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ARRIVÉE

Screening for stress tolerant genotypes via microspore *in vitro* culture

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ABSTRACT

Some of the limitations of the conventional screening methodologies for stress tolerance in cereal could be overcome by selection via microspore in vitro culture techniques. Highly variable gametophytic cell population can be developed. Large-scale microspore selection system allows (1) to screen millions of genotype in a short time, (2) to identify the expression of dominant as well as recessive traits, (3) to apply a controlled and homogenous selection pressure, and (4) to rapidly develop new cultivars from the selected mutants. However, further efforts need to be directed towards the increase of microspore culture response and the development of selecting procedure compatible with subsequent plant regeneration. Since only some mechanisms involving in stress tolerance would be affected by this selection technique, this methodology should be considered as a complementary tool of the field test techniques.

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INTRODUCTION

Breeding for environmental stress tolerance to increase and stabilize crop productivity has been reasonably successful. Useful heritable variation for stress tolerance has been exploited through conventional breeding techniques. However, any additional techniques which could aid the breeder for screening and/or selecting stress tolerance genotype would be highly desirable.

Cell-culture techniques have proved useful in many areas of cereal plant research. Its application ranges from basic physiological studies of biochemical pathways at the cellular level, to plant regeneration from in vitro tissue culture. Two aspects of plant breeding in which cell-culture is particularly applicable are the production of haploid plants (Baenziger 1984) and the selection of mutants (Wersuhn 1989).

Haplodiploidistation procedures offer the possibility of developing completely homozygous lines from heterozygous parents in a single generation. Regarding crop improvement for stressed environments, the production of doubled haploid lines provides a rapid technique for the incorporation of stress tolerance into agronomically superior germplasm. Furthermore, more efficient selection for stress tolerance is possible because of a greater contribution of additive effects to the variation and the absence of within-family segregation. Cereal haploid plants can be produced by various techniques. Some of these techniques (i.e. in vitro androgenesis, interspecific cross) have been introduced into several research and commercial breeding programs in winter cereal such as bread wheat (Picard et al 1989) and barley (Choo et al 1985).

Use of cell culture techniques can (1) significantly increase the variation available to the breeder, and (2) help him select for unique and specific modifications in a crop. So far, most of the experimental data on *in vitro* screening have been obtained with cell suspension and callus culture form diploid tissues (e.g. embryos, leaf segments) but recent advances in the microspore *in vitro* culture techniques should greatly increase the interest concerning the use of male gametophytic cell culture systems. Large populations of microspores may allow to efficiently combine selection at cellular level and use of double haploid in plant breeding. The purpose of this paper is to outline the potentialities and difficulties of utilizing *in vitro* screening via microspore culture for improving abiotic stress tolerance in winter cereal.

MICROSPORE *IN VITRO* CULTURE TECHNIQUE

Present status

The principle of *in vitro* androgenesis is to divert the normal development of the male gametophyte by an abnormal pathway, to a sporophytic pathway resulting in callus or embryo formation. The culture of microspores (contained in the anther or separated from it) can, under appropriate conditions, result in cell division and growth of gametophytic cells and eventually in plant regeneration.

The culture responsiveness varies considerably among and within cereal species, and is strongly influenced by the genotype of the donor plants. Several other parameters were found to influence the general culture response (Dunwell 1985). These

include environmental conditions during growth of donor plants, cold pretreatment of anthers prior to culture initiation, stage of microspore development, culture medium, and incubation conditions. Although progress has been made, induction frequency of pollen embryos remains low in comparison with the number of available microspores. As an example there are about 3.000 microspores within a single anther of barley (Ye. et al 1987). However, Kohler and Wenzel (1985) reported that only 0.2% divide during microspore culture and only 30% of these develop into callus. Nevertheless, recent advances in the technique used for barley (Sorvari and Shieder 1987, Hunter 1987) as well as for bread wheat (Chu and Hill 1988) are very promising. Yields as high as 200 green plants per 100 anthers have been reported for barley, and devising an efficient selection procedures during the microspore culture becomes necessary. On the other hand anther culture response in durum wheat is still too low to be of a value for selection (Hadwiger and Heberle-bors 1985). Table 1 summarizes the present stages of development of the anther and isolated microspore culture techniques in winter cereal.

