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Anther culture of tetraploid plants of asparagus (*Asparagus officinalis* L.)

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Abstract

Anther culture was carried out using tetraploid asparagus (Asparagus officinalis L.) which were obtained through diploid anther cultures and partly through colchicine treatment in order to detect the origin of the anther-derived tetraploid plants, and to produce dihaploid, aneuploid and polyploid plants for providing stock plants for breeding asparagus.

1. Best callus formation(82%) was observed when anthers at meiosis to uninuclear developmental stage of microspores were incubated. The rate of callus formation less than 3% was observed in the culture of anthers at binuclear to mature developmental stage of microspores.

2. Concerning the culture of anthers at uninuclear developmental stage of microspores, best callus formation (80% or more) was obtained on the medium containing 0.2mg/l of 6-benzylaminopurine(BA) and 0.2mg/l of α-naphthalene acetic acid(NAA) under both of light(16 hr-photoperiod) and dark condition. Kinetin and 2,4-dichlorophenoxyacetic acid(2,4-D) were inferior in callus formation to BA and NAA, respectively.

3. Shoot differentiation from anther-derived calluses was observed at the rate of 20 to 25% on the medium containing 1.0mg /l of BA and 0.5mg/l of NAA. Sequential transferring of the differentiated shoots to the rooting medium with 0.1mg/l of indole-3-butyric acid(IBA) resulted in regeneration of plantlets with a high frequency(80% or more). All the plantlets obtained so far were tetraploid male plants.

Introduction

Asparagus (*Asparagus officinalis* L.) is a dioecious plant and has male and female plants separately with a sex ratio of 1:1. Female plants consume some of the photosynthetic products for the production of berries in the fall, in which 6 seeds were normally contained. This may be why female plants neither can have high productivity nor can live as long as male plants. Therefore, commercial fields consisting of only male plants have been desired for an increased productivity, and the breeding of all-male F_1 hybrid have tried.[9] Many in vitro plants have been regenerated through anther culture of diploid asparagus plants[4, 5, 11], which have been cultivated in many countries as one of the most popular green and yellowish vegetables. Diploid plants are the most common among the plants which were obtained through the anther culture, but a few haploid, polyploid(3X, 4X, 5X), and aneuploid plants have also been obtained[6, 9]. Among them tetraploid plants have been obtained most frequently when cultured on the medium supplemented with high concentrations of growth regulators(cytokinin and auxin: 1-10mg / 1). Up to now, we have obtained tetraploid plants which consisted of only male plants, but we could not yet determine whether they were originated from microspores(n) or from anther tissues(2n). Moreover we were unable to produce F_1 seeds from crosses between 2X female plants and 4X male plants, which were induced through diploid anther culture, for progeny test. Many fruits resulted from 2X(♀) × 4X(♂) contained empty seeds. This phenomenan was also observed in the fruits from

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2X(♀) × 4X(♂) which was produced with colchicine treatment of 2X seedlings.[2)]

If tetraploid male plants induced through anther cultures are originated from microspores, they can have 4 male determining superior genes, MMMM. Hence dihaploid plants(2X) obtained through the anther cultures of tetraploid with a genotype of MMMM should result in production of all male plants. On the other hand, if tetraploid male plants are originated from somatic cells(anter tissues) with a genotype of Mm, the induced dihaploid can be expected to be consisting of 1 super-male plant with "MM" genes, 4 male plants with "Mm' genes, and 1 female plant with "mm" genes. Consequently, 5 male plants of 2 genotypes and 1 female plant can be induced. We intended to produce dihaploid plants through anther culure of diploid anther-derived tetraploid of asparagus in order to detect the origin of anther-derived tetraploids, and also to examine the probability to induce haploid, triploid, and aneuploid for applying them in asparagus breeding systems. In anther culture of tetraploid plants diversities of chromosome segregation at meiosis of tetraploid microspore mother cells can be expected.

Materials and Methods

Tetraploid anthers obtained through the anther culture and colchicine treatment of diploid asparagus plants(cv. 'Mary Washington 500') were prepared. Murashige and Skoog medium supplemented with 20g / 1 of glucose and 100mg / 1 of inositol, and ajusted to pH 5.5 before autoclaving was provided as the basal medium in the experiments. Plant growth regulators were added to the medium when needed.

Experiment 1. Effect of the developmental stage of tetraploid anthers on callus formation.

