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Contributions of Biotechnology to Asparagus Breeding in Argentina

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ABSTRACT

Garden asparagus (*Asparagus officinalis* L.) is an economically important horticultural crop, susceptible to pathogenic soil fungi of the genus *Fusarium.* These fungi cause stem, crown and root rot, a major disease all over the world that is difficult to control by the use of chemicals and cultural practices. The development of resistant/tolerant cultivars appears to be the best alternative for control; however, the asparagus-*Fusarium* pathosystem is complex and advances in this regard have not been very successful. The species is perennial and dioecious, which hinders the breeding process, but it can be asexually and sexually reproduced and *in vitro* manipulated. In Argentina, imported open-pollinated cultivars and F_1 and F_2 clonal hybrids are grown, which are susceptible to *Fusarium* and not necessarily adapted to local conditions. In 1992, an asparagus breeding program was started in Balcarce, in collaboration with another national research group, to (a) generate adapted and good yielding cultivars and (b) explore potential sources of *Fusarium* resistance. Various biotechnologies were used in an attempt to generate and/or introduce genetic variability for the trait (gametophyte selection, *in vitro* selection of plants and calluses, interspecific hybridization followed by *in vitro* ovule culture) and to clone elite genotypes (micropropagation and somatic embryogenesis) to produce clonal hybrids. The principal results of the application of these biotechnologies are summarized in this review.

Keywords: *in vitro* cloning, *in vitro* evaluation, *in vitro* interspecific embryo rescue, *Fusarium* resistance, gametophyte selection, somaclonal variation

Abbreviations: AFLP, amplified fragment length polymorphism; **CF**, fungal toxic culture filtrate; **cpDNA**, chloroplast DNA; **2,4-D**, 2,4-dichlorophenoxy-acetic acid; **FCA**, Facultad de Ciencias Agrarias; **INTA**, Instituto Nacional de Tecnología Agropecuaria; **ITS**, internal transcribed spacer region; **HSF**, half-sib family; ***mye***, *Asparagus densiflorus* cv. Myersii; ***off***, *Asparagus officinalis*; **PCR**, polymerase chain reaction; **RAPD**, random amplified polymorphic DNA; **RFLP**, restriction fragment length polymorphism; ***spr***, *Asparagus densiflorus* cv. 'Sprengeri'; **UNMdP**, Universidad Nacional de Mar del Plata; **UNR**, Universidad Nacional de Rosario

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INTRODUCTION

Garden asparagus, *Asparagus officinalis* L. (*off*, 2n=2x=20), is the economically most important species in the genus due to its edible shoots. The species is dioecious (that is, individual plants bear flowers with either female or male organs) and, therefore, an obligate outcrosser. As a consequence of the reproductive system, individual plants are highly heterozygous and the populations are genetically heterogeneous.

Fortunately for commercial purposes, this species can also reproduce asexually (cloning) by *in vivo* crown division (i.e. the crown, formed by shoot and root primordia, can be cut into sections, to form new plants) and by *in vitro* culture of various explant types (buds, meristems, shoot sections, etc.). The crop can be established in the field by means of crowns or seedlings, and can be in production for 10-15 years or more, without important decrements in yield.

Old commercial cultivars, such as Mary Washington,

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Invited Review

Martha Washington and Argenteuil, are actually open-pollinated populations. Later on, several types of hybrids were introduced in the market (see Corriols and Doré 1988): (a) double hybrids, originated from four parental heterozygous plants chosen by their good specific combining ability, (b) clonal hybrids, originated from two heterozygous micropropagated parents, (c) mixed-F_1, and (d) all-male F_1 hybrids, originated from two double haploid parents (Corriols-Thevenin 1979 in Ellison 1986; Falavigna 1979; Qiao and Falavigna 1990; Peng and Wolyn 1999). In (c), haploid female plants are obtained either from polyembryonic seeds or from *in vitro* anther culture (from gametes carrying one X chromosome). After chromosome doubling, these female plants are backcrossed successively to a male to obtain a pure line composed of male and female plants, each isogenic for the initial haploid female genotype. The intercrossing of two such pure lines produces a homogeneous F_1 hybrid composed of 50% male and 50% female plants. In (d), haploid males are obtained through *in vitro* anther culture (from gametes carrying one Y chromosome) and then diploidized to produce supermales (that is, plants with two Y chromosomes). Crossing one of these plants to a homozygous female plant results in the production of an all-male F_1 hybrid. This procedure can be modified by pollinating a heterozygous female plant with a homozygous supermale plant to produce (e) three-way hybrids.

