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Regeneration of a Tetraploid Clone from Callus Culture of *Asparagus officinalis* L. through Somatic Embryogenesis

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Summary A tetraploid callus line of *Asparagus officinalis* L. was identified from 4 regenerated callus lines. Plants were regenerated from these callus lines following somatic embryogenesis and the tetraploid clone of this species was established in the field with 80% survival rate. The embryogenic callus was induced in Murashige and Skoog's (MS) medium in presence of α-naphthaleneacetic acid (NAA) (0.2 mg/l) and kinetin (0.02 mg/l) and proliferated as well as maintained in 2,4-dichlorophenoxyacetic acid (2,4-D) (1.0 mg/l) containing MS medium. Somatic embryos were initiated in Gelrite-solidified MS medium with variable levels of NAA and 6-(γ-γ-dimethylallylamino) purine (2ip). A higher level of carbohydrate enhanced embryo conversion efficiency. The embryos induced in presence of 10% glucose for 2 weeks and subsequently transferred to 2% sucrose level showed higher conversion rate than those maintained in 3% sucrose concentration. Karyotype analysis of diploid and tetraploid clones revealed exact duplication of the diploid set in tetraploid plants.

Key words *Asparagus officinalis* L., Karyotype analysis, Micropropagation, Plant regeneration, Somatic embryogenesis, Tetraploid.

Asparagus officinalis L. of the Liliaceae is an economically important vegetable crop, used worldwide. The plants are dioecious and almost all cultivars possess high heterozygosity. Clonal propagation of elite varieties is, therefore, important to obtain high yield. *Asparagus* has a very low propagation efficiency rate using conventional propagation methods (Yang and Clore 1973) and somatic embryogenesis may be a potential system for mass propagation of elite *Asparagus* crowns (Mukhopadhyay and Desjardins 1994). Clonal propagation of *Asparagus* has been reported earlier by shoot bud multiplication (Desjardins 1992). An effort has also been made to improve the quality of roots from the multiplied regenerated shoots using glucose (2%) and high level of Gellan Gum (8%) in the medium (Shigeta *et al.* 1996). On the other hand, the phenomenon of clonal variation *in vitro* was first reported long back among regenerates from callus cultures (Sacristan and Melchers 1969). Later it was considered as a new and useful source of genetic variation. Since then, chromosome structure changes in cells in culture under different conditions were extensively studied (Bayliss 1980, Mukhopadhyay and Sharma 1990, Mukhopadhyay *et al.* 2000) despite the fact that genetic stability has also been recorded in several plant species (Swedlund and Vasil 1985, Mukhopadhyay *et al.* 1989, 2002). Therefore, the most widely employed technique for creating genetic variation through tissue culture has been the use of callus culture. However, micropropagation through somatic embryogenesis from callus culture has been found to induce polyploidization in cultivar 'Mary Washington' of *A. officinalis* L. using both solid and liquid cultures (Komura *et al.* 1990, Odake *et al.* 1993). The liquid culture technique, though very complex and time consuming, has been found to be responsible for complete tetraploidization in *Asparagus* (Odake *et al.* 1993). In the present communication, we have reported a simple protocol for selection and development of a tetraploid line from callus culture of an Indian cultivar of high productivity of *Asparagus offici-*

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nalis L. and subsequent plant regeneration *via* somatic embryogenesis.

Material and methods

The shoot segments from *in vitro* germinated plants from seeds of *Asparagus officinalis* L. (Strain No-JL-991) (kindly provided by RRL, Jammu) were cultured on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose, 0.25% (w/v) Gelrite® and different levels of NAA (0.2, 1.0, 2.0 mg/l) in combination with a constant level of kinetin (0.02 mg/l) for callus induction.

The induced calli (soft and friable) were sub-cultured on Gelrite-solidified MS medium with 2,4-D (1.0 mg/l) after 6–8 weeks for further proliferation. Subsequently, calli were further transferred to MS basal medium with different concentrations and combinations of growth regulators and sugar concentrations for somatic embryo initiation and subsequent regeneration. At first the calli were transferred to MS medium supplemented with 0.25% (w/v) Gelrite®, 3% (w/v) sucrose, NAA (0.1 mg/l) and varying concentrations of 2ip (0.15, 0.3, 0.6 mg/l) (embryo induction medium) (Table 1) and maintained for 4 weeks.

