アルファルファ(Medicago sativa L.)のアルミニウム耐性選抜における培地条件

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Medium Conditions for *In Vitro* Selection of Aluminum-tolerant Cells of Alfalfa (*Medicago sativa* L.)

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Synopsis

SUGINOBU, K. and T. TAKAMIZO (1991) : Medium conditions for *in vitro* selection of aluminum-tolerant cells of alfalfa (*Medicago sativa* L.). *J. Japan. Grassl. Sci.* **37**, 157-168.

In order to establish suitable medium conditions for *in vitro* selection of aluminum (Al)-tolerant cells of alfalfa, the effects of the concentration of phosphate, pH and the concentration of Al in the medium were examined.

With low concentrations of phosphate in the MS medium, the growth of cells in suspention culture was greatly suppressed. Addition of phosphate at intervals of two days accelerated proliferation of suspension-cultured cells when 1/8, 1/4 and 1/2 of the standard concentrations of KH_2PO_4 were added in this way.

Proliferation of cells in suspension culture in standard MS medium was more rapid than that in MS with 1/10 KH_2PO_4 at all pH values tested. In standard MS, the proliferation of suspension-cultured cells increased with increases in pH from 3.0 to 4.5. In contrast, MS with 1/10 KH_2PO_4 and pH 4.0 gave the highest proliferation rate of suspension-cultured cells.

Wet weight of the cells in suspension on the seventh day after culture initiation increased in proportion to the increase in the wet weight of cells added at culture initiation. However, the increase in wet weight per gram of cells added at culture initiation was highest with 0.5 g of cells per 50 ml of liquid medium.

The increase in wet weight of suspension-cultured cells up to the fourth day after culture initiation was suppressed with increased concentrations of Al in MS medium with 1/10 KH_2PO_4. There were no differences in packed volume of suspension-cultured cells between the control and cells cultured in 0.05 or 0.1 mM $Al_2(SO_4)_3 \cdot 18H_2O$ on the eighth day after culture initiation. However, proliferation of suspension-cultured cells was suppressed at 0.2, 0.4 and there was no proliferation at 0.8 mM $Al_2(SO_4)_3 \cdot 18H_2O$. Residual Al in the medium in treatments with initial concentrations of 0.05, 0.1, 0.2 and 0.4 mM $Al_2(SO_4)_3 \cdot 18H_2O$ was almost absent one day after culture initiation. We conclude from the proliferation of suspension-cultured cells, the Al content of cells and residual Al in the medium, that MS medium with Al concentrations of 0.2 mM to 0.4 mM $Al_2(SO_4)_3 \cdot 18H_2O$ will be the most suitable for future attempts to isolate Al-tolerant cells.

Key words : Alfalfa, Aluminum tolerance, *In vitro* selection, Medium conditions.

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Introduction

Stunting of alfalfa growth under acid soil conditions is mainly attributed to the effects of aluminum or manganese toxity. Among them, the effects of alminum (Al) seems to be more serious than manganese[13]. Therefore, selection for Al tolerance in alfalfa is desirable in Japan and elsewhere. Plant species and genotypes within species differ widely in their tolerance to excess Al, and some of these differences are genetically controlled[10,11].

Principal effects of Al and phosphate in solution culture are consistent with the effects at similar concentrations as those observed in soils. Al toxicity is found at 10 to 100 μM concentrations of Al in soil solution[16,17]. The effects of various concentrations of Al on developing alfalfa seedlings have also been studied. Size of unifoliate leaves and dry weight of young seedlings, when grown at an Al concentration of 20 ppm, can be used as a valuable screening technique[8]. Selection for tolerance to acid soils or high Al concentrations would aid attempts to increase productivity or to lower the cost of production of alfalfa in many environments. Furthermore, Al tolerance is a heritable trait in alfalfa populations[2-4,9].

