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Influences of Auxin and Cytokinin on the Relationship between Dedifferentiation and Redifferentiation in Hypocotyl Culture of Tomato.

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Introduction

There are only a few papers reporting success of shoot formation from tomato tissue and callus cultured in vitro, although we have heard that tomato plant is comparatively easier to induce organs, shoot and root. NORTON et al (1954) obtained complete plant from callus culture of root in *Lycopersicon peruvianum*, but did not indicate the culture procedures in detail. GRESSHOFF & DOY (1972) also obtained haploid plantlet originating from male gamete in anther culture, but the rate in obtaining a haploid plantlet was very low. YAKUWA et al (1973) investigated in detail the effect of auxin (NAA) and cytokinin (BA) on callus and organ induction from segments of cotyledon, hypocotyl and primary root of young seedling in tomato. They reported that the three parts of seedling easily induced callus and root, but no shoot was formed from root or callus from root in spite of a successful formation of shoot from cotyledon and hypocotyl. Furthermore, their data showed that several combinations of auxin and cytokinin favoured callus growth but did not induce shoot organ. In this study, shoot formation from the callus with no indication of shoot organogenesis during prolonged culture on the same medium with callus induction was attempted as a main purpose.

Materials and methods

Five cultivars of tomato, *Lycopersicon esculentum* L. cv. Hikari, Fukuju No. 2, Tōkō K, Kyōryokushūhō and BF Okitsu No. 101, were used as materials in this study.

Aseptical germination : In order to obtain segments of hypocotyl in young seedling, sterile seeds were germinated on an agar medium containing 3% of sucrose in 200 ml Erlenmyer flask.

Hypocotyl culture for dedifferentiation : The media used in the present

experiments contained defined inorganic salts and organic constituents, various amounts of plant growth substances, 3 % of sucrose and 1 % of agar. The pH was adjusted to 5.8 with 1N NaOH and 1N HCl prior to the addition of agar. The solution containing agar, after preliminary heating, were distributed 20 ml in each 100 ml Erlenmyer flask or 10 ml in each Pyrex culture tube and then autoclaved for 15 min. at 1.2 kg/cm² pressure. Excised segments of hypocotyl (1 cm) were planted on the media, either one explant in a Pyrex culture tube or two in an Erlenmyer flask.

Callus culture for redifferentiation : The media were prepared by the same procedure with the preparation in the hypocotyl culture. The calluses induced by the hypocotyl culture were excised about 50 mg in fresh weight and planted three pieces in a 100 Erlenmyer flask.

The plant growth substances added to the media were the auxin α-naphthalene acetic acid (NAA) and the cytokinin 6-benzyladenine (BA). Another substance,

Table 1. Composition of basal medium. (mg/l)

Medium / Constituent	MS	Madified MS	White	Modified White
$Ca(NO_3)_2 \cdot 4H_2O$			300	300
Na_2SO_4			200	200
KNO_3	1900	640	80	640
KCl			65	65
$NaH_2PO_4 \cdot 2H_2O$			16.5	16.5
$MgSO_4 \cdot 7H_2O$	370	370	720	720
$ZnSO_4 \cdot 4H_2O$	8.6	8.6	3	3
H_3BO_3	6.2	6.2	1.5	1.5
KI	0.83	0.83	0.75	0.75
$MnSO_4 \cdot 4H_2O$	22.3	22.3	7	7
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25		
$CuSO_4 \cdot 5H_2O$	0.025	0.025		
$CoCl_2 \cdot 6H_2O$	0.025	0.025		
KH_2PO_4	170	170		
NH_4NO_3	1650	550		550
$CaCl_2 \cdot 2H_2O$	440	440		
$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.8	27.8
Na_2-EDTA	37.3	37.3	37.3	37.3
Glycine	2	2	2	2
Nicotinic acid	0.5	0.5	0.5	0.5
Pyridoxine-HCl	0.5	0.5	0.5	0.5
Thiamine-HCl	0.1	0.1	0.1	0.1
Myo-inositol	100	100	100	100

Note : MS medium : Murashige & Skoog (1962).
White medium : White (1963).

adenine, was used as nitrogen base in the callus culture.

