Optimizing anther culture for barley breeding

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Barley anther culture methods were optimized for the production of doubled haploid lines from Finnish spring barley (*Hordeum vulgare*) breeding material. 22 F_1 -progenies of two-rowed barley cultivars ('Bonus', 'Inari', Jo1610, 'Kustaa', 'Kymppi', 'Prisma') and six-rowed barley cultivars ('Arve', 'Botnia', 'Larker Mutant', OB264, 'Rolfi', WW7860) were used for the experiments. The effect of basic induction media, pretreatment on mannitol medium, density of anthers, incubation temperature and light regime were tested. Pretreatment of anthers for 4 days on medium containing 0.175 M mannitol was beneficial for all 8 genotypes tested and increased production of green plants per 100 anthers from 26% to 74% for the best genotype ('Inari' x 'Kymppi' F_1). A lower anther density (1.6 anthers per cm^2) was better than a more dense one. A modified MS-medium with ammonium nitrate partly replaced with glutamine (MMS-MG) was slightly better than a medium based on N_6 salts (N_6-MG), and addition of 100 µM silver nitrate reduced both plant and green plant production. No significant differences were observed between the effects of incubation temperatures (20°C vs. 25°C) or the light regime (darkness vs. weak light) during incubation of anthers. In each experiment the genotypic effect was prominent and the recalcitrance of some genotypes was apparent. Green plants were produced however from all genotypes.

Key words: doubled haploid, *Hordeum vulgare*, lighting, mannitol, pretreatment, temperature

Introduction

Breeding new barley varieties is based on creating new gene combinations by controlled crosses and subsequent testing and selection during the selfing generations. This takes 12–15 years using conventional methods. The early generations following crossing are highly heterozygous, making reliable selection difficult until an acceptable level of homozygosity is reached. In heterozygous plants recessive genes are not expressed in the phenotype and heterosis may influence performance. Such effects are lost during the later generations. To secure stable and homogeneous new barley varieties single head selections must be made during later generations when adequate homozygosity has been reached,

and subsequent multiplication of seed on a commercial scale requires several years.

A short cut to homozygosity can be achieved by producing doubled haploid lines from the microspores of the early, segregating generations (F_1–F_3). Success in production of microspore-derived barley plants through anther culture was first reported 25 years ago by Clapham (1973). Since then barley anther culture methods have been developed into a practical breeding method by modifying the donor plant growth conditions (Foroughi-Wehr and Mix 1979), by using a cold pretreatment of tillers or spikes (Powell 1988b, Huang and Sunderland 1982), or mannitol pretreatment of anthers (Roberts-Oehlschlager and Dunwell 1990), and by replacing ammonium nitrate with glutamine in the induction medium (Olsen 1987). A major breakthrough was achieved when alternative sugars, including maltose, were used in induction media inplace of sucrose (Hunter 1987, Sorvari and Schieder 1987, Finnie et al. 1989). Much of the work with barley anther culture was done with one highly responsive model cultivar, Igri. Genotypic differences in anther culture capacity complicate its practical use in breeding. To be useful for breeders, the anther culture method should be easy to use, efficient and applicable to all breeding lines. In addition, the anther culture methods used should not invoke any unconscious selection of the variation existing within the barley material.

The aim of this study was to optimize the anther culture method to be able to produce an adequate number of lines from crossing combinations used in the Finnish spring barley breeding programme. In addition, several methods for statistical analysis of data were compared.

Material and methods

22 F_1 -progenies of two-rowed barley cultivars ('Bonus', 'Inari', Jo1610, 'Kustaa', 'Kymppi', 'Prisma') and six-rowed barley cultivars ('Arve',

'Botnia', 'Larker Mutant', OB264, 'Rolfi', WW7860) from the Finnish barley breeding programme were used for the experiments. Different genotypes were used in each of the experiments. Plants were grown in the greenhouse in pots with peat soil mix and fertilized regularly (11% N, 4% P, 25% K). Natural light was supplemented with high pressure sodium lamps (Sylvania SHP-T-400) for 16 h per day and the day/night temperatures were approximately 18°C/12°C. The first lot of plants was planted in August and the second in December. Plants from both lots were used in each experiment.

