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Anther Culture Potential of Linseed (*Linum usitatissimum* L.): Effects of Genotypes and Pretreatment on Callus Formation and Differentiation

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Abstract: This study is concerned with the production of haploids of linseed. Anthers of eight genotypes were cultured both on solid and in liquid medium. It was found that callus induction rate was low in both media and also the cultivars significantly affected callus induction rate both on solid and in liquid medium. The cultivar Blue-Chip gave, on average, the maximum (3.67%) callus induction rate on solid medium and the cultivar Antares gave, on average, the maximum (3.35%) callus induction rate in liquid medium. The cultivar Norman produced callus on solid medium but did not produce any callus in liquid medium. On the other hand, the cultivar McGregor did not produce any callus in either solid or liquid medium. Pre-treatment of flower buds with cold significantly reduced callus induction rate by about four fold compared with the no pre-treatment on solid medium but increased (not statistically significant) callus induction rate by 25.8% in liquid medium. Although some callus induction was achieved in both on solid and in liquid media, no differentiation was observed when calli were transferred to regeneration medium.

Keten (*Linum usitatissimum* L.)'de Anter Kültürünün Potansiyeli: Kallus Oluşumu ve Farklılaşmasına Genotip ve Ön Muamelenin Etkisi

Özet: Bu araştırma haploid keten bitkilerinin elde edilmesi ile ilgili o lup, 8 keten çeşitinin anterleri katı ve sıvı kültür ortamında kültüre alınmıştır. Araştırma sonucu her iki ortamda da kallus teşekkül oranının düşük olduğu belirlenmiştir. Ayrıca, kallus teşekkül oranı bakımından her iki ortamda da çeşitler arasında farklılık bulunmuştur. Katı kültür ortamında Blue-Chip keten çeşidi, sıvı kültür ortamında ise Antares keten çeşidi en yüksek kallus teşekkül oranına (sırasıyla, %3.67 ve 3.35) sahiptir. Norman keten çeşidinin sadece katı kültür ortamında kalluş oluşumu sağladığı, McGregor keten çeşidinin ise her iki kültür ortamında da kallus oluşumu sağlamadığı belirlenmiştir. Soğukla muamele edilmiş olan anterlerin kallus oluşum oranı katı kültür ortamında kontrole göre dört kat azalmasına karşın sıvı kültür ortamında ise istatistiki anlamda önemli olmamakla beraber, %25.8 oranında artmıştır. Genel olarak, her iki kültür ortamında da kallus oluşumu sağlanmış ise de farklılaşma ortamına transfer edilen kalluslardan herhangi bir farklılaşma elde edilmemiştir.

Introduction

Haploid is a general term that refers to a plant containing the gametophytic number of chromosomes, that is, a single set of chromosomes in the sporophyte. Although in some special cases individual workers obtained haploids spontaneously or experimentally by special methods, until 1964 the large scale production of haploids in higher plants was only a theoretical possibility. In 1964, Guha and Maheshwari reported direct development of embryos from microspores of *Datura innoxia* by the culture of excised anthers (1). Basically, this technique involves the isolation and sterilizition of flower buds, aseptic removal of anthers with subsequent culture either on agar-solidified medium or in liquid medium a tissue culture room. The development of this technique for the induction of androgenesis by the culture of excised anthers played a very important role in the resurgence of interest in haploids. This method has since been successfully applied throughout the world with many different species and genera. With intensive application to species of economic importance, progress has been made on the cereals, such as barley (2), wheat (3, 4), triticale (5), rice (6), maize (7), rye (8) and *Solanaceous* crops, namely tomato (9) and patotoes (10) and also *Brassica* (11, 12). In fact, anther cultureas been attempted in almost all important crops during the last 20 years.