Organ induction was checked by visual observation through the present experiments. Together with shoot itself, shoot-like organ with chlorophyl was included in shoot formation.

Results

1. HYPOCOTYL CULTURE FOR DEDIFFERENTIATION

Difference of basal media in callus and organ induction

In order to know the effect of basal medium on callus and organ induction from hypocotyl, four kinds of basal media, MS medium (MURASHIGE & SKOOG, 1962), modified MS medium (Concentrations of NH_4NO_3 were reduced to one third of MS medium). White medium (WHITE, 1963) and modified White medium (550 mg/l of NH_4NO_3 was added to White medium and KNO_3 was augmented to 640 mg/l), were employed, as shown in Table 1. The concentrations of auxin and cytokinin combined with each basal medium in this experiment were all combinations of 0.1 and 1.0 ppm NAA and BA. Three cultivars, Hikari, Fukuju No. 2, and Tōkō K, were used as materials. Conditions of culture were about 25°C in temperature and natural light in a room.

The results of callus and root induction in the four basal media are compared in Table 2. Best development of callus growth was observed in the modified MS medium, and the MS and modified White media followed in order and no callus

Table 2. Callus and root induction in four basal media.

Basal medium	Concentration of ingredient		Callus	Root
	NAA (ppm)	BA (ppm)		
MS medium	1	1	++++	−
do.	1	0.1	+++++	++
do.	0.1	1	++++	±
do.	0.1	0.1	++++	+++
Modified MS medium	1	1	++++	−
do.	1	0.1	+++++	+++
do.	0.1	1	+++++	+
do.	0.1	0.1	++++	++++
White medium	1	1	−	−
do.	1	0.1	−	±
do.	0.1	1	−	−
do.	0.1	0.1	±	+
Modified white medium	1	1	+	−
do.	1	0.1	++	±
do.	0.1	1	++	−
do.	0.1	0.1	+++	+++

tissues were visible in the White medium. Similar observation was obtained in root induction. The difference of callus and root growth between the modified MS and MS media was observed during two or three weeks after planting of hypocotyl, and gradually disappeared. There was basically no interaction between the four levels of NAA and BA concentration and the four basal media on root formation from hypocotyl. On the other hand, shoot formation was not observed in any 16 media.

Difference of cultivars in callus and organ induction

Four cultivars, Hikari, Fukuju No. 2, Tōkō K and BF Okitsu No. 101, were examined on callus and organ induction in the four levels of NAA and BA concentration that consisted of all combinations of 0.025 and 0.25 ppm NAA and BA and were combined with the MS medium. One explant of hypocotyl (1 cm) was planted onto the slant agar medium in a Pyrex culture tube. Conditions of culture were about 25℃ in temperature and 16 hr. photoperiod in illumination from fluorescent lamps for plant culture giving about 2000 lux.

Difference of the cultivars in callus and root growth was enough small to ignore (Table 3). Rate of shoot formation at 6 weeks after planting of hypocotyl

Table 3. Veriety difference of callus growth in hypocotyl culture of tomato.

NAA (ppm) / BA (ppm) / Cultivar	0	0.025	0.025	0.25	0.25
	0	0,025	0.25	0.025	0.25
Fukuju No.2	0.5	2.1	2.5	2.3	3.5
Tōkō K	1.2	2.4	2.7	2.5	3.4
Hikari	0.8	1.8	2.5	2.0	4.0
BF Okitsu No. 101	1.2	2.0	2.7	2.4	3.9

Note 1 : Callus growth was recorded at 4 weeks after planting.
Note 2 : Standard of callus size is as follows ; 1 : Rice seed, 2 : Azuki bean, 3 : Soybean, 4 : Horsebean.

Table 4. Variety difference of shoot formation in hypocotyl culture of tomato.