Donor spikes were collected when the distance between the flag leaf ligule and the penultimate leaf was 3–7 cm and the microspores were at the mid- or late uninucleate stage. If not otherwise stated, freshly collected spikes were used for anther isolation. Usually MMS-MG medium was used for induction. This was modified MS-medium (Murashige and Skoog 1962) with ammonium nitrate partly replaced with glutamine as proposed by Olsen (1987). All induction media contained 175 mM maltose as their carbohydrate source, 4,4 µM 6-benzylaminopurine and 5,7 µM indolate-3-asetic acid, and were solidified with 2 g/l Gelrite. All media were sterilizer by autoclaving 20 min. at 120°C. If otherwise stated, the dishes were first kept in darkness for four weeks and then in light, the temperature being +25°C.

Induction media

Anthers from the F_1 generation of two six-rowed and four two-rowed barley crosses were used. Two different basic media were used: MMS-MG and N_6-MG. The latter contained the macro- and micro-salts and vitamins of the N_6 medium (Chu et al. 1975) inplace of those of the MS- medium. The third medium tested was MMS-MG supplemented with 100 mM silver nitrate. Anthers from each spike were distributed to all three media, 50 anthers per Petri dish (5 cm Ø). A total of 700 anthers per genotype x medium combination were used.

Light and temperature conditions during embryoid induction

Anthers from the F_1 generation of four six-rowed and four two-rowed barley crosses were used. Anthers from several spikes were randomly distributed to four 5 cm diameter Petri dishes, 50 anthers per dish, containing the MMS-MG medium. One of the four dishes was incubated in darkness at +20°C, the second in darkness at +25°C, the third in dim light at +20°C, and the fourth in dim light at +25°C. A total of 600 anthers per genotype x induction treatment combination were used.

Pretreatment

Anthers from the F_1 generation of four six-rowed and four two-rowed barley crosses were used. Anthers from several spikes were randomly distributed to four treatments: 1) MMS-MG medium, 5 cm dishes, 2) MMS-MG medium, 9 cm dishes 3) MMS-MAN medium, 5 cm dishes, 4) MMS-MAN medium, 9 cm dishes. 100 anthers were isolated per plate. MMS-MAN was the same medium as MMS-MG except that maltose was replaced by 175 mM mannitol. Anthers from the mannitol dishes were transferred to normal MMS-MG medium after four days pretreatment. A total of 600 anthers per genotype x pretreatment combination were used.

The number of green and albino plantlets induced during 8 weeks after anther isolation were counted in each experiment. The efficiency of three transformations to homogenize variances was tested with one of the data sets and variance analysis results were compared between different transformations. All datasets were analysed by variance analysis (ANOVA) using the arcsin√ transformed numbers of plants per 100 cultured anthers (PPA), green plants per 100 cultured anthers (GPA) and proportion of green plants (PGP). When needed, normalized indices were produced by dividing all data by the maximum value in the experiment. The model included the main effects of genotype, treatment and season plus all the two-way interactions. The data from all experiments were analysed as completely randomised designs. Pairwise comparisons between significantly different treatment means according to the results of ANOVA, were done with Tukey's test. Results of parametric tests were compared with those of the non-parametric Friedmans test in one of the experiments. All statistical analyses were run using SPSS®, version 6.1.

Results

Effect of induction media

The per plate values for plant production ranged from zero to 172.0% and for green plant production from zero to 126.0%. All the main effects for PPA and GPA were statistically significant (P<0.05), but the propotion of green plants was effected only by genotype and season, not the treatment used. No statistically significant interactions were detected. The MMS-MG medium was the best induction medium for most genotypes (Fig. 1). The addition of silver nitrate had no positive effect on any of the variables measured, but hindered the formation of green plants. Large genotypic differences were seen in anther culture response and the treatments used could not overcome the recalcitrance of some genotypes (Fig. 1.). The second sowing time (December) was significantly better for anther culture response than the first (August).

