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BRIEF COMMUNICATION

# Shoot organogenesis from immature zygotic embryo cultures of *Ginkgo biloba*

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## Abstract

Immature zygotic embryos were cultured on Murashige and Skoog's medium (MS) supplemented with various combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), benzyladenine (BA) and zeatin or with various concentrations of 2,4-D alone. The maximum number (8 per embryo) of adventitious buds formed from cotyledons of heart stage embryos cultured on MS medium with 1 mg $dm^{-3}$ BA and 0.01 mg $dm^{-3}$ NAA. The adventitious buds originated from procambial strands of immature embryo cotyledons and then developed into adventitious bud primordia within 20 d. Adventitious buds transferred to hormone free MS medium grew into shoots, but did not produce plantlets because the shoots failed to root.

*Additional key words*: adventitious shoot formation, benzyladenine, 2,4-dichlorophenoxyacetic acid, naphthaleneacetic acid, zeatin.

Immature embryos are the most frequently used explant source for plant regeneration *via* organogenesis and somatic embryogenesis from many plants (Ashis and Deepesh 1990, Darlene and Stephene 1991). Plantlet regeneration from tissue cultures of immature zygotic embryos has been achieved *via* organogenesis in *Calotropis giganta* (Ashis and Deepesh 1990), and somatic embryos were developed from immature zygotic embryo of *Zamia integrifolia* (Norstog 1965) and of *Pinus palustris* (Sommer *et al.* 1975). Thus, immature zygotic embryos seem to be the most suitable source from which embryogenic or organogenic cultures in both angiosperms (Vasil 1987) and gymnosperms (Attree and Fowke 1993) may initiate. Since the callus induction from the *in vitro* culture in *Ginkgo biloba* reported by Tulecke (1967), somatic embryogenesis was investigated from immature embryos (Laurain *et al.* 1996), from isolated microspores (Laurain *et al.* 1993a) and from female prothallus protoplasts (Laurain *et al.* 1993b), but, to our knowledge, the adventitious bud formation has not been reported. Likewise, little work has been done on *in vitro* culture of this plant. The aim of this study was to determine the optimal stage of immature zygotic embryo development for adventitious shoot formation.

Seeds of *Ginkgo biloba* with different developmental stages of immature embryos were harvested from the end of August to the end of September in Chonbuk National University every year for three years. They were washed in running water to eliminate the sarcotesta, and then stored in the dark at 4 °C for four weeks. The seeds were disinfected by treating with 70 % alcohol for 1 min, followed by 1 % sodium hypochlorite solution for 15 min. After rinsing three times with sterile double distilled water, the immature zygotic embryos at globular (< 1.0 mm), heart (1.0 - 1.5 mm), torpedo (1.5 - 2.5 mm) and cotyledonary stages (> 2.5 mm) were excised from gametophytic tissues using forceps and placed onto solid Murashige and Skoog (1962, MS) induction medium to

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*Abbreviations*: MS - Murashige and Skoog's medium, 2,4-D - 2,4-dichlorophenoxyacetic acid, NAA - naphthaleneacetic acid, BA - benzyladenine; IBA - indolebutyric acid, FAA - formaldehyde acetic acid.
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induce adventitious buds. The induction medium was composed of MS medium supplemented with various combinations of cytokinins (BA and zeatin at 0.1, 0.5 or 1.0 mg $dm^{-3}$) and auxins (2,4-D and NAA at 0.01 or 0.1 mg $dm^{-3}$) or with 0.1, 0.5, 1.0, 2.0 or 5.0 mg $dm^{-3}$ 2,4-D alone. All media were adjusted to pH 5.8 before adding 8 g $dm^{-3}$ agar and then autoclaved at 121 °C for 15 min. Five immature zygotic embryos were placed in each Petri dish and then were maintained at 25 °C under 16-h photoperiod at irradiance of 46 μmol $m^{-2}s^{-1}$, provided by cool-white fluorescent lamps. After five weeks of culture, adventitious bud numbers per embryo were recorded. Three replicates were prepared for each treatment. For histological studies, heart stage embryos cultured on the MS medium supplemented with 1 mg $dm^{-3}$ BA and 0.01 mg $dm^{-3}$ NAA were isolated from the induction medium at 0, 5, 10, 15 and 17 d of culture, and fixed in a solution of FAA for 24 h, dehydrated in a tertiary-butanol series and embedded in paraffin. Serial sections were cut at 10 μm and stained with 0.5 % hematoxylin and 1 % safranin (Sass 1971). The sections were observed under a light microscope.