Studies have been reported on the response to Al of cultured cells from several plants. Al toxicity in tomato is not exclusively a whole-plant phenomenon, and Al-resistant variants were obtained from cultured tomato cells by challenging callus and plated suspension cultures with 200 μM Al for several months[13,14]. Two lines of carrot cells tolerant to Al and manganese (Mn) were selected from suspension cultures by subculturing cells in excessive amounts of $AlCl_3$ or $MnCl_2$ for several months. A cell line tolerant to both Al and Mn was obtained by subculturing the Al-tolerant cell line in the presence of excess Mn[19]. Similarly, selected calluses of sorghum grew better in the presence of Al, and Al-tolerant plants were regenerated from a single cultivar[21]. In *Nicotiana plumbaginifolia* Viv., fertile plants were regenerated from 40 of the 67 variants that retained stable resistance to Al in callus culture. All 40 plants transmitted resistance to Al to their seedling progeny at segregation ratios that could be expected for a single dominant mutation[5,15]. Strategies have been developed for selecting and characterizing Al-resistant variants from cell cultures of *Nicotiana plumbaginifolia* Viv.[6]. For plant cell cultures, simulation of the mineral environment of Al-toxic soils has been proposed[7].

In alfalfa, Parrot and Bouton[20] indicated that plants from an Al-tolerant (AT) germplasm rapidly express tolerance to Al at the tissue level. With increasing time in culture, the AT germplasm also expresses tolerance to acid conditions in culture. The differences in callus growth shown by genotypes within germplasms indicate that the cell culture procedure can be used to screen genotypes for tolerance to acid soils or high Al concentration of soils.

In the present study, medium conditions, the Al and phosphate concentration and pH, for *in vitro* selection of Al-tolerant alfalfa cells were examined. The effect of the wet weight of cells added at culture initiation was also examined.

Materials and Methods

Cells from the cell line of Alfalfa K 21 were cultured in 200-ml erlenmeyer flasks with

50 ml of liquid MURASHIGE and SKOOG[18] basal medium (MS medium) supplemented with 2 mg/l each of 2, 4-dichlorophenoxyacetic acid (2, 4-D), α-naphthaleneacetic acid (NAA) and kinetin (K) on a gyratory shaker operated at 110 rpm throughout all experiments. The temperature was 25-27°C with 16 hours of illumination per day at 3,800 lux.

1. Dissolution of Al in MS medium

Three concentrations of Al sulfate ($Al_2(SO_4)_3 \cdot 14\text{-}18\,H_2O$) were tested for their dissolution at different pH levels and phosphate concentrations of MS medium with two replications. The weights of Al sulfate added to the medium were 16.6 mg/l (designated as 0.05 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$), 66.5 mg/l (0.2 mM) and 266.0 mg/l (0.8 mM) of $Al_2(SO_4)_3 \cdot 14\text{-}18\,H_2O$. Phosphate concentrations in MS medium were standard (170 mg/l KH_2PO_4) and 1/4, 1/16 and 1/64 of the standard. PH of media was adjusted to 3.0, 4.0, 5.0 and 6.0 with HCl or NaOH after autoclaving. Then, each medium was filtered (Advantec No. 2 paper) for 10 hours after pH adjustment. The Al concentration of each medium was examined twice by colorimetric measurement using aluminon[1]. Analysis of variance was carried out on the data of 0.05, 0.2 and 0.8 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$, respectively.

2. Effect of phosphate concentration in medium

The standard medium used in this trial contained 170 mg/l of KH_2PO_4. We tested the effects of different KH_2PO_4 concentrations using 1/2, 1/4, 1/8 and 1/16 of the standard concentration. Effects of addition at two-day intervals of the same solutions were also examined. These treatments referred to as phosphate supplement treatments, received 4 times more phosphate than unsupplemented treatments. Suspension-cultured cells at given concentrations of phosphate were cultured for eight days with two replications. Changes in the concentration of phosphate in each flask were examined by a modified TRUOG method[22] on the first, second, fourth and eighth days after culture initiation. Packed volume of suspension-cultured cells was measured using a graduated cylinder at two-day intervals after culture initiation. Analyses of variance were carried out on the data of pH of medium and packed volume. Significance was tested by the Duncan's multiple range test.

3. Effects of medium pH

Standard MS and 1/10 concentration of KH_2PO_4 MS media were adjusted to pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 with HCl or NaOH. Suspension-cultured cells in medium adjusted to each pH were cultured for 10 days with two replications. Changes in pH in each flask were examined with a pH meter on the first, second, fourth, sixth, eighth and tenth days after culture initiation. The pH of one of two replicate flasks was re-adjusted with HCl or NaOH to the chosen pH at intervals of two days after culture initiation. Packed volumes of suspension-cultured cells were measured on the second, sixth and tenth days after culture initiation.

