Review

Plant Biotechnology, 15(2), 51-61(1998)

Somatic Embryogenesis and Its Application for Breeding and Micropropagation in Asparagus (*Asparagus officinalis* L.)

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Received 10 February 1998; accepted 20 March 1998

1. Introduction

Asparagus (*Asparagus officinalis* L.) ($2n=20$) is a monocotyledonous perennial species belonging to the Liliaceae family, and is cultivated as a vegetable. Plants of asparagus are usually raised from seeds and cultivated continuously in the field for 10 years or more. Young stems, known as spears, emerging through the ground are harvested as the edible part from the plants from spring to autumn in Japan.

Asparagus originates from the Mediterranean area and is especially well adapted to mild and sunny climates and sandy soil. Recently, its production has increased year by year in several countries as one of the most refined and healthy vegetables. Nowadays, the main producers are USA, Spain, France and Taiwan. White asparagus production predominates in Europe (France and Spain) and Taiwan, while green asparagus is mainly produced in North America, Australia and Japan.

The increment of the cultivation area has increased the demand for the uniformity of the plants cultivated. In asparagus, a population of any cultivars exhibits a high degree of natural heterozygosity causing very irregular yield, since asparagus is a cross-pollinated dioecious species with a sex ratio of 1: 1. Therefore, many attempts at clonal propagation of elite stocks have been made using shoot-tip culture. However, the propagation efficiency is not high and the procedures used are very labor intensive.

Another problem recently encountered is a disease syndrome known as asparagus decline, primarily caused by *Fusarium oxysporum*, *F. moniliforme* and *Phomopsis asparagi*. These diseases are found throughout the world, and decrease annual yields of asparagus. As disease-resistant germplasms, *Asparagus densiflorus*, an ornamental species, was reported to be resistant to *F. oxysporum* and *F. moniliforme*[1], and *A. macowanii* to *Phomopsis asparagi*[2]. However, sexual cross of *A. officinalis* with these resistant species have been unsuccessful, probably due to incompatibility barriers[3, 4].

Recently, plant biotechnologies have developed into very powerful tools for the micropropagation, crop breeding and physiological studies. Particularly, somatic embryogenesis induced by tissue culture has a great potential for rapid and efficient clonal propagation, and embryogenic calli are promising materials for protoplast culture, somatic hybridization and genetic transformation.

Asparagus is the first monocotyledonus species which showed regeneration of plantlets *in vitro* through somatic embryogenesis, and the embryogenic callus culture was first produced from the hypocotyls of sterile seedlings[5]. Since then, many studies have been conducted on the culture requirements for somatic embryogenesis, and asparagus has been chosen as one of the model plants in many laboratories. The embryogenic callus has efficiently been utilized as the source of protoplasts from which plant regeneration was achieved[3, 6, 7]. Furthermore, interspecific somatic hybrids and genetically manipulated plants have been produced using the protoplast culture system[4, 8].

Although fundamental studies have been made on micropropagation and breeding of asparagus using somatic embryogenesis, several problems remained to be solved. In this article, therefore, we describe several important factors responsible for the induction of normal somatic embryogenesis and plant regeneration of asparagus, and discuss the application of somatic embryogenesis for micropropagation and breeding using somatic hybridization and genetic transformation.

2. Somatic embryogenesis

Somatic embryogenesis is the process by which somatic cells develop into differentiated mature embryos through characteristic embryological stages without fusion of gametes. Somatic embryos, which are morphologically and physiologically similar to zygotic embryos, develop bipolarity with vascular system at an early stage attain[9, 10]. In this review, we defined the term 'embryogenic calli' as the calli

with the ability to initiate somatic embryos on a specific medium.

2. 1 Induction of embryogenic calli

Somatic embryogenesis of asparagus was first reported in 1968 by Wilmar and Hellendoorn[5] who described hypocotyl-derived callus forming a large number of dense, globular embryo-like bodies when placed on Linsmaier and Skoog (LS) [11] medium containing 1.0 mg/*l* 2,4-D and 0.315 mg/*l* kinetin. Since then, numerous reports have been published on somatic embryogenesis of asparagus. The culture conditions of asparagus so far reported are summarized in **Table 1**, that includes the following important factors for inducing the embryogenic calli.

2. 1. 1 Visual selection

In asparagus, 2 types of calli are usually induced at the callus induction steps from shoot tips on Murashige and Skoog (MS) medium [12]containing 2,4-D. Primary calli with compact and yellow appearance are induced from these explants after 1 month of culture, and embryogenic calli secondarily emerge from the surface of primary calli. Embryogenic calli of asparagus have a yellowish-white, friable and nodular appearance. Careful visual selection is required to establish the callus lines with high potential of somatic embryo formation.

2. 1. 2 Explants

The most suitable explants to induce embryogenic calli of asparagus are tissues or organs with shoot meristems such as internal bud, lateral bud and bud clusters. Up to date, embryogenic calli have also been obtained from hypocotyl, stem, cladodes, internode and immature embryo. Although the use of immature embryos results in a high frequency of embryogenic callus formation, the calli are not genetically identical to the mother plants because of the heterozygous nature of this crop. The calli originated from seedlings with unknown genetical traits are usually not suitable as the material for breeding through somatic hybridization and genetic transformation. The other explants are not used as the source for inducing embryogenic calli due to the low efficiency of the induction.

