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A review on genome study and *in vitro* culture of *Asparagus officinalis* L

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Abstract
Asparagus officinalis L. of Asparagaceae (formerly Liliaceae) is an important vegetable crop. It is also called as garden Asparagus and cultivated worldwide for its edible spears which are basically modified shoots. These spears have high nutritional value in addition to its beneficial effects on human health due to its contents of different bioactive compounds. The present review has described its genomic constitution to develop high yielding varieties through breeding program. The different *in vitro* culture techniques have also been included in the present review for propagation, conservation and development of disease resistant varieties of this species through biotechnological approaches.

Keywords: *Asparagus officinalis* L, chromosome study, genome size, tissue culture

Introduction

Asparagus is a large genus under Asparagaceae of Monocot plant comprising of herbaceous perennials, tender woody shrubs and vines. In a broad sense, the genus covers species that have been segregated in several genera. The genus Asparagus includes approximately 240 species, most of which is *Asparagus officinalis*L. (garden Asparagus), as it is a vegetable crop cultivated worldwide for its edible spear. These spears or modified shoots have a special taste and texture that is very unique among vegetables, in addition to their high nutritional value owing to the presence of several bioactive compounds that exhibits beneficial effects on human health (Guo *et al.,* 2020) [25]. It is low in calories and contains low cholesterol with very low amount of sodium. It is a good source of different vitamins, minerals and amino acids. The amino acid, asparagines get its name from Asparagus and the plant is very rich in this amino acid. Rhizomes and roots contain saponin and asparagines and are used in dropsy and cystitis as folk medicine. Out of 240 species, 17 species are distributed throughout the tropical and sub-tropical regions of India, expanding from West Bengal, Bihar, Orissa to Kashmir and Himalayan regions (up to 5,300 ft) (Mukhopadhyay and Ray 2013) [39]. *A. officinalis* is a perennial herb growing up to 50-150 cm in height with feathery foliage. The leaves are modified stems with needle-like cladodes in the axis of scale leaves. In this genus, the true leaves are reduced to inconspicuous scales and the green photosynthetic leaves are actually flattened or needle like modified branches (cladodes) developed in the axils of the scale like leaves. Roots are tuberous. The morphological characters are important in the classification of different species and varieties of Asparagus (Kamble, 1997) [27]. This genus has characteristics underground root systems which are tubers or bulbs. *Asparagus officinalis*L. of Asparagaceae (earlier Liliaceae) is dioecious and almost all cultivars possess high heterozygosity. Being dioecious, it bears either conspicuous male flowers or less conspicuous female flowers. The species of Asparagus have important medicinal, ornamental, and horticultural values. For example, *A. officinalis* is an important vegetable crop grown for its herbaceous, newly emerged shoots (commonly referred to as spears) used worldwide. It is cultivated in many countries including Taiwan, China, USA, Canada and several European countries are the largest producers of Asparagus spears (Xu *et al*., 1996) [50]. In India, its cultivation is limited and mostly restricted to Kashmir and other Himalayan states.