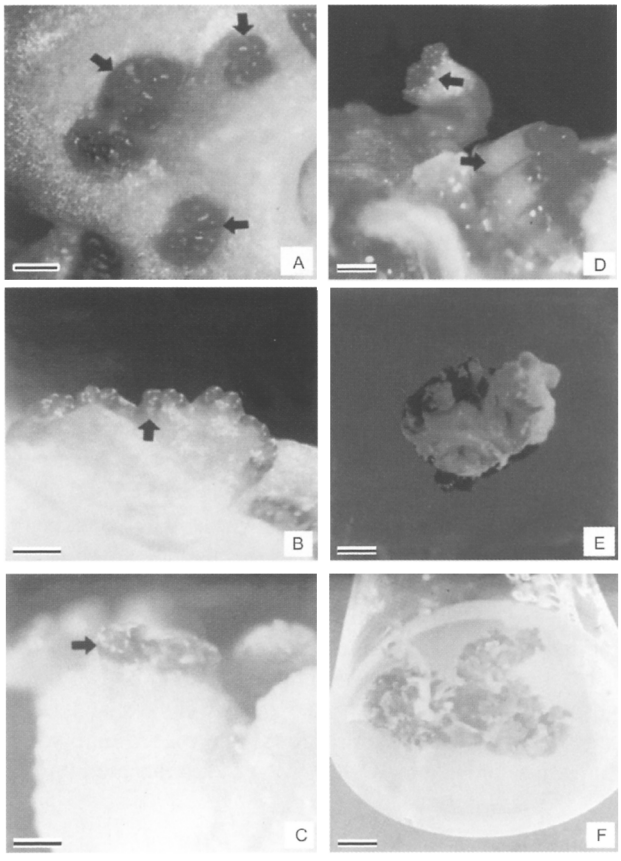


Fig. 1. Shoot organogenesis from the immature embryo cotyledon cultures of *Ginkgo biloba* L. on MS medium containing 1.0 mg $dm^{-3}$ BA and 0.01 mg $dm^{-3}$ NAA: *A* - green spots (*bar* = 1 mm) ; *B*, *C*, *D* - adventitious shoot primordia (*bars* = 1 mm) ; *E*, *F* - adventitious shoots (*bars* = 2 cm).

Table 1. Effects of plant growth regulators and developmental stage on the adventitious bud formation from immature zygotic embryos of *Ginkgo biloba* (number of adventitiuos shoots per immature zygotic embryo). Means ± SE of three replicates from about 15 explants per treatment. No shoots were formed from zygotic embryos at cotyledonary stage as well as on embryos at all stages on medium with only 2,4-D.