Effect of light and temperature conditions during embryoid induction

The plants per 100 anthers values ranged from zero to 106.0% and the green plants per 100 anthers values from zero to 46.0%. A significant interaction for genotype x treatment was detected for GPA (P=0.032). The genotypic effect was significant for PPA, GPA and proportion of green plants when tested against genotype-treatment

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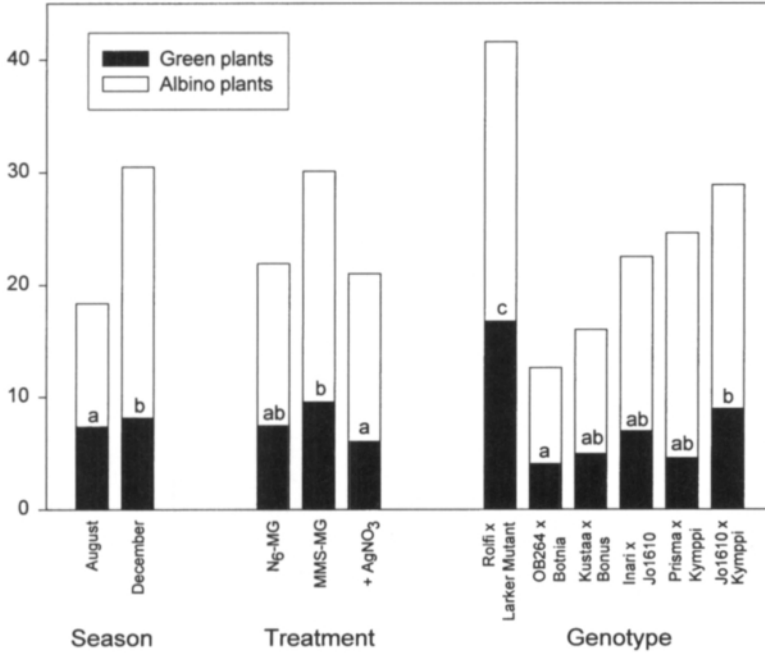
Plants per 100 anthers

Fig. 1. Effect of induction media, season and genotype. Black bars represent the production of green plants per 100 anthers, bars with different letters are significantly different (P<0.05) from others within the same main effect.
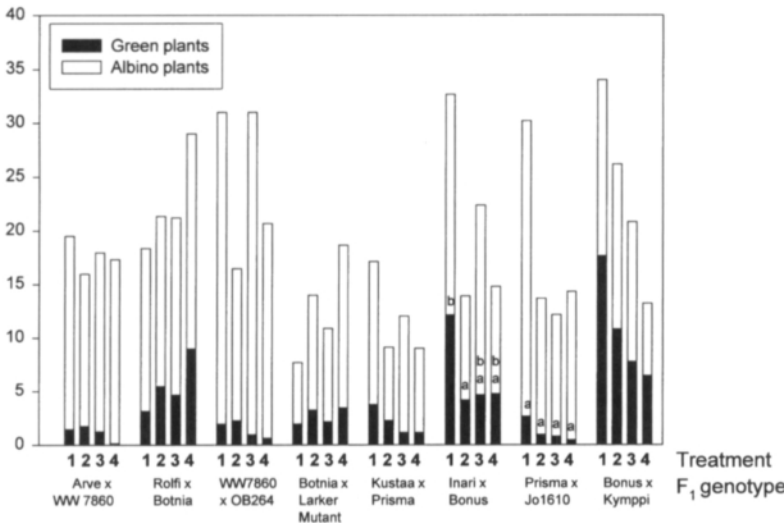
Plants per 100 anthers

Fig. 2. Effect of light and temperature conditions during embryoid induction. Treatment 1) 20°C, weak light, 2) 20°C, darkness, 3) 25°C, weak light, 4) 25°C, darkness. Results from pairwise comparisons of treatments are shown for only those genotypes, were one way ANOVA showed significant differences.Treatments with different letter are significantly different (P<0.05) for their green plant production.

Table 1. Mean squares and P-values for main effects and two-way interactions in pretreatment experiment.