Genome Study

Chromosome Analysis

Chromosomes are considered as nuclear genome and analysis of chromosomes is extremely useful for classification of different plant species as well as to evaluate phylogeny at the cytological level. Study of genetic diversity within and among different species and populations of different plant species is necessary for crop improvement, conservation, and management. The chromosome number of several species of Asparagus has been reported previously (Mukhopadhyay and Desjardins, 1994a; Mukhopadhyay and Ray, 2013) [36, 39]. Cytological analysis on different species and varieties of Asparagus has clearly indicated the presence of polyploidy series including diploids, tetraploids and hexaploids (Jena *et al*., 2007) [26]. Karyotype analysis has revealed the presence of different chromosome types in the species of Asparagus. *A. officinalis* is a diploid species under the genus Asparagus with 2n=2x=20 chromosomes. The individual chromosome morphology studied in *Asparagus officinalis*is quite distinct with asymmetric karyotypes (Mukhopadhyay and Ray, 2013) [39] Karyotype analysis has revealed the presence of different chromosome types in the species of Asparagus. *A. officinalis* is a diploid species under the genus Asparagus with 2n=2x=20 chromosomes. The individual chromosome morphology studied in *Asparagus officinalis*is quite distinct with asymmetric karyotypes (Mukhopadhyay and Ray, 2013; Mukhopadhyay and Desjardins, 1994a, b) [36, 37, 39]. This may reveal that polyploidy has played an important role in the evolution of different species of Asparagus. In plants, polyploidy occurs most commonly through the production of variable gametes, caused by different environmental factors. In Asparagus, research on the origin of polyploids is scanty and it may be supposed that the same phenomenon has occurred probably during evolution of different species (Sheidai and Inamdar, 1992) [48]. It is essential to detect the nature of polyploidy in such taxa, like autopolyploid or allopolyploid origin that may be revealed

from the pairing behavior of the chromosomes during meiosis. However, the karyotypic details of the species of Asparagus may suggest the fact that the process of autopolyploidy may have been responsible for species evolution (Jena *et al.,* 2007) [26]. In addition, the importance of chromosomal structural alteration is indicated (Mukhopadhyay and Ray, 2013) [39]. This species has revealed asymmetric karyotype with the presence of medium to short chromosomes. The presence of tetraploid species in *A. officinalis* may indicate the duplication of both centromeric chromosomes and nucleolar chromosomes at an early stage of evolution (Lahiri *et al.*, 2010) [29].

Nuclear DNA Analysis

The amount of nuclear DNA in a haploid genome remains constant and is a characteristic feature of each and every living species. This is called the C value which may be referred to as haploid genome size. The G1 phase of cell cycle with two copies of un-replicated DNA in the nucleus is referred to as 2C DNA amount (Dolezel and Bartos, 2005) [18]. Therefore, the analysis of DNA contents in different plant species is very important in different facets of chromosome research. The available data indicates both phylogenetic and ontogenetic increments of DNA content during evolution (Nagl, 1977) [43]. A large number of studies on in situ DNA quantification at the intergeneric and interspecific levels have indicated contradictory results, like within a genus there is wide range variation in the nuclear DNA (Sharma and Mukhopadhyay, 1984) [47]. It has been observed that the amount of nuclear DNA varies 3-6 folds in the closely related species of the same genus (Cavallini and Natali, 1990) [5]. There are also simultaneous reports where different species of the same genus do not necessarily show any difference in the DNA contents (Resslar *et al.,* 1981) [44]. Some of the DNA content variations may originate out of polyploidy. However, other phenomena like gene duplication or loss or amplification of DNA sequence within the chromosome may also account for such variation (Cionini, 1989) [11]. A steady increase in genome size with increasing complexity of the genome has been noted. This may be due to the fact that there is an increase in non-coding DNA in the larger genomes. It was subsequently noted that there is no relationship between C value and complexity of the genome. The lack of such correlation was later termed as C value paradox. A widely used method for genome size determination is Feulgen densitometry (Bennett and Leitch 1997) [4]. However, most modern and efficient method for nuclear genome size determination has been through flow cytometry. In situ cytophotometric estimation has revealed nearly equal amount of 4C nuclear DNA in diploids *A. officinalis* (approx 31 pg) whereas, the tetraploid species had little higher nuclear DNA (38 pg) content indicating the role of non-disjunction of chromatids during anaphase in the origin of tetraploids from diploid species. The genome size of plant species as reflected through its nuclear DNA value may provide information for molecular geneticists and plant breeders. This is also important in cytotaxonomy and phylogenetic studies of different plant species (Lee and Lin, 2005) [32]. DNA flow cytometry (Fluorescence Activate Cell Sorter or FACS) is a very popular and effective method for ploidy screening, cell cycle analysis, assessment of degree of polysomaty and estimation of absolute DNA amount or genome size (Dolezel and Bartos, 2005) [18]. The genome size of *A. offcinalis* has been found to be variable, as revealed from their 2C nuclear DNA values (Stajner *et al.,* 2002) [49]. A survey of genome size variation has shown interest in the variation below species level (Greilhuber, 1998) [24]. Genome size is basically a quantitative character that should be constant between individuals of a population as long as there is enough inbreeding to mix up the gene pool. Genome size variation may occur when the population is split up by geographical barriers (Greilhuber, 1998) [24]. The species and populations of *Asparagus officinalis*have revealed a variation in 2C nuclear DNA contents indicating differences in genome size among diploids and tetraploids. Genome size data is available for few Asparagus species (Jena *et al.,* 2007) [26]. A study was carried out on 173 double haploids of *A. officinalis*, five wild relatives and three tetraploid interspecific genotypes using amplified fragment length polymorphisms (AFLPs) has revealed that the latter groups showed largest amount of genetic diversity. On the other hand, cultivated Asparagus genotypes showed a narrow genetic base (Riccardi *et al.,* 2011) [45]. Similarly, the narrow genetic basis in cultivated garden Asparagus was observed using sequence related amplified polymorphism (SRAP) technique for studying genetic diversity (Amato *et al.,* 2021) [1]. Such narrow genetic basis of cultivated Asparagus germplasms has been found to be a contributing factor preventing the development of hybrids with higher yield and disease resistance (Garcia *et al.,* 2021) [21].