| Growth regulators [mg $dm^{-3}$] | Globular | Heart | Torpedo |
|---|---|---|---|
| BAP 0.1 + 2,4-D 0.01 | 0.4 ± 0.1 | 1.5 ± 0.7 | 0.2 ± 0.1 |
| BAP 0.1 + 2,4-D 0.10 | 0 | 1.3 ± 0.1 | 0.5 ± 0.3 |
| BAP 0.1 + NAA 0.01 | 0.3 ± 0.1 | 1.9 ± 0.1 | 0.4 ± 0.2 |
| BAP 0.1 + NAA 0.10 | 0 | 1.7 ± 0.8 | 0.1 ± 0.1 |
| BAP 0.5 + 2,4-D 0.01 | 0.5 ± 0.2 | 1.4 ± 0.3 | 0.2 ± 0.1 |
| BAP 0.5 + 2,4-D 0.10 | 0 | 1.6 ± 0.1 | 0.4 ± 0.3 |
| BAP 0.5 + NAA 0.01 | 1.1 ± 0.7 | 4.8 ± 0.5 | 0.5 ± 0.2 |
| BAP 0.5 + NAA 0.10 | 1.3 ± 0.4 | 3.7 ± 0.7 | 0.7 ± 0.1 |
| BAP 1.0 + 2,4-D 0.01 | 0.9 ± 0.1 | 2.8 ± 0.2 | 1.8 ± 0.5 |
| BAP 1.0 + 2,4-D 0.10 | 0.7 ± 0.3 | 2.5 ± 0.9 | 0.8 ± 0.3 |
| BAP 1.0 + NAA 0.01 | 3.0 ± 0.9 | 8.0 ± 1.2 | 1.2 ± 0.4 |
| BAP 1.0 + NAA 0.10 | 0 | 6.8 ± 2.7 | 0.6 ± 0.5 |
| Zeatin 0.1 + 2,4-D 0.01 | 0 | 0.3 ± 0.1 | 0 |
| Zeatin 0.1 + 2,4-D 0.10 | 0 | 0.6 ± 0.1 | 0 |
| Zeatin 0.1 + NAA 0.01 | 0 | 1.0 ± 0.5 | 0 |
| Zeatin 0.1 + NAA 0.10 | 0 | 0.9 ± 0.0 | 0 |
| Zeatin 0.5 + 2.4-D 0.01 | 0 | 1.3 ± 0.4 | 1.1 ± 0.7 |
| Zeatin 0.5 + 2,4-D 0.10 | 0 | 0.5 ± 0.1 | 1.1 ± 0.8 |
| Zeatin 0.5 + NAA 0.01 | 0.5 ± 0.1 | 0.8 ± 0.2 | 0.2 ± 0.1 |
| Zeatin 0.5 + NAA 0.10 | 0.3 ± 0.1 | 0.5 ± 0.1 | 0.4 ± 0.1 |
| Zeatin 1.0 + 2,4-D 0.01 | 0 | 2.3 ± 0.6 | 0 |
| Zeatin 1.0 + 2.4-D 0.10 | 0 | 0.9 ± 0.3 | 0 |
| Zeatin 1.0 + NAA 0.01 | 0 | 1.2 ± 0.4 | 0.3 ± 0.2 |
| Zeatin 1.0 + NAA 0.10 | 0 | 0.7 ± 0.2 | 0.5 ± 0.4 |

After two weeks of culture, the cotyledons of immature zygotic embryos on the induction media with combinations of zeatin and auxins (2,4-D and NAA) or 2,4-D alone became hypertrophic with green colour and then developed greenish compact callus. However, on the medium with combinations of BA and NAA, the cotyledons only became swollen and no callus was produced. The radicles degenerated on all media (data not shown). Adventitious bud formation from immature zygotic embryos was greatly influenced by the embryo developmental stages and plant growth regulators. Within three weeks of culture on the induction medium, green spots were only initiated on the surface of the swollen cotyledon part, which developed in most cases into buds and leaf-like structures within 30 d (Fig. 1). The greatest adventitious bud formation capacity was obtained from heart stage embryos, and was lower for globular and torpedo embryos and did not occur with cotyledonary embryos (Table 1). These results indicated that the stage of embryonic development was an important factor in the success of adventitious bud formation of *Ginkgo biloba*. Similarly Maheswaran and Williams (1986) emphasized

the importance of the immature embryo stage in somatic embryogenesis of *Brassica campestris*.

