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Origin and Development of Single- and Poly-embryos formed Directly on Excised Cotyledons of Ginseng Zygotic Embryos

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Abstract : Excised cotyledon segments of ginseng zygotic embryos cultured on MS basal medium without growth regulators produced somatic embryos near the basal excised portion at a high frequency. The frequency of somatic embryo formation on the segments declined along with advancing zygotic embryo maturity. In immature cotyledons, all the cells of the epidermis and subepidermis were smaller and more densely cytoplasmic than those in mature cotyledons, and from which multiple cells participated in embryogenic division to form somatic embryos with multiple cotyledons and fasciated radicles (poly-embryos). But in germinating cotyledons, only the epidermal cells were densely cytoplasmic and singularly competent to develop into somatic embryos resulting in single-embryos with closed radicles. This result means that the origin and development of somatic embryos is determined according to whether the cells participating in embryonic division are in a single state or a massive state relative to cotyledon maturity.

Key words : Origin of somatic embryo, Cotyledon segment, Maturity of explant, *Panax ginseng*.

Introduction

Somatic embryogenesis can occur directly from cultured explants without an intervening callus formation.[1,2,3] Such direct embryos from explants form due to a predetermined embryogenic competency of the explants.[4,5] Somatic embryos originate from a single cell[1,3] from multiple cells,[3] or from both types of cells on the same explants.[4,6] And, somatic embryos can develop into single-embryos[1,3] or into poly-embryos.[2] The factors producing the above diverse origins and development of somatic embryos has not been yet clearly elucidated.

In general an exogenous growth regulator, usually 2,4-D, on the culture medium is prerequisite to induce somatic embryos in a tissue culture system.[7] On the other hand, somatic embryos can form on medium without any supplementary growth regulators.[8,9] In *Panax ginseng* also, we observed that the excised cotyledon segments directly produce polar developing somatic embryos near the basal excised portions without an intervening callus formation on medium without any growth regulator addition.[9,10,11] The direct somatic embryogenesis from ginseng cotyledons occurred under the following circumstances: the embryo axis contains a substance suppressing somatic embryo development, thus detachment of the cotyledon from the embryo axis is required for somatic embryogenesis[11] and the polar somatic embryogenesis from cotyledon segments was induced by the coorporating action of both wound response and tissue polarity of explants.[10] This system is very convenient for clarifying the origin and development of somatic embryos anatomically since nonembryogenic division did not occur.

This experiment was carried out to study the origin and developmental patterns of poly- and single-embryogenesis by culturing ginseng cotyledons at different stages of maturity.

Materials and Methods

Freshly harvested Korean ginseng (*Panax ginseng* C.A. Meyer) seeds with globular stage embryos were stratified in humidified sand for further maturation. Dur-

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ing stratification the zygotic embryos differentiated into three states for culturing explants: immature (3 mm in length); fully mature (5 mm in length); and germinating stage (7 to 30 mm in length).

The seeds were immersed in 70% ethanol for 1 minute and then sterilized in 1% sodium hypochlorite solution for 1 hour and washed three times with distilled water. After carefully dissecting the zygotic embryos from the seeds, the abaxial side of the excised cotyledons were placed on the medium surface. The culture medium was composed of MS basal salts containing 5% sucrose, 0.7% agar and adjusted to pH 5.8 before autoclaving at 120°C for 15 minutes. The cultures were performed using 10×1 cm glass Petridishes containing 30 m*l* of medium. The culture room was maintained at 24±2°C under 16 : 8 hour photoperiods with 1900 lux cool white fluorescent tubes. The production rate of somatic embryos was evaluated by counting cotyledon explants showing the somatic embryos from total cultured cotyledon explants. About thirty explants were cultured in each experiment which was repeated three times.

For anatomical examination of somatic embryogenesis, cotyledon explants from each stage of somatic embryo development were fixed in FAA (formalin, acetic acid and alcohol) and dehydrated in ethyl alcohol and then embedded in paraffin. After the samples were cut into 10 μm size, they were stained with hematoxylin. For observing by scanning electron microscope, the samples were fixed in 1% glutaraldehyde, dehydrated with ethyl alcohol and dried in a critical point drier. After being coated with gold, the samples were observed by scanning electron microscope (JSM T330A).