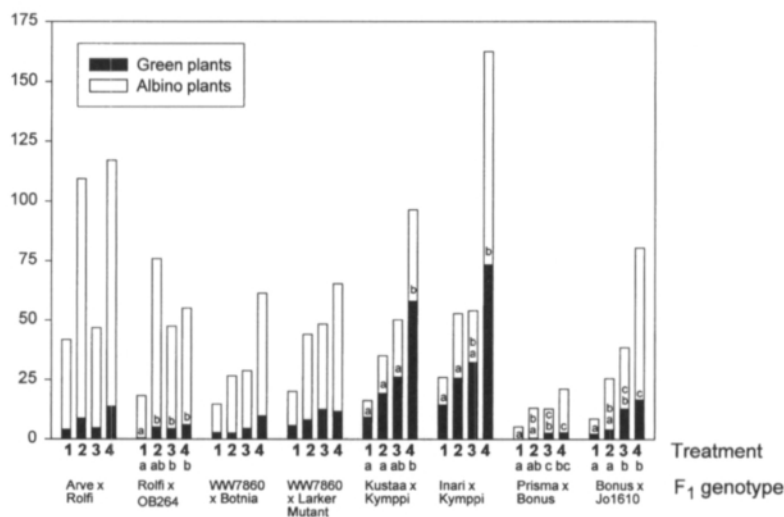|	df	Plants per 100 anthers		Green plants per 100 anthers		Proportion of green plants	
		MS	P	MS	P	MS	P
Genotype (G)	7	0.260	0.001[a]	0.426	<0.001[a]	1.286	<0.001[a]
Treatment (T)	3	0.785	<0.001[a]	0.387	<0.001[a]	0.061	0.407[a]
Season (S)	1	0.158	0.01	0.140	0.003	0.027	0.323
G x T	21	0.048	0.006	0.035	0.004	0.060	0.004
G x S	7	0.012	0.816	0.030	0.075	0.022	0.588
T x S	3	0.038	0.187	0.003	0.889	0.013	0.693
Residual	139	0.023	0.016	0.028			

[a] Tested against G x T interaction term.

interaction (P-values 0.007, <0.001 and <0.001 respectively), but the treatment effect was not (P-values 0.095, 0.091 and 0.057). Values of PPA and GPA for each genotype x treatment combination are shown in Fig. 2. Treatment 1 (induction in dim light, 20°C) had a positive effect on green plant production for a few crossing combinations. Several genotypes remained recalcitrant after the treatments used. The second sowing time (December) was significantly better for anther culture response than the first one (Au-

gust). The effect of three transformations to normalize the distribution of residuals for GPA was compared graphically. The arcsin√-transformation was the most effective. None of the transformations equalized variances satisfactorily. When ANOVA was performed on all transformed data as well as on untransformed data, no substantial differences were observed. Even though the P-values varied, this did not alter the conclusions drawn from the analyses.

Fig. 3. Effect of pretreatment and plating density. Treatment 1) no pretreatment, 5 cm dish, 2) no pretreatment, 9 cm dish, 3) mannitol pretreatment, 5 cm dish, 4) mannitol pretreatment, 9 cm dish. Results from pairwise comparisons of treatments are shown for only those genotypes, where one way anova / Friedmans test showed significant differences. Treatments with different letter are significantly different (P<0.05) for their green plant production. Results from the parametric tests are shown above the black bars and those of non-parametric ones below the treatment numbers.

Plants per 100 anthers

Effect of pretreatment

Plants per 100 anthers values ranged from zero to 279.0% and green plants per 100 anthers from zero to 151.0%. All main effects and the genotype x treatment interaction had a statistically significant effect on PPA and GPA (Table 1). accounted for 43% and 22% respectively. In this experiment only genotype and G x T interaction had a statistically significant effect on the proportion of green plants. The mean values of PPA and GPA for each genotype x treatment combination are shown in Fig. 3. The mannitol pretreatment promoted PPA and GPA in several genotypes, especially when the lower anther density (1.6 anthers per cm^2) was used. The second sowing time (December) was significantly better for anther culture response than the first one (August).

The results from one-way ANOVA and nonparametric Friedmans test for GPA are shown in Fig. 3. Friedmans test appeared more conservative and did not eshtablish significant differences between treatments in the cross 'Inari' x 'Kymppi' when ANOVA did. Some differences were also found in pairwise comparisons.

Discussion

Genotypic effects

In all experiments the genotype effect was highly significant for all characters measured and accounted for 8–21% of the variation in plant production per 100 anthers, 17–40% in green plant production per 100 anthers and 8–62% in proportion of green plants. The genotype effect on anther culture response is widely reported for various plant species. In barley, Knudsen et al. (1989) tested the anther culture response of 17 varieties and reported that the component of variance from genotypes accounted for 60% of the total variation in embryo formation. In their barley material PPA ranged from 1 to 47% and the

