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Callus Induction and Growth with Reference to Acid-tolerance in Calli and Seedlings of Spinach (*Spinacia oleracea L.*)

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Summary

In the present experiments, the following are examined, 1) callus induction from plant organ and seed, 2) callus growth, 3) callus selection for acid-tolerance and 4) plant and/or organ regeneration.

Addition of 7.0 mg/l of NAA to the basal MS medium was most efficient for callus induction from spinach seeds and hypocotyl segments. However, difference of the rate of callus induction was observed among cultivars and organs used.

The highest value of callus growth was observed on the medium into which 5.0 mg/l of 6 BAP and 7.0 mg/l of NAA were added. Difference of the growth rate of seed-origin calli was observed among cultivars.

On the media containing low concentration of NAA in combination with high concentration of 6 BAP, hair-like roots and green spots were formed on the calli, but no adventitious bud.

In the acid-tolerance test of callus on the different pH media, calli from all the cultivars grew on the acidic media. Especially, Atlas calli showed a relatively high value on the media of pH 4.0 and 5.0. In the acid-tolerance test for seedlings, root and hypocotyl in five cultivars except for Atlas were depressed in the soil of pH 4.0 and 5.0. However, root and hypocotyl of Parade were slightly longer than those of other cultivars. Callus and root of Atlas seedlings grew parallel in the pH range of 4.0 to 9.0.

Introduction

Spinach is one of the most nutritious vegetables[24], however, it is so vulnerable to acid soil that it can not grow and/or it's growth decreases below pH = 6.0[28,30,35]. Unfortunately, acidic soils are widely distributed especially in developing countries where farmers could not afford to use an enough amount of lime due to its high cost[3]. Acidity of soil increases the availability of aluminum, manganese and iron[4,29,31], indicating that complicated factors take part in acid tolerance of crop plants. Of the complicated factors, tolerance to aluminum which disturbs cellular metabolism inside the roots have been intensively examined using crop plants grown in water culture[25,28~30,33], seedlings[26,27] and calli[16~18]. To facilitate the study on acid-tolerance, biotechnological techniques may be useful to improve the acid-tolerance of spinach. In this study, the following are examined：1) callus induction from hypocotyl and seed, 2) callus growth, 3) callus selection for acid-tolerance and 4) plant and/or organ regeneration.

Expriment I : Callus Induction

Materials and Methods

Spinach cultivars used were Atlas, Sunlight, Titan, Pioneer, Parade and Hoyo which were bred by Sakata Seed Co. Ltd. To eliminate bacterial and fungal contamination and to increase germination rate, seed pericarp was carefully taken off by forceps. The seeds were sterilized in 2 % sodium hypochlorite for 40 min. and washed with sterilized distilled-water.

Murashige and Skoog[9] (MS) medium which contains 30 g/l of sucrose and 8 g/l of agar at pH 6.5 was used as the basal medium throughout the experiment I, II, III and IV. Temperature at 26～28℃ and continuous illumination of about 3,000 lux were also maintained throughout the experiments. In callus induction from seeds, the sterilized seeds were inoculated onto the MS basal medium containing different concentrations of plant hormones, i.e. Naphthaleneacetic Acid (NAA) : 1.0, 4.0, 7.0, 10.0 mg/l, Indole-3-acetic Acid (IAA) : 10.0, 20.0, 30.0, 40.0 mg/l and 2, 4-Dichlorophenoxyacetic Acid (2, 4-D) : 1.0 mg/l, respectively. Of these plant hormones, IAA and 2, 4-D have been used for callus induction in spinach although the effect of concentration of the hormones on callus induction has not yet been examined[2,12].

In callus induction from hypocotyl segments, the seeds were inoculated onto the MS basal medium without plant hormones. The hypocotyl segments were excised from seedlings 7 days after incubation. Then, they were cultured on the MS basal media containing the plant hormones mentioned above. The rate of callus induction was determined by the number of calli to that of seeds or hypocotyl segments 45 days after incubation.

Results

(1) Callus induction from hypocotyl segments

The induced calli are shown in Photo. 1. The calli appeared to be white and friable. The rate of callus induction from hypocotyl segments is also shown in Figs. 1 and 2. In all the cultivars, high values of callus induction were observed on the media into which 4.0 to 10.0 mg/l of NAA were added. Especially, addition of 7.0 mg/l of NAA was most efficient for callus induction. Difference of the rate of callus induction was observed among cultivars used. That is, in callus induction from hypocotyl segments, Atlas showed 92 %, but Pioneer 56 % . Although callus induction was observed on the media supplemented with IAA and 2, 4-D, the rate of callus induction was lower than on the media supplemented with NAA.