These calli were then placed on the same induction medium with elevated (10%) glucose level for 10 d and further transferred to the same medium with 2% sucrose where bipolar embryonic structures appeared. These embryos were subsequently transferred to 1/2 strength of MS medium with 0.25% (w/v) Gelrite®, 3% (w/v) sucrose and varying concentrations of kinetin (0.5, 1.0, 1.5 mg/l) and abscisic acid (ABA) (0.5 mg/l) for maturation (embryo maturation medium). Embryos that converted to complete plantlets were kept in 1/2 strength of Gelrite-solidified MS medium with 3% sucrose before transferring to the soil. All cultures were incubated in a culture room at 24 ± 1°C under a photoperiod of 16 h, supplied by cool white fluorescent tubes giving light intensity of 48 μmol m^{-2} s^{-1}.

Ploidy level of callus tissue and the regenerates was determined through chromosome analysis. Callus tissue and root tips were taken after 10–14 d of subculture and pretreated in a saturated aqueous *p*-dichlorobenzene solution at 18°C for 3.5 h followed by fixation in Carnoy's fixative (ethanol : chloroform : acetic acid, 6 : 3 : 1) for overnight at 20°C. The tissue was then hydrolyzed in 5N HCl for 15 min at 18°C, stained in 2% acetic-orcein staining solution after a brief treatment in 45% acetic acid and subsequently squashed for observation (Mukhopadhyay and Sharma 1990).

Results and discussion

The yellowish, friable calli initiated in presence of NAA/kinetin (0.2/0.02 mg/l) grew rapidly after transferring to MS medium containing 1.0 mg/l 2,4-D with 3% (w/v) sucrose. Cytological observations revealed tetraploid cells in one of the 4 callus lines. Those calli were further maintained in MS+2,4-D (1.0 mg/l) medium up to 4 months and cytological analysis in a 4–5 weeks interval showed a stable tetraploidy. Such callus tissue was further induced to somatic embryo formation for plant regeneration. Somatic embryogenesis is generally found to be triggered by auxin and in many species, 2,4-D is the most potent for induction of somatic embryos (Mukhopadhyay and Desjardins 1994). Similarly the calli, when kept only in NAA/kinetin did not show embryogenic responses but when transferred to the media with 2,4-D showed favorable results. These calli showed little embryogenic response when they were maintained in the same media for longer period but shows positive embryogenesis in embryo induction medium or in a lower auxin containing media. Similar results were observed in other plants (Mukhopadhyay and Desjardins 1994) where auxin at a lower concentrations was more effective for embryo induction.

Some embryonic structures were recorded in MS medium with NAA and 2ip (0.1/0.15 mg/l) when they were sub-cultured in the same medium (Fig. 1a) as compared to the other 2 combinations

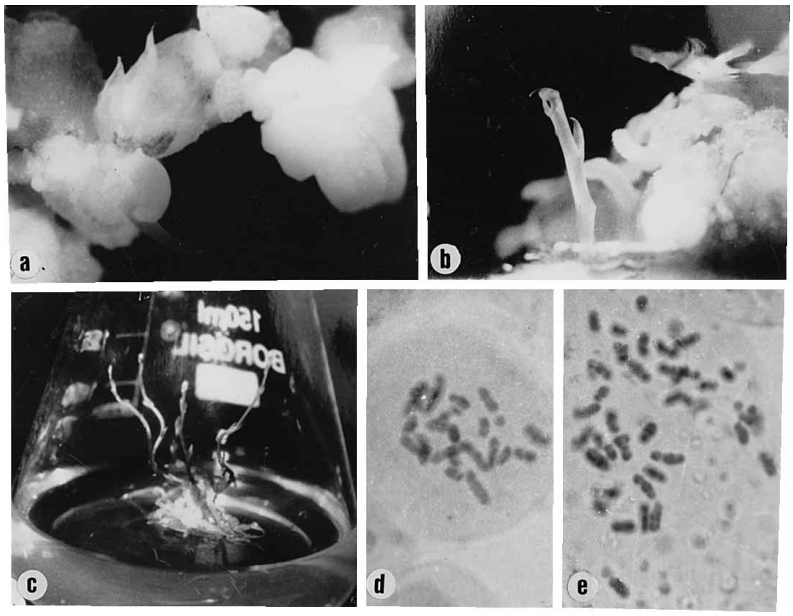

Fig. 1. Somatic embryo induction and plant regeneration *in vitro* of *Asparagus officinalis* L. a) Stereo-microscopic view of somatic embryo initiation in NAA (0.1 mg/l) and 2ip (0.3 mg/l) containing medium (*ca.* ×3.85). b) Stereo-microscopic view of shoot and root formation from germinated somatic embryos in 1/2 MS medium with 1 mg/l kinetin (*ca.* ×3.85). c) Complete plant regeneration in MS basal medium (*ca.* ×0.63). d) Somatic metaphase plate of the mother plant showing $2n=20$ chromosomes (*ca.* ×1375). e) Somatic metaphase plate from a tetraploid regenerate showing $2n=40$ chromosomes (*ca.* ×1500).