NAA (ppm) / Cultivar / BA (ppm)	0				0.025				0.25			
	F	T	H	B	F	T	H	B	F	T	H	B
0	0	50	10	50								
0.025					30	70	30	30	0	0	0	0
0.25					20	20	40	50	60	40	30	10

Note 1 : Rate of shoot formation (%) was recorded at 6 weeks after planting.
Note 2 : F : Fukuju No. 2, T : Tōkō K, H : Hikari, B : BF Okitsu No. 101.

is presented in Table 4. Remarkable observation was no shoot formation at all of the four cultivars on the media with lower BA concentration than NAA (0.25 ppm NAA and 0.025 ppm BA). In comparison between the cultivars, interaction between cultivar and plant growth substance on shoot formation was observed, that is, the rate of shoot formation of Fukuju No. 2 increased at higher concentration of NAA and BA (0→30→60%), but that of BF Okitsu No. 101 conversely decreased (50→30→10%).

Effect of NAA and BA on root and shoot formation

Effects of various levels of NAA and BA concentration on root and shoot formation are presented in Table 5. Root formation was promoted by higher NAA concentration than BA, or eqaul with (for example, 0.1 ppm NAA and BA, 1.5 ppm NAA and 0.1 ppm BA), and conversely suppressed by higher BA concentration than NAA (for example, 0.1 ppm NAA and 1.0 ppm BA, 0.01 ppm NAA and 5.0 ppm BA). By way of exception, root formation was also observed at higher BA concentration than NAA at the lower levels (0.025 ppm NAA and 0.25 ppm BA).

Shoot formation was not observed at any combinations of NAA and BA concentration within a range of higher NAA than BA (for example, 1.0 ppm NAA

Table 5. Effects of NAA and BA on root and shoot formation in hypocotyl culture of tomato.

Basal medium	Light	NAA (ppm)	BA (ppm)	Cultivar	No. of hypocotyl	Root	Shoot
MS, MS′, W, W′	N	0.1	0.1	H, F	60	+++	−
do.	N	0.1	1	do.	60	±	−
do.	N	1	0.1	H, F, T	120	++	−
do.	N	1	1	do.	150	+	−
MS, W	L, N	0	0	H, F, T, B	80	+	+++
do.	L	1	0	H	40	+	−
MS	L	1.5	0.1	S	20	++	−
do.	L	2	0.1	do.	20	++	−
do.	N	0	5	F	10	−	+
do.	N	0.01	1	do.	10	−	++
do.	N	0.01	5	do.	10	−	++
do.	N	0.01	10	do.	10	−	+
do.	L	0.025	0.025	H, F, T, B	40	+	++++
do.	L	0.025	0.25	do.	40	+	+++
do.	L	0.25	0.025	do.	40	+	−
do.	L	0.25	0.25	do.	40	+	+++

Note 1 : Basal medium ; MS : Murashige & Skoog (1962), W : White (1963), MS′ : Modified MS, W′ : Modified white.

Note 2 : Light condition ; N : Natural light in a room, L : Artificial light.

Note 3 : Cultivar ; H : Hikari, F : Fukuju No. 2, T : Tōkō K, S : Kyōryokushuhō. B : BF Okitsu No. 101.

Table 6. Organ induction from callus derived from hypocotyl of tomato.

Hypocotyl culture		Organ induction culture			Shoot formation			Root formation	Light
NAA (ppm)	BA (ppm)	NAA (ppm)	BA (ppm)	AD (ppm)	No. of Calluses	No. of shoots	%		
0.1	0.1	0.01	1	0	40	1	3	−	N
0.1	0.1	0.01	10	0	40	0	0	−	do.
0.1	1	0.01	1	0	40	3	8	+	do.
0.1	1	0.01	10	0	40	6	15	−	do.
1	0.1	0.01	1	0	40	0	0	+	do.
1	0.1	0.01	10	0	40	0	0	−	do.
1	1	0.01	1	0	40	7	18	−	do.
1	1	0.01	10	0	40	5	13	−	do.
1	1	0	0	0	8	0	0	+++	L
1	1	0	10	0	8	6	75	−	do.
1	1	0	15-20	0	16	5	47	−	do.
1	1	0	10	1-15	32	20	63	−	do.
1	0.1	0	0	0	8	0	0	++	do.
1	0.1	0	10	0	8	0	0	−	do.
1	0.1	0	15-20	0	20	3	15	−	do.
1	0.1	0	10	1-15	49	15	31	−	do.
1.5-2	0.1	0	0	0	7	0	0	+++	do.
1.5-2	0.1	0	10-20	0	21	1	5	−	do.
1.5-2	0.1	0	10	1-15	24	2	8	−	do.
1	1	0	0	0	3	0	0	+	do.
1	1	0	10-20	0	9	5	56	−	do.
1	1	0	10	1-15	13	3	23	−	do.
1	1*	0	0	0	6	0	0	±	do.
1	1*	0	10-20	0	18	0	0	−	do.
1	1*	0	10	1-15	24	0	0	−	do.

