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Plant Regeneration from Leaf Disks and Stem Segments of Sweet Potato Using Only NAA as Supplementary Growth Regulator

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To determine the most suitable culture medium for regeneration from explants of sweet potato, various culture media were tested on leaf disks, stem and petiole segments of line KT, a clone previously regenerated from stem callus of Kokei 14, and cultivar Jewel. Two media, R6 and R7, both of which contain only NAA as supplementary growth regulator, gave high root and shoot regeneration rates particularly in leaf disks and stem segments. Other media exhibited either no or a very low frequency of shoot formation except for moderate rooting in some media. Among the explants tested, petioles had the lowest regeneration rate. In experiments conducted to determine appropriate levels of NAA, leaf disks and stem segments responded to a narrow range of NAA concentrations (0. 1-0. 6 mg/*l*) for shoot regeneration. Addition of either *trans*-zeatin or BAP to the regeneration medium, even at a concentration as low as 0. 01 mg/*l* reduced the shoot regeneration rate, particularly from leaf disk. The decline in shoot regeneration rate with increase in cytokinin level was more gradual in stem segments than in leaf disks. In treatments where roots and shoot were observed, shoot regeneration was preceded by root regeneration, and most of the shoots arose directly from explants close to the cut ends and only few arose from regenerated roots.

Introduction

An efficient *in vitro* plant regeneration system is an essential component in most laboratory work involving genetic manipulations. This is particularly true in plant genetic transformation where success in regenerating transgenic plants is greatly affected by the regeneration system being employed. In addition to being efficient, a regeneration system also needs to be reproducible in order to be of wider practical use.

Unfortunately, there have been reports of regeneration procedures that were not reproduced[1]. A number of reasons may account for this observation, but perhaps a major reason is because numerous factors affect regeneration and that it is difficult to control and duplicate all these factors, or at least the most critical of these factors, in a trial. For explant-related factors alone, for instance, Brown and Thorpe[2] cited about 10 of these factors including culture condition and genotype of donor plant. Therefore, empirical tests of regeneration systems on available materials and under existing laboratory conditions are necessary to ascertain their effectiveness.

In sweet potato, several regeneration trials with varying degrees of success have been reported using different explants of a number of genotypes. These include regeneration from leaf sections[3-6], stem sections[4,7], storage and adventitious roots[4,8-10], shoot tips[9,11,12] and even

protoplasts[13-15]. In addition, there are a number of reports on regeneration from the related wild species[5,16,17]. The regeneration media used in these reports, however, were different from each other in terms of nutrient and/or hormone composition even where the same explant was used. Moreover, the relative regeneration efficiencies in most of these media have not yet been determined. We therefore set out to test and verify the relative regeneration efficiencies of various explants of sweet potato on some of these media, as an initial step for developing a transformation system for this species. In this report we show that a medium containing only NAA as a supplementary growth regulator is sufficient for plant regeneration from leaf disks and stem segments at