In linseed, Sun (13), Sun and Fu (14) and Nichterlein *et. al.,* (15 and 16) have investigated the potential of such in vitro anther culture technique and Nichterlein *et. al.,* (15 and 16) were able to regenerate some plants from *Linum usitatissimum* anthers. However, the anther culture protocol for linseed needs to be improved allowing a more efficient production of haploids if it is to

be of any use in plant breeding.

The objective of the present study was to assess the possibility of anther culture of linseed as a method of producing haploids and, in particular, to establish the effect of genotype and pretreatment on callus formation and regeneration of plantlets on a solid and in a liquid medium.

Materials and Methods

The overall protocol for anther culture is now fairly well defined. Anthers containing microspores at a specific stage of development are dissected out under aseptic conditions and cultured on/in an appropriate solid or liquid medium. Evidence from the published work (15, 16) indicated that linseed microspores at mid to late uninucleate stage give the best response in culture and consequently this stage was aimed at in the present experiment. It was also decided to include a short cold pretreatment of anthers prior to culture and that the one medium in both solid and liquid form would be used in the trial. Details are given below.

Plant Materials for anther Culture

The plant material chosen for the present study consisted of the eight cultivars, namely Linda, Lidgate, Cristal, Antares, Barbara, Blue-Chip, Norman and McGregor.

Seeds of donor plants were sown on October 6, 1992 in 24-hole (7 cm diameter) multitrays containing John Innes potting compost No. 2 with 4 plant to each pot. The trays were kept in a heated glasshouse cubicle. Five weeks after sowing, the seedlings were transplanted in to 13 cm diameter pots of John Innes potting compost No. 2, still with 4 plants per pot. The pots were watered daily and a liquid fertiliser applied (containing N 10%, P_2O_5 10% and K_2O_5 27%) at a rate of 4.4 g per liter water. The culture conditions throughout consisted of 16 hours photoperiod at 30 klx and a temperature of between 12˚C (night) and 22˚C maximum (day).

In the third week of January 1993, the donor plants were judged to be at the optimum stage. Healthy and vigorously growing flower buds were collected four days before flowering when the microspores were estimated to be at the uninucleate stage. The exact stage of pollen grain development was also ascertained by a cytological test as follows. Anthers were dissected from one flower bud of each cultivar, the anther length measured using a binocular microscope and then squashed in a drop of acetocarmine stain and the microspores examined under the microscope. Microspore development of linseed was examined.

Pretreatment of Flower Buds

For the pretreatment of flower buds, healthy and vigorously growing buds were collected every two days from the donor plants and kept in a plastic jar filled with up to 2 cm of water for half an hour. They were then placed in a 60 mm petri dishes on filter paper and about 15 ml distilled water applied. The petri dishes were kept in the dark room at 4˚C for three days.

Culture Media

The callus induction media used for anther culture are given in Table 1. They were liquid G23 and solid G23 (15, 16) which was solidified using Gelrite (3.5 g/l). For the preparation of the media, 4 g of N6 basal salt mixture was dissolved in 900 ml distilled water in a 1 litre beaker on a magnetic stirrer. After adding and mixing remaining ingredients from the stock solutions with the exception of Zeatin, the P^H of the medium was adjusted to 6.4. The total volume was then made up to 999 ml litre using distilled water and poured into medical flats. The meda were sterilised by autoclaving at 121˚C for 25 minutes (17), and, after cooling, filter sterilised Zeatin (1 ml/l) added. The medium containing Gelrite was poured into 35 mm petri dishes (10 ml/dish) and the liquid medium into 5 by 5 multiwell plates (2.5 ml/well) in a laminar air flow cabinet. The petri-dishes and multi-well plates were stored in a refrigerator until required.

For shoot regeneration, P20 medium (15, 16) (Table 1) was used in solid form. The P20 media were prepared using the same method as for G23 media but omitting the 2,4-D and NAA, and poured into 35 mm petri-dishes (10 ml/dish).

