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Studies on Tissue Culture of *Vigna sinensis* Endl. with Reference to Callus Formation, Subculture and Habituation

SATOSHI MATSUBARA

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Tissue culture of *Vigna sinensis* Endl. was studied with reference to callus formation, subculture and habituation. Callus formation was observed on cotyledon, hypocotyl and root sections, when cultured on Linsmaier and Skoog basal medium supplemented with IAA or 2,4-D. 2,4-D showed higher stimulative activity than IAA. Cotyledon sections required higher auxin level for callus formation than hypocotyl and root ones. Kinetin and nicotinic acid were not so effective to the callus formation. Yeast extract caused vigorous callus formation, when used with auxin and kinetin. No bud formation was observed in any section on any medium. In the subculture of the callus, IAA ($10^{-7} \sim 4\times10^{-5}$M) gave brown callus with roots, possibly containing differentiated tracheal elements, while 2,4-D (except 10^{-8} and 10^{-9}M) did yellow friable and homogenous callus without root. Kinetin somewhat promoted the callus growth, and significantly affected the tissue texture. High level of kinetin gave compact callus, while high level of auxin did friable callus. Nicotinic acid promoted much the callus growth, but had no effect on the tissue texture. Yeast extract strongly stimulated the callus growth. After more than 15 transfers of subculture in the media added with 2,4-D, kinetin, nicotinic acid and yeast extract, alone or in combination, at least three distinguishable stock calli with different requirement for growth factors were obtained.

Introduction

Plant tissue cultures have been successfully used to investigate factors regulating plant growth [1-6]. Studies on the tissue culture of *Vigna sinensis* revealed that the growth of the callus was regulated by nicotinic acid and its derivatives in addition to 2,4-D, kinetin and yeast extract [5]. During these studies it was found that the growth response of the callus was unstable and often sporadic especially in early subcultures after isolation, and that requirement of the callus for the growth factors was changeable on many transfers in the subculture, resulting in different strains of stock cullus. Under these circumstances, it seemed important to investigate the callus formation, subculture and habituation and to obtain more detailed informations.

Materials and Methods

Sterilized seeds of *Vigna sinensis* Endl. cv. Akadane Onaga were germinated in 22 ×220 mm test tubes containing 20 ml of 7 g/l semisolid agar medium. The seedlings in the test tubes were grown at 28°C under 3000 lux fluorescent light for 3 days. Cotyledon, hypocotyl and root sections were isolated aseptically from seedlings, and used

Laboratory of Applied Biology, Kyoto Prefectural University, Shimogamo Hangi-cho, Sakyo-ku, Kyoto 606, Japan

for the experiment of callus formation and for the preparation of stock callus. The basal medium for all the cultures was composed of salts, vitamins and 30 g/l sucrose as reported by Linsmaier and Skoog (1965) [4] and was supplemented with 1.14×10^{-5}M IAA, 2.21×10^{-6}M 2,4-D, 10^{-6}M kinetin, 10^{-5}M nicotinic acid and 1 g/l yeast extract alone or in combination, unless otherwise stated. After all the ingredients were added, the medium was adjusted to pH 5.6 with 1 N HCl or NaOH and 9 g/l agar was added. After the agar was melted in a steamer, the medium was distributed in aliquats of 10 ml in test tubes (18×180 mm) and autoclaved at 1.0 kg/cm^2 (1 bar) for 15 min. To study on callus formation, the cotyledon, hypocotyl and root sections were implanted in 10 or 20 test tubes containing 10 ml of the medium to be tested. The test tubes were maintained under continuous darkness at 28°C for 2 weeks, and callus formation was observed. To establish the stock culture, callus tissue induced on cotyledon, hypocotyl and root sections in the medium supplemented with various growth factors as described above was isolated and subcultured as stock culture on the same medium under same conditions. Tissues were transferred to fresh medium every 4 weeks. For the studies on callus growth, callus pieces weighing about 10 mg in fresh weight were dissected from the stock culture and implanted on the agar surface of the medium to be tested in the test tubes. The cultures were maintained at 28°C in continuous darkness for 4 weeks, and then the average fresh weight was determined.

