Plant Tissue Culture and Breeding of Asparagus

Subjects: Horticulture

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In vitro plant tissue culture and biotechnology used to assist and support the development of plant breeding when classical methods of propagation must be accelerated or it was necessary to overcome barriers inaccessible by classical approaches. In asparagus, to improve multiple breeding tasks, a high number of *in vitro* methods have been used, such as plant regeneration methods through organogenesis, embryogenesis, manipulation of ploidy, protoplast isolation, genetic manipulation (protoplast fusion, genetic transformation), embryo rescue and germplasm preservation (*in vitro*, *in vitro* slow growth, cryopreservation). Plant tissue culture methods can overcome multiple problems in asparagus breeding such as, barriers of self and cross-incompatibility between asparagus species through embryo rescue of interspecific hybrids and protoplast fusion or genetic transformation, introgression of new genes, clonal propagation of elite genotypes of asparagus, mass screening, and the generation of haploid and polyploid genotypes, among others, becoming the tool of choice for asparagus breeding programs. Some of these *in vitro* methods are still under development.

Keywords: electroporation ; embryo rescue ; genetic transformation ; micropropagation ; ploidy modifications ; protoplasts

1. Introduction

The genus *Asparagus* belongs to the Asparagaceae family and includes approximately 200 species [1]. This genus is native of Europe, Northern Africa, and Western Asia [2], and taxonomically, it is divided into three subgenera: *Asparagus*, *Protoasparagus*, and *Myrsiphyllum* [3]. All the species belonging to the subgenus *Asparagus* are dioecious with a basic number of chromosomes (x = 10), and this chromosomic number varies depending on the species due to changes in the ploidy level. *Asparagus* genotypes with a chromosome number: diploid (2x = 20), triploid (3x = 30), tetraploid (4x = 40), pentaploid (5x = 50), he-xaploid (6x = 60), octoploid (8x = 80), decaploid (10x = 100), and dodecaploid (12x = 120) can be found [4][5]; Castro et al. [6] indicate that this frequent occurrence of ploidy changes by polyploidization in this genera could be a possible evolutionary strategy of asparagus species.

Asparagus species are economically important as ornamental plants, such as *A. asparagoides*, *A. densiflorus*, *A. plumosus*, and *A. virgatus*, as medicinal plants, such as *A. adscendens*, *A. racemosus*, and *A. verticillatus*, and as edible vegetables, such as A. *albus*, *A. acutifolius*, *A. maritimus*, *A. aphylus*, and *A. officinalis* (2n = 2x = 20), the most important species for human nutrition.

Asparagus officinalis is cultivated worldwide, and the world production is estimated at 8.451.689 t/year, China being the world's major producer [7].

The genetic base of the cultivated *A. officinalis* is quite narrow [4] because all modern commercial varieties of asparagus come from a breed of unique origin from the Netherland's population, 'Violet Dutch' [8][9], which becomes a limiting factor for further asparagus breeding.

Commercially, asparagus is propagated by elite seeds obtained by aimed crossing between selected parentals, and sometimes vegetatively, through mechanical division of the plant rhizome to obtain a very limited number of clonal copies from the selected genotypes, but this method is expensive and involves a sanitary risk of spreading diseases (e.g., *Fusarium* sp.) to new plantations [10].

The dioecious character of *Asparagus* makes it impossible to use sexual reproduction for the generation of new elite genotypes, varieties and the emerging interspecific hybrids.

The classic breeding methods to introgress foreign genes through interspecific sexual crossing are also extremely difficult due to the incompatibility barriers existing between *A. officinalis* and *Protoasparagus* and *Myrsiphyllum* species, and even inside the *Asparagus* genera, due to the different ploidy levels between the species [2][6][11][12][13][14][15][16]. Still, there are some wild relatives of *A. officinalis*, such as *A. prostratus*, *A. maritimus*, *A. pseudoscaber*, *A. brachyphyllus*, *A.*

kasakstanicus, *A. tenuifolius*, *A. macrorrhizus*, *A. persicus*, *A. breslerianus*, *A. verticillatus*, *A. kiusianus*, *A. oligoclonos*, that could be a source of genetic variability and new varieties through interspecific hybridization [17][18][19].

