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Influence of medium and cold pretreatment on androgenetic response in *Lolium perenne* L.

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Summary. Genotypes of *Lolium perenne* L. with different androgenetic responses were used to test effects of induction medium composition. The media tested were potato II (pII), 190-2, and modified Linsmaier and Skoog media, LS-1, LS-2, and LS-3. The effect of different gelling agents, activated charcoal in a 'double layer' design, and casein hydrolysate were also studied. From 36,696 anthers, 25,906 embryo-like structures, 1,959 albino and 173 green plants were generated. Significant differences were found between media, genotypes and medium-genotype interactions studied. All three media commonly used, pII, 190-2, and LS-3, were equivalent in production of green plants. Cold pretreatment of the anthers (4°C) significantly increased the number of embryo-like structures, the number and proportion of albino plants produced, but not the production of green plants.

Abbreviations: ELS, embryo-like structures; ALB, albino plants; ANT, anthers; GRP, green plants; DH, doubled haploid plants; AC, activated charcoal; CH, casein hydrolysate; 2,4-D, 2,4-dichlorophenoxyacetic acid; LS, Linsmaier and Skoog (1965) basal medium; MS, Murashige and Skoog (1962); PL, plants; pII, potato II induction medium; DL, double layer.

Introduction

Anther culture has been applied in breeding programmes of some *Poaceae* species, mainly for the cereals rice (*Oryza sativa* L.) and barley (*Hordeum vulgare* L.), but also for wheat (*Triticum aestivum* L.). In forage grasses, doubled haploid plants (DH) make the production of F_1 hybrids feasible, and may make the construction of synthetic varieties more precise. However, there are two major problems in anther culture of forage grasses compared with self-pollinated cereals: firstly, forage grasses are cross-pollinated, suffering from inbreeding depression when made homozygous by anther culture; and secondly, less work has been done to develop the technique in forage grasses compared to cereals. Perennial ryegrass (*Lolium perenne* L.) is the most important forage grass in Europe, and breeders are about to apply anther culture in breeding programmes in this species. Even though breeding companies are working with several cell and tissue culture techniques in ryegrass, so far they have had little apparent success (Bante et al. 1990a; Creemers-Molenaar et al. 1992).

Although there are similar effects of cold pretreatments, sugars and auxins on green plant production in barley, rice, wheat and perennial ryegrass, each species has nevertheless required a specific protocol to optimize its androgenetic response. Cold pretreatment is used routinely in barley anther culture, and has also been reported to have positive effects in rice, wheat and ryegrass (Tsay et al. 1988; Lazar et al. 1985; Bante et al. 1990b). However, the effect is likely to be genotype dependent (Olesen et al. 1988; Lazar et al. 1990).

Despite many studies in this area, it seems difficult to obtain a general improvement in androgenetic response by changing *in vitro* culture conditions, since, to a large extent, the response depends on genotype (Henry and de Buyser 1985; Tuvesson et al. 1989; Opsahl-Ferstad et al. 1994). Few systematic studies of medium requirements for anther culture of perennial ryegrass have been done, and there is much variation in the composition of the media published (Olesen et al. 1988; Bante et al. 1990b). Unmodified induction media for wheat anther culture, e.g. potato II (pII) and 190-2 (Wang and Hu 1984), have been used in perennial ryegrass (Olesen et al. 1988). There are several potentially important factors related to the medium that may influence the outcome of anther culture. These factors fall into three categories: chemical

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properties, physical properties and repeatability.

A problem with systematic approaches to the development of new media is the large experimental variation often seen in tissue culture experiments, and the large number of factorial combinations that need to be tested. Some of the induced random variation may be due to ill-defined organic additives, e.g. potato extract and casein hydrolysate (CH), which sometimes make it difficult to reproduce experimental results and detect significant effects of different factors. Parallels and replicated experiments are therefore required in order to remove random variation.

Important chemical factors of induction media are the total nitrogen content which, along with the ratio of NO_3^-:NH_4^+ and the content of organic versus inorganic nitrogen, have been studied in several experiments in barley and perennial ryegrass (Olsen 1987; Bante et al. 1990b).