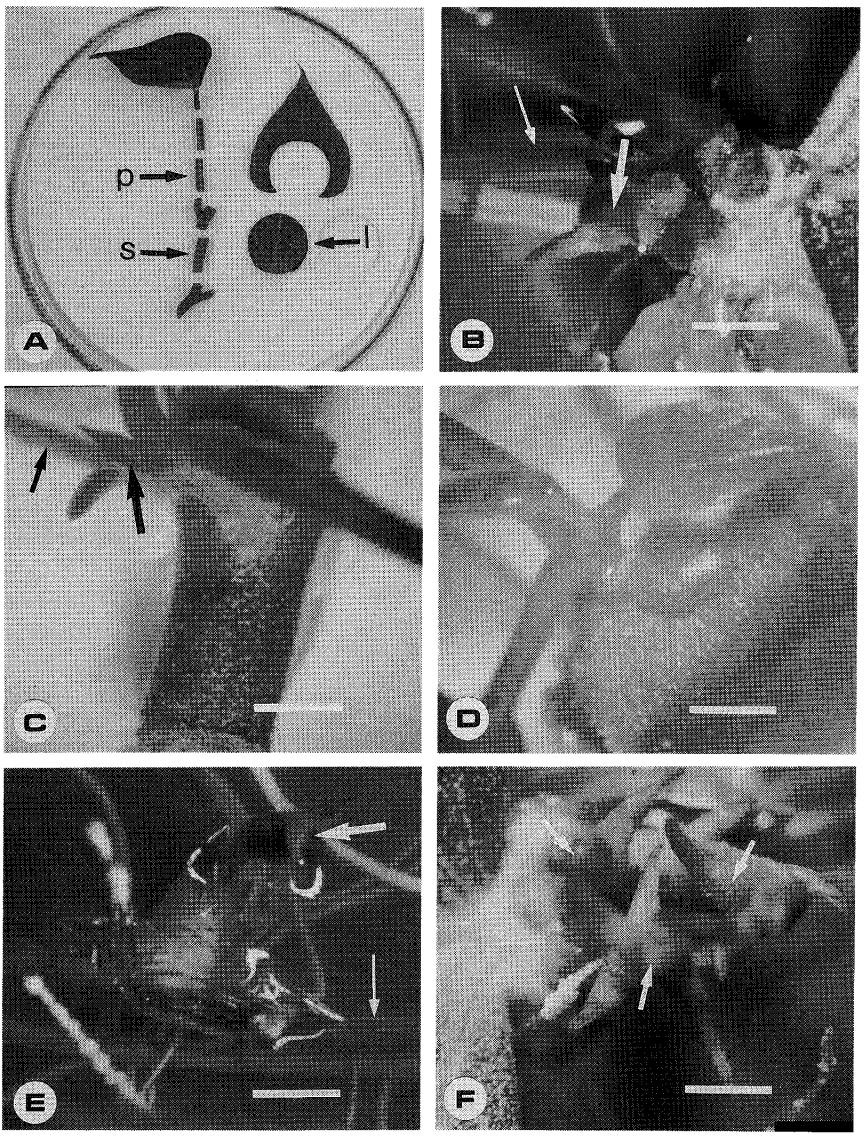

Fig. 1 Regeneration from different explants of sweet potato.
A, the different explants used in this study: p, petiole segment; s, stem segment; l, leaf disk. B, shoot (thick arrow) and root (thin arrow) arising from petiole end of leaf disk of Jewel. C, shoot (thick arrow) and root (thin arrow) arising from thickened end of a stem segment of KT. D, shoot arising near the middle of a stem segment of KT. E, shoot (thick arrow) arising from thick root (thin arrow) in Jewel. F, multiple shoots (arrows) arising from leaf disk of Jewel. Bars are 3 mm long.

rates comparable to or higher than those previously reported, and that addition of cytokinin has an inhibitory effect on regeneration in the plant material tested.

Materials and Methods

1. *Source and preparation of explants*

Two sweet potato genotypes, KT and Jewel, were tested in this study. The line KT is a clone regenerated from Kokei 14 through adventitious embryo formation from stem callus[18]. Both KT and Jewel plants were maintained as aseptic shoot cultures in liquid MS medium. Three to 5 weeks after subculture, the cultures were used as source of explants, the same age group being used in the same experiment.

Explants were obtained from the upper part of the shoot bearing the first 5 fully expanded leaves as shown in **Fig. 1-A**. Leaf disks were obtained from the expanded leaves as described by Carelli *et al.*[6], that is, 1.5 cm disks were punctured out of the leaf base including the petiole end using a borer. Petiole and internode segments measuring 1 cm long were cut from the same shoot region at least 2 mm away from the nodes to exclude lateral buds.

Table 1. Composition of culture media tested for regeneration of roots and shoots from leaf disks and stem and petiole segments.

