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Variation for improved protein and yield from rice anther culture*

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Summary. The anther culture technique is useful for the recovery of haploids which when doubled provide homozygosity. Additionally, beneficial as well as deleterious genetic and epigenetic changes are promoted by the in vitro procedures. The majority of plants recovered from anther calli of the cultivar 'Calrose 76' were similar to the starting cultivar but plants regenerated from microspore calli had a wider range of responses than controls for several characteristics. Plants with larger seeds, higher levels of seed protein, shorter stature and more highly tillered than the starting cultivar were obtained from selfed anther-derived plants. The data also support the concept that in vitro procedures including anther culture of specific cultivars of rice promoted the recovery of phenotypes with increased seed storage proteins.

Key words: Androgenesis – Anther culture – Genetic stability – Germplasm – Haploids – Homozygosity – Protein – Rice, *Oryza sativa* – Tissue culture – Variation

Introduction

The anther culture of rice, *Oryza sativa* L. (Guha-Mukherjee 1973; Oono 1975; Niizeki and Oono 1971; Schaeffer 1982), has progressed rapidly in recent years. The procedures are sufficiently advanced for the application of the techniques to plant breeding (Suenoga et al. 1982). Even though hundreds of doubled haploid plants may be recovered for selection and breeding in single experiments, there is only preliminary documentation based on rigorous statistical evaluations for the recovery of doubled haploids of rice with superior performance characteristics over the parental lines. Both useful and deleterious phenotypes may be recovered by in vitro techniques (Larkin and Scowcroft 1981; Schaeffer 1982; Oono 1975, 1978; Niizeki and Oono 1971; Suenoga et al. 1982). Our work has produced genotypes for increased seed storage proteins and improved levels of protein lysine (Schaeffer 1981; Schaeffer and Sharpe 1981, 1983). Both of these parametes are useful in germplasm and are highly beneficial when yield is maintained. However, frequently, variation recovered in vitro impacts negatively upon yield.

In anther culture as in mutation breeding it is not difficult to generate variants and most will be deleterious for yield by virtue of some metabolic imbalance which may be expressed as reduced vigor or infertility. The work with tobacco anthers produced lines of anther-derived doubled haploids that were inferior in leaf yield and other characteristics to selfed diploids or conventionally derived parents. The reduced vigor was expressed in F_1's (Brown 1982) and F_2's in flue cured tobacco (Arcia et al. 1978; Burk and Matzinger 1976). On the other hand Deaton et al. (1982) found no reduction in vigor with burley tobacco. In cereal grains particularly rice, the seed yields are frequently less from anther-derived doubled haploids than in analogous controls without in vitro histories. Even though deleterious effects do occur they are not caused, in many instances, by haploidy or the homozygosity generated by doubling the haploids but are probably caused by the *in vitro* conditions prescribed for callus growth and plant regeneration.

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Yield is a multigenic character with highly interdependent metabolic relationships. The disturbance of any one component may easily decrease the yield. However, individual components of yield may be recovered in vitro and doubled haploid plants with improved components may then be backcrossed to the original cultivar to restore characteristics inherent in the original cultivar including full fertility. Anther culture, with or without selection pressure, cannot by definition select for broad agronomic characteristics but the *in vitro* protocol can be utilized to modify and recover specific components of quantitatively inherited characteristics.

Recently Schaeffer and Sharpe (1981) recovered cell lines of rice resistant to the analog of lysine, S-aminoethyl cysteine (S-AEC). Most plants were partially sterile due to the past in vitro histories. Thus, dihaploids may have reduced seed number, caused by infertility induced simply by passage through the in vitro procedures. For example, control plants with similar in vitro histories but without exposure to S-AEC had reduced yields or expressed unexpected infertility. However, plants recovered from calli resistant to S-AEC had improved levels of protein and lysine within the protein. This was true for plants with full seed set (Schaeffer and Sharpe 1983). Unexpectedly the tissue culture control was intermediate between the field control, which had no in vitro history and the in vitro S-AEC resistant line in protein content. This led to the hypothesis that the in vitro procedures, either anther culture or tissue culture, promoted the recovery of plants with elevated levels of seed storage protein. While the in vitro conditions may predispose cells toward the capacity for increased protein synthesis the full expression required the selection pressure from the analog and the full increase in protein content did not occur until the third or fourth generation out of tissue/anther culture (Schaeffer and Sharpe 1983).