Garden asparagus became economically important in Argentina during the 1990s because of the excellent prospectives for off-season export to Northern Hemisphere countries with high income levels. By 1991, only open-pollinated populations derived from a European cultivar ('Argenteuil') and the first and second generation of a clonal hybrid from the USA ('UC157 F_1' and 'UC157 F_2') were grown in different ecological regions of Argentina, for white and green spears production, respectively. Plants of the open-pollinated populations and segregating hybrids lack homogeneity in spear yield, caliber and quality due to gene segregation and both the populations and hybrids are susceptible to a fungus complex of the genus *Fusarium* that causes stem, crown and root rot, the main cause of asparagus decline in the world. The most important asparagus pathogenic fungi in the *Fusarium* complex are *F. proliferatum* (syn. *moniliforme*), *F. oxysporum* f.sp. *asparagi*, *F. redolens* f.sp. *asparagi* and F. *roseum* (Graham 1955; Grogna and Kimble 1959; Lewis and Schoemaker 1964; Endo and Burkholder 1971; Johnston *et al.* 1979; Elmer 1990; Bruna 1991). Besides asparagus, *F. proliferatum* can also colonize corn stalks and persist on these and other residues as a saprophyte, sporulating above ground. In our country, this pathogen is widely present in asparagus growing areas and other areas of potential use for this purpose, including Southeastern Buenos Aires province. The effectiveness of controlling methods for the pathogen in asparagus is low due to the abundance of inocula in different agroecological regions, rapid colonization of young plants by the pathogen, and the perennial nature of the plant species. Therefore, the development of resistant cultivars appears to be the most viable long-term strategy for disease control. In fact, this has been, and continues to be, a central goal in asparagus breeding programs worldwide. Some cultivars characterized as tolerant to *Fusarium* have been developed by classical breeding approaches, although this tolerance is generally limited, and the breeding process is very slow due to the complex nature of the pathosystem, as described earlier.

In order to develop cultivars with *Fusarium* resistance and good yield and adaptation to our harvesting conditions, a breeding project was initiated at the Instituto Nacional de Tecnología Agropecuaria (INTA) in collaboration with the Universidad Nacional de Mar del Plata (UNMdP) and the Universidad Nacional de Rosario (UNR), all of them public institutions.

Several potential resistance/tolerance sources were considered in this project: (a) populations (land races) that grow spontaneously along wire fences and roads in various ecological regions of our country, (b) plants from old asparagus fields no longer under cultivation, (c) related species and (d) somaclonal variation induced by *in vitro* culture. For (a) and (b), genetic variability for *Fusarium* resistance/tolerance was expected to be present because, as Sprague (1967) and Leppik (1970) had stated, pathogen pressure in natural environments is presumably high because hosts and parasites may have been associated as reciprocal selective factors over many years.

This project produced the first five Argentine registered asparagus cultivars (clonal hybrids): 'Lucero FCA-INTA', 'Mercurio FCA-INTA', 'Neptuno FCA-INTA', 'Pampeano FCA-INTA' and 'Sureño INTA-FCA'. Besides traditional breeding techniques, other approaches -which made use of various biotechnologies- were investigated and applied during the breeding process. These were:

1. Adjustment and development of protocols for *in vitro* cloning (micropropagation and somatic embryogenesis);
2. Use of cytogenetics and molecular markers to analyse the genetic similarity of somatic embryo-derived plants with regard to donor plants, to eventually use somatic embryogenesis as an alternative method for micropropagation;
3. *In vitro* evaluation of landraces of Southeastern Buenos Aires province for resistance to *Fusarium proliferatum*;
4. Gametophyte selection for resistance to *F. oxysporum* f.sp. *asparagi*;
5. Identification of interspecific hybridization barriers between garden asparagus and the ornamental *Asparagus densiflorus* (Kunth) Jessop cv. 'Sprengeri', reported as resistant to *Fusarium* by Stephen and Elmer (1988) and *in vitro* embryo rescue to circumvent the post-zygotic barriers in otherwise pollen-pistil compatible genotypic combinations, in an attempt to develop a strategy to incorporate the resistance once it were detected;
6. Induction of somaclonal variation and *in vitro* selection of variants for resistance to *Fusarium proliferatum* toxins.

Here, we concisely review the achievements and limitations derived from the application of these approaches to asparagus breeding in Argentina over the last 15 years.

ADJUSTMENT AND DEVELOPMENT OF *IN VITRO* CLONING PROTOCOLS

Micropropagation

Many protocols have been developed for fast and massive cloning of specific asparagus genetic materials (among the first, by Murashige *et al.* 1973; Yang and Clore 1973; Tendille and Lecerf 1974; Chin 1982; Conner and Fallon 1990; Conner *et al.* 1992; Falavigna *et al.* 1985). In contrast with other easily micropropagated species, in which explants can develop both shoots and roots in the same medium, asparagus explants have to be cultured in an initiation medium for shoot induction and, subsequently, in shoot multiplication and rooting media. An additional drawback is that there are strong genotype × environment interactions in asparagus *in vitro* cultures. In fact, and although most protocols were developed on some similar premises and restrictions, the methodologies are not coincident due to these interactions. Thus, we tried to adjust a protocol for our own breeding materials, that was successful for the first two stages (induction and multiplication) but that yielded regular results for the third (rooting) that, in further studies, could not be improved above 57% (Raimondi *et al.* 1995).

Somatic embryogenesis

As proposed by Reuther (1984), somatic embryogenesis appeared to be a valid alternative to overcoming the limitations of micropropagation protocols. In fact, asparagus was one of the earliest plant species to be regenerated by this

technique (Wilmar and Hellerdon 1968; Steward and Mapes 1971; Reuther 1977). The influence of genotype, explant type and plant growth regulators on callus induction (first step), and growth and embryogenic differentiation (in the following steps), was reported by various authors (see Raimondi *et al*. 2001). In our laboratory, we failed to generate somatic embryos in two selected clones of our breeding materials when following most of the published protocols (Raimondi *et al*. 2001).

