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APPLICATION OF ANTHER CULTURE TO HIGH VOLUME RICE BREEDING

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Recent advances resulting in increased callus induction and plant regeneration combined with modifications in methods for handling large numbers of anthers/calluses have reduced the problems that formerly restricted the application of anther culture to a high volume rice improvement program. The difficulties of screening/selecting at the level of callus induction, plantlet regeneration, or R_1 plants are discussed. The problems involved in generating and mass screening 20,000 or more R_2 diploid lines are presented.

Although it has been 17 years since Niizeki and Oono (6) demonstrated that diploid rice plants could be regenerated from haploid anthers, the practical application of this technique for varietal improvement has been largely neglected. The exception has been China, where several commercial varieties have been produced via the anther culture method. The slow adoption of anther culture as a rice breeding tool is a result of the many difficulties involved in generating useful diploid plants. The low rate of callus induction from anthers combined with the small percentage of calluses that produce useful plants certainly has not been appealing to a plant breeder. Furthermore, the methods employed in extracting anthers and placing them on media for callus induction, with subsequent transfer to regeneration media are suitable for conducting small experiments but are not appropriate for a breeding program that requires tens of thousands of regenerated plants. Significant progress has been made in recent years in anther culture technology but the technique still remains largely in the laboratory in the hands of the tissue culturalists and not in the field with the rice breeder.

Rice anther culture research at the Centro Internacional de Agricultura Tropical (CIAT) in Colombia began only a few years ago. Initial efforts mainly were to familiarize ourselves with the existing techniques and to construct the necessary infrastructure. More recently, we have concentrated on developing methodology to produce large numbers of regenerated plants. This reflects our interest within the Rice Program in incorporating anther culture into the main stream of our existing breeding program.

CIAT Rice Program responsibilities include providing improved lines to the national programs of Mexico and the countries of Central America, the Caribbean and South America. Experience has taught us that it is not feasible to breed plants in Colombia that are adequately adapted to all the diverse environments in these regions. A more practical approach may be the development of regional breeding programs that should be more capable of addressing localized production constraints. For example, the Southern Cone of South America which includes southern Brasil, Uruguay, northern Argentina and central Chile is a major rice producing area, with a complex of problems most of which cannot be addressed at CIAT. Rice varieties in these areas must possess cold tolerance, be reasonably resistant to the local blast complex as well as have tolerance to Fe toxicity (southern Brazil) and resistance to the straighthead disease (Argentina). The principal limitation to rice varietal improvement in this region is its slowness, given only generation per year with the

conventional breeding methods resulting in 10 or more years to produce a commercial variety. Theoretically, the anther culture procedure could reduce the time required to generate a commercial variety to under 4 years. Consequently, our ultimate goal at CIAT is to develop anther culture technology that can be used by national programs in areas where conventional breeding methods are inadequate. This may take one or two forms -- provide for local evaluation regenerated R_2 lines from CIAT crosses between parents offering appropriate traits and/or assisting in the establishment of regional anther culture facilities for local production of regenerated lines.

CONSIDERATIONS FOR ANTHER CULTURE BREEDING

Induction problem

A primary limitation in using anther culture in varietal improvement has been the low percentage of anthers that form calluses. Although this problem remains, considerable advances have been made during the last 10 years. Anthers having pollen in the mid to late uninucleate stage are the most suitable for callus induction and this stage is easily identified in the field. Cold shocking anthers at 8° C for 4-8 days stimulates callus formation. Improvements in media composition, including liquid nutrient cultures containing 3-4% sucrose and 1-2 ppm 2,4-D or NAA, have resulted in further increases in callus induction. Through the use of these improved methods, it is not uncommon to observe 25-50% callus induction in many varieties. This is a remarkable achievement as 15 years ago reports of less than 1% callus

formation were common. However, the low rate of callus induction of indica types is still a major obstacle.

The influence of media composition has been one of the most researched areas in anther culture. However, there is no general agreement on which media are superior, as variety x medium interactions are always significant. The universal medium, in which anthers from all varieties are capable of high percent callus induction, has not been developed, nor is there reason to believe that such a medium exists. Varietal differences in response to soil nutrient levels is a well known phenomenon, and one would be surprised not to observe a similar response for callus induction in media of varying nutrient and hormonal composition. Another problem is that almost all research conducted on media has utilized japonica types. Consequently, it is not surprising that the media presently available are more suitable for japonica than indica types. Chinese scientists have reported that anthers from F_1 hybrids of japonica x indica crosses cultured on the N_6 medium (originally developed for japonicas) resulted in a biased callus induction and subsequent regeneration of japonica types (2). The generalization that anthers from japonicas are easier to culture may well be due to the extensive research conducted on these rice types. If equal research efforts were devoted to indicas, we may find that indicas respond to anther culture as well as japonicas.