Physical properties of the medium, such as texture, may also affect androgenetic response. Float culture has been reported to increase the embryogenic response in wheat (Lazar et al. 1985), even though solid medium is used most frequently for the grasses. However, very different concentrations of a range of gelling agents have been used to solidify media. Further, in liquid culture for grass anther culture, thickening agents such as Ficoll and wheat starch have been used to ensure aeration of the anthers (Simonson and Baenziger 1992). This appears to be important for obtaining good embryogenesis and subsequent regeneration (Kao 1981). The physical properties of the medium may further affect the production and diffusion of phenolic compounds, produced by the anthers, and thought to be responsible for the browning of tissues commonly observed in ryegrass anther culture. Adsorption of such compounds by activated charcoal (AC) is one explanation for the positive effect of AC on androgenetic response in potato (*Solanum tuberosum* L.) (Johansson 1986), and on callus culture in red fescue (*Festuca rubra* L.) (Zaghmout and Torello 1988). By using a double layer system, Johansson (1986) combined the positive effect of AC and the physical properties of liquid medium. The bottom layer is solid and contains AC, while the upper layer is liquid and without AC. This allows the phenolic compounds to be adsorbed by the AC when they diffuse into the solid layer.

Because of the variable and contradictory results reported from experiments with perennial ryegrass, we wanted to undertake a more systematic comparison of the effects of the very different induction media commonly used in this species. We also studied the effect of different modifications of some of these media.

Materials and Methods

Medium - Experiment 1 (1990). The compositions of the three basic induction media, previously unpublished, are given in Table 1, and a summary of the compositions of the total number of ten media, which were used in the experiments, is presented in Table 2. The pII and 190-2 induction media are well known (Wang and Hu 1984). For the sake of brevity we named the media which were studied in experiment 1; M1, M2, M3, M7, and M8.

Table 1. Chemical composition of the basic induction media not previously reported (mg/l), pH 5.8.

Medium	LS-1[1]	LS-2[1]	LS-3[2]
KNO_3	1900	1900	
NH_4NO_3	165	165	165.2
KH_2PO_4	170	170	170
$MgSO_4*7H_2O$	370	370	370
$CaCl_2*2H_2O$	440	440	440
$MnSO_4*4H_2O$	22.3	22.3	22.3
KI	0.83	0.83	0.83
$CoCl*6H_2O$[4]	0.03	0.03	0.03
$ZnSO_4*7H_2O$	8.6	8.6	8.6
$CuSO_4*5H_2O$[4]	0.03	0.03	0.03
H_3BO_3	6.2	6.2	6.2
$Na_2MoO_4*2H_2O$	0.25	0.25	0.25
$FeSO_4*7H_2O$	27.8	27.8	27.8
$Na_2EDTA*2H_2O$	37.3	37.3	37.2
myoInositol	100	100	100
Thiamine HCl	1	1	1
Pyridoxine HCl	5	5	5
Nicotinic acid	5	5	5
Glutamine		750	292.2
Glycine	20	20	20
Casein hydrolysate	1000	1000	
2,4-D	1.5	1.5	1.5
Kinetin	0.5	0.5	0.5
Maltose[3]	90000	90000	90000
Gelrite	3000	3000	3000

[1] Bante et al. 1990b, [2] van Ark pers comm. Barenbrug AS,
[3] Hunter 1987. [4] The amount is rouned off from 0.025 to 0.03 of layout reasons. Activated charcoal was tested with 10 g/l in a solidified bottom layer with a liquid layer on top. When using Ficoll as a solidifying agent, 20 g/l was added.

Four Norwegian genotypes of perennial ryegrass, 1-1, 6-6, 7-5, and 9-5, with very different androgenetic responses, were applied in an initial experiment to study main effects of medium composition and effects of medium-genotype interactions on androgenetic response. Genotype 1-1 had a low embryogenic ability, 9-5 was very embryogenic and produced only albino plants, 6-6 regenerated a high percentage of green plants, and 7-5 was the best producer of green plants from 90 Norwegian genotypes screened (Opsahl-Ferstad et al. 1994). The clones were vernalized at 4°C for five months. Donor plants (6 ramets of each clone) were grown at 15°C/12°C day/night-temperatures (12h cycles) in natural day light (early summer), with additional light (115 $\mu molm^{-2}s^{-1}$, Hy Pressure Mercury Halogen), in controlled greenhouse conditions. Replicates were obtained by growing two sets of clones (of all

