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Effect of exogeneous plant growth regulators on morphogenetic response *in vitro* by embryo and leaf cultures of *Camellia sinensis* (L.) O. Kuntze

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차나무 잎과 배 배양에 있어서 식물 생장조절물질이 형태형성에 미치는 영향

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Morphogenetic responses were investigated by culturing embryo and leaf explants of Korean wild type tea plant, *Camellia sinensis* (L.) O. Kuntze. Induction of direct somatic embryogenesis as well as adventitious and/or axillary shoots was obtained from mature zygotic embryo cultures on Murashige and Skoog (MS) basal medium having 5 to 20 μM cytokinin a lone. Morphogenetic response was decreased dramatically by the addition of auxins tested. One hundred percent of induced and isolated shoots formed roots after four weeks of culture on half-strength MS or quarter-strength Schenk and Hildebrandt (SH) media supplemented with 10 μM indole-3-butyric acid (IBA). Immature zygotic embryos were shown to be a suitable explant for embryogenic callus formation in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) in basal medium. Mature zygotic embryo originated leaves were used to test their ability for mophogenesis by incorporating plant growth regulators such as IBA, naphthyl-1-acetic acid (NAA), and 6-benzylaminopurine (BAP). Apparently, the morphogenetic responses of the cultured explant sources on the types and/or levels of plant growth regulators tested were observed visually.

Key words: *Camellia sinensis*, embryo culture, leaf culture, endogenous phytohormones, somatic embryogenesis, organogenesis.

In breeding and cultivation of tea plant (*Camellia* spp.), much attention should be given to original wild type populations retaining original gene pool. It seems to be reasonable as an initial point of the creation of new varieties having particular useful traits. Another aspect of tea plant breeding represents a significant impetus both for conservation and for multiplication of tea plant germplasm through cell and tissue culture system. Several studies on high degree of responsiveness of *Camellia* spp. *in vitro* were demonstrated (Kato, 1986, 1989: Nakamura, 1988: Pedroso and Pais, 1993). Comparison of those results suggested that there are some relationships between the media compositions and the species tested. In addition, distinctive responses related to morphogenesis within species and /or within varieties were also introduced. These observations partly represent the possible reasons on significant genetic distance among Japanese, Korean and Chinese tea plants (Yamaguchi et al., 1996).

In this paper the factors controlling morphogenetic responses of cultures of Korean wild type of *Camellia sinensis* were investigated to meet final goals for tea plant improvement programs as well as mass propagation of tea species.

MATERIALS AND METHODS

Plant material

Seeds of Korean wild type *Camellia sinensis* (L.) O. Kuntze were collected from the bushes growing in the seed orchard of Tea Experimental Station (Posong, Korea) in the August, 1993 (immature seeds), and during the autumn, 1995 (mature seeds). Seeds used for embryo culture were stored on refrigerator at 4°C for less than ten months.

After removing seed coat endosperm containing zygotic embryo was surface-sterilized consecutively with 70% (v/v) ethanol for 1 min and 3% (v/v) sodium hypochlorite for 20 min. Each sterilization step was followed by five rinses in sterile distilled water. Intact zygotic embryos were dissected out aseptically from the surrounding endosperm and placed onto solidified nutrient medium.

Shoot cultures derived from cultured mature zygotic embryo were used as explant sources for leave culture.

Zygotic embryo culture

Basal medium for culturing mature and immature zygotic embryos was MS (Murashige and Skoog, 1962). Effect of growth regulators on morphogenesis from mature zygotic embryos was assessed in two series of experiments. At first, BAP at 5, 10 and 20 μM were combined with either IBA or NAA, both at 2.5, 5 and 10 μM. In the second series of experiments, cytokinins BAP and 2iP ranging from 5 to 20 μM were incorporated to compare their capacities related to induce and promote morphogenesis in the absence of exogenous auxin supply. Both series of experiments were conducted under dark or light condition (16 h day photoperiod: 20 $\mu E \cdot m^{-2} \cdot s^{-1}$) provided by cool fluorescent lamps (General Electric, FL 400). Thirty zygotic embryos were tested in each experiment with more than two replication.