Medium	Saltbase	Vitamins/Amino Acid (mg/*l*)	Sucrose (g/*l*)	Growth Regulator (mg/*l*)	pH	Reference
R 1	MS	MS	30	—	5.8	19
R 2	B 5	B 5	20	—	5.5	1
R 3	MS	LS	30	IAA, 0.5 BAP, 10.0	5.8	16, 20
R 4	MS	MS	30	IAA, 0.4 BAP, 5.0	5.8	17
R 5	MS	TH, 0.1 NA, 0.5 PH, 0.5 MY, 100.0 GC, 2.0	30	IAA, 1.0 kinetin, 1.0	5.7	12
R 6	B 5	MS	30	NAA, 0.1	5.8	Ochi *et al.* (unpublished)
R 7	MS	TH, 1.68 NA, 1.23 PH, 1.03 MY, 90.10	16	NAA, 0.19*	5.8	21
R 8	MS	Staba vitamins MY, 100.0 AA, 50.0	30	NAA, 0.25 BAP, 0.06	5.7	6, 22
R 9	MS	MS	30	NAA, 2.0 BAP, 0.1	5.8	5
R10	NN	NN	20	NAA, 0.1 BAP, 0.1	5.7	23, 24
R11	MS	MS	20	NAA, 1.0 BAP, 0.1	5.6	25

Note: Abbreviations: MS, Murashige and Skoog; LS, Linsmaier and Skoog; B 5, Gamborg; NN, Nitsch and Nitsch; TH, thiamin hydrochloride; NA, nicotinic acid; PH, pyridoxine hydrochloride; MY, myo-inositol; GC, glycine; AA, ascorbic acid; NAA, alpha naphthalene acetic acid; IAA, indole-3-acetic acid; BAP, 6-benzylaminopurine. * Increased from 0.019 mg/*l* (0.1 μmoles) in original medium.

2. *Culture media and conditions*

Three experiments were conducted in this study. In Experiment 1, efficiency of plant regeneration was compared among the different culture media using KT and Jewel. In Experiment 2, the effects of different levels of NAA on regeneration from leaf disks and stem segments were examined using Jewel, and in Experiment 3, the effects of supplementary cytokinin on regeneration from leaf disks and stem segments of Jewel were tested.

The composition of the various media tested in Experiment 1 are shown in **Table 1.** R3 to R11 had been tried with some success in regenerating plants from various explants of *Ipomoea* species, including *I. batatas, I. triloba* and *I. trichocarpa.* Subsequent Experiments 2 and 3 were based on R6 medium with modifications on composition and concentration of growth regulator. Each plot consisted of 20 or 21 explants. Explants were placed on 0.8% agar-solidified media, 5 in a plate for leaf disks and 7 or 10 in a plate for stem or petiole segments. Leaf disks were placed on media abaxial side down. Both explant sources and explants were cultured under 16 hr light/8 hr dark cycle provided by white fluorescent lamps. Temperatures as a rule ranged from 26°C to 28°C.

Results and Discussion

1. *Evaluation of different regeneration media*

A higher proportion of regenerated roots and shoots was obtained from Jewel than from KT, and from stem segments and leaf disks than from petiole segments (**Table 2**). Noticeable differences in

Table 2. Regeneration and callus formation in leaf disks and petiole and stem segments of KT and Jewel after 2 month culture in different regeneration media.

Medium	% Explant with Roots			% Explant with Shoot			Callus Formation		
	leaf disk	petiole	stem	leaf disk	petiole	stem	leaf disk	petiole	stem
				KT					
R 1	0	0	14	0	0	0	−	−	+
R 2	0	0	0	0	0	0	−	+	+
R 3	0	0	24	0	0	0	+++	+++	+++
R 4	5	0	5	0	0	0	+++	+++	+++
R 5	0	0	5	0	0	0	+	++	++
R 6	5	0	33	0	0	0	+	+	+
R 7	10	0	90	0	0	38	−	+	+
R 8	0	0	0	0	0	0	−	+	+
R 9	5	14	0	0	0	0	+	++	++
R10	0	0	29	0	0	0	+	++	++
R11	0	0	24	0	0	0	+	++	++
				Jewel					
R 1	55	5	0	0	0	0	−	+	+
R 2	5	0	0	0	0	0	+	+	+
R 3	0	0	0	0	0	0	++	+++	+++
R 4	0	10	14	0	0	0	++	+++	+++
R 5	45	29	81	0	0	0	++	+++	+++
R 6	100	52	100	30	0	29	+	++	++
R 7	85	24	100	5	10	29	−	+	+
R 8	0	0	14	0	0	0	+	+	+
R 9	20	19	14	0	0	0	++	+++	+++
R10	14	0	81	0	0	5	+	+	+
R11	20	10	95	0	0	0	++	+++	+++