genotypes) at different times during the summer. Spikes were collected when microspores were at the mid- to late-uninucleate stage (He and Ouyang 1984), and surface sterilized with 0,1 % mercuric chloride for 8 minutes. Anthers from flowers of the five intermediate spikelets on each spike were plated on induction media in a 'triple-dish-design' (Lyne et al. 1986). Each Petri-dish contained 8 ml medium, except in the case of medium pII-AC (M2), where 4 ml liquid pII medium was added on top of 5 ml of solid pII medium containing AC. Twentyfour anthers were plated in each dish and approximately 20 parallels (20 spikes) were plated for each combination of genotype, medium and replicate. A total number of 17,760 anthers were plated. Petri dishes were incubated at 26°C in continuous white fluorescent light at low light intensities (15 $\mu molm^{-2}s^{-1}$) (Bjørnstad et al. 1989). Embryo-like structures (ELS) were transferred to regeneration medium 190-2 with 0.5 mg/l kinetin and 0.5 mg/l naphthaleneacetic acid (NAA) at three intervals, four to eight weeks after incubation of anthers. Plants were grown further on rooting medium 190-2 (without growth regulators) before being transplanted into soil. Chromosome counts were performed on Feulgen stained root tips.

Medium - Experiment 2 (1991). Based on the good results obtained with induction medium 190-2 in exp. 1, new combinations of this medium were tested in addition to a modified LS-medium (LS-3) in 1991 (Linsmaier and Skoog 1965, van Ark pers. comm.). We named these media M3, M4, M5, M6, M9 and M10 (Table 2). The Norwegian genotypes 6-6 and 7-5, in addition to the highly responsive Danish genotype 245 (Olesen et al. 1988) were used, all of which had previously shown ability to produce green plants. Three replicates of the donor plants were grown under controlled greenhouse conditions at 18°C, 8h/ 12°C, 16h, at ambient daylength conditions in May-July. A total number of 18,936 anthers were plated.

Cold pretreatment - Experiment 3 (1990 and 1991). Eight Norwegian genotypes (1-1, 1-5, 4-10, 5-5, 6-6, 7-5, 8-3, 9-5) with very different androgenetic responses were selected from a screening experiment and used to test the effect of cold pretreatment in 1990. It was repeated in 1991 with three of these genotypes (6-6, 7-5, 9-5) in addition to the Danish genotype 245, which was used as reference. Pretreatment for two and three weeks at 4°C were compared with controls without cold pretreatment. Prior to the cold pretreatments, the donor plants were grown in the same growth chambers at the same time as the two experiments described earlier. Standard anther culture conditions were applied as described above, and induction medium 190-2 with gelrite and maltose (M3) was used.

Table 2. An overview of the induction media (M1-M10) tested in the experiments.

M [a] no	M1	M2	M3[3]	M4	M5	M6	M7	M8	M9	M10
M type	pII	pII	190-2	190-2	190-2	190-2	LS-1	LS-2	LS-3	LS-3
Sol [4]	Gel	Gel	Gel	Gel	None	Fic	Gel	Gel	Gel	Gel
		None		None						None
		DL[1]		DL						DL
Add [2]	Pot	Pot		AC	*	*	CH	CH	*	AC
		AC		*						*

[a] M, medium. [1] DL, double layer; Fic, Ficoll; Gel, gelrite; [2] AC, activated charcoal; Add, additions; CH, casein hydrolysate; Pot, potato. [3] M3 was tested in both experiments. [4] Solidifying agent. * Media tested in experiment 2.