Since reports in the literature on statistical analyses in asparagus of interactions between genotype, explant type and plant growth regulators (PGR) were lacking, possibly due to the difficulties encountered in carrying out an experiment that would include all these variables, we decided to analyze the interaction effects of several factors on callus induction, growth and embryogenic differentiation in the previously mentioned selected genotypes, in order to develop our own protocol (Raimondi *et al*. 2001). The interactions among genotypes, explant types, PGRs and culture batches were conspicuous at the initial culture stage, with their significance (and the significance of the main factors) decreasing in the following stages. This meant that other factors not considered in our study increasingly accounted for the observed variability after culture initiation. Our results differed from others in the literature (e.g. Levi and Sink 1991) possibly due to the levels of endogenous hormones and/or sensitivity to the same types and concentrations of exogenous PGRs of the genetic materials used in each study. The best combinations of PGRs revealed significant differences between explants obtained from *in vivo* and *in vitro* donors, with the first having the highest callus growth rates as did spear sections over lateral buds. Histological analyses of developing callus revealed that it originated from the upper third of the spear, in a subepidermic parenchymatous zone with high mitotic activity that surrounded the vascular tissue. The average frequency of embryogenic differentiation was low (< 2%), but with the best factor combination this value increased to 11%, which is similar to the reported for explants taken from minicrowns of seed-derived plantlets (10%, Saito *et al.* 1991), various explant types and genotypes (0-19%, Delbreil *et al.* 1994) and bud clusters (Kohmura *et al.* 1994). The differentiation took place in similar periods of time (70-90 days) for bud clusters; these periods were remarkably shorter than the required when the initial explants were lateral buds and spear sections (above 200 days). As it was also observed by Levi and Sink (1991), lateral buds and *in vitro* crowns formed embryogenic callus more rapidly than did spear sections in a basic medium with low auxin levels.

In summary, important interactions between genotype, explant type and PGRs determined callus performance during the initial culture stages. Thus, despite the constantly growing literature on the subject, ideal conditions for callus induction, growth and embryogenic differentiation should be empirically established for each specific asparagus genotype. The analyses of interactions as the one carried out in our study could be of help in accomplishing this goal.

The hypothesis that somatic embryogenesis would circumvent the difficulties encountered in micropropagation cannot be accepted under the current knowledge. The applicability of published *in vitro* micropropagation protocols, being also dependent on the genotype, has to be ascertained in each breeding program, as has been done in France, USA, Italy, New Zealand and Argentina, among other countries (see previously cited literature).

GENETIC SIMILARITY BETWEEN DONOR AND SOMATIC EMBRYO-DERIVED PLANTS

In asparagus, somatic embryogenesis has not been widely applied for commercial purposes due to the occurrence of inherited variations in genotypes expected to be identical to the donor. This type of variation – that can be observed after a genotype has undergone tissue culture – is known as somaclonal variation (Larkin and Scowcroft 1981). Although protocols have been published for cloning different asparagus genotypes using somatic embryogenesis protocols (see Raimondi *et al*. 2001), somaclonal variation has been reported in only a few of them, for morphology (Kohmura *et al.* 1996) and chromosome number (Araki *et al.* 1992; Odake *et al*. 1993; Kunitake *et al*. 1998).

We tried to assess somaclonal variation with two alternative approaches: use of molecular markers to detect changes at the DNA level and cytogenetic techniques to detect changes in chromosome structure and number. Ours (to our knowledge) was the first report in asparagus on the applicability of RAPD markers as an alternative or complementary method for assessing genetic conformity of embryogenic tissues and derived somatic seedlings to the genotypes of donor plants (Raimondi *et al*. 2001).

The regenerants analyzed were obtained from three distinct embryogenic lines, two of them derived from the same genotype. Morphologically, two plants of the second genotype exhibited a dwarf phenotype similar to the reported by Kunitake *et al*. (1998), whereas the rest of the plants exhibited a true-to-type phenotype. For the molecular analyses, and as a first step, 45 arbitrary 10-mer primers were screened using two independent DNA samples of each donor genotype. Of these primers, only 17 (37.8%) gave consistently reproducible banding patterns among samples and repeated PCR (Polymerase Chain Reaction) runs, and generated 157 consistent bands. Polymorphisms between the two donor genotypes were detected with 16 of the 17 primers, revealing that they were highly polymorphic as expected from the dioecious nature of the species, and that RAPD markers had high discriminatory power in this species. By combining a total of 77 DNA samples from the regenerants and two from the donors and the 17 selected markers, 1343 RAPD profiles were obtained and scored. Despite this large number of samples and markers, no intraclonal variation was observed regardless of whether the phenotypes of the somatic embryo-derived plants were variants or true-to-type.

Cytogenetic analyses were performed in 33 of the 77 regenerants that had been molecularly analyzed to examine if the phenotypic variants were the result of genomic changes (variations in ploidy level that were undetectable by the conventional RAPD analysis) or large chromosomal changes (also not detected with those markers).