Induced shoot cultures were multiplicated using 2/3 MS basal medium supplemented with 5 μM BAP. Shoot cultures were maintained under 16 h day photoperiod and subcultured onto the fresh medium at every four-week interval.

To induce roots from individual shoot cultures half-strength MS and quarter-strength SH (Schenk and Hildebrandt, 1972) media were associated. Both media were supplemented with 5 or 10 μM IBA. Shoot cultures were incubated for one week in the dark and then transferred to the light conditions. Each rooting treatment included 40 to 60 shoots.

To initiate embryogenic callus, immature zygotic embryos were cultured onto MS medium supplemented with 2,4-D ranging from 0.5 to 25 μM. After four weeks of culture under dark, the calli were transferred to the light condition. For the development of somatic embryos, plant growth regulator-free medium was associated. Thirty zygotic embryos were cultured in each with more than two replications.

All media were supplemented with 1 mM L-glutamine, 2.5% (w/v) sucrose and 0.3% (w/v) Phytagel. The pH of the media was adjusted to 5.8 prior to autoclaving for 20 min at 150 kPa. Aqueous stock solution of L-glutamine was filter-sterilized and added to sterilized medium.

All the chemicals used for culture medium preparation were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Temperature of culture incubation was automatically adjusted to 25 ± 1°C.

Zygotic embryos and callus tissues were cultured onto Petri dishes (diameter in 90 mm), whereas maintenance of shoot cultures for elongation, multiplication or rooting were performed in 200 mL baby jars with opaque plastic closure (light intensity reduced to 30%).

Leaf culture

Entire leaves were immersed into liquid solution containing plant growth regulator for 20 min under slight vacuum condition. To eliminate deteriorated effects possibly caused by sedimentation under the solution pH was adjusted to 5.8. Three induction treatments were tested, mainly with NAA (3 mM), IBA (3 mM), and BAP (3 mM). After the treatments, leaves were placed with their abaxial surfaces down on growth regulator-free 2/3MS medium and incubated in darkness for two weeks. The cultures were then transferred to 16 h day photoperiod conditions. After six weeks of culture, the leaves were placed onto BAP-containing 2/3 MS medium of the same composition used for the maintenance of shoot cultures. Five leaf cultures on plastic Petri dishes with 20 replications were distributed to each treatments.

Monitoring of cultures

In experiments with mature zygotic embryo cultures, continuous and/or discontinuous developmental responses such as zygotic embryo germination, callus formation, and development of somatic embryos, adventitious and axillary shoots were documented. Cultures were monitored under binocular microscope after six weeks from starting experiments

and thereafter. The rates of all the developmental responses were expressed as percent of zygotic embryos possessing positive responses. Parameter denoted as "the total morphogenetic activity" was used in this study by representing the percent of morphogenetically active zygotic embryos which reveal at least one of the three morphogenetic responses (i.e., development of somatic embryos, adventitious, or axillary shoots).

Root formation from individual adventitious or axillary shoots was assessed after four weeks of culture on rooting medium.

With immature zygotic embryos the number of globular staged somatic embryos per 1 g fresh weight of callus was determined after seven weeks of culture and thereafter consecutively with 5-day intervals. The frequency of secondary somatic embryo formation was determined after ten weeks of culture as a percent of calli giving secondary embryos.

Four types of responses of cultured leaves on different treatments were recorded.

RESULTS

Developmental responses in mature zygotic embryo culture

Wide spectrum of developmental responses of mature zygotic embryos were recognizable after six weeks of culture. All the embryos cultured on BAP-supplemented MS medium germinated slightly and produced primary radicle. This process was partly or completely suppressed by the addition of either IBA or NAA (Fig. 1). Sometimes formation of adventitious roots from the hypocotyl, accompanied by rupturing epidermal layer, was observed (Fig. 2A).

Contrary to germination, callus formation was significantly promoted by exogenous auxins tested. NAA gave higher rates of callusogenesis than IBA (Fig. 1).

Three types of morphogenetic processes were recorded: direct somatic embryogenesis (Figs. 2B, C), adventitious shoot formation (Fig. 2D), and axillary shoot induction (Fig. 2E). All these processes initiated directly from enlarged cotyledon and hypocotyl tissues, and were not preceded by unorganized callus growth. More than one type of morphogenetic processes were occurred simultaneously from the same zygotic embryos.

