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Studies on the Mericlonal Protocorm of Orchid (II)

Protocorm development from cultured explants

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洋蘭의 生長點培養에 關한 硏究 (II)

培養生長點에서 原塊體의 發生過程

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ABSTRACT

Using several varieties of *Cymbidium*, investigations were carried out to make clear how the protocormic tissue develops from the cultured explant. Explant to be cultured were prepared in several ways: exclusively apical meristem, apical meristem dissected out with the basal part attached, axillary bud primordia in their initial stage of development, or apical or axillary bud dissected out as a whole etc.

It was observed that protocorms or protocormic tissues were developed from the explant's meristematic tissues regardless of where these tissues were located. Apical meristem, leaf primordia, leaf axil, or internodal part of young bud turned easily protocormic, while the scaly leaves of axillary bud or stem tissue of mother shoot turned quickly brwonish and died away.

Both in axillary and apical bud explant alike, whether they were cultured whole or divided, some took quickly green color while others were slower, and some developed protocorms easily while others remained unchanged for months. Varietal difference as well as environmental factors seemed to be responsible for it. Further details should be clarified by histogenetical investigations.

INTRODUCTION

When Morel conducted the sterile culture of the apical meristem of certain *Cymbidium* variety in 1960 (Morel) for the purpose of freeing the virus from infected individual, he observed that the apical meristem, instead of differentiating shoot and root as in the meristem cultures of such plants as potato, tulip, carnation, and many others, gave rise to small globular bodies resembling the ordinary protocorm developing from the seed of orchid, and that these bodies when solid cultured developed finally into plantlets.

A few years later Wimber (1963) and Morel (1964) each conducted further researches on the proliferation of this globular bodies which were by then called protocorm-like bodies. By these earlier works it has been made clear that many other orchid varieties, also, have the same specific character as Cymbidium, i.e., their apical meristems or buds are easily modified into the protocorms (for convenience

protocorm like body is descrided hereafter merely as portocorm) and the protocorms whether cultured whole or thinly sliced biget new protocorms. And yet unlike the callus in the *in vitro* culture of tissue of other plants, the gene constitution of the protocorm is considered exactly identical to the original individual from which the protocorms arose.

Thus the means of endlessly vegetative-propagating orchid varieties through protocorm has been established and workers' attention, being drawn away from the production of virus-free stock as Morel originally attempted, has been concentrated more on the applying this new technique to the rapid multiplication of commercial orchid varieties (Bertsch, 1967, Kohl, 1965, Morel, 1960, 1964, 1965, 1966, Sagawa, 1966, 1967, Scully, 1964.) Recently many varieties of excellent performance have been increased profusely, throwing light and confusion on the orchid growing world.

No morphological and histogenetical researches, however, have been reported on how the mericlonal protocorm develops from the explanted bud, bud tissue, or sliced protocorms (Kohl, 1962). Present deals with part of the veries of investigation on the developmental changes of cultured explants and other paper related observations.

MATERIALS AND METHODS

Materials used were several varieties of *Cymbidium*, *Cym. pumillum* 'Shinree' x *Cym. Rosanna* 'Pinkie' *Cym*, *Hawtescens*, and *Cym. Swallow* 'Exbury.' As no varietal differences were observed in the present studies description on the results was made on only one variety, *Cym. punillum* 'Shinree' x *Cym. Rosanna* 'Pinkie'. In order to observe clearly how the protocormic tissue develops from the bud meristem explants to be cultred were dissected out in various ways:

1. Apical meristem exclusively (Fig. 8). Apical meristem consisting only of apical dome and one or two leave primordia adjacent to the dome were dissected out under dissecting microscope and used as explant as in the case when the apical meristems were excised for the purpose of producing virus-free stocks (Figs. 1–8) Apical meristems were obtained not only from apical buds, but also from axillary buds.

2. Apical meristem dissected out with the basal part attached:

a) Apical meristems from axillary buds on approximately 3 cm long new shoot(Fig. 2). By removing the leaves of the shoot, axillary buds were exposed(Fig. 3). The young scaly leaves of the axillary bud, were removed until the dome of apical meristem appeared(Figs. 4–6). All the remains of the young leaves were trimmed away neatly naking the meristem consisting only of dome and one or two leaf primordia below (Fig. 6). Then removed the apical meristem together with all the basal part of the axillary bud and some of the stem tissue of original mother shoot where the axillary bud has been attached.

b) Axillary bud primordia from the half-open new shoot about 15 cm long: By removing the open young leaves of the shoot, in the axils of upper nodes were found tiny axillary bud primordia, which had just started to develop, with only apical dome and one or two leaf primordia. Dissected out the bud primordia with bulk of the axil of the shoot attached. In this kind of long shoot, on the nodes of under part were found several large axillary buds, but on the upper noder were one or two bud primordia which had just started to form apical cone and leaf primordia were suited as material for this purpose.

c) Meristem of apical bud of approximately 3 cm long new shoot: By removing all the leaves, the

dome of apical meristem and one or two leaf primordia were exposed. The apical meristem with bulk of the stem part attached were dissected out.