Note: Callus formation rating: −, absent; +, slight; ++, moderate; +++, vigorous.

root and shoot regeneration rate were observed among the culture media tested. In KT, R7 medium gave the highest root regeneration rate (90%) in stem segments and produced shoots only from stem segments (38%). In Jewel, the highest shoot regeneration rate was observed in R6 and R7. Both media also gave the highest root regeneration rates from leaf disks and stem segments.

Both R6 and R7 media were based on B5 medium and MS medium, respectively and contained only NAA as a supplementary growth regulator (**Table 1**). The other media tested, both the hormoneless ones (R1 and R2) and those containing different combinations of auxin and cytokinin (R3, R4, R5, R8, R9, and R11), were unable to produce shoots in any of the explants used, although in R10 only one shoot was produced from the stem segment. The experimental results clearly show that R6 and R7 are the most suitable media for regeneration of roots and shoots from leaf disks and stem segments of sweet potato, and that addition of NAA alone as a supplementary growth regulator is sufficient for plant regeneration in at least two genotypes of sweet potato, KT and Jewel. The present study also shows that there are differences in regenerability between KT and Jewel as well as among explants. Differences in regenerability have been observed among different genotypes in sweet potato[9] and among different explants in *I. triloba*[17]. In the latter report, petiole explants had the lowest regeneration rate, as was the case in the present study.

In all cases where shoots were observed, roots were always formed prior to the shoot formation. Roots usually started emerging within 5-7 days in leaf disks and within 6-8 days in stem segments, while shoots started emerging within 4-5 weeks and were produced mainly up to the seventh week of culture. Most of the shoots arose directly from the thickened cut ends of explants (**Fig. 1-B** and **1-C**), but a few shoots also arose occasionally near the middle of a stem segments (**Fig. 1-D**) and from the regenerated thick roots (**Fig. 1-E**). In leaf disks, multiple shoots were sometimes observed (**Fig. 1-F**). Regenerated shoots grew readily when transferred to liquid MS medium.

2. *Effect of NAA concentration on plant regeneration*

Since R6 was one of the two media which gave the highest shoot and root regeneration in the initial trial, we used this medium in testing the response of leaf disk and stem segments of Jewel to different levels of NAA (**Table 3**). For shoot regeneration, leaf disks of Jewel responded to a narrower range of NAA concentrations than stem segments. The optimum NAA concentrations

Table 3. Root and shoot regeneration from leaf disks and stem segments of Jewel after 2 month culture in R6 medium with varying levels of NAA.

NAA Level (mg/*l*)	% Explant with Root		% Explant with Shoot	
	leaf disk	stem	leaf disk	stem
0	100	10	15	10
0.1	100	20	40	20
0.2	100	75	55	60
0.3	100	75	40	55
0.4	100	80	10	50
0.5	100	75	10	45
0.6	100	80	10	55
0.7	100	60	10	20
0.8	100	55	0	15
1.6	90	70	0	0
3.2	80	50	0	0

for leaf disk explants ranged from 0.1-0.3 mg/*l* (40-55% regeneration) whereas 0.2-0.6 mg/*l* for stem segments (45-60% regeneration). Root regeneration from leaf disks, however, was observed in a wider range of NAA, indicating that NAA in the range of 0.1-3.2 mg/*l* has no clear effect on rooting from leaf disks. Cantliffe[21] reported that a very low concentration (0.019 mg/*l*) or no addition of NAA was sufficient for plant regeneration from embryogenic callus of sweet potato. The present study, however, indicates that about 10-fold concentration of NAA (0.1～0.6 mg/*l*) is efficient for shoot regeneration from the explants.