TABLE 1. PRESENT STAGES OF DEVELOPMENT OF THE ANTHER AND ISOLATED MICROSPORE CULTURE TECHNIQUE IN WINTER CEREAL

	ANTHER CULTURE	ISOLATED MICROSPORE CULTURE
BREAD WHEAT	+++	+
DURUM WHEAT	+	-
BARLEY	+++	++
TRITICALE	+++	-
RYE	+	-

\- UNSUCCESSFUL
\+ PLANT REGENERATION REPORTED
++ LARGE PLANT PRODUCTION REPORTED FROM SPECIFIC GENOTYPES.
+++ APPLIED TO PLANT BREEDING PROGRAMS

Compared with the anther culture, the isolated microspore culture technique presents several advantages. It allows the production of a single-celled population with consequent advantages for selection purposes (homogeneity, synchroniztion etc.), and the removal of undesirable interactions between anther wall and microspores. It also reduces the laborious and time consuming effects due to the handling of entire anthers. There are several reports of successful culture of isolated microspores in wheat (Datta et al 1987), barley (Hunter 1987), and rice (Cho and Zapata 1988). However, two major problems limit the effective utilization of microspore culture in cereal: (1) low embryo yield, and (2) poor plant recovery from microspore-derived embryos.

Genetic Variability

Selection for tolerance to any environmental stress depends closely on the available genetic variability. Microspore culture can allow the use of both in vitro induced variability and the natural variability following meiotic recombination. For selection of tolerant genotypes it is of no importance whether the new character has resulted from an induced mutation or a segregation.

The phenomenon of gametoclonal variation (Evans et al 1984) has been observed in several species including wheat (Parisi and Picard 1986, Rode et al 1987) and barley (Powell et al 1984). The mechanisms causing the variation are not yet well understood, but may include individual changes at the nucleotide level, large chromosomal modifications (duplications, deficiencies, inversions, and translocations), and the production of aneuploids and polyploids. Nevertheless, the microspore culture system appears relatively stable and variations occurring after in vitro culture are limited. The genetic variability can be increased by

treatment with chemical or physical mutagens. This potential has been successfully investigated by mutating microspores immediately after isolation in Brassica (Swanson et al 1988) or during the anther culture in tobacco (Medrano et al 1985). Although the gametoclonal variation may allow to obtain directly modified variations of already widely adapted varieties, its usefulness as a source of variation for selection for stress tolerance remains to be proven.

Genetic variability resulting from recombination and segregation appears as the preferential source of variation in microspore in vitro culture. The amount of variability is determined by the level of heterozygosity of the donor plant. F_1 hybrid plants appears as a very attractive source of heterogeneous gametophytic cell populations. The haploid state of the population presents additional interests. Recessive characters are expressed and could be identified. Additive genetic effects constitute a large part of the genetic variance in the population.

BASIS OF THE SELECTION AT THE MICROSPORE LEVEL

Selection at cellular level using microspore in vitro culture requires that the genes expressed during gametophyte growth to be related to sporophytic gene expression and that the selected character is completely or partially expressed at the cellular level.