(2) Callus induction from seeds

The seeds germinated 2 to 4 days after incubation. Hypocotyls started to swell 1 to 2 weeks after incubation and then yellowish and friable calli were induced from the swelled regions (Photo. 2). The degree of callus induction, however, was influenced by the plant hormones and/or their concentrations. The rate of callus induction from seeds is shown in Figs. 3 and 4. High values of callus induction were observed on the media containing the same concentrations of NAA as in the callus induction from hypocotyl segments. Difference of the rate of callus induction was observed among cultivars used. That is, in callus induction from seeds, Parade and Hoyo showed about 96 % , but Pioneer 68%. The effect of IAA and 2, 4-D addition on callus induction from seeds was also the same as that from hypocotyl segments.

Experiment II : Callus Growth

Materials and Methods

Based on the results in experiment I, the concentration of NAA was fixed at 7.0 mg/l which showed the highest callus induction in both cases of seeds and hypocotyl segments. The MS basal culture media which contained 7.0 mg/l of NAA in combination with four levels of 6-Benzylaminopurine (6 BAP) , i.e. 0.0, 5.0, 10.0 and 30.0 mg/l respectively were prepared for callus growth. Forty five-day calli which were induced from seeds and hypocotyl segments were inoculated onto the culture media. The rate of callus growth was determined by a relative size increment of the calli 30 days after incubation.

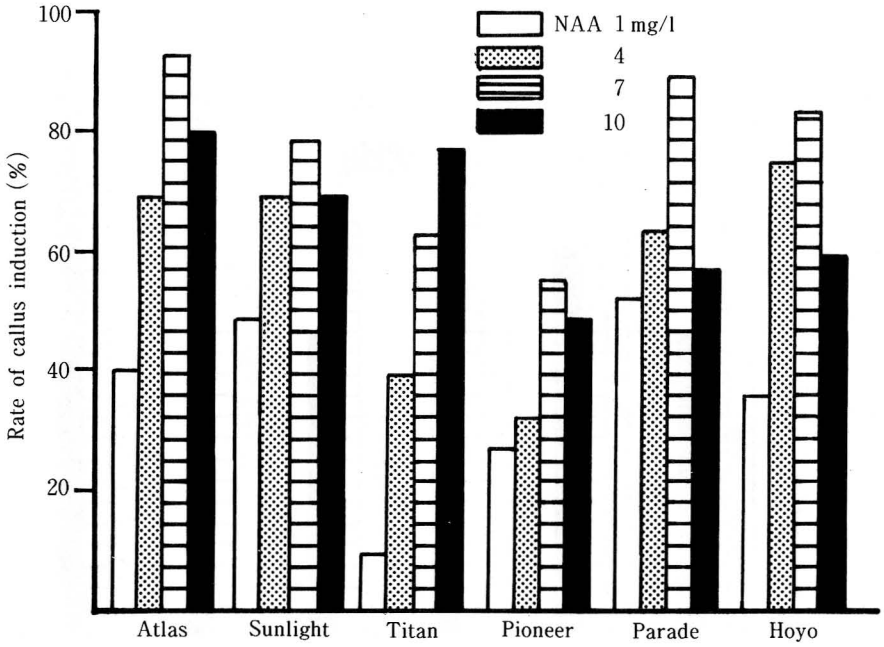

Fig. 1. Effect of concentration of NAA on callus induction from hypocotyl segments.

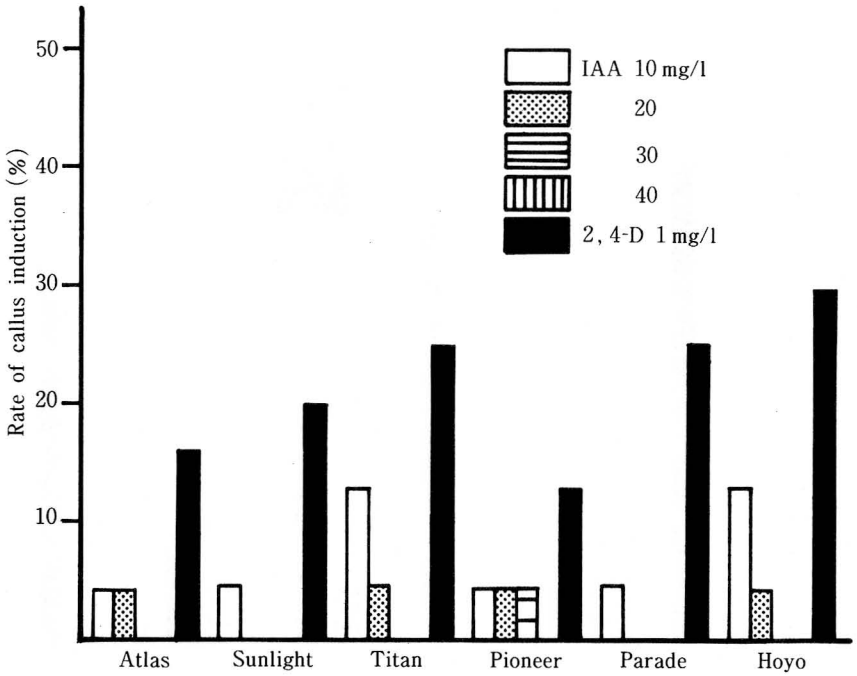

Fig. 2. Effect of concentration of IAA and 2, 4-D on callus induction from hypocotyl segments.