Sterilization of Flower Buds and Anther Plating

Flower buds were dipped in 96% ethanol for 30 seconds and then in 20% Hypochlorite solution for 10 minutes and subsequently rinsed three times with sterilized distilled water (15, 16). Thereafter, the anthers were carefully and gently dissected from flower buds under the binocular microscope in a laminar flow cabinet and placed on the induction media. A total of 30 anthers were plated on the medium in each petri dish and a total of 100 anthers were plated on the medium in each multi-well plate (four anthers/well). For each cultivar, 5 petri dishes of solid medium and two multiwell plates of liquid media were used. Finally, all of the petri dishes and multiwell plates were sealed with parafilm and labelled. The dishes were then incubated in the dark at 25˚C in a a tissue culture room.

Scoring and Transferring of Calli

Table 1. Culture media used for anther culture of linseed.

Content	Induction Media		Regeneration Media
	G23*	G23**	P20*
Macro Nutrient	N6	N6	N6
Micro Nutrient	N6	N6	N6
Vitamins	N6	N6	N6
My-Inostol (mg/l)	100.00	100.00	100.00
L-Glutamine (mg/l)	375.00	375.00	375.00
L-asparagine (mg/l)	250.00	250.00	250.00
L-Serine (mg/l)	125.00	125.00	125.00
NNA (mg/l)	0.05	0.05	-
2,4-D (mg/l)	0.05	0.05	-
Zeatin (mg/l)	0.10	0.10	1.00
Maltose (g/l)	60.00	60.00	30.00
Gelrite (g/l)	-	3.50	-
PH	6.4	6.4	5.8

*Solid medium (16); **Modified liquid medium (16); N6=Chu's N6 Basal Salt Mixture.

The cultures were examined after one week for any contamination and these were discarded. Callus counts started from the fourth week of culturen solid medium and the fifth week of culture in liquid medium. The dishes were scored for callus producing anthers at intervals of three days. Subsequently, calli with a diameter of more than 1 mm were transferred from the induction medium to a regeneration medium. One callus was placed in each petri dish and all petri dishes were sealed and labelled and cultured at 26°C under continuous fluorescent light in the tissue culture room.

Statistical Analysis

Callus induction ability was measured as the number of calli induced per 100 anthers in anther culture. All data were transformed by square root transformation, $(X+0.5)^{1/2}$ (18) to improve their normality. The transformed data were analysed as a completely randomised experimental design. Analysis of variance and the LSD tests were done using a statistical programs by computer.

Results

Cultured anthers are expected to by pass tehir normal pathway of development with the result that individual pollen grains divide repeatedly to form a callus. This was so in this experiment. After five weeks of implantation, the initation of growth varied from anther to anther. With time, the growth increased forming a distinct callus tissue of irregular shape and size. This phenomenon was observed until the ninth week of culture in some of the anthers. The colour of the callus varied from creamy white, creamy to light yellow. The texture was smooth or rough and loose or compact. Afterwards some of the calli became brown and then black and finally died.

Initially the number of anthers forming calli was recorded every three days. The data given in column 2 of Table 2(*i*) and 2(*ii*) are, however, the final count (nine weeks). Since slightly different numbers of anthers were placed, especially in the liquid medium, these values have been converted to number of responsive anthers per 100 plated (i.e., %). These are given in column 3 of Table 2(*i*) and 2(*ii*). The range is from 0 to 6.7% in solid medium with no pretreatment of anthers, from 0 to 2.0% in solid medium with pretreatment, 0 to 2% in liquid medium with no pretreatment and 0 to 4.7% in liquid medium with pretreatment.

The different induction rates of the different cultivars on both solid and liquid media are illustrated graphically in Fig 1(a).