Morphogenetic activity decreased dramatically by the addition of auxin. NAA completely suppressed morphogenesis when applied at 2.5 μM, while the inhibitory concentration of

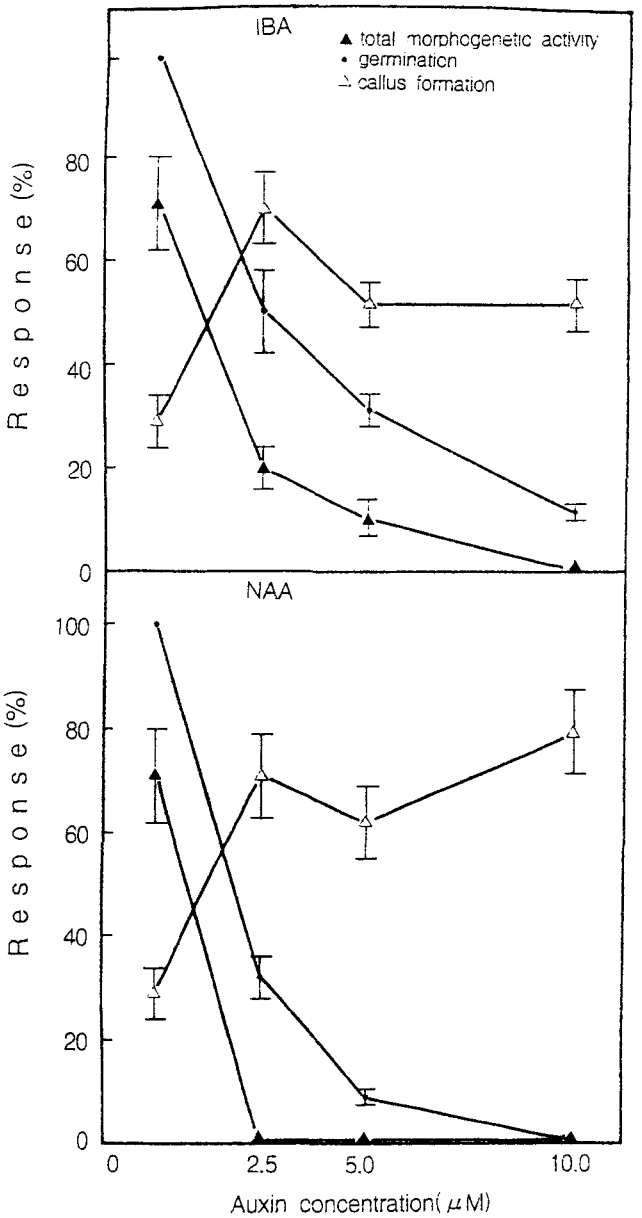

Figure 1. Effect of exogenous auxins on developmental response of mature zygotic embeyos of *Camellia sinensis* cultured in the presence of 10 μM BAP under dark condition.

IBA was about ten times higher (Fig. 1) than just mentioned above level.

Effect of cytokinin as a sole growth regulator on morphogenesis depended on its concentration and type (BAP *versus* 2iP) as well as on the light regimen of incubation (16 h day photoperiod *versus* dark). On the whole, BAP was more effective than 2iP in inducing and promoting morphogenesis especially in direct somatic embryogenesis (Fig. 3). Dark cultivation significantly inhibited adventitious and/or axillary shoot formation, however, no pronounced effect on