Results and Discussion

Cotyledon, hypocotyl and root sections were isolated aseptically from 3-day-old seedlings and cultured on the basal medium supplemented with various concentrations of IAA or 2,4-D. As shown in Table 1, the sections did not induce callus tissue without auxin, but they did when IAA or 2,4-D was supplemented to the medium. On the whole, 2,4-D was more stimulative to callus induction than IAA. Cotyledon sections required higher level of auxin than hypocotyl and root ones. The hypocotyl sections responded most readily. They induced callus at 10^{-8}M 2,4-D, while cotyledon sections

Table 1. Effect of various concentrations of IAA and 2,4-D on the callus formation and root development of cultured sections isolated from cotyledon, hypocotyl and root of *Vigna sinensis*.

Additives (M)		Percent of cotyledon section with		Percent of hypocotyl section with		Percent of root section with	
		callus	root	callus	root	callus	root
—		0	0	0	91.7	0	95.0
IAA	10^{-8}	0	0	0	90.9	0	95.0
	10^{-7}	0	0	16.7	100	0	100
	10^{-6}	21.0	15.8	94.4	89.9**	93.3	46.7
	10^{-5}	52.9	0	100	100**	100	35.7
	10^{-4}	61.1*	0	100*	91.7**	88.9	22.2
2,4-D	10^{-8}	0	0	40.0	100	0	94.1
	10^{-7}	0	0	100	100	78.6	78.6
	10^{-6}	61.1	0	100*	31.6	100*	22.2
	10^{-5}	100*	0	100	0	50.0	0

* Vigorous callus formation was observed. ** Vigorous root development was observed.

required 100 times higher level of 2,4-D. This may be ascribable to high levels of endogenous auxin and cell division potency in hypocotyl sections and low ones in cotyledon sections.

Hypocotyl and root sections induced roots on the basal medium without auxin. In hypocotyl sections 10^{-6}M 2,4-D was inhibitory to root development. In root sections, higher levels of IAA over 10^{-6}M and of 2,4-D over 10^{-7}M were inhibitory to root development. Cotyledon sections did not develop roots except those at 10^{-6}M IAA. Some unknown gradient for root initiation through cotyledon, hypocotyl and root may be existent. In the present experiment no bud formation was observed in any sections.

Cotyledon, hypocotyl and root sections were cultured on the media added with 1.14×10^{-5}M IAA (I), 2.21×10^{-6}M 2,4-D (D), 10^{-6}M kinetin (K), 10^{-5}M nicotinic acid (N) and 1 g/l yeast extract (Y), alone or in combination, namely on the K, I, IK, IKN, IKY, D, DK, DKN and DKY media, and callus formation and root development were examined. Photographs presented in Fig. 1 were partly cited from a previous report [5]. Hypocotyl sections showed more vigorous callus formation and root development than cotyledon and root ones. Kinetin alone was ineffective to callus induction, but not inhibitory to root development. Kinetin, when added to the medium with IAA or 2,4-D, slightly increased the callus formation. In a preliminary separate experiment, nicotinic acid added alone to the medium without auxin did not induce callus formation. The present experiment revealed that nicotinic acid somewhat augmented the callus formation, when added together with auxin and cytokinin. Poor response of the sections to kinetin and nicotinic acid might be a reflect of rich endogenous level in these factors. A separate experiment indicated that yeast extract, when added alone, was inactive to callus induction. The present experiment revealed that an addition of yeast extract to the medium with auxin and kinetin promoted remarkably the callus formation except that in cotyledon sections on the IKY medium. The growth factors such as kinetin, nicotinic acid and yeast extract did not induce bud formation in any sections.