4. Effect of wet weight of cells added at culture initiation

Wet weight (grams) of suspension-cultured cells was measured after filtration with a tea strainer. The cells of 0.25, 0.50, 0.75, 1.00, 1.25, 1.50 and 2.00 g in wet weight were added to 50 ml of medium. After addition, pH of the medium was adjusted to 5.9-6.0 with NaOH and then suspension cells were cultured with two replications. The packed volume of the suspension-cultured cells was measured on the eighth day after culture initiation.

5. Effect of Al concentration in the medium

Six Al concentrations, 0.0, 16.6, 33.3, 66.5, 133.0 and 266.0 mg/l Al sulfate ($Al_2(SO_4)_3 \cdot 14\text{-}18\,H_2O$) were used for the test. The concentrations of Al added in 1/10 KH_2PO_4 MS medium were designated as 0, 0.05, 0.1, 0.2, 0.4 and 0.8 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$ and pH was adjusted to 4.0 with HCl or NaOH. Suspension-cultured cells at given concentrations of Al were cultured for eight days with two replications. Changes in Al concentration of the media with different Al concentrations and the suspension-cultured cells were examined by colorimetric measurement using aluminon[1] on the first, second, fourth and eighth days after culture initiation. The packed volume of suspension-cultured cells was measured using a graduated cylinder at two-day intervals after culture initiation.

Results

1. Dissolution of Al in MS medium

Dissolutions of Al in the media with different phosphate concentrations and pH values were tested. Dissolution of Al in MS medium was greatly influenced by pH level at all phosphate concentrations. Al dissolution was also influenced by phosphate concentrations. At pH 3.0, there were no significant differences in Al dissolution among the media of standard, 1/4, 1/16 and 1/64 of standard phosphate concentration (Fig. 1 A-C). In the medium of

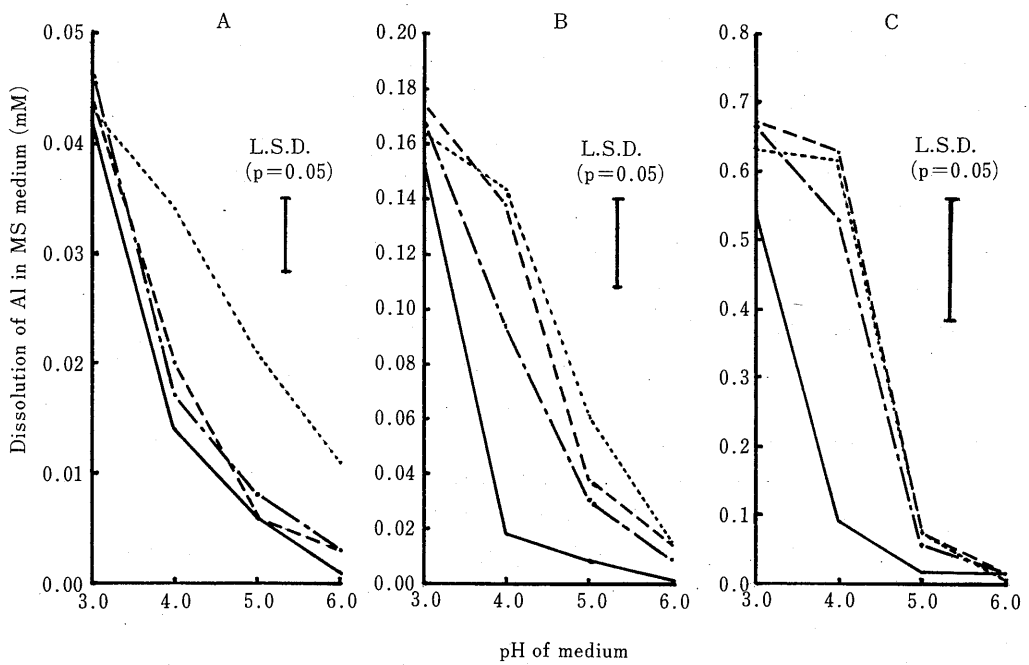

Fig. 1. Effect of phosphate concentration and pH on dissolution of Al in MS medium, ———, 170 mg/l KH_2PO_4 (Standard); -·-·-·-, 1/4 of standard; - - - - - -, 1/16 of standard; ·····, 1/64 of standard.
A : 0.05 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$,
B : 0.2 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$,
C : 0.8 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$.