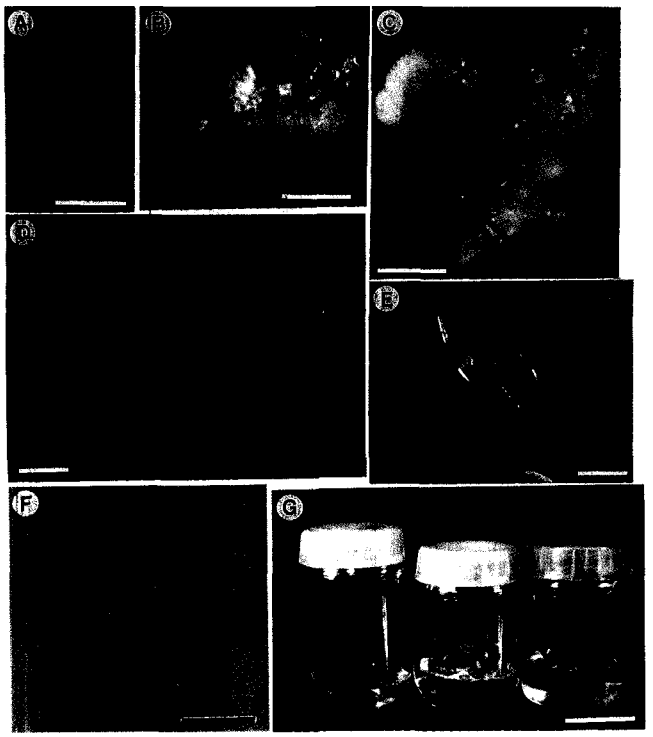

Figure 2. *In vitro* developmental responses of mature zygotic embryos of *Camellia sisnensis*.
(A) Formation of adventitious shoots (arrowed) from hypocotyl. Bar = 2 mm
(B) Globular staged somatic embryos. Bar = 1 mm
(C) Direct somatic embryos at advanced development stages. Bar = 2 mm
(D) Numerous adventitious shoots. Bar = 2 mm
(E) Axillary shoot (as) formation. Bar = 2 mm
(F) Established shoot cultures. Bar = 4 mm
(G) Rooting of individual shoots. Bar = 4 cm

somatic embryogenesis in comparison with 16 h day photoperiod (Fig. 3) was observed.

Actively growing shoot cultures were established after six weeks of culture and maintained routinely for more than six months without reduction in their capacity related to multiplication (Fig. 2F). The dominant morphogenetic process in long-term shoot cultures of *C. sinensis* was axillary shoot formation.

One hundred percent of isolated shoots formed roots after four weeks of culture on 1/2 MS or 1/4 SH media supplemented with 10 μM IBA (Fig. 2G). However, subsequent root growth was better on 1/4 SH compared with 1/2 MS media.

Complete cycle of propagation from initial embyros culture to intact plant regeneration of *C. sinensis* takes three months.

Somatic embryogenesis in immature zygotic embryo culture

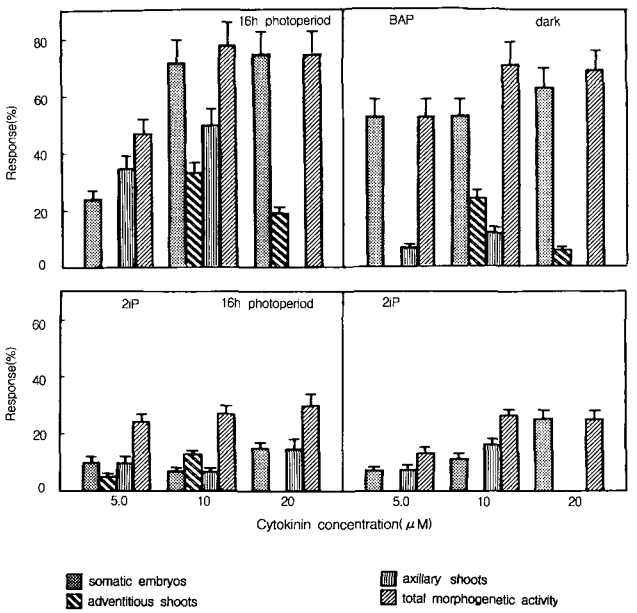

Figure 3. Interaction between types of cytokinins and light illumination on morphogenesis from the cultured mature zygotic embryos of *Camellia sinensis*.