The callus tissue induced on cotyledon, hypocotyl and root sections in the I,

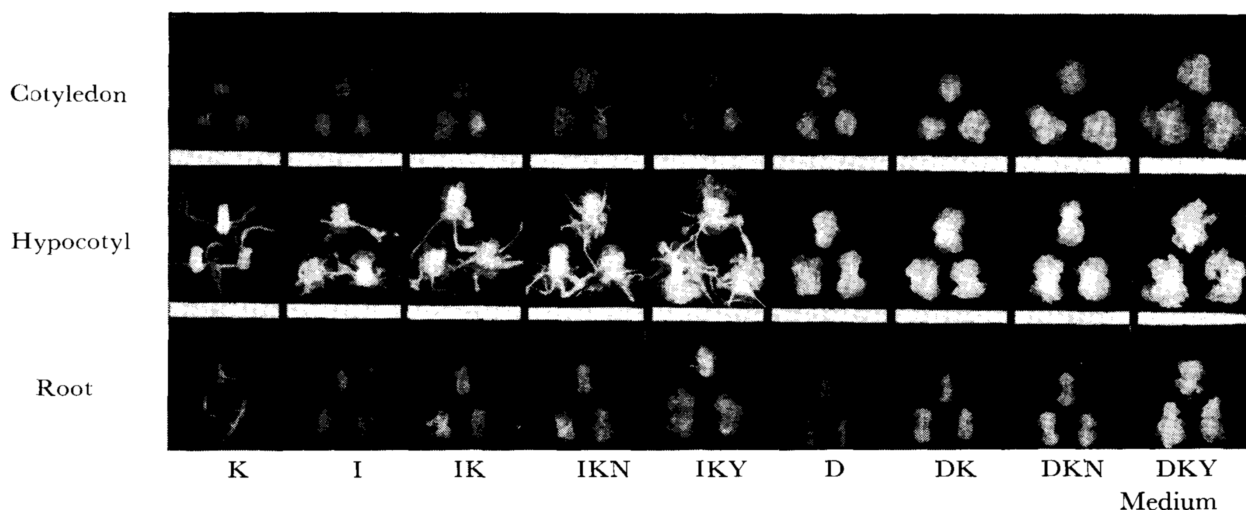

Fig. 1. Callus formation and root development on cotyledon, hypocotyl and root sections. Sections were cultured on the medium added with 10^{-6}M kinetin (K), 1.14×10^{-5}M IAA (I), 2.21×10^{-6}M 2,4-D (D), 10^{-5}M nicotinic acid (N) and 1 g/l yeast extract (Y), alone or in combination.

Table 2. Growth of *Vigna* callus cultured on the medium added with 1.14×10^{-5}M IAA (I), 2.21×10^{-6}M 2,4-D (D), 10^{-6}M kinetin (K), 10^{-5}M nicotinic acid (N) and 1 g/l yeast extract (Y), alone or in combination. Callus used for inoculum was isolated originally from cotyledon, hypocotyl and root sections on the I, IK, IKN, IKY, D, DK, DKN and DKY media and subcultured on the same medium respectively for two transfers.

Medium	Fresh weight (mg) of callus derivated from		
	cotyledon	hypocotyl	root
I	—	—	—
IK	65.9	31.3	52.8
IKN	31.8	110.2	76.0
IKY	214.6	309.0	341.0
D	30.6	8.5	15.0
DK	23.1	33.5	31.1
DKN	250.8	206.6	130.6
DKY	293.1	288.6	281.9

IK, IKN, IKY, D, DK, DKN and DKY media was isolated and subcultured on the same medium respectively. A problem was whether or not these calli can be subcultured for many transfers. On the I medium containing 1.14×10^{-5}M IAA the callus derived from cotyledon, hypocotyl and root became necrotic during 1 or 2 transfers and did not grow. Therefore, no subculture was carried out further. During early subcultures within 10 transfers or so callus growth on any medium was usually unstable and often sporadic. Table 2 offered an example of the callus growth. Tissues on the IK, IKN and IKY medium were mostly brown heterogenous, sometimes a little compact, often initiating roots, irrespective of the source of the callus. On the other hand, the D, DK, DKN and DKY media produced yellow friable callus without root. As kinetin stimulated slightly the callus growth, the callus derived from any source on the IK medium gave a little growth during several transfers. Although some IK calli were able to be subcultured for 10 transfers, most of them became necrotic after 3 or 4 transfers. Additional subcultures were nearly impossible. The callus on the DK medium grew slowly, and some calli were able to be subcultured. Nicotinic acid promoted considerably the callus growth, and the IKN callus cultured on the IKN medium sometimes grew well. However, most of calli were unfavorable for subculture, because of their unstable growth. The DKN callus grew well and was easily subcultured. Yeast extract caused a vigorous growth of the callus. Therefore, the IKY and DKY callus grew rapidly and was easily subcultured for many transfers, although the former callus was brown and heterogenous and often initiated roots (Fig. 2).

