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Micropropagation of *Spinacia oleracea* L. through Culture of Shoot Primordia

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Tissue-cultured shoot primordia of *Spinacia oleracea* were induced from apical domes of shoot tips in MS liquid medium supplemented with 0. 2, 2. 0 or 4. 0 mg/*l* IAA and 0. 2, 2. 0 or 4. 0 mg/*l* BAP at pH 5. 8 by shaking at 2 cycles/minute on rotary culture equipment(1 m diameter). Multiplication of those shoot primordia was the most effective in MS liquid medium supplemented with 0. 2 mg/*l* IAA and 0. 02 mg/*l* BAP. Vigorous regeneration of plantlets was accelerated in MS supplemented with 0. 2 mg/*l* BAP and 4. 0 mg/*l* IAA ; 2. 0 mg/*l* BAP and 0. 02, 0. 2, 1. 0 or 4. 0 mg/*l* IAA ; or 4. 0 mg/*l* BAP and 0, 0. 02, or 0. 2 mg/*l* IAA in combination, and their root formation was easily promoted in MS supplemented with no hormone or 0. 2 mg/*l* IAA.

Introduction

Spinacia oleracea L. (Chenopodiaceae), native to Middle East Asia, from Afghanistan to the eastern part of the Mediterranean area, is an annual, alkaliplant. Since this species is dioecious, any mutated individual-plant, pure homozygous, individual-plant, etc. is difficult to maintain and necessary alleles are difficult to take in if they are placed in different individual-plants of the same sex.

The shoot primordium method of Tanaka and Ikeda[4] has been applied to numerous plant species for rapid mass-propagation of clonal plants without any chromosome aberration, genic unbalance, or virus contamination. The cultured shoot primordia can be maintained and multiplied only in liquid media stirred in test tubes by rotary culture equipment (2 cycles/minute).[4]

In this study, the shoot primordium method was utilized to develop and improve a system of gene storage for genetically important, individual strain and genetic diversity of *Spinacia oleracea*.

Materials and Methods

Seeds of two cultivars of *Spinacia oleracea* L., 'Minster Land' and 'Jiromaru' purchased from a commercial source were sterilized with 1/100 benzalkonium chloride (Takeda) solution for ten minutes and then in 1/5 Purelox (sodium hypochlorite solution : 5% active chlorine) with a drop of Tween 20 for 30 minutes. Then, they were rinsed with sterile distilled water several times, before they served as starting material for the culture.

After germination *in vitro*, apical domes of their shoot tips approximately 1 mm long were harvested and placed in test tubes (30 mm X 200 mm, 25 m*l*) containing Murashige and Skoog media (MS)[2] supplemented with auxin [1-naphthaleneacetic acid (NAA) or indole-3-acetic acid

(IAA)] at concentrations of 0, 0. 02, 0. 2, 2. 0 and 4. 0 mg/*l* and cytokinin [6-benzylaminopurine (BAP)] at concentrations of 0, 0. 02, 0. 2, 2. 0 and 4. 0 mg/*l* at pH 5. 8 for shoot primordia induction. Then, the proliferation of masses of shoot primordia and regeneration of plantlets from those shoot primordia were tested in MS media containing IAA at concentrations of 0, 0. 02, 0. 2, 2. 0 and 4. 0 mg/*l* and BAP at concentrations of 0, 0. 02, 0. 2, 2. 0 or 4. 0 mg/*l*. All of the cultures were incubated at 22°C under 6300 lux illumination by fluorescent lamps for 16 hours of photoperiod and shaken at 2 cycles/minute on a rotary culture equipment (1 m diameter). They were subcultured at two weekintervals. During the subcultures, rates of proliferation of shoot primordia and regeneration of plantlets in the liquid media conditioned at pH 6. 5, 7. 0, 7. 5 and 8. 0 were compared with those in the media adjusted at pH 5. 8 as the standard. Gibberellic acid (GA_3) at concentrations of 0, 0. 01, 0. 1, 0. 5, 1. 0, 3. 0, 5. 0, 7. 0 and 10. 0 mg/*l* was applied in the MS liquid media for a test of growth acceleration of masses of *Spinacia oleracea* shoot primordia.