Results

The majority of somatic embryos developed directly

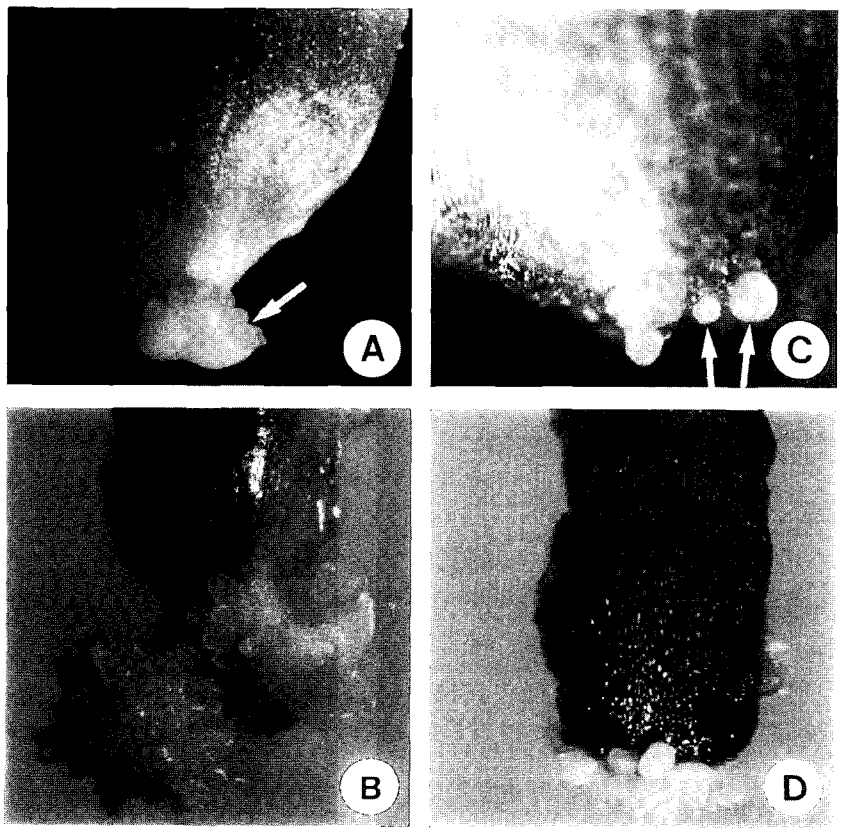

Fig. 1. Poly- and single-embryos formed from the cotyledon of zygotic embryos at different stages. A,B, poly-embryos (arrows) formed from cotyledons of a immature zygotic embryo of 3 mm in length. C,D, single-embryos (arrow) formed from cotyledons of a germinating zygotic embryo of 7 mm in length.

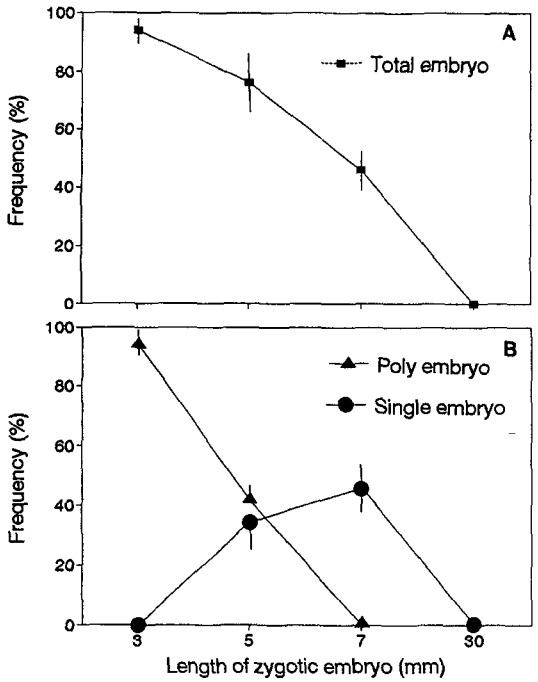

Fig. 2. Forming frequency of total embryos(A), poly- and single-embryos(B) from culture cotyledons of zygotic embryos at various mature stages.