Thus, brown compact heterogenous callus with root as observed in the IKY medium (Fig. 2) was inconvenient for successful subculture and for study on the effect of growth factors. It was interesting to know whether brown callus was attributable to IAA itself or low level of auxin activity. In order to clarify this problem the IKY callus was cultured on the media added with various concentrations of IAA together with 10^{-6}M kinetin and 1 g/l yeast extract (Table 3). Without IAA the callus was necrotic and did not grow. With 10^{-7}M IAA, very compact granular brown callus was ob-

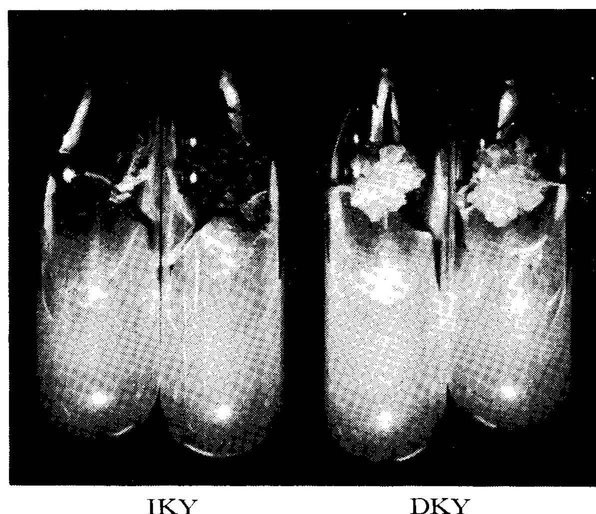

Fig. 2. The IKY and DKY callus cultured on the IKY and DKY medium respectively. These calli were isolated from root sections and subcultured. For further details see text.

Table 3. Effect of various concentrations of IAA on the growth of the IKY callus. The medium was supplemented with 10^{-6}M kinetin and 1 g/l yeast extract in addition to IAA. The IKY callus had been subcultured on the medium added with 1.14×10^{-5}M IAA, 10^{-6}M kinetin and 1 g/l yeast extract for more than 10 transfers after isolation from root sections.

IAA (M)	Fresh weight (mg)	Remark
0	25.0	BN
10^{-7}	82.6	BG
10^{-6}	113.9	BC
4×10^{-6}	—	BC
10^{-5}	242.6	BC
4×10^{-5}	477.9	YF and BC
10^{-4}	460.7	YF
4×10^{-4}	144.0	YF
10^{-3}	—	BN

BN: brown necrotic callus, BG: brown granular callus, BC: brown compact callus, YF: yellow friable callus.

tained. With increase of IAA concentration until 4×10^{-5}M, the fresh weight of the callus increased with the maximum growth at 4×10^{-5}M IAA, although the callus was brown compact, and no yellow callus was obtained. At 10^{-4}M and 4×10^{-4}M IAA yellow callus, still with roots, was obtained. In this case, the callus was very watery and friable, and seemed to be unfavorable for subculture. The highest concentration, 10^{-3}M, resulted in brown necrotic callus.

Low level of 2,4-D was also examined for the DKN callus with 10^{-6}M kinetin and 10^{-5}M nicotinic acid (data have not been shown). 2,4-D at 10^{-9} and 10^{-8}M gave brown callus with roots. With increase of 2,4-D concentration, yellow friable callus without roots was obtained. These results indicated that low auxin activity caused to produce brown tissue and high level of auxin gave yellow friable tissue. With high level of kinetin (10^{-5}M), 2,4-D at lower than 10^{-6}M caused compact callus.