***In Vitro* Culture**

In vivo clonal propagation of *Asparagus officinalis*is only possible by seeds obtained by crossing between selected elite parental plants and vegetatively through mechanical cutting of the plant rhizomes to obtain a very limited number of plants from the selected genotypes. However, such methods are quite expensive, laborious and may involve the risk of spreading diseases (*Fusarium* sp.) to new plants (Encina and Regalado, 2022) [20]. The use of sexual reproduction for the generation of new elite genotypes, varieties and interspecific hybrids is almost impossible due to dioecious nature of Asparagus *officinalis*.

The biotechnological approaches can overcome many problems, and methods such as immature embryo rescue, micropropagation, and regeneration through organogenesis, embryogenesis can be applied successfully to obtain and preserve outstanding new genotypes. Tissue culture technique has been an important tool for propagation, conservation and improvement of different plant species of economic importance. There are different approaches for regeneration of genetically stable and variable plants. The shoot bud/meristem culture produces mostly genetically uniform plants like the mother plant. On the other hand, regeneration through callus culture has the possibility of producing genetically variable plants of interest. However, the age of the callus tissue is the determinant factor for regenerating variable plants of different degrees (Mukhopadhyay and Desjardins, 1994a, b; Lahiri *et al.,* 2012a, b) [30, 31, 36, 37]. Two factors are responsible for successful plant regeneration in culture: a) the choice of initial explants and b) the composition of nutrient medium with specific type and concentration of growth regulators. In Asparagus, extensive research has been carried out including mass propagation using shoot bud culture, organogenesis and somatic embryogenesis (Mukhopadhyay and Desjardins, 1994a, b; Dasgupta Nag *et al.,* 2003, 2007)

[14, 15, 36, 37] as well as development of transgenic plants (Mukhopadhyay and Desjardins, 1994c; Mukhopadhyay *et al.,* 2002) [38, 41]. Several diseases cause severe losses for Asparagus growers of which the disease caused by a fungus, *Fusarium oxysporum* and *F. moniliforme* results in maximum loss of Asparagus yield (Desjardins, 1992) [17]. *A. officinalis* is highly sensitive to *Fusarium* infections. These species of *Fusarium* cause a complex array of Asparagus diseases including wilts and root rots (Schreuder *et al.,* 1995) [46]. On the other hand, a wild species of Asparagus, *A. densiflorus* var. *sprengeri* is resistant to *Fusarium*. However, sexual incompatibility between *A. officinalis* and *A. densiflorus* has been the major factor in the failure of transfer of resistance through classical breeding technique (Elmer *et al.,* 1989) [19]. It has been found that the cross between these two species is being unsuccessful due to development of abnormal endosperm (Marcellan and Camadro, 1999) [35]. The unconventional techniques including somatic hybridization and genetic transformation (Mukhopadhyay and Desjardins 1994c; Mukhopadhyay *et al.,* 2002) [38, 41] may be useful alternatives to develop *Fusarium* resistant varieties of *A. officinalis*.