0.05 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$ concentration, there were no significant differences in Al dissolution among the media of standard, 1/4 and 1/16 standard phosphate concentrations at pH 3.0 to 6.0. Al dissolution in the medium of 1/64 standard phosphate concentration was significantly higher than those of standard, 1/4 and 1/16 standard phosphate concentrations at pH 4.0 to 6.0 (Fig. 1 A). With 0.2 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$ concentration, Al dissolution in the media of 1/16 and 1/64 standard phosphate concentrations at pH 4.0 were significantly higher than those of standard and 1/4 standard phosphate concentrations. At pH 5.0, only the Al dissolution in 1/64 standard phosphate concentration was significantly higher than that in standard (Fig. 1 B). With 0.8 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$ concentration, Al dissolution in the media of 1/4, 1/16 and 1/64 standard phosphate concentrations at pH 4.0 were significantly higher than that in stadard, while there were no significant differences in Al dissolution among the media of all phosphate concentrations at pH 3.0, 5.0 and 6.0 (Fig. 1 C).

2. Effect of phosphate concentration in the medium

There were no significant differences in pH of the media containing different levels of phosphate (Table 1). In contrast, there were significant differences in proliferation of suspension-cultured cells in media containing different levels of phosphate. At low concentrations of phosphate in the medium, proliferation of suspension-cultured cells was greatly suppressed. Addition of phosphate at two-day intervals accelerated proliferation of suspension-cultured cells in 1/8, 1/4 and 1/2 of phosphate concentration present in the standard MS medium. At 1/16 standard phosphate, additions of phosphate at two-day intervals resulted in a proliferation rate equal to that in the standard cultures. The residual concentration of phosphate on the eighth day after culture initiation in the standard was 15.32 μg/ml, while it was 0.12 μg/ml when lower levels of phosphate were used. With addition of phosphate at two-day intervals, residual levels were 68.96, 0.20, 0.13 and

Table 1. The effects of phosphate concentration in the medium on the pH of the medium and proliferation of suspension-cultured cells.

Phosphate concentration	pH of Medium				Packed cell volume (ml)				Residual phosphate in medium (μg/ml)			
	Days after culture initiation				Days after culture initiation				Days after culture initiation			
	1	2	4	8	1	2	4	8[a)]	1	2	4	8
Standard	4.49	4.46	4.53	4.60	2.6	3.6	5.4	9.2d	121.86	77.59	48.86	15.32
1/2	4.58	4.56	4.52	4.64	2.7	3.5	5.5	8.7d	55.06	25.24	3.27	0.12
1/4	4.64	4.54	4.54	4.60	2.7	3.6	5.7	8.4d	18.42	1.09	0.22	0.12
1/8	4.67	4.55	4.53	4.64	2.8	3.5	5.3	7.5e	5.45	0.12	0.12	0.12
1/16	4.58	4.47	4.51	4.69	2.8	3.2	4.6	6.5f	0.42	0.12	0.12	0.12
1/2 Suppl.[b)]	4.58	4.56	4.54	4.63	2.7	3.5	6.5	9.9c	55.06	25.24	36.17	68.96
1/4 Suppl.	4.64	4.54	4.54	4.70	2.7	3.6	6.3	13.4a	18.42	1.09	0.33	0.20
1/8 Suppl.	4.67	4.55	4.57	4.71	2.8	3.5	6.5	12.1b	5.45	0.12	0.13	0.13
1/16 Suppl.	4.58	4.47	4.51	4.71	2.8	3.2	4.5	9.2d	0.42	0.12	0.12	0.12

a) : Data with the same letter are not significantly different at the 5% level, as analyzed by DUNCAN's multiple range test.
b) : Phosphate supplement added at two-day intervals.