Overlaping of Gametophytic and Sporophytic Gene Expression

In Tomato (Tanksley et al 1981), maize (Sari-Gorla ,et al 1986) and barley (Pedersen et al 1987) it is estimated that

respectively 58%, 73% and 60% of the genes coding for enzymatic proteins are expressed in both pollen and sporophyte. Moreover, through gametophytic selection (i.e. pollen competition), positive results have been obtained for a number of important agronomical characters such as plant vigor, endosperm development, and tolerance to heavy metals and salinity (Ottaviano and Sari-Gorla 1988). As a consequence, it is reasonable to assume that a substantial overlap between active genes in the gametophytic and sporophytic tissue exists, and a large part of the genetic variation is potentially accessible to selection at the gametophytic cell level. Additionally, the microspore will be engaged in an embryogenic pathway during the microspore in vitro culture process and the embryoid structure is more likely to have a genomic expression similar to that of the whole plant.

Cellular Components of Environmental Stress Tolerance

Stress tolerance can result from a large spectrum of mechanisms acting at different levels in the organization of the plant and at different stages of its life cycle. Any single component at cell or tissue level accounts generally for only a portion of the total mechanism of tolerance. However, it is reasonable to assume that cellular components of even complex phenotypes such as drought, salt, cold, and heat tolerance can play an important role (Petolino and Collins 1984) and are accessible to selection at the cellular level.

In order to develop efficient cellular selection strategies, it is important to consider the physiology of the trait and the relative importance of cellular components to overall stress tolerance. Certain stresses will be more responsive to in vitro approaches than others since the mechanisms associated with

sensitivity and resistance are largely determined at the cellular level.

For example, cellular selection for improving tolerance to temperature stress appears promising. Although tolerance may be a function of whole plant structure, the injurious effects of temperature are directly affecting cells themselves. Cold tolerance in plants appears to be related to the chemical and physical properties of cellular membranes. Increased cellular solute concentration and changes in membrane structure are frequently reported as mechanisms of freezing tolerance (Steponkus 1980). Membrane stability and heat shock protein response are important for high temperature tolerance (Mascarenhas 1984). Similarly, tolerance to metals in plants usually results from the ability of plant tissues to tolerate large amounts of toxic ions. Cell wall structure and metal binding properties, membrane function and intracellular compartmentalization as well as internal ligand and metal binding protein production should all be amenable to cellular manipulation (Petolino and Collins 1984).

On the other hand, tolerance to water stress is largely a result of plants avoiding internal water deficits via morphological and physiological adaptations, and the relative importance of cellular components such as osmotic adjustment and proline or abscisic acid accumulation remain to be evaluated. Although many of the basic mechanisms associated with salt tolerance operate at the cellular level and comparative studies on the susceptibility of whole plants and callus cultures to salt have suggested that this trait is exhibited in <u>in vitro</u> culture (Orton 1980, Smith and McComb 1981), relative contribution of whole plant and cellular components remains to be seen.

Successful Cell Selection

It is well established that mutants can be selected from plant somatic cell cultures, Although many variations for agronomicaly important traits have been selected from cell culture, examples demonstrated plants and transmission to sexual progeny are limited to a few instances of herbicide resistance, pathotoxin resistance, amino-acid over production, and heavy metals (Ahloowalia 1986). Regarding environmental stress tolerance, Conner and Meredith (1985) selected a large number of aluminum resistant variants which have expressed the trait in regenerated plants. Salt tolerant cell lines have been selected from cell cultures of several species, but tolerant plants have been regenerated only in few cases such as in tobacco (Nabors et al 1982) and in rice (Yano et al 1982).

Few attempts have been made to select using microspore culture systems. In barely for example, it has been indicated that the screening for salt tolerant genotypes via F_1 anther culture in salt stress media induced by high concentration of Na_2SO_4 is feasible and effective (Ya et al 1987). However, there was a sharp decrease in callus formation and plant regeneration frequencies in salt stressed media. Many potentially salt tolerant individuals were eliminated. In Brassica napus, advances in media and donor plant maintenance have permitted consistently higher frequencies of embryogenesis from isolated microspores (10-40 embryos per anther). The ability to rapidly generate large populations of microspore-derived haploid embryos makes it possible to use the microspore culture system as a tool for selection in oil seed rape breeding programs (Polsoni et al 1988). As an example, rapid and clear separation of herbicide (Chlorsulfuron) tolerant genotypes

from segregating microspore populations isolated from hybrid plants has been reported (Swanson et al 1988).