2. 1. 3 Genotype

In genus *Asparagus*, induction of embryogenic calli has been reported only in 2 species of *A. officinalis* L. and *A. cooperi* Baker [33], although there are many species including ornamental plants. In *A. officinalis*, induction of embryogenic calli has been reported in many cultivars and selected superior clones (**Table 1**). However, not all of the genotypes showed the ability to induce embryogenic calli. Delbreil and Jullien [23] described that differences were observed for the frequency of embryogenic callus induction from shoot apices among 12 genotypes. Li and Wolyn [28]reported that embryogenic calli were induced from only 2 among 5 genotypes. We also observed that embryogenic calli were induced from crown buds in 5 cultivars among the 10 leading cultivars in Japan, and that the induction frequencies were 2.1～28.0% (unpublished data). Differences were also shown for the frequency of embryogenic callus induction among several polyploids (haploid to tetraploid) (Kunitake *et al.* [34]).

2. 1. 4 Growth regulators

Generally, synthetic auxins such as 2,4-D, picloram and dicamba have been used for induction of embryogenic calli in many monocotyledonous plants. In asparagus, a type of auxin is also an important factor and addition of only 2,4-D is effective for efficient induction of embryogenic calli. While, Odake *et al.* [22] reported that embryogenic calli were induced from internode segments on MS medium containing 3 mg/*l* NAA and 1 mg/*l* kinetin, and that most of the regenerated plants were tetraploids. They suggested that the use of NAA and kinetin might be responsible for chromosome doubling in the induced embryogenic calli. Therefore, growth regulators in media used for both callus induction and subculture must be carefully examined to avoid the occurrence of somaclonal variations for applying the embryogenic callus culture system to micropropagation and genetic manipulation.

2. 2 Induction of somatic embryos

Detailed morphological analysis of asparagus somatic embryogenesis in contrast with zygotic embryogenesis has already been described by Bui Dang Ha *et al.* [35] and Levi and Sink [36]. They revealed that developmental process of somatic embryos in asparagus is a typical one which is observed in monocotyledonous plants. Proembryos induced from embryogenic calli become mature embryos through several stages such as globular embryos, elongated forms and cotyledonary embryos (banana-shaped embryos) (**Fig. 1**). Intensive studies on induction of somatic embryos have been carried out using embryogenic calli of asparagus, and modification of culture media and several treatments have contributed to major advances in the normalization of somatic embryo development. Several factors such as growth regulators, carbohydrates, and partial desiccation (concentration of gelling agents and relative humidity) were revealed to be important for inducing normal somatic embryo formation.

2. 2. 1 Growth regulators

In asparagus, somatic embryogenesis can be induced by transferring embryogenic calli to plant growth regulator-free medium, which has been used

Table 1. Culture conditions reported for somatic embryogenesis and plant regeneration of *Asparagus*.

Genotype	Explant	Basal medium	Growth regulators and other factors			References
			Callus induction	Embryo induction	Germination	
—	hypocotyl	LS*1	1 mg/*l* 2,4-D −0.315 mg/*l* K	0.1 mg/*l* K	—	[5]
XX, YY varieties	shoot segment	LS	1 mg/*l* NAA +1 mg/*l* K	1 mg/*l* IAA +0.1 mg/*l* BA	HF	[12]
Haidel	immature seed	MS*2	2 mg/*l* 2,4-D	HF*4	1/2MS	[13]
A-9(male)	lateral bud	MS	0.5 mg/*l* NAA+ 0.2 mg/*l* 2iP	1.5 mg/*l* NAA+ 0.3 mg/*l* 2iP (0.15M suc)*5	1.5 mg/*l* NAA+ 0.3 mg/*l* 2iP (0.28M fru)	[14]
MW500W Poultom	stem	MS	0.2 mg/*l* 2,4-D	HF (0.15～0.3M suc)	0.02 mg/*l* zeatin	[15] [16]
MW500W	crown or young spear	LS	0.1 mg/*l* 2,4-D (filtrated cultures)*3	HF (1% gellan gum)	1/2LS(HF) (Vessel with aseptic ventilative filter)*7	[17]
Welcome	internode root	MS	2 mg/*l* 2,4-D +1 mg/*l* 2iP	HF (0.025M suc) (0.8% gellan gum)	1/2MS(HF) (>0.01M suc) (Vessel with Milliseal)	[18]
M.S.U. male C3	crown lateral bud	MS	10 mg/*l* NAA	—	1～10 mg/*l* NAA +0～1 mg/*l* K	[19]
male M.S.U. 88-10	stem	MS	0.01 mg/*l* NAA +0.03 mg/*l* 2iP	0.01 mg/*l* NAA +0.02 mg/*l* 2iP (0.24M suc or 0.33M fru or glu)*6	0.01 mg/*l* NAA +0.02 mg/*l* 2iP (0.06M suc or 0.11M fru or glu)	[20]
Haidel	immature embryo	MS	2 mg/*l* 2,4-D (filtrated cultures)	HF (1% gellan gum)	1/2MS(HF) (cold pretreatment)*8	[21]
MW500W	internode	MS	3 mg/*l* NAA +1 mg/*l* K	HF	HF	[22]
F1 hybrids, XX(511A)	lateral shoot	MS	1 mg/*l* NAA	HF	—	[23]
Hiroshima green	bud clusters	MS	0.2 mg/*l* 2,4-D	HF	HF	[24]
6 clones, 7 hybrids 511A	cladodes crown	MS	10 mg/*l* NAA or 1 mg/*l* NAA+ 0.1 mg/*l* zeatin (or BA)	HF	HF	[25]
Welcome	crown	MS	2 mg/*l* 2,4-D (filtrated culture)	HF (0.1M maltose) (1% gellan gum)	1/2MS(HF) (cold pretreatment) (CO_2;1,500 μmolmol^{-1}) (light;150 μmol·m^{-2}s^{-1}) (Vessel with Milliseals)	[26]
G447	stem	MS	1 mg/*l* 2,4-D	0.1 mg/*l* NAA +0.5 mg/*l* K (0.12M suc)	0.1mg/*l*NAA+0.1mg/*l*K +0.75 mg/*l* ancymidol +40 mg/*l* adenine sulphate +0.17 mg/*l* sodium phosphate (0.24M suc)	[27]
Jersey Giant G203, G447	stem	MS	1 mg/*l* 2,4-D	800 mg/*l* glutamine +500 mg/*l* casein acid hydorolysate+0.75 mg/*l* ancymidol+0.1 mg/*l* NAA +0.5 mg/*l* K (0.12M suc)	800 mg/*l* glutamine +500 mg/*l* casein acid hydorolysate+0.75 mg/*l* ancymidol−0.1 mg/*l* NAA +0.5 mg/*l* K (0.09M suc)	[28] [29] [30]
Welcome	crown	MS	2 mg/*l* 2,4-D (filtrated culture)	HF (0.1M maltose) (1% gellan gum)	1/2MS(HF) (cold pretreatment)	[31]

