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Relationship between Ontogenic Age and Formation of Bud from Leaf Segments in Japanese Persimmon, *Diospyros kaki*, THUNB.

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Leaf segments of 1-year-old Japanese persimmon, *Diosyros kaki*, were cultured aseptically on various agar media to determine suitable media for bud formation. The favorable medium to initiate new buds found was as follows: a half strength of minerals of Murashige and Skoog's medium supplemented with vitamins, inositol, 19 kind of amino acids, 1 mg/*l* BA and 0.1-1 mg/*l* IAA. Bud was formed only on the adaxial side of the leaf segments and the histological observation indicated that the bud meristem was directly developed from the palisade parenchyma without callus formation. Bud formation occurred in the leaf segments of the trees only younger than 4-year-old, whereas the same attempt to induce bud formation was not successful on the leaf segments of the trees older than 5 years, suggesting a strong influence of plant age on bud formation from leaf segments.

In some deciduous woody species including Japanese persimmon, the organ formation in tissue culture was largely influenced by their ontogenic age. The embryonic explants generally show a great capability to initiate organs such as embryos or buds.[1,3,5-8]

However, the information on the capacity of the formation of embryo or bud in the transitional ontogenic age between embryo and adult was very little about woody species including both angiosperms and gymnosperms.

In this experiment, the favorable agar medium for the direct bud formation was found, and early developmental stages of the bud formation were morphologically studied in the leaf segments of Japanese persimmon, *Diospyros kaki*, and the relationship between bud formation and the ontogenic age was clarified.

Materials and Methods

The seeds of Japanese persimmon, *Diospyros kaki* cv. "Fuyu" were harvested in the late fall, sterilized with 3% sodium hypochloride for 20 min, and germinated in the autoclaved garden soil. The plantlets were grown in the green house under natural light condition. Leaves of 5-8 weeks old (between 5th and 7th from the top of the trees) were colleted, respectively, from 15 trees in every summer for 7 years, sterilized with 3% sodium hypochloride for 15 min, and rinsed with autoclaved distilled water for several times. After taking off the midrib, the leaves were cut into small pieces (10 mm × 10 mm). The leaf segments were placed horizontally on the surface of the agar medium and cultured under continuous light (3,000 lux) at 27±1°C for about 3 months.

Murashige and Skoog's inorganic salts[4] was used in the original concentration (BM) or a half strength (1/2 BM). To each medium, 1 mg/*l* thiamin·HCl, 1 mg/*l* pyridoxine·HCl, 4 mg/*l* nicotinic acid, and 0.1 mg/*l* biotin were added. Inositol was added at 100 mg/*l* to the medium. Sucrose was

added to the medium at 3% in BM, and 2% in 1/2 BM. The amino acid constituent was as follows; 5 mg/*l* glycine, 2 mg/*l* L-alanine, 2 mg/*l* L-valine, 2 mg/*l* L-isoleucine, 2 mg/*l* L-leucine, 2 mg/*l* L-serine, 2 mg/*l* L-threonine, 2 mg/*l* L-proline, 2 mg/*l* L-aspartic acid, 5 mg/*l* L-glutamic acid, 2 mg/*l* L-lysine, 1 mg/*l* L-arginine, 2 mg/*l* L-asparagine, 2 mg/*l* L-glutamine, 2 mg/*l* L-methionine, 2 mg/*l* L-tryptophan, 2 mg/*l* L-phenylalanine, 2 mg/*l* L-tyrosine, 2 mg/*l* L-histidine. After the pH of the medium was adjusted to 5.7-5.8 with 1 N NaOH or 1 N HCl, agar (7 g/*l*) was added to the medium and dissolved in the boiling water bath. The test tubes (15 cm × 2 cm) containing 15 ml medium was covered with aluminum foil and autoclaved at 121℃ for 15 min.

The cultured leaf segments were fixed with FAA (formalin : acetic acid : ethylalcohol, 1 : 1 : 18), dehydrated through ethylalcohol series, embeded in paraplast, and cut into 10 μm thick. The sections were stained with Heidenhain's iron-alum-hematoxylin and observed histologically under a microscope and photographed at various stages of the bud formation.

