response but in 1989 fewer lines were responsive but some of these had much higher response rates (see Table 20). Two plants in particular showed good response rates, $3^1/_2$-63 with 38.41% response and 3001 with 26.73%.

All embryoids were subcultured (placed onto Gamborgs B5 and cultured in the light) when they reached a size exceeding 2mm. As with those cultured in 1988 many went through an undifferentiated state prior to producing plantlets. Furthermore several of these undifferentiated clumps produced many plantlets these were further subcultured and subsequently rooted and weaned. In some cases it was impossible to judge whether a developing clump from an anther was derived form a single embryoid or from a cluster of embryoids. Thus it is difficult to say with certainty in some instances whether plantlets obtained from a single undifferentiated clump are simple clones or whether they are genetically different by virtue of being derived from different original embryoids. The different structural forms are depicted in Figure 1. A summary of the plantlet regeneration data is given in Table 21.

Table 20 - Embryoid Results for 1989

Plant number	Number of responsive anthers	Number of embryoids produced	% Response rate per 100 anthers cultured
BSG			
1-21	3	3	0.52
$1^1/_2$-9	1	6	1.45
$1^1/_2$-18	1	2	0.61
$1^1/_2$-21	3	5	1.49
$1^1/_2$-24	1	1	0.30
$3^1/_2$-47	2	2	0.84
$3^1/_2$-54	16	26	6.47
$3^1/_2$-63	33	121	38.41
$3^1/_2$-80	1	13	1.33
TOTAL	**61**	**179**	**4.57**
CODEBRIC			
C/ND 9	8	16	4.36
C/ND 10	2	6	0.73
C/M 1	1	1	0.11
C/M 7	1	1	0.30
TOTAL	**12**	**24**	**1.00**
TRINITY			
1921	20	39	2.31
2507	2	9	2.78
3001	15	85	26.73
TOTAL	**37**	**133**	**5.70**
OVERALL TOTAL	**110**	**336**	**3.88***

* Note - when the numbers of anthers from non-responsive plants is included then the overall response rate is reduced to 1.45%.

Table 21 - Regeneration of embryoids to weaned plants

Plant number	shoots produced	shoots weaned	% success
BSG			
$1^1/_2$ - 21	37	2	5.4
$1^1/_2$ -24	1	0	0
$3^1/_2$ -54	96	75	78.1
$3^1/_2$ -63	148	107	72.3
TRINITY			
1921	158	96	60.8
3001	718	509	70.9
CODEBRIC			
C/M 7	1	1	100
C/ND 9	17	12	70.6
OVERALL	**1176**	**802**	**68.2**

Note: not all embryoids responded to sub-culturing - some turned black and died, some proliferated in an undifferentiated manner but failed to produce shoots.

Media responses were also different to the 1988 season (Table 22). Best response was obtained on the BAII medium which did not contain silver nitrate but did contain activated charcoal. This medium was the second most responsive in the 1988 season.

Table 22 Media Responses for 1989

	Number of embryoids				
Medium code	% BSG	CODEBRIC	TRINITY	TOTAL	response
OCK II	0	0	0	0	0
OCK V	46	20	54	120	1.07
BA II	125	4	79	208	2.63
BA IV	8	0	0	8	0.71

Ploidy determination

Results of the stomatal length investigations are presented in Table 23 and Figure 2.

Table 23 Ploidy determination results.

	MEAN	RANGE	NUMBER
Diploid control plants	22.95	20.00 - 27.30	10
(Ockendon controls*	-	20.00 - 27.00)	-
(Brussel Sprout controls*	-	20.00 - 28.00)	-
Anther Derived Plants			
Possible Haploids	18.51	14.79 - 19.88	33 (32%)
Possible Diploids	23.41	20.00 - 27.88	56 (54%)
Possible Tetraploids	32.85	29.10 - 40.85	15 (14%)

* - data taken from published literature.

Statistical analysis of the gaurd cell length data showed highly significant plant to plant differences (Appendix 2). Using the Studentised Range Test significant differences between individual plants are given in Table 24. The calculated Least Significant Range for the analysis was 3.24 - thus mean values of gaurd cell length had to differ by more than 3.24 to be significantly different.

Figure 1. CULTURE MORPHOLOGY

Embryoid Types.