Culture conditions of somatic embryogenesis in protoplast culture were not listed in this Table. *1Murashige and Skoog [32] *2Linsmair and Skoog [11] *3Embryogenic calli were sieved through mesh and were transferred to embryo induction medium. *4HF; Plant growth regulators-free medium *5Conditions on carbohydrates and gelling agents were described in the cases where they were particularly important. *6suc; sucrose, glu; glucose, fru; fructose *7Culture vessels with aseptic ventilative filters or Milliseals were used for promoting the growth. *8Somatic embryos were pretreated at 4°C for 7 to 14 days.

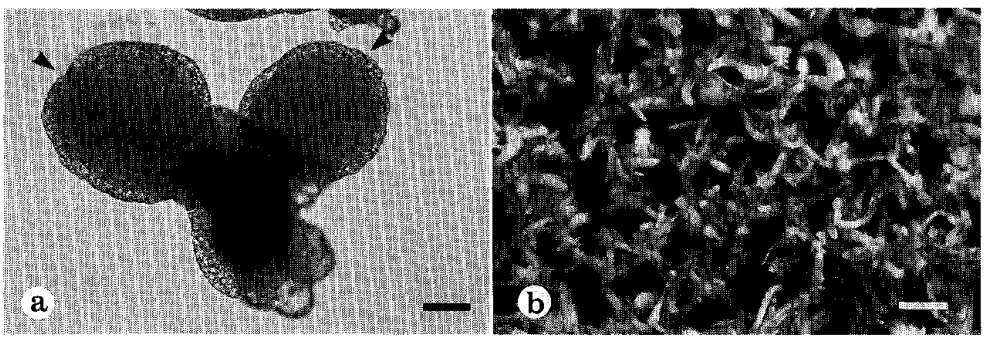

Fig. 1 Development of somatic embryos from embryogenic calli of *Asparagus officinalis* L. (Kunitake *et al.* [31])
a) Proembryos 7 days after transfer to the embryo induction medium (arrowheads). Bar=100 μm.
b) Mature embryos (banana-shaped embryos) 30 days after transfer to growth regulators-free MS medium containing 1.0% gellan gum. Bar=2 mm.

for micropropagation [15-17, 24]. However, several reports showed superior effects of plant growth regulators for enhancing somatic embryogenesis. NAA at 1 mg/*l* or 10 mg/*l* enhanced the formation and development of somatic embryos [19]. The combination of 0.1 mg/*l* NAA with 0.75 mg/*l* ancymidol in the embryo induction medium produced the somatic embryos with high germination rate [30]. Furthermore, inclusion of ancymidol and ABA in the embryo induction medium was more effective than uniconazole and paclobutrazol for enhancing somatic embryo formation, and the effect of ancymidol on somatic embryo formation was affected by sucrose and NAA concentration [28]. Considering these published results, plant growth regulators might be effective for normalization of somatic embryo formation. However, the occurrence of somaclonal variations has been observed to increase by adding plant growth regulators in embryo induction medium. Relation between plant growth regulators and somaclonal variation in asparagus tissue culture is unclear. Further studies would be needed for the practical use of somatic embryogenesis.