Shoot Bud Culture

The shoot tip/bud culture is most convenient and efficient for clonal propagation of Asparagus *officinalis*. Any micropropagation (*in vitro* clonal propagation) system has the advantage of producing large number of uniform plants that are genotypically same as the mother plant. Direct organogenesis of mother shoot bud has been most suitable for multiplication of daughter shoot bud with stable genotypes. The other advantage of this technique is maintaining stable environmental and nutritional conditions which can be easily manipulated and controlled. The genetic stability in micropropagated plants is very important for further breeding process as the growers always prefer stable genotypes of certain economically important characters for commercial exploitation. There are reports of micropropagation of different species of Asparagus including *A. officinalis* L. using modified Murashige and Skoog's medium with different concentrations and combinations of auxin and cytokinin (Encina and Regalado, 2022; Nag *et al.,* 2004; Mukhopadhyay *et al.,* 2006) [20, 40, 42]. Conventional propagation methods using basal media without growth regulators have very low propagation efficiency. The vegetative propagation by fragmentation of crown is possible on a small scale and almost impossible on commercial basis (Desjardins, 1992) [17]. The most efficient auxin in shoot bud multiplication has been found to be α-napphthalene acetic acid (NAA) in low concentration (0.1 mg/l) in combination with either kinetin (0.2-0.5 mg/l) or 6-benzylaminopurine (BAP) (0.2-0.5 mg/l). However, in certain genotypes, BAP has been found to be more suitable than kinetin (Mukhopadhyay *et al.,* 2006) [40]. The effectiveness of kinetin in shoot bus multiplication in association with low concentration of NAA has been observed in certain genotypes of *A. officinalis* L. (Nag *et al.,* 2004) [42]. However, the most challenging aspect of micropropagation of *A. officinalis* is the induction of roots from multiplied elongated shoots. The main problem in micropropagation of Asparagus is root induction as it is very much species and genotype specific and the rate of root induction varies in similar condition in different genotypes. However, it has been observed that high level of sucrose along with NAA and kinetin improves rooting process (Conner and Faloon, 1993) [12]. The use of plant growth retardants, like ancymidol by Chin (1982) [10] dramatically improved the rooting of *in vitro* grown shoots of *A. officinalis* by disrupting the apical dominance and it has become the only choice for rooting of Asparagus shoots. Chang and Peng (1996) improved the rooting of Asparagus shoots (78%) by supplementing the medium with ancymidol, 6% sucrose and 162 mg/l phloroglucinol. The roots initiated by application of auxins like indole butyric acid (IBA) were found to be morphologically and anatomically poor than those induced by the application of ancymidol (Nag *et al.,* 2004) [42]. The incorporation of ancymidol in the culture medium enhanced the production of more plantlets, promoted the development of stronger roots and shoots. These plantlets were morphologically different from those initiated on the other rooting media. They had shorter, more vigorous and more numerous shoots which formed a small compact and woody structure resembling a crown, as also found by Chin (1982) [10]. The roots also differed from the untreated cultures, and were thick, large and non-ramified, resembling storage roots. Encina and Regalado (2022) [20] reported the use of rhizome bud as an alternative to the shoot bud for micropropagation of Asparagus. These researchers used NAA, kinetin and ancymidol for both shoot multiplication and root induction from regenerated shoots with high success rate.