Chromosome counts revealed that the two dwarf plants were mixoploid, with diploid and tetraploid cells in both buds and roots. Reuther and Becker (1987) had reported variations in mixoploidy in non-organogenic calluses, but had not detected mosaicisms among the regenerants. In other studies, mixoploidy was detected in regenerants derived from haploid donors (Kunitake *et al.* 1998) and in anther and microspore tissue cultures (Feng and Wolyn 1993a, 1993b). Two phenotypically normal plants derived from one of the donor genotypes that were regenerated after a long period of subculture (200 days) were aneuploid ($2n=2x+12=32$ and $2n=2x+14=34$), that exhibited meiotic irregularities such as multivalent configurations, lagging chromosomes in anaphase II, dicentric bridges in anaphase II, and micronuclei and restitution nuclei in telophase II. Kunitake *et al*. (1998) indicated that chromosome variations in embryogenic-derived plants increased with the duration of the subcultures, particularly when the donor plants had low chromosome levels (haploid and diploid). In contrast, the regenerants from the other two lines were chromosomically uniform at all ploidy levels analyzed.

Somaclonal variation is a very complex problem that requires the use of various approaches to be correctly appreciated (Fourré *et al.* 1997). Depending on the species under study, molecular marker analysis can be useful for genetic discrimination and to locate and isolate mutations linked to this type of markers. However, the absence of intraclonal molecular marker polymorphisms, as revealed in our study, is not a warrant of genetic stability because morphological, epigenetic, genomic and chromosomal changes may remain undetected. For this reason, it is advisable to use complementary approaches, such as morphological and

cytogenetic analyses, to obtain more reliable information than the generated with molecular markers alone. Even though the RAPD analysis in our study was performed rigorously and only consistent band patterns were taken into account, other molecular markers available today, such as AFLP and microsatellites, should yield consistently reproducible band patterns without the need to screen (and discard) a large set of primers.

IN VITRO EVALUATION OF LANDRACES FOR RESISTANCE TO *FUSARIUM PROLIFERATUM*

Major constrains in screening for *Fusarium* resistance are the quantitative control of the trait (Ellison 1986), the genetic variability of the pathogen (various species and specific forms as well as asexual, sexual and parasexual modes of reproduction), and the dioecy and perennial nature of the crop. In addition, field and greenhouse screening techniques for this pathogen require large spaces, have high labor costs and can give inconsistent results due to cross-contaminations (Stephen and Elmer 1988). In contrast, *in vitro* assays in controlled environments allow the measurement of even slight quantitative differences in polygenically controlled resistance and the handling of large number of individuals in small spaces (Wenzel 1985; Bolik *et al*. 1986; McCoy 1988) with a reduction of costs and an increase in efficiency, provided the *in vitro* results have good correlation with the behavior of plants in the field.

As previously stated, truly resistant cultivars are not available in the market, however some of them are considered "tolerant" because, even though they exhibit various degrees of susceptibility to the pathogen, the reduction in yield is not economically relevant. To search for a source of *Fusarium* resistance/tolerance, the genetic variability of three asparagus populations of different origins (two land races of Southeastern Buenos Aires province and one commercial cultivar, 'UC 157 F_1') were screened using an *in vitro* test, and the genetic gains expected under two recurrent selection methods were estimated (Lassaga *et al*. 1998).

Seedlings of 69 half-sib families (HSF) from each land race and 60 HSF from the cultivar, grown in test tubes in a controlled environment (26-28°C and 16-h photoperiod), were inoculated with a 0.5 ml suspension of 10^7 conidia/ml of *F. proliferatum* and visually scored 30 days later for percentage of affected root tissue. Due to the complexity of the breeding problem, the study was carried out with a single-spore isolate under the following assumptions: (1) that the resistance to a toxic metabolite from one *Fusarium* species was accompanied by a general increase of the resistance to other species of the same fungal complex and (2) the average percentage of affected tissue *in vitro* was a good indicator of the general behavior of the plant in the field.

Estimated variance components and genetic variation coefficients for the land race populations were rather similar and higher than for the cultivar, as expected because the first two are open-pollinated populations and the cultivar is the open-pollinated progeny of a clonal hybrid. Expected gains were calculated according to two selection methods, individual in both sexes and combined within and between HSF. A greater progress in breeding was expected from the land race populations because of their estimated additive variances (221.86 ± 111.55 and 325.56 ± 128.27 versus 86.72 ± 78.38).

Although the advantages of using *in vitro* selection techniques seem to be clear, it is necessary to establish the correlation between the *in vitro* and *in vivo* performances before making a recommendation on the technique(s) to be used in breeding programs. In fact, and in further studies (not published), the genotypes that, *in vitro*, had the lowest percentage of affected tissue, actually developed the disease in the field. It is important to point out in this regard that, once a selected genotype is transplanted in the field, new interactions occur since the environment can affect the plant, the pathogen and their interactions.