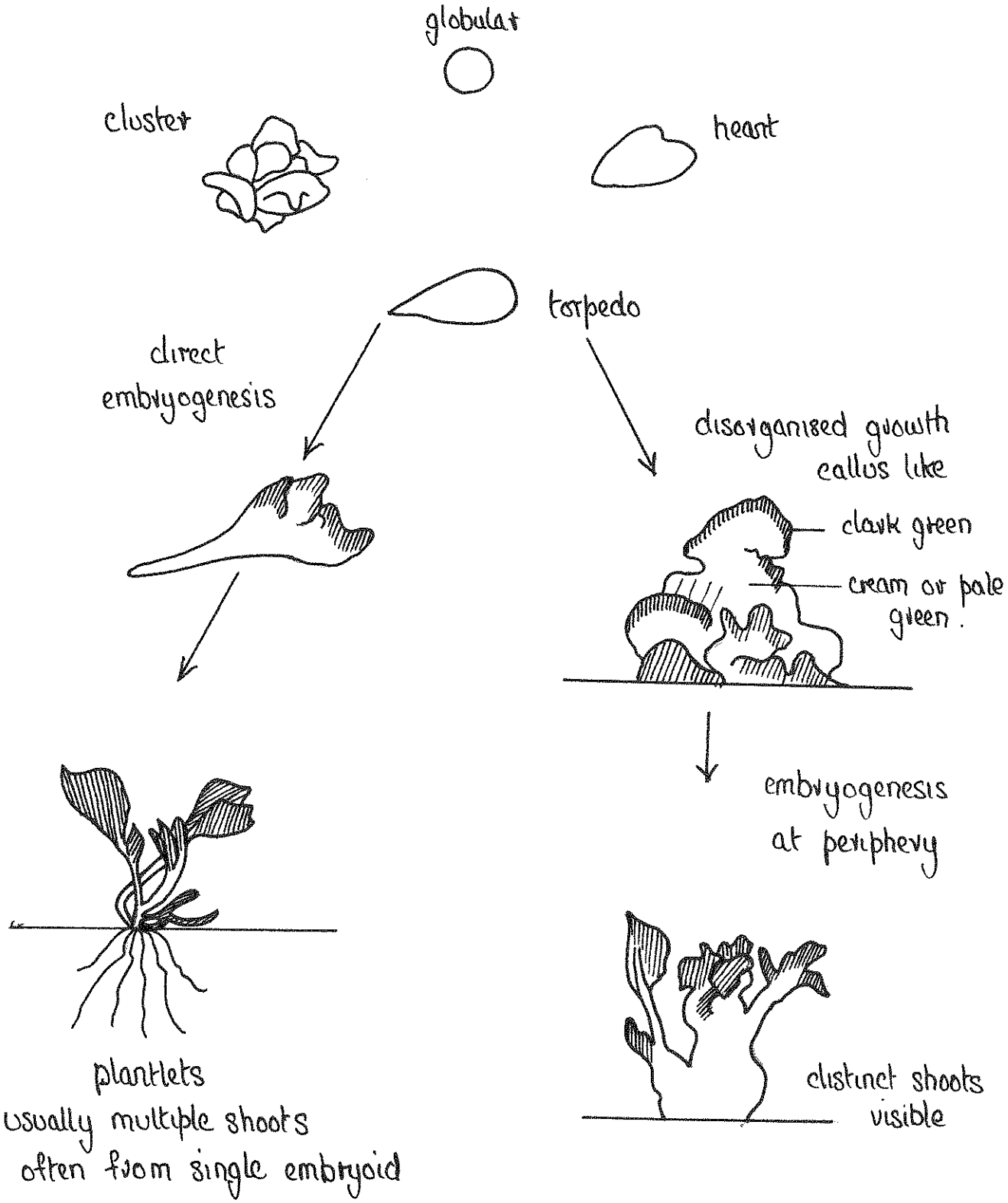


Figure 2 – **Stomatal lengths of anther derived plants**

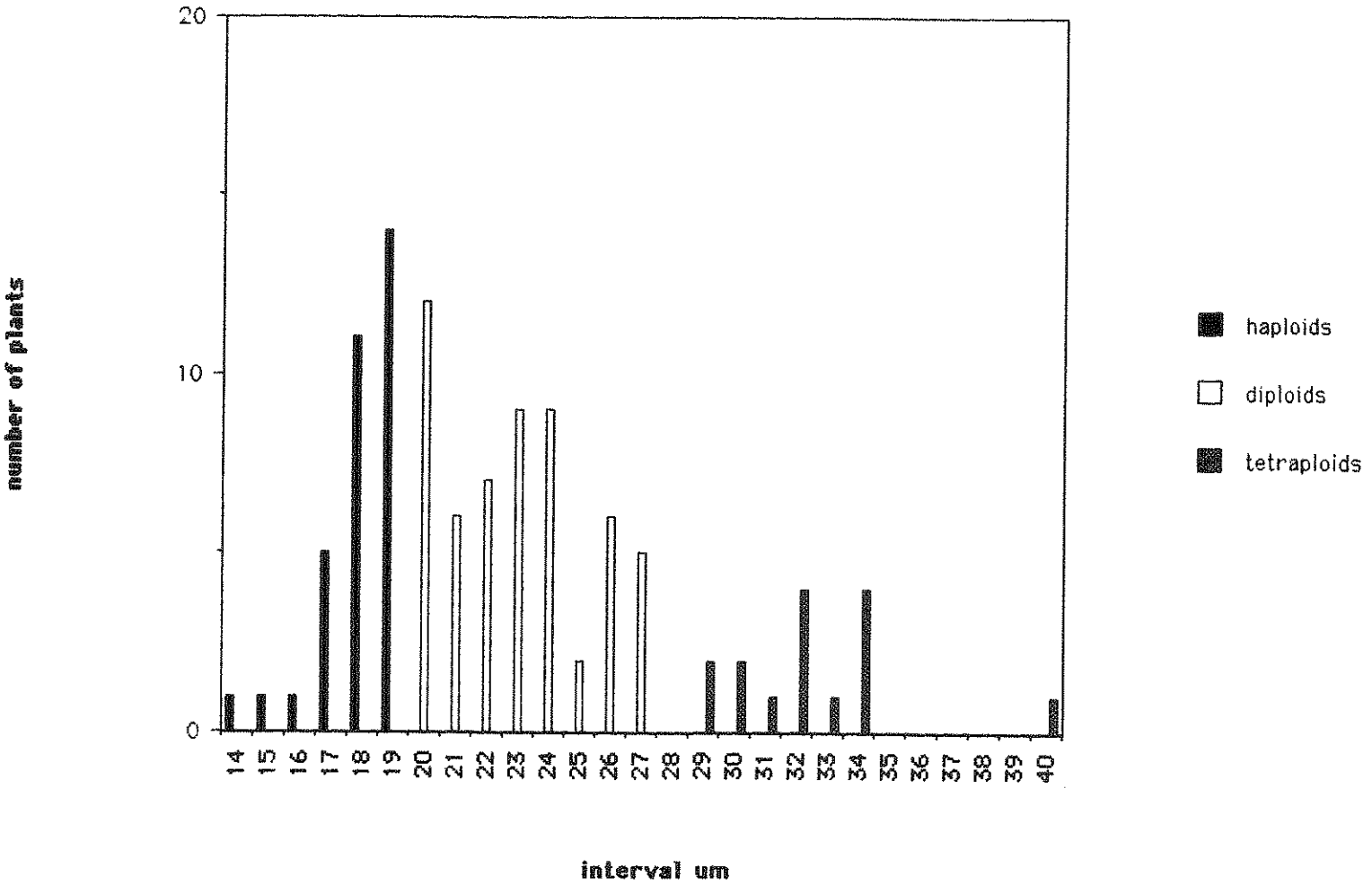
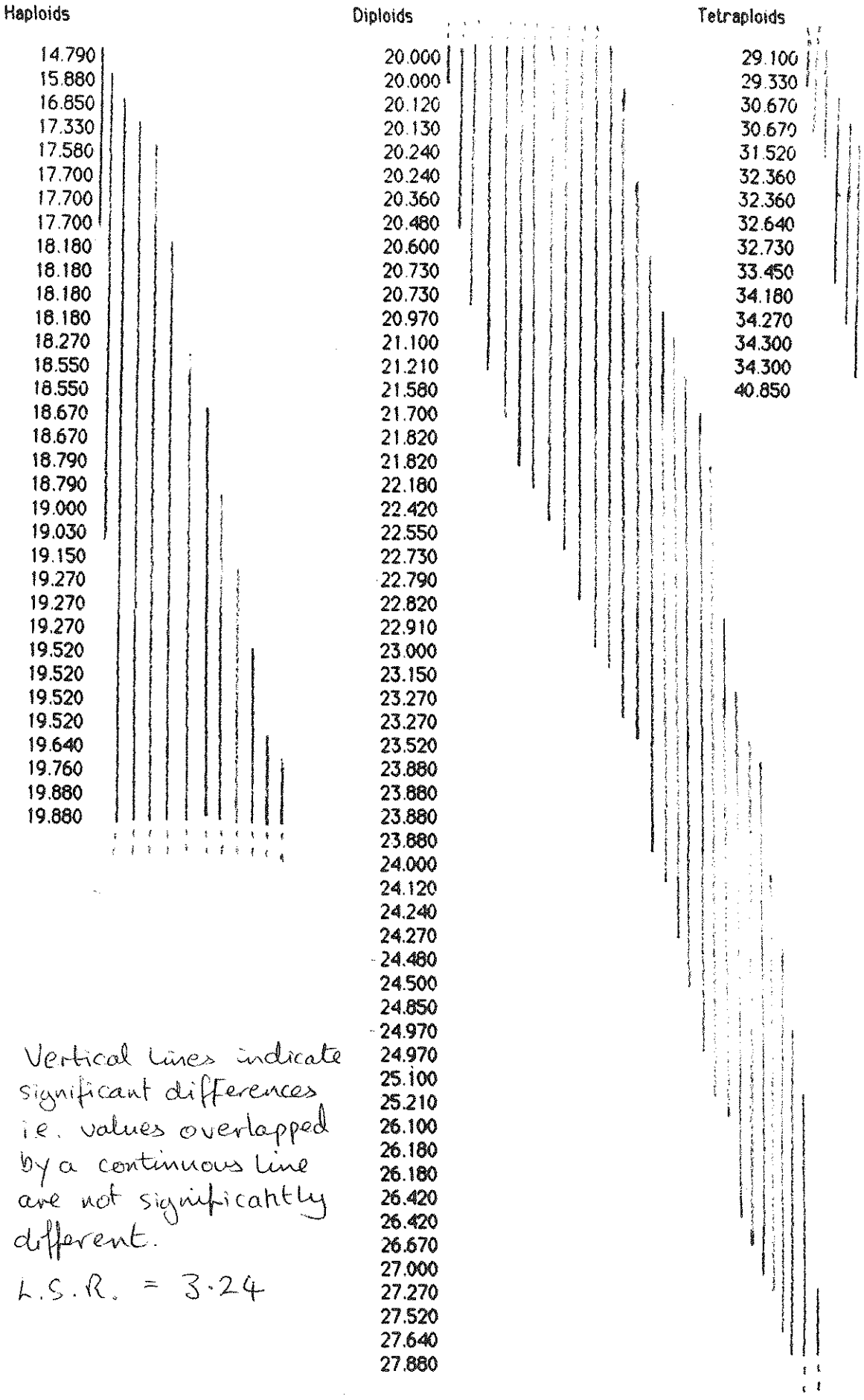


Table 24 - Ranked Guard Cell lengths



Vertical lines indicate significant differences i.e. values overlapped by a continuous line are not significantly different.

L.S.R. = 3·24

DISCUSSION

The 1989 season was considered to be more successful than the 1988 season when judged by the number of responsive anthers although the overall rate of response was very similar in both '88 and '89. However, the number of embryoids produced in 1988 was 152 compared to 336 in 1989. With the greater number of embryoids obtained in '89 it was possible to wean more plantlets from culture. However the weaning process was not as straight-forward as with curd culture derived material and plantlets were very vulnerable to dessication damage. It is suggested that the potentially most useful plantlets, the haploid ones, may be more difficult to wean than diploid or indeed tetraploid ones. If this is indeed the case then it is obvious that the weaning protocol needs to be carefully refined so as to recover as many haploid plants as possible.

The response rates obtained for the different plants would indicate that there is still some as yet undefined restriction to response in winter-heading cauliflower. This appears to be a genetic component and as such is reported to exist in other Brassica systems. The identification of good responding plants is significant. If these plants can be cloned and shown to respond as well in a second season of culturing then the genetic nature of response can be confirmed. Selective crossing with such individuals can then be carried out in order to transfer anther culture response into other individuals with other desirable traits but with no response to anther culture.
Overall response rates are still low and when this is coupled to the weaning problems then the process cannot be classified as an efficent haploid generating system. If the response rate could be improved further it would undoubtedly be of great benefit to the commercialisation of this technique. This would involve a painstaking degree of experimentation varying the numerous media ingredients until an optimum is reached. Consultation with the other research teams in the U.K. and in Europe confirms the recalcitrant nature of winter-heading cauliflower.

The findings of the ploidy investigation were in line with other workers in the field. There was a high frequency of diploid plants weaned from anther culture suggesting that a high freqency of spontaneous diploidisation occurs with cauliflower. This adds a time factor on to the overall process since it becomes necessary to flower each plant, self it and then examine the progeny to ensure that it is homozygous. With haploid plants this is not necessary although may be recommended in order to double check the material. The uniform and vigorous nature of the material derived from anther culture suggests that the surviving plants could be of direct use to plant breeders and consultation with other workers in the field suggests that more useful plants are obtained from anther culture than from the apparently more responsive method of microspore culture. The frequency of haploids in our studies was higher than that reported by Ockendon (1989). In field trials diploid derivatives of cauliflower anther culture showed that most of the diploids obtained were indeed homozygous

(Ockendon pers. comm.). This gives further confidence in the material raised through our experimentation.