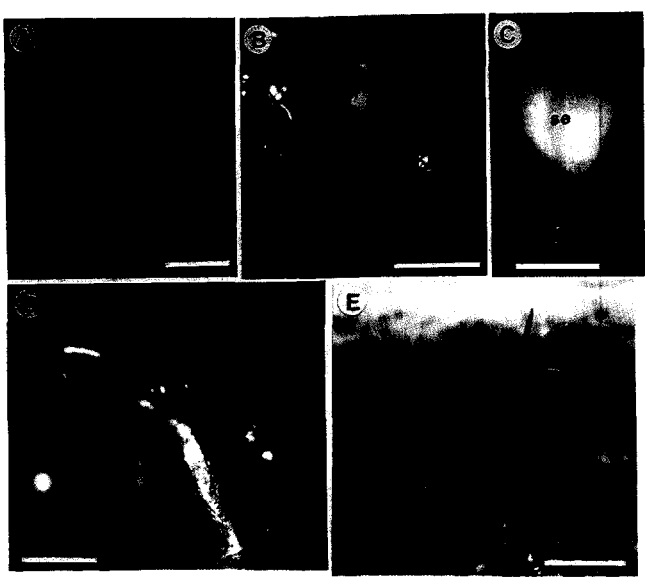

Figure 4. Indirect somatic embryogenesis from immature zygotic embryos of *Camellia Sinensis*.
(A) Embryogenic callus. Bar = 2 mm
(B) A heart-shaped somatic embryo (se). Bar = 1mm
(C) A torpedo-shape somatic embryo (se). Bar = 1mm
(D) A germinating somatic embryo. Bar = 2 mm
(E) Somatic embyro-originated plants after *ex vitro* transfer. Bar = 2 cm

Callus obtained from culture medium containing 2,4-D was compact, globular, and white to yellowish in colour (Fig. 4A). Globular staged somatic embryos were visible by naked eye

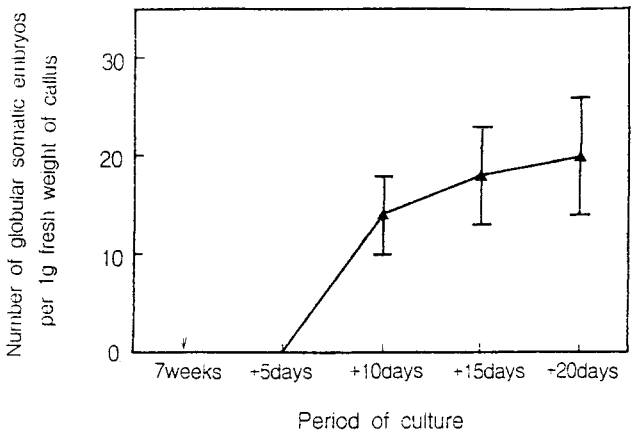

Figure 5. The dynamics of somatic embryogenesis in callus cultures derived from immature zygotic embryos of *Camellia sinensis*.

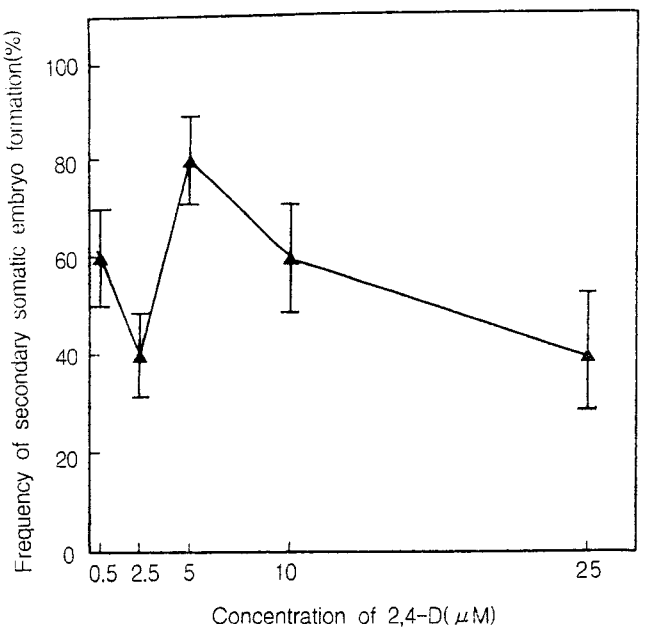

Figure 6. The influence of 2,4-D in the medium on secondary somatic embryo formation in callus cultures of *Camellia sinensis*.