Results

The components of the medium for the bud formation in the leaf segments of 1-year-old trees

The leaf segments were cultured on BM with/without amino acids, or 1/2 BM with amino acids in the presence of vitamins, inositol, 1 mg/*l* BA, and 0.3 mg/*l* IAA to survery suitable media for bud formation. Buds were formed only on 1/2 BM with amino acids (**Table 1, Figs. 1, 2**).

Buds were mainly formed, when the leaf segments were cultured on 1/2 BM combined with inositol (100 mg/*l*), amino acids, 1 mg/*l* BA and 0.3 mg/*l* IAA (SF-medium). The withdrawing inositol or amino acids from SF-medium led to a remarkable decrease in the rate of bud formation (**Table 2**).

When the segments were cultured on SF-medium containing various combinations of BA and IAA, bud formation occurred only on the media containing 1 mg/*l* BA and 0.1, 0.3 or 1 mg/*l* IAA (**Table 3**).

The initiation of the bud was recognized on the leaf segments on SF-medium about 10 days after

Table 1. Formation of buds on the various combinations of the minerals and amino acids.

Medium	Rate of the bud formation
BM	0/35
BM+amino acids	0/35
1/2 BM+amino acids	11/20

BM showed the minerals of Murashige and Skoog's medium with vitamins and inositol as described in Materials and Methods. 1 mg/*l* BA and 0.3 mg/*l* IAA were added to each medium.

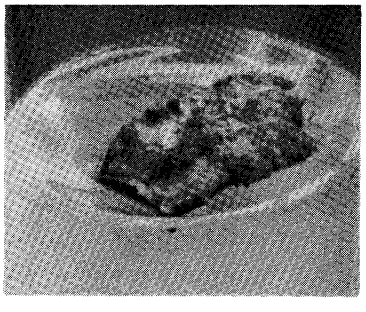

Fig. 1. Cultured leaf segment with regenerated bud meristem.

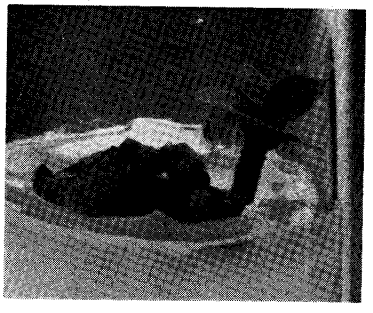

Fig. 2. A new shoot emerged from the leaf segment.

Table 2. The effects of amino acids and inositol added to 1/2 BM on bud formation.

Medium	Bud formation
1/2 BM	1/12
1/2 BM+amino acids	1/10
1/2 BM+100 mg/*l* inositol	1/13
1/2 BM+amino acids+100 mg/*l* inositol	10/18

The 1/2 BM was composed as described in Materials and Methods and contained 1 mg/*l* BA, and 0.3 mg/*l* IAA.

Table 3. The effects of various combinations of BA and IAA on bud formation.

BA	0	0.1	1	5	0	0	0.03	0.03	0.1	0.1
IAA	0	0	0	0	0.1	1	0.1	1	0.03	0.1
Bud formation	0/25	0/25	0/20	0/20	0/20	0/15	0/15	0/20	0/25	0/20
BA	0.1	0.1	1	1	1	1	5	5	5	5
IAA	0.3	1	0.03	0.1	0.3	1	0.1	1	3	10
Bud formation	0/20	0/25	0/25	4/25	12/20	5/25	0/25	0/24	0/25	0/25

Basal medium was the same as SF-medium.

the inoculation. Leaves of the new buds began to appear within 3 weeks from the inoculation. Both the number of the regenerated buds per a cultured leaf segments and the rate of the leaf segments with buds to the inoculated leaf segments reached to a maximum within 7 weeks of culture.

The regenerated buds developed into plantlets easily on the basal medium of SF-medium.

The histological observation of the new buds on the cultured leaf segments

For the determination of a suitable medium to initiate adventitious buds, the abaxial side of the leaf segments was faced to the surface of agar medium. In this experiment, abaxial or adaxial side of the leaf segments was faced to the agar surface of SF-medium, respectively. Regeneration of buds on the adaxial side of the leaf segments occurred with remarkably high frequency (60%). On the other hand, bud formation was very low (5%) on the abaxial side. Microscopic observation of the cultured leaf segments with new buds showed that the epidermis of the adaxial side remained, and that the callus induction was not recognized on the palisade parenchyma, and also showed that many bud meristems developed on the margin of the palisade (**Figs. 3, 4**).