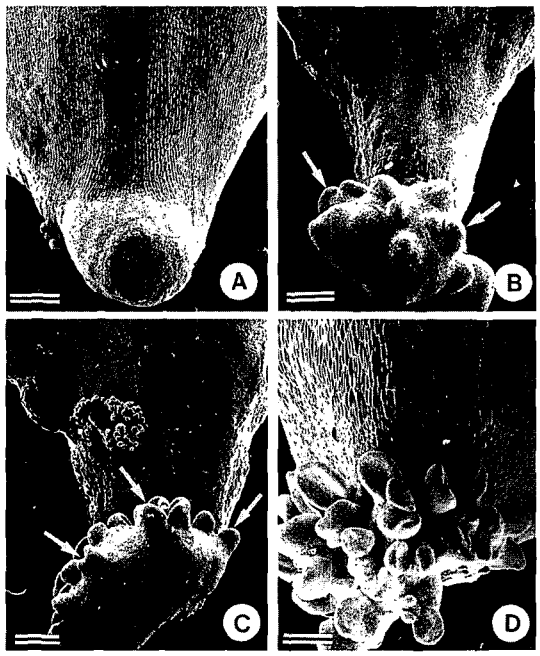

Fig. 3. Scanning electron microscopy of poly-embryogenesis from cotyledons of immature zygotic embryos. A, active cell division occurs from broad area of cotyledon base after 1 week of culture (Bar; 183 μm). B, hemispherical nodular tissue (arrows) formed after 2 weeks of culture (Bar; 232 μm). C, numerous cotyledon primodia (arrows) formed directly from on the surface of nodular tissue after 3 weeks of culture (Bar; 232 μm). D, Poly-embryos fused with each other formed after 4 weeks culture (Bar; 238 μm).

from the basal portions of cotyledons without an intervening callus formation when cultured on MS basal medium without growth regulators (Fig. 1). The highest frequency of somatic total embryos formation was observed in the cotyledons of immature zygotic embryos (Fig. 2A). Then the embryogenic potential decreased rapidly as the zygotic embryos matured and germinated (Fig. 2B). No somatic embryos formed in the cotyledons of seedlings grown to 30 mm in length (Fig. 2B). Two types of somatic embryos: poly- and single-embryos formed at different rates according to the degree of maturity of the explants (Fig. 2). In the culture of immature cotyledons, most of the somatic embryos developed into poly states fused to each other (Fig. 1A, B). While, somatic embryos were developed in single state as the cotyledons of the zygotic embryo source materials matured (Fig. 1C, D). In cotyledons of germinating embryos, only single-embryos were formed. The cotyledons of immature zygotic embryos were yellow green in color after the somatic embryos matured (Fig. 1A). But in the cotyledons of germinating ones, red pigment accumulated on the cotyledon surface within one week of culture and became dark brown after the somatic embryos matured (Fig. 1C).

Somatic embryogenesis from cotyledon explants was observed by scanning electron microscope. In immature cotyledons (Fig. 3), somatic embryo development was initiated from a broad area of the cotyledon base, which formed hemispherical nodules after about 7 days of culture (Fig. 3A). Numerous cotyledon primordia developed from this nodular tissue after 12 days (Fig. 3B) and eventually formed poly-embryos after 20 days of culture (Fig. 3D). The hypocotyl and radicle regions of the poly-embryos were fused to each other and to the parent cotyledon explants (Fig. 3C, D). In cotyledons of germinating zygotic embryos (Fig. 4), somatic embryos originated from single epidermal cells (Fig. 4A, arrows). Globular (Fig. 4B), heart-shaped (Fig. 4C, arrows) and torpedo stage (Fig. 4D) somatic embryos formed after 21, 28 and 35 days of culture, respectively.

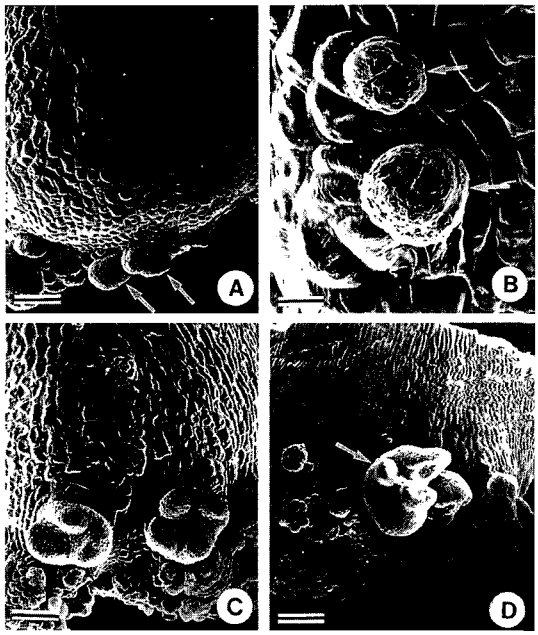

Fig. 4. Scanning electron microscopy of single-embryo formation from cotledons of germination zygotic embryos. A; globular somatic embryos (arrows) formed from surface of cotyledon base after 3 weeks of culture (Bar; 153 μm), B;somatic embryos (arrows) developed from single cell of cotyledon epidermis (Bar; 59 μm). C; heart-shaped somatic embryos embryos after 4 weeks of culture (Bar; 153 μm). D; torpedo stage somatic (arrow) embryos after 5 weeks of culture (Bar; 238 μm).