2.2.2 Carbohydrate requirements

In tissue culture of several higher plants, type and concentration of carbohydrates are important for inducing somatic embryos. For example, somatic embryo formation from nucellar calli of *Citrus* species could be stimulated when the sucrose in the induction medium was replaced by several carbohydrates such as galactose and lactose [37]. The superior effect of maltose over sucrose as a carbohydrate has also been reported for somatic embryo formation in alfalfa [38], indica rice [39]and creeping bentgrass [40]. In asparagus, the importance of carbohydrate type and concentration in the embryo induction medium has been described [8, 20, 31]. Levi and Sink [14]reported that sucrose was more effective than glucose and fructose for somatic embryo formation. Kunitake *et al.* [31]described that high production of somatic embryos (approximately 1,500 somatic embryos per 0.1 m*l* p.c.v.) was obtained when embryogenic calli were cultured on MS medium containing 0.1M maltose, glucose or sucrose, among 11 carbohydrates tested. Particularly, most of the somatic embryos induced on maltose-containing medium had normal morphology with greenish epidermis. Furthermore, induction of somatic embryos was affected by the concentration of maltose, and the maximum number of somatic embryos was obtained on 0.1M maltose-containing medium.

2.2.3 Partial desiccation

Generally, partial desiccation of embryogenic calli in higher plants increases the frequency of normal somatic embryo formation. Two methods have been used to partially desiccate embryogenic calli, *i.e.* increasing the concentration of gelling agents and decreasing of relative humidity in tissue culture vessels. In asparagus, development of normal somatic embryos was enhanced when the embryogenic calli were cultured on growth regulator-free MS medium containing 0.8~1% gellan gum using the culture vessels capped with aseptic ventilation filters [17, 18]. The effectiveness of this method for normalization of somatic embryo formation has also been confirmed in other asparagus cultivars and selections [21, 31].

2.3 Germination

Induction of normal germination of somatic embryos is the most important step for decreasing the price of the plantlets per a nursery in commercial micropropagation and for obtaining genetically

manipulated plants. Placing globular somatic embryos on embryo induction medium with a high carbohydrate level (0.12M~0.3M sucrose, glucose or fructose) for 2 weeks, followed by transfer to the same medium with a low carbohydrate level (0.06M), significantly increased the percentage of germinating somatic embryos [20]. Carbohydrates are involved in regulating the osmotic potential of plant cells *in vitro*, in addition to their role as carbon sources [41, 42]. Therefore, the osmotic potential in the somatic embryos may be increased during embryo development and maturation by a higher carbohydrate level in the medium and transfer of the embryos to the medium with a lower level of carbohydrate enhances the uptake of water and subsequent germination [20].

Cold pretreatment of somatic embryos at 4°C for more than 7 days increased the germination rate in asparagus [21]. The cold pretreatment of somatic embryos was successfully applied for obtaining a somatic hybrid between *A. officinalis* and *A. macowanii* by overcoming the difficulty in inducing normal germination [31]. Furthermore, somatic embryos induced on maltose-containing medium had a high potential for normal germination as compared with those induced on media containing other carbohydrates after cold pretreatment [31]. Synchronization of germination is also important for intentional nursery production. Kunitake *et al.* [26] reported that water pretreatment of cold-stored somatic embryos promoted synchronous high frequency germination in asparagus.

2.4 Somaclonal variation

Somaclonal variations such as chromosome doubling and genetic variation are critical problems for commercial micropropagation. Vasil [43] suggested that genetic stability found in plants regenerated from callus or tissue in many species of cereals and grasses was considered to be due to the regeneration through somatic embryogenesis. Kobayashi *et al.* [44] also described morphological and cytological stability in citrus protoplast-derived plants regenerated by somatic embryogenesis. In asparagus, the plantlets regenerated through somatic embryogenesis were also all diploids [13, 16, 21, 45]. While, chromosome variations were observed in callus cells and embryogenic calli-derived plants of several asparagus cultivars. Araki *et al.* [46] described that chromosome numbers in asparagus callus cells varied widely and showed a possibility of producing polyploids by continuing the callus culture for a long term, although all of plants regenerated from callus cultures were diploid. In contrast, Odake *et al.* [22] described that embryogenic calli-derived plants using liquid culture medium were all tetraploids.

Kunitake *et al.* [34] investigated in detail the chromosome variations of embryogenic calli-derived plants of gynogenic haploid, diploid, triploid, and tetraploid asparagus. Eight percent of the plants regenerated from diploid embryogenic calli which had been subcultured for 6 months after callus induction were tetraploids, and all of the regenerated plants derived from the calli subcultured for one year became tetraploids. While, haploid embryogenic calli-derived plants were diploid (32%), triploid (6%), tetraploid (6%) and chimeric (53%) when they were regenerated immediately after callus induction. However, no chromosome variations were observed among plants regenerated from triploid and tetraploid embryogenic calli which had been subcultured for 6 months after callus induction. Consequently, it is difficult to perfectly control somaclonal variation of asparagus long-term subcultured callus cultures using the present techniques. Therefore, the following techniques of long or short-term preservation of callus cultures will be important for the micropropagation and improvement of asparagus.