3. *Effect of cytokinin on regeneration*

To determine the effect of cytokinin on shoot and root regeneration in leaf disks and stem segments of Jewel, varying levels of two cytokinins, *trans*-zeatin and BAP, were incorporated into R6 medium containing 0.2 mg/*l* NAA (**Table 4**). The results of these experiments showed that addition of either of these cytokinins inhibited root and shoot formation: the higher the cytokinin concentration, the greater the inhibitory effect, although a corresponding increase in callus growth was also noted. Inhibition of shoot regeneration was particularly sharp in leaf disks where addition of as little as 0.01 mg/*l* *trans*-zeatin or BAP completely or almost completely inhibited shoot formation. In stem segments, inhibition of shoot formation with increasing levels of cytokinin was more gradual. Shoot regeneration appeared to be more sensitive to addition of cytokinin than root regeneration.

Reduction of regeneration rate with increase in cytokinin concentration has also been observed in sweet potato by Ozias-Akins and Perera[10], Otani and Shimada[26] and Carswell and Locy[4]. Thus inhibitory action of cytokinin on regeneration of sweet potato seems to be a widespread observation and runs counter to the usual practice of using this hormone in combination with an auxin in regeneration trials. Cytokinins are thought to be synthesized primarily in the roots[27] and thus rooted explants should conceivably be able to synthesize some amount of cytokinins. In fact, some tissues are considered to be cytokinin independent[28], and the results of the present study suggest that it is not necessary to supplement the regeneration medium with cytokinin for plant regeneration from leaf disks and stem segments.

Table 4. Regeneration rate in leaf disks and stem segments of Jewel after 2 month culture in R6 medium containing 0.2 mg/*l* NAA and different levels of cytokinin.

Cytokinin Level (mg/*l*)	% Explant with Root		% Explant with Shoot	
	leaf disk	stem	leaf disk	stem
trans-Zeatin				
0	100	80	35	30
0.01	65	25	0	10
0.05	15	15	0	0
0.10	10	5	0	0
0.15	0	20	0	0
0.20	0	5	0	0
BAP				
0	100	85	65	35
0.01	100	85	5	25
0.05	65	50	5	20
0.10	30	30	0	20
0.15	5	5	0	0
0.20	20	15	0	0

The regeneration rates obtained in the present study are comparable to or somewhat higher than those reported previously in similar explants of sweet potato[4,7,23]. The results of the present study, however, differ from the other studies in shoot regeneration patterns. Most of the shoots obesrved in the present study were directly regenerated from the primary explants rather than through the regenerated roots of the primary explants. The regenerated shoots, arising at or near the cut ends of explants, are a favorable indication that stem segments and leaf disks might be suitable materials for transformation in sweet potato using *Agrobacterium*. This has, in fact, already been tried by Carelli *et al.*[6] and Otani *et al.*[29] using leaf disks, and the latter group was successful for obtaining transformants from roots of the *A. rhizogenes*-infected primary explants. The trial by Prakash and Varadarajan[25] using the smaller leaf and petiole pieces was more successful, but the transgenic plants were obtained indirectly from the calli transformed by *A. tumefaciens*. It is thus left for further studies to determine whether the plant regeneration system established in the present study can be successfully applied for the *Agrobacterium*-mediated transformation of sweet potato.

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《和文要約》

植物ホルモンとしてNAAのみを含む培地を用いたサツマイモの葉片および茎断片からの植物体再生

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サツマイモの形質転換系を確立することを目的として，高系14号の再分化系統(KT)および栽培品種Jewelの葉片や茎断片からの植物体再生に最も適する培地について検討した．調査した11種類の培地のうち，2種類の培地(R6, R7)では植物体再生率が最も高く，有効な培地であることが判明した．これら2種類の培地は植物ホルモンとして0.1-0.2 mg/lのNAAのみが添加されており，IAAやカイネチンを含む他の培地では再生率が低いか植物体再生が全く認められなかった．またNAAの最適濃度について検討した結果，葉片では0.1-0.3 mg/l，茎断片では0.2-0.6 mg/lであった．さらにゼアチンやBAPを添加した場合には，0.01 mg/lの低濃度でも植物体再生率を低下させることが明らかとなった．本研究で得られた大部分の苗条(shoot)は，外植体の切断部分から分化しており，アグロバクテリウムによる形質転換体作成に適した植物体再生系であると考えられる．