Studies on the Differentiation in Cultured Cells I. Embryogenesis in Three Strains of *Solanum* Callus*

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Abstract

The two strains of callus, one strain BD that forms "embryo" and the other strains BND that shows no differentiation, were isolated from the callus of the common origin after two years of culturing. The BD strain formed chlorophyll, while BND, which is a translucent callus with a good rate of multiplication, showed no chlorophyll formation. The KA, a different strain, showed no differentiation of organs on the basic culture medium.

Using these three strains, the effect of IAA and kinetin on organ formation was experimented. The BD differentiates "embryos" on the basic medium, its formation being controlled by IAA. In the KA, bud differentiation is induced under white fluorescent light in the range of suitable combinations of IAA and kinetin.

The embryogenesis of BD is similar to that of the embryo in an ovule.

In KA, development of spherical colonies precedes adventive bud formation. In this case, a base for buds is formed which then grows into adventive buds.

In BND, cultured cells are variable in nature, so the "embryo" forming portion is maintained by selection.

The conditions for inducement of buds differ with types, but even in the same type they differ with strains or callus age, so that generalizations on the bud inducement process are now difficult.

The previous papers^{1,2)} reported studies on *Tradescantia* and *Paeonia* calli and revealed that the calli were generally mixoploidal cell colonies which were lacking cytological uniformity. Hitherto the studies of inducing certain organs from colonies have been attempted for the purpose to observe whether or not uniformity of calli is gained to form an entire plant.

In the present paper the effects of auxin and kinetin on the embryogenesis of the three strains of calli derived from the two forms of *Solanum* will be reported.

Materials and Methods

Two forms of the eggplant (*Solanum melongena*), Sinkro (B, black variety) and Kantôao (KA, green variety) were used. Two callus strains were isolated from an

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embryo callus of the B variety in July 1961. One strain (BD) of them formed embryo-like structures and chlorophyll under light, while the other strain whose callus mass is translucent showed no indication of embryo or chlorophyll formation. The KA callus was isolated from embryos in December 1965.

The White's medium with 1 mg/l α -naphthalene acetic acid (NAA) was used from June 1961 to December 1965 and the Murashige's medium was used since January 1966.

Observations

1. *Embryogenesis in the BD callus.*

An immature seed embryo usually grows directly into a young plant on auxin-free-medium, while a callus is formed from the embryo on auxin medium. In July 1961 calli were formed from immature embryos on auxin medium. Neither root nor bud formation were observed in these calli until March 1963 when the BD strain appeared. The ability for embryogenesis of this BD strain is still maintained at present, but it is variable and not stable. Some portions of it show degeneration of differentiating ability, thus showing practically no indication of embryogenesis, while the other forms numerous embryos (Figs. 1 and 2). The differentiating portion of the strain may be maintained by a subculture of the embryo forming calli. When these "embryos" were transplanted onto the auxin medium they multiplied as calli, and embryo formation followed.

The period of embryogenesis was affected by cultural conditions. The dark culture, without transplantation, required 2-3 months, but the period could be shortened by transplanting to a low auxin medium (0.01-0.001 mg NAA/l). BD callus growth could be continued to a certain extent by transplanting to a low auxin medium, while "embryo" formation could be induced on the low auxin medium. The entire callus under white fluorescent light (1500-2000 luxes) turned lime green and embryogenesis occurred within 3-4 weeks. On a subculture of this portion, the "embryos" which did not dedifferentiate, became an incomplete callus. Callus formation and embryogenesis were taking place simultaneously (Fig. 2).

Embryogenesis occurred on the surface of the callus. The embryos formed were of various shapes, but those which required a long period for formation bore a close resemblance to the embryos developed from the fertilized egg cell (Figs. 3 and 4). A vessel formation was observed in some embryos. An even growth was shown by the embryos originated from the free cells that flowed out away from the callus mass onto the surface of the agar or the container (Figs. 5 and 6). However the embryos originating directly from the callus cells showed an uneven growth (Figs. 3 and 6). By transplantation of these "embryos" to auxin-free-medium, roots came out and cotyledon unfolded and finally a normal plant developed (Figs. 7 and 8).

2. *Inducement of buds from the KA callus.*

The BND callus, isolated from the same origin as the BD callus, is translucent and deficient in the ability to form chlorophyll. It had a rapid rate of increase in size without embryogenesis. It is a strain possessing entirely different characters from the BD callus. An attempt was made to induce bud formation in BND by the use of auxin, kinetin and adenine, but no satisfactory results were obtained.