The use of biotechnological approaches can overcome these problems, and methods such as immature embryo rescue, micropropagation, and regeneration through organogenesis, embryogenesis, storage, and preservation (cool incubation, cryopreservation) can be applied successfully to obtain and preserve outstanding new genotypes. Due to climate change, there is a growing demand by the asparagus sector for the release of new varieties wearing higher yields and resistant against biotic (e.g., pest and diseases) and abiotic (e.g., drought, arid/hot climate conditions) stresses.

2. Micropropagation

Loo in 1945 [20] published the first report about *in vitro* culture of asparagus, and since, multiple methods for micropropagation of asparagus species have been published. Authors have reported different micropropagation methods for *Asparagus officinalis* L. [21][22][23][24][25][26][27][28][29] and other wild species of asparagus [30][31][32][33][34][35][36][37][38]. According to them, three types of methods have been used: direct organogenesis, indirect organogenesis, and embryogenesis.

2.1. Direct Organogenesis

The high genetic stability of the asparagus progenies micropropagated *in vitro* through direct organogenesis is the most important characteristic of these methods for breeding purposes, resulting in the clonal multiplication of the selected genotypes, always identical to the original elite one [29][36].

The growth and development of asparagus shoots from shoot tips or lateral buds was developed in the 70s and 80s in the 20th century [21][22][23][39][40][41][42][43][44].

The main problem during the micropropagation of asparagus is the induction of rooting. It is species-dependent as the rate of rooting varies from almost null to perfect (e.g., in *A. stipularis* (9%) vs. 100% in *A. cochinchinensis*) in identical conditions [38][45].

Thus, the most researchers working with this micropropagation method focus on obtaining high rates of rooting. High doses of sucrose or glucose (6–7%) combined with NAA and KIN improve rooting success [23][46][47]. The application of plant growth retardants such as ancymidol (ANC) by Chin in 1982 [22] substantially improved the rooting of asparagus shoots obtained *in vitro*, and even today it is the choice of treatment for asparagus root induction *in vitro*. Chang and Peng [48] improved the rate of rooting by supplementing the medium with ANC and high doses of sucrose (6%) and 162 $mg \cdot L^{-1}$ phloroglucinol (PG), reaching a 78% rooting in *A. officinalis*; and in the case of *A. racemosus*, an 85% rooting was reached by supplementing the basic medium with PG alone [49][50].

The development of a new method of micropropagation based on the use of asparagus rhizome buds as primary explants by Encina et al. in 2008 [28][29] has opened a new opportunity for asparagus micropropagation by direct organogenesis. The possibilities for the use of that type of explant were indicated by Aynsley and Marston in 1975 [51], but until 2014, reports of that micropropagation method involving the culture of rhizome bud explants were not published [29]. The method consists of explant dissection, disinfection, and the *in vitro* establishment and incubation of rhizome bud explants of *A. officinalis* in the MS medium supplemented with 0.7 $mg \cdot L^{-1}$ KIN, 0.5 $mg \cdot L^{-1}$ NAA, 2 $mg \cdot L^{-1}$ ANC and 6% sucrose. The rates of shoot growth range from between 70 to 100%, with rooting rates of over 70%. With minor modifications, this method has been used successfully to micropropagate other *Asparagus* species, such as *A. brachyphyllus*, *A. densiflorus* cv. Sprengeri, *A. maritimus*, *A. macrorrhizus*, and *A. pseudoscaber* [36][37]. The rhizome bud explants are versatile and have also been used as initial explants in studies of polyploidization and cryopreservation [52][53].