Histological observations revealed that in immature cotyledons, the cells of both the epidermis and subepidermis were densely cytoplasmic and isodimetrical in size (Fig. 5A-B). Cell division occurred synchronously from both the epidermal and subepidermal layers of the cotyledon base (Fig. 5C), then formed embryogenic nodules of hemispherical shape (Fig. 5D). Numerous cotyledon primordia developed from the nodules (Fig. 5E), which eventually developed into poly-somatic embryos fused to each other (Fig. 5F). The procambial strands (Fig. 5C-D) of poly-embryos revealed that the nodules were not embryogenic callus but an intermediate stage of poly somatic embryogenesis. When the nodules were not large in size, single-embryos with radicles fused to the explants formed at a low frequency (Fig. 5G-I). In germinating cotyledons, only some epidermal cells (arrows) of the cotyledon base were small and densely cytoplasmic (Fig. 6A-B). These single cells divided periclinally and anticlinally (Fig. 6C) and formed early globular somatic embryos (Fig. 6D). Subsequently, somatic embryos developed to the globular (Fig. 6E) and heart-shaped stages (Fig. 6F).

Discussion

The potential of cotyledon explants to produce somatic embryos decreased as the cotyledon explants of zygotic embryos matured and germinated. Similar results were reported in zygotic embryos of *Zey may, Theobroma cacao, Glycine max* and *Ouercus rubra,* in which the explants of immature zygotic embryos showed the highest production of somatic embryos and the production capacity was reduced as the zygotic embryos matured.[4,12] In the present experiment, the embryogenic potential of cultured explants was closely related to the cellular differentiation of the cotyledon explants since somatic embryogenesis occurred primarily from small and densely cytoplasmic undifferentiated cells. The high frequency somatic embryo formation in immature cotyledons might be the result of the large quantity of predetermined embryogenic cells within the cotyledon tissues. However, somatic embryogenesis from cotyledon segments of ginseng occurred only near the excised portion situated the cotyledon base.

From histological observation, the origin of somatic embryos was closely related to the cellular differentiation of explants. In immature cotyledons, both the epidermis and subepidermis of immature cotyledons were composed of small, isodimetrical cells, and somatic embryogenesis occurred from multiple cells of both the epidermis and subepidermis. But in cotyledons in a germinating state, only certain single cells of the epidermis were small and densely cytoplasmic and somatic embryos originated from single epidermal cells. The result indicates that cellular origin of somatic embryos may be affected by whether the cells having embryogenic competency were in single state or massive state secondary to the maturity of the cotyledon explants. The present result is an experimental proof of Williams and Maheswarans postulation[13] in which they suspected that the factors influencing the origin of somatic embryos corresponded to the coordinated behavior of cells participating in embryonic development.