The purpose of this work was to further test the hypothesis that the anther culture and the in vitro cycle provide techniques for the recovery of rice lines with beneficial characteristics including higher levels of seed storage proteins. Experiments were designed to characterize the variants and apply statistical estimates of significance to deviations from the controls. Additionally the study provides quantitative comparisons of anther-derived dihaploids with conventionally selfed diploids of 'Calrose 76' a commercial cultivar currently used in rice production.

Materials and methods

The data presented in this report was from the rice, (*Oryza sativa* L.), cultivar 'Calrose 76' developed by Rutger et al. (1977). The anther culture techniques, the greenhouse growth conditions, the colchicine doubling technique and the description of the genotype for enhanced short stature (dwarf) of this cultivar were described earlier (Schaeffer 1982). The following protocol is abstracted for the convenience of the reader:

The procedures included the culture of anthers of 'Calrose 76' in Blaydes (1966) inorganic salts, yeast extract, coconut milk, 2,4-dichlorophenoxy acetic acid (2,4-D) and 3% sucrose as prescribed by Guha-Mukherjee (1973). Anthers in the intact panicle surrounded by the leaf sheath were given a temperature shock at 4°–7 °C for 7 days. They were then excised and cultured on 0.8% agar for 35–45 days. Developing calli were lifted from the anther and placed on a tissue increase medium, and then transferred to a plant regeneration medium. Regenerated plants, following a 3–5 day conditioning period in high humidity were placed into the greenhouse for seed collection from spontaneously doubled dihaploids.

Seeds for progeny analyses were germinated in jiffy mix in 7.6 cm pots. Plants were transplanted into 25-cm plastic pots approximately 14 days after germination into a soil/clay mixture consisting of two parts compost soil and one part clay. Plants were maintained in the greenhouse under flooded conditions for the remainder of the life cycle. During seasons in which the day length was less than 12 h, daylight was supplemented with approximately 20–30 μE of cool white fluorescent plus incandescent light for 5 h starting at 1,600 h. Individual experiments were maintained on single or adjoining benches in a completely random design in which each plant within a treatment was considered a replication.

Protein levels were calculated from percent total nitrogen determined by standard Kjeldahl procedures. Thirty seeds were dehulled, ground in a mortar and pestle and dried to constant moisture. One hundred fifty mg samples were used for the digestion. Kjeldahl nitrogen, expressed as percent of sample weight was multiplied by 5.95 to determine percent protein (Jones 1931).

R_0 is the designation for the original plant regenerated from callus cultured on a regeneration medium. S_1 designates the seed grown from the original plants and is first generation material. S_2 represents the seed from plants grown from S_1 seed. SD designates plant types spontaneously doubled during anther culture procedures, whereas the CD designation is for plants derived from haploids treated with colchicine.

The experimental material utilized are designated as follows:

a) ANC 2-1 represents data from 26 different second generation (S_2) lines originally regenerated from anther-derived callus. The 26 lines represent random variation among plants regenerated from three different in vitro experiments.

b) SD-S_3-1 through 15 (AC group); the materials utilized in the AC experiments were selected for seed protein content and are independent and different from AnC 2-1 unselected materials; S_3 designates third generation plant and seed data from 4 anther-derived sublines and one control. Data reported in Table 2.

c) SD-S_3-1 and SD-S_3-14 designate third generation plant and seed selected for short stature and normal yield from AC group above.

d) SD-S_4-7, SD-S_4-11 and SD-S_4-14; represent fourth generation plants and seed from three different SD-S_3 plants, namely -7, -11, and -14 from AC group above. Plants were selected for short stature, normal yield and protein content. The three types are designated in Table 3 as AC-7, AC-11 and AC-14 for the three numbers, respectively.

e) CD-S_4-28-3. Fourth generation plant and seed from anther-derived colchicine doubled dihaploids. Original plant was selected for dwarf character and normal seed production. It is abbreviated in Table 3 as CD-28-3.

f) Controls. All controls were from bulk field grown seed of the cultivar, 'Calrose 76', grown in the greenhouse under the same cycles as the experimental anther-derived lines. The controls had no in vitro history.