Fortunately, it is not necessary to rely upon only one induction medium for a program where mass production of R_2 lines is the goal.

We routinely use 2 or 3 media for cultivating anthers of F_1 hybrids - the N_6 medium supplemented with sucrose and auxin, and two versions of the the potato extract media also containing auxins. These media are also used extensively by Chinese scientists. By employing several media and staggering plantings of F_1 hybrids and ratooning, one can significantly increase the probability of generating a sufficient number of calluses. The staggered plantings/ratooning permit assessment of callus induction in the various media. If callus induction is superior in one of the media, anthers from the later planting are cultured only in the best medium.

Successful in vitro selection for desireable plant traits at the callus induction level has not been demonstrated. The relatively low inherent percentage of callus induction combined with the differential responses of F_1 hybrids and varieties to media composition and other unknown factors render selection at this stage extremely difficult.

Plant Regeneration

Callus growth and the subsequent regeneration of plants have received much less attention than callus induction. This may be due to the large amount of information already available on regeneration of plants from callus tissue in several crops. The most significant achievement in rice has been the employment of the two-step process in which calluses are induced on liquid media containing auxins and are transferred to solid media containing cytokinins (4). The stage of callus transfer appears to be critical, as calluses allowed to grow

excessively on induction media seldom regenerate plants when transferred to regeneration media. There is without a doubt a strong varietal influence on the efficiency of plant regeneration from callus. However, recent work, again with japonica types, routinely reports 50% regeneration, but regeneration of indica types is significantly less.

The possibility of applying _in vitro_ selection pressure at the regeneration level is frequently proposed. Differences in callus growth in media containing Al have been demonstrated in several crops, e.g. carrots, tomato and sorghum (5,7,10). However, varietal response to Al at the regenerative level may not be related to field reactions. Even at the whole plant level, there does not appear to be a significant correlation between the reaction in nutrient solutions containing Al and responses in the field on acid soils. This is not surprising, since soil acidity is complex, involving toxic levels of Al combined with deficiencies in Ca and/or P as well as other micronutrients. It is doubtful that one can simulate such a complicated factor as the soil acidity complex _in vitro_.

It has also been suggested that amending anther culture media at the callus or regeneration stage with pathogen-produced toxins could be an effective means of pre-screening regenerated plants for disease resistance (8). While this is an attractive idea, and should be explored, we feel that the evidence is lacking to support a large-scale effort in this area. Very few pathogen-produced toxins are primary determinants of disease and thus are inappropriate for

such a method. We suspect that screening *in vitro* for toxin tolerance would merely select for easily overcome resistance (akin to a hypersensitive response). Furthermore, it is highly unlikely that diseases with very complex etiology will be overcome by exposing callus *in vitro* to only one or a few of the hundreds or thousands of pathogen products that result in a disease relationship between the rice plant and the pathogen. A final concern is how well the response of undifferentiated tissue will correspond to that of a maturing plant in the field. Can we expect a plant regenerated from callus and tolerant to one or more toxins to be resistant to neck blast? In a field situation even adult plants resistant to leaf blast may be very susceptible to neck blast.

Parental selection

Evaluation of parents for callus induction and regeneration abilities is often considered important. However, convincing data are lacking to support this conclusion. Generally, pollen from the F_1 exhibits hybrid vigor for regeneration, but callus induction does not appear to follow any consistent pattern. More data are required to demonstrate the benefits of parental selection before one can justify restricting the crossing program based upon callus induction and regeneration capabilities. At CIAT, we prefer to increase either the number of crosses or the number of anthers cultured as opposed to confining the breeding program to a few crosses of known parental capability.

R_2 lines

With the presently available techniques and knowledge of anther culture, the only currently feasible breeding approach involves evaluation and selection of homozygous R_2 lines. Consequently, the anther culture procedure becomes no more than a means of reaching homozygosity in a reduced period of time. In this light, breeding via anther culture is similar to the single seed descent (SSD) method (1). The SSD method has been shown to be an effective breeding procedure for many important crops, especially in areas where only one generation/year can be advanced. Both methods also have the same limitation in that thousands of fixed lines are produced that have not received prior selection pressure. Consequently, success in using anther culture for varietal improvement depends to a large extent upon the development of screening techniques capable of handling thousands of fixed lines using only small quantities of R_2 seed. Another serious problem confronting breeding via anther culture is the capacity to regenerate sufficient material to have a reasonable chance of encountering large numbers of useful R_2 lines.