2.5 Long or short-term preservation

Long-term preservation of plant cells, somatic embryos, meristems and embryogenic calli using liquid nitrogen (cryopreservation) has become an important tool for conservation of many germplasms and experimental materials without causing genetic variations [47, 48]. In asparagus, Uragami *et al.* [49, 50] first demonstrated the successful results on cryopreservation of cultured cells, somatic embryos and axillary buds by vitrification method with the regeneration of plantlets. Since then, the usefulness of cryopreservation in asparagus was confirmed in bud clusters [51], embryogenic calli [21, 45, 52, 53] and somatic embryos [21]. Vitrification technique by a simple freezing method [47] was used in these cryopreservation studies. Cryopreservation of meristems will be needed to maintain a large number of cultivars, elite selections and experimental materials in asparagus. Cryopreservation of asparagus embryogenic calli would be also important for commercial micropropagation, since embryogenic calli have the potential to produce polyploids during the prolonged subculture of the calli, as mentioned above.

Nakashima *et al.* [21] reported that somatic embryos with high potential of germination (more than 80%) could be maintained for 8 months at 4°C in the dark. They suggested that short term-preservation of numerous somatic embryos in a small space is possible using this technique, and elite asparagus plantlets derived from somatic embryos could be supplied at any time according to the demands of the farmers.

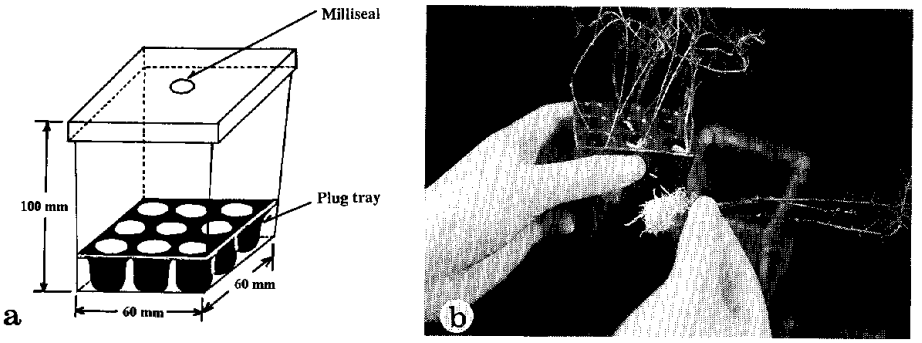

Fig. 2 A schematic diagram of *in vitro* plug nursery box. (Kunitake *et al.* [26])
a) *In vitro* plug nursery box consisting of polycarbonate vessel, heat-resistant plug tray and Milliseal.
b) Plantlets of asparagus 30 days after transfer the mature embryos to the plug nursery box. These plantlets were easily transferred to the pots.

3. Micropropagation

Asparagus is a cross-pollinated dioecious plants with a sex ratio of 1: 1 [54]. Consequently, considerably large differences are observed among the seed-propagated cultivars in various traits such as sprouting and flowering times, stem and spear types, and stem and cladode color [24]. Therefore, much work has been carried out on micropropagation of elite asparagus clones. As the result, highly efficient system for normal somatic embryo formation has been established by improving several cultural factors as described above. However, commercial use of the system is still restricted due to its relatively high production costs mainly caused by high labor costs, limited growth rate during multiplication, and poor rooting and low survival rates of the plantlets during acclimatization. Recently, extensive research has been conducted on the control of *in vitro* environmental factors for increased plant productivity at different culture stages [55, 56]. In asparagus, however, only a few studies have been conducted on the effect of *in vitro* environmental factors on the growth of somatic embryo-derived plants.

Kunitake *et al.* [26] developed an *in vitro* plug nursery box combined with a polycarbonate vessel (60 mm×60 mm×100 mm, air volume; 370ml, IWAKI, Co. Ltd.), heat-resistant plug tray (MINORU, Co. Ltd.) and Milliseals (microporous polypropylene film, pore size; 0.5 μm, Millipore, Co. Ltd.) (**Fig. 2-a**). They showed that *in vitro* plug nursery plants with compact root systems were obtained one month after transfer the somatic embryos to the pores of the tray. The plantlets were then easily transferred into the pots (**Fig. 2-b**). Furthermore, the improvements of *in vitro* environmental factors such as CO_2 enrichment (1,500 μmolmol^{-1}), increased number of air exchanges (3 hr^{-1}) and high light intensity (150 μmol·m^{-2}s^{-1}) were effective for promoting plantlet growth (**Fig. 3**). Approximately 80% of the plantlets cul-