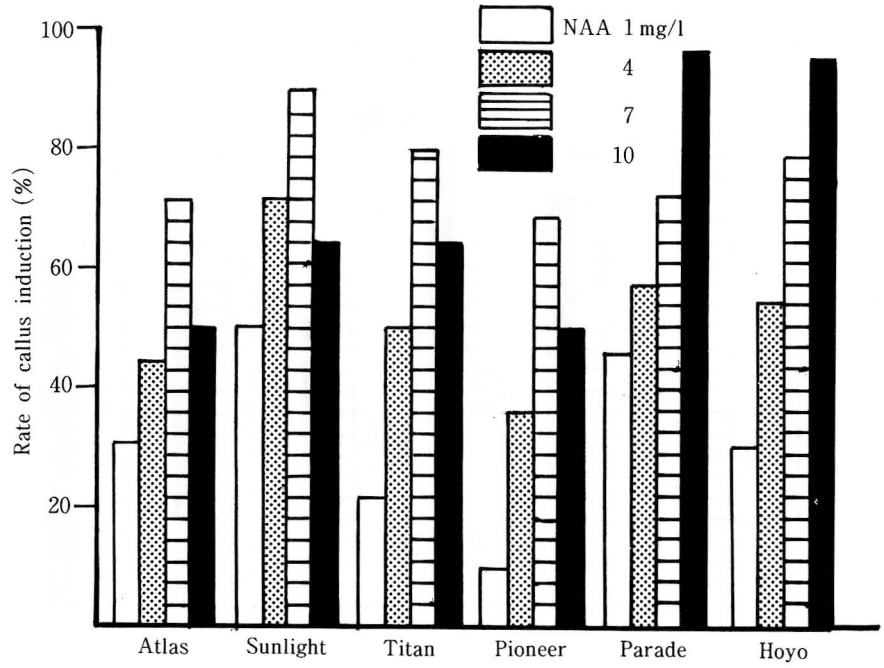

Fig. 3. Effect of concentration of NAA on callus induction from seeds.

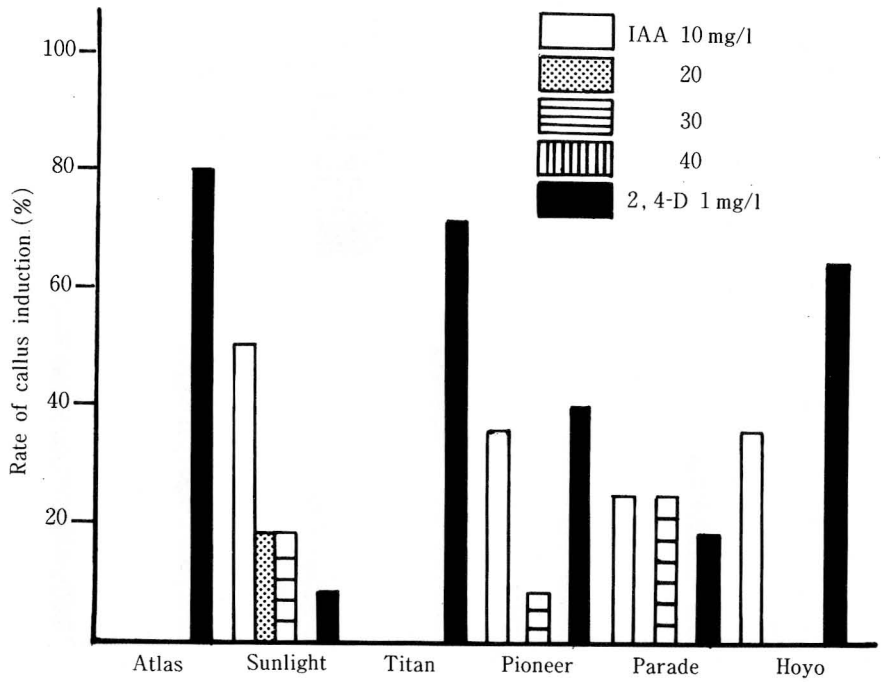

Fig. 4. Effect of concentration of IAA and 2,4-D on callus induction from seeds.

Results

(1) Increase of callus induced from hypocotyl segments

The increased calli appeared to be light-green or white and friable (Photo. 3). The rate of callus growth from hypocotyl segments is shown in Fig. 5. In all the cultivars, the highest value of callus growth was observed in the medium into which 5.0 mg/l of 6 BAP and 7.0 mg/l of NAA were added. There was no increase of calli on the medium containing 30.0 mg/l of 6 BAP and 7.0 mg/l of NAA. The calli turned necrotic and died 3 weeks after incubation. However, increase of calli was observed on the medium with only 7.0 mg/l of NAA, but the value of callus increment was lower than that on the medium mentioned above.

(2) Increase of callus induced from seeds.

