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Plant Regeneration from Hypocotyl-derived Calli of Spinach (*Spinacia oleracea* L.) and Anatomical Characteristics of Regenerating Calli

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Plant regeneration and/or embryogenesis from callus and/or protoplast are necessary for selection of various tolerances such as acid- and pesticide-tolerance and for the formation of transgenic plants. In this experiment, we studied callus induction from hypocotyl segment, plant regeneration from hypocotyl-derived calli and anatomical structures of regenerating calli of spinach cv. Sunlight. Calli were induced on MS medium containing 7.0 mg/*l* of NAA. Shoots without roots were obtained from calli, which were precultured on 0.1 mg/*l* of IAA without GA_3, upon transfer on to hormone-free MS medium. Contrastingly, calli which were precultured with the following hormone combinations, *i.e.* 1.0 mg/*l* of IAA and 1.0 mg/*l* of GA_3, 5.0 mg/*l* of IAA and 0.1 mg/*l* GA_3 and 5.0 mg/*l* of IAA and 1.0 mg/*l* of GA_3 showed plant regeneration. In these calli, vascular bundle tissues that connect shoots and adventitious roots were observed. The present results indicate the significant effect of GA_3 in the culture medium in assuring plant regeneration in spinach.

Introduction

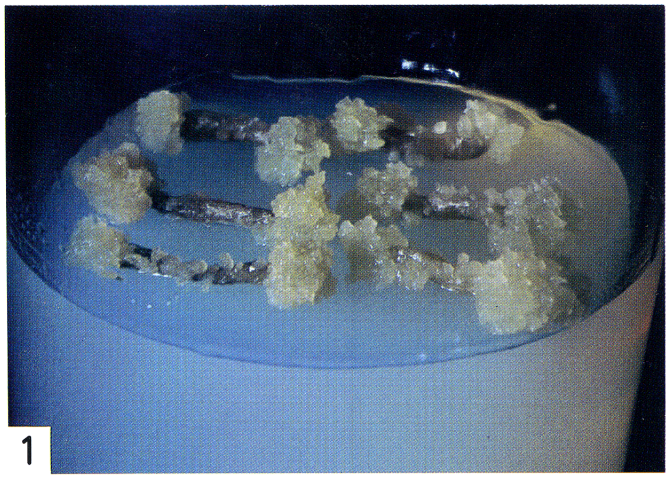
Spinach is one of the most nutritious vegetables[1]. However, spinach is vulnerable to acid soil, and hence cannot grow and/or growth decreases extremely below pH 6.0[2-4]. Unfortunately, acid soils are widely distributed especially in developing countries where farmers cannot use enough amount of lime due to its high cost[5]. Soil acidity increases the availability of aluminum, manganese and iron[3,6-8], indicating that complicated factors take part in the acid tolerance of a crop plant. Of these factors, tolerance to aluminum, which disturbs cellular metabolism inside the roots has been intensively examined using plants grown in water culture [2,3,7,9,10], seedlings[11-13] and calli[13-16]. Thus, biotechnological techniques also appear to be efficient for producing acid tolerant spinach. However, studies for high frequency plant regeneration from spinach calli or protoplasts are lacking.

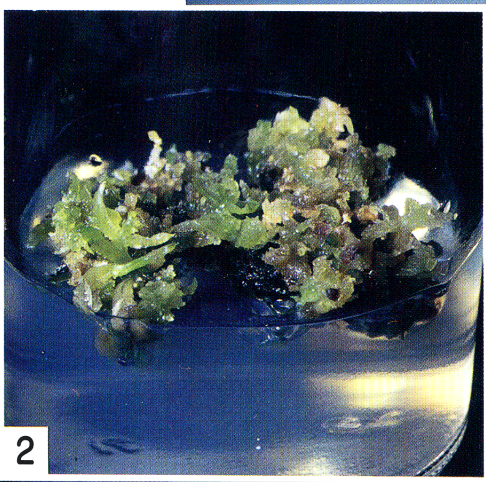
In this study, callus induction from hypocotyl, plant regeneration from calli and anatomical structures of regenerating calli were examined.