3. Apical or young axillary bud excised whole:

In the case of axillary bud no leaf scales were removed. Whole bud was transferred with part of stem tissue of mother shoot attached, while in apical bud the older leaves were removed with younger leaves enveloping the bud proper intact.

4. Divisions of whole buds:

The axillary buds exised as in 3 were divided into two before transplanted. The apical buds removed as in 3 were cross-divided into four and transplanted.

Medium was prepared according to the usual method. Details of it were described elsewhere in the series of this paper. Details on other operations such as transplanting, subculture, and other precausions were also described elsewhere.

RESULTS AND DISCUSSIONS

1. The explant consisting exclusively of apical meristem prepared as in the 1 of "Method and Meterial" turned into a protocorm or protocormic body ten days after culture. Whole part of the meristem including the apical dome and leaf primordia below the dome turned into a mass of protocormic tissue, leaving no part remaining unchanged. Later this protocormic tissue developed into a single globular protocorm or several protocorms. "Protocormic" or "Protocormic body", in this paper, meant the mass of modified irregular growth of the bud meristematic tissue. It was not the globular body of protocorm in the embryogeny of orchid, nor the globular protocorm-like body of meristem culture. This protocormic body or tissue was destined to be a single protocorm-like body or as in many cases continued to grow and later several outgrowth of protocorms arose from it.

For the observation of the protocorm development from the apical meristem, dissecting out the meristem with the bulk of stem part of mother shoot attached was preferrable, because this had much better chance of survival when transplanted than excising the thin slice of apical meristem only.

2 a. When the explants were prepared as indicated in 2 a of the "Method and Material" in a week the basal part of axillary bud and bulk of mother shoot stem turned gradually brown but the apical meristem, upper nodal and internodal parts of the axillary bud remained unchanged. Usually ten or fifteen days after culture the basal part turned deeper brown while the apical meristem, upper nodal parts, some of the remnants of upper leaf bases had activated, turned protocormic, and by growing rapidly emerged above the surronding mother stem tissue(Fig. 9). In most cases the emerging body appeared colorless, or transparent at first.

About 20 days after culture, the parts swelled and turned into clearly dinstinguishable. The apical meristem proper of dome and leaf primordia, remains of upper leaves, and upper nodal and internodal parts of the axillary bud became protocormic(Fig. 9). The parts turning brown were the extreme lower part of axillary bud, remains of most of leaves, and bulk of mother shoot stem tissues (Fig. 9).

About one month after initial culture the protocormic body or protocorms turned into green color with the basal brown part eventually dying away.

2 b. When the explant was prepared as indicated in the 2 b, that is, the material to be explanted was

in the initial stage of bud primordial development consisting only of dome of apical meristem and one or two leaf primordia dissected out with bulk of the axillary tissue of mother shoot attached, the stem axillary tissue quickly turned brown and by two weeks after culture completely died away.

The meristem with one or two leaf primordia, on the other hand, grew slowly to be protocormic and about ten days after culture the whitish semi-transparent protocormic body projected out of the dying axillary tissue of mother shoot(Figs. 10 a, 10 b). This protocormic tissue soon developed chloroplast and underwent usual developmental changes to be a single protocorm or several protocorms exactly in the same way as those described in 1 and 2 a.

2 c. When the materials were prepared as indicated in the 2 c, i.e., the apical meristem with its basal part and stem tissue of mother shoot were excised and transplanted, the apical meristem and most of the internodal or nodal parts became quickly, or in some cases slowly, protocormic as in other cases described above. But in certain cases the explant became green far earlier than those above mentioned. No emergence of apical meristem and no protocorm formation were observed in this case. The meristem remained unchanged inside the leaf remains of the explant for a long time, in some cases as long as about two months. Outwardly this explant seldom indicated any signs of change. Only change perceivable was the green color in the entire body of the explant. But inwardly it seemed that certain changed had been taken placed. The entire explant became swollen and by two months after culture it turned into a swollen irregular protocormic mass of considerable size. Further later with the leaves withering away and further protocormic growth in the internodal parts and slower growth in the nodal part, the whole bud turned to an irregular protocormic body looking much like a larva of certain Coleoptera insect.