Note 1 : AD : Adenine
Note 2 : * : One subculture.
Note 3 : Light condition ; N : Natural light in a room, L : Artificial light.

and 0.1 ppm BA, 0.25 ppm NAA and 0.025 ppm BA). In the culture under natural light in a room, no media with eqaul, or small difference of, NAA and BA concentration regenerated shoots (1.0 ppm NAA and 1.0 ppm BA), but media with lower NAA and higher BA concentration regenerated shoot organs (for example, 0.01 ppm NAA and 10.0 ppm BA). On the other hand, the culture under artificial light favoured the rate of shoot formation, as shoot was formed at 0.25 ppm NAA and BA.

2. CULTURE OF REDIFFERENTIATION (CALLUS CULTURE FROM HYPOCOTYL)

Callus tissues planted onto the media for redifferentiation were distinguished

from each other by the derivation of the hypocotyl culture. Concentrations of NAA and BA in the hypocotyl culture, culture conditions for redifferentiation and results of organ induction are presented in Table 6. The culture of this experiment was carried out under two conditions of culture, that is, one was about 25°C in temperature and natural light in a room and the other the same temperature and 16 hr. photoperiod with about 1500 lux from the fluorescent lamps.

Root formation

In the culture under natural light, root formation was poorly observed only in the culture condition in which callus induction was carried out on the media containing 0.1 ppm NAA and 1.0 ppm BA and 1.0 ppm NAA and 0.1 ppm BA and redifferentiation culture followed on the medium containing 0.01 ppm NAA and 1.0 ppm BA. In the culture under artificial light, root formation was observed only at the culture on media in the absence of NAA, BA and adenine, regardless of any combinations of NAA and BA concentration in the culture for callus induction.

Shoot formation

In the culture under natural light, shoot formation was observed on the callus from the callus induction with lower NAA concentration than BA, or eqaul with at the high level of NAA and BA (0.1 ppm NAA and 1.0 ppm BA, 1.0 ppm NAA and BA), but not induced from the callus induction with higher NAA concentration than BA, or eqaul with at the low level of NAA and BA (0.1 ppm NAA and BA, 1.0 ppm NAA and 0.1 ppm BA). In the culture under artificial light, shoot formation was generally promoted when compared with the culture under natural light and observed even on the media with higher NAA concentration than BA in the callus induction. However, the rate of shoot formation was evidently higher in from the callus induction containing 1.0 ppm NAA and BA than 1.0 ppm NAA and 0.1 ppm BA.

Addition of adenine to the medium for organ induction seemed to give a little favour for shoot formation.

Shoot formation from the callus obtained by the subculture was not successful in spite of the addition of BA and adenine.

Discussions

We should first examine the effect of various basal media and varieties in a species, when a plant tissue is used for an attempt of dedifferentiation and redifferentiation in vitro. The present experiment suggested that the basal medium of MURASHIGE & SKOOG (1962) was superior to that of WHITE (1963) in the culture for dedifferentiation and redifferentiation from hypocotyl of tomato and basal medium did not interact with combination of auxin and cytokinin. From

this result, the basal medium of MURASHIGE & SKOOG (1962) was employed for the following experiments. Also, we have a knowledge that dedifferentiation and redifferentiation might be influenced by variety in a species. GRESSHOFF & DOY (1972) reported that successful organ induction to complete plantlet in anther culture of tomato was possible only from two out of fourty-three varieties used as materials. Variety difference of callus and root induction was very small in this experiment. But in shoot formation an observation was obtained that the rate of shoot formation was a little influenced by the interaction of variety with concentration of auxin and cytokinin. However, no shoots were formed from the four varieties used on a medium containing 0.25 ppm NAA and 0.025 ppm BA. This fact suggests that at least the four varieties are generally controlled by a general rule mentioned below.