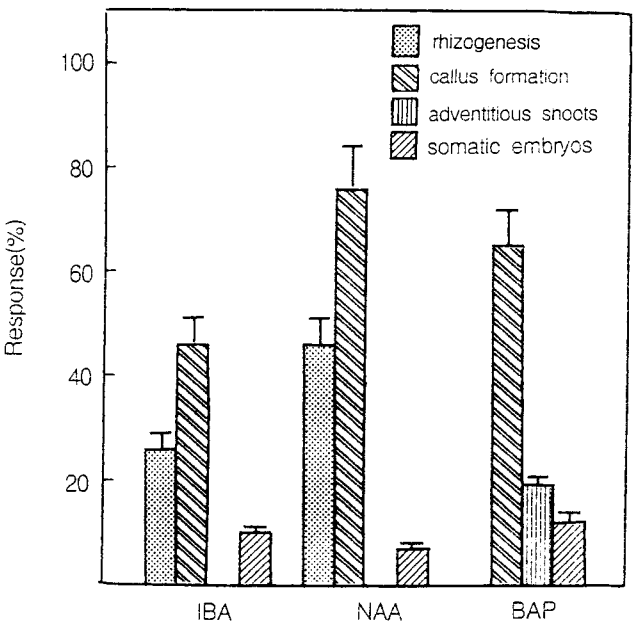

Figure 7. Effect of growth regulators in induction on developmental reponses in leaf culture of *Camellia sinensis*.

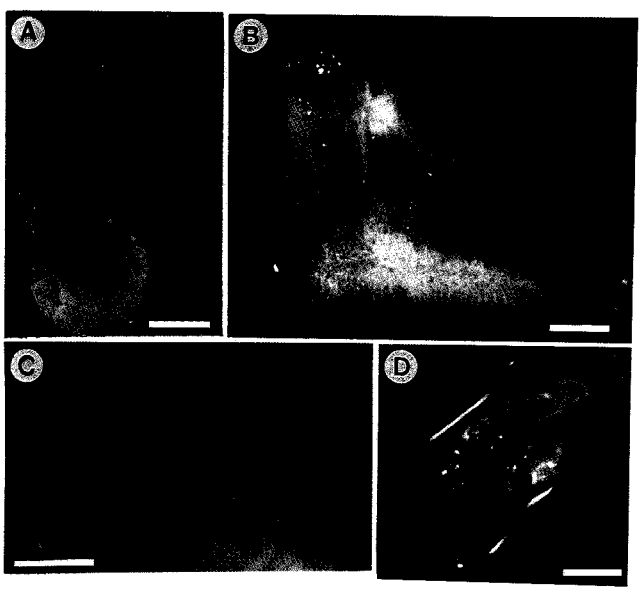

Figure 8. *In vitro* developmental responses of the leaf cultures of *Camellia sisnensis*.
(A) Callus formation from base and tip position of leaf. Bar = 2 mm
(B) Rhizogenesis from base and tip position of a leaf. Bar = 2 mm
(C) Globular staged somatic embryos formed (arrowed) from the leaf margins. Bar = 1 mm
(D) Adventitious shoot formation from a leaf midrib Bar = 2mm

and the rate of that staged embryos after eight weeks of culture (i.e., four weeks after transfer of calli from induction to growth regulator-free medium) was relatively high. Maximum number of embryos attained at ten weeks of the culture (Fig. 5). In the same time, development of secondary somatic embryos occurred from the tissues of primary embryos (Fig. 6).

The development of somatic embryo was true-to-type and resemble to most of the main steps observed in angiosperm zygotic embryoenesis (Fig. 4B, C). After germination (Fig. 4D), plantlets derived from somatic embryos were transferred *ex vitro* for further adaptation to non-sterile conditions (Fig. 4E).

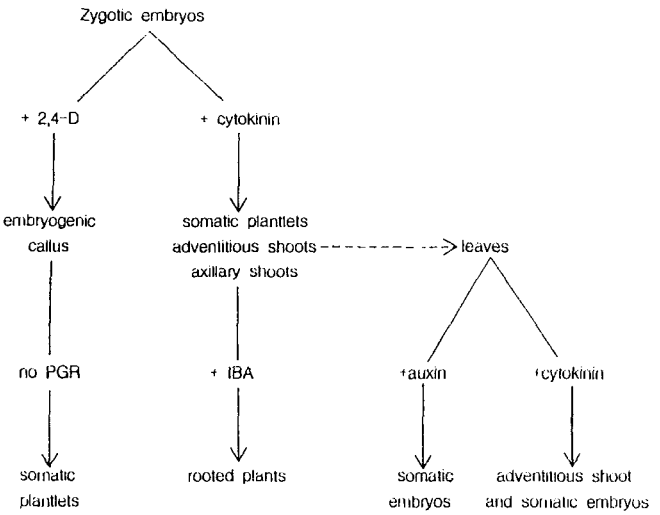

Figure 9. Possible morphogenetic processes in embryo and leaf culture of *Camellia sisnensis*.