Despite the low response rate of anther culture of winter-heading cauliflower the technique is not without use since useful plant material can be obtained as it stands provided that large numbers of anthers are cultured. Whilst this may be labour intensive and therefore costly, the cost may be justifiable if the end point leads to potential F1 hybrid parents. If the goal is to obtain a pool of say 1,000 inbreds for screening then at a response rate of 1.5% then about 65,000 anthers need to be cultured which would take two technicians working full-time for 5-6 months to achieve comfortably. Such an investment may therefore be priced at say £10,000 and must be weighed against the potential value of the F1 hybrid returns.

SUMMARY PROCEDURES AND RECOMMENDATIONS

Growth of Donor Plants

Raise donor plants in 25 to 30cm diameter pots filled with peat based compost. Add a persistent soil acting insecticide (such as "Temic") to the compost to give long term protection from aphid, cabbage root fly and caterpillar.

Irrigate the plants as necessary by drip (do not irrigate overhead) and provide a liquid feed of N, P and K with trace elements. Avoid luxury feeding with Nitrogen.

Grow the donor plants in a plastic tunnel or glasshouse over winter to allow them to become vernalised. Artifical vernalisation may be carried out if access to a low temperature (2 to 4 ^{o}C) growth room is possible. Thereafter, where possible, maintain the plants in a controlled temperature environment and attempt to keep cool in summer. Ideally a temperature of between 10 to 15 ^{o}C is desirable as this slows development down and gives large buds on which to work.

Avoid the subsequent flowering of unwanted buds by decapitation, this will allow secondary flushes of buds from non-aborted curd fragments.

Culture of Anthers

Select bud clusters from the donor plants. Remove from the plant and transport to the laboratory in a closed vessel containing moist tissue paper so as to avoid the dessication of buds.

Surface sterilise the buds as follows:
1. Immerse in bleach (5% domestic bleach) for 5 minutes shaking for 5-10 seconds every 30 seconds or so.
2. Remove the buds from the sterilant in a laminar flow cabinet and immerse in sterile distilled water. Shake for 10 to 20 seconds and then remove. Repeat 3 times in fresh distilled water. Place sterile buds on a sterile surface to drain (e.g. sterile petri dish)

Select as many buds as possible with a length of 3 to 6 mm. Using fine forceps, remove the sepals and immature petals (note the length of the petals in relation to the length of the anthers - ideally the petals should be about half the length of the anthers). Remove the anthers by bending the anther backwards so as to snap the filament off and leave it attached to the bud. If this is unsuccessful then the filament will need to be removed using a second pair of forceps or with a fine needle.

Place the anther onto the medium in the culture vessel (usually a petri

dish). Great care is needed to avoid contamination since a petri dish can take a protracted length of time to fill.

The two media types recommended are OCK V and BA II (see Table 7). These should be prepared in advance in petri dishes.

Once a petri dish is full then seal it with a strip of "Nescofilm". Using a full-proof coding system label the dish with an indelible non-fade marker pen.

Completed petri dishes should be transferred to an incubator running at 35°C and left for 15 to 24 hours. After removal from the incubator add a further strip of Nescofilm (the heat can cause the original strip to become brittle) before being placed in the dark in a culture room at 23°C.

A work-rate of 400 to 600 anthers cultured per day per operator is satisfactory with an upper limit of about 1,000 anthers per day. Such culturing needs however to be interspersed on an alternate day basis or on a rota basis using more than one operator to avoid fatigue and to maintain a high culturing rate over a period of several weeks flowering.
BEAR IN MIND - flowering of plants is concentrated in the summer months despite differing times of curding!

Inspect petri dishes routinely every week. Embryoids normally appear after 3 to 6 weeks but can take up to 12 weeks. Record the number and shape of all embryoids and the number of responding anthers at each inspection. Embryoids can be removed from the petri dishes as soon as they are big enough to be handled without damaging them (normally about 2mm diameter). Reseal the petri dish after the removal of embryoids and replace in the culture room to allow any further embryoids to develop.

Handling of Embryoids

The removal of embryoids from culture vessels should be carried out in a laminar flow cabinet. Handle the embryoids with great care and transfer them to a culture tube or tub containing G2 medium (see Table 7). Transfer to a culture room running at 23 °C with a photoperiod of 16 hours.

Embryoids should turn green and begin to grow and develop. Frequently the embryoid will increase in size with no apparent organisation and then begin to produce shoot like structures from its periphery. When these shoots are large enough they can be removed and sub-cultured back onto Gamborgs B5 or onto a rooting medium. If the sub-cultured plantlets become excessively "leggy" then reculture them by cutting through a node near the apical region and placing this portion into fresh medium. This will aid the later weaning of the plants.

A sub-cultured embryoid can in this manner produce several plants. It is

possible that these will be clones of a single embryoid but experimentation is still in progress to confirm this. If the enlarging lump which came from the embryoid is haploid in nature then it is possible that plants derived from it could be of varying ploidy levels.

Weaning

When the plants have rooted in culture weaning can be attempted, a propagator based weaning system is recommended.
Plants derived from anther culture via the method described above appear to be more vulnerable than similar plants obtained through a curd micropropagation system and therefore require more careful handling.

Prick out the plants into a peat based compost in seed trays. Spray the plants with a fungicide (e.g. Benomyl) and place in a well shaded propagator with a very high humidity. Maintain the plants in this humidity for 10 to 14 days before beginning to lower the humidity gradually over the next 10 to 14 days. Finally transfer to a shaded glasshouse with frequent irrigation and begin a weak nutrient feed regime until plants are fully weaned and can be potted-on without risk of loss.

Ploidy Determination

The ploidy level of the plants derived from anther culture can be determined by the measurement of the length of the guard cells on the lower epidermis of the leaf. Whilst this is not considered a "foolproof" method it is never the less relatively quick and reasonably accurate.

When looking at the guard cell lengths it is important to standardise your procedures particularly with respect to the developmental stage of the plants to be examined. Avoid examining leaves that have been produced during the culture stage or that were produced early on in the weaning process. Instead take leaves that have been produced once plants have been fully weaned and allowed to establish in compost for 4 to 6 weeks.
Select only fully expanded leaves for examination and use say the third or fourth leaf back from the youngest leaf (just visible and still expanding).
Take a leaf sample using a 1 cm cork borer (or similar) and place this in an easily labelled container for transport to the laboratory. Always take the leaf sample from the same location on the leaf - centrally on the blade avoiding any veins. Sample some seed derived plants to use as controls.

In the laboratory practise removing the lower epidermis of discs with a fine pair of forceps by cracking the disc in half and then peeling exposed pieces or by damaging the epidermis with the points of the forceps until a small section can be seized and peeled off. Avoid getting mesophyl lumps attached (green bits) as these will impede the cover-slip and the section will be too thick to observe easily.

Transfer the peeled epidermal strips to a microscope slide bearing a drop of water (sometimes a drop of washing-up liquid in a litre of water can improve things) and add a cover-slip. Gently press the cover-slip to exclude excess water and soak this away with tissue paper.
Examine the prepared slide under a microscope at x400.

The stomatal lengths can be measured by using an eyepiece graticule. This will give the lengths in arbitrary units. The arbitrary units can converted to metric units by reference to a calibration slide which is engraved with graduations of a known size.
Measure at least 15 stomatal lengths per slide and average the measurements.

less than 20 um - Haploid
20 to 27 um - Diploid
over 27 um - Polyploid

Plants with average measurements of 19 to 21 um may need rechecking in order to say with any confidence whether they are haploid or diploid.

Ovule Culture - This part of the report is extracted from the Report submitted to Brunel University as part of the Sandwich Placement of Miss Karen Pulford.

Ovule culture of Brassica oleracea var. botrytis

In spite of the recent advances in ovary and ovule culture as yet no work has been published on unfertilised ovary culture of any Brassica species. Although work has been published on culture of fertilised ovaries for use in hybrid breeding programmes. The technique described below is derived from what is known about Brassica oleracea var. botrytis and its response in culture (especially with repsect to anthers) and methods which have so far yielded haploid plants in other species.

Method - Materials : flower buds were taken from open pollinated varieties of cauliflower, provided by the grower grpups (as per the anther culture project).For this investigation a single plant was used which had already proved to be of a responsive genotype during the anther culture project.

Buds were initially dissected and the ovary and anthers from the same bud were squashed using acetocarmine. A 5 mm bud, showed pollen with pitted exine but in the ovary, although the nucellus was visible no nuclear staining could be seen. A comparative squash was made of an ovary from an open flower. In this, much nuclear staining was apparent with the fertilised ovules showing clearly stained nucleii. A further 20 5 mm buds were then stained and gave the same results so it was concluded that a 5 mm bud size would be investigated provided that the bud was still completely unopened so that fertilisation had been unable to take place.

Sterilisation : as per the anther cultuere technique.

Dissection : when preparing the flower buds for staining it had been decided that ovules could be dissected out from the ovary without causing large amounts of damage. Ovules were dissected out from the buds in a lamina flow cabinet using a dissecting microscope.

Media : three basic media types were investigated based on Ockendon (1984),Bagga (1982) and Turton and Fuller (1989). These were named OCK,,BA., and BA-Petal repectively and were all used in solid form. Additionally a liquid media version of the BA-Petal was used in two forms,filter bridges and flasks which were constantly agitated. Ovules were plated out onto 9 cm Petri dished with approximately 30 ovules per dish. Also ovules were placed into conical flasks, approximately 10 per flask which

which were then constantly shaken and into Universal tubes containing filter bridges (one ovule per tube). It was decided to investigate the one media type in liquid form as its sucrose content of the OCK and BA medias of 10% would be hypotonic with respect to the ovules and so tissue death would result.