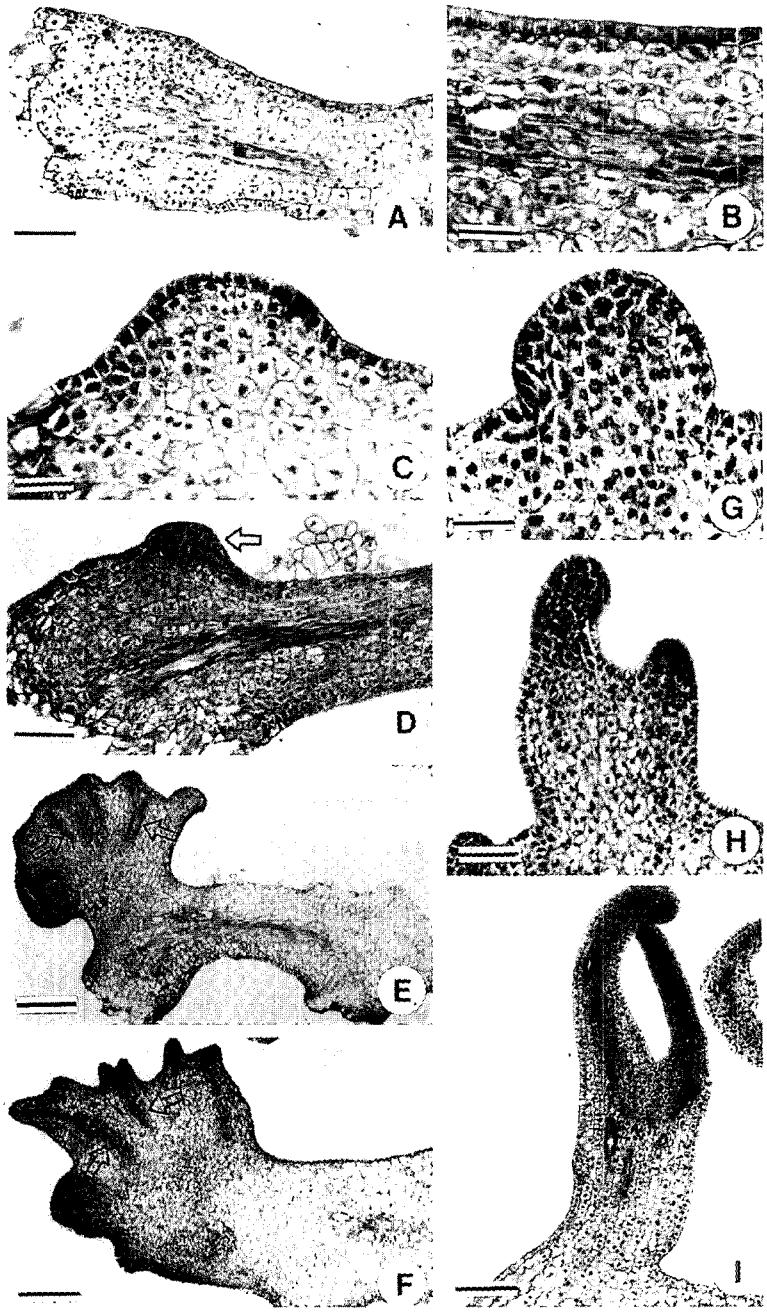

Fig. 5. Histological observation of somatic embryogenesis from immature cotyledons. A-B; median longitudinal section of a immature cotyledons showed both epidermal and subepidermal cells are isodimetric in size. (Bar; 230 μm in A, Bar; 82 in B). C; somatic embryo development from division of multiple cells of both epidermis and subepidermis (Bar; 82 μm), D: hemispherical structure formed on the surface of cotyledon (Bar; 230 μm), E: several cotyledon primodia developed from a nodulus structure (arrows indicate procambial strands, Bar; 430 μm), F: poly-embryos at cotyledonary stage (arrows indicate procambial strands, Bar; 430 μm), G-I: single-embryos from hemispherical nodular structure but showing fused radicle to cotyledon explants (G) which were formed from multiple cells of epidermis and subepidermis (Bars; 82 μm in G, 230 in H, 430 μm in I).