GAMETOPHYTE SELECTION FOR RESISTANCE TO *FUSARIUM OXYSPORUM* F. SP. *ASPARAGI*

Provided that some adaptive traits are determined by genes that are expressed in both the sporophytic and the gametophytic generations (reviewed in Ottaviano and Mulcahy 1989), the utilization of a selective agent on male gametophytes is likely to be positively correlated with changes in the next sporophytic generation; consequently, the selection process in plant breeding could be accelerated (Hormaza and Herrero 1996). Based on this hypothesis, and on the fact that *Fusarium* spp. produce toxins that are involved in pathogenesis and induce lesions *per se*, we investigated the viability of increasing resistance to *Fusarium* in asparagus with the use of a fungal toxic culture filtrate (CF) as the selective agent on gametophytes. As a preliminary study, and because no information was available in asparagus, the effects of a toxic CF obtained from a *F. oxysporum* f.sp. *asparagi* strain cultured *in vitro* were evaluated on four land race genotypes – two susceptible female genotypes and one tolerant and one susceptible male genotypes – both *in vitro* and *in vivo* (Pontaroli *et al*. 2000). *In vitro*, the CF did not affect either pollen germination or tube growth of the tolerant genotype, but decreased pollen germination of the susceptible genotype as compared with the respective control. This suggested that the percentage of pollen germination in a toxic medium might be correlated with the plant response to the pathogen. *In vivo*, pollen germination and tube growth was negatively affected by the CF in all combinations; furthermore, several abnormalities in pollen tube growth were observed in some combinations. Hence, it was observed that asparagus pollen was not insensitive to the CF *in vivo*.

Gametophyte selection was then evaluated in the asparagus/*Fusarium* pathosystem to determine whether the application of the selective agent *in vivo* could increase selection efficiency (Pontaroli and Camadro 2001). Two susceptible female plants and one tolerant and one susceptible male plant were used in controlled crosses. Before pollination, a drop of a germination vehicle with CF or without it was applied to the stigmas. Some pollinated pistils were fixed and analyzed by fluorescence microscopy; the rest were left on the plant for seed production. Fifty to 200 seeds were obtained per treatment combination (male plant × female plant × pollination vehicle). The derived plantlets were inoculated *in vitro* with a conidia suspension (Lassaga *et al*. 1998) and evaluated for disease symptoms.

As a result, the application of CF to stigmas prior to pollination reduced pollen germination and tube growth as compared with untreated controls, regardless of the genotypes. It also decreased the number of seeds per pollination as compared with the controls, but only when the susceptible genotype was the pollinator. Moreover, the application of CF to the stigma increased both the resistance to *Fusarium* in the progenies and the percentage of selected individuals with respect to the controls only when the tolerant genotype was the pollinator, independently of the genotype of the female parent. This could indicate that the toxic CF affected mainly the male gametophytes, and that the tolerant male genotype was segregating for polygenes conferring resistance.

Our results, in line with those obtained for other traits and species (reviewed in Ottaviano and Mulcahy 1989 and Hormaza and Herrero 1996), provided the first evidence regarding the feasibility of increasing resistance to *Fusarium* crown and root rot in asparagus by gametophyte selection. However, important issues remain unanswered, i.e. whether the reported response is widely observed in the species, and whether the progenies derived from gametophyte selection and further selected *in vitro* are actually resistant to *Fusarium* when put in the field. Future investigations on this matter are warranted.

INTERSPECIFIC HYBRIDIZATION BARRIERS BETWEEN GARDEN ASPARAGUS AND THE ORNAMENTAL *A. DENSIFLORUS* CV. 'SPRENGERI'

Breeding barriers

The genus *Asparagus* is composed of 150 species that vary in ploidy level, sex expression, morphology, and utility, among other traits. The only source of genetic resistance to *Fusarium* reported in the literature is the ornamental monoecious species *Asparagus densiflorus* (Kunth) Jessop cv. 'Sprengeri' (*spr*, 2n=6x=60) and cv. 'Myersii' (*mye*, 2n=4x=40) (Stephens and Elmer 1988). Both botanical cultivars exhibited hypersensitive cell death in soil inoculation experiments and this response was associated with restriction of fungal growth and activation of two enzymes involved in defense mechanisms: peroxidase and phenylalanine ammonia-lyase (He *et al*. 2001).

Unfortunately, controlled crosses between the two species either directly or involving bridge species have not been successful (McCollum 1988). It has to be taken into account that the process of speciation leads to the development of reproductive isolation barriers that maintain the integrity of species by restricting the flow of genes from one to another. These barriers can be classified into two categories: pre- and post-zygotic. In the first type, pollen does not germinate on the stigma, the pollen tube does not completely traverse the style, or the male gamete does not fuse with the egg, even though the pollen tube reaches the ovary. In the second type of barrier, the embryo, endosperm or both do not develop normally, or if they do, the F_1, F_2 or more advanced segregating generations are either sterile or too weak (Hadley and Openshaw 1980). The breeder who wishes to temporarily break down these barriers should at least investigate their type in order to develop strategies to improve the chances of success. Therefore, we carried out studies to identify the pre- and post- zygotic barriers to hybridization in asparagus.