Appearance of calli was light-green and friable (Photo. 4). The rate of callus growth is shown in Fig. 6. As the calli induced from hypocotyl segments, four cultivars, i. e. Atlas, Sunlight, Titan and Hoyo showed high values of callus growth on the medium with 5.0 mg/l of 6 BAP and 7.0 mg/l NAA, and also on the medium with 1.0 mg/l of 6 BAP and 7.0 mg/l of NAA except for Hoyo. Furthermore, two cultivars ; Pioneer and Parade showed high values of callus growth on the medium with 1.0 mg/l of 6 BAP and 7.0 mg/l of NAA. The response of callus on the medium containing 30.0 mg/l of 6 BAP and 7.0 mg/l of NAA and on the medium containing only 7.0 mg/l of NAA was the same as that of the hypocotyl segment-derived callus.

Experiment III : Approach to Plant Regeneration from Spinach Callus

Materials and Methods

Calli of Atlas, Sunlight , Parade and Hoyo which

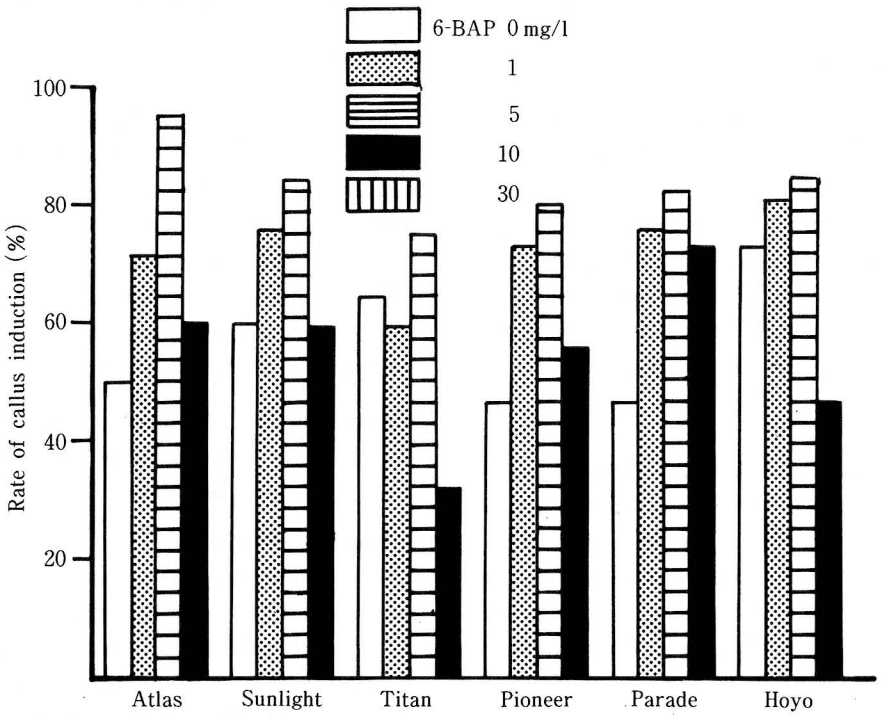

Fig. 5. Effect of 6BAP in combination with NAA (7 mg/l) on the growth of hypocotyl-derived callus.

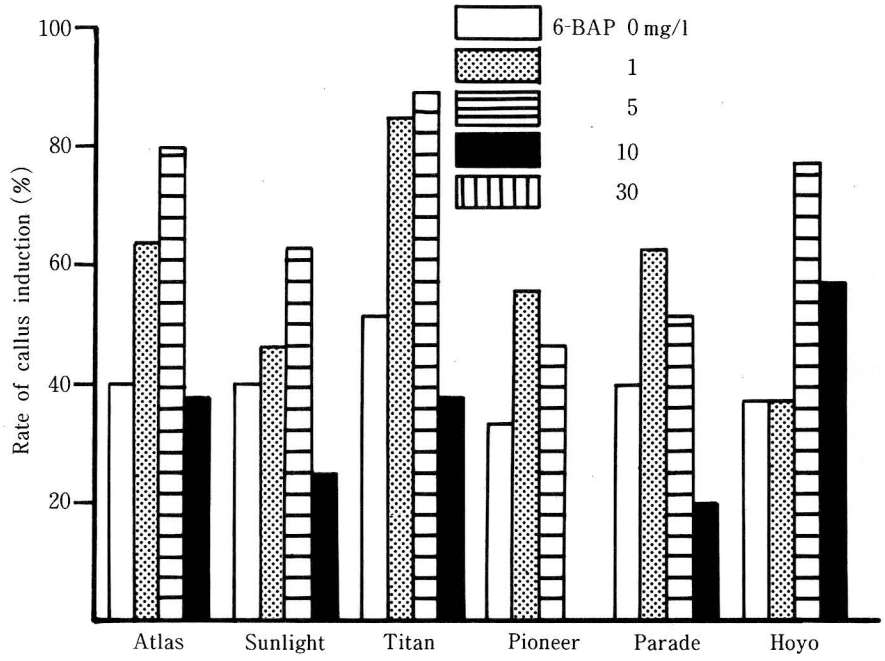

Fig. 6. Effect of 6BAP in combination with NAA (7 mg/l) on the growth of seed-derived callus.

were induced from hypocotyl segments and then subcultured as described in the experiment I and II were used for this experiment. Two media were prepared to determine the response of calli for plant regeneration. Medium I contains low concentrations of NAA, i. e. 0.1, 0.5, 1.0 and 3.0 mg/l, in combination with high concentrations of 6 BAP, i. e. 5.0, 10.0, 20.0 and 50.0 mg/l, respectively. Medium II is hormone-free.