Different combinations of cytokinins and auxins in the induction medium resulted in different adventitious bud formation capacity. Bud numbers per embryo in the medium containing BA and NAA were generally higher than in the medium containing zeatin and 2,4-D. With 1 mg $dm^{-3}$ BA and 0.01 mg $dm^{-3}$ NAA, adventitious bud numbers formed per embryo were 3.0 at the globular stage, 8.0 at the heart stage and 1.2 at the torpedo stage. None were formed at cotyledonary stage. Similar observations have been reported for immature zygotic embryos of *Calotropis giganta* (Ashis and Deepesh 1990), and were consistent with the report that the cytokinins such as BA are essential for adventitious bud formation from immature zygotic embryos (Ozgen *et al.* 1996). On the basis of these results, we suggest that the organogenic potential of immature zygotic embryos is closely related to the presence of exogenous BA or zeatin and to the embryo developmental stage for *Ginkgo biloba*. From the histological studies, the adventitious bud primordia originated from the procambial strands of the cotyledons of immature zygotic embryos, and were developed into adventitious bud primordia within 20 d (Fig 2). When the adventitious buds were transferred to hormone-free MS medium, they grew into shoots (Fig. 1), but the adventitious shoots mechanically isolated from their tissue of origin and cultured on solid MS medium with 0.5 mg $dm^{-3}$ indolebutyric acid (IBA) failed to root. However, we expect that these results provide basic and essential information for plant scientists to study *in vitro* mass propagation of *Ginkgo biloba*, and that the adventitious bud formation system can be used for production of whole plantlets.

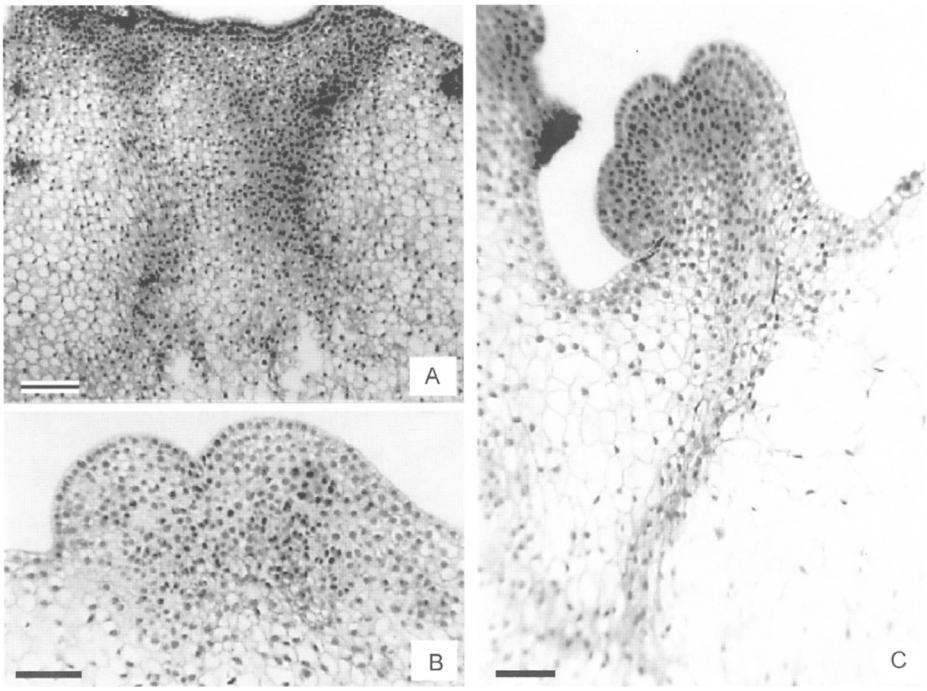


Fig. 2. Histological observations on the adventitious shoots formation from immature embryo cotyledon cultures of *Ginkgo biloba* L.: *A* - procambial cells of the cotyledon at 10 d of culture (*bar* = 200 μm); *B*, *C* - shoot primordia linked vascular tissue (*bars* = 95 μm).

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