Results

Dwarf phenotype

The data from progeny of anther plants of 'Calrose 76' illustrate the recovery of both useful and deleterious characteristics from the in vitro procedures. The most prominent result from anther culture is the recovery of the dwarf phenotype reported earlier and further characterized here (Tables 1–3). Table 1 shows that 76% of the plants recovered were significantly shorter than the control. However, the response is irregular.

In two experiments not detailed here 38 and 30% of the plants recovered were dwarf. There is substantial variation between experiments on rate of calli production and on the type of variation recovered. Most anthers produce multiple calli but whether the calli come from single microspores cannot be easily determined except by anther culturing hybrids with two distinct markers. On some occasions all plants regenerated from a single anther had similar characteristics, e.g., all were putative tetraploids. Similarly the propensity to differentiate may be strong in a majority of calli from single anthers but calli from other anthers will not regenerate at all. Thus the condition of individual anthers and the condition of the plants from which the anthers are excised may influence the nature and vigor of the variants recovered. The enhanced dwarf character probably represents a change in the expression of a short stature gene (Schaeffer 1982) for which 'Calrose 76' was developed (Rutger et al. 1977). The expression of the dwarf gene reflected in reduced spike height is complete in lines selfed three (Table 2) and four times (Table 3).

The reduction in height may be 26% (Table 3) or up to 33% reported earlier (Schaeffer 1982). Crosses have been made to define the nature of the dwarf gene. Its genetic nature will be described in a later communication.

Table 1. Comparisons between randomly recovered second generation anther-derived rice plants and the original cultivar, 'Calrose 76'. Data from 25 doubled haploid lines were statistically analyzed and evaluated with the Duncan's Multiple Range test at $P=0.05$. Doubled haploid lines and the controls were replicated 7 times in a completely randomized design. Each plant per line represents one replication

Parameters	No. of lines equal to or significantly different from the control mean at $P=0.05$.		
	Higher than	Equal to	Lower than
Seeds/plant, no.	0	23	2
Seed wt, mg	4	20	1
Yield, gm/plant	0	24	1
Seed protein, %	2	21	2
Protein, mg/seed	3	20	2
Total seed protein, gm/plant	0	22	3
Tillers, no. plant	11	14	0
Spike height, cm	0	6	19
Panicle length, cm	1	19	5
Days to flower	1	18	6

Table 2. Range of plant responses and 15 plant mean of 'Calrose 76' (controls) and 14 plant mean of third generation plants derived from anther culture (AC). AC-1 (SD-S_3-1) and AC-14 (SD-S_3-14) represent single plant responses from the AC group

Plant characteristics units	Rice lines							
	Range of responses in populations						Single plant responses from AC group	
	Control			AC group				
	Lowest	Highest	Mean	Lowest	Highest	Mean	AC-14	AC-1
Seed/plant, no.	564	854	747	627	941	774	745	745
Seed wt, mg	21.4	23.7	22.8	20.3	22.3	21.6	20.6	22.3
Yield, gm/plant	12.1	20.2	17.0	12.7	21.0	16.7	15.4	16.6
Seed protein, %	7.3	9.9	8.9	8.1	11.0	10.0	11.0	11.0
Protein, mg/seed	1.56	2.25	2.04	1.73	2.45	2.16	2.27	2.45
Seed protein, total/plant, gm	1.26	1.85	1.53	1.30	1.88	1.66	1.69	1.83
Lysine, total amino acids, %	3.38	3.60	3.45	2.94	3.60	3.25	3.31	3.60
Tillers, no. plant	9	12	10.3	13	18	15.1	14	16
Spike height, cm[a]	75	81	79	45	69	52	56	51
Lysine, mg/seed	0.064	0.079	0.071	0.059	0.088	0.071	0.075	0.088
Total lysine, mg/plant	43	63	54	43	66	54	56	66