The data from both solid and liquid media was initially analysed statistically as two separate experiments because different culture vessels had to be used and consequently the layout of the experiment was different. The LSD values used in Table 2(*i*) and 2(*ii*) were also based on

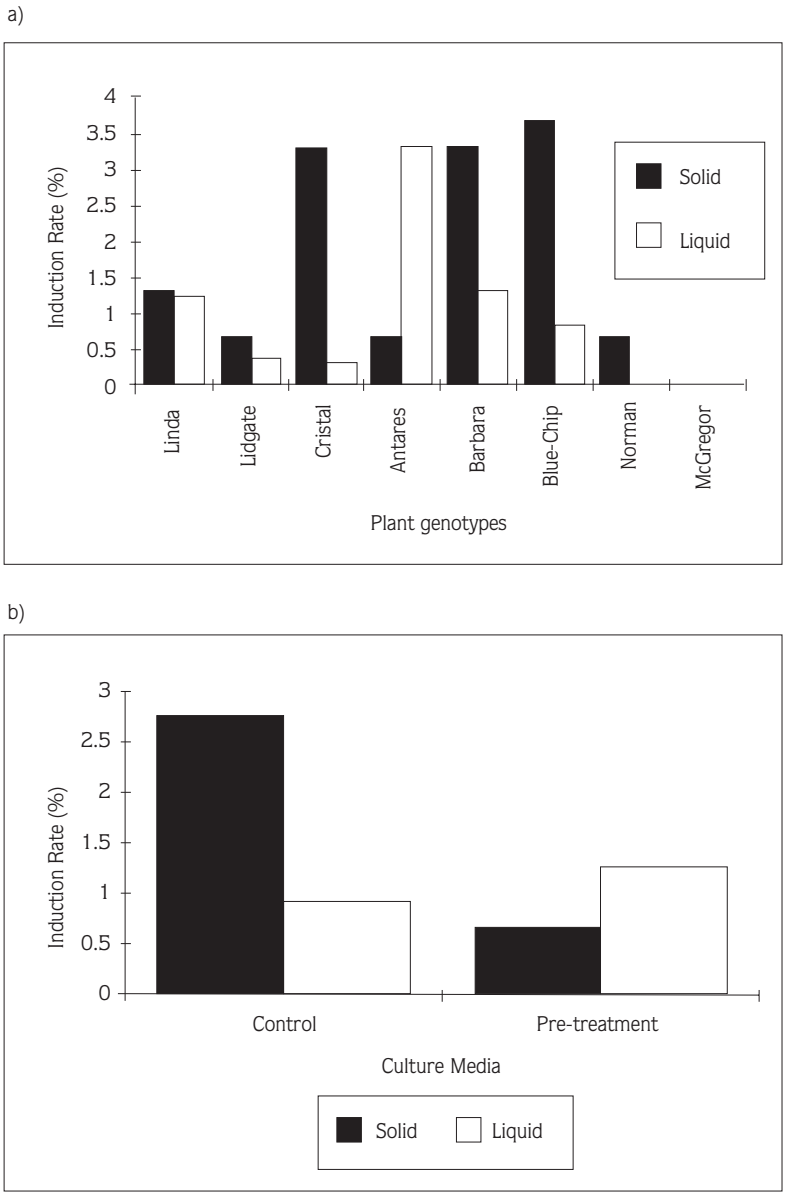

Figure 1. The effect of genotype (a) and culture media (b) on callus induction rate (%).

separate analyses. However, a combined analysis was also done to compere culture media. While it is appreciated that there is some imprecision in this combined analysis because of the different replication within the two media, it was felt that it would, nevertheless, give some indication whether three wereny significant differences between the media.

Effect of Cultivar on Callus Induction

Variable responses were expressed by all of the eight cultivars tested on the two different induction media. The separate analyses showed that cultivar significantly affected callus induction in both solid and liquid ($P \leq 0.05$) medium. As shown in column 2 of Table 2(*i*) and 2(*ii*), the highest number of callus induction was 10 from cultivar Barbara in solid medium with no pretreatment and 3 from cultivar Cristal in solid medium with pre-treatment, and 4 from cultivar Barbara in liquid medium with no pre-treatment and also 8 from cultivar Antares in liquid with pre-treatment. The different response ($P \leq 0.01$) overall is confirmed by the combined analysis.