but those embryos showed very low response during maturation. Consistent high embryogenic efficiency (65–70%) from those calli was achieved only in presence of NAA/2ip (0.1/0.3 mg/l) with a carbon source shock (10% glucose to 2% sucrose) (Table 1). The higher sugar level for a limited period found to favor somatic embryogenesis in several species (Williams and Maheswaran 1986, Lazzeri *et al.* 1988, Mukhopadhyay and Desjardins 1994).

All those embryonic bipolar structures were transferred to 1/2 MS medium with 3% (w/v) sucrose and 4 different hormone combinations (kinetin and ABA) for maturation and maximum number of shoots and roots were recorded in 1/2 MS with 1 mg/l kinetin within 20–30 d (Fig. 1b). Addition of ABA (0.5 mg/l) in the same medium showed formation of shoots and roots after 50–60 d of subculture only in stressed condition (exhausted medium). The complete plants grew profusely in 1/2 strength of Gelrite-solidified MS basal medium (Fig. 1c). The most critical step in somatic embryogenesis is the maturation process which leads to germination and complete plant formation. The importance of ABA (Li and Wolyn 1996) and kinetin (Sink *et al.* 1990) in the differentiation media has been well demonstrated. In our experiment, kinetin favored germination to a great extent and finally resulted in complete plant regeneration showing higher percentage of germination (more than 50%) than other combinations.

Our results suggested that though both NAA and 2,4-D favored induction of embryogenesis, a carbon source shock was found to be most important for a consistent and rapid embryogenesis. The embryo formation was found to be enhanced largely when sucrose (as a carbon source) level was reduced to 2% from 10% glucose. Previous reports also showed that the carbohydrate type and its concentration during induction, maturation and germination affected the process and product of embryogenesis in *A. officinalis* (Levi and Sink 1990, Mukhopadhyay and Desjardins 1994).

The plantlets were potted in vermiculite to acclimatize for 2–3 weeks, after which they were

Table 1. Regeneration of plants from callus of *Asparagus officinalis* L. following somatic embryo induction and maturation

Conc. of growth regulators (mg/l) in induction medium with 10% glucose	Responses as revealed by developed structures	Conc. of growth regulators (mg/l) in maturation medium with 2% sucrose	Responses	Conc. of growth regulators (mg/l) in 1/2 MS medium for plant development	No. of regenerated shoots*	No. of regenerated roots*
NAA/2ip (0.1/0.15)	Globular	NAA/2ip (0.1/0.15)	Globular	—	—	—
NAA/2ip (0.1/0.3)	Many bipolar	NAA/2ip (0.1/0.3)	shoots and roots developed in high number	Kn (0.5)	5.4±0.46	12.4±0.73
				Kn (1.0)	11.6±0.67	14.2±0.10
				Kn (1.5)	6.2±0.18	6.6±0.22
				Kn/ABA (1.0/0.5)	5.8±0.33	8.0±0.49
NAA/2ip (0.1/0.6)	Less bipolar	NAA/2ip (0.1/0.6)	Shoots and roots developed in less number	Kn (0.5)	—	—
				Kn (1.0)	1.4±0.22	7.2±0.33
				Kn (1.5)	1.2±0.18	5.2±0.33
				Kn/ABA (1.0/0.5)	—	—

* Data represents as mean±S.E. from five replicates.

transferred to soil with 80% survival rate. The tetraploid clone was morphologically different from the diploid ones with dense cladodes and short stature. Chromosome analysis from all regenerated plants thus obtained showed $2n=40$ chromosomes (Fig. 1e). Comparative karyotype analysis of the mother plant (diploid) (Fig. 1d) with the present tetraploid line revealed exact duplication of the diploid set with 4 different types of chromosomes (Fig. 2). The length of the chromosomes has been found to be reduced with increase in chromosome number. Out of the 4 callus lines, one was found to be tetraploid after 1 month of callus induction. Successive frequent cytological analysis of these 4 callus lines in 2,4-D containing medium showed stability in respective chromosomal status, *i.e.* the diploid lines with $2n=20$ chromosomes and the tetraploid one with $2n=40$ chromosomes. These results suggest that chromosome doubling might have occurred during callus induction in presence of NAA and kinetin at very low levels in Gelrite-solidified medium. In a previous report, tetraploidy in *Asparagus* was obtained in liquid

Fig. 2. Karyotypes of somatic metaphases of the diploid ($2n=20$) mother plant and a tetraploid ($2n=40$) plant, regenerated *in vitro* of *Asparagus officinalis* L.

medium containing high levels of NAA and kinetin (Odake *et al.* 1993). In our study, it was also observed that intermediate phase in association with 2,4-D did not alter the chromosome number of the callus lines. 80% of the regenerated plants from these callus line survived in the field and were all tetraploids.