The KA callus possessed a character intermediate to BD and BND. Chlorophyll formation occurred, but no differentiation of bud or root took place on the basic culture medium (Fig. 9). The experiments to induce buds from the KA callus with

various combinations of kinetin and IAA were carried out under white fluorescent light (1500–2000 luxes) (Table 1). Bud formation was not induced by kinetin or auxin

Table 1. The effects of IAA and kinetin on bud formation of the KA callus.

These were cultured under white fluorescent light.

Embryos were formed in about one month.

	Kinetin concentration (ppm)					
		8	4	2	0.2	0
IAA con- centration (ppm)	10	++++ ○○	++++ ○○○○	++++ ○○	++++ —	++++ —
	2	++++ ○○○	++++ ○○○	++++ ○○	++++ —	++++ —
	0.4	++ ○○○	++++ ○○○	++++ ○○○	++++ ○	++++ —
	0	+ —	++ ○	++ ○	+* —	+* —

+ Callus formation. — None. ○ Bud formation. * Necrosis after one month.

alone, but certain combination of kinetin and auxin led to embryogenesis. The effect of kinetin varied with the concentration of IAA and the impediment of growth caused by kinetin could be checked by raising the concentration of IAA. The formation of numerous undifferentiated spherical colonies, having rich chlorophyll and protocorm-like hard callus, preceding embryogenesis was characteristic of the calli in which buds were inducible (Fig. 10). Thereafter 1–3 “embryos” were formed in that portion (Figs. 11–14). When a colony with “embryos” was transplanted to culture medium containing gibberellin 2 mg/l to elongate the shoot and then transplanted in the basic medium without auxin, roots germinated and a young plant was formed (Figs. 15 and 16). As this kinetin-induced-embryo differs from that of the BD callus, which possesses a root-shoot axis, it may be more suitable to call it a primordial bud. No “cotyledons” like those of the buds in the BD callus, were formed in these bud. The shapes of early leaves and their attachments were irregular, e. g., verticillate, opposite, etc. (Fig. 12). This irregularity became normal and the leaves resumed their normal pattern in the course of growth.

Discussion

Though embryos normally originate from zygotes, the production of “embryo” is also observed in the cultured cells. These may be classified into two types. In the first type small meristematic cell colonies which are found as a regenerative phenomenon form roots and buds by way of a structure resembling a growing point, while in the second type a process similar to normal embryogenesis leads to “embryo” formation. The KA callus belongs to the first type and the BD to the second in the present experiment. Embryo-like structures in callus are known in *Daucus*³⁻⁶⁾, *Citrus*⁷⁾, *Cuscuta*⁸⁾, *Chicorium*⁹⁾ and others.

The ability to differentiate organs in the callus differs with the species and strains and it may be divided into an easily differentiating type like the BD, a not so easily

Figs. 1-8. Embryogenesis and plantlet formation in the BD callus of *Solanum*. 1. A callus mass showing no embryogenesis. 2. Differentiation of a large number of "embryos". (1,2, from the common source of BD callus, after 16 days in culture on Murashige's medium under white fluorescent light.) 3. "Embryo" formation on the callus. 4. Various shapes of "embryo" on the surface of the callus. 5,6. "Embryo" originated from the free cells showing an even growth. (3-6. "Embryo" formation after 3 months in culture on White's medium in the dark.) 7a. Unfolded "cotyledons". 7b. Plantlets with roots and shoots. 8. A plantlet, transferred to soil, which has grown to the normal B plant.

Figs. 9-16. Inducement of buds from KA callus. 9. Friable callus with chlorophyll cultured for 30 days on kinetin-free-Murashige's medium. 10. Spherical colonies cultured on Murashige's medium containing 8 ppm kinetin and 2 ppm IAA (after 30 days in culture). 11. Spherical colonies with primordial buds transferred to Murashige's medium containing 2 ppm gibberellin. 12-14. Bud initiation in the colonies transferred to gibberellin-medium and grown under white fluorescent light (after 14 days in culture.) 15. A plantlet with roots and an abnormal stem and leaves. 16. A plantlet with a normal leaf.

differentiating type like the KA and a hardly differentiating type like the BND. The remarkable point here is that the BD and the BND have a common origin of B callus. The BD strain appeared unexpectedly after two years in culture. The reason for origin whether by mutation or by other factors such as metabolism change is not yet clarified. The ability for embryogenesis of the BD callus is not stable but varies. Even the callus having the ability for embryogenesis, which was selected and cultured, also indicates the nature of variability of cultured cells in general. The same may be said of the chromosome numbers. In the cultured cells of *Paeonia*, variation in the chromosome numbers is observed, subsequent to each subculture, occurring at about the same frequency²⁾. It may be stated that cultured cells are unstable and variable due to the occurrence of deteriorated differentiating ability or of differentiating and undifferentiating types, although the calli may be of a single cell origin¹⁰⁾.