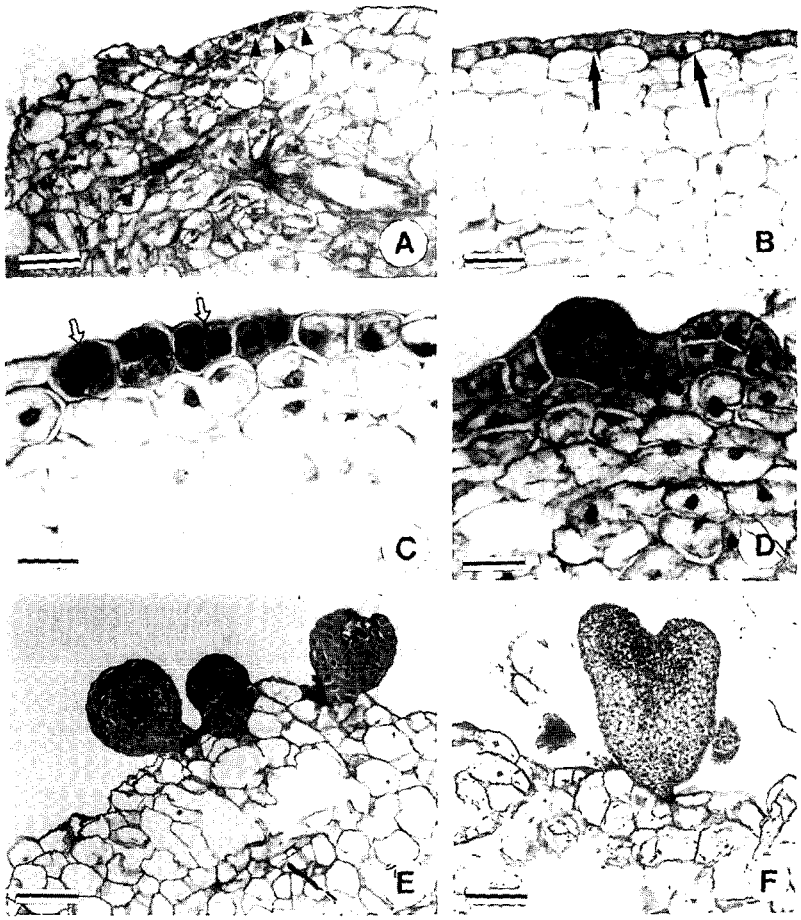

Fig. 6. Histological observation of somatic embryogenesis from germinating cotyledons. A; median longitudinal section of a cotyledon of germinating embryos showed only some epidermal cells (arrows) were small and densely cytoplasmic (Bar; 82 μm). B, magnified view of cotyledon tissue showed cortical cells were highly vacuolated (Bar; 73 μm). C; early embryogenic cell division from single cells of epidermis (Bar; 73 μm), D; preglobular embryos formed from consecutive division of epidermal single cells (Bar; 73 μm), E; globular stage embryos (Bar; 82 μm), E; a heart stage embryo (arrow) (Bar; 82 μm).

Somatic embryos can develop into a single state with a closed radicle[1,3] or into a poly state fused to each other and fused to cultured explants.[2] In the present experiment, poly or single somatic embryogenesis was determined by the cellular origin as poly-embryos always originated from from multiple cells but single-embryos were derived mainly from independent single cells. There is some evidence, especially in direct somatic embryogenesis, that undifferentiated tissue such as immature zygotic embryos of *Ilex* and *Trifolium* produces poly-embryos[2] and differentiated tissue such as seedling *Ranunculus* produces single embryos.[1] In the embryo culture of *Acanthopanax senticosus*, poly somatic embryos formed from the cotyledonary node in which active cell division occurred but single somatic embryos formed from rapidly elongating radicles.[6]

In the formation of single-embryos, the developmental pattern was similar to zygotic embryogenesis. But in poly-embryogenesis, the origin and development was similar to adventitious buds in the point of multicellular origin. But there are some differences between poly-embryos and adventitious buds as poly-embryos have obvious cotyledons after maturity and the procambial strands of them are not interconnected to the vascular strands of the cotyledon explants. The hemispherical nodular tissues during the early stage of poly-embryo-

genesis in the present experiment may raise the question whether the nodular tissue is merely embryogenic callus. But the nodular tissue was an intermediate stage of somatic embryo development because the nodular tissue directly became poly-embryos. If the nodular tissue were merely embryogenic callus, somatic embryos would have formed from the surface of the tissue and the procambial strands would not have resided within the nodular tissue.

There is some discrepancy between poly-embryogeny in seeds and poly-embryogenesis in the present experiment. Cleavage polyembryogenesis in seeds occurs spontaneously or can be induced by exogenous growth regulators, acid treatment or X-ray treatment[14], in which cases, cleavage polyembryogeny indicates the formation of multiple-embryos without respect of cellular origin. However, in the present tissue culture, poly somatic embryogenesis occurred owing to coordinated embryogenic division of multiple cells of cotyledons. Therefore, it is not certain whether the poly somatic embryogenesis in the present experiment is a variation of cleavage polyembryogeny or merely an occurrence of multicellular origin.

요 약

인삼 접합자배의 자엽을 식물호르몬이 전혀 첨가되지 않은 MS기본배지에 배양한 결과 자엽의 기부에서 높은 빈도의 체세포배가 유기되었다. 체세포배의 형성 빈도는 접합자배 자엽의 성숙도에 따라서 차이가 있었는데 미숙배에서 성숙배로 진행됨에 따라 감소되는 경향을 보였다. 미숙자엽의 경우에는 표피세포의 윗 아래층들이 모두 성숙자엽의 세포보다 더 적거나 더 촘촘하였으며, 많은 세포들이 체세포배의 형성에 관여하였으나 뿌리의 형성이 어려운 다배상태로 유기되었다. 그러나 발아직적의 성숙자엽은 표피세포의 윗층만이 촘촘한 세포로 이루워 졌으며 뿌리의 형성이 가능한 체세포 단일배로 유기되었다. 이런 결과는 체세포배의 기원과 발육이 배발생시 사용한 자엽의 성숙도에 따라서 배발생에 관여한 세포들이 단일 혹은 다량상태인지에 따라서 결정된다는 것을 의미한다.

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