To study the prezygotic barriers, controlled intra- and inter-specific crosses were made using 32 plants of two commercial cultivars of *off* grown in Argentina, 'UC 157 F_2' and 'Argenteuil', and 17 plants of three introductions of *spr*, from the USA, Africa and local nurseries, following an incomplete diallel mating design.

Since it was difficult to synchronize the flowering periods of all these plants, pollen stored at low temperatures had to be used in some pollinations. Thus, previously, we carried out an experiment to determine the period of time over which pollen retains its viability, as estimated by *in vitro* germination. Based on this experiment, stored pollen could be used during most of the flowering period; however, for storage periods of over 1 month viability had to be checked before pollination, because the rate of viability decline turned to be genotype-dependent in asparagus (Marcellán and Camadro 1996a).

The examination of pollinated pistils via fluorescence microscopy revealed pollen-stigma and pollen-style incompatibility reactions which prevented F_1 zygote formation; however, pollen tubes were seen growing among the ovules in most of the interspecific crosses (Marcellán and Camadro 1996b). Since seeds were not formed, a stronger post-stylar barrier was involved in pollen-pistil compatible genotypic combinations.

To study this post-stylar barrier, intra- and inter-specific crosses were made using 25 plants of *off* and 29 plants of *spr*. Pollinated pistils were fixed at different intervals from 1 h to 15 days after pollination and, after processing, serial sections were microscopically examined. Events in the embryo sacs of intraspecific crosses were used as controls. In interspecific crosses, fertilization took place 8 to 16 h after pollination but the endosperm failed to develop normally 3 and 5 days after pollination, respectively, in *spr* x *off* and *off* x *spr* crosses, leading to subsequent embryo abortion (Marcellán and Camadro 1999).

The failure of the endosperm to develop normally is of common occurrence in interploid intra- and inter-specific crosses in some genera such as *Solanum* (Johnston *et al*. 1980; Camadro and Masuelli 1995), *Lycopersicon* (Cooper and Brink 1945), *Avena* (Nishiyama and Yabuno 1978), *Gossypium* (Stephens 1942), *Zea* (Cooper 1951), *Brassica* (Nishiyama and Inomata 1966), *Triticum* (Watkins 1927 in Brink and Cooper 1947). The collapse of the endosperm has also been observed in 2x × 4x crosses in *off* (Wagner and Ellison 1964).

In vitro embryo rescue to circumvent post-zygotic barriers

In vitro immature embryo rescue, by culturing zygotic embryos, ovules or ovaries in a medium that substitutes for the collapsed endosperm, appears to be a very promising technique to circumvent endosperm barriers and produce interspecific hybrids. In fact, there have been several examples of successful application of this technique over the last 50-60 years, in genera such as *Brassica* (Inomata 1977, 1979), *Glycine* (Broué *et al*. 1982), *Helianthus* (Espinasse *et al*. 1991; Serieys 1992), *Lilium* (Van Tuyl *et al*. 1991; Lim *et al*. 2008) and *Lycopersicon* (Alexander 1956 in Raghavan 1976) to cite a few examples. Since no information was available on the use of this technique in *Asparagus*, we carried out experiments to test *in vitro* embryo rescue. Both ovule and ovary culture were attempted in this work because the rescue of interspecific embryos in *Asparagus* has to be done early, when the embryos are very small (25 µm long in the *spr* × *off* crosses and 35 µm long in the *off* × *spr* ones) and difficult to manipulate. In addition, the *off* × *spr* direction was preferred to the *spr* × *off* one, due to the larger ovule size of *off*, the greater degree of embryo development before abortion and the lack of cross-incompatibility at the pollen-pistil level. Controlled interspecific crosses were made and 2,032 ovules and 826 ovaries were cultured three days after pollination under various culture media and incubation conditions. Ovaries cultured for 60 days became red (similar to mature fruits), but seed formation was incomplete. Transfer of ovules to other media was necessary to promote embryo development. The interspecific embryos increased their length from 35 µm at the initiation of culture to 1,900 µm after 120 days of culture, but seedlings were not obtained. Histological studies revealed differentiation of protoderm only. The failure of the embryos to complete differentiation and morphogenesis was probably due to a lack or unbalance of critical metabolite(s) in the media or to a genetic cause (Marcellán and Camadro 2000).

Recent molecular phylogenetic studies have provided new insights into relationships among *Asparagus* species. The first studies carried out by Lee *et al*. (1997) and Stajner *et al*. (2002) already revealed that *off* and *spr* belong to distinct monophyletic groups. These studies were performed on the basis of restriction fragment length polymorphism (RFLP) analysis of chloroplast DNA (cpDNA) and nuclear DNA internal transcribed spacer region (ITS), respectively; but they had some limitations such as low number of sample species and characteristics studied, and the lack of appropriate outgroup taxa. Fukuda *et al*. (2005) using cpDNA sequences applied to a greater number of species, drew a more complete picture about the taxonomy and evolution of the genus *Asparagus*. More recently, Ito *et al*. (2008) constructed a phylogenetic tree of *Asparagus* species based on ITS, in which *spr* was placed at the most basal position in the genus. This could explain our failure to obtain interspecific hybrids between *off* and *spr*. These authors also showed that *off* was closely related to species such as *A. schoberioides*, *A. kiusianus* and *A. maritimus*, and that hybrid descendants could be obtained with these species. However, whether these hybrids are useful to incorporate resistance to *Fusarium* is still unknown.