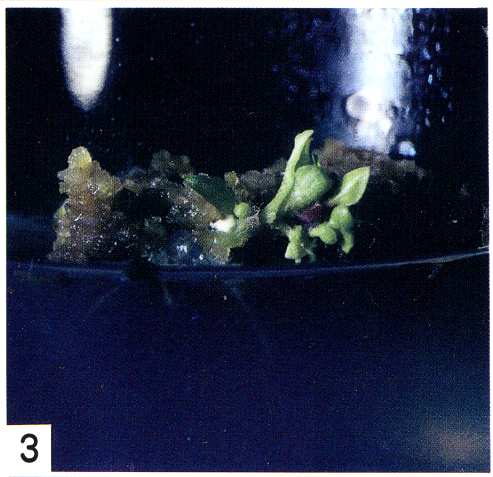
Materials and Methods

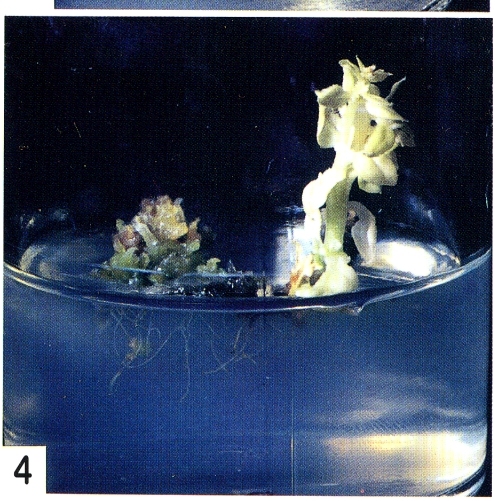
Spinach cv. Sunlight from Sakata Seed Co., Ltd. was used. To decrease bacterial and fungal contamination and to increase germination rate, seed pericarp was carefully taken off using forceps. The seeds were sterilized in 2% sodium hypochlorite for 45 min. and washed with sterilized distilled-water.

Murashige and Skoog's[17] (MS) medium which contains 30 g/*l* of sucrose and 8 g/*l* of agar was used for callus induction. MS medium with 30 g/*l* of sucrose and 2 g/*l* gellan gum was used for









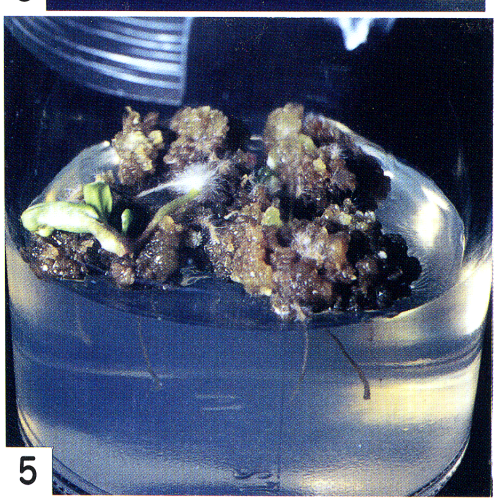


Fig. 1 Callus formation from hypocotyl segment on MS medium containing 7.0 mg/*l* of NAA.

Fig. 2 Formation of shoots from hypocotyl-derived calli which were precultured with 0.1 mg/*l* of IAA.

Fig. 3 Formation of shoot and adventitious roots from hypocotyl-derived calli which were precultured with 5.0 mg/*l* of IAA and 1.0 mg/*l* of GA_3.

Fig. 4 Chlorophyll degradation on some regenerated plant. Calli were subcultured on hormone-free MS medium after preculturing with IAA and GA_3 containing medium.

Fig. 5 Regenerated plant on hormone-free MS medium after preculturing with IAA and GA_3 containing medium.

preculture and plant regeneration. The pH was adjusted to 6.5 throughout the experiments. Throughout callus induction, preculture and plant regeneration experiments, 20°C temperature and 16 hr. day length at about 3,000 lux light intensity were maintained.

In callus induction from hypocotyl segments, seeds were cultured on MS basal medium without plant hormones. Hypocotyl segments from 7-day old seedlings were excised and cultured on MS basal medium containing 7.0 mg/*l* of naphthaleneacetic acid (NAA).

After 45 days of culture, hypocotyl-derived calli were transferred for preculture on MS basal medium containing indole-3-acetic acid (IAA) at 0.1, 1.0 and 5.0 mg/*l* and gibberellin (GA_3) at 0, 0.1 and 1.0 mg/*l*, and in all combinations. The 30-day old calli were subcultured onto hormone-free MS basal medium for plant regeneration. The rate of plant regeneration was evaluated 30 days later.

Hypocotyl-derived calli were fixed in formalin, acetic acid and alcohol (FAA solution) for 24 hr. Following dehydration in *n*-butanol, the materials were embedded according to the usual paraffin method, and were cut to 10 micrometers in thickness. The materials were stained with hematoxylin for 30 min., and microphotographs were made.

Results

Hypocotyl-derived calli are shown in **Fig. 1**. The calli appeared to be yellowish and friable. In the medium containing 7.0 mg/*l* of NAA, the rate of callus induction was 80% and the calli growth was vigorous.