Statistical analyses. In all experiments the following characters were observed: number of embryo-like structures (#ELS), albino (#ALB), and green (#GRP) plants produced. Number of ELS, ALB and GRP per 100 anthers (#ELS/100ANT, #ALB/100ANT, #GRP/100ANT), total plant regeneration (#PL/100ELS), and albino versus green plant production (%ALB/PL and %GRP/PL) were calculated, based on the sums over parallels. Residual plot analysis showed that the data were not normally distributed. The calculated variables were therefore square root transformed before the analyses of variance were carried out using the SAS/STAT GLM procedure (SAS 1987). Differences between means were found by Duncan's Multiple Range Test (Duncan 1975). Differences between media were further investigated by decomposition of the mean squares for media in orthogonal linear contrasts (Steel and Torrie 1987). The following contrasts were estimated: 1) Ficoll and liquid versus (vs.) others (M5, M6 vs. M3, M4, M9, M10), 2) 190-2 (M3,M4) vs. LS (M9, M10), 3) activated charcoal in a double layer system vs. gelrite for 190-2 (M3 vs. M4) and for LS (M9 vs. M10), 4) Gelrite (M3) vs. liquid (M5), and 5) Ficoll (M6) vs. liquid (M5).

Results and Discussion

Medium, genotype, and G - M interactions

In exp. 1 there were significant effects of media on all characters tested (Table 3), while differences between genotypes were significant only for embryogenic ability (ELS/100ANT). The significant differences between media were due to the non or low responsive media LS-1 and LS-2 (Bante et al. 1990b).

Table 3. Mean squares from the analysis of variance, testing the experiments from 1990 and 1991 separately and with contrasts between different groups of media.

Source		ELS/100 ANT	PL/100 ELS	ALB/100 ANT	GRP/100 ANT	ALB/ PL[6]	GRP/ PL[6]
	df	MS	MS	MS	MS	MS	MS
Exp I							
Geno	3	42.8**	0.6	2.6	0.3	10.8	2.1
Medium	4	74.8***	27.5***	10.5***	0.5*	130.6***	6.9*
Inter.[1]	12	9.1	1.1	0.8	0.1	7.6	1.4
Error	19	7.5	3.1	0.9	0.1	15.3	1.7
Exp II							
Geno	2	16.1	2.7***	11.5***	2.9***	8.9	28.4***
Medium	5	49.0**	12.2***	10.6***	1.3***	8.7	9.3**
Contrasts:							
Fic/liq-R[2]	1	5.3	1.4*	0.1	0.9**	12.6	15.2**
Ficoll -L[3]	1	104.7**	1.7*	0.5	0.0	0.0	0.1
190-2 -LS	1	48.7*	0.1	0.5	0.0	10.5	1.9
Gel -AC[4]	1	52.9*	25.8***	20.5***	0.7*	0.9	0.4
Gel -AC[5]	1	33.2	32.1***	31.6***	4.8***	19.4*	29.0***
Inter.	10	19.2	0.9*	1.5***	0.6***	3.1	8.7***
Error	28	9.7	0.3	0.2	0.1	3.8	2.0

*, **, ***; significant at the 5, 1 and 0.1 % level, respectively.
[1] Inter.: interaction genotype-medium, [2] R: rest, [3] L: Liquid, [4] 190-2, [5] LS, [6] in percent.

There was no significant genotype-medium interaction. This experiment showed that the pII medium can be replaced by the 190-2 medium, which is preferable because it does not contain the variable potato extract. The following discussion is mainly based on the results from experiment 2, because this experiment compared media 190-2 and LS-3 without undefined organic additives.

As shown in Table 3, embryogenic ability (ELS/100ANT) in exp. 2 was not significantly affected by genotype, probably due to the use of genotypes with a similar phenotype regarding this character (Opsahl-Ferstad et al. 1994). The magnitudes of the mean squares (MS) (Table 3) show that the medium effects are more important sources of variation in embryogenic ability and total plant regeneration (ELS/100ANT, PL/100ELS) than genotype. Medium and genotype were equally important in causing variation in albino and green plant production. Necessary alleles for androgenetic response have to be present (Opsahl-Ferstad et al. 1994), however when present, the environment regulates their expression. The contrast 190-2 (M3, M4) vs. LS (M9, M10) was significant for embryogenic ability (Table 3), and significantly more ELS were produced on the 190-2 media than on the LS media. Table 4 shows mean values for media tested in experiment 2. The media 190-2 (M3) and LS-3 (M9), both containing gelrite, gave the best total plant regeneration and green plant production. The pII medium (M1) of exp. 1 gave a comparable response in terms of regeneration of green plants as these two media (M3, M9).