The pattern of developmetal changes to protocorm or protocormic body from apical meristem described above were same in any of the material used although varietal differences exist in their rapidity in development, some varieties becoming easily protocormic while others were gradual in changes. The differences of protocorm formation among the four methods of explant preparation, i.e., excised apical meristems, apical meristem with basal part of axillary bud, axillary bud primordia, and apical meristem of apical bud with basal part attached, seemed to lie not in the way of protocorm develoment, but in the fact which tissuses become protocormic and which parts die away finally. In some cases it has been observed that at first the basal part as well as the apical meristem of apical or axillary bud became protocormic and swollen, but later with the vigorous growth of the protocormic tissue of apical meristem the basal protocormic tissues died away along with the non-protocormic tissue of the explant. It may, also, be assumed that explant's meristematic tissues form protocorm regardless of where they may be located---in dome, leaf primordia, internode, or in young leaf base; certain cells are more quickly activated while others are slower or remain dormant according to the locations where these cells or tissue originate. Further details will be clarified later by histogenetical observation.

3. When the apical buds were exised whole and transplanted some took green color immediately while others remained colorless. Usually the colorless bud by two weeks after culture would give rise to typical globular protocorms from various parts such as apecies or internodes of the bud. The easily green coloring buds would remain unchanged outwardly, for more than 2 weeks at times. But inside there were changes gradually taking place in every meristematic part as in 2 c.

Accoring to the observation about 20 days after the initial transplanting, the apical meristem, leaf

primordia, young leaf base, and internodal part of the bud turned protocormic and became swollen. The parts which did not undergo protocormic changes were the leaves and the nodal lines. After a month the leaves mostly turned brown and finally faded away, the whole irregularly swollen bud looking just like Coleoptera larva with alternate swelling in internodes and concaving at the nodal lines.

The colorless bud which did not take up green color at first but later turned as much green as the bud mentioned above took nearly the same course when observed a few months later, and turned into a large wrinkled mass of green protocormic body more irregular in surface due to the early emergence of several protocorms. From this mass later developed many globular protocorms. As for the description on the "Easily greening" or "Tardy coloring bud," further observation and confirmation are needed. It is true that certain apical buds easily gave rise to protocorms while others were slow in becoming protocormic. The slow protocormic bud tended to become more readily green. Whether these phenomena are due to varietal differences or environmental factors should be clarifies by further observation.

When the axillary buds were cultured as a whole, they immediately took green color and became swollen with the lapse of time. Usually no recognizable changes occurred outwardly except for the swelling inside. These condition continued in many buds for about 2 months(Fig. 11). In certain buds protocorms would pop out from various parts of the bud as observed in apical bud culture. In some other buds, apical and axillary alike, the protocorms popped out here and there rapidly covering the whole bud.

The changes occuring inside the bud were the same as in apical bud. Meristematic tissue of the apical meristem, leaf primordia, internodal part, and upper young leaf base underwent protocormic changes. Most of the leaves and nodal lines remained unchanges. As the leaves turned brown and finally withered away, the axillary bud became the large wrinkled protocormic mass(Fig. 12). The original stem tissue which had been dissected out along with the axillary bud turned brown soon after culture and died away by the time the bud started to swell. The upper young leaves near the apical meristem of axillary bud as in the case of apical bud turned protocormic.

Usually the axillary buds appeared to be slower in protocormic changes than the apical buds, taking longer time in swelling or giving rise to protocorm outgrowth. Some cases were observed where the excised bud gradually grew into usual shoot and later root. No protoocorm developed from such a bud.

4. When the apical and axillary buds were used for protocorm production, cross-dividing the apical bud into four or symmetrical division of axillary bud has been practiced by many worker. No result is available at present on the merits of the divided or non-divided whole bud explant, but it was observed that when the unchanging swollen axillary buds planted as a whole for more than a month were taken out, divided and replanted, the divided sections were accelerated to develop many protocorms.

When the buds were divided from the start, they formed protocorm or protocormic tissue taking exactly the same course of development as when they were cultured as a whole. The divided sections of bud often turned brown and gradually died, but after a few months tiny protocorms would emerge from the outwardly dead-looking explant. It seems that one or two cells which have happened to remain alive in the gradually dying bud explant were activated and developed into protocorm.