The flower buds of 1.5-2, 2.5-3, 4, and 5mm in length were collected from tetraploid male plants For sterilization, they were immersed in 70% ethanol for 30 seconds, followed by 1% sodium chloride solution for 15 minuts, and then rinsed 3 times with sterile double distilled water. Five anthers dissected from a flower bud were incubated in a vessel containing the medium supplemented with 0.2mg/l of BA and 0.2mg/l of NAA. Ten vessles were used for each teatment. The cultures were placed under 16 hr of daylength with 1,500 lux illuminance provided by cool-white fluorecent lamps at 25-27℃.

Experiment 2. Effects of cytokinin and auxin of callus formation from anthers.

The anthers of 2mm in length at uninuclear developmental stage of microspores were incubated on media supplemented with 10 combinations of NAA and/or 2,4-D with BA or kinetin. The cultures were

Table 1. Relationships between the length of flower bud, the developmental stage of microspore, and starch accumulation in microspores and anther tissues.

Length of flower bud (mm)	Developmental stage of microspore	Starch accumulation**	
		Microspore	Anther tissue
1	Pollen mother cell ~ meiosis	−	+
2	Early uninuclear	−	−
3	Late uninuclear ~ binuclear	+~+++	−
4	Binuclear ~ mature	+++	−
5	Mature*	++	−

* : generative cell started dividing into two sperm cells.
** : starch accumulation was expressed as follows ; − : no starch, + : a trace, ++ : a fair amount, +++: full amount (pollen grains were stained strongly with an iodine reagent)

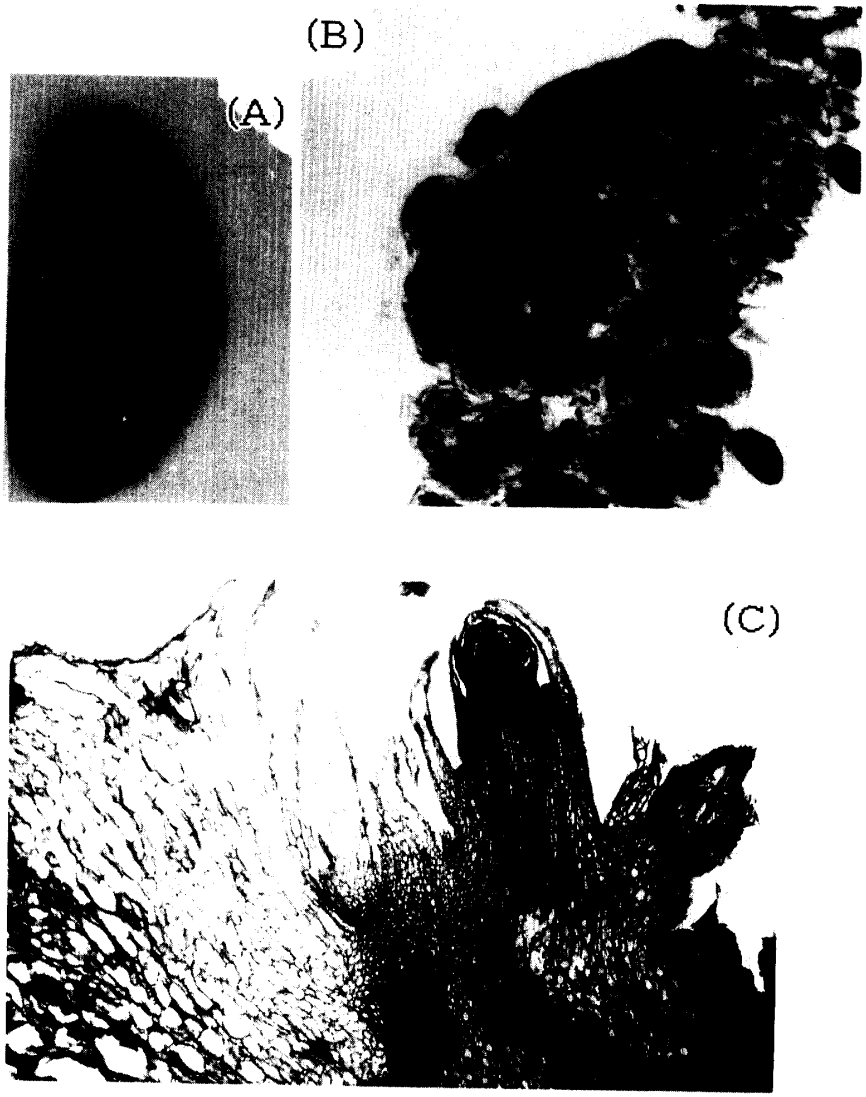

Fig. 1 Microspores (A) and callus cells (B) observed in early stages of incubation on the medium with 0.2mg/l of BA and 0.2mg/l of NAA, and shoots (C) differentiating from the callus incubated on the medium with 1.0mg/l of BA and 0.5mg/l of NAA.