As shown in Table 2(*i*) and 2(*ii*), cultivar Barbara gave the highest callus induction rate (6.7%) in solid medium with no pre-treatment and cultivar Cristal in solid medium (2.0%) with pretreatment. However, cultivar

Table 2. Effects of genotype, pre-treatment and culture medium on callus induction in linseed.

	(*i*)			SOLID MEDIUM					TOTAL			
CULTIVARS	NO PRE-TREATMENT				PRE-TREATMENT							
	1	2	3	4	1	2	3	4	1	2	3	4
LINDA	150	4	2.7	1.50b	150	0	0.0	0.71d	300	4	1.33	1.35
LIDGATE	150	1	0.7	0.96cd	150	1	0.7	0.96cd	300	2	0.67	1.08
CRISTAL	150	7	4.7	2.11a	150	3	2.0	1.22bc	300	10	3.33	1.96
ANTARES	150	1	0.7	0.96cd	150	1	0.7	0.96cd	300	2	0.67	1.08
BARBARA	150	10	6.7	2.32a	150	0	0.0	0.71d	300	10	3.33	1.96
BLUE-HIP	150	9	6.0	2.35a	150	2	1.3	1.21bc	300	11	3.67	2.04
NORMAN	150	1	0.7	0.96cd	150	1	0.7	0.96cd	300	2	0.67	1.08
McGREGOR	150	0	0.0	0.71d	150	0	0.0	0.71d	300	0	0.00	0.71
TOTAL	1200	33	2.75		1200	8	0.67		2400	41	1.71	
	(*ii*)			LIQUID MEDIUM								
CULTIVARS	NO PRE-TREATMENT				PRE-TREATMENT							
LINDA	156	3	1.9	1.14bcd	156	1	0.6	0.97cd	312	4	1.28	1.33
LIDGATE	200	1	0.5	0.97cd	100	0	0.0	0.71d	300	1	0.33	0.91
CRISTAL	200	1	0.5	0.97cd	96	0	0.0	0.71d	296	1	0.33	0.91
ANTARES	156	3	1.9	1.55b	172	8	4.7	2.26a	328	11	3.35	1.96
BARBARA	200	4	2.0	1.42bc	116	1	0.9	0.97cd	316	5	1.58	1.44
BLUE-HIP	200	1	0.5	1.34cd	40	1	2.5	1.53b	240	2	0.83	1.15
NORMAN	100	0	0.0	0.71d	164	0	0.0	0.71d	264	0	0.00	0.71
McGREGOR	200	0	0.0	0.71d	44	0	0.0	0.71d	244	0	0.00	0.71
TOTAL	1412	13	0.92		888	11	1.24		2300	24	1.04	
Total of Treatment	2612	46	1.76		2088	19	0.91		4700	65	1.38	

1=Numbers of placed athers; 2=Numbers of callus induced anthers; 3=Callus induction rate (%); 4=Callus induction rate (%), after (X+0.5)1/2 transformation (Gomez and Gomez, 1984). Result shown by the same letter for each genotype is not significantly different at the 0.05 probability level according to LSD's multiple range test.

Blue-Chip gave highest callus induction rate in solid medium with combined non pretreatment and pretreatment by 3.67%. Cultivar Barbara gave the highest callus induction rate (2.0%) in liquid medium with no pretreatment and cultivar Antares gave the highest callus induction rate (4.7%) in liquid medium with pretreatment and also in liquid medium with combined no pretreatment and pretreatment by 3.35% (in column 3 of Table 2(*i*) and 2(*ii*)).

Anthers from cultivar McGregor did not form any callus in either solid or liquid medium (in column 2 of Table 2(*i*) and 2(*ii*)). Cultivar Norman was only able to produce a total of 2 calli in solid medium (in column 2 Table 2(*i*) with callus induction rate of 0.67% (in column 3 of Table 2(*i*) with no callus induction at all in liquid medium (in column 2 of Table 2(*ii*)).