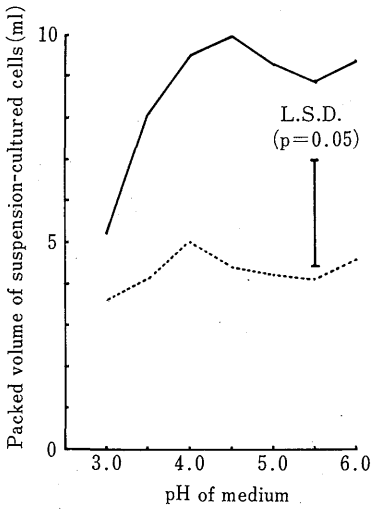

Fig. 2. Effect of pH in medium on packed volume of suspension-cultured cells in standard MS medium (———) and 1/10 standard KH_2PO_4 (·····).

0.12 μg/ml, respectively, for MS with 1/2, 1/4, 1/8 and 1/16 standard concentration of KH_2PO_4.

3. Effect of medium pH

Proliferation of suspension cells in standard MS was more rapid than that in MS with 1/10 standard phosphate at all pH values tested (Fig. 2). In standard MS, the proliferation of cells in suspension accelerated when the pH was raised from 3.0 to 4.5. In contrast, the suspension cells cultured with 1/10 standard phosphate at pH 4.0 gave the highest proliferation rate.

4. Effect of wet weight of cells added at culture initiation

Wet weight of suspension-cultured cells on the seventh day after culture initiation increased with the increase in the wet weight of cells added at culture initiation (Fig. 3). However, the increase in wet weight per gram of suspension-cultured cells added at culture initiation was highest with 0.5 g of suspension-cultured cells per 50 ml of liquid medium. With more than 0.5 g of cells added per 50 ml of liquid medium at culture initiation, the increase in wet weight per gram of cells at culture initiation decreased with increases in the initial wet weight of cells at culture initiation.

5. Effect of Al concentration in the medium

Whitish turbidity was observed in the medium of 0.8 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$ immediately after autoclaving, but it diminished when the medium cooled. The increase in packed volume of suspension-cultured cells up to the fourth day after culture initiation was suppressed with increasing concentrations of Al but there were no differences between cells cultured in the standard and in 0.05 or 0.1 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$ on the eighth day after culture initiation (Fig. 4). However, at eight days after culture initiation, proliferation of suspension-cultured

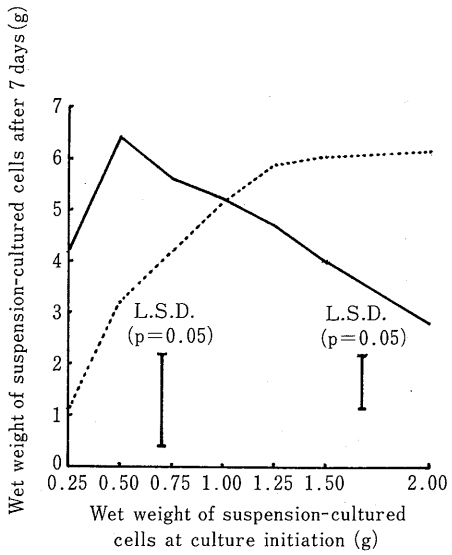

Fig. 3. Effect of quantity of suspension-cultured cells at culture initiation on wet weight of supension cultured cells after seven days per gram of initial wet weight (———) and per 50 ml of suspension-cultured cells (·····).

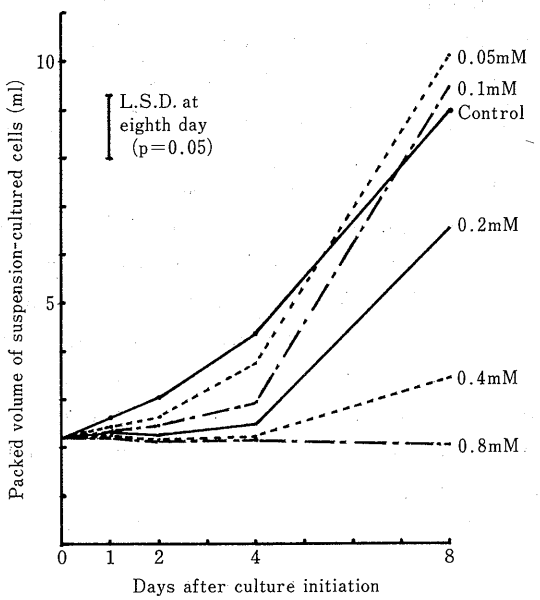

Fig. 4. Effect of Al concentration ($Al_2(SO_4)_3 \cdot 18H_2O$) in the medium on packed volume of suspension-cultured cells.