[a] Mean of control significantly different from AC-group mean at $P=0.05$

Table 3. Mean plant characteristics of greenhouse grown 'Calrose 76' and several fourth generation anther-derived lines selected from the third generation material for protein level and yield characteristics. Means are from 15 plants of each type grown in a completely randomized design. Seeds for the fourth generation were selected from the population described in Table 2

Characteristics and units	Rice lines and means				
	Control	AC-11	AC-7	AC-14	CD-28-3
Seeds/plant, no.	1782	1703	1560*	1665	1634*
Seed wt, mg	20.7	19.5*	20.6	20.7	21.0
Seed protein, %	8.16	8.36	8.34	8.55**	8.25
Protein, mg/seed	1.69	1.63	1.71	1.77	1.74
Seed protein, total/plant, gm	3.0	2.8	2.7*	3.0	2.9
Spike height, cm	95	68*	72*	70*	71*
Tillers, no./plant	28	35**	34**	32**	33**
Ratio, grain/straw weight[a]	0.31	0.32	0.32	0.34	0.33

* Significantly lower than control at $P=0.05$; ** Significantly higher than control at $P=0.05$
[a] Straw weight not used in statistical analyses

Table 4. Range of plant responses in 15 plants of 'Calrose 76' controls and 15 plants of a fourth generation anther-derived line (AC-14) selected for short stature, and seed protein content compared with a single plant (AC-14-9) response recovered from the AC-14 (SD-S_4-14) population

Characteristics and units	Rice lines		
	Range of responses		Single plant responses from AC-14 group
	Control[a]	AC-14[a]	AC-14-9
Yield, gm/plant	31.4 – 43.8	30.6 – 45.2	45.2
Seed/plant, no.	1414 – 2045	1305 – 1957	1957
Seed wt, mg	19.1 – 22.4	19.8 – 23.1	23.1
Seed protein, %	7.44 – 8.74	7.56 – 9.47	9.47
Protein, mg/seed	1.52 – 1.82	1.52 – 2.19	2.19
Seed protein, total/plant, gm	2.5 – 3.5	2.1 – 4.3	4.3
Time to flower, days	103 – 112	103 – 109	–

[a] See Table 3 for population means

Tiller number

The dwarf phenotype is associated with increased tillering. Forty-two percent of plants recovered from anther culture had significantly higher tiller number (Table 1) which can be greater than 20% over the control (Tables 2 and 3). The high tiller number is most frequently associated with the dwarf phenotype.

Yield and protein levels

Anther culture in rice permits the recovery of germplasm with improvements in two components of yield as well as some quality characteristics. Some lines recovered from in vitro techniques and repeatedly selfed had increased tillers per plant and increased seed weight (Table 1 and Fig. 1). Others had increased protein over the controls (Table 3). Progeny from one anther-derived plant AC-14-9 detailed in Table 4 showed superior performanc in seed size, seed number and protein level.

The genetic nature and the specific biochemical components contributing to increased yield and protein content are unknown at present but the germplasm is derived from a small population of plants (AC-14), with significantly higher protein content and with seed number equal to the control (Table 3). AC-14-9 seems unique because the seed size and percent seed protein are higher in this line than in all plants of the experiment (Table 3) (Fig. 2), and in spite of these characteristics had seed numbers approximately equal to the control.

