



Review

Aspects of In Vitro Plant Tissue Culture and Breeding of Asparagus: A Review

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Abstract: 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



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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 chromosomal 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 ($5x = 50$), hexaploid ($6x = 60$), 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. aphyllus*, 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 Nether-

land'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 [2,6,11–16]. Still, there are some wild relatives of *A. officinalis*, such as *A. prostratus*, *A. maritimus*, *A. pseudoscaberrimus*, *A. brachyphyllus*, *A. kasakstanicus*, *A. tenuifolius*, *A. macrorrhizus*, *A. persicus*, *A. breslerianus*, *A. verticillatus*, *A. kiusianus*, *A. oligoclonus*, that could be a source of genetic variability and new varieties through interspecific hybridization [17–19].

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. [21–29] and other wild species of asparagus [30–38]. 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 [21–23,39–44].

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·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 $0.7 \text{ mg}\cdot\text{L}^{-1}$ KIN, $0.5 \text{ mg}\cdot\text{L}^{-1}$ NAA, $2 \text{ mg}\cdot\text{L}^{-1}$ 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 [25,26,62–65], 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 [34,35,66–68], 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 [24,25,69–73] 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 [26,34,63,64,74–84].

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 [24,25,60,62,63,65,70,72,73,76–81,84–86].

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 [59,91,92,97,102–104] 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* [59,100–103]. 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 [107–109]. 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 de-

veloped 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*.

3.2. Polyploid Induction

Moreno et al. [5] report that, in general, species belonging to *Asparagus* genera are diploid with a basic chromosome number ($x = 10$). *A. officinalis* L. is diploid ($2n = 2x = 20$), but inside the genus *Asparagus*, several wild species show different ploidy levels, *A. prostratus*, *A. acutifolius*, *A. maritimus* and some landraces of *A. officinalis* such as ‘Violetto d’Albenga’ and ‘Morado de Huetor’ (MH) are tetraploids ($4x$), *A. maritimus* is hexaploid ($6x$) and *A. macrorrhizus* is dodecaploid ($12x$). Moreno et al. [4,5] recorded that the landrace ‘Morado de Huetor’, normally tetraploid, also includes a reduced number of genotypes with non-standard ploidy levels ($2n = 2x, 3x, 5x, 6x, 8x = 20, 30, 50, 60, 80$).

At present, flow cytometry is the method to study ploidy in asparagus [113,117–119].

In *Asparagus* breeding, the different levels of ploidy are both an advantage and an obstacle. The hybridization of genotypes with different ploidy levels for breeding purposes in asparagus [41,120–122], and/or the induction of genotypes autotetraploids, autooctoploids or triploids [59,119,123], sometimes result in the appearing of morphological and agronomical traits that are interesting for asparagus cultivation. Different levels of ploidy also involve diverse problems for asparagus breeding, such as crossing incompatibility between genotypes and genetic instability [11,52,117,124].

Mishiba et al. [125] reported that it is possible to find differences in ploidy level depending on the physical location of tissues. At least in the case of asparagus plantlets growing in vitro, the spatial distribution of the ploidy in vitro is heterogeneous (this is important when it is necessary to maintain polyploid lines induced in vitro). These polyploid genotypes need continuous screening and selection until they reach the stability of polyploid lines [52].

4. Protoplast Isolation and Culture

In *Asparagus* breeding, protoplasts can be used for multiple purposes in different ways: to obtain cellular lines or genotypes with resistance/tolerance to plant pathogens (*Fusarium*) through mass protoplast screening [126], to achieve direct gene transfer by protoplast electroporation [57], to create interspecific somatic hybrid plants through protoplast electrofusion [127], to generate transgenic plants by protoplast electroporation [56], and to induce sex-conversion during protoplast isolation and culture [73].

All these uses of protoplasts in *Asparagus* breeding are supported by previous works and studies focused on the development of efficient systems of isolation, culture and regeneration of full plantlets from protoplasts. Since the 70s of the 20th century, different authors [54,128] developed efficient methods of protoplast isolation with cellulase and

mace-rozime from cladodes and callus, and methods for protoplast development and regeneration.

During the next decades, other authors have developed new systems for protoplast isolation and culture, such as: isolation of protoplasts with pectolyase and cellulase followed by cell division and development of microcalli in agarose droplets on a porous polypropylene membrane [129,130], isolation of protoplasts with Cellulysin, Macerase, and Rhozyme, and regeneration from callus [131–133]. Kunitake and Mii [24] used mace-rozime, pectolyase and cellulase, to isolate protoplasts from embryogenic callus cultures, as later did Mukhopadhyay and Desjardins [71,134], using Cellulysin, Macerase and Rhozyme. Dan and Stephens [55,135] efficiently developed protoplasts through a bead culture method. Kunitake et al. [136] used macerozime plus cellulase, to isolate and develop protoplasts from microspores. Chen et al. [64,137] isolated protoplasts from etiolated shoots with pectinase, cellulase and hemicellulase evaluating the effect of enzymes. Benmoussa et al. [33] isolated protoplasts from the callus of *A. densiflorus* cv. Sprengeri with a mix of Cellulysin, Rhozyme and Macerase, and successfully regenerated full plants from these protoplasts.

5. Genetic Manipulation

5.1. Protoplast Fusion

Protoplast fusion of different species of *Asparagus* is another underutilized in vitro resource in asparagus breeding, as just one study of regeneration of the heterokaryons obtained through protoplasts electrofusion between *A. officinalis* and *A. macowanii* was reported [127]. The goal of that work was to obtain interspecific hybrid genotypes to transfer the resistance to the stem blight caused by *Phomopsis asparagi* from *A. macowanii* to *A. officinalis*. The authors succeeded in the regeneration of an interspecific hybrid between these species, showing the feasibility of the method, but the hybrid line showed some abnormalities such as lack of vigor and problems in flowering.

The systems for protoplast isolation, culture, and regeneration, together with the methods for protoplast fusion, have the potential to be used for breeding purposes and could help to develop new elite genotypes of asparagus, widening the genetic pool of *A. officinalis*, but these methods are technically difficult and at present can only be used on a few species of *Asparagus*.

5.2. Genetic Transformation and Genome Editing

Along with the 90s of the 20th century, several authors developed methods for the genetic transformation of asparagus through *Agrobacterium* [138–140], protoplast electroporation [56], and biolistic systems [141,142], but this line of research stopped for years, until in recent times a novel impulse took place [143–145]. In all these works, the gene transferred was a reporter gene (β -glucuronidase (*uidA*) gene). Chen et al. in 2019 [146], transferred a gene interesting for breeding, the hevein-like gene, to increase the tolerance to diseases such as stem blight in *Asparagus*. Harkess et al. (2020), working with *A. officinalis* and using genetic transformation, were able to change the sex of flowers from male to hermaphrodite (knocking out *soft*), from male to neuters (knocking out *tdf1*) and from male to female (knocking out *soft* and *tdf1*) [147].

Different targeted genome editing strategies, such as CRISPR/CAS9 (clustered regularly interspaced short palindromic repeats-associated 9 endonuclease system), have been recently applied to vegetable species and have an enormous potential in plant breeding [148]; however, limited information was found about genome editing of *Asparagus* sp. [144,148], except for the works on asparagus sex determination of Harkess et al. [149], revised by Renner and Müller in 2021 [150].

6. Immature Interspecific Embryo Recovery

The crossability barriers to interspecific hybridization are well known in *Asparagus*. The problem of self- and cross-incompatibility between *A. officinalis* and *A. densiflorus* cv. Sprengeri was studied by Marcellán and Camadro [12]. The second species is known to

be resistant to several diseases produced by pathogens of the genus *Fusarium*, such as: *Fusarium oxysporum* f. sp. *Asparagi*, *Fusarium proliferatum*, *Fusarium moniliforme* and *Fusarium solani* [10,151]. The authors reported that sometime after fertilization, the endosperm collapsed, and the hybrid embryo died.

There is a technique in vitro, the immature embryo rescue [152], apparently never used in asparagus breeding, that could be applied when fertilization is possible and could be a useful technique to obtain interspecific hybrids of asparagus with outstanding agronomic traits between cross-incompatible asparagus species.

7. Germplasm Preservation

There are three basic systems to preserve the germplasm resources existing in nature and the genetic resources generated by the breeding programs in asparagus species.

7.1. In Vitro Preservation

Cultivated varieties [28,153], wild species [36,154] and asparagus landraces all around the world are at risk of disappearing due to changing habitats and environmental conditions, human activities or simply from the competition with new varieties developed through breeding or biotechnological ways [59,118].

The advances in micropropagation of different asparagus species and genotypes [29,36,37,154] open the possibility to preserve in vitro the asparagus material in safe and controlled conditions, avoiding the biotic and abiotic risks of the in situ or ex situ preservation of asparagus collections in field plantations. The in vitro collections have lesser requirements and fees than the germplasm collections in field repositories and are quite expensive and space- and time-consuming. The seed banks are also difficult to maintain due to the progressive loss of viability of seeds and the expensive method of renovation to obtain fresh seeds [155,156].

The in vitro preservation of endangered *Asparagus* species such as *A. macrorrhizus*, *A. arborescens*, *A. fallax*, *A. nesiotis*, *A. usambarensis*, *A. officinalis* subsp. *rostrates*, *A. plocamoides*, *A. racemosus*, and many other *Asparagus* species only requires the development of specific micropropagation and biotechnological methods able to reduce the growth rate: slow growth/low temperature incubation and cryopreservation [53,157,158], detailed in Sections 7.2 and 7.3.

7.2. In Vitro Slow Growth Preservation

This method is useful for the mid-term preservation of asparagus genotypes, and it involves the slowdown of the development of the plants in vitro. It restricts the availability of nutrients in the culture medium to suboptimal amounts, and/or reduces the light intensity or changes the light spectrum and/or the temperature level of incubation in the culture room, and decreases the metabolic activity of the plants in vitro to a minimum, reducing costs of cultivation and materials. This mid-term preservation method, allows for maintenance for long periods of time without losses of viability and is a very good option to maintain an active collection of germplasm, always available to quickly produce plant material for breeding purposes [154,159,160].

From the 80s of the 20th century, different authors developed methods for slow growth in vitro of asparagus through low-temperature incubation of cellular suspensions [157]; incubation at low temperature of aerial crowns in a medium supplemented with ancymidol and sorbitol, with a perfect rate of regeneration after two years [158]; incubation of shoots at a low temperature in dark conditions with good rates of survival [159]; incubation of *A. racemosus* shoots at a low temperature in a medium supplemented with a high level of sucrose/mannitol/sorbitol [160]; incubation of asparagus shoots in a minimal culture medium, consisting of reducing salts plus sucrose and an osmoticum (mannitol, sorbitol) [154].

7.3. Cryopreservation

This system is appropriate for the non-active collections of germplasm, due to the difficulty and the time necessary to obtain plants from freezing explants in Liquid Nitrogen (LN). This method requires a reduced space for storage, and the costs of maintenance are low. It is adequate for the long-term preservation of germplasm [53,161].

The first works on asparagus cryopreservation were reported in the early 80s of the 20th century and were based on the use of different explants (shoot tips, somatic embryos, embryogenic cells, bud clusters, and nodal sections) and several approaching methods, such as: the use of preculture (sorbitol) and cryoprotectants (PVS) on shoot tips with a survival of 55% [162]; use of a vitrification method with somatic embryos [163]; use of preculture and desiccation methods on stem explants [164]; use of a vitrification method, consisting in a treatment of explants with a vitrification solution (PVS2, PVS3: a mix of glycerol and sucrose) 45–120 min before the immersion in LN. The rates of recovery are high (80–90%) depending on the type of explant used (aerial-bud clusters or embryogenic cells) [165–167]; use of a preculture system on shoot tips and nodal section explants [168,169]; use of a droplet method on shoot tips, consisting of the suspension of the explants in a cryo-protective solution (DMSO) before their immersion in LN, with an average rate of survival of 80% [170]; use of a preculture method with high levels of sucrose on embryogenic cell suspensions [171], and the most recently published method (2018), to use rhizome-bud explants and apply a pretreatment (48 h) with high levels of sucrose (0.3 M) and then encapsulate them in alginate beads and desiccate them until reaching a 35% water content before the pre-freezing treatments (1 h at 0 °C plus 1 h at –20 °C) followed by the immersion in LN. This system is efficient (84% survival, 42% recovery) and the regenerated plants never show somaclonal/genetic variations [53].

The main problem of using cryopreservation to preserve asparagus germplasm is to succeed on the development of explants to obtain full plantlets after the freezing in LN, because freezing is an aggressive method damaging to the plant material. Apparently, the best explants for freezing in LN are the bud clusters (aerial crowns) [165] or rhizome bud clusters, [53] because that explant material allows for a drastic reduction in the time (2 months) of recovery into full plantlets due to the easier rooting of those explants, never needing an additional step of rooting.

Fortunately for asparagus breeding, the last studies of somaclonal variation through ploidy analysis by flow cytometry and EST-SSR markers confirm the lack of somaclonal variation of the recovered material after freezing in LN [53]. This fact validates cryopreservation as an adequate method for the long-term preservation of asparagus genotypes.

The development of reliable, versatile, and efficient methods of cryopreservation appropriate for different species of asparagus will improve the long-term preservation of asparagus in LN at a low cost, which is a big advantage for asparagus breeding and the preservation of this genetic resource.

8. Conclusions and Prospects

The biotechnological breeding of asparagus still needs to reach a higher level of success by developing new technologies *in vitro* and methods focused on the development of new genotypes of asparagus able to face the new challenges in asparagus cultivation (effect of climate change, new and old resilient pests and diseases, quality, productivity, etc.).

There are some tasks waiting for development:

- To increase the number of micropropagation protocols specifically adapted to different wild asparagus species and hybrid genotypes, with special attention to endangered species at risk of extinction to preserve the genetic pool of the asparagus family.
- To develop more efficient, versatile, and reliable methods for the preservation of asparagus germplasm, such as slow growth *in vitro* and cryopreservation methods.
- To overcome the barriers of interspecific sexual incompatibility, allowing the introgression of genes from wild species of asparagus to transfer tolerance to pests and diseases or agronomic traits of interest.

- To develop methods for widening the genetic pool of *Asparagus officinalis* cultivars through the use of landraces and wild species to create interspecific hybrids and generate new varieties able to tolerate and thrive against biotic and abiotic stresses. Some of these methods still to be developed are: intergeneric somatic fusion of protoplasts and the rescue of immature interspecific hybrid embryos, genetic transformation and genetic editing.

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