Results

All the calli became necrotic and died on medium I containing 50.0 mg/l of 6 BAP. However , the calli on the other media formed fine and hair-like roots (Photo. 5). The rate of hair-like root formation is shown in Table 1. The hair-like root formation in Hoyo-derived calli was higher than that in other calli, especially on the media with 3.0 mg/l of NAA and 5.0 or 10.0 mg/l of 6 BAP. Compact and deep-green regions, i. e. green-spots were formed on some of the calli (Photo. 6) , although no adventitious buds were formed. All the calli showed high rates of green-spot formation on the media with 5.0 mg/l of 6 BAP although the concentration of NAA was different. That is, the maximum rate of green-spot formation was 80% in Atlas-derived calli, 60% in Sunlight-derived, 110% in Parade-derived, 140% in Hoyo-derived (Table 2). On hormone-free medium, no plant regeneration was observed in all the cultivars and all the calli died 60 days after incubation.

Experiment IV : Selection of Acid-Tolerant Calli and Acid-Tolerance Test of Seedlings.

Materials and Methods

The hypocotyl segment-origin calli were used in experiment IV. The calli were induced and subcultured as in experiment I and II. The basal medium for acid-tolerance selection was prepared by adding 7.0 mg/l of NAA and 5.0 mg/l of 6 BAP to the MS basal medium. The pH of the basal medium was adjusted to 4. 5, 5.0, 6.0, 7.0, 8.0 and 9.0. Acid-tolerance of calli was

Table 1. Rate of hair-like root formation.

Atlas

	6BAP 5 mg/l	10	20	50
NAA 0.1 mg/l	0	0	0	0
0.5	0	0	0	0
1.0	5	0	0	0
3.0	0	0	0	0

Sunlight

	6BAP 5 mg/l	10	20	50
NAA 0.1 mg/l	0	0	0	0
0.5	0	0	0	0
1.0	0	0	0	0
3.0	20	0	0	0

Parade

	6BAP 5 mg/l	10	20	50
NAA 0.1 mg/l	0	0	0	0
0.5	0	0	0	0
1.0	0	0	0	0
3.0	0	0	0	0

Hoyo

	6BAP 5 mg/l	10	20	50
NAA 0.1 mg/l	10	30	0	0
0.5	20	20	20	0
1.0	0	0	0	0
3.0	110	60	0	0

Table 2. Rate of green-spot formation.

Atlas

	6BAP 5 mg/l	10	20	50
NAA 0.1 mg/l	40	50	10	0
0.5	40	35	20	0
1.0	80	40	40	0
3.0	70	35	50	0

Sunlight

	6BAP 5 mg/l	10	20	50
NAA 0.1 mg/l	60	10	0	0
0.5	30	30	0	0
1.0	40	50	0	0
3.0	20	0	0	0

Parade

	6BAP 5 mg/l	10	20	50
NAA 0.1 mg/l	70	20	10	0
0.5	110	50	30	0
1.0	60	0	20	0
3.0	30	0	30	10

Hoyo

	6BAP 5 mg/l	10	20	50
NAA 0.1 mg/l	40	60	20	0
0.5	70	80	20	0
1.0	90	60	30	0
3.0	140	40	10	0

evaluated by a relative size increment. The first evaluation was carried out 30 days after incubation and the second 30 days after subculture which started after the first evaluation.

The acid-tolerance test for spinach seedlings was carried out in sandy soils of which pH was adjusted to 4.0, 5.0, 6.0, 7.0 and 8.0. The seeds were germinated and sown in sandy soils. The sandy soil was fertilized with 1/1000 Hyponex of which pH was also adjusted as that of the soil. Length of root and hypocotyl was measured 30 days after sowing.