All cultures were sealed,(solid with Nescofilm,liquid with autoclave tape) and transferred to a culture environment of 23 C with a 16 hour photoperiod. In addition duplicates of the solid media plates and filter bridge tubes were incubated in darkness. Unfortunately that was not possible for the agitated liquid media cultures.

Results - after approximately one week green ovules could be seen to be present in all cultures in the light. This indicated that an increase in size had occurred in the ovules as previously they had not been visible to the naked eye. However the size increase appeared to be more pronounced on the ovules on solid media. Cultures in the dark did not appear to be responding. At this time photo 1 was taken showing an ovule in culture (size 0.42 mm).

After approximately four weeks the ovules in the dark appeared to have taken on a shrivelled brown appearance. However those in the light were still surviving. The ovules in liquid media and on filter bridges had undergone no further size increase and did not appear to be developing any further, as did the ovules on BA.

At this time the ovules on the OCK and BA-Petal were showing a survival rate of some 50% and many had white attachments, often at the point where the ovule had been attached to the ovary. Also on the OCK one ovule appeared to have almost doubled in size and a shoot of some kind could be seen.(photo 2). The plates were left in the light to see if any further development took place.

After another four weeks, ovules exhibiting growth of any kind were moved onto three proliferating media types with equal numbers of ovules from each of the three initial media being put on the new media. No subsequent development was seen.

Discussion - the initial success of the transfer of the ovules from the bud to an <u>in-vitro</u> situation proved that ovule culture of buds of this size is feasible. Additionally culture method resulted in approximately 50% of the ovules surviving the initial stages although the figure is likely to rise as the technique became used more frequently. A major problem in transfer of such

minute pieces of plant tissue is the maintainance of sterility in the working environment. Much equipment is needed inside the cabinet and it all must be sterilised thoroughly to avoid contamination.

The survival of some of the cultures through the initial stages of the process shows that the media used is basically correct although solid media appears to be favoured over liquid, even when filter bridges are used. Also the presence of light in the environment seems to encourage growth and ovule development.

It is likely that the ovules remained on the initial media for too long a period of time. Ideally a period of four to five weeks should be spent on this media before transfer to media of a different type. After this time period the ovules did not undergo any further changes and by the actual time of transfer they appeared to be less healthy. Unfortunately as no further development occurred on the proliferation media the suitability of the media types used could not be assessed.

Conclusions - Although no definitive results were obtained from this project, this preliminary study into the ovule culture of Brassica oleraeea var. botrytis seems to indicate the following points ;

(i) it is possible to dissect out the ovules of this species and use this tissue in culture without incurring very high levels of infection or large amounts of tissue damage.

(ii) solid media types appear to be favoured over liquid media, either as filter bridges or by flotation in the liquid media).

(iii) light culture conditions favour the growth of ovules.

(iv) a Gamborgs B5 based media seems to be a suitable media type.

(v) the establishment media needs to contain hormones. The best development was seem on media containing NAA and 2,4-D.

(vi) The ovules must not remain on the establishment media for a prolonged period of time (greater than four weeks). It is not clear whether after this point the ovules should be moved onto fresh establishment media of a media of a different type

OVERALL CONCLUSIONS

1. Anther culture of winter-heading cauliflower is possible but at present the response rates are low and unpredictable. The highest response rate obtained was 38.4% (embryoids produced per 100 anthers cultured).

2. There appears to be a high genetic component involved in determining the response rate to anther culture.

3. Media used for the anther culture of other Brassica species appear to yield responses for winter-heading cauliflower although minor modification in hormone level and in the addition of silver nitrate improved response rates overall.

4. Embryoids can be stimulated to produce shoot structures by sub-culturing onto a Gamborgs B5 medium with 2% sucrose and no hormones. A single embryoid frequently gives rise to a disorganised "callus" like structure before producing shoots.

5. Shoots from the embryoids can be rooted in a standard cauliflower rooting medium (M & S with 3% sucrose and 2mg/l IBA) prior to weaning.

6. Weaning of anther culture derived plants can be achieved by direct transfer to peat-based compost, treating with Benlate and by using a propagator based weaning system.

7. Most of the material produced through this protocol was either haploid (32%) or diploid (54%) in nature but a significant percentage (14%) were polyploid (tetraploid or greater).

8. The technique as developed in this investigation requires further refinement and modification before it can be properly commercialised for use in a plant breeding programme. However, if the need to obtain homozygous lines quickly is very urgent then the technique can be used in its present format provided that the investment in labour in forthcoming.

9. The alternative haploid derivation route, ovule culture, also requires further investigation but preliminary examination showed that the technique is possible.

10. An alternative micropropagation method, that of petal culture, was discovered and refined during the course of this investigation.

REFERENCES

Bagga, S., Bhalla-Sharin, N., Sopory, S.K. & Guha-Mul-herjee, S. (1982). Phytomorphology 32, 152-156.

Biddington, N.L., Sutherland, R.A. & Robinson, H.T. (1989). Ann. Bot. 62, 181-185.

Chen, Y. (1986). In. 'Haploids of Higher Plants in Vitro'. Hu, H. & Yang, H. (eds), Springer Verlay, Heidelberg, 3-25.

Dunwell, J.M. (1986). In. 'Plant Tissue Culture and its Agricultural Applications'. Withers, L.A. & Alderson, P.G. (eds), Butterworths, London, 377-404.

Fuller, M.P. & Turton, S. (1989). Acta. Hort. (In press).

George, L. & Rao, P.S. (1982). Pl. Sc. Kett. 26, 111-116.

Guha, S. & Maheshwari, S.C. (1966). Nature 212, 97-98.

Henry, T. & de Buyser, J. (1985). Pl. Cell Rep. 4, 307-310.

Keller, W.A. & Armstrong, K.C. (1977). Can. J. Bot. 55, 1383-1388.

Keller, W.A. & Armstrong, K.C. (1979). Theor. Appl. Genet. 55, 65-67.

Keller, W.A. & Armstrong, J.C. (1981). Can. J. Genet. Cytol. 23, 259-265.

Keller, W.A., Armstrong, K.CV. & de la Roche, A.I. (1983). In, 'Plant Cell Culture in Crop Improvement', Sen, S.K. & Giles, K.L. (eds), Plenum Press, New York, 169-183.

Kumasewska, K. & Keller, W.A. (1983). Z.Pflanzenphysiol. 109, 235-149.

Kuo, C.G., Dellafield, S., Choug, C. & Chiang, M.S. (1983). Crucif. Newslett. 8, 565.

Ockendon, D.J. (1984). Ann. Appl. Biol. 105, 285-291.

Ockendon, D.J. (1985). Ann. Appl. Biol. 107, 101-104.
Ockendon, D. J. (1989). Ann. Appl. Biol. 113, 319-325
Uhrig, H. (1985). Theor. Appl. Genet. 71, 455-460.

APPENDICES

1. Reprints of relevant papers published by the authors

    a. Anther culture of winter-heading cauliflower

    b. Petal culture of winter-heading cauliflower

2. Statistical analysis of ploidy data

Reprints of relevant papers published in 1989 by the authors

Acta Horticulturae (In Press) Proc. I.S.H.S. Syposium
Cesena, Italy, June 1989.

ANTHER CULTURE OF WINTER-HEADING CAULIFLOWER

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Abstract

Fifteen cultivars of cauliflower originating from the coastal growing regions of England were used to investigate the response to anther culture. Thirteen of the cultivars gave embryos in culture. There were differences in the responsiveness of cultivars and in the response on various media. Embryo yields were low ranging from 0 to 16 embryos per 100 anthers cultured.

1. Introduction

The winter-heading cauliflower crop grown in the coastal regions of north-west Europe is currently comprised of open-pollinated cultivars. It has been recognised for some years that crop uniformity and quality could be improved by the production of F_1-hybrids. As with many cross-pollinated species the production of useful inbred lines of winter-heading cauliflower for use as F_1 parents is a time-consuming process. This, coupled with the long growing season of the crop (9 to 12 months) means that suitable inbreds can take 10 to 15 years to produce.

Homozygous lines with all the attributes of inbreds can be produced in several species by in-vitro culture methods (anther, pollen, ovule, ovary) followed by chromosome doubling (Dunwell, 1986). Such techniques have been applied to several species of the Brassica genus (Keller & Armstrong, 1977,1979 & 1981, Klimaszewska & Keller, 1983, Ockendon, 1984) and even to some varieties of cauliflower (Ockendon, 1988). In many of these species these techniques are now used as routine in breeding programmes.

2. Materials and method

2.1. Plant material

All of the plants used were supplied by sponsoring seed groups and were from open-pollinated cultivars. Plants were grown in pots and maintained in an unheated tunnel. A total of 95 plants were used in the 1988 season.

2.2. Anther sampling

Unopened bud clusters were removed from plants over the period 21 April 1988 to 11 September 1988, surface sterilised with 5% bleach (0.6% active Cl), rinsed and dissected in a laminar flow cabinet. Two bud size categories were investigated, 2.5 to 4.0 mm and 3.0 to 6.0 mm. Filaments were removed from the anthers and anthers placed onto solidified media in 9 cm diameter petri-dishes. The number of anthers per petri-dish was 35 to 45.