Table 4. Average production from media tested in 1991 (Exp. 2), with Duncan contrasts.

MEDIUM	ELS/ 100ANT	PL/ 100ELS	ALB/ 100ANT	GR/ 100ANT	%ALB/ PL	%GRP/ PL
LS-3_{GE} (M9)	103.7^{ab}	13.3^{a}	12.3^{a}	1.3^{a}	87.6^{a}	10.0^{a}
190-2_{GE} (M3)	92.1^{ab}	13.8^{a}	12.9^{a}	0.7^{a}	91.7^{a}	4.8^{ab}
190-2_{AC-DL} (M4)	165.6^{a}	0.9^{c}	1.1^{c}	0.1^{b}	86.2^{a}	5.1^{ab}
190-2_{Li} (M5)	127.5^{a}	5.9^{b}	5.3^{b}	0.2^{b}	95.9^{a}	1.4^{b}
190-2_{Fi} (M6)	47.9^{b}	8.3^{b}	3.6^{b}	0.1^{b}	94.7^{a}	1.7^{b}
LS-3_{AC-DL} (M10)	53.2^{b}	0.6^{c}	0.3^{d}	0.0^{b}	70.0^{b}	0.6^{b}

Entries with the same letters are not significantly different at $p \leq 0.05$.

As to the genotypic differences, genotype 7-5 had the highest total plant regeneration (PL/100ELS) and produced significantly more albino as well as green plants compared to the other genotypes. Genotype 6-6 produced relatively more albino plants than 245, while 245 produced more green plants. A more detailed study of the genotype-medium interactions, showed that interactions did not involve the most responsive genotype (7-5) or media (M3 and M9), except for the percentage green plants produced (data not shown). Genotype-medium interaction and genotype-environmental interactions have previously been found in other anther culture studies, i.e. Liang et al. (1987) and Lazar et al. (1984). The presence of genotype-medium interactions is not unexpected, it only makes it more difficult to find a single optimal induction medium.

Nitrogen and unspecified organic additions

Differences between media could only partly be explained by different nitrogen content and NO_3^-: NH_4^+ (inorganic nitrogen) ratios (c.f. Tables 1, 4 and 5). The reduction of ammonium nitrate in LS-2 compared to LS-1, and the addition of glutamine (Bante et al. 1990b) was tested, but neither of these media gave any response with the genotypes used. LS-1 has an extremely high total inorganic N content and LS-2 has a high glutamine content, which may be the reasons why these media did not give any androgenetic response. LS-3, however, gave a good androgenetic response, even though it has a very low total inorganic N content as well as a low NO_3^-:NH_4^+ ratio compared to the media 190-2 and pII. However, the inorganic to organic N content is quite high in this medium and closer to the others (Table 5), which may explain why this medium gave such a high androgenetic response.

Table 5. Inorganic and inorganic : organic nitrogen ratio in the media.

Medium	Inorganic N Ratio NO_3^-: NH_4^+	Inorganic: organic N Ratio
pII	88 : 12	90 : 10
190-2	78 : 22	91 : 9
LS-1	66 : 34	[1]
LS-2	91 : 9	[1]
LS-3	50 : 50	67 : 33

[1] Not calculated because of the casein hydrolysate content in these media.

Inorganic to organic N ratio is much lower in the two media LS-1 and LS-2. Mordhorst (pers. comm.) obtained the highest regeneration from a inorganic nitrogen ratio of 90:10, and a inorganic to organic nitrogen (being glutamine) ratio from 71:29 to 90:10. Mordhorst and Lörz (1993) further found that 20-35 mM total nitrogen content was optimal for regeneration from barley microspore cultures, while Grimes and Hodges (1990) did not find any difference in regeneration between 25, 35 or 45 mM total nitrogen in rice embryo cultures. Total