Population Requirements

Experienced rice breeders using conventional methods normally attempt to obtain an F_2 population of 4,000 to 5,000 plants/cross. Although it is difficult to make an analogy with anther culture, Chinese scientists have calculated that an equivalent population would be in the order of 100 to 150 regenerated plants (9). Although we question the equivalence of a large F_2 with a reduced number of unevaluated

regenerated plants, we accept 125 R_2 plants/cross as a reasonable target. Thus, a rough estimate can be made of the number of anthers and calluses required to regenerate the desired amount of R_2 material. One panicle taken from the field when the distance between the auricle of the flag leaf and the subtending leaf is 4 to 7 cm will produce approximately 25 florets containing pollen in the mid to late uninucleate stage. From each floret approximately 2 to 3 anthers can be separated with ease, resulting in approximately 60 anthers/panicle. Assuming a 25% callus induction rate, each panicle would produce about 15 calluses. If a 25% regeneration rate is obtained, 4 plants would result, and approximately half of these R_1 plants would be diploid. Consequently, from each panicle 2 diploid plants can be expected. However, only about 50% of the diploid R_1 plants might be agronomically acceptable, resulting in about one useful R_2 plant from each panicle harvested. To obtain the target population of 125 R_2 plants, roughly 100 F_1 panicles will be required from each cross. For a national program, it may be considered that 100 crosses/year is adequate. Consequently, if anther culture is to be used as a breeding tool, one must be capable of handling approximately 10,000 panicles from F_1 hybrids, 600,000 anthers, 150,000 calluses, and 75,000 R_1 plants per year. The 75,000 R_1 plants would result in about 15,000 useful diploid R_2 lines. This illustrates the problems of how to regenerate such a large volume of lines and how to expose them to selection pressures to identify elite lines.

METHODS FOR GENERATING R_2 LINES

Induction Phase

The techniques normally used for isolating anthers from panicles and placing them on induction media are laborious. The most time consuming step is the cutting of individual florets to extract the anthers. The method we employ consists of the following:

- Approximately 100 panicles are harvested from each cross at the uninucleate stage, wrapped in aluminum foil, and cold shocked for 3-10 days at 8° C.
- Immmature spiklets are surface sterilized, and the top and bottom portions of the panicle discarded, leaving approximately 25 florets, which are removed and dissected at the base.
- Anthers are extracted from the cut florets by tapping the cut floret on the induction flask. Another method in which anthers are extracted in mass appears to be promising but more work is required to reduce the high amount of contamination.
- The induction flask is sealed and placed in the dark at about 20° C for callus induction.

Each flask contains the anthers from one panicle. All procedures are conducted under a flow hood using sterile conditions. Using this method approximately 150 panicles (9,000 anthers) can be prepared daily.

Regeneration

In each induction flask approximately 15 calluses are normally produced. Close observation is required, as callus formation is never uniform. When there are more than 10 small calluses/flask they are transferred to the regeneration medium. When only a few calluses are present it is necessary to decide to transfer or wait for more callus formation. If the latter is the case and new calluses appear, the first formed calluses are discarded, since large calluses seldom regenerate plants. Calluses are transferred in mass by filtering the entire contents of the induction flask on a Buchner funnel apparatus. It is important to use a flat vacuum filter to obtain a uniform dispersion of the calluses on the filter paper. Uninitiated anthers and small calluses are collected on a 9 cm filter, which is the same size as the regeneration container. The filter paper is inverted and placed on top of the induction medium and tapped lightly to embed the calluses in the medium. The filter paper is removed, and the calluses remain on the medium. The regeneration container is sealed and placed under artificial lighting until regeneration. With this method, one person can prepare approximately 150 to 200 flasks/day containing a total of 2,000 to 3,000 calluses. This is equivalent to 1.5 to 2 crosses/day. The advantage of this method is that the unit of work is the panicle, eliminating the need to transfer individually thousands of calluses to test tubes.

In summary, two laboratory assistants using the previously indicated methods can handle 100 crosses per year. In developing

these techniques a deliberate attempt has been made to employ locally available glassware. This is important as imported scientific glassware is expensive, and with the large volume of material the total cost would be prohibitive for many developing countries. Baby food jars (125 ml) are ideal for callus induction flasks and are readily available. Regular fruit jars (9 cm diameter) can be cut and made into regeneration containers. In Colombia, we have purchased sufficient glassware to handle 100 crosses/year at a total cost of approximately US$1,200.

Evalution of R_2 Lines

R_1 plants are inappropriate for screening as they are weak, and diploids cannot be distinguished until flowering. Once R_2 seeds (approximately 20 g/plant) are obtained, the procedure for selecting elite lines is similar to any other breeding method. Due to the large number of lines involved, screening in the field under heavy pressure is required. The material is first screened for tolerance to the easiest character, eliminating many of the lines. Material selected in the first test is further screened for tolerance to the next easiest constraint, and the process continues until the number of lines has been reduced to a sufficiently small number that can be handled in an observation nursery.