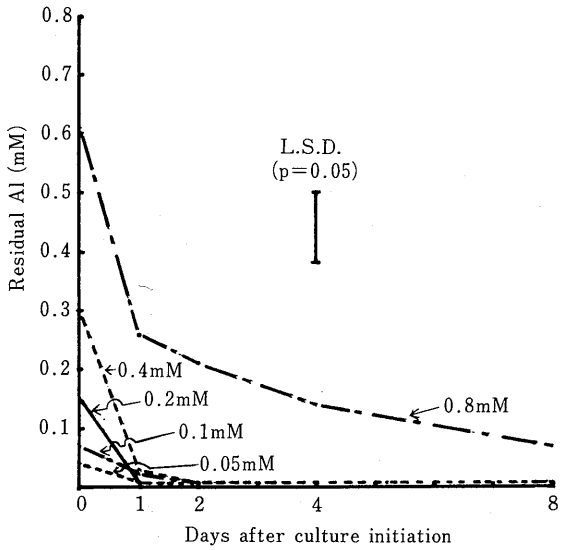

Fig. 5. Residual Al in medium containing different concentrations of Al at culture initiation.

cells was much suppressed at 0.2, 0.4 and 0.8 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$ and no proliferation of suspension cells occurred at 0.8 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$ and no proliferation of suspension cells occurred at 0.8 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$.

Residual Al in the medium in treatments with initial concentrations of 0.05, 0.1, 0.2 and 0.4 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$ in the medium was almost absent on the first day after culture initiation (Fig. 5). Residual concentrations of Al in the medium supplemented with 0.8 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$ were 0.26, 0.21, 0.14 and 0.07 mM on the first, second, fourth and eighth days, respectively.

The concentration of Al in 1 mg of dried suspension-cultured cells increased significantly ($p<0.05$) with increases in the concentration of Al in the medium on the first, second and fourth days after culture initiation. The concentration of Al in 1 mg of dried suspension-cultured cells cultured in 0.8 mM $Al_2(SO_4)_3 \cdot 18\,H_2O$ was extremely high on the first day and it continued to increase up to the eighth day after culture initiation. The concentration of Al in 1 mg of dried suspension-cultured cells at the other concentrations of Al tested increased up to the first or second day in culture, but after that there were either no changes or the the first or second day in culture, but after that there were either no changes or the concentration of Al decreased (Fig. 6).

Discussion

1. Effect of phosphate concentration in the medium

The concentration of phosphate should be as low as possible to prevent precipitation of Al in the medium. CONNER and MEREDITH[7] concluded that the concentration of phosphate

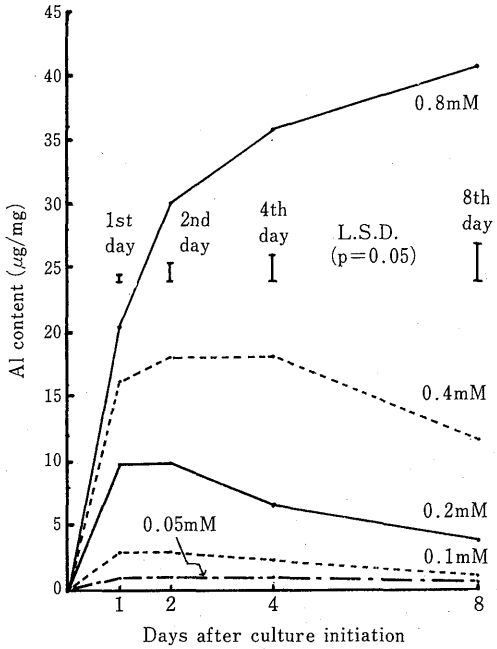

Fig. 6. Concentration of Al in dried suspension-cultured cells grown in medium containing different levels of Al at culture initiation.