INDUCTION OF SOMACLONAL VARIATION AND *IN VITRO* SELECTION FOR RESISTANCE TO *FUSARIUM PROLIFERATUM* TOXINS

Among the strategies adopted to address the complexity of the asparagus/*Fusarium* pathosystem and to facilitate the breeding process, we investigated the development of *in vitro* selection techniques. These techniques have allowed the achievement of significant progresses in other species and diseases (reviewed in Crinò 1997, and Remotti 1998 among others). Many of them aim to exploit somaclonal variation, an otherwise undesirable phenomenon when cloning elite genetic materials through micropropagation or somatic embryogenesis, as it was discussed earlier in this review. However, there was little information as to whether useful somaclonal variation could be induced and selected for increasing *Fusarium* resistance in asparagus. Therefore, as a first step, we adjusted callus growth and organogenesis (i.e. shoot and root development) from asparagus long-term callus cultures to establish a suitable protocol for a prospective *in vitro* selection program (Pontaroli and Camadro 2005a). Two elite clones of cultivar Argenteuil, both susceptible to *Fusarium* (the same as used by Raimondi *et al.* 2001), were used in this study. After initiating callus cultures from spear sections in media with different auxin levels, an adequate growth rate and appearance of calluses was obtained when using 1.5 $mg.l^{-1}$ 2,4-D in the media. Shoot primordia induction from >18-months-old calluses was evaluated on several media, varying in auxin and cytokinin type and concentration, sucrose, agar and salt concentration, and inclusion of antioxidants. As a result, the percentage of shoot primordia induction ranged from 0 to 89%, and the average number of shoot primordia per callus, from 0 to 8.6, depending on the media × genotype combination. Once shoot primordia were induced and shoots developed, several different rooting media were tested. The percentage of root induction varied between 0 and 100%, again depending on the media × genotype combination. Overall, a suitable protocol for the establishment of long-term callus cultures and further plant regeneration by organogenesis was produced for two of our elite breeding genetic materials. In parallel with the results obtained in our laboratory when adjusting the micropropagation and somatic embryogenesis techniques for mass propagation of such materials (Raimondi *et al.* 1995, 2001), important medium × genotype interactions were detected here, adding evidence to the need of adjusting *in vitro* culture protocols for specific asparagus genotypes.

Before applying *in vitro* selection it is essential to identify the conditions which favor somaclonal variation and also to characterize such variation. Whereas information on somaclonal variation in asparagus was available for plants regenerated by somatic embryogenesis (e.g. Kunitake *et al.* 1998; Raimondi *et al.* 2001), only a few cytogenetic studies had been published for plants regenerated by organogenesis from long-term callus cultures (Reuther and Becker 1987; Reuther 1990). Based on the hypothesis that organogenesis favors the occurrence of somaclonal variation as compared to somatic embryogenesis (Duncan 1997), we considered that the results obtained by Raimondi *et al.* (2001) would not necessarily apply to our situation, even though the same genotypes were used in both their and our works. Therefore, somaclonal variation in plants regenerated by organogenesis from long-term cultured calluses (Pontaroli and Camadro 2005a) was characterized by plant phenotype, ploidy, meiotic behavior, pollen viability, fruit and seed set, and amplified fragment length polymorphism (AFLP) profiles (Pontaroli and Camadro 2005b). Phenotypic deviations from the donors were detected in foliage color, flower size, and cladode and flower morphology. Ploidy changes were observed in 37.8% of the 37 regenerants studied. Meiotic alterations in 12 out of 21 regenerants included laggards, dicentric bridges, micronuclei, restitution nuclei and polyads. Of the 408 AFLP markers screened in 43 regenerants and the donor clones, 2.94% showed polymorphism between regenerants and their respective donor clone. High pollen viability was observed in the 22 regenerants analyzed. All crosses between one female plant and 35 regenerants, as well as the controls, produced fruits and seeds; however, no plump seeds resulted in 35.3% of the crosses with regenerants, and no seeds germinated in 12.5% of those with apparently normal seeds. Fruit and seed set was similar in crosses with diploid regenerants with normal meiosis and the controls but was lower in crosses with diploid and polyploid regenerants with abnormal meiosis.

In some cases, differences in several to all the traits studied were detected among regenerants derived from the same callus. It is particularly important to consider this variation when regenerating plants from callus after *in vitro* screening because, according to our results, many calluses seem to exhibit a heterogeneous (epi)genetic and/or chromosomal constitution after long-term culture that can eventually be manifested in the regenerants.

In summary, the different tools used by us in this study (Pontaroli and Camadro 2005b) proved useful and reliable for assessing somaclonal variation in asparagus, and the conspicuous variability detected led us to conduct further research on the establishment of an *in vitro* selection program (Pontaroli 2005).