Results and Discussion

Masses of shoot primordia of *Spinacia oleracea* cv. 'Minster Land' and 'Jiromaru' were induced in MS supplemented with IAA or NAA at concentration of 0. 2, 2. 0 or 4. 0 mg/*l* and BAP at concentration of 0. 2, 2. 0 or 4. 0 mg/*l* 30 days after the beginning of primary culture (Table 1). Masses of shoot primordia of both cultivars grew within two weeks up to approximately twice as large as those at the initiation of subculture in MS supplemented with 0. 2 mg/*l* IAA and 0. 02 mg/*l* BAP (Fig. 1A).

Vigorous regeneration of leaves was displayed when the masses of shoot primordia were transferred to MS media supplemented with 0. 2 mg/*l* BAP and 4. 0 mg/*l* IAA ; 2. 0 mg/*l* BAP and 0. 02, 0. 2, 1. 0 or 4. 0 mg/*l* IAA ; or 4. 0 mg/*l* BAP and 0, 0. 02 or 0. 2 mg/*l* IAA in combination (Table 2 ; Fig. 1B-D).

Spinacia oleracea is an alkaliplant which shows weak growth against acid soil-condition. Although MS supplemented with 0. 2 mg/*l* IAA and 0. 02 mg/*l* BAP at pH 5. 8 was a sufficient medium for shoot primordium growth of this species, that with 0. 2 mg/*l* IAA and 0. 02 mg/*l* BAP and conditioned pH 6. 5, 7. 0, 7. 5 and 8. 0 stimulated the species to induce shoot formation.

Table 1. Induction of masses of shoot primordia of *Spinacia oleracea* from apical domes 30 days after culture in various MS media supplemented with auxin and cytokinin for primary culture.

Auxin (IAA or NAA) (mg/*l*) \ Cytokinin (BAP) (mg/*l*)	0	0. 02	0. 2	2. 0	4. 0
0	F	F	F	F	F
0. 02	F	F	F	F	F
0. 2	F	F	SP	SP	SP
2. 0	F	F	SP	SP	SP
4. 0	F	F	SP	SP	SP

SP=formation of a mass of shoot primordia F=failure
Five apical domes were used in each experiment.

Table 2. Plant regeneration from tissue-cultured shoot primordia of *Spinacia oleracea* 30 days after transplantation to various MS media supplemented with BAP and IAA.

IAA (mg/*l*) \ BAP (mg/*l*)	0	0. 01	0. 02	0. 2	2. 0	4. 0
0	F	B	B	B	A, B	A
0. 02	F	F	B	B	A	A
0. 2	F	F	B	A, B	A	A
1. 0	F	B	B	A, B	A	F
2. 0	F	B	B	A, B	F	F
4. 0	F	B	F	A	A	F

A=shoot primordia producing well several broad, thick, green leaves
B=shoot primordia producing poorly linear, thin, yellowish green leaves
F=no regeneration
Three masses of shoot primordia were used in each experiment.