should be 1/100 of that in the standard medium used for cultures of *Nicotiana* in cases where 1.0 mM Al was present. MUNNS[16] reported that if the concentration of phosphate was kept below 50 μM at pH 4.0, then concentrations of Al of the order of 100 μM could be maintained without obvious reaction between Al and the phosphate in solution. From residual concentrations of phosphate in the medium and the proliferation of cells recorded in this study, 1/8 and 1/16 of standard concentrations of phosphate without supplementation were inadequate. However, 1/4 and 1/8 of standard concentrations of phosphate with supplementation of the same quantities of phosphate at two-day intervals improved cell proliferation compared to the standard. However, as CONNER and MEREDITH[7] and MUNNS[16] pointed out, the concentration of phosphate should be as low as possible to prevent precipitation of Al in the selection medium. Thus, 1/8 to 1/16 of standard concentrations of phosphate, with supplementation of phosphate at two-day intervals, can be used to screen alfalfa cells for tolerance to Al.

2. Effects of medium pH

Without supplementation of phosphate, proliferation of suspension-cultured cells in the medium with 1/10 KH_2PO_4 was suppressed at all pH values tested compared to standard. As CONNER and MEREDITH[7] found, pH of the selection medium should be 4.0 or below to prevent precipitation of Al. As alfalfa cells in suspension proliferated relatively well at pH 4.0 in the medium with 1/10 KH_2PO_4, this condition appears to be favorable for selection for

Al-tolerant cells.

3. Effect of wet weight of cells added at culture initiation

As the wet weight of suspension-cultured cells after eight days increased with the increase in the initial wet weight of cells added up to 2 g per 50 ml of liquid medium (40 mg/ml), this high level is recommended, if maximum multiplication of the suspension-cultured cells per flask is required. However, to achieve maximum multiplication per mg of initial suspension-cultured cells, 0.5 g of suspension-cultured cells per 50 ml of liquid medium (10 mg/ml) is recommended.

4. Effect of Al concentration in the medium

It was clear that the proliferation of suspension-cultured cells was suppressed with increases in the concentration of Al in the medium up to the fourth day after culture initiation. Then proliferation of suspension-cultured cells recovered rapidly at low concentrations of Al, such as 0.05 and 0.1 mM, but not at high concentrations of Al such as 0.8 and 0.4 mM $Al_2(SO_4)_3 \cdot 18H_2O$. The extent of recovery of proliferation of suspension-cultured cells at 0.2 mM $Al_2(SO_4)_3 \cdot 18H_2O$ was intermediate. These results are supported by the residual concentrations of Al in the medium and levels of Al in suspension-cultured cells.

Acceleration of proliferation at low concentrations of Al has been reported previously. The dry weight of whole plants of sunflower increased after addition of 1 ppm Al to the nutrient solution[12]. On the first day after culture initiation, levels of Al in suspension-cultured cells were maximum and residual Al was almost absent at concentrations of 0.05 and 0.1 mM $Al_2(SO_4)_3 \cdot 18H_2O$ in the medium indicating total up take of Al from the medium. These cells started to proliferate rapidly after the second day of culture initiation. Proliferation of suspension-cultured cells seemed to accelerate after the Al concentration decreased in the medium. In contrast, the levels of Al in the suspension-cultured cells cultured at 0.8 mM $Al_2(SO_4)_3 \cdot 18H_2O$ increased up to the eighth day after culture initiation and residual Al was present in the medium even on the eighth day. Levels of Al in suspension-cultured cells cultured at 0.2 and 0.4 mM $Al_2(SO_4)_3 \cdot 18H_2O$ increased up to the second day but residual Al in the medium was almost absent on the second day after culture initiation. The levels of Al in suspension-cultured cells decreased after the fourth day at 0.4 mM $Al_2(SO_4)_3 \cdot 18H_2O$ and after the second day at 0.2 mM $Al_2(SO_4)_3 \cdot 18H_2O$.

Judging from the proliferation of suspension-cultured cells, the levels of Al in suspension-cultured cells and the residual Al in the medium, concentrations of 0.2 to 0.4 mM $Al_2(SO_4)_3 \cdot 18H_2O$ in the selection medium seem to be suitable for selection of Al-tolerant alfalfa cells at a medium of pH 4.0.