Results

The rate of callus growth on different pH media and the degree of acid-tolerance of spinach seedlings in different pH soils are shown in Fig. 7. Large differences of response to pH of the culture medium were observed among calli of different cultivars. The growth

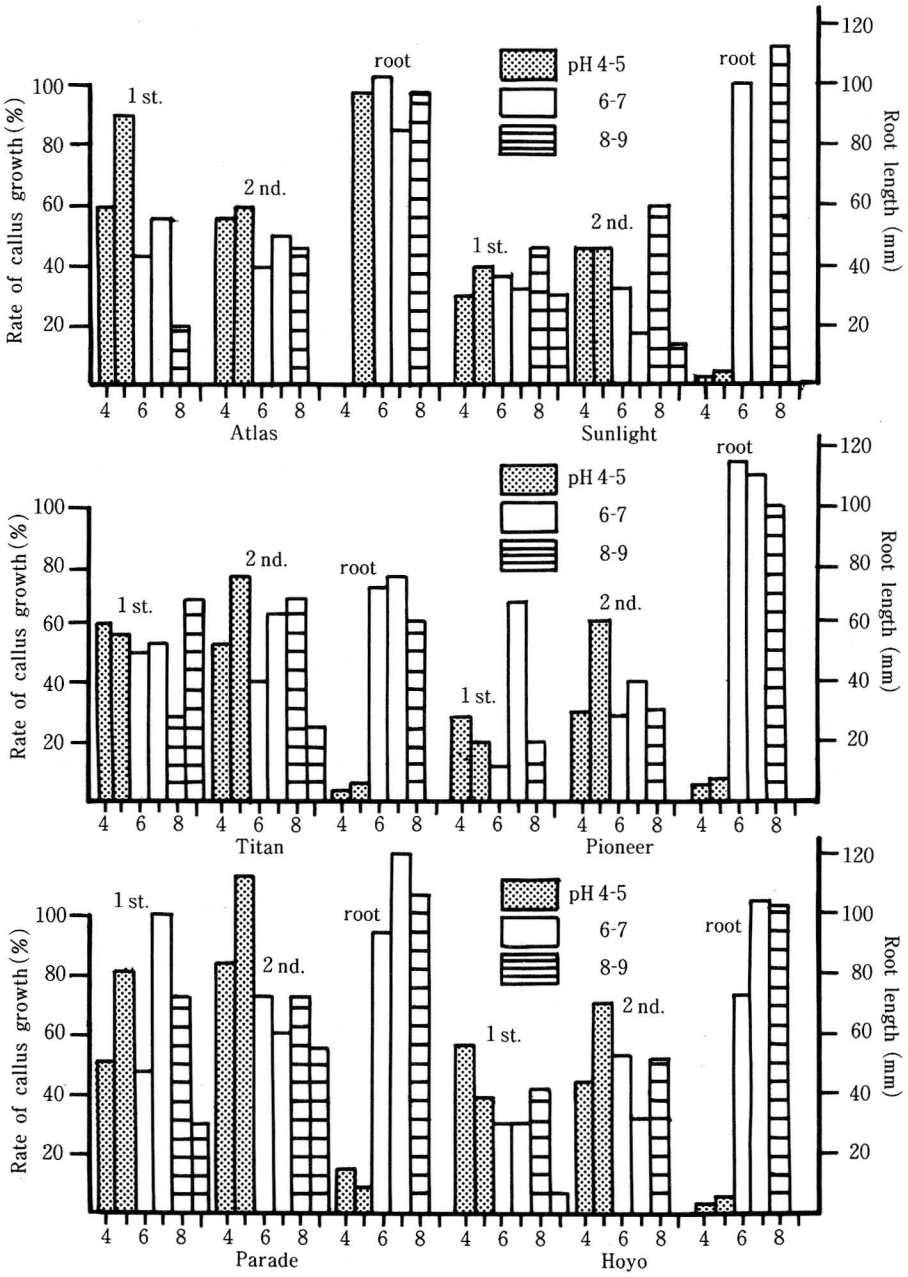

Fig. 7. Effect of different pH media and soils on callus and root growth.
1st. and 2nd. indicate the observation time of callus growth, i. e. 30 days after the first culture and 30 days following subculture respectively.

rate of Atlas-derived calli showed a relatively high value on the medium of pH 4.5 and 5.0, especially 90 % on the pH 5.0 medium at the first evaluation. In contrast, all the Atlas-derived calli became necrotic and died on the pH 9.0 medium 3 weeks after incubation (Photo. 7). The Parade-derived and Titan-derived calli showed a relatively stable growth in a wide pH range of acidic to alkaline medium. The growth rate of Pioneer-derived calli was 20% at the first evaluation, but 60 % at the second evaluation on pH 4.0 to 5.0 medium. On pH 7.0 medium, the growth rate of Pioneer-derived calli was 70 % at the first evaluation, but 40 % at the second evaluation. On pH 9.0 medium, all the Pioneer-derived calli became necrotic and died like Atlas-derived ones. The growth rate of Hoyo-derived calli was generally low on all the media as compared with the other cultivars.

The growth of root and hypocotyl in five cultivars except for Atlas was severely depressed in pH 4.0～5.0 soils (Photo. 8) although root and hypocotyl of Parade were slightly longer than those of other cultivars. All the Atlas seedlings died in pH 4.0 soil, but the growth of root and hypocotyl in pH 5.0 soil was almost similar to that in pH 6.0 to 8.0 soils.