2.3. Culturing

Petri-dishes were sealed with nesco-film, incubated at 35 °C for 16 to 24 hours in the dark and then cultured face-up in the dark at 23 °C for 6 to 9 weeks.

2.4. Media

Two basic media were investigated which from the literature showed promise for this application (Ockendon, 1984; Bagga, 1982). Two adaptations of these media were incorporated (Table 1).

3. Results

A total of 16,145 anthers from the bud size 2.5 to 4 mm and 9,400 from the bud size 3 to 6 mm were cultured. From the 2.5-4 mm range a total of only 3 embryos were obtained but from the 3 to 6 mm range a total of 155 embryos were obtained. This represents an overall response rate for the bud range 3 to 6mm of 1.62 embryos per 100 anthers cultured (1.62 %).

Of the 15 cultivars tested, 13 were responsive to anther culture but considerable cultivar reponsiveness existed (table 2). There was a variation in media responsiveness with the OCK III giving the highest response (Table 2). However, embryos were recovered on all media tested. There was evidence that a genotype x media interaction existed with most cultivars responding on OCK III but, for some, a higher response was obtained on BA I (table 2).

4. Discussion

The results of this investigation indicate that anther culture of winter-heading cauliflower is possible, but as with other species there is variation in response with genotype and media. Whilst the response rate was low in most instances some recovery rates were as high as 10 to 16 %. Ockendon (1988) has reported a recovery rate of 99.6 % for one cultivar of autumn cauliflower but at the same time also reported recovery rates of 0.3, 0.7 and 7.6 % for others.

In agreement with Ockendon, the results of this investigation showed a lack of consistency of response within a cultivar. This hampers greatly the ability to statisically test important components such as media, anther development stage and plant and anther pretreatments. However, there is an indication from the results that a bud size range of 3-6mm is better than smaller buds. Furthermore, it would appear that a medium based on that of Ockendon (1984) but without activated charcoal and with added silver nitrate yields embryoids from larger numbers of test plants. This supports the work of Biddington et al. (1989) who found that silver nitrate improved the responsiveness to anther culture of Brussels sprouts. The precise nature of this effect is not known but it may implicate a role for ethylene suppression of anther culture.

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References

Bagga,S., Bhalla-Sarin,N., Sopory,S.K., & Guha-Mukherjee,S.,1982. Comparison of in-vitro plant formation from somatic tissues and pollen grains in *Brassica oleracea* var. *botrytis*. Phytomorphology. 32:152-156.

Biddington,N.L., Sutherland,R.A. and Robinson,H.T., 1989. Silver nitrate increases embryo production in anther culture of Brussels sprouts. Ann.Bot. 62,181-185

Dunwell,J.M.,1986. Pollen, ovule and embryo culture as tools in plant breeding. In Plant Tissue Culture and its Agricultural Applications, eds. Withers L.A. and Alderson P.G. Butterworths, London:377-404.

Keller,W.A. and Armstrong,K.C.,1977. Embryogenesis and plant regeneration in *Brassica napus* anther cultures. Can.J.Bot. 55:1383-1388.

Keller,W.A and Armstrong,K.C.,1979. Stimulation of embryogenesis and haploid production in *Brassica campestris* anther culture by elevated temperature treatment. Theor.Appl.Genet. 55:65-67

Keller,W.A. and Armstrong,K.C.,1981. Production of anther derived dihaploid plants in autotetraploid marrowstem kale (*Brassica oleracea* var. *acephala*). Can.J.Genet.Cytol. 23:259-265.

Kilmaszewska,K. and Keller,W.A.,1983. The production of haploids from *Brassica hirta* Moench (*Sinapsis alba* L.) anther culture. Z.Pflanzenphysiol. 109:235-249.

Ockendon,D.J.,1984. Anther culture in Brussels sprouts (*Brassica oleracea* var. *gemmifera*). I. Embryo yields and plant regeneration. Ann.Appl.Biol. 105:285-291.

Ockendon,D.J.,1988. The ploidy of plants obtained from anther culture of cauliflowers (*Brassica oleracea* var *botrytis*). Ann.Appl.Biol. 113:319-325.

Table 1 - Media and codes used

Code	Media recipe
OCK I*	Gamborgs B5 + CaCl.2H2O (600mg/l), Glutamine(800mg/l) Activated charcoal (0.5%), Sucrose(10%) 2.4-D (0.1mg/l), Agar (0.8%).
OCK II	As OCK I without charcoal
OCK III	As OCK II + AgNO3 (5ppm)
BA I	Gamborgs B5 + Sucrose (10%), Activated charcoal(0.5%) B.A.P. (0.5 mg/l), Agar (0.8%).
BA II*	As BA I without activated charcoal
BA III	As BA II + AgNO3 (5ppm)

* As used in references Ockendon (1984) and Bagga (1982).

Table 2 - Response rates of cultivars of cauliflower to anther culture on different media (embryos per 100 anthers cultured)

Cultivar Code	Media						Mean
	BAI	BAII	BAIII	OCKI	OCKII	OCKIII	
TG08	0	0	0	0	0	1.1	0.18
TG11	11.1	0	0	7.7	0	2.2	3.50
TG14	0	0	0	0	0.2	3.6	0.65
TG21	0	0	2.2	0	0	4.9	1.18
TG22	0	1.3	4.1	10.0	3.5	16.3	5.87
TG24	9.8	0	0	0	0	0.8	1.77
TGAT	2.4	0	0	0	0	0	0.40
CBF	0	0	0	0	0	0	0.00
CBFM	0	0	0	1.2	0	0	0.20
CBM	0	0	0	0	10.5	5.3	2.63
CBMA	0	0	0	1.6	0	0	0.27
BSG1.5	9.5	0	2.5	2.4	1.4	0	2.63
BSG2	0	0	0	0	0	4.0	0.67
BSG3	0	1.2	0	2.2	0.8	13.9	3.02
BSG4	0	0	0	0	0	0	0.00
Mean	2.2	0.2	0.6	1.7	1.1	3.5	

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Petal culture of winter-heading cauliflower

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Abstract

Clones of winter heading cauliflower plants were successfully raised from immature petals by in-vitro culture using two media types. Plants did not show high levels of somaclonal variation and were able to produce commercial crops in the field.

1. Introduction

In-vitro culture techniques are being used increasingly for the rapid multiplication of cauliflower plants for breeding programmes and seed production (Anderson & Carstens 1977, Grout & Crisp 1977, Pow 1969, Walkey et al 1977, Walkey & Woolfitt 1970). Conventional cloning uses direct shoot formation from curd meristem tissue with explants being taken when the curd is at a marketable stage which coincides with the optimal culture response stage(Crisp & Walkey 1974). Other methods involving adventitious shoot production, through the mediation of callus, using explants such as young leaves (Parkeek & Chandra 1978), hypocotyls (Dietert et al 1982) or internode segments (Trimboli et al 1977) are encumbered by the risk of somaclonal variation (Clare & Collin 1974, Dunwell 1981). For commercial seed production a high degree of somaclonal variation is unacceptable but for plant breeding it may prove a useful source of novel variation.

The seed producers of winter-heading cauliflower use both adventitous basal shoot production (stump cuttings) and curd meristem micropropagation from mother plants to obtain clones for bulking-up seed production. However, both of these methods are constrained by the duration of their respective development phases. Supplementary cloning techniques which extend the opportunities to obtain cultures from mother plants at alternative development phases would be of benefit to the seed industry.

Initial observations - Axillary shoot production was first noticed in culture dishes of anthers during an investigation of anther culture (Fuller & Turton 1989). Where anthers had been cultured with filaments attached shoot initials frequently appeared at the basal end. Close examination showed that this only occurred when pieces of petal were left attached to the filament.

2. Materials and methods

2.1 Plant material

Open-pollinated varieties of winter-heading cauliflower were supplied by sponsoring seed groups. Plants were grown in pots and maintained in an unheated tunnel with drip-fed irrigation. Flowering occurred over the period April to September 1987.

2.2 Bud sampling

Unopened buds, 2.5 to 3.0mm in size were removed from the plants, surface sterilized in 5% bleach for 3-4 minutes, rinsed and dissected in a lamina flow cabinet. A total of 63 buds from 3 genotypes were tested for responsiveness to petal culture. In addition, from one genotype 4 bud sizes were chosen (3.5mm, 4.5mm, 6.5mm and 7.5mm) and the responsiveness of basal sections (1mm slices through the bud at the point of attachment of the corolla to the floral axis) were assessed.

2.3 Culturing

Immature petals were plated onto solidified media in 9cm petri dishes with 28-32 petals per dish. These were sealed with Nescofilm and cultured at 23°C in the light (16h photoperiod) for 4 weeks. Clumps of shoots were removed and sub-cultured on proliferating medium. After a further 4 weeks individual shoots were taken off and sub-cultured on rooting medium for 2 weeks prior to weaning. Several plants were grown to maturity in the field in the 1988/89 season. Two media were used to test the response of petals to culture and a further two media for sub-culturing (table 1).