placed either under the same condition as in Experiment 1 or under continuous darkness.

Experiment 3. Shoot differentiation from anther-derived calluses, and root formation from the shoots.

Rice grain-sized callus pieces, which were divided from soybean-sized calluses grown on the medium containing 1mg/l of BA and 1mg/l of NAA, were transferred onto the medium supplemented with 1.0mg/l of kinetin or BA. and 0.5mg/l of NAA.

Shoot apexes of 0.5-1cm in length were transferred into the medium supplemented with 0.1mg/l of IBA for direct rooting. For the experiments of shoot differentiation and rooting, 3 explants per vessel were incubated under a 16 hr daylength with 4,000 lux of illuminance at 25-27℃.

Results

Experiment 1. Effect of the developmental stage of anthers on callus formation.

When male flowers of tetraploid plants were compared with those of diploid plants at the same developmental stage of anthers, flower buds of the tetraploid plants were a little longer, and much wider

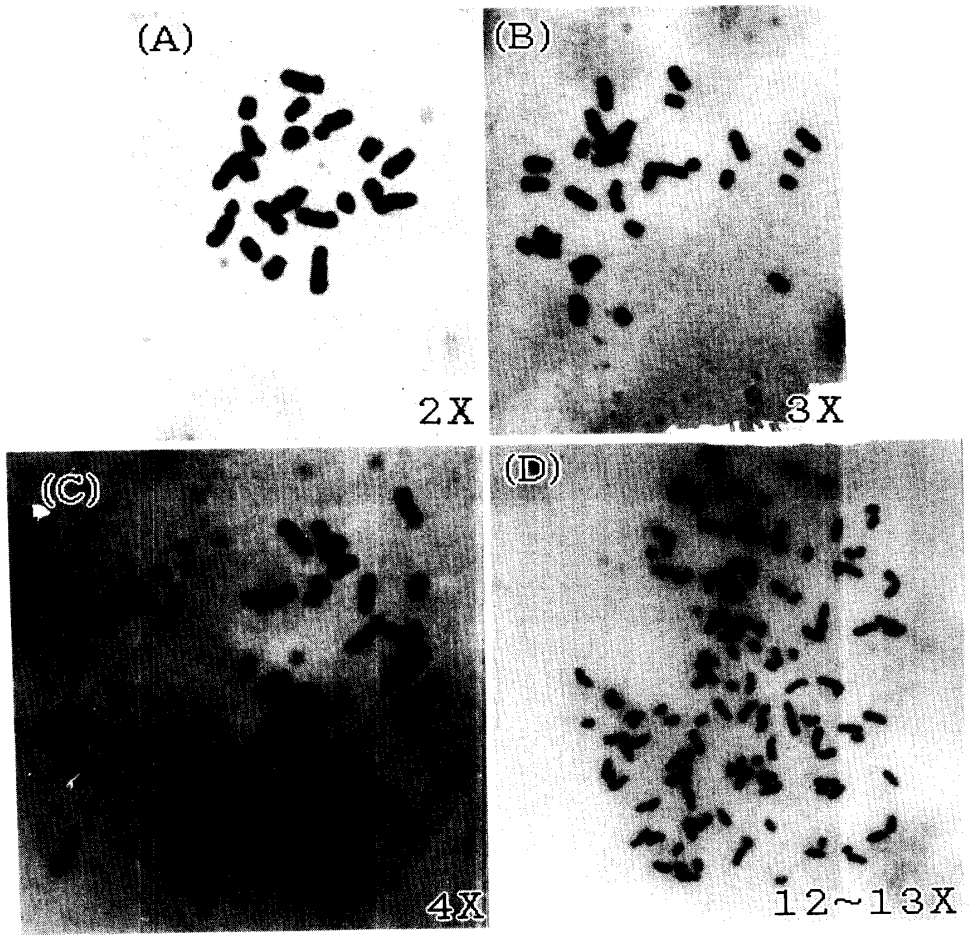

Fig. 2 Chromosomes observed in callus cells produced through tetraploid anther cultures of asparagus
(A) : 2n=2X (20) : Cells with 2X chromosomes were considered to be microspores.
(B) : 2n=3X (30) Cells with 3X chromosomes were also considered to have the origin in microspores.
(C) : 2n=4X
(D) : 2n=12~13X Prolonged incubation of calluses on the identical medium resulted in the induction of cells with high ploidy levels. Cells with 8X ore more chromosomes were observed in this experiments.

than those of the diploid plants. Consequently, the tetraploid plants could be distinguished from diploid plants. Table 1 shows relationships between the length of flower buds, the developmental stage of microspores, and starch accumulation in microspores. The relationships between them in tetraploid plants were almost the same as those in diploid plants.

In the early stage(7 to 10 days after incubation) of cultured anthers, some pollens were observed to develop into multicellar tissues(Fig.1-B). We also observed cells with 20-40 chromosomes(FIg.2-A,B,C) in calluses induced in a few weeks of incubation, which were considered to be originated from microspores(Fig.1-A). Longer incubation of calluses resulted in increases of many polyploid and aneuploid cells with 70 to 80 or more

chromosomes(Fig.2-D).

The rate of callus formation was highest(82%) for anthers at tetrad to early uninuclear stage, followed by anthers at late uninuclear to binucler stage(28%) (Fig. 3). Incubation of mature anthers of 4mm and 5mm in length resulted in low callus formation at 3 and 0%, respectively.

Experiment 2. Effects of cytokinin and auxin on callus formation from anthers.