The present investigation has, therefore, been very important as it has established a new elite tetraploid clone of a new cultivar of *A. officinalis via* somatic embryogenesis. The present protocol is very simple, less time consuming and is able to regenerate tetraploids, in addition to normal diploids.

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References

Bayliss, M. W. 1980. Chromosomal variation in plant tissue in culture. Int. Rev. Cytol. (Suppl.) **11A**: 113–144.

Desjardins, Y. 1992. Micropropagation of Asparagus (*Asparagus officinalis* L.). In: Bajaj, Y. P. S. (ed.). Biotechnology in Agriculture and Forestry, Vol. 19. Springer-Verlag, Berlin, pp. 26–41.

Komura, H., Chokyu, S. and Ikeda, Y. 1990. Micropropagation of asparagus through somatic embryogenesis. (2) Condition of somatic embryogenesis, plant regeneration and habituation from Mary Washington 500W seedling. Bull. Hiroshima Pref. Agr. Exp. Stn. **53**: 50–61.

Lazzeri, P. A., Hildebrand, D. F., Sunega, J., Williams, E. G. and Collins, G. B. 1988. Soybean somatic embryogenesis: interaction between sucrose and auxin. Plant Cell Rep. **7**: 517–520.

Levi, A. and Sink, K. C. 1990. Differential effects of sucrose, glucose and fructose during somatic embryogenesis in asparagus. J. Plant Physiol. **137**: 184–189.

Li, B. and Wolyn, D. J. 1996. Abscisic acid and ancymidol promote conversion of somatic embryos to plantlets and secondary embryogenesis in *Asparagus officinalis* L. *In Vitro* Cell Dev. Biol.-Plant **32**: 223–226.

Mukhopadhyay, M. J., Ray, T. and Mukhopadhyay, S. 2000. Ploidy level variation in callus culture of *Pisum sativum* L. Nucleus **43**: 24–26.

—, — and Sen, S. 2002. *In vitro* propagation of *Iphigenia indica*, an alternative source of colchicine. Plant Cell Tiss. Org. Cult. **69**: 101–104.

Mukhopadhyay, S. and Desjardins, Y. 1994. A comparative study on plant regeneration from protoplasts of two genotypes of *Asparagus officinalis* L. Plant Science **100**: 97–104.

— and Sharma, A. K. 1990. Chromosome number and DNA content in callus culture of *Costus speciosus* (Koen) Sm. Genetica **80**: 109–114.

—, Banerjee, N. and Sharma, A. K. 1989. Chromosome behaviour in regenerants from excised leaf-discs of *Solanum sarrachoides* Sendt. Caryologia **42**: 147–152.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15**: 473–497.

Odake, Y., Udagawa, A., Saga, H. and Mii, M. 1993. Somatic embryogenesis of tetraploid plants from internodal segments of a diploid cultivar of *Asparagus officinalis* L. grown in liquid culture. Plant Science **94**: 173–177.

Sacristan, M. D. and Melchers, G. 1969. The karyological analysis of plants regenerated from tumorous and other callus cultures of tobacco. Mol. Gen. Genet. **105**: 317–333.

Shigeta, J., Sato, K., Tanaka, S., Nakayama, M. and Mii, M. 1996. Efficient plant regeneration of asparagus by inducing normal roots from *in vitro* multiplied shoot explants using gellan gum and glucose. Plant Science **113**: 99–104.

Sink, K. C., Elmer, W. H. and Stephens, C. T. 1990. Regeneration of asparagus plants from callus-derived protoplasts. Acta Hortic. **271**: 117–127.

Swedlund, B. and Vasil, I. K. 1985. Cytogenetic characterization of embryogenic callus and regenerated plants of *Pennisetum americanum* (L.) K. Schum. Theor. Appl. Genet. **69**: 575–581.

Williams, E. G. and Maheswaran, G. 1986. Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. Ann. Bot. **57**: 443–462.

Yang, H. J. and Clore, W. J. 1973. Rapid propagation of *Asparagus* through lateral bud culture. HortScience **8**: 141–143.