3. Results

Shoot initials appeared 7-10 days after petals were put into culture and continued to initiate for 4 weeks. From an initial 247 petals from 63 buds cultured, 330 plants were derived in 12 weeks. Plants were successfully obtained from all genotypes tested and on both media used.

Bud sections responded to culture at the same rate as intact petals. There were however, differences in reponsiveness with respect to bud size with the 7.5mm buds being less responsive.

Plants grown on and planted in the field showed extreme uniformity and enhanced vigour when compared to a seed derived population. Clonally derived populations showed less variation than a seed population for the character of total leaf number (figure 1) with curd clones having slightly less variation than petal clones.

4. Discussion

It was shown that plants can be successfully derived from immature petals taken from unopened flower buds in the size range 2-6mm. Furthermore, it was found that the shoots appeared from the basal end

of the petal suggesting that their origin was meristematic rather than adventitious in contrast to other reports where an intermediary callus stage was reported (Walkey & Woolfitt 1970, Anderson & Carstens 1977).

Shoot proliferation from petal culture occurred on media with a high sucrose content but further study is necessary to determine the optimum media requirements. Shoots which were removed from the initial cultures and sub-cultured on to curd culture media responded in a similar manner to curd cultures. Populations of petal derived plants did not show a high incidence of soma-clonal variation and were comparable in all respects to curd derived clones.

The technique of petal culture offers to the seed producers and plant breeders another opportunity for obtaining clones from valuable plants. Sampling time for petal culture is substantially later than for curd culture and gives a second opportunity for culture in the event of initial curd cultures failing. Petal culture will be useful to the plant breeder who needs to select plants after screening at flowering for traits such as male-sterility.

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References

Anderson W.C. & Carstens J.B. (1977). Tissue culture propagation of Broccoli (Brassica oleracea) for use in F1 hybrid seed production. J.Amer. Hort. Sci. 102:69-73.

Clare M.V. & Collin H.A. (1974). The production of plantlets from tissue cultures of Brussel Sprout (Brassica oleracea L. var gemmifera) Ann. Bot. 38:1067-1076.

Crisp P. & Walkey D.G.A. (1974). The use of aseptic meristem culture in cauliflower breeding. Euphytica 23:305-313.

Dietert M.F., Barron S.A. & Yoder O.C. (1982). Effects of genotype on in-vitro culture in the genus Brassica. Plant Sci. Lett. 26:233-240

Dunwell J.M. (1981. In-vitro regeneration from excised leaf disc of three Brassica species. J. Exp. Bot. 32:789-799.

Fuller M.P., Turton S. & Grout B.W.W. (1989). Anther culture of winter-heading cauliflower. Acta Hort. (in press)

Grout B.W.W & Crisp P. (1977). Practical aspects of the propagation of cauliflower by meristem culture. Acta Hort. 78:289-296.

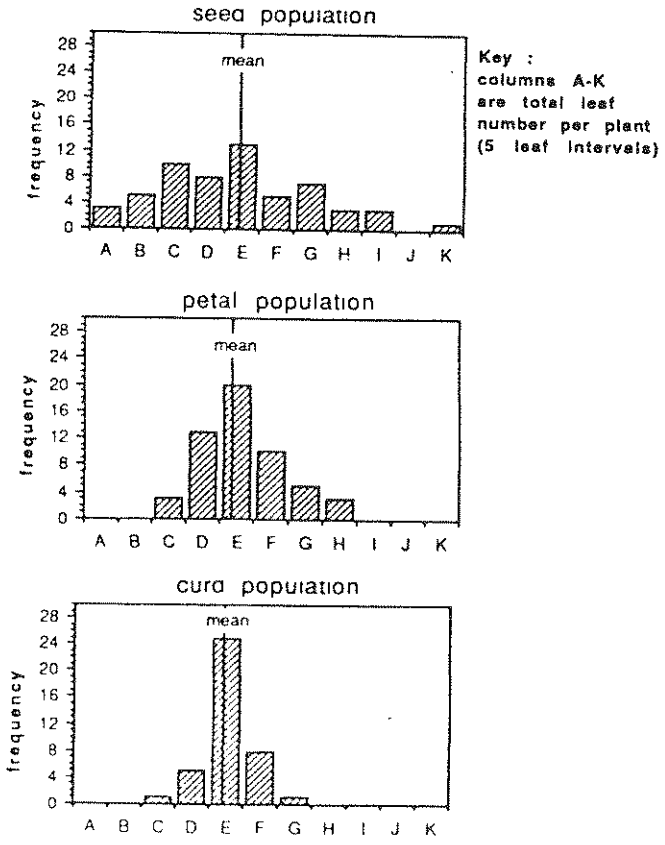
Parkeek L.K. & Chandra N. (1978). Somatic embryogenesis in leaf callus in
cauliflower (Brassica oleracea var. botrytis). Plant Sci. Lett. 11:311-316.

Pow J. (1969). Clonal propagation in-vitro from cauliflower curd. Hort. Res. 9:151-152.
Trimboli D.S., Prakash N. & De Fossard R.A. (1977). The initiation, rooting and establishment of cortical buds in cauliflower. Acta Hort. 78:243-248.
Walkey D.G.A. & Woolfitt J.M.G. (1970). Rapid clonal multiplication of caulilower by shake culture. J. Hort. Sci.45:205-206
Walkey D.G.A.,Cooper V.G. &Crisp P. (1977). The production of virus-free cauliflower by tissue culture. J. Hort. Sci. 49:273-275

Table 1 - Media and code used

Code	Media recipe
Test I	Gamborgs B5 + Sucrose (10%), BAP (0.5 mg/l), Agar (0.8%)
Test II	As Test I with Sucrose at 5%
Proliferation	Murashige & Skoog + Adenine sulphate (80mg/l), Sodium dihydrogen orthophosphate (170 mg/l), Sucrose (3%), Kinetin (4 mg/l), IBA (2 mg/l), Agar (0.8%)
Rooting	Murashige & Skoog (half strength) + Sucrose (1.5%), IBA (2 mg/l), Agar (0.8%)

Figure 1 - Frequency histograms for total leaf number of three populations of cauliflower.



Statistical analysis of ploidy data

DATA SET FOR PLOIDY ANALYSIS

DATA 1

Plant 1	21.816	23.634	25.452	23.634	23.634	21.816	25.452	21.816
	23.634	25.452	27.270	21.816	21.816	27.270	23.634	18.180
Plant 2	23.634	18.180	16.362	19.998	19.998	16.362	19.998	21.816
	18.180	19.998	19.998	19.998	18.180	18.180	45.450	36.360
	29.088	38.178	18.180	36.360	27.270	25.452	36.360	39.996
	36.360	32.724	39.996	36.360	36.360	27.270	23.634	21.816
	25.634	21.816	23.634	21.816	25.452	19.998	18.180	23.634
	21.816	27.270	21.816	19.998	27.270	23.634	29.088	21.816
	25.452	29.088	27.270	23.634	23.634	25.452	25.452	25.452
	23.634	25.452	23.634	27.270	21.816	19.998	21.816	23.634
	23.634	23.634	21.816	21.816	34.542	25.452	19.998	27.270
	21.816	23.634	25.452	27.270	29.088	27.270	25.452	27.270
	25.452	29.088	25.452	30.906	25.452	29.088	27.270	29.088
	30.906	19.998	21.816	19.998	19.998	21.816	23.634	18.180
	18.180	19.998	16.362	19.998	18.180	18.180	19.998	16.362
	18.180	16.362	18.180	16.362	18.180	16.362	14.544	14.544
	18.180	18.180	16.362	18.180	14.544	16.362	18.180	18.180
	18.180	18.180	19.998	21.816	18.180	21.816	18.180	19.998
	21.816	21.816	19.998	16.362	14.544	19.998	18.180	19.998
	19.998	19.998	18.180	18.180	23.634	19.998	19.998	18.180
	19.998	18.180	19.998	21.816	25.452	23.634	19.998	25.452
	23.634	19.998	23.634	18.180	21.816	19.998	29.088	18.180
	23.634	21.816	21.816	23.634	18.180	19.998	19.998	18.180
	18.180	19.998	21.816	21.816	19.998	21.816	21.816	21.816
	23.634	19.998	23.634	25.452	29.088	25.452	29.088	25.452
	30.906	29.088	27.270	32.724	34.542	36.360	29.088	27.270
	25.452	29.088	25.452	25.452	23.634	23.634	27.270	23.634
	23.634	23.634	23.634	23.634	27.270	25.452	25.452	23.634
	27.270	32.724	36.360	25.452	29.088	36.360	34.542	36.360
	27.270	32.724	29.088	29.088	29.088	32.724	32.724	29.088
	30.906	30.906	25.452	32.724	25.452	23.634	27.270	19.998
	32.724	21.816	25.452	25.452	34.542	36.360	25.452	34.542
	29.088	34.542	23.634	30.906	27.270	25.452	34.542	36.360
	32.724	25.452	27.270	30.906	34.542	32.724	23.634	21.816
	18.180	19.998	18.180	23.634	21.816	18.180	16.362	19.998
	21.816	16.362	19.998	16.362	16.362	29.088	23.634	25.452
	23.634	21.816	23.634	25.452	23.634	23.634	27.270	23.634
	21.816	21.816	23.634	23.634	23.634	29.088	27.270	25.452
	23.634	23.634	27.270	21.816	25.452	23.634	29.088	29.088
	29.088	25.452	29.088	27.270	19.998	23.634	19.998	19.998
	18.180	18.180	18.180	21.816	19.998	21.816	19.998	19.998
	21.816	19.998	29.088	30.906	34.542	34.542	32.724	34.542
	32.724	32.724	32.724	32.724	34.542	34.542	32.724	38.178
	34.542	19.998	29.088	23.634	27.270	29.088	19.998	27.270
	21.816	29.088	27.270	29.088	27.270	25.452	34.542	25.452