SKOOG & MILLER (1957) first pointed out that the two plant growth substances, auxin and cytokinin, determined not only whether plant tissue grows or not, but also how it grows, in callus tissues derived from the pith parenchyma of tobacco shoots. On an agar medium containing 2 mg/l of auxin and 0.1 mg/l of kinetin only an undifferentiated callus resulted. However, the kinetin concentration was lowered to 0.02 mg/l without changing the auxin level, roots were formed in the cultures. Higher concentrations of kinetin led, conversely, to shoot formation and root formation was then suppressed. Such a general trend is considered to apply to the hypocotyl tissue of tomato. This conclusion is unhesitatingly affirmed by the relative relationship of auxin and cytokinin on callus induction, root and shoot formation which was observed in the present experiments and by the results reported by YAKUWA et al (1973).

The second conclusion will be led from the present experiments and the results of YAKUWA et al (1973) also that hypocotyl culture, even if continued over prolonged periods, will fail to form shoot on media containing NAA and BA with combinations of 0.1 and 1.0 ppm, although callus grows successfully. In the culture for redifferentiation, shoot formation was attempted from such a callus mentioned above. The concentration of auxin and cytokinin in the media of redifferentiation consisted of the combinations of low NAA (0 and 0.1 ppm) and high BA (1.0 and 10.0 ppm) concentration, as referred to the results of YAKUWA et al (1973). As the result, shoot formation was successful both under the culture conditions of natural light and artificial light. Furthermore, the most prominent observation was obtained that whether callus cultures regenerated shoot or not was destined by the medium for callus induction. It will be very difficult for shoot formation to be induced on the callus derived from medium containing higher NAA concentration than BA. This indicates that researcher's attention should be paid on not only medium for redifferentiation but also for dedifferentiation in an attempt for shoot induction from callus tissue.

MURASHIGE & NAKANO (1968), NEBEL & NAYHOR (1968) pointed out the necessity of light for shoot formation in tissue cultured in vitro. Shoot formation seems to be positively correlated with light intensity within untill an optimum light intensity. However, researches for light quality have not yet arrived to indicate which portion of light spectrum is the most effective for shoot formation, although light quality definitely holds the key for organogenesis. In the present experiments, the culture under artificial light showed a higher rate of shoot formation than the culture under natural light, probably, because of the increase of light intensity.

The effective result of the addition of adenine on shoot formation was reported by SKOOG & TSUI (1948) and NITSCH et al (1967). However, further tests will be necessary to point out whether adenine is important for shoot induction from redifferentiation culture in tomato or not.

MURASHIGE & NAKANO (1965) obtained an evidence of change in the totipotency of plant tissue and callus. In tobacco, the ability to form root was lost within one and a half years and that to regenerate shoot gradually diminished within from one and a half years to three years after the beginning of culture. In tomato, the callus from subculture clearly reduced the capacity to form root and at all lost that to form shoot. REINERT & BACKS (1968), however, showed that carrot cultures retained the capacity to produce root for years. Our unpublished data also showed the ability to produce complete plant from the callus of subculture for three months in tomato. It may be to recognize that the loss of the capacity for organogenesis in cultures can be reversible.

Summary

The purpose of the present experiments was, at first, to know the effects of basal medium and cultivar on callus and organ induction in hypocotyl culture of tomato, *Lycopersicon esculentum* L., and, secondly, to confirm the results reported by YAKUWA et al (1973) which described the effects of auxin and cytokinin on callus and organ formation from tissue cultures in vitro in tomato and, thirdly, also using hypocotyls of tomato, to examine culture conditions on the relationship between dedifferentiation and redifferentiation.

The culture procedures were arbitrarily divided into two steps : hypocotyl culture for dedifferentiation and callus culture for redifferentiation.