GPA from zero to 40%. In our experiments green plants could be produced from each of the 22 crossing combinations tested and the best genotypes on the best treatments gave plants per 100 anthers values as high as 176% and green plants per 100 anthers values of 74%, although for many crossing combinations the green plant production remained low (5–10%). The capacity for green plant production did not depend on row type in any of our experiments and it also appears to be independent of the spring/winter growth habit (Larsen et al. 1991). The genetic background of anther culture response is complex and environment often affects gene action. Both additive and dominance effects for embryoid formation, total plant production and green plant production have been observed (Hou et al. 1994). Powell (1988a) reported a significant reciprocal effect in anther culture response in a diallel study, which indicated that the direction of the cross may be important for the development of microspore-derived plants. Reciprocal effects may indicate either cytoplasmic or maternal effects on anther culture response. Large genotypic differences hinder effective use of anther culture for breeding purposes. Hou et al. (1994) reported that most of the F_1's studied were intermediate in response between the parents even though transgressive segregation was also observed for some combinations. In practical breeding it is worthwhile testing the anther culture response of the parents of the crosses used in doubled haploid production to allocate resources most effectively. Improvement in anther culture response by crossing and selection is possible, but from the barley breeders' point of view this may be of marginal interest.

Treatment effects

The most commonly used pretreatment in barley anther culture is placing tillers or spikes at +4°C for 14 to 28 days (Huang and Sunderland 1982, Powell 1988b). This has been shown to been beneficial for several barley genotypes, but not invariably (Szarejko and Kasha 1991). In our earlier studies we consistently obtained a lower

response in anther culture of cold pretreated tillers or spikes compared with anthers isolated from fresh tillers (data unpublished). In the present study all anthers were isolated from fresh tillers. The mannitol pretreatment for four days was beneficial for most genotypes studied. The genotype x treatment interactions detected were not crossover interactions, which means that mannitol did not suppress anther culture response in any genotype. In practical use of anther culture it is impossible to optimize methods for each genotype, and the methods used should be applicable to a wide range of genotypes. The beneficial effect of mannitol pretreatment in barley anther culture was first detected on cv. 'Igri' (Roberts-Oehlschlager and Dunwell 1990), which is a widely used model genotype for barley anther culture. Mannitol pretreatment has been considered as a starvation treatment (Hoekstra et al. 1992), but in barley mannitol treatment induces enrichment of glucose into anthers, which may promote the early stages of development (Roberts-Oehlschlager et al. 1990). Mannitol pretreatment has also been found to be beneficial in barley microspore culture (Kasha et al. 1992, Hoekstra et al. 1996). However, Hou et al. (1993) reported that 28 days cold pretreatment of spikes was more efficient than 3 days pretreatment on 0.3 M mannitol.

Anther density can affect green plant production, especially when numerous embryoids are formed. In our experiment, 1.6 anthers per cm^2 was better 5.1 anthers per cm^2. Xu and Sunderland (1982) found that the optimal anther density for callus formation was as high as 8.5–17 anthers per cm^2. Subsequently barley anther culture methods have undergone several developments and in accordance with our results 1–1.5 anthers per cm^2 was found to be the optimal density for mannitol pretreated anthers (Roberts-Oehlschlager and Dunwell 1990).

N_6-MG and MMS-MG differ in their macro- and microsalt contents. In MMS ammonium nitrate is partly replaced by an organic nitrogen source, glutamine. No statistically significant differences were detected between these two media, although MMS-MG was slightly better for most genotypes. The addition of silver nitrate to MMS-medium hindered the formation of green plantlets in anther culture. Silver nitrate is an inhibitor of ethylene production and Purnhauser et al. (1987) were able to enhance shoot formation in wheat callus culture using silver nitrate. Cho and Kasha (1989) studied ethylene production and the effect of ethylene inhibitors and promotors in barley anther culture and concluded that an optimum level of ethylene may be required for embryogenesis. Genotypes may differ in their production of ethylene in anther culture. Evans and Batty (1994) found that silver nitrate hindered green plant production in anther culture of barley but positive effects were achieved with another ethylene inhibitor, silver thiosulphate. It may be that silver nitrate is not appropriate for barley anther culture or that the genotypes used in our experiment were of the low ethylene production type.

Incubation of anthers in weak light, together with the lower incubation temperature (+20°C), was beneficial for a few genotypes. It has been found that green plant production increases in wheat anther culture by incubating isolated anthers in weak, diffuse light (Bjørnstad et al. 1989).