Each plant is represented by 15 data records reading from left to right

29.088	29.088	36.360	39.996	43.632	39.996	41.814	45.450
45.450	39.996	41.814	45.450	41.814	45.450	47.268	27.270
29.088	21.816	25.452	23.452	27.270	25.452	23.634	21.816
21.816	23.634	23.634	23.634	25.452	21.816	27.270	21.816
21.816	29.088	21.816	21.816	25.452	21.816	25.452	29.088
25.452	19.998	25.452	23.634	21.816	36.360	34.542	27.270
36.360	34.542	30.906	36.360	29.088	29.088	27.270	29.088
36.178	27.270	32.724	36.360	25.452	25.452	19.998	21.816
19.998	23.634	23.634	19.998	21.816	21.816	19.998	21.816
21.816	23.634	25.452	18.180	18.180	21.816	21.816	21.816
19.998	21.816	19.998	19.998	21.816	23.634	23.816	23.634
21.816	19.998	18.180	21.816	16.362	18.180	16.362	16.362
18.180	19.998	19.998	21.816	21.816	34.542	18.180	16.362
16.362	19.998	16.362	18.180	18.180	19.998	29.088	18.180
14.544	16.362	19.998	14.544	18.180	19.998	18.180	19.998
27.270	30.906	30.906	30.906	34.542	32.724	32.724	30.906
32.724	34.542	32.724	34.542	32.724	32.724	34.542	27.270
30.906	23.634	23.634	21.816	19.998	27.270	27.270	23.634
25.452	23.634	25.452	23.634	21.816	29.088	14.544	18.180
18.180	16.362	18.180	16.362	14.544	18.180	14.544	12.726
16.362	16.362	14.544	12.726	16.362	19.998	19.998	19.998
21.816	19.998	23.634	21.816	18.180	19.998	21.816	18.180
19.998	19.998	19.998	19.998	32.724	29.088	25.452	29.088
23.634	23.634	25.452	23.634	23.634	27.270	30.906	29.088
27.270	29.088	25.452	19.998	19.998	19.998	18.180	21.816
19.998	18.180	18.180	19.998	19.998	21.816	18.180	19.998
18.180	18.180	21.816	21.816	23.634	21.816	27.270	23.634
27.270	27.270	27.270	29.088	23.634	25.452	23.634	27.270
25.452	18.180	14.544	18.180	12.726	18.180	19.998	19.998
18.180	14.544	19.998	18.180	18.180	18.180	19.998	16.362
21.816	18.180	21.816	18.180	21.816	19.998	18.180	21.816
21.816	23.634	18.180	18.180	18.180	19.998	23.634	19.998
18.180	18.180	18.180	19.998	19.998	19.998	18.180	19.998
19.998	18.180	21.816	16.362	19.998	21.816	29.088	32.724
27.270	30.906	29.088	30.906	27.270	25.452	32.724	34.542
30.906	25.452	23.634	32.724	27.270	25.452	29.088	25.452
29.088	23.634	25.452	27.270	23.634	29.088	23.634	27.270
23.634	27.270	25.452	25.452	29.088	23.634	23.634	25.452
27.270	23.634	27.270	29.088	30.906	27.270	27.270	29.088
30.906	29.088	29.088	21.816	19.998	27.270	21.816	19.998
25.452	21.816	23.634	25.452	27.270	27.270	25.452	23.634
23.634	23.634	21.816	21.816	19.998	19.998	19.998	21.816
21.816	21.816	21.816	23.634	21.816	19.998	23.634	23.634
21.816	16.362	19.998	21.816	19.998	18.180	19.998	18.180
16.362	18.180	18.180	19.998	19.998	19.998	16.362	14.344
21.816	23.634	18.180	19.998	19.998	18.180	21.816	19.998

18.180	19.998	18.180	21.816	21.816	21.816	21.816	27.270
25.452	21.816	21.816	21.816	21.816	23.634	23.634	21.816
19.998	19.998	21.816	21.816	25.634	21.816	25.452	21.816
18.180	21.816	18.180	21.816	18.180	21.816	19.998	19.998
23.634	21.816	21.816	19.998	21.816	21.816	19.998	19.998
21.816	23.634	23.452	27.270	27.270	21.816	23.634	23.634
21.816	23.634	23.634	21.816	18.180	21.816	19.998	18.180
21.816	21.816	19.998	21.816	19.998	23.634	21.816	23.634
18.180	18.180	19.998	21.816	21.816	21.816	19.998	19.998
21.816	19.998	23.634	19.998	18.180	19.998	21.816	23.634
21.816	18.180	34.542	25.452	34.542	36.360	38.178	32.724
32.724	34.542	30.906	34.542	38.178	38.178	34.542	36.360
32.724	18.180	21.816	19.998	18.180	18.180	18.180	21.816
19.998	16.362	19.998	19.998	19.998	18.180	16.362	18.180
25.452	30.906	27.270	23.634	23.634	27.270	23.634	21.816
23.634	25.452	23.634	23.634	21.816	27.270	25.452	32.724
30.906	23.634	21.816	21.816	25.452	25.452	21.816	29.088
30.906	25.452	29.088	29.088	27.270	21.816	19.998	25.452
25.452	23.634	29.088	23.634	21.816	25.452	21.816	29.088
23.634	19.998	25.452	23.634	29.088	19.998	18.180	21.816
19.998	19.998	19.998	18.180	19.998	21.816	21.816	19.998
19.998	18.180	18.180	19.998	19.998	19.998	18.180	19.998
21.816	21.816	19.998	23.634	21.816	21.816	19.998	18.180
23.634	23.634	23.634	16.362	18.180	18.180	18.180	18.180
18.180	18.180	16.362	19.998	16.362	18.180	18.180	14.544
18.180	18.180	25.452	29.088	19.998	23.634	21.816	25.452
21.816	19.998	21.816	27.270	23.634	27.270	21.816	19.998
19.998	18.180	18.180	19.998	19.998	18.180	19.998	19.998
18.180	18.180	16.362	18.180	18.180	19.998	19.998	21.816
19.998	18.180	18.180	18.180	18.180	16.362	18.180	18.180
19.998	18.180	18.180	18.180	18.180	16.362	18.180	21.816
23.634	21.816	19.998	23.634	23.634	29.088	21.816	21.816
23.634	23.634	25.452	21.816	25.452	25.452	21.816	25.452
21.816	23.634	21.816	23.634	21.816	21.816	29.088	21.816
23.634	21.816	19.998	21.816	25.634	19.998	18.180	18.180
18.180	18.180	18.180	18.180	16.362	16.362	18.180	19.998
18.180	18.180	18.180	18.180	16.362	18.180	18.180	18.180
18.180	16.362	19.998	18.180	18.180	14.544	14.544	18.180
18.180	18.180	18.180	18.180	18.180	18.180	19.998	16.362
19.998	18.180	19.998	18.180	19.998	19.998	18.180	18.180
18.180	19.998	18.180	18.180	19.998	19.998	18.180	19.998
19.998	19.998	21.816	19.998	18.180	18.180	18.180	19.998
21.816	18.180	19.998	21.816	18.180	19.998	19.998	16.362
16.362	19.998	19.998	18.180	19.998	18.180	16.362	18.180
29.088	27.270	25.452	27.270	23.634	23.634	19.998	21.816
21.816	25.452	23.634	23.634	23.634	25.452	21.816	12.726