Effect of Pretreatment on Callus Induction

The pretreatment of flower buds (at 4°C for three days) significantly lowered the number of calli induced in solid medium ($P \leq 0.01$) but it did not have a statistically significant effect in liquid medium. Pretreatment of flower buds decreased the callus induction rate from 2.75% (no pretreatment) to 0.67% (pretreatment) in solid medium whereas it increased (although not significantly) from 0.92% (no pretreatment) to 1.24% (pre-treatment) in liquid medium (in column 3 of Table 2(*i*) and 2(*ii*)).

Overall, the combined analysis show that the number

of calli induced anthers were significantly lowered by the pretreatment of flower buds. A total of 46 (an average of 1.76% induction rate) calli induced anthers from 2612 placed anathers with no pre-treatment and a total of 19 (an average of 0.91% induction rate) calli induced anthers from 2088 placed athers with pretreatment was found (in column 1 and 2 of Table 2(*i*) and 2(*ii*)). However, it is shown above that this is entirely due to the response in solid medium. This differential response is confirmed by the highly significant mediaxtreatment interaction found in the combined analysis. The large decrease in callus induction on solid medium due to pretreatment is illustrated graphically in Fig. 1(b) as is the different response in liquid medium.

Effect of Culture Medium on Callus Induction

Clearly this comparison can only be effected in a combned analysis. It has to be pointed out again that the control of error for this test is far from rigid. The more accurate error variance to use for testing differences between media is that of variance between replicates within media. Consequently the test is not very sensitive. Despite a relatively large difference overall in the induction rate in the two media (1.71% in solid against 1.04% in liquid) this is statistically not significant. A less accurate test of media mean square against overall error mean square gives a significant difference.

Regeneration of Calli

After transfer of the calli onto the regeneration (P20) medium (Table 1), shoot regeneration could not be achieved. It was observed that transferred calli were able to continue to grow in the regeneration media for some time forming a callus tissue of irregular shape and size. They eventually lost colour from green to brown and finally died. Clearly they were still undifferentiated calli and this was confirmed by examination of surface cells under a stereomicroscope. Moreover, the observation of stained cells under a high powered microscope showed that there were very few cells in each observed area. It was also apparent that the number of cells at division at any one time was extremely low and it was not possible to clearly identify and count chromosomes.

Discussion

The main objective of this experiment was to determine the effects of cultivars (genotype) and culture medium on the response of linseed plants to anther culture. As reported earlier, anthers of eight cultivars were cultured in two media. As it is obvious from the previous section the experiment was only partly successful. Some of the anathers responded well to culture in both the liquid and solid induction media resulting in well developed and healthy calli. However, no differentiation into embryoids and plantlets was observed on the regeneration medium and to this extent the results were somewhat disappointing. Nevertheless, it was observed that the anthers of different cultivars responded differently in terms of callus induction. In general, all cultivars were low callus producing (except cultivar McGregor).

Relatively low induction rates are a feature of most anther culture experiments. In previous work with linseed a maximum anther induction rate of 21.6% was reported by Nichterlein *et. al.*, (15) with cultivar Hella and 39.1% (16) with cultivar Atalante. In wheat, Hassawi and Liang (4) found only 9.8% callus induction with cultivar Pavon-76. Part of the reason is that many of the anthers will not have been at the ideal developmental stage when they were cultured. Cytological examination of a sample of anthers only serves as a general guide to the size of anthers to implant. In the end the whole procedure is, to a certain extent, subjective and many of the anthers cultured are too young or too old. The variation in callus producing ability between the cultivars was significant both on solid and in liquid medium ($P \leq 0.05$). This difference in the level of culturability in both culture media is strongly indicative of a genetic component to the response rate. A crossing experiment in involving responsive and non-responsive genotypes would be needed to confirm this. In this particular experiment the cultivar Blue-Chip was shown to be on average to have the highest callus induction rate while cultivar McGregor did not produce any calli at all. This result corresponds with that obtained by Nichterlein *et. al.*, (15 and 16) where they found that anther induction rate of linseed genotypes varied according to the plant genotype. Similar anther response has been observed in many other species. For example, anther response was obtained in only 5 of 12 *Nicotiana* species (19), in only 10 of 21 cultivars of *Triticum aestivum* (20) and also Phippen and Ockendon (21) also found a variation between and within the 11 hybrids of *Brassica spp.*