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アルファルファ（*Medicago sativa* L.）のアルミニウム耐性選抜における培地条件

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要 約

酸性土壌におけるアルファルファの生育阻害要因は，主に土壌中に含まれるアルミニウムイオンによるとされており，アルミニウム耐性品種の育成が望まれている。細胞選抜法によりアルミニウム耐性選抜をする場合，通常の MS 培地の塩類濃度や pH 条件では所定のアルミニウム塩を添加しても沈澱してしまい，選抜に適さない。そこで細胞選抜法によりアルミニウム耐性アルファルファを選抜する場合の培地条件及び培養法について試験し，次のような結果を得た。

1. MS 培地に加える KH_2PO_4 濃度を，標準の 170 mg/l を対照区とし，処理区として対照区の 1/2，1/4，1/8 及び 1/16 に，さらにそれぞれの量の KH_2PO_4 を 2 日ごとに補給する補給区を設け，合計 9 区の懸濁細胞の増殖量及び培地の pH を比較した。この結果，培地の KH_2PO_4 濃度が細胞の増殖量に及ぼす影響は顕著で，濃度が低いほど細胞の増殖量は抑制されたが，2 日ごとの補給区ではいずれも標準濃度の対照区以上の増殖量を示した。

2. 培地の pH 条件を，標準濃度の MS 培地及び KH_2PO_4 が 1/10 濃度の MS 培地について，それぞれ 3.0，3.5，4.0，4.5，5.0，5.5 及び 6.0 の 7 段階の pH 処理区を設け，懸濁細胞の増殖量を比較した。この結果，培地の pH の影響は，標準 MS 区でとくに顕著に認められ，pH 3.0 及び 3.5 区の増殖量が著しく抑制された。1/10-KH_2PO_4 MS 区の細胞の増殖量は標準 MS 区と比較して著しく抑制されたが，1/10-KH_2PO_4 MS 区内では pH 4.0 区が最も増殖が良かった。

3. 培養開始時の細胞量によるその後の増殖程度を明らかにするため，MS 液体培地 50 ml 当たりの細胞量を，0.25，0.5，0.75，1.0，1.25，1.5 及び 2.0 g の 7 段階として，各区の細胞の増殖量を比較した。この結果，培養開始時に選抜培地 50 ml に加える細胞量が多いほど増殖量も多いが，細胞 1 g 当たりの増殖量は 0.5 g 区が最も多く，それ以上は量が増えるにしたがって増殖率は低下した。

4. 選抜培地に添加する適正アルミニウム濃度を明らかにするため，培地に添加する硫酸アルミニウム（$Al_2(SO_4)_3 \cdot 14\text{-}18\,H_2O$）を，0（無添加），16.6，33.3，66.5，133.0 及び 266.0 mg/l（$Al_2(SO_4)_3 \cdot 18\,H_2O$ として，それぞれ 0.05，0.1，0.2，0.4 及び 0.8 mM 濃度）の 6 段階として，細胞の増殖量，培地のアルミニウム残存量及び培養細胞のアルミニウム含量を比較した。培地に添加するアルミニウムの濃度が細胞の増殖に及ぼす影響は，培養 4 日目までは，アルミニウム添加区が無添加区と比較して増殖が抑制されたが，8 日目では 0.05 及び 0.1 mM の低濃度区ではむしろ増殖が助長された。一方，0.4 及び 0.8 mM の高濃度区の細胞の増殖量は著しく抑制され，とくに 0.8 mM 区では増殖がみられなかった。培地内のアルミニウムの残存量は，0.8 mM 区を除いては培養 1 日後にすでに大半が吸収されてしまうが，0.8 mM 区では 8 日後にもかなり残存していた。また，懸濁培養細胞のアルミニウム含量は，培養 4 日目までは培地のアルミニウム濃度が高いほど高かった。その後は，0.4 mM 区までの濃度区の細胞のアルミニウム含量は低下したが，0.8 mM 区の高濃度区では培養 8 日目でも細胞のアルミニウム含量は増加していた。

キーワード：アルファルファ，アルミニウム耐性，細胞選抜，培地条件.

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