Hypocotyl culture for dedifferentiation

In the hypocotyl culture, segments of hypocotyl (1 cm) in young seedling were cultured in vitro on the agar media containing various concentrations of auxin (NAA) and cytokinin (BA).

The results were as follows ;

1. The basal medium of MURASHIGE & SKOOG (1962) was superior to that

of WHITE (1963) in callus and root induction.

2. Shoot formation seemed to be influenced by interaction between variety and concentration of NAA and BA, but this was not beyond the general rule first pointed out by SKOOG & MILLER (1957) that root and shoot induction was basically regulated by interaction between auxin and cytokinin.

3. Root formation was more induced on media with higher NAA concentration than BA, and shoot was favourably regenerated on media with lower NAA and higher BA concentration.

4. On the media containing NAA and BA with all combinations of 0.1 and 1.0 ppm, callus grew favourably but shoot was not at all formed in spite of prolonged culture.

5. The results of 3 and 4 coincided with those reported by YAKUWA et al (1973).

Callus culture for redifferentiation

In the callus culture, callus from each combination of NAA and BA concentration in the hypocotyl culture was differentially planted on medium for redifferentiation. The results were as follows ;

1. Whether shoot was induced from callus or not was destined by the medium for callus induction, that is, shoot formation was very difficult on the callus derived from medium containing higher NAA concentration than BA.

2. Increase of light intensity in the culture under artificial light gave a higher rate of shoot formation than the culture under natural light in a room.

3. Callus derived from subculture clearly reduced the capacity to form root and showed no ability to regenerate shoot.

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要　　約

トマトの胚軸培養における脱分化と再分化におよぼすオーキシン，サイトカイニンの影響

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本研究の目的は，まず最初に，基本培地の種類と品種の如何がトマトの胚軸からのカルス形成と器官分化におよぼす影響を知り，つぎに，八鍬ら (1973) の報告 —オーキシンとサイトカイニンがトマト幼苗の胚軸組織からのカルス形成および器官分化におよぼす影響— を確認し，第3に，同じくトマトの胚軸を用いた培養試験を行ない，カルス形成すなわち脱分化とそのカルスからの再分化の培養条件について検討することであった．

培養試験の手順は脱分化のための胚軸培養と，再分化のためのカルス培養に分けて行なった．

胚軸培養によるカルス形成と器官分化

胚軸培養では，オーキシン (NAA) とサイトカイニン (BA) が種々の濃度で含まれる寒天培地で稚苗の胚軸切片 (1 cm) を無菌培養した．結果はつぎのように要約される．

1. カルス形成と根の分化には Murashige および Skoog (1962) の基本培地が White (1962) の基本培地よりもすぐれていた．

2. 茎葉分化は NAA および BA 濃度の相互作用と品種の如何に影響されるようであったが，これは Skoog および Miller (1957) によって最初に指摘された一般的法則，すなわち，根と茎葉の分化はオーキシンとサイトカイニンの相互作用によって基本的に制禦されるという法則の範囲を越えるものでなかった．

3. 根の分化は BA より NAA 濃度が高い培地で良好であったが，茎葉分化は逆に NAA 濃度が低く，BA 濃度が高い培地でより良好であった．

4. NAA と BA 濃度の0.1 ppm と 1.0 ppm をそれぞれ組合せた培地では，カルン形

成は良好であったが，茎葉分化は培養を長く続けても全く認められなかった．

5．3と4の結果は八鍬ら（1973）によって報告された結果と一致した．

カルスの再分化培養

カルスの再分化培養では，胚軸培養の際に設定したNAA，BAの濃度区にしたがって，それぞれのカルスを区別して再分化培地に植付けた．

1．再分化培地でのカルスからの茎葉分化の有無はカルス形成培地の影響を受けた．すなわち，茎葉分化はBAよりもNAA濃度が高い培地で形成されたカルスからは非常に困難であった．

2．人工照明による明るさの増大は室内自然光での培養に比べて茎葉形成率を増加させた．

3．継代培養を経たカルスは明らかに根の分化能が低下し，茎葉分化能も欠けていた．