An obvious prerequisite for carrying out *in vitro* selection is the availability of an effective selective agent. In asparagus, it had been shown that the culture filtrate (CF) of *F. oxysporum* f.sp. *asparagi* displayed toxicity to *in vitro*-grown plantlets (Nik 1993), mesophyll cell cultures (Jullien 1988) and pollen (Pontaroli *et al.* 2000, described earlier in this review). However, we did not know whether a CF obtained from *F. proliferatum*, the other *Fusarium* species we were working with, would display such toxicity. Therefore, a CF of *F. proliferatum* was characterized regarding its mycotoxin content and toxicity to asparagus *in vitro* cultures (Pontaroli 2005). The CF, in which fumonisin B1 (0.88 μM) was detected, (1) diminished callus induction, fresh weight and growth rate, and increased the percentage of callus area with necrosis; (2) was highly toxic to cell suspensions; and (3) induced typical symptoms in roots of *in vitro*-grown plantlets. This demonstrated that the CF contained metabolites and/or toxins involved in pathogenesis. This assertion was reinforced by the fact that fumonisin B1, a toxin present in asparagus plants naturally infected with *F. proliferatum* (Logrieco *et al.* 1998), and known to produce necrotic lesions in several species such as tomato and corn (Lamprecht *et al.* 1994) and *Arabidopsis* (Stone *et al.* 2000), was determined in the CF.

Long-term callus cultures in the presence of CF and plant regeneration from selected calluses were then performed, aiming to increase *Fusarium* resistance in the same two (susceptible) clones used in our previous studies. Fifty-nine calluses of clone '265' and 115 of '357' were selected after the culture period; of these, respectively seven and 26 showed stability in their resistance to the CF. Similar results were reported in other pathosystems, as cucumber/*F. oxysporum* f.sp. *cucumerinum*, tomato/*F. o. f.sp. lycopersici* R1 and alfalfa/*F. o. f.sp medicaginis* (reviewed in Remotti 1998).

Plant regeneration was attained from two selected callus lines of clone '357' and from three control lines (one from '357' and two from '265'). These plants, along with the donor clones, were tested for *in vivo* resistance to the pathogen after inoculation with a conidial suspension (Lassaga *et al.* 1998). The percentage of root area with symptoms did not significantly differ, on average, between lines and donor clones, contrarily to what was observed in other pathosystems (see reviews by Crinò 1997, Duncan 1997 and Remotti 1998, among others). Callus resistance to the CF and plant response to the fungus *in vivo* could be only partially correlated. As the CF contained at least some toxin(s) related to pathogenesis, it could be hypothesized that the surviving calluses were resistant to these toxin(s), as were likely the derived plants. However, the action of other mechanisms of fungal pathogenesis *in vivo* could have masked

this resistance and resulted in a susceptible phenotype. Callus selection for resistance to fungal metabolites not related to pathogenesis but present in the CF could have also occurred (Remotti 1998). Although there are several reports on the generation of pathogen resistant materials through *in vitro* selection in a high number of species, it could be additionally hypothesized that callus resistance to the CF and plant response to the fungus *in vivo* are independent characters.

Even though no significant differences in the average percentage of root area with symptoms were detected between lines and donor clones, an important variation between replications was observed in some of the lines, showing that, if analyzed individually, some of those regenerants had a much more resistant phenotype than the donor clones. The experimental error was minimized by a strict environmental control and an adequate design; rather, it is feasible that the regenerants derived from the same line were not precisely 'replications' (as they were considered in this study), but exhibited a certain level of (epi) genetic and/or chromosomal variation, as it was observed by Pontaroli and Camadro (2005b). Overall, our results somewhat discourage the use of *in vitro* selection for *Fusarium* resistance in asparagus, at least under the experimental framework applied in our studies. However, these studies provide original information that corroborates the extreme complexity of the pathosystem *Asparagus officinalis/F. proliferatum*, and contributes to the design of strategies for increasing disease resistance. Future research should focus on specific studies that help elucidate and characterize the nature of the plant-pathogen interaction.

CONCLUDING REMARKS

Asparagus breeding is a long process when conventional methods are used because the species is dioecieus and, being also a perennial, field evaluations have to be conducted for several years to obtain reliable data on the expected performance over longer periods of time. Notwithstanding, asparagus breeders can take advantage of the two modes of reproduction of the species and a handful of already tested biotechnologies both to introduce and/or generate genetic variability (*in vitro* embryo rescue after wide hybridization, callus induction for selection and/or generation of somaclonal variation) and to clone elite genotypes (micropropagation and somatic embryogenesis). In doing so, it is important to take into account that (a) important genotype × environment interactions occur *in vitro*, establishing the need to adjust already published protocols to specific genotypes; (b) the asparagus / *Fusarium* pathosystem is very complex, and (c) the *in vitro* results do not necessarily correlate with the *in vivo* behavior.

The *in vitro* techniques described in this paper can be carried out with simple facilities and equipment. They should be incorporated into breeding programs if the advantages they entail (high multiplication rates of elite genotypes in small spaces, production of pathogen-free planting genetic materials, possibility of generation of genetic variability not present in the crop) outcome the disadvantages they could pose by genotype × environment (medium composition and culture conditions) interactions (with strong interactions, the time required to adjust a protocol could demand one full time person for an undetermined period of time).

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