Organ formation is controllable to a certain extent in the BD callus by auxin and in the KA callus by light, kinetin or auxin, while in the BND callus it is not induced by these agents. However, as Braun¹¹⁾ observed the disappearance of tumorous characters of the crown gall in its isolated single cell, it should not be concluded that the BND is a strain which has completely lost its differentiating ability. The appearance of cells that merely multiply due to mutation or to change in metabolic systems may be considered to be the major cause for the special character of the BND strain.

The pattern for organ formation is different in the BD and the KA calli. "Embryos" of BD are formed on the surface or in places isolated from the callus body as in the similar embryological pattern in zygote. The isolation of cells from the meristematic zone of callus seems to be one of the factors for "embryo" formation. The formation of spherical colonies is observed in KA. It precedes the adventive bud formation. This resembles the protocorm formation in an orchid seedling. By dissection and transplantation of the protocorms, numerous protocorms are reformed, on each of which buds appeared. In the case of regeneration of buds from the primary tissue, the tissue mass needs to be of certain size.

The isolation of cells from the callus mass has been stated previously as one of the conditions for inducing "embryo" formation. But the isolated culture of a single cell does not necessarily lead to embryogenesis. The isolated single cell first becomes a cell mass and then buds are formed¹²⁾. This indicates that the isolation of the cell is not the sole condition. "Embryo" formation seems to be a result of some unknown effects from callus tissues or cells. Direct effects from the callus are considered to disorganize the shape of "embryos" and result in a bud-like form, while indirect effects are thought to induce "embryo" formation. Either may occur according to the existing conditions. In fact, in *Nicotiana* there are reports¹³⁾ on "embryo" formation under light conditions, though only the bud forming types are generally known. Organs were similarly induced in *Nicotiana*¹⁴⁾, *Daucus*¹⁵⁾, and *Convolvulus*¹⁶⁾ by the IAA and kinetin combination.

In the inducement of buds, the cultured cells are highly variable, easily changed and the conditions differ with species, strains or the age of callus, so that generalizations on the inducement process are difficult.

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摘 要

山田卓三・中川裕子・篠遠喜人：培養細胞の分化の研究 I. ナスのカルスからえられた 3 系統の胚形成

先に *Tradescantia* や *Paeonia* のカルスは混倍数性をしめし、細胞学的には不均一な細胞集団であることをたしかめた。このカルスの不均一性が個体の変異となってあらわれるかどうかをたしかめるためにナスを用いて器官の誘導をこころみてきた。ナスは真黒 (B) と関東青 (KA) の二つの品種を用いた。B カルスは 1961 年 6 月に胚よりカルスをえ、1963 年分離した二つの系統、すなわち一つは光条件下で葉緑素を形成し“胚”をつくる系統 (BD)、他は半透明のカルスで増殖率はよいが葉緑素形成も“胚”形成もみられない系統 (BND) であり、KA カルスは 1965 年に胚より分離したもので基本培地の上では“胚”の形成はみられないが葉緑素を形成する。

この 3 系統を用いてオーキシン、カイネチンその他の条件のもとにおこなわれる器官形成の状態をみた。

BD は基本培地の上で“胚”を分化し、オーキシンで“胚”の形成を或る程度調節でき、KA は光条件下で IAA とカイネチンとの適当な組合せの範囲で芽を誘導することができる。BD は種子胚と同様な胚形成をおこない、KA は芽の原基の形成にさきだって球形のプロトコーム状のコロニーを多数生じ、これに原基がつくられて不定芽となる。BND では芽の誘導は現在のところ成功していない。

培養細胞は変りやすい。BD の“胚”形成能は選択によって維持され、分化しやすいカルスと分化しないカルスとが分離してくる。誘導は種類によってはもちろん、同一種類でも系統やカルスの古さによっても条件が異なるので、誘導過程の一般理論化を困難にしている。現在までにナスではカルスより育成した個体について変異植物はえられていない。(国際基督教大学生物学教室)