As shown in Fig. 4, callus formation was achieved most successfully(82.2%) on the medium supplemented with 0.2mg/l of NAA in combination with 0.2mg/l of BA. As compared with NAA, 2.4-D had less callus forming ability. Kinetin had less callus forming ability than BA in a low concentration(0.2mg / l), but more callus forming ability in a high concentrarion(3.0mg / l) . Calluses induced on the medium supplemented with 0.2mg / l of BA and 0.2mg / l of NAA under the light condition was green, hard compact in contrast to pale yellow and friable under the dark condition. After 8 to 12 weeks of culture, shoot formation was observed in most of caluses induced under the dark condition(Fig. 1-C).

Also, pale yellow, soft and compact calluses were induced through the anther culture of tetraploid plants produced by colchicine treatment.

Experiment 3. Shoot differentiation from anther-derived calluses, and root formation on the shoots.

The percentages of calluses differentiating shoots were 20% and 25% on media with 0.5mg/l of NAA in combination with 1.0mg/l of BA and 10.mg/l of kinetin, respectively(Fig. 5). Calluses which were able to differentiate one shoot had a tendency to produce more shoots succesively.

Transplanting 5-10mm apical end(stem tip) of shoots induced from calluses to a medium with 0.1mg/l IBA resulted in rooting of 80% or more, i.e., regeneration of plantlets occurred in 80% or more of the transplanted shoots(Fig.6).

All the regenerated plantlets were male tetraploids, of which the stem had cell sizes and stractures similar to those in diploid anther-derivied tetrapliod. Also it had larger cortical layer cells than those in diploid(Fig.7).

Discussion

Diploid, aneuploid, and tetraploid plants were produced through diploid anther cultures of asparagus(*Asparagus officinalis* L.) . As shown in Fig.7, the stem of tetraploid was regular in structure, and had larger cortical layer cells than those in diploid; on the other hand, the stem of aneuploid showed irregular structure, and contained larger intercellular spaces. Calluses from anther cultures of tetraploid plants, which were produced through dipoid anther culture, grew and proliferated more rapidly than diploid anther-derived calluses, and differentiated many shoots on the same medium as calluses were induced. Shoot differentiation was observed remarkably on the calluses which proliferated to the size of soybean seed or bigger under the dark condition. However, the anther-derived tetraploid calluses which were produced with colchicine treatment were slightly soft and compact as compared with the meristematic friable calluses procuced through diploid anther culture, and did not differentiate any shoots.