Discussion

Callus induction in spinach was examined by a combination of IAA and Kinetin [12] and by 2, 4-D[2] only. The rate of callus induction in these studies was low as shown in the present experiment in which a wide range of IAA concentrations was examined. However, we reported that a combined use of IAA with GA_3 was efficient for callus induction[21]. In contrast, addition of NAA to the medium in the range of concentrations from 7.0 to 10.0 mg/l brought about high rates of callus induction from seeds and hypocotyl segments. However, the rate of callus induction showed differences among cultivars and organs. These facts indicate that some genetic factors and organ specificity may take part in the callus induction. The effect of GA_3 in combination with a constant concentration of IAA on callus growth was examined using five spinach cultivars[19]. However, GA_3 showed no stable effect on callus growth of different cultivars. In the present experiment, in contrast, addition of 5.0 mg/l of 6 BAP in combination with 7.0 mg/l of NAA showed a relatively stable effect on callus growth across cultivars in case of calli induced from hypocotyl segments. In case of calli induced from seeds, however, there exist differences of callus growth among cultivars. Thus, the present results appear to indicate existence of organ specificity concerning callus growth which may be related with genetic background.

Studies on plant regeneration from callus[22,23] showed that concentrations and combinations of hormones for plant regeneration from callus differed with plant species and organs used. In spinach, there are a few studies on plant regeneration from plant tissues[8,10,19,20,32] and a report of plant regeneration (embryogenesis) [1]. In the present results, no adventitious bud formation was observed, although hair-like roots were formed. Thus, a combination of plant hormones such as low auxin plus high cytokinin which was efficient in tobacco[22] may be not valid for plant regeneration from spinach callus. Green-spots formed very frequently in the present study although they did not develop into plantlets in contrast with rice callus[1]. This fact indicates that the physiological and/or morphological traits of green-spots in spinach calli may differ from those of rice-calli. There remain many problems for plant regeneration in spinach to study in future, i. e. use of different plant hormones and organs, examination of effect of osmotic pressure and temperature on plant regeneration and so on[6,15].

In the present experiment, callus and root of seedlings grew parallel in Atlas in the pH range of 4.0 to 9.0. Furthermore, callus growth in all the cultivars used were more superior than root growth in the low pH range. These facts indicate that selection for acid-tolerance may be possible using calli, based on the genetic traits of cultivars concerning acid-tolerance. That is, Atlas may be genetically acid-tolerant, but the acid-tolerance of calli of other cultivars may be adaptional. However, we have to examine the acid-tolerance in spinach using cultured cells like carrot, tomato and sorghum[7,16,23]. The protoplast isolation[13,14,35] and callus

formation from protoplast[11] have been established in spinach. Based on these studies, we have also to establish the system of protoplast selection, callus formation and plant regeneration for acid-tolerance selection. Aluminum becomes absorbable in acid soil and deposited in root xylem, resulting in the wilting of plant[5,26,34]. Thus, aluminum is one of the most detrimental factors in acid soil. Hence, examination for aluminum-tolerance may be needed in acid-tolerance selection in spinach.

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ホウレンソウ（*Spinacia oleracea L.*）の植物体器官・種子からのカルス誘導・増殖・再分化および耐酸性選抜に関する研究

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（山形大学農学部育種学研究室）

摘 要

本実験は，ホウレンソウの6品種を用い，種子および胚軸からのカルスの誘導・増殖，酸性調整培地での耐酸性能カルスおよび幼植物の品種間差異を知るために行なった．さらに，再分化培地における植物体再分化を検討した．

種子および胚軸からのカルスの誘導は，MS 基本培地に NAA 7 mg/l 添加した時にもっとも旺盛であったが，品種間差異も認められた．また，カルスの増殖は，NAA 7 mg/l と6 BAP 5 mg/l 添加した時にもっとも旺盛であった．この時，胚軸由来カルスでは品種間差異が認められなかったが，種子由来カルスでは品種間差異が認められた．

カルスを，低濃度のオーキシンと高濃度のサイトカイニンが添加された培地に移植すると compact で濃い緑色の部位（green-spot）が観察されたが，不定芽の形成は観察されなかった．また，ホウヨウでは，細かい hair-like root の形成が観察された．

酸性調整培地におけるカルスの耐酸性選抜の結果，すべての品種で pH 4.5～5.0 の酸性域でのカルスの増殖が認められた．幼植物の耐酸性検定の結果，pH 4.0～5.0 の酸性域では，根または胚軸の伸長が著しく阻害された．しかし，アトラスでは阻害の程度が比較的低かった．これらのことから，酸性域でのカルス増殖は，遺伝的要因によって支配される場合と，適応的要因によって支配される場合があることが推察された．

Explanation of Photograph

Photo. 1. Callus formation from hypocotyl segments on the MS basal medium containing 7.0 mg/l NAA. Three weeks after incubation.
cv. Atlas.

Photo. 2. Callus formation from seeds on the MS basal medium containing 7.0 mg/l of NAA.
Three weeks after incubation.
cv. Hoyo.

Photo. 3. Growth of hypocotyl-derived callus on the basal medium containing 7.0 mg/l of NAA, and 5.0 mg/l of 6 BAP.
Three weeks after incubation.
cv. Atlas.