Developmental responses in leaf culture

Both the rate and the type of *in vitro* response of isolated leaves were determined by the type of growth regulator applied in induction treatment. Callus formation and direct somatic embryogenesis occurred after all treatments, whereas rhizogenesis and adventitious shoot formation were specifically dependable for the plant growth regulators tested (Fig. 7).

Different leaf regions revealed different patterns of competence. The results partially suggested that *in vitro* responses were restricted to specific regions of the leaf cultured. Callus and roots were preferentially formed on the basis and on the tip of the leaf (Figs. 8A, B), whereas direct embryo formation occurred only in the marginal leaf regions (Fig. 8C). Only the midrib of the leaf had a competence to form adventitious shoots (Fig. 8D).

DISCUSSION

These experiments have shown a high degree of morphogenetic plasticity of embryo and leaf cultures of *C. sinensis*. These observation remind us that considerable promise for the development of micropropagation systems at the aim for cloning elite individuals of *C. sinensis* is possible. In addition, cell and tissue culture of *C. sinensis* might be ideal system for the researches on experimental morphogenesis of plants (see also Pedroso and Pais, 1995).

Morphogenetic processes observed in this study as well as their exogenous controlling factors are summarized in Fig. 9. Noteworthy is that depending on the starting plant material, somatic embryogenesis may be controlled either by auxin (in immature zygotic embryo culture), or by cytokinin (in mature zygotic embryo culture), or by both the kinds of growth regulators (in leaf culture). Contrary to somatic embryogenesis, adventitious budding is always controlled by cytokinin only. It is reasonable to speculate endogenous auxin/cytokinin activity. The capabilities of cultured tissues of *C. sinensis* decreases were the highest with mature zygotic embryos followed leaves by immature zygotic embryos.

Moreover, even within the individual leaves, the difference in morphogenetic competence between the different leaf regions was existed. The same deduction may be reached from the data obtained by Pedroso and Pais (1993: 1995) with leaf culture of *C. japonica*. Analytical data on endogenous levels of phytohormones in different tissues of *Camellia* spp. are required for supporting this presumption.

This is the first report on regulation of morphogenesis in leaf culture of *C. sinensis*, as well as on description of a whole spectrum of morphogenetic processes in wild type of *C. sinensis* growing in Korean peninsula.

Future efforts will be directed to combining leaf culture experimental protocols with 'leaf disk' method of transformation (Horsh et al., 1985) for the application of genetic engineering in *Camellia* spp.

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적 요

한국 야생차나무의 잎절편과 배배양에서 생장조절물질의 첨가에 따른 형태형성 과정의 변이를 조사하였다. 그 결과 접합자배는 싸이토키닌을 5-20 μM을 첨가한 MS 배지에서 직접적인 체세포 배와 부정아 및 액아 발생률이 높았으며 옥신의 함량이 높아질수록 형태형성율이 급격히 저하되었다. 1/2 MS 및 1/4 SH배지에 10 μM IBA가 첨가된 배지에서 모든 줄기가 발근 되었다. MS배지에 2,4-D를 첨가한 배지에서 미성숙 접합자 배를 배양한 결과 체세포배성 캘러스가 유발되었다. 성숙된 접합자 배를 발아시킨 후 어린잎을 채취하여 고농도의 옥신 (IBA와 NAA) 또는 싸이토키닌 (BAP)이 함유된 MS배지에 배양한 결과 체세포배 형성 캘러스가 발생되었으며 또한 직접적인 체세포배가 발생하였다. 그러나 뿌리와 줄기 형성에는 각각 요구하는 옥신의 농도와 종류가 각각 달랐다.

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