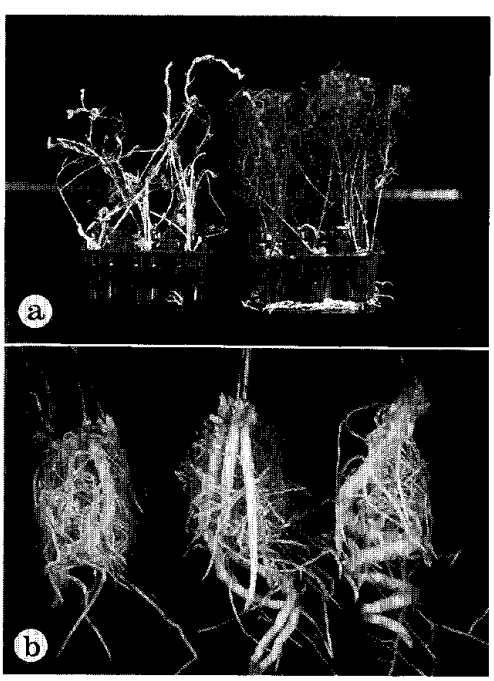

Fig. 3 Somatic embryo-derived plantlets of asparagus cultured in *in vitro* plug nursery box. (Kunitake *et al.* [26])
a) Comparison of somatic embryo-derived plantlets cultured under conventional culture conditions (left) with improved ones (1,500 μmolmol^{-1} CO_2, 150 μmol·m^{-2}s^{-1} light intensity and 3h^{-1} of air exchanges) (right).
b) Vigorously growing compact root systems produced under the improved culture conditions.

tured under the improved condition for 10 days showed normal growth without wilting, and more than 95% survived after they were cultured for 30 days. While, all of the control plantlets cultured in the conventional environmental condition died immediately after transplanting to soil, and even plantlets cultured for 30 days under the same conditions had survival rate of approximately 50% (unpublished results). Therefore, it can be concluded that the production costs of elite asparagus clone can be reduced through the improvements of environmental conditions of *in vitro* cultures.

4. Protoplast culture

For conferring disease resistances, production of interspecific hybrids between asparagus and other wild relative species has been considered a useful strategy. However, it is very difficult to produce interspecific hybrids using conventional breeding methods in genus *Asparagus*. Therefore, somatic hybridization through protoplast fusion is expected to be an alternative way to obtain the hybrids. Establishment of plant regeneration system from protoplasts is a prerequisite for conducting somatic hybridization. Plant regeneration from asparagus protoplasts was first reported by Bui Dang Ha *et al.* [35, 57]. They isolated protoplasts from cladodes, and succeeded in regenerating whole plants via somatic embryogenesis. However, these protoplasts isolated from cladodes were difficult to divide and the plating efficiency was very low. To overcome the difficulty, Kong and Chin [6] and Chin *et al.* [58] succeeded in establishing a plant regeneration system from protoplasts with stable and high plating efficiency by using embryogenic calli which were obtained by culturing crown tissue of two week-old seedlings. Since then, various studies have been reported on cell division, differentiation and plant regeneration from protoplasts with different cultivars of asparagus.

4. 1 Isolation of protoplasts

For the isolation of cladode protoplasts, an enzyme solution containing 1% Macerozyme SS and 3% Cellulase SS [35] was adopted. However, embryogenic callus and its suspension culture are now generally used as protoplast sources, because of the high yield and high potential to regenerate plants. Some of the enzyme solutions used for protoplast isolation were the combination of 1% Cellulase RS and 1% Pectolyase Y-23 for embryogenic suspension cultures [58], and that of 1% Cellulysin, 0.2% Macerase and 1% Rhozyme for embryogenic calli. Mannitol at 0.9M [3, 35], 0.6M glucose [6, 8, 59], or 0.6M sorbitol [7] was supplemented to those enzyme solutions as an osmoticum. Kong and Chin [6] reported that protoplasts isolated in an enzyme mixture containing mannitol or sorbitol as an osmoticum showed poor growth, whereas, those obtained with an enzyme mixture containing glucose grew faster. CPW salts [3, 8, 59], $CaCl_2 \cdot 2H_2O$ [7], MS salts [6] were added to those enzyme solutions as a stabilizing agent of protoplast membrane. Duration of enzymatic maceration of embryogenic calli was approximately 16hr in most of the published conditions. After enzymatic maceration, freshly released protoplasts were rinsed several times with washing solution and purified. Protoplast yields isolated from embryogenic calli were $10^6 \sim 10^7$/g of callus [3, 8, 59, 60]. Elmer *et al.* [3] described that the genotype of the callus donor as well as the age of the calli (10, 20, 30 days) after subculture did not greatly affect the number of protoplasts released.

Most of the purified protoplasts of asparagus were 15 to 30 μm in diameter and exhibited a dense cytoplasm. Protoplast viability (more than 95% as assessed by fluorescein diacetate staining) and division frequency could be dramatically increased by a pretreatment of the calli in half strength MS liquid medium containing 1% sucrose for 4 to 7 days [7].