2.2. Indirect Organogenesis

Methods involving the regeneration of adventitious shoots or full plantlets of asparagus from callus tissues or cells of somatic origin [33][54][55] are normally applied for biotechnological breeding, frequently involving protoplasts cultures, an ideal material to develop works of a mass selection of protoplasts/cells able to tolerate different biotic and abiotic stresses, such as diseases, pests, toxins, extreme climate conditions, soil acidity, etc. [56], to introgress genes in asparagus protoplasts [57], and in studies of regeneration, the heterokaryons through electrofusion of protoplasts obtained from different species of asparagus [10].

The application of organogenesis for asparagus breeding from callus of different origins has been possible due to previous works on plant regeneration of different species, such as *A. robustus* [58], *A. officinalis* [54][59][60], and *A. densiflorus* cv. Sprengeri [33].

The rooting of adventitious shoots regenerated from callus is still a problem. In general, methods of adventitious regeneration in asparagus require a specific rooting step to root the regenerated shoots [27][52][61].

The regeneration of adventitious shoots or full plantlets is a method scarcely used in the micropropagation of selected genotypes due to the possible genetic variability resulting in a high rate of progenies without the parental characteristics, which is unsuitable.

2.3. Somatic Embryogenesis

The use of somatic embryos in asparagus breeding can be the screening against pathogens or toxins and the induction/regeneration of genetically modified cells through biotechnological methods (recovery of homokaryons/heterokaryons products of protoplast fusion, regeneration of mutant or elite cells, or genetically modified genotypes).

The use of somatic embryogenesis in asparagus breeding is burdened by the strong influence of the genotype [25][26][62][63][64][65], making it difficult to obtain an efficient system of somatic embryogenesis with high levels of induction of somatic embryos (SE), maturation, development, rooting and plantlet recovery. Another negative fact of using somatic embryos in asparagus breeding is the low genetic stability of the progenies obtained after the long and aggressive process of the induction of SE.

Studies of somatic embryogenesis have been reported for more than 80 varieties of *A. officinalis* and for some wild species of asparagus such as *A. breslerianus*, *A. cooperi* and *A. densiflorus* cv Sprengeri [34][35][66][67][68], and shoot apices obtained from seedlings recently germinated are the explant of choice to induce somatic embryos, without discarding other types of explants (vg., spear sections, hypocotyls, internodal pieces, protoplasts, bud clusters, *in vitro* stems, roots. and cladodes).

Several authors [24][25][69][70][71][72][73] reported that the method to induce asparagus somatic embryos is transferring embryogenic callus to a medium lacking plant growth regulators (PGR), and that other changes in PGRs (e.g., ancymidol) and/or in carbohydrates levels can improve the growth and maturation of somatic embryos [26][34][63][64][74][75][76][77][78][79][80][81][82][83][84].

The main concern with somatic embryos is the germination: since the 90s, several authors achieved the conversion of the somatic embryos into plants with different degrees of success, but the bottleneck on plant conversion persists [24][25][60][62][63][65][70][72][73][76][77][78][79][80][81][84][85][86].

3. Manipulation of Ploidy

3.1. Anther Culture: Development of “All-Male” Asparagus Varieties

A. officinalis is a dioecious species, generating in nature a sex ratio of 1 male: 1 female in open-pollination conditions. However, male plants present better agronomic traits than female plants: the lack of seeds in females turns them into weeds the farmer must eliminate, and male plants show higher yields, longevity and tolerance to diseases than the female plants [87][88]. For all these reasons, the “all-male” cultivars are very appreciated by farmers.

A unique dominant gene (M), located on the homomorphic chromosome pair L5, determines the sex in asparagus ([89][90]. In diploid asparagus (2n = 2x = 20), the female genotypes are homozygous recessive (*mm*) and the male genotypes are heterozygous (*Mm*). Andromonoecious flowers may be present in some male plants, and the self-pollination of those flowers can produce “super males” (*MM*) that can be used to develop “all-male” cultivars [91][92], just by being crossed with a female genotype, because all the resulting progeny consist exclusively of male plants [93].