We have made approximately 100 triple crosses designed for southern Brazil, which should result in approximately 15,000 R_2 lines. A 3g sample is evaluated for blast resistance, resulting in about 50%

rejection. Since there is a high correlation between leaf blast and panicle blast resistance, this screening is done only at the leaf stage requiring only 50 days. Duplicate 3 g samples of the blast resultant R_2 lines are screened for Fe tolerance. Again, 50% of the entries are discarded; this test requires approximately 60 days. White belly determination is conducted using remnant 3g samples of the 3,000 R_2 lines possessing blast resistance and Fe toxicity tolerance. The remaining 10 g of remnant R_2 seed of about 1,500 selected lines are planted in 4 row plots in southern Brazil, where the lines are evaluated for phenotypic characters and cold tolerance. Elite selected lines are sent to Uruguay, which has the same yield constraints. This whole process commencing with panicles of F_1 hybrids requires approximately 1.5 months for callus induction, 1.5 months for regeneration, 4 months for R_1 growth, 2 months for testing for blast resistance, 2 months for Fe tolerance and 1 month for white belly determination. Consequently, in 1 year lines are available for observation nurseries. Using conventional breeding methods to arrive at the same stage, at least 4 years would be required at CIAT, where 2 generations are produced/year. If the anther culture process were applied to Brazil which has only 1 field generation/year, the savings in time would be considerably greater.

A similar scheme is being used for Chile and Argentina. In the latter case, a 3g sample of R_2 seeds is used to screen for blast and the selected lines are tested for straighthead resistance. Resistance to the straighthead disease can be evaluated using arsenic which has

been shown to mimic the disease (3). Lastly, white belly is determined. Again, the remnant R_2 seeds are tested in observation nurseries in Argentina.

PERSPECTIVES FOR BREEDING VIA ANTHER CULTURE

The varied rice ecologies in Latin America offer distinct examples of possible application of anther culture to varietal improvement. These include the Southern Cone, upland rice for acid savanna soils, and tropical Mexico. These examples are characterized by vast production potential addressed by conventional breeding programs restricted to one generation/year.

We consider that anther culture has the potential to reduce considerably the time required to produce fixed lines. Its utility in high volume breeding programs will depend upon resolving remaining limitations and uncertainties, including:

° Continued improvement in the efficiency of callus induction and plant regeneration in indica crosses is essential to reduce the volume of panicles, florets and anthers processed.

° Field experience with R_2 lines from many crosses is required to estimate the population desired/cross to simulate progeny sizes used in conventional rice breeding.

° Similarly, only extensive field experience will indicate whether doubled haploids give goodly numbers of normal, agronomically useful lines.

The CIAT Rice Program is concentrating on these and other problems with the goal of moving anther culture from the laboratory to rice breeders' fields. If success is achieved, we believe that serious consideration should be given to establishing anther culture facilities within selected national programs. We would continue to improve methods and train national scientists for local application of anther culture to high volume rice breeding programs.

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REFERENCES CITED

1. Brim, C.A. 1966. A modified pedigree method of selection in soybean. Crop Sci. 6:220

2. Chen Ying. 1983. Anther and pollen culture of rice in China. Pages 11-26 in Proc. of workshop on cell and tissue culture techniques for cereal crop improvement. Science Press. Beijing; China and IRRI, Manila, Philippines.

3. Gilmour, J. T. and B. Wells. 1980. Residual effects of MSMA on sterility in rice cultivars. Agron. J. 72:1066-1067.

4. Inoue, M. and E. Maeda. 1981. Stimulation of shoot bud and plantelet formation in rice callus cultures by two-step culture method using abscisic acid and kinetin. Jpn. J. Crop Sci. 50: 318-322.

5. Meredith, C.P. 1978. Response of cultured tomato cells to aluminum. Plant Sci. Letters 12:17-24.

6. Niizeki, H. and K. Oono. 1968. Induction of haploid rice plant from anther culture. Proc. Jpn. Acad. 44:554-557.

7. Ojina, K. and K. Ohira. 1982. Characterization and regeneration of an aluminum-tolerant variant from carrot culture. Proc. 5th. Intl. Cong. Plant Tissue and Cell Culture. Pages 475-476.

8. Oono, K. 1981. In vitro methods to rice. Pages 273-29 in T.A. Thorpe (ed.) Plant tissue culture methods and applications in agriculture. Academic Press.

9. Shen Jim-Lua, Li Mei-fang, Chen Yin-quan and Zhang Zhen-hua, 1983. Improving rice by anther culture. pp. 183-205 in Proc. of workshop on cell and tissue culture techniques for cereal crop improvement. Science Press, Beijing; China and IRRI, Manila, Philippines.

10. Smith, R.H., S. Bhaskaran, and K. S. Schertz. 1983. Sorghum plant regeneration from aluminum selection media. Plant Cell Reports 2:127-132.