There was also some indication that productivity of the tested entries was different in the two media although the statistical test was inconclusive. The callus induction rate was 19.3% higher on solid medium than in liquid medium. It will be recalled that the composion of the both culture media was the same apart from the fact that one had been solidified with gelrite. The evidence relating to the use of solid and liquid media for anther culture is contradictory. Claims have been made for both

types although some of the comparisons have been confounded with differences in the basic composition of the media themselves. Nevertheless, therereell documented examples which can be quoted. In some specific cases, liquid medium gives the better anther responses as, for example, in *Nicotiana tobaccum* (22), in *Datura innoxia* (23) and in *Brassica napus* (11, 24). However, in other cases it has been shown that solid medium gives the better results. In cauliflower, embryo production from anather culture was greater on solid medium than liquid medium, with yield increases of up to 100% (21). It has been suggested that anaerobic conditions may occur in calli beneath the liquid surface thus inhibiting growth (21). Although, there was indication from this experiment that the solid medium was more suitable than liquid medium for linseed anther culture further work needs to be done to confirm this.

The other variable tested in this experiment was that of pre-treatment of flower buds. Although the overall level of callus induction was low, there was, nevertheless, a strong indication of a different response to cold pre-treatment from anthers cultured on solid and in liquid media. The effect of pre-treatment was a negative one. As indicated earlier anthers cultured in solid medium without pre-treatment produced a four fold higher callus induction rate than pre-treated anthers. This was highly significant ($P \leq 0.01$). On the face of it this result is at odds with others. In many case it has been shown that pre-treatment increases callus induction ability. For example, in *Dature innoxia,* Nitsch and Norreel (25) found that cold treatment of flower buds prior to culture greatly enhanced the yield of haploid plants. However, there is no standard pretreatment which can be recommended for all plant species and there are many contrasting results. For instance, *in Dature innoxia,* a cold pre-treatment of 48 h at 3°C has been recommended by Nitsch (26) but Tyagi *et. al.*, (23) claim that 4 days at 4°C to be optimum. The exact temperature and duration must be determined for each species. Although in the present work, pre-treated anthers (in liquid medium) produced a higher callus induction rate (25.8%) than non pretreated anthers, it was non statistically significantly different. It is tempting to suggest that pretreatment might be recommended for liquid medium but not solid medium but the evidence is not conclusive. Part of the problem is that the overall level of callus induction was low, especially in the liquid medium.

Low yield of embryos and regenerated shoots has often been a problem in the production of haploids in many crops. They few previous efforts at anther culture in linseed have also encountered this problem. Nichterlein *et al.*, (15) obtained a few entire plants form cv. Antares only but were able to improve on this in later work (16). Although a similar protocol was used in the present experiment no differentiation of callus was observed. All aspects of the procedure have to be critically examined in any future attempt to obtain an adequate yield of embryoids. Even the growing conditions of the donor plants can not be ruled out and as a possible cause despite the positive callusing response of many anthers in the induction medium. Other factors are the nature of the species itself, the genotypes used and the regeneration medium chosen in this particular case.

In conclusion, the results presented here show that:

1) The plant genotype significantly influenced the callus induction rate in linseed.

2) Cold pre-treatment of flower buds significantly reduced callusing in the solid medium but slightly increased it (although statistically not significant) in liquid

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