Up to now, all the plantlets obtained through the anther culture of tetraploids were tetraploid male plants. Chomosome numbers of these plants were considered to be doubled in the periods of callus proliferarion and / or shoot differantiation(Fig. 1-C), because microspore-derived calluses(Fig .1-B) were induced on the medium for callus formation contaning 0.2mg / l of BA and 0.2mg/l of NAA. It was reported in many plant species such as sugar cane[1] , tobacco[8] , and pea[7] that long periods of incubation on callus inducing medium resulted in remarkable increases of polyploid and aneuploid cells in the calluses. Malnassy[6] reported that 29 tetraploid plants were produced among 31 plants obtained from diploid calluses. We observed many callus cells with 100 or more chromosomes(Fig. 2-D), and even with 150-160 chromosomes in this experiments. Since the concentrations of BA and NAA that we used in this experiments were considered to be too high for regenaraion of dihaploid plants. We should investigate on the lower concentrarions of BA and NAA.

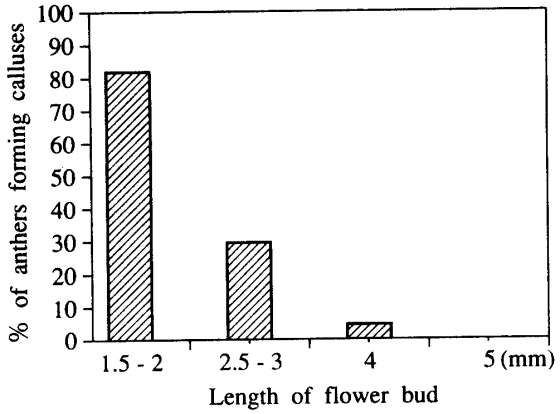

Fig. 3 Effect of developmental stages of anthers on callus formation (incubated for 7 weeks)

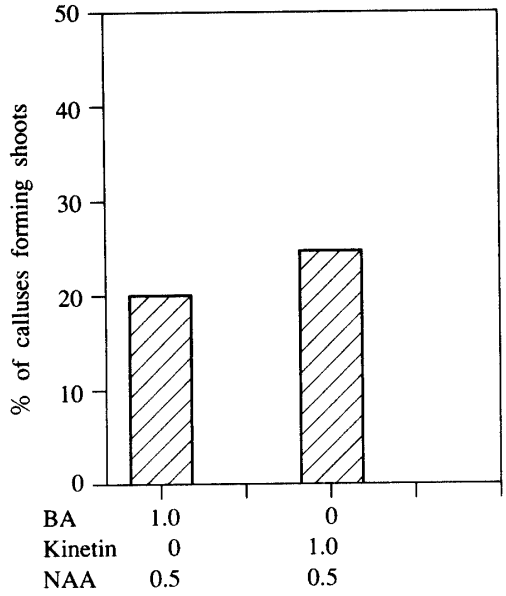

Fig. 5 Effect of BA, kinetin, and NAA on shoot formation from anther-derived calluses (incubated for 7 weeks)