Callus Organogenesis

The indirect organogenesis from callus culture involves regeneration of shoots or complete plants by rooting the regenerated shoots for biotechnological purposes. The process of organogenesis involves many cells which take part to form a single shoot bud and so it is a multicellular event. The use of only cytokinin may be suitable for shoot bud regeneration from callus culture, though in some cases, higher cytokinin level along with an auxin at very low concentration has been effective in organogenic response. The callus organogenesis can be regulated by exogenous growth regulators (Ghosh and Sen, 1994, 1996) [22, 23]. Exogenous *p*-chlorophenoxyacetic acid (*p*CPA) in association with high level of BAP has been observed to regenerate shoots from internodal callus tissue in Asparagus *densiflorus* cv *Sprengeri* (Benmoussa *et al.,* 1996) [2]. However, in *A. officinalis*, the hormone *p*CPA has not been effective in organogenic response from callus tissue. Root induction from the regenerated shoots has been only possible by using ancymidol (Chin, 1982) [10] or GA_3 (Chen and Zhou, 1994) [8] in *A. officinalis*. However, the regeneration of adventitious shoots or plantlets through callus organogenesis is usually not practiced in micropropagation of selected genotypes due to the possible genetic variability resulting in a high rate of progenies without the parental characteristics which is not desirable.

Somatic Embryogenesis

The somatic embryogenesis is a process by which a single somatic cell is transformed into an embryo (somatic embryo) in culture in presence of specific growth regulator/s that is eventually developed into a bipolar structure, like zygotic embryo with distinct plumular and radicular ends in specific levels of exogenous growth regulators. Such structures develop into a complete plantlet after transferring to a nutrient medium without growth regulators. Therefore,

the somatic embryogenesis process is unicellular in origin. The advantage of this process is that the regenerated plant will be a true clone where all the cells of the plant body are genetically similar to the mother plant. Also, once induced successfully, the somatic embryogenesis will produce a large number of plants from a single culture. As it is unicellular in origin, this process may produce plants of variable ploidy levels. In *A. officinalis*, a single tetraploid clone has been produced from multiple diploid clones (Dasgupta Nag *et al.,* 2003) [14]. However, in *A. officinalis*, the induction of somatic embryogenesis is difficult as it is strongly influenced by the genotypes (Dasgupta Nag *et al.,* 2003; Mukhopadhyay and Desjardins, 1994a, b; Encina and Regalado, 2022) [14, 20, 36, 37]. It has been observed that the induction of somatic embryo formation and its maturity are greatly influenced by the type and concentration of exogenous growth regulators. In certain genotypes, only 2,4-Dichlorophenoxyacetic acid (2,4-D) is required to induce somatic embryo formation and the withdrawal of this auxin induced bipolar structure formation and ultimately complete plants were formed. There are reports where, in a particular genotype of *A. officinalis*, somatic embryos are induced in half concentration of MS medium without growth regulators and converted into plants by culturing embryos in MS medium with GA_3 (Mukhopadhyay and Desjardins, 1994a) [36]. In certain genotypes, the use of 2 isopentyl (2iP) as cytokinin is essential to induce somatic embryos (Mukhopadhyay and Desjardins, 1994b; Dasgupta Nag *et al.,* 2003) [14, 37]. The type of carbon source has played an important role in inducing somatic embryos. MS medium with 10% glucose along with NAA and 2iP produced maximum number of somatic embryos. The conversion of these embryos to form complete plants occurred in the same medium with similar growth regulators (both types and concentrations) along with only 2% sucrose instead of glucose (Mukhopadhyay and Desjardins, 1994b) [37]. In conclusion, the somatic embryogenesis has been an effective way for micropropagation of *Asparagus officinalis*plants of both stable and variable genotypes in large numbers.