That strategy was used to develop “Lucullus”, the first commercial “all-male” variety [94]. However, “super-males’’ are very rare in asparagus populations (less than 2%), and these plants don’t always feature the best agronomic traits for breeding [95]. The introgression of andromonoecy into a good genetic background requires a long time [16][96]. Hence, a faster alternative is necessary to obtain these “super-males’’ from selected males with outstanding agronomic traits. The development of di-haploids (DH) males (MM) through *in vitro* culture techniques offers a solution [97][98]. Moreover, the “all-male” cultivars obtained from “super-males’’’ di-haploids are F_1 hybrids, which are more uniform than the “all-male”

cultivars generated with “super males” derived from the self-pollination of andromonoecious plants [16]. The first F_1 all-male hybrid obtained was “Andreas” [99], and in that case, the “super-male” parent was obtained by anther culture, but asparagus “super-male” have also been obtained with success from the culture of isolated microspores [100][101]. Most authors opted for anther culture [59][91][92][97][102][103][104] because the important technical requirements of the culture of isolated microspores limited the application of this technique. The microspore’s isolation from the anther and the inoculation in a liquid medium are the most challenging stages of this method [105].

The success of anther culture is highly influenced by the genotype used [102][106] and by the developmental stage of the microspores and the anther culture conditions. The late microspore stage, just before its asymmetrical division, is the appropriate developmental stage for microspores to be successful in anther culture in many species [105], including *A. officinalis* [59][100][101][102][103]. However, the appropriate flower bud size to obtain microspores in late microspores is genotype-dependent, and varies from 1 mm to 3 mm, which implies that to succeed in anther culture, it is necessary to run a previous study of the relationship between the flower bud size and the developmental stage of the microspores for each asparagus male genotype [59][100][101][103]. To succeed in anther culture, it is necessary to induce the embryogenic growth pathway in microspores. The beginning of this pathway consists of a symmetric division in microspores instead of the asymmetric division that defines the first pollen mitosis [107][108][109]. To induce this change in microspores’ growth pathway, microspores in the late stages are submitted to different types of physicochemical stress (e.g., high/low temperatures, carbon starvation, chemical inductors, auxins) [110], and these stress treatments are sometimes more important than the use of plant growth regulators in the culture media to succeed in anther culture [59][105][111]. In the case of asparagus, the best stress treatment is a cold pretreatment at 4°C for a week followed by incubation at 32°C for four weeks, being the optimal combination to obtain a high rate of callus proliferation from anthers [58][102][105]. Different protocols have been developed to regenerate plantlets from callus induced from anthers [102][103][106]; however, these protocols are very long and involve several steps, basically the induction of embryogenic callus, induction of somatic embryos, proliferation of somatic embryos, maturation, and germination of full plantlets derived from microspores. The combination of para-chlorophenoxyacetic acid (pCPA) and BA induce shoot and root regeneration together with the callus proliferation [59], shortening the time needed to obtain full plantlets derived from microspores and the number of subcultures necessary for it, and reducing the possibilities of somaclonal variation [112]. The occurrence of endoreduplication is very usual in asparagus cells during the proliferation of callus obtained from anther culture [59][103][106][113], which makes it unnecessary to use polyploidization to obtain dihaploid genotypes from anther culture; even tetraploid and octoploid asparagus have been regenerated from anther culture [59]. The sex of asparagus regenerated from anther can be determined with the sex-linked marker Asp1-T7 while waiting for their flowering [96][114]. However, one of the drawbacks of anther culture is that many of the male plants regenerated are heterozygous, originating from somatic cells present in the walls and filaments of anthers [16][59][115]. Molecular markers (RAPDs [116] and EST-SSRs [59]) have been used to determinate the origin of the callus from which the asparagus has been regenerated (microspore or somatic cells) and to select “super male” genotypes of *Asparagus*.

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