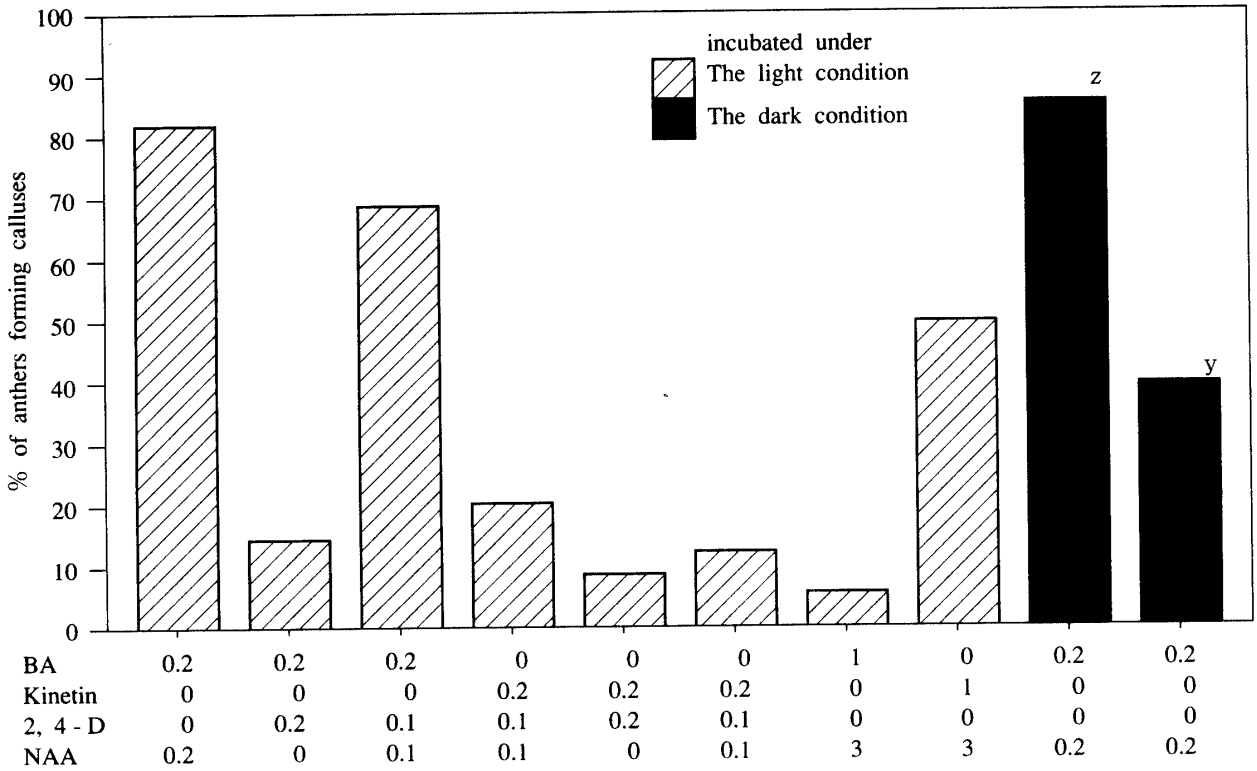

Fig. 4 Effect of cytokinin and auxin on callus formation from anthers of tetraploid plant (incubated for 8 weeks)
z : Most of calluses differentiated shoots.
y : Anthers of tetraploid produced with colchicine treatment were incubated.