Genetic Transformation

The yield of *Asparagus officinalis*L. is drastically reduced in the field due to *Fusarium* infection. There were several attempts to obtain resistance in *A. officinalis* through classical breeding process. However, there was no success due to sexual incompatibility (Mukhopadhyay and Desjardins, 1994c) [38] between *A. officinalis* and *A. densiflorus* cv *Sprengeri*. The natural resistance against *Fusarium* infection has been observed in *A. densiflorus* cv *Sprengeri*. Due to presence of sexual incompatibility between these two species, the researches started to search an alternative method to transfer the gene from the resistance species to the sensitive species against *Fusarium* infection using biotechnological approaches. The only possibility for transferring the resistance would be either protoplast fusion or genetic transformation (Encina and Regalado, 2022) [20]. In Asparagus, protoplast cultures have been utilized by different researchers to obtain specific genotypes resistant to pathogen (*Fusarium*) infection through mass protoplasts screening, to obtain interspecific hybrids through somatic hybridization following electrofusion (Kunitake *et al.,* 1996) [28] or to regenerate transgenic plants using eletroporation of protoplasts (Mukhopadhyay and Desjardins, 1994c; Mukhopadhyay *et al.,* 2002) [38, 41]. Also, attempts were made to obtain transgenic plants through *Agrobacterium*-mediated gene transfer technology to develop *Fusarium*-resistance lines of *A. officinalis* (Delbreil *et al.,* 1993) [16].

The successful protoplast culture and its regeneration were studied by different researchers using different cell wall degrading enzymes, like cellulysin, macerozyme and Rhozyme H 150 in different combinations and concentrations (Dan and Stephens, 1991; Mukhopadhyay and Desjardins, 1994a, b; Benmoussa *et al.,* 1997) [3, 13, 36, 37]. All these studies also tried different culture methods to obtain efficient protoplast regeneration, like bead culture (Dan and Stephens, 1991) [13] and agarose droplet culture on porous polypropylene membrane (Chin *et al.,* 1988) [9]. However, the monitoring of protoplasts division and growth was quite difficult under the microscope for selection of those responding protoplasts for further culture to produce embryogenic callus tissue. Mukhopadhyay and Desjardins (1994a, b) [36, 37] developed a very simple methodology using protoplast culture in a semisolid medium with 0.1% Gellan gum for efficient plating density and plant regeneration that can be easily monitored under the microscope.

Protoplast fusion between different species of Asparagus has not been carried out elaborately, except one report by Kunitake *et al.,* 1996 [28]. This study has established regeneration of heterokaryons following electrofusion of protoplasts between *A. officinalis* and *A. macowanii*. The attempt was to regenerate interspecific hybrids to transfer resistance to the stem blight caused by *Phomopsis asparagi* from *A. macowanii* to *A. officinalis*. The researchers only succeeded in the regeneration of an interspecific hybrid between these species, but the hybrid line showed some abnormalities including lack of vigor and in flowering. However, gene transfer methodology has been successful in obtaining transgenic plants of *A. officinalis* with desired gene. The indirect method of gene transfer following co-cultivation of stem segments and embryogenic calli with *Agrobacterium* has been established (Limanton-Grevet and Julien, 2001) [34]. The transgenic plants of *A. officinalis* have been developed from electroporated protoplasts (Mukhopadhyay and Desjardins, 1994c; Mukhopadhyay *et al.,* 2002) [38, 41] as well as by partial gun bombardment of somatic cells (Li and Wolyn, 1997) [33]. There is a report of transferring a gene of interest for breeding to increase the tolerance to diseases such as stem blight in Asparagus (Chen *et al.,* 2019) [7].

Conclusion

The studies of genomic constitution and tissue culture of *A. officinalis* have been presented in the present article, as a guide to focus on other facets of research of this economically important vegetable crop. The development of *Fusarium*-resistance genotypes of this species through genetic manipulation as well as development of super males through haploid culture has to be studied for successful breeding program of *A. officinalis* L.

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