4. 2 Culture of protoplasts

The first mitotic cell division of protoplasts occurred after 3~4 days of culture and colonies consisting of as many as 20~30 cells were obtained after 20 days of culture. The composition of the culture medium, selection of suitable growth regulators and the concentrations, and the addition of vitamins and amino acids were critical factors in obtaining high plating efficiency. In previous reports on protoplast culture of asparagus, Bui Dang Ha and Mackenzie [57] obtained 4% frequency of cell division after 7 days of culture by using protoplasts isolated from cladodes. However, they could not get consistent results and the plating efficiency varied among culture attempts. In contrast, Kong and Chin [6] obtained greater than 10% plating efficiency in agarose-droplet culture supported on a polypropylene membrane, using embryogenic calli. Although this method may be a useful technique for protoplast culture of asparagus, it is inconvenient for observation of protoplasts during culture, and for obtaining accurate plating efficiency. Kunitake and Mii [7] constantly obtained 7% plating efficiency by embedding the protoplasts in gellan gum-solidified 1/2MS medium containing 1 mg/l NAA, 0.5 mg/l zeatin, 1,000 mg/l L-glutamine and 0.6M glucose, and suggested the importance of the addition of L-glutamine. Dan and Stephens [60] reported a higher plating efficiency (19%) in bead culture with replacement of surrounding liquid medium every week, than in the culture with liquid medium or

agarose layer method. They suggested that effectiveness of bead culture for increasing protoplast division might be attributed to maximized conditioning effects. A large volume of liquid culture medium might act as a reservoir and dilute substances released by the developing cells that may be inhibitory or toxic to the protoplasts [61].

Generally, genotypes also affect viability, division and colony formation of protoplasts. Difference in plating efficiency was shown among the 5 cultivars and 8 genotypes tested, and the plating efficiencies ranged from 0.2% to 40.8% [62]. A similar result of plating efficiency was obtained in 4 cultivars [59].

4.3 Plant regeneration

The process of plant regeneration from protoplast-derived colonies is almost the same as that adopted for embryogenic callus. Embryogenic callus-derived protoplasts of asparagus usually developed into visible microcolonies after 2 months of culture. After transfer onto the proliferation medium (MS medium containing 2 mg/*l* 2,4-D, 3% sucrose and 0.2% gellan gum), they grew rapidly into friable and nodular calli that closely resembled the original embryogenic calli. They initiate to produce somatic embryos several weeks after transferring again to the embryo induction medium (MS medium containing 1.0% gellan gum). Mature somatic embryos thus obtained developed roots and shoots a week after transfer to the germination medium.

For the biotechnological uses of the protoplast culture system, genetic stability of protoplast-derived plants is required. The somatic chromosome analysis from the root tip cells of protoplast-derived plants of asparagus revealed that they had normal diploid ($2n=20$) chromosomes [6-8, 57]. In other studies, however, some protoplast-derived plants were aneuploids with 22 to 38 chromosomes [3], whereas 2 out of 56 protoplast-derived plants became tetraploids, and pollen fertility decreased in some of the plants [62]. As the number of plants examined in these studies were relatively few, more research will be needed to understand the causal factors for inducing these aneuploids and polyploids.

5. Somatic hybridization

Production of somatic hybrids in genus *Asparagus* has only been reported in combination between *A. officinalis* and *A. macowanii* [4]. *A. macowanii*, a wild species used for ornamental purposes, *A. macowanii* was reported to be resistant to *Phomopsis asparagi* which is a causal fungus of a serious disease, stem blight of asparagus [2], it is expected that the resistant trait of this species to *P. asparagi* could be incorporated into asparagus by interspecific hybridization. However, no interspecific hybrid has been produced between *A. officinalis* and *A. macowanii* by conventional breeding methods until now. Therefore, we tried to produce somatic hybrid plants between these 2 species using electrofusion [4].

At present, protoplast culture system of genus *Asparagus* has only been established in *A. officinalis*, and plant regeneration from protoplasts has not been reported in wild species of asparagus. Therefore, we used the protoplast culture system of *A. officinalis*, for producing somatic hybrids. In our preliminary experiments, we tried to produce somatic hybrids between embryogenic callus-derived protoplasts of *A. officinalis* and mesophyll or callus protoplasts of several wild species such as *A. macowanii*, *A. plumosus* and *A. sprengeri* by adopting the protoplast culture system established for *A. officinalis*. Consequently, interspecific somatic hybrid plants between *A. officinalis* and *A. macowanii* have been produced by using electrofusion. In this somatic hybridization experiment, embryogenic callus-derived protoplasts of *A. officinalis* were pretreated with iodoacetamide (IOA) for inactivation and electro-fused with non-embryogenic callus-derived protoplasts of *A. macowanii*. Electrofusion was carried out by producing "pearl-chains" at 1 MHz, 100 V/cm for 20 sec of alternating current followed by the application of direct current at 1.0 kV/cm for 40 μs. Maximum frequency of protoplast fusion was approximately 9%. In the fusion-treated cultures, somatic embryos were produced from some protoplasts through callus formation. However, most of the somatic embryos obtained showed abnormal germination with only root development. Normal germination of the embryos was achieved in those derived from one callus line by transferring to the germination medium after cold pretreatment at 4°C for 14 days. The regenerated plants showed intermediate morphological characteristics but grew abnormally with difficulty in transferring to soil. The hybrid nature of the plants of this callus line was confirmed by isozyme, nuclear rDNA and random amplified polymorphic DNA (RAPD) analyses. However, the interspecific somatic hybrid had a chromosome number of $2n=50$ which was more than the sum of the parents ($2n=40$), suggesting that it was the product of multifusion followed by reduction of some chromosomes. Abnormal growth of this somatic hybrid may be partly due to such an aberrant chromosome number. More detailed studies are required to evaluate the rate of production of somatic hybrids in the genus *Asparagus* as a tool for breeding of asparagus.