Season

Season had a small, but significant effect on green plant production, December being better for sowing the donor plants in all experiments. Even though a controlled greenhouse environment was used for growing the donor plant material, the amount of natural light differred between seasons. Kuhlman and Foroughi-Wehr (1989) detected large seasonal variation in anther culture response of 'Igri', and speculated that temperatures occasionally exeeding 25°C during summer would make the donor plants less suitable for anther culture. In the practical use of anther culture for breeding, the yearly cycle of crossing and field testing will determine the suitable season for anther culture. In our conditions anthers are mainly isolated during October which leaves enough time for the doubled haploid plants to set seed before sowing in the spring.

Statistical analysis of anther culture data

Statistical analysis of anther culture data is often problematic since data may not be normally distributed and the variances are often heterogeneous. Data often contain many zeros and due to occasional contamination may be unbalanced. One way to avoid problems with non-normality and heterogeneous variances is to use transformations. From the transformations tested with our data, the arcsin√ was the most efficient one. Despite the fact that untransformed data did not fulfil the expectations needed when using parametric tests, results from ANOVA did not differ markedly from those of transformed data, which may be a sign of the robustness of variance analysis. Instead of using transformations, non-parametric tests, which do not require a normal distribution can be used. Unfortunately, non-parametric equivalents to variance analysis with more complicated models seldom exist in commercial computer packages. We compared the results from Friedmans test with those from the one-way analysis of variance. Some differences existed among the pairwise comparisons and the Friedmans test seemed to be little more conservative than ANOVA. When analysing results from a tissue culture experiment, one should keep in mind that it is often more important to establish the existence of differences large enough to be of practical importance rather than to reveal small statistically significant, and in practice meaningless, differences.

Conclusions

The best method indicated by the results of our experiments is isolation of fresh anthers on the mannitol pretreatment medium. Transfer of anthers after 4 days pretreatment to MMS-MG medium can be aided with a polyester net, which has been placed on the MMS-MAN medium before isolation of anthers. Induction of embryoids and germination of embryoids into plantlets occur on the same medium, and there is no requirement to transfer the embryoids to a new medium. The present method is efficient enough for most barley breeding lines and an adequate number of doubled haploid lines can be produced for the Finnish barley breeding programs.

A practical method for production of doubled haploids provides the breeder with a possibility to produce homozygous lines from crossing combinations within a year. Selection is more efficient when done on homozygous and homogeneous lines. While using anther culture, one should be aware of the possibility of distorted segregation in the doubled haploid lines since segregation distortion has been detected in several crosses with molecular markers (Graner et al. 1991, Heun et al. 1991).

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SELOSTUS

Ponsiviljelymenetelmän optimointi ohranjalostuksen käyttöön

Outi Manninen

Maatalouden tutkimuskeskus

Ohran ponsiviljelymenetelmää optimoitiin kaksoishaploidien ohralinjojen tuottamiseksi suomalaisesta ohran jalostusmateriaalista. Emokasveina käytettiin ohralinjojen ja -lajikkeiden ('Arve', 'Bonus', 'Botnia', 'Inari', Jo1610, 'Kustaa', 'Kymppi', 'Larker Mutant', OB264, 'Prisma', 'Rolfi', WW7860) välisten risteytysten F_1-sukupolvea. Tutkimuksessa selvitettiin induktioalustan, mannitoli-esikäsittelyn, ponsien maljaustiheyden sekä inkubaatiolämpötilan ja -valaistuksen vaikutusta ponsiviljelyvasteeseen. Ponsien esikäsittely 0,175 M mannitolialustalla osoittautui hyväksi useilla genotyypeillä, parhaalla genotyypillä ('Inari' x 'Kymppi') vihreiden taimien tuotto 100 pontta kohden parani 26 %:sta 74 %:iin. Pienempi ponsitiheys maljalla (1,6 pontta/cm²) tuotti enemmän vihreitä taimia kuin suurempi (5,1 pontta/cm²). MMS-MG induktioalusta oli jonkin verran N_6-MG alustaa parempi, kun taas 100 µM hopeanitraatin lisäys heikensi taimien tuottoa. Inkubaatiolämpötila ja -valaistus eivät vaikuttaneet ponsiviljelyvasteeseen. Sen sijaan kaikissa kokeissa genotyyppi vaikutti voimakkaasti ponsiviljelyvasteeseen. Kaikista tutkituista genotyypeistä kyettiin tuottamaan vihreitä kasveja.