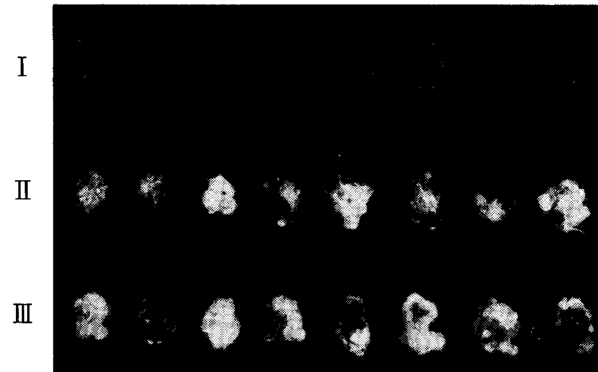

Fig. 3. *Vigna* callus cultured on the medium with 1.14×10^{-6}M IAA, 4×10^{-6}M nicotinic acid and 10^{-6}M kinetin (I), 10^{-5}M kinetin (II) or 4×10^{-5}M kinetin (III). For further details see text.

However, higher level of 2,4-D over 2.21×10^{-6}M gave yellow friable callus. This result indicated that the friability of the callus was attributable to levels of auxin and cytokinin. High level of auxin gave friable callus, while high level of cytokinin did compact callus.

Thus, it was obvious that kinetin had a significant role in the callus growth and also an effect on tissue texture. Kinetin in various concentrations was examined additionally for its effect on tissue texture in the presence of 1.14×10^{-5}M IAA. Kinetin lower than 10^{-7}M gave again brown friable callus, but at 10^{-6}M did brown compact callus. Unusually high concentrations of kinetin (10^{-5} and 4×10^{-5}M) caused more compact callus, resulting in somewhat whitish callus with decrease of rooting (Fig. 3). In the presence of low level of 2,4-D (2.21×10^{-7}M), the highest concentration of kinetin (4×10^{-5}M) gave white compact tissue. Such white callus was similar to tobacco callus in supra-optimum concentration of kinetin [1].

It is interesting to learn whether or not the brown callus on the medium with IAA as auxin is composed exclusively of brown necrotic cells. It is otherwise possible that the color of brown callus is attributable to differentiated cells, because a preliminay microscopic observation on the brown tissue revealed distribution of a number of brown tracheal elements as shown previously [5]. Moreover, brown callus was mostly accompanied by root initiation, while yellow friable callus on the medium 2,4-D scarcely developed roots. It was supported by another observation that an increase of kinetin concentration from 10^{-6}M to 4×10^{-5}M in the presence of 1.14×10^{-5}M IAA caused a change of the tissue texture from brown callus with roots to white callus without rooting.

As reported previously [5] and mentioned above, nicotinic acid tested at 10^{-5}M was ineffective to callus induction, but promoted strongly the callus growth. In the present experiment, the effect of various concentrations of nicotinic acid on the tissue texture as well as callus growth was examined in detail in the presence of 10^{-6}M kinetin and 2.21×10^{-7}M 2,4-D, 2.21×10^{-6}M 2,4-D or 1.14×10^{-5}M IAA (data have not been presented). The maximum callus growth observed at 4×10^{-6} or 10^{-5}M nicotinic acid. Even at the highest concentration (10^{-4}M) no effect on the tissue texture was observed. The tissue texture and its color were dependent exclusively on the levels of auxin and cytokinin. This was clearly suggestive that auxin and cytokinin had important roles in cell differentiation of *Vigna* callus as well as in cell

Table 4. Growth of the D, DK, DN, DKN and DKY calli on the various media added with 2.21×10^{-6}M 2,4-D (D), 10^{-6}M kinetin (K), 10^{-5}M nicotinic acid (N) and 1 g/l yeast extract (Y), alone or in combination. For further details see text.