Protocorms whether they originated from bud explants or sliced protocorms, when left untransferred for months or put on such adverse conditions as dryness and malnutrition, gave rise to tremendous numbers of protocorm outgrowth (Figs. 13, 14).

摘 要

洋蘭 *Cymbidium* 의 數品種을 사용하여, 培養材料에서 어떻게 原塊體(實은 原塊體狀球體)가 形成되는가를 究明하였다. 原塊體形成過程을 細密히 追求하기 爲해서 培養材料를 다음과 같이 만들었다.

1. 生長點의 頂端分裂組織만을 摘出하여 培養
2. 頂端分裂組織과 Leaf Primordia 로 된 幼芽原基만을 培養
3. 腋芽의 生長點을 母體葉腋의 一部를 붙여서 摘出 培養
4. 腋芽 및 頂芽全部를 培養

原塊體는 培養材料의 分裂組織에서 形成된다. 分裂組織은 그것이 頂端分裂組織이든, 葉原基이든, 幼芽節間의 것이든 모두 原塊體를 形成할 수 있다.

腋芽나 頂芽가 原塊體를 發生時, 어떤 것은 빠르고, 어떤것은 늦으며, 또 어떤것은 多數發生하는 等 區區한데, 이것이 品種間의 差異만인지, 環境의 影響도 있는지는 今後 더 檢討해야 겠다.

原塊體發生過程의 詳細한것은 今後 組織發生學的調査에 依해 明白해질 것이다. 本 實驗에 使用한 培養基의 調製, 材料의 摘出, 無菌操作, 移植等은 常法에 依하였고 詳細는 本論文의 Series 에 旣히 報告하였다.