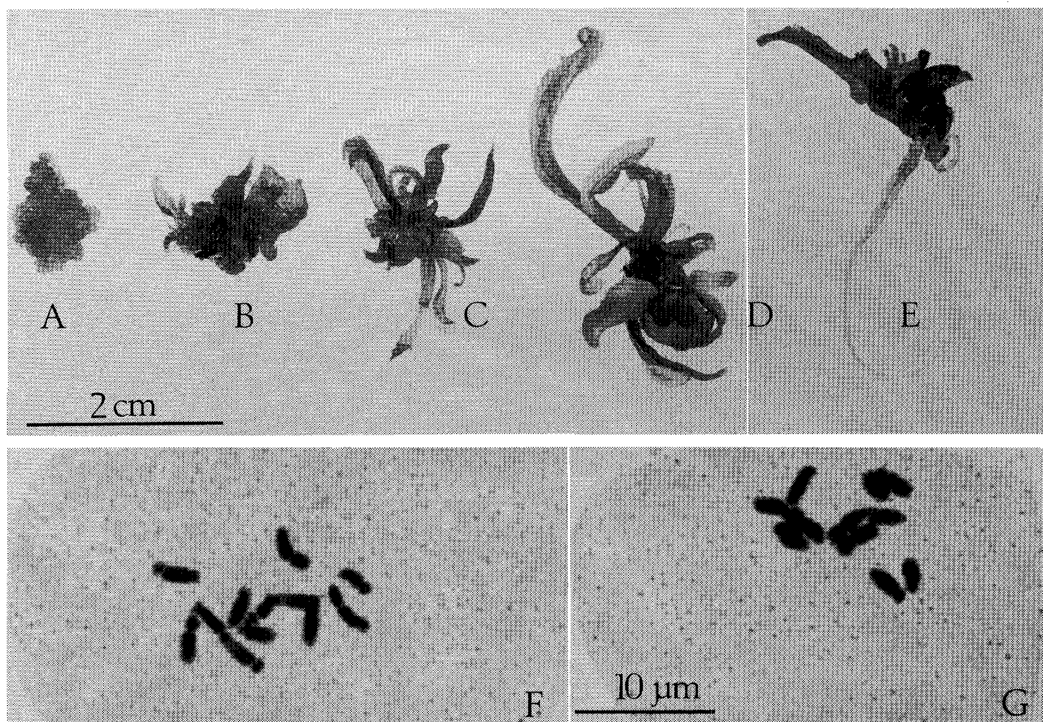

Fig. 1 Culture progress of *Spinacia oleracea* through the tissue-cultured shoot primordium method. A. A mass of shoot primordia at the stage of two weeks culture. B-D. Developmental stages of leaf regeneration. E. Root regeneration for a complete plantlet. F and G. Mitotic-metaphase cells of present subcultured shoot primordia of the female *Spinacia oleracea* cv. 'Minster Land' (F) and 'Jiromaru' (G) showing the standard chromosome number of 2n=12.

Furthermore, masses of shoot primordia cultured in MS medium supplemented with 0. 01 mg/*l* BAP and 1. 0 mg/*l* GA_3 and on MS media supplemented with 2. 0 mg/*l* BAP and conditioned at pH 6. 5, 7. 0, 7. 5 and 8. 0, especially pH 6. 5 synthesized a red-pigmented area on every shoot primordium. The pigment was similar to that of the base of the above-ground portion of the plant *in vivo*. The cultures with red pigment regenerated leaves more easily than those cultured in the media at pH 5. 8. However, the shoot primordia and regenerated plantlets quickly weakened after awhile and later died, if they were cultured in a medium with a pH higher than 5. 8. Therefore, the pH should be adjusted at 5. 8 for healthy plantlet formation. On the other hand, some shoot primordia of *Spinacia oleracea* planted on MS gelrite (2g/*l*) media supplemented with 0. 02 mg/*l* BAP and 0. 02 mg/*l* IAA or with 0. 01 or 2. 0 mg/*l* BAP and 0. 1 mg/*l* GA_3 at pH 5. 8 in flasks (100 m*l*) exhibited regeneration of several plantlets with numerous leaves 18 days after transplantation. Their roots formed readily in MS supplemented with or without 0. 2 mg/*l* IAA (Fig. 1E).

The clonal plants finally selected in the two cultivars used were determined female by their flowered inflorescences.

These female strains of shoot primordia of the two cultivars of *Spinacia oleracea* have been continuously subcultured for five years and so far have not displayed any chromosome number (Fig. 1F, G) different from the standard chromosome number of 2n=12, although endopolyploidy is common in *S. oleracea*.[1,3]

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《和文要約》

組織培養苗条原基によるホウレンソウの大量クローン増殖

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一年生，雌雄異株，アルカリ植物であるホウレンソウの，安定したクローンレベルの増殖，遺伝子保管を目的に，組織培養苗条原基誘起ならびに維持のための培養条件を検討した．0.2 または 8.0 mg/*l* IAA と 0.2, 2.0 または 4.0 mg/*l* BAP を添加した MS 液体培地中で，茎頂ドームを回転培養して苗条原基を作出した．苗条原基塊の増殖には 0.1 mg/*l* IAA と 0.02 mg/*l* BAP を添加した MS 液体培地がよかった．葉の再分化には 0.2 mg/*l* BAP と 4.0 mg/*l* IAA, 2.0 mg/*l* BAP と 0.02, 0.2, 1.0 または 4.0 mg/*l* IAA，そして 4.0 mg/*l* BAP と 0，0.02 または 0.2 mg/*l* IAA の組合せで添加した MS 液体培地がよく，発根には 0.2 mg/l IAA 添加またはホルモン無添加 MS 液体培地でよい結果が得られた．