Callus	Medium	Fresh weight (mg)
D callus	D	65
	DK	169
	DN	381
	DKN	409
	DKY	365
DK callus	D	42.5
	DK	68.2
	DN	94.6
	DKN	417.8
	DKY	319.6
DN callus	D	23
	DK	120
	DN	770
	DKN	713
	DKY	591
DKN-1 callus	D	11.1
	DK	31.6
	DN	117.8
	DKN	424.2
	DKY	438.6
DKN-2 callus	D	10.5
	DK	227.3
	DN	20.9
	DKN	350.8
	DKY	498.2
DKY callus	D	12.0
	DK	23.0
	DN	22.2
	DKN	220.3
	DKY	529.3

proliferation.

As described above, IAA was unfavorable to establish vivid subculture with homogenous tissue. Therefore, 2,4-D was employed as auxin for subculture of the callus isolated from root sections. The stock cultures were established on the D, DK, DN, DKN and DKY medium after more than 15 transfers. They were designated as the D, DK, DN, DKN and DKY callus respectively. The growth response of these habituated calli was examined in the D, DK, DN, DKN and DKY media (Table 4).

The D callus was able to grow, although slowly, on the medium with only 2,4-D. Addition of kinetin promoted slightly the growth, and nicotinic acid strongly stimulated it. No additive effect by these two factors was observed. Yeast extract was also stimulative to the callus growth, but less effective than nicotinic acid. The growth