PROTOCOL FOR IN VITRO SELECTION

Choosing Selection Unit

Selection can be performed at different stages of the microspore culture procedure and, consequently, different selection units will be involved: microspore, multicellular structure, embryoid or plantlet. The last stage (plantlet) provides a selection unit which is closer to the whole plant and allow to practice selection in situations where cell specialization, cell communication or plant integrity are fundamental. However this approach requires a large amounts of labour (plant regeneration) and presents no determining advantages over the conventional methods using seedling test. On the other hand, earlier stages (i.e. microspore, embryoid) offer the possibility of selection using a larger number of genotypes.

Selecting the Screening Agent

So far, cell selection strategies have used the ability of the selection unit to grow or survive upon exposure to a given stress. Several different screening agents have been used in in vitro selection for stress tolerance. In the case of salt tolerance, various salts (i.e. Nacl, Na_2SO_2,$MgCl_2$) can be directly added to the culture medium. Similarly, excessive amounts of aluminum and manganese have been used. Drought tolerance may be co-selected with salt tolerance, otherwise needs to be approached indirectly using a compound which simulates osmotic stress, for

example, polyethylene glycol (PEG). Temperature stress can be applied to the culture. In addition, observations on cell culture suggest that temperature stress shares some common features with cadmium (Huang and Goldsbrough 1988) and salt tolerances (Harrington and Alm 1988), and could be simultaneously selected for.

Dosage-Time-Response Studies

Regardless of the nature of the screening agent, the optimum dosage-time for selection must be determined. Effective screening must be obtained without sacrificing plant regeneration independently of the sizes of selection units. Moreover, the length of time of the selection pressure application must be considered. Short-term exposure could be favorable to selection for genetically determined traits, and minimize the risks of selecting cells with only transient physiological adaptation.

Integration in crop improvement program

The possibilities to screen a very large number of genotypes, and subsequently to rapidly develop new cultivars from the selected mutants are the major interests in a large-scale microspore selection system in a breeding program (Fig.1). The wide use of the doubled haploid lines in breeding program is handicaped by the absence of selection during the production process. The availability of a selection procedure among the microspores may allow to overcome or reduce this principal limitations. Moreover, since large variability is analyzed and rare recombinant detected, introgression of desired characters via interspecific cross or conventional backcross could be greatly facilitate and accelerate.