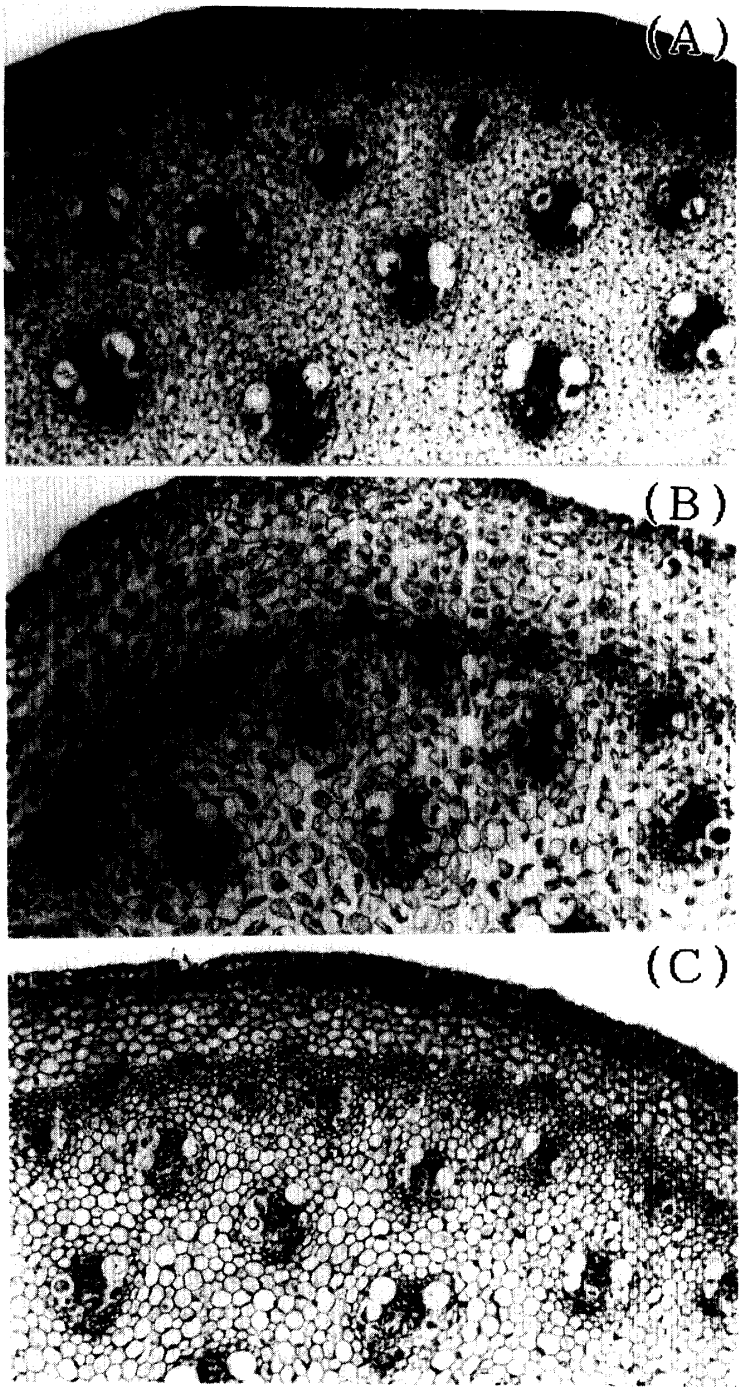

Fig. 7 Cross sections of the stems diploid (2n=2x=20), aneuploid (2n=33−36), and tetraploid (2n=4x=40) plant obtained through diploid anther cultures of asparagus
(A) : DIPLOID (B) : ANEUPLOID (C) : TETRAPLOID

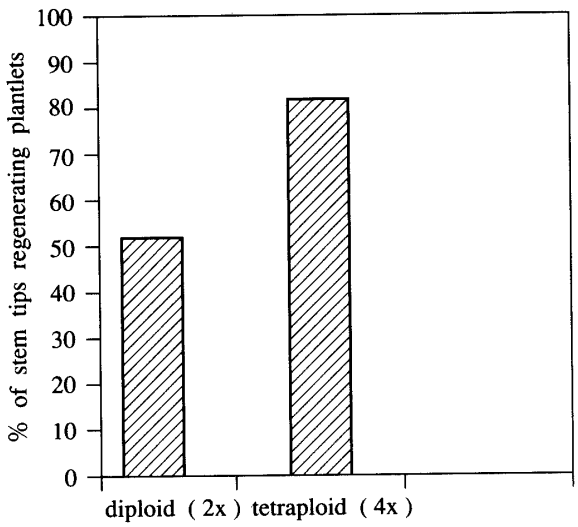

Fig. 6 Plantlet regeneration from stem tips of shoots obtained by the anther culture of diploid (2x) and tetraploid (4x) plants (incubated for 8 weeks)

Root formation from shoots regenerated from tetraploid anther-derived calluses was obtained at the higher frequency(80%) than that from diploid anther-derived callus, and resulted in plantlet regeneration. The tetraploid plantlets obtained in this way were transplanted successfully in soil, and grew as normaly as the mother plants. Consequently, we can consider the tetraploid anther culture as the method of mass production suitable for the field-sized cultivation of tetraploid plants in asparagus.

Up to now, we have not been able to induce dihaploid plants of asparagus successfully. However since we were able to induce microspore-derived calluses, it would be possible to regenarate dihaploid plants if polyploidization of chromosomes in callus cells could be suppressed.

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アスパラガス4倍体の葯培養

稲垣　昇

要　約

2倍体株の葯培養によって得られた4倍体株の起源の確認、及び多様な育種素材の作出を目的として4倍体の葯培養を行った。

1）葯の発育度とカルス形成率：葯からのカルス形成率は減数分裂期～1核期初期の葯を用いた場合最も良好（82%）で、花粉成熟期の葯からはほとんど（3%）形成されなかった。

2）カルス形成率に及ぼすサイトカイニン及びオーキシンの影響：小胞子1核期の葯からのカルス形成率は、BA0.2+NAA0.2mg/l添加区で、明所及び暗所培養とも高い値を示した。カイネチンはBAに比較して、また2，4－DはNAAに比較してカルス形成率の点で劣っていた。

3）カルスからのシュート分化及びシュートからの発根：シュート分化培地（BA1.0+NAA0.5mg/l）でのカルスからのシュート分化率は20～25%であった。またシュートを発根培地に移植すると80%以上で発根が認められ、植物体にまで生長した。

現在のところ、作出された植物は全て4倍体の雄株であった。