The rate of shoot formation from hypocotyl-derived calli is shown in **Table 1** and plant regeneration from hypocotyl-derived calli is shown in **Table 2.** The calli which were precultured on the

Table 1. Rate of shoot formation from hypocotyl-derived calli on hormone-free MS medium.

IAA \ GA_3	0 mg/*l*	0.1	1.0
0.1 mg/*l*	40.0	8.0	0.0
1.0	0.0	0.0	16.0
5.0	0.0	22.1	20.0

Regenerating-calli which were precultured on IAA and GA_3 combinations. Concentrations of IAA and GA_3 which were included in the preculture medium. Numerical values indicate percentage (%) shoot per callus.

Table 2. Rate of plant regeneration from hypocotyl-derived calli on hormone-free MS medium.

IAA \ GA_3	0 mg/*l*	0.1	1.0
0.1 mg/*l*	4.0	0.0	0.0
1.0	0.0	0.0	12.0
5.0	0.0	10.0	14.0

Plant regenerating-calli which were precultured on IAA and GA_3 combinations. Concentrations of IAA and GA_3 which were included in the preculture medium. Numerical values indicate percentage (%) plantlet per callus.

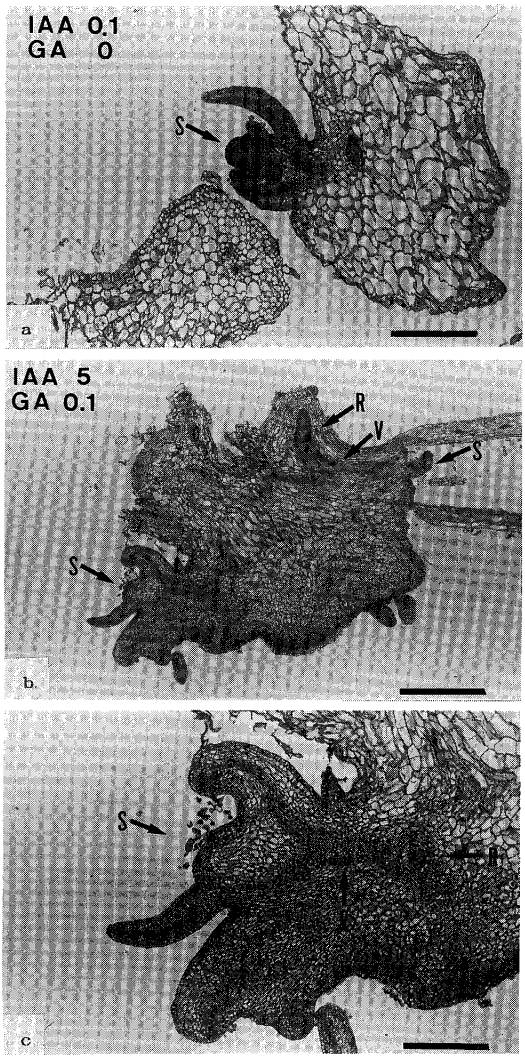


Fig. 6 Anatomical observation of calli.
Shoot formation on regenerated calli which were precultured with 0.1 mg/*l* of IAA (a). Shoot, aventitious root and vascular bundle on regenerated calli which were precultured with 5.0 mg/*l* IAA and 0.1 mg/*l* of GA_3 (b and c). Arrows indicates Shoot (S), adventitious root (R) and vascular bundle (V). Bar is equal to 300 micrometers in **Fig. 6-a** and **b** and 120 micrometers in **Fig. 6-c.**

medium with 0.1 mg/*l* IAA exhibited a rate of 40% shoot formation characterized by thick leaves (**Fig. 2**). However, root formation was not observed.

On the other hand, calli which were precultured on culture medium with the following hormone combinations, *i. e.* 1.0 mg/*l* of IAA and 1.0 mg/*l* of GA_3, 5.0 mg/*l* of IAA and 0.1 mg/*l* of GA_3, and 5.0 mg/*l* of IAA and 1.0 mg/*l* of GA_3 showed a rate of about 20% shoot formation. This rate was lower than shoot formation in calli precultured on 0.1 mg/*l* of IAA. However, Hypocotyl-derived calli which were precultured with the hormone combinations mentioned above developed shoots and adventitious roots, *i. e.* plantlet regeneration (**Fig. 3**). The calli which were precultured on IAA and GA_3 containing media exhibited a rate of about 10% plant regeneration (**Table 2**). However, the plantlets showed chlorophyll degradation (**Fig. 4**). Furthermore, some calli also regenerated plantlets when transferred onto hormone-free MS medium (**Fig. 5**).