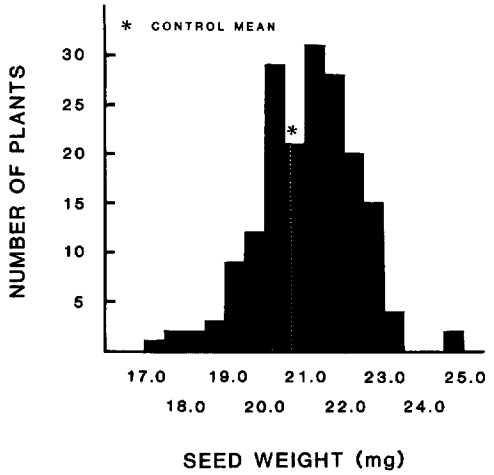

Fig. 1. Distribution of seed weight from an unselected population of 168 second generation doubled haploid plants of 'Calrose 76'. * indicates mean seed weight of the cultivar, 'Calrose 76'

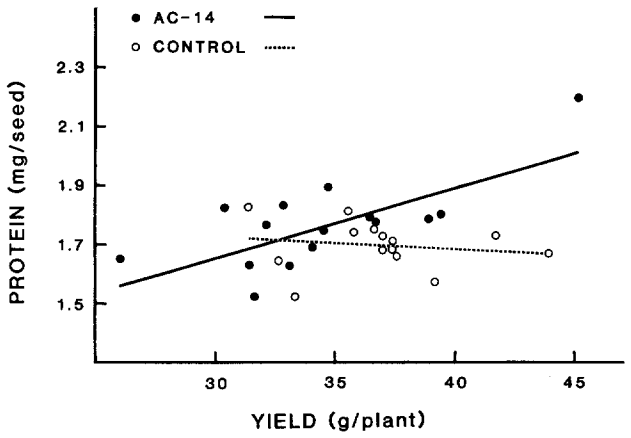

Fig. 2 Relationship between seed protein content and yield of individual plants of fourth generation anther-derived line (*AC-14*) and controls

Yields equal to controls on a statistical basis and higher than controls on single data points from some segregants in the third and fourth generation out of anther culture are reported here (Table 4 and Fig. 2). Clearly beneficial as well as deleterious variants may be recovered from in vitro techniques with commercial cultivars. In wheat 16% of doubled-haploids at S_2 had significantly lower yields than the starting cultivars (Baenziger et al. 1983). In these field grown lines 84% had yields equal to the control. Thus the recovery of the AC-14 line and AC-14-9 plant in rice (Tables 2 and 4) (Fig. 2) may be highly significant and may represent a valuable genotype.

Seed number and seed size

Seed size is a heritable characteristic and improved seed size may be recovered by the in vitro techniques. Figure 1 shows the mean for seed size is less in the controls than the mean of the experimental anther-derived population. The anther-derived population is skewed toward higher seed weights.

Also, in Table 1 note that four lines had significantly higher seed weight and only one had smaller seeds than the controls. There was little difference in seed number. None of the doubled haploids had more seed than the control but two lines had significantly less. The majority of lines had seed yields equal to the controls on a statistical basis. Frequently, the range of responses is wider in doubled haploids or plants regenerated from tissue culture than in similar populations of the controls (Tables 2 and 4). However, some of the lower yielding types were indeterminate in flowering habit and were atypical in overall stature perhaps reflecting some chromosomal abnormalities. Many observations on floret infertility across numerous lines at second, third and fourth generations out of anther/tissue culture suggest that some in vitro derived lines are less tolerant to environmental extremes than the original cultivars. However, cultivars probably differ in their predisposition towards stability or instability.

Even though seed number is frequently inversely proportional to seed size there are exceptions and the second generation anther derived lines (Table 1), and selected lines (Table 4) illustrate the phenotype. The anther culture of rice produces plants with a wider range of responses including the range for seed size (Tables 1 and 4, and Fig. 1). This occurs in some plants with full seed set and is dramatically illustrated in AC-14-9 (Table 4).

Progeny with improved protein

Perhaps the most valuable phenotype recovered from anther culture is the increased protein levels over the starting cultivar in these experiments. Evidence in Tables 1–4, and Fig. 2, show that seed protein is significantly higher in some anther-derived lines than in controls of the cultivar, 'Calrose 76'. This is particularly true for materials several generations removed from the in vitro treatment (Schaeffer 1981; Schaeffer and Sharpe 1983) and data presented here.