14.544	14.544	16.362	12.726	16.362	14.544	12.726	14.54
18.180	16.362	14.544	14.544	12.726	16.362	14.544	19.99
19.998	14.544	18.180	18.180	19.998	21.816	19.998	16.36
16.362	19.998	19.998	19.998	19.998	21.816	18.180	21.81
19.998	18.180	18.180	19.998	18.180	18.180	19.998	16.36
19.998	18.180	10.908	18.180	18.180	16.362	19.998	16.36
19.998	19.998	21.816	16.362	16.362	16.362	12.726	19.99
18.180	18.180	16.362	14.544	19.998	19.998	19.998	23.63
23.634	19.998	19.998	16.362	19.998	21.816	23.634	19.99
23.634	18.180	19.998	19.998	18.180	18.180	18.180	18.18
18.180	18.180	18.180	19.998	16.362	18.180	16.362	18.18
18.180	18.180	16.362	18.180	19.998	21.816	21.816	21.81
19.998	16.362	21.816	21.816	21.816	21.816	19.998	18.18
18.180	21.816	18.180	19.998	19.998	18.180	19.998	19.99
18.180	19.998	19.998	21.816	18.180	19.998	21.816	18.18
19.998	19.998	19.998	18.180	18.180	18.180	16.362	18.18
19.998	19.998	21.816	16.362	18.180	16.362	18.180	21.81
21.816	19.998	21.816	23.634	19.998	21.816	19.998	21.81
23.634	19.998	19.998	21.816	21.816	23.634	18.180	18.18
18.180	18.180	18.180	18.180	18.180	19.998	16.362	16.36
18.180	18.180	19.998	18.180	18.180	18.180	19.998	16.36
16.362	19.998	16.362	16.362	12.726	14.544	18.180	18.18
16.362	14.544	19.998	21.816	21.816	19.998	18.180	19.99
19.998	21.816	21.816	18.180	18.180	18.180	21.816	19.99
21.816	19.998	21.816	18.180	19.998	21.816	19.998	23.63
18.180	19.998	18.180	19.998	21.816	18.180	19.998	18.18
23.634	21.816	21.816	23.634	19.998	27.270	21.816	21.81
21.816	23.634	19.998	21.816	21.816	19.998	21.816	18.18
32.724	34.542	25.452	23.634	23.634	29.088	27.270	25.45
29.088	25.452	32.724	27.270	21.816	21.816	29.088	36.36
36.178	34.542	29.088	32.724	36.360	36.360	34.542	29.08
34.542	32.724	36.360	36.360	32.724	32.724	29.088	30.90
23.634	30.906	23.634	27.270	29.088	23.634	29.088	23.63
25.452	29.088	27.270	23.634	23.634	21.816	21.816	21.81
21.816	25.452	23.634	19.998	18.180	21.816	23.634	19.99
21.816	21.816	21.816	21.816	23.634	21.816	25.452	23.63
25.452	29.088	25.452	21.816	27.270	30.906	27.270	29.08
21.816	30.906	29.088	19.998	21.816	21.816	23.634	21.81
21.816	21.816	21.816	19.998	25.452	25.452	23.634	23.63
23.634	21.816	32.724	30.906	30.906	30.906	29.088	29.08
34.542	30.906	25.452	29.088	27.270	29.088	30.906	34.54
34.542	23.634	21.816	29.088	21.816	21.816	23.634	21.81
23.634	23.634	21.816	21.816	21.816	23.634	19.998	21.81
34.542	32.724	32.724	32.724	34.542	32.724	30.906	34.54
27.270	34.542	34.542	32.724	29.088	32.724	29.088	32.72
36.360	36.360	32.724	36.360	34.542	32.724	34.542	36.36

32.724	38.178	32.724	32.724	38.178	34.542	21.816	35.452
21.816	23.634	23.634	25.452	19.998	23.634	21.816	21.816
27.270	27.270	21.816	19.998	23.634	23.634	23.634	25.452
23.634	23.634	23.634	25.452	23.634	27.270	25.452	27.270
19.998	21.816	23.634	21.816	21.816	18.180	18.180	16.362
19.998	18.180	19.998	14.544	19.998	14.544	21.816	19.998
14.544	16.362	21.816	36.360	30.906	30.906	32.724	32.724
32.724	32.724	30.906	36.360	29.088	32.724	32.724	32.724
32.724	36.360	18.180	19.998	21.816	23.634	21.816	25.452
25.452	23.634	25.452	23.634	23.634	25.452	21.816	25.452
23.634	23.634	25.452	21.816	19.998	23.634	21.816	21.816
23.634	21.816	21.816	23.634	25.452	23.634	21.816	19.998

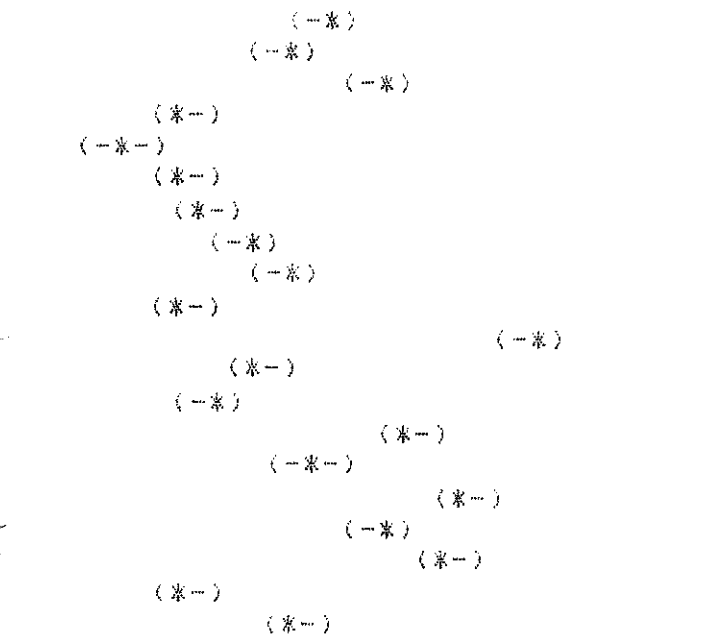
ANALYSIS OF VARIANCE ON DATA 1

SOURCE	DF	SS	MS	F	P	
PLANT	103	38465.89	373.46	59.37	0.000	***
ERROR	1456	9159.16	6.29			
TOTAL	1559	47625.05				

LSR = 3.24

LEVEL	N	MEAN	STDEV
2	15	25.331	2.114
3	15	23.876	3.692
4	15	27.634	1.968
5	15	19.513	1.999
6	15	16.847	1.452
7	15	19.271	2.151
8	15	20.119	2.114
9	15	22.301	2.872
10	15	23.876	1.927
11	15	19.271	1.919
12	15	34.300	6.798
13	15	22.786	2.559
14	15	20.725	1.792
15	15	29.088	3.366
16	15	24.846	1.484
17	15	31.512	3.481
18	15	27.876	4.843
19	15	30.664	4.057
20	15	19.513	2.614
21	15	24.119	1.999

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV



22	15	26.179	2.553	(-*)
23	15	20.725	2.360	(-*)
24	15	33.451	2.038	(-*)
25	15	26.422	3.879	(-*-)
26	15	40.844	5.577	(
27	15	24.482	2.265	(-*)
28	15	24.119	2.872	(*-)
29	15	32.360	3.959	(*-)
30	15	22.422	2.023	(-*-)
31	15	21.089	1.655	(*-)
32	15	19.634	4.620	(-*)
33	15	18.786	3.413	(*-)
34	15	32.360	1.968	(*-)
35	15	24.967	2.953	(*-)
36	15	15.877	1.878	(-*)
37	15	20.362	1.408	(*-)
38	15	27.028	2.904	(-*)
39	15	19.513	1.279	(*-)
40	15	25.088	2.400	(*-)
41	15	17.695	2.223	(*-)
42	15	20.362	2.084	(*-)
43	15	19.392	1.484	(*-)
44	15	29.330	3.213	(-*)
45	15	26.058	2.023	(-*)
46	15	27.512	2.465	(*-)
47	15	23.876	2.465	(-*)
48	15	21.695	1.279	(*-)
49	15	18.544	1.968	(*-)
50	15	20.483	1.747	(-*)
51	15	22.543	1.919	(*-)
52	15	21.089	2.038	(*-)
53	15	23.149	2.223	(-*-)
54	15	20.604	1.903	(-*)
55	15	20.968	1.664	(*-)
56	15	34.300	3.286	(-*)
57	15	19.028	1.664	(-*)
58	15	24.967	2.426	(*-)
59	15	26.422	3.755	(-*-)
60	15	24.482	2.985	(-*)
61	15	19.877	1.279	(-*)
62	15	21.210	1.903	(-*)
63	15	17.695	1.279	(*-)
64	15	23.270	3.011	(-*-)
65	15	19.028	1.351	(-*)
66	15	18.180	0.972	(-*)
67	15	23.513	2.223	(*-)

68	15	22.907	2.151	(-*)
69	15	18.160	0.972	(-*)
70	15	17.574	1.484	(-*-)
71	15	18.786	1.122	(*-)
72	15	19.513	1.279	(*-)
73	15	18.786	1.636	(*-)
74	15	24.240	2.446	(*-)
75	15	14.786	1.664	(*-)
76	15	18.665	2.223	(*-)
77	15	18.344	2.389	(*-)
78	15	17.816	2.300	(*-)
79	15	20.362	2.679	(*-)
80	15	18.301	1.079	(-*)
81	15	19.998	2.061	(-*-)
82	15	19.756	1.351	(-*)
83	15	18.665	1.607	(*-)
84	15	21.210	1.484	(-*)
85	15	18.544	1.711	(*-)
86	15	17.089	2.151	(*-)
87	15	20.240	1.516	(*-)
88	15	20.240	1.927	(*-)
89	15	21.816	2.061	(*-)
90	15	27.270	3.947	(-*-)
91	15	34.176	2.679	(-*)
92	15	26.664	2.888	(*-)
93	15	21.816	1.683	(*-)
94	15	26.179	3.208	(-*)
95	15	22.543	1.655	(*-)
96	15	30.664	2.649	(*-)
97	15	22.786	2.046	(*-)
98	15	32.360	2.300	(*-)
99	15	34.784	2.046	(*-)
100	15	23.270	2.300	(-*-)
101	15	23.998	1.968	(-*-)
102	15	18.422	2.649	(-*-)
103	15	32.845	2.114	(-*-)
104	15	23.270	2.195	(-*-)
105	15	22.664	1.664	(*-)

POOLED STDEV = 2.508

16.0 24.0 32.0 40.0