The anatomical characteristics of regenerating calli are shown in **Fig. 6-a** which were precultured on the medium with 0.1 mg/*l* of IAA, **Fig. 6-b** which were precultured on the medium with 5.0 mg/

l of IAA and 0.1 mg/l of GA_3, and **Fig. 6-c** a magnification of **Fig. 6-b**. Hypocotyl-derived calli which were precultured on MS medium containing 0.1 mg/l of IAA produced shoots, and were confirmed anatomically to have no adventitious root formation (**Fig. 6-a**). In contrast, hypocotyl-derived calli which were precultured on MS medium containing 5.0 mg/l of IAA and 0.1 mg/l of GA_3, produced both shoots and adventitious roots, and vascular bundles which connected shoots and adventitious roots were confirmed.

Discussion

Generally, studies on plant regeneration from callus[18,19] showed that concentrations and combinations of plant hormones for plant regeneration from callus differed with plant species and organ used. In spinach, there are few studies on plant regeneration from plant tissue culture[20-22] and on embryogenesis[23,24]. The adventitious bud formation[20] and embryogenesis[23,24] from hypocotyl segments of spinach was stimulated by simultaneous application of IAA and GA_3. Embryogenesis in spinach was achieved by transferring hypocotyl-derived calli, previously cultured on IAA and GA_3 containing medium on MS hormone-free medium. However, these studies revealed that the rate of plant regeneration was low. In the present experiments, a relatively high rate of shoot formation without adventitious root from hypocotyl-derived calli of spinach which were precultured with 0.1 mg/l of IAA without GA_3 were observed even on the hormone-free MS medium. Furthermore, a relatively high rate of shoot and adventitious root formation, *i. e.* plant regeneration was also observed in calli cultured on hormone-free MS medium when the calli were precultured on the media with 1.0 mg/l of IAA and 1.0 mg/l of GA_3, 5.0 mg/l of IAA and 0.1 mg/l of GA_3 and 5.0 mg/l of IAA and 1.0 mg/l of GA_3. Thus, these results suggest that preculturing of calli in adequate concentrations of IAA and GA_3 combination and subsequent transfer onto hormone-free MS medium enhance plant regeneration from hypocotyl-derived calli of spinach. Furthermore, addition of GA_3 was efficient for inducing plant regeneration. This fact appears to agree with GA_3 effectiveness for adventitious bud[21] and embryogenesis[23,24] in spinach. Although, the rate of plant regeneration was relatively high in the present experiment, the plants appeared to experience some mutations as shown in the development of thick leaves and chlorophyll degradation.

Calli, shoots and roots were independently derived at a relatively high rate from hypocotyl segments[20]. Contrastingly, we derived calli from hypocotyl segments, and then shoots, roots and plantlets were derived from calli, i. e. plantlet regeneration. This information will contribute to further genetic manipulation for improvement of tolerance to acid-soil, quality and so on through callus and/or cell selection as shown in carrot[14-16,25]. Selection for tolerance to acid soil was also reported in other crop plants[14-19,26]. Spinach is also one of the vegetables most susceptible to acid soil. However, genetic manipulation through tissue culture and gene-engineering is lacking. Thus, the information which was obtained in the present experiments will contribute to these studies.

Furthermore, there remain many problems for further study to increase the rate of plant regeneration from calli in spinach, *i. e.* use of different plant hormones or their combinations, examination of the effect of osmotic pressure, temperature, moisture and medium composition as indicated by Fry and Street[27] and Nishimura *et al.*[28]. The protoplast isolation[29-31] and callus[32] and shoot[33] formation from protoplast have been established in spinach. However, the rate of plantlet regeneration was very low[33]. Thus, establishment of a system for protoplast selection, subsequent callus formation and plant regeneration still remain an important experimental target.

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《和文要約》

ホウレンソウ(*Spinacia oleracea* L.)の胚軸由来カルスからの植物体再分化と再分化カルスの組織学的観察

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植物組織培養手法を用いた植物育種をおこなうためには，不定芽・不定胚を誘導する植物体再分化系の確立が必要である．本実験は，ホウレンソウ品種のサンライトを用い，胚軸からのカルスの誘導，植物体再分化，及び再分化カルスの組織学的観察をおこなった．

胚軸からのカルスの誘導は，MS培地にNAA 7 mg/l添加しておこなった．得られたカルスをIAA 0.1 mg/l添加した培地で前培養した後に，ホルモンフリー培地に継代する事により不定芽が得られた．一方，再分化植物体は，胚軸から誘導したカルスをIAA 1.0 mg/lとGA_3 1.0 mg/l，IAA 5.0 mg/lとGA_3 0.1 mg/l，またはIAA 5.0 mg/lとGA_3 1.0 mg/lをそれぞれ添加した培地で前培養した後にホルモンフリー培地に継代する事により得られた．また，その組織学的観察により，不定芽と不定根を連絡するように維管束組織が観察された．