Table 1 shows more plants with significantly higher protein/seed than plants with lower values than the control. Table 3 shows significantly higher percent protein in AC-14 than the other lines and Table 4 and Fig. 2 show that one of the components of the AC-14 mean, i.e., AC-14-9, is superior among the samples in the experiment and represents the highest value in the range of responses for yield, seed weight, percent seed

protein, mg protein per seed, and total plant protein among controls as well as the anther-derived plants. AC-14 also had the highest grain/straw ratio (Table 3). Additionally, Fig. 2 illustrates the relationship between protein level and seed number. We consider it significant that the correlation coefficient between mg protein/seed and yield is +0.73 for the AC-14 line and non-existent, −0.15, for the controls. Clearly the increase in seed size and yield do not decrease protein content in this AC-14-9 selection under greenhouse conditions. We do not yet have data on the field performance of this material. The data show, however, that anther culture and in vitro selection will produce progeny with improved protein over 'Calrose 76' based on percent protein in seed in the greenhouse.

Discussion

The short stature genes in wheat and other cereals have been associated in the past with remarkable increases in productivity highlighted in a recent FAO/IAEA (1982) report. Even though the dwarf doubled haploids may be too short for direct commercial use they should be useful germplasm for the transfer of short stature genes into existing cultivars and new crosses.

From the experiments reported here, unpublished observations and the work of others discussed in the introduction plants recovered from anther or tissue cultures of rice sometimes have reduced seed yields. The reduction in yield is not exclusively associated with anther culture or the haploid origin in anther culture but rather with infertility caused by passage through the in vitro regime. Plants regenerated from callus tissue derived from excised embryos also acquired substantial infertility in selfed progeny (unpublished observations). Loss of fertility due to in vitro conditions are minimized during anther culture if developing calli approximately 1 mm^2 are lifted from the anthers and recultured in an increase medium for only 2–3 weeks. Regeneration of plants from vigorous calli will produce stable and fully fertile dihaploid plants. Prolonged time in vitro and unfavorable growth conditions favor the production of plants predisposed towards infertility. Thus calli exposed to intense selection pressure could produce plants with substantial infertility. Recent unpublished experiments show that the backcross of plants with in vitro-derived infertility to the parental cultivars will produce segregants of the fertile and infertile type. Rice lines recovered from cells exposed to the inhibitor, S-AEC, produced lower yields (Schaeffer 1981, 1982; Schaeffer and Sharpe 1983) first generation out of in vitro culture and in subsequent progeny as well. Other plants from anther culture appeared normal after the first generation. In this respect our work is similar to the work of Oono (1975) and Suenoga et al. (1982) and others.

Inasmuch as the original selection with an analog of lysine produced increased levels of protein (Schaeffer and Sharpe 1981) with 'Assam 5', an introduction from an indica subspecies, the response reported here with 'Calrose 76', a cultivar derived from the japonica subspecies, suggests that progress in the selection for improved seed protein may not be due exclusively to unique characteristics of 'Calrose 76' carrying genes for short stature and abundant tillering.

Analyses of progeny for percent lysine in seed storage protein of these 'Calrose 76' derivatives showed no statistical difference between anther-derived lines and the control. This result supports the hypothesis that the biochemical environment during in vitro culture and regeneration from culture may itself provide some selection pressure for greater RNA and protein synthesis, for which the potential is more fully expressed in the presence of the analog or in the presence of the selection pressure. An examination of the DNA coding for ribosomal RNA synthesis might be a fruitful research approach with the high protein lines. However, other cultivars may require greater and more specific selection pressures, such as aminoethylcysteine used in the 'Assam 5' selection. Thus, with two protocols, i.e., anther culture and anther culture followed by analog selection, we have recovered genotypes with the capacity for higher levels of seed storage protein from in vitro procedures in rice under non-field conditions.

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