Photo. 4. Growth of seed-derived callus on the MS basal medium containing 7.0 mg/l NAA, and 5.0 mg/l 6 BAP.
Three weeks after incubation.
cv. Parade.

Photo. 5. Hair-like root formation in hypocotyl-derived callus on the basal medium containing 3.0 mg/l of NAA and 5.0 mg/l of 6 BAP, after preculture on the medium containing 7.0 mg/l of NAA and 5.0 mg/l of 6 BAP.
cv. Hoyo.

Photo. 6. Green-spot formation in hypocotyl-derived calli on the medium containing 0.1 mg/l of NAA and 5.0 mg/l of 6 BAP, transferred from the preculture medium containing 7.0 mg/l of NAA and 5.0 mg/l of 6 BAP.
cv. Sunlight.

Photo. 7. Atlas-derived calli on the pH 9.0 medium.
Three weeks after incubation.
cv. Atlas.

Photo. 8. Growth of root and hypocotyl in the pH 4.0 soil.
No. 1. : cv. Sunlight.
No. 2. : cv. Hoyo.
No. 3. : cv. Pioneer.
No. 4. : cv. Atlas.

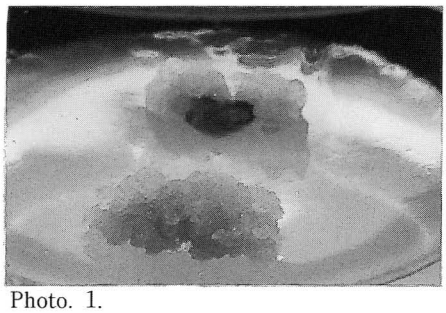
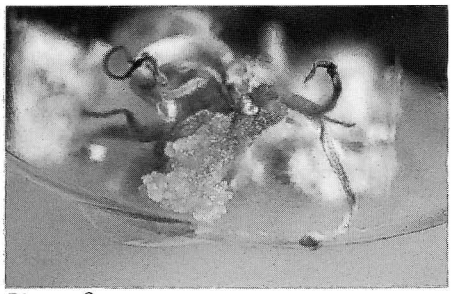


Photo. 1.



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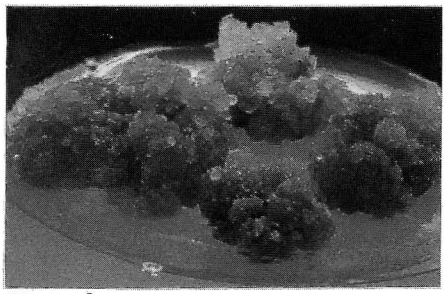

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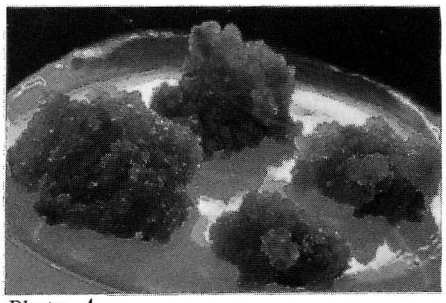

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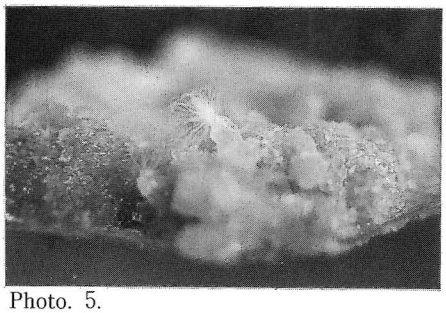

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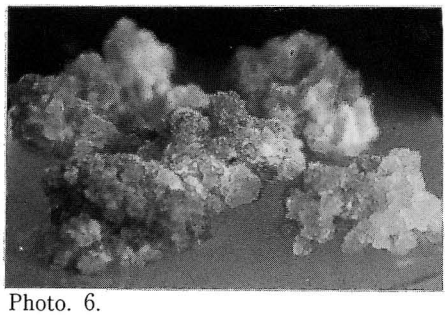

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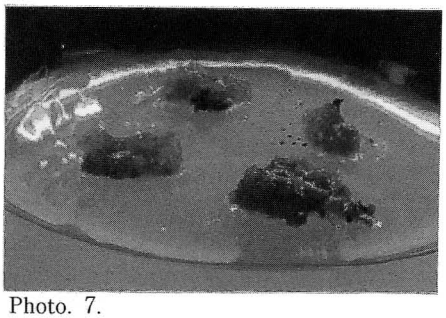

Photo. 7.

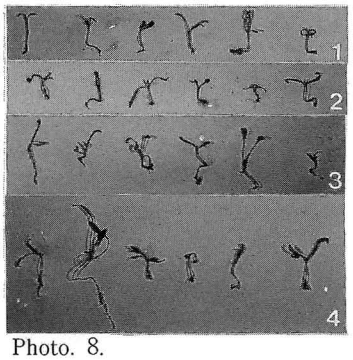

Photo. 8.