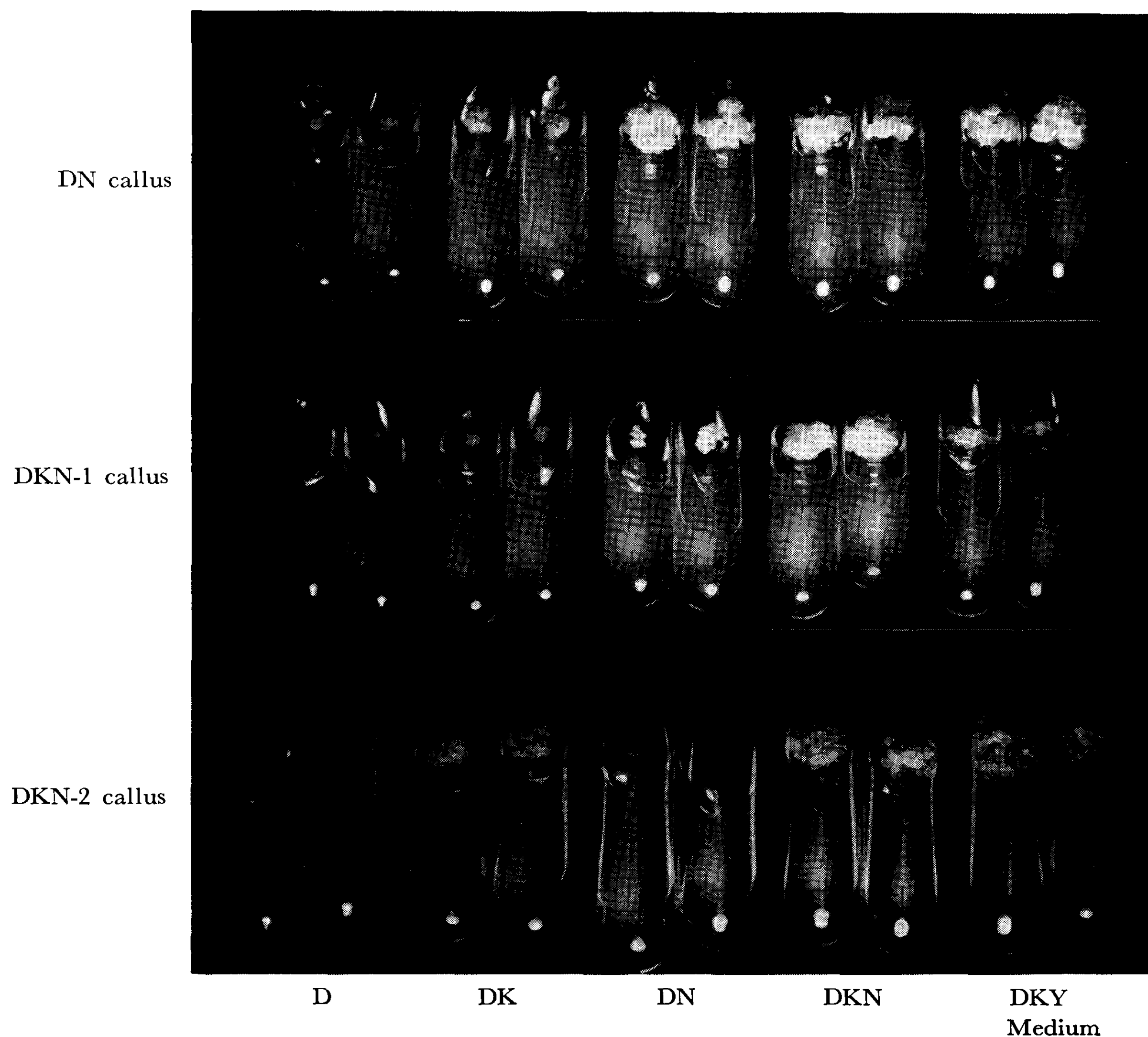

Fig. 4. The DN, DKN-1 and DKN-2 callus cultured on the D, DK, DN, DKN and DKY media. For further details see text.

of the DK callus was promoted by kinetin and nicotinic acid to some extent, but a synergistic ebect of these factors was remarkable. Like the D callus, the growth of the DN callus was promoted strikingly by nicotinic acid (Fig. 4). No additive effect by kinetin and nicotinic acid was observed. Yeast extract was rather less effective than nicotinic acid.

During subculture of the DKN callus, two distinguishable calli with different requirements for kinetin and nicotinic acid were obtained. One was the DKN-1 callus, the growth of which was promoted somewhat by kinetin and nicotinic acid and a synergistic effect between two factors was clear. Another was the DKN-2 callus, the growth of which was certainly promoted by kinetin, but not by nicotinic acid, nevertheless synergistic effect of them was observed. Yeast extract was effective to the growth of these DKN-1 and -2 calli. The DKY callus grew very poorly on the D, DK and DN media. However, a synergistic effect of kinetin and nicotinic acid was remarkable. This callus responded strongly to yeast extract.

As shown in Table 4 and Fig. 4, the growth of the D and DN callus was promoted much by nicotinic acid. They did not show any additive effect by kinetin and nicotinic

acid. On the other hand, the DKN-2 callus was quite different from other calli. The DK, DKN-1 and DKY calli responded similarly to the growth factors, although not identically. Now three different growth responses were presented in Fig. 4. To study further the requirement of the callus for growth factors, single cell culture from these different types of stock callus should be necessary.

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要　　旨

ササゲ (*Vigna sinensis* Endl.) 組織培養のカルス形成，継代培養，馴化について調べた．子葉，胚軸，根の切片を IAA か 2,4-D を含む Linsmaier-Skoog 培地で培養するとカルスを形成した．カルス形成は IAA より 2,4-D の方が強い活性を示した．子葉の切片は胚軸や根の切片より高濃度のオーキシンを必要とした．カイネチンおよびニコチン酸はカルス形成に大きな効果を示さなかった．酵母抽出物はオーキシンおよびカイネチンと共に使用すると，旺盛なカルス形成をひこおこした．

継代培養では，2,4-D を含む培地では黄色の柔らかいカルス組織を生じ，発根は見られなかった．一方，オーキシンとして IAA を含む培地では組織は発根し褐色を呈した．多数の分化した細胞を含むためと考えられる．カイネチンは組織の生長を促進し，さらに組織の性状に影響した．低濃度のカイネチンでは柔らかい組織を生じたが，高濃度のカイネチンは硬い組織を生じた．逆に高濃度のオーキシンは柔らかい組織を生じた．オーキシンおよびサイトカイニンの量はカルス組織の細胞の分化の程度を支配していることを示すものと考えられる．ニコチン酸および酵母抽出物はカルス組織の生成を著しく促進するが，組織の性状に影響しなかった．

2,4-D およびカイネチン，ニコチン酸，酵母抽出物を選択的に加えた培地で15回以上継代培養したカルス組織から，これらの生長因子に対する要求性の異なったカルス組織が少なくとも 3 種得られた．

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