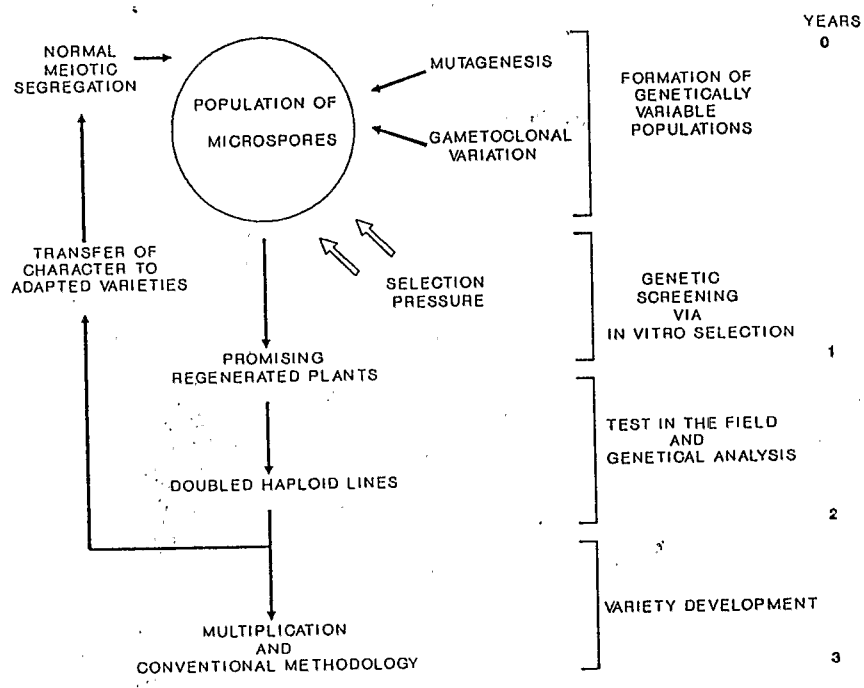

Fig. 1. Schematic representation of a general procedure for the development of stress tolerant cultivars using microspore in vitro culture.

However, we must keep in mind that only the components of environmental stress tolerance which are expressed in microspore culture can be selected. Screening for stress tolerant genotype via microspore culture must be considered as a pre-screening. Field test techniques are absolutely necessary to confirm and study the expression at the whole plant level of the selected trait and to continue the selection process as well.

CONCLUSIONS

Some of the typical limitations of conventional breeding and selection for stress tolerance could be overcome by selection at cellular level using microspore <u>in vitro</u> culture techniques. In

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the small space of a flask or petri dish, cell-culture provide large numbers of cells that can be screened in a single experiment. The in vitro culture provides a homogeneous and controlled environment which greatly simplified selection procedures as compared to those used with plants grown in a open field.

Additionally, a large scale microspore selection system offers several advantages over traditional somatic tissue selection systems:

- Gametoclonal variation, mutagenesis as well as recombination and segregation events can be used as source of genetic variability.
- Haploid embryoid structure express both dominant and recessive traits during the selection process.
- The embryoid as a selection unit is more likely to respond to selection pressure in a manner similar to a whole plant, in contrast to differentiated cells in suspension or callus culture.
- In association with double haploid breeding methodology, rapid development of new cultivars is possible.

The availability of a system that produces large numbers of regenerated haploid selection units is of considerable importance. In cereals, current techniques of microspore in vitro culture are not efficient enough and further technical improvements are needed to use fully the potentialities of the system. Another aspect that limits its application is the lack of knowledge in the areas of plant physiology and plant genetic regulation. Better

understanding of the molecular basis of the tolerance mechanisms, and of the relationship between cellular and whole plant phenotype will allow to optimize the system and develop highly efficient selecting procedure compatible with subsequent plant regeneration.

Since some major physiological processes (e.g. translocation, photosynthesis, etc.) are absent, screening stress tolerant genotypes via microspore in vitro culture would operate only on tolerance mechanisms expressed at a cellular or embryo level. This methodology should be considered as a complementary technique within the framework of an integrated breeding program to improve the efficiency of conventional approaches.

Résumé

CRIBLAGE DE GENOTYPES TOLERANTS AUX STRESS PAR CULTURE DE MICROSPORES *in vitro*

Quelques-unes des limitations des méthodologies conventionnelles de criblage pour la tolérance aux stress chez les céréales pourraient être surmontées grâce à des techniques de sélection par culture *in vitro* de microspores. Des populations cellulaires à haute variabilité gamétophytique peuvent être développées. Un système de sélection de microspores à grande échelle permet 1/ de cribler des milliers de génotypes dans des délais brefs, 2/ d'identifier l'expression de caractères tant dominants que récessifs, 3/ d'appliquer une pression de sélection contrôlée et homogène, et 4/ de créer rapidement de nouveaux cultivars à partir des mutants sélectionnés. Des efforts supplémentaires doivent toutefois être consacrés à l'amélioration de la réponse à la culture de microspores, et au développement de protocoles de sélection compatibles avec la régénération ultérieure des plantes. Compte tenu du fait que seuls quelques mécanismes impliqués dans la tolérance aux stress seraient affectés par cette technique de sélection, cette méthodologie devrait être considérée comme un outil complémentaire des techniques utilisées au champ.

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