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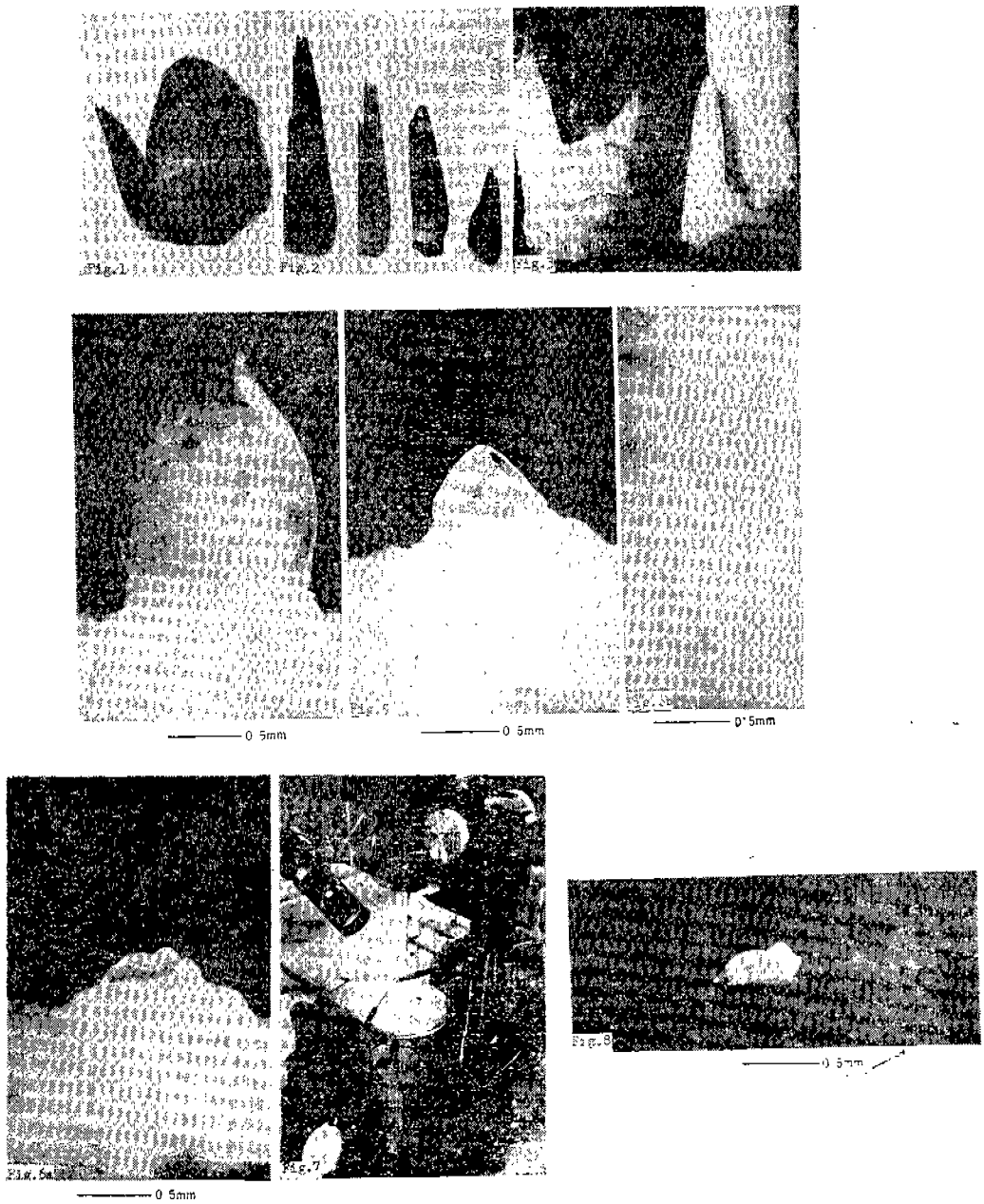
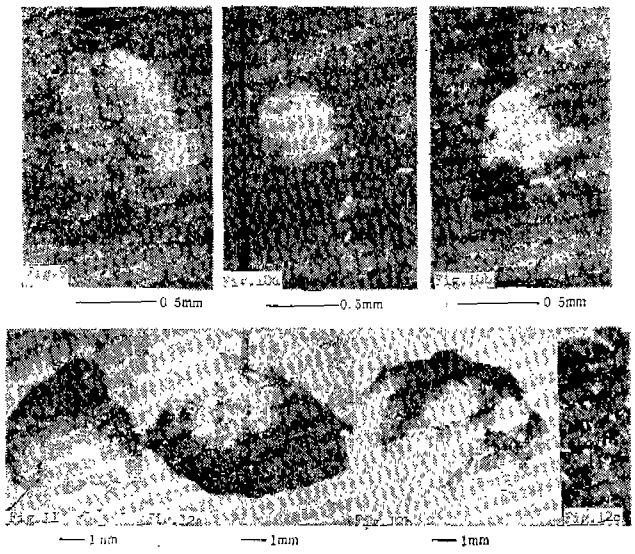
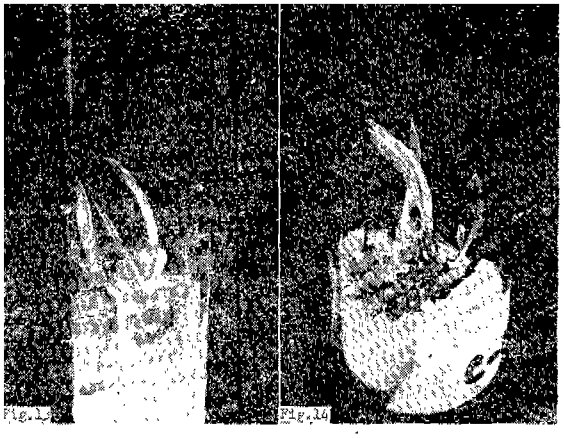
Explanation of figures



Figs. 1—8. Order of dissecting out the apical meristem:
Fig. 1, new shoot arising from pseudobulb; Fig. 2, shoots suitable for exising apical meristems; Fig. 3, peeling off the scale leaves, axillary buds are exposed. These are also good materials for obtaining apical meristems; Fig. 4, leaves of one of the axillary buds in Fig. 3 are removed; Fig. 5, further removal of leaves from Fig. 4; Fig. 6, white-glowing cone of apical meristem is exposed; Fig. 7, operations are done aseptically under dissecting-microscope; Fig. 8, dissected-out apical meristem prior to transplant.



Figs. 9—12. protocormic tissue development from explanted apical meristems and buds:
Fig. 9, apical meristem of axillary bud changing to protocormic body. Below the protocormic body is the browning basal part of the axillary bud; Fig. 10, bud primordia turning to protocormic body. Axil of mother stem quickly degenerates; Fig. 11, green colored axillary bud becomes swollen without changing much surface appearance for months; Fig. 12, whole bud modified into wrinkled protocormic masses.



Figs. 13, 14, proliferaton of protocorms and plantlets from a single meristem(Fig. 13) or from a single sliced-protocorm (Fig. 14). Fig. 13, variety, C-8; Fig. 14, variety, C-4.