nitrogen is reported to be less important than the NO_3^-: NH_4^+ ratio for regeneration from barley and rice tissue cultures (Olsen 1987, Grimes and Hodges 1990). Sensitivity to organic nitrogen and auxins may depend on inorganic nitrogen ratios. A high ratio such as 80:20 in the N6 medium (Chu et al. 1975), increased auxin sensitivity in rice callus compared to a ratio of 50:50 (LS-3). The effects of organic nitrogen differ depending on the source i.e. casein hydrolysate (CH) or tryptophan (Grimes and Hodges 1990). According to Olsen (1987), a lower concentration of ammonium in MS induction medium (Murashige and Skoog 1962) was beneficial in barley, given that glutamine was added simultaneously at high concentrations (5.1 mM). However, glutamine concentrations above 3,4 mM have been reported to be toxic in wheat anther culture (Henry and deBuyser 1981). This ratio may have an influence on auxin response through regulation of uptake, altered cell sensitivity to auxins, or metabolism of hormones (Grimes and Hodges 1990).

There was no significant difference between the effect of pII and 190-2 on green plant production. This indicates that the variable potato extract can be avoided as a component of induction media. Potato extract contains organic nitrogen in addition to other components. It has been shown that potato extract can be replaced by glutamine in wheat pII medium. Glutamine may function as an organic N source (Henry and de Buyser 1981). Optimum glutamine concentration depends on nitrogen concentration, myo-inositol content and whether the medium is liquid or solid (Olsen 1987). CH also contains glutamine, among other components, and has been shown to stimulate somatic embryogenesis in grasses. It has been reported to have no effect on rice when added to MS media (Koetje et al. 1989), although it affected the optimal inorganic N-ratio (NO_3^-: NH_4^+). When CH was added, the optimal inorganic N-ratio was 50:50, as in the LS-3 medium (Grimes and Hodges 1990).

Gelling agents and activated charcoal in a double layer system (AC-DL)

Liquid medium and activated charcoal in a double layer system (AC-DL) of the 190-2 medium (M4, M5) gave the best results for embryogenic ability (Table 4). This is presumably due mostly to the liquid nature of both media and to a lesser extent to the activated charcoal, which is shown by the contrasts in Table 3. The presumed positive effect of AC in absorbing toxic compounds released after autoclaving does not seem to be significant in these experiments. The media 190-2 and LS with gelrite (M3, M9) were best for the other characters of androgenetic response. This is mainly a result of gelrite being better than AC-DL, Ficoll or liquid media with respect to regeneration and production of green plants. However, the effect of gelling agents may be physical rather than chemical (Lazar et al. 1990).

Significant differences were detected between gelling agents in the media. Liquid 190-2 medium (M5) gave more ELS than when Ficoll was added (M6), shown by the contrasts liquid vs. Ficoll (Table 3). By including Ficoll, a higher total plant regeneration was obtained. These two media were equivalent for all other characters studied. The contrast AC-DL vs. Gelrite, was significant for total plant regeneration, albino production, percent albino and green plant production for both media 190-2 and LS-3 (Table 3). For the character embryogenic ability (ELS/100ANT) this contrast was only significant for the media 190-2 (M3 vs. M4), while for percent green plants only media LS (M9 vs. M10) showed a significant contrast. AC-DL had a positive effect on embryogenic ability, but a negative effect on regeneration. The effect of AC-DL depended on genotype in addition to medium. When added to the induction medium 190-2, AC-DL significantly increased embryogenic ability in genotype 6-6, but not in 7-5. AC has been reported to stimulate embryogenesis in recalcitrant genotypes, but not in responsive ones (Lazar et al. 1990), which may explain the differential effects of AC-DL in the present genotypes. With respect to regeneration, AC-DL had a significant negative effect (Table 4). This is a strong argument for excluding AC in induction media, because increased embryogenesis without increased regeneration of green plants does not improve output from anther culture. Johansson (1986) found L-cysteine-HCl, which inhibits synthesis of phenolics, to increase embryogenesis in potatos; but polyvinylpyrrolidone, a phenol-absorbing agent, did not show this effect. He reported AC to increase embryogenic ability, and believed that it absorbed phenolics to some extent. However, Weatherhead et al. (1978) reported that AC absorbed certain auxins, cytokinins, hydroxymethyl-furfural, and some minor compounds present in tissue culture media, but not phenolics. It is now well established that an auxin of some kind is needed to induce embryogenesis in wheat, and it has even been reported that there will be no embryo formation if 2,4-D is absent from the medium for the first 12 days after the start of culture (Henry and de Buyser 1981). Since AC-DL increased embryogenic ability in this study, AC may not remove auxins as fast as assumed, or 2,4-D may be needed only transiently in ryegrass.

Cold pretreatment

From 52,752 anthers plated in experiment 3; 46,173 ELS, 5,466 albino and 401 green plants were generated, giving an average of 87.5 ELS, 10.4 albino and 0.8 green plants per 100 anthers. There were significant differences between genotypes for all characters tested (Table 6). Genotypes 1-5 and 5-5, previously classified as nonembryogenic produced ELS and regenerated albino

plants after cold pretreatment. Cold pretreatment only had significant effects in 1991 for the characters embryogenic ability (ELS/100 ANT) and albino plant production (ALB/100 ANT, % ALB/PL). There was no significant effect of the duration of cold pretreatment.

These results apparently contradict those reported by Bante et al. (1990b), who reported two weeks cold pretreatment to be best for androgenetic response. However, these authors did not analyse their results statistically, and only obtained ELS without any regeneration of plants. Their conclusions may therefore have little relevance in predicting green plant formation. It should be mentioned that a one week cold pretreatment, which was tested by Bante et al. (1990b), might have given better results than the two or three weeks used in the present investigation. Tsay et al. (1988) reported increased embryogenesis and albino plant generation from rice anthers, cold pretreated at 10°C for two and three weeks, but with the highest number of green plants after one week of treatment.

Table 6. Mean squares from the analysis of variance of cold pretreatment.

		Characters					
Source	df	ELS/ 100ANT	ALB/ 100ANT	GRP/ 100ANT	PL/ 100ELS	%ALB/ PL	%GRP/ PL
1990:							
Genotype	8	63.4***	8.7***	1.6***	9.2*	1.5	13.1
Coldpr.	1	3.4	0.1	0.0	2.1	0.9	2.8
Error	8	3.9	0.2	0.1	4.0	0.5	1.5
1991:							
Genotype	3	90.4***	22.4***	3.1***	10.6***	0.9***	16.2***
Coldpr.	2	34.2*	7.7**	0.0	0.4	0.1	0.7
Error	6	10.4	1.2	0.2	0.5	0.2**	2.7*

*, **, ***; significant at the 5, 1 and 0.1% level, respectively.

Conclusions

Three media commonly used, pII, 190-2 and LS-3, appear to be equal in their ability to produce green plants. However, pII should be avoided in order to reduce uncontrollable variation connected with the use of potato extract. LS-3 may be less tolerant to changes in chemical composition, since AC-DL had a more positive effect on embryogenesis and less negative effect on regeneration of green plants in 190-2 than in LS-3. Further studies should be conducted in order to compose a better induction medium which may improve the androgenetic response in perennial ryegrass. Medium 190-2 may be the best basis for further investigations on media improvements. Cold pretreatment only increased number of ELS and albino plants regenerated, and can therefore not be considered beneficial for use in anther culture of perennial ryegrass.

Several grass breeding companies are using anther culture at the moment, and it seems likely that synthetic varieties or F_1-hybrids based on DH may be released in the forseeable future. We have tested DH in field trials, and although inbreeding depression is severe, as expected, it will be possible to establish fertile, highly inbred lines from DH of *Lolium perenne*. However, in order to use anther culture on a large scale, it is necessary to breed for green plant response, by crossing highly responsive genotypes into promising breeding populations (Opsahl-Ferstad et al. 1994). Anther culture is nowadays used quite extensively in breeding programmes of several species within the *Poaceae*, and expecially in allogamous grass species it opens new possibilities both for genetic studies and plant breeding. Application of molecular markers may make anther culture more useful, and used in conjunction, these techniques are powerful in genetic manipulation of allogamous grasses.

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Erratum

Due to an unfortunate error in volume 13, issue 7, 1994, pp. 394–396, the dates of submission and acceptance and the name of the Communicating Editor of the article "Isolation and transformation of rice aleurone protoplasts" by S. Sadasivam and D. R. Gallie were omitted. The correct information is given below:

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Isolation and transformation of rice aleurone protoplasts

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