6. Genetic transformation

Genetic transformation to introduce foreign genes into plant genome has now become a routine technique in many plant species including crops [63]. In asparagus, a lot of transgenic plants have been produced using *Agrobacterium*-mediated transformation and electroporation. Although it had long been considered that *Agrobacterium*-mediated transformation was only applicable to dicotyledonous plant, the method is now efficiently used for transferring foreign DNA into cells of monocotyledonous plants.

The first successful transformation in monocotyledonous plants was achieved in *Asparagus officinalis* L. by Hernalsteens *et al.* [64] who obtained a tumor tissue that grew on growth regulator-free medium and produced two opines, after inoculation with a wild-type *Agrobacterium tumefaciens* strain C58. Molecular proofs of the T-DNA insertion into asparagus were reported by Bytebier *et al.* [65] and Prinsen *et al.* [66]. Bytebier *et al.* [65] obtained calli exhibiting kanamycin resistance after inoculation of stem slices with *A. tumefaciens* strain C58C1pGV3850 :: 1103neo(dim). The callus showed multiple T-DNA insersions and the plants regenerated from the callus showed no major T-DNA rearrangements in comparison with the transformed callus. Delbreil and Jullien [67] described that 25 kanamycin-resistant callus lines were obtained after co-cultivation of long-term-subcultured embryogenic calli of asparagus with an *A. tumefaciens* strain C58p35SGUSINT harboring β-glucuronidase (GUS) and neomycin phosphotransferase II (NPT II) genes. All of these callus lines showed GUS activity by histological staining, and DNA analysis of the calli and of the regenerated plants confirmed the integration of the T-DNA.

While, Mukhopadhyay and Desjardins [8] described direct gene transfer to asparagus protoplasts using electroporation. Embryogenic calli-derived protoplasts were electroporated to introduce GUS gene. The level of GUS transient gene expression was influenced by the voltage and duration of the electric pulse, and the optimum condition obtained was 500 V/cm exponential decay pulse for 94ms. The transient expression level was also enhanced by increasing the plasmid DNA concentration and by the presence of polyethylene glycol (PEG) in the electroporation medium. They obtained a large number of kanamycin resistant colonies, but plant regeneration from these colonies was not described.

Recently, whole transgenic plants of asparagus with introduced NPT II-GUS genes [68] and HPT-GUS or bar gene [69] were produced using a particle gun. Embryogenic calli, bud clusters and somatic embryos were also used as materials. Transformation of the plants were confirmed by histochemical GUS assay and Southern analysis of GUS gene.

7. Conclusion and perspective

In the present review, we described several important factors for inducing normal somatic embryogenesis of asparagus, and discussed the application of somatic embryogenesis for micropropagation and breeding through somatic hybridization and genetic transformation. There is no doubt that somatic embryogenesis is a powerful tool for micropropagation of asparagus. By the improvement of culture conditions, frequency of embryogenic callus induction, normal somatic embryo formation, germination and acclimatization have dramatically been increased. As a result, the price of plants micropropagated through somatic embryogenesis has decreased up to one tenth, as compared to that of plants propagated by *in vitro* cutting (unpublished data). In Hiroshima and Saga prefectures in Japan, approximately 1,000 plants micropropagated through somatic embryogenesis have experimentally been cultivated by several farmers for 2 years which resulted in good evaluations of the products with respect to commercial qualities (personal communications from Mr. H. Kohmura). However, the price of the micropropagated plants is still not low enough for commercial growing of asparagus. Therefore, applications of automation, robotization and computerization in the micropropagation process would be necessary for decreasing the production cost. In the future, production of elite asparagus plants through the use of artificial seeds would probably be realized, as Rendenbaugh *et al.* [70] suggested advantages of artificial seeds for propagation such as ease of handling and potential long-term storage. Ghosh and Sen [71] actually made artificial seeds from somatic embryos of *Asparagus cooperi* Baker, and showed plant regeneration from the artificial seeds *in vitro*.

Embryogenic calli have also attained an important role for obtaining transgenic plants. Although production of asparagus transgenic plants has been achieved using *Agrobacterium tumefaciens*, electroporation and particle gun[8, 67-69], the production efficiency of transgenic calli or plants is not high enough to employ the methods for asparagus breeding. Among these methods, particle gun is easy to handle, and will be the most efficient method for obtaining transgenic plants if embryogenic callus is used as a target. Further studies on bombardment conditions and improvements of hardware design for easy manipulation will be required. At present, transformation of asparagus cells using *Agrobacter-*

ium and particle gun to produce disease resistant plants is being attempted ([72], personal communication from Mr. H. Kohmura). These studies will have significant impact on asparagus improvement in the near future.

Acknowledgments

The authors are grateful to Mr. T. Nakashima, Mr. K. Mori and Mr. M. Tanaka of Saga Prefectural Agricultural Research Center for their advice and experimental help about asparagus tissue culture. We wish to thank Mr. H. Kohmura of Hiroshima Prefectural Agricultural Research Center, Dr. T. Matsumoto of Shimane Agricultural Experiment Station and Mr. Y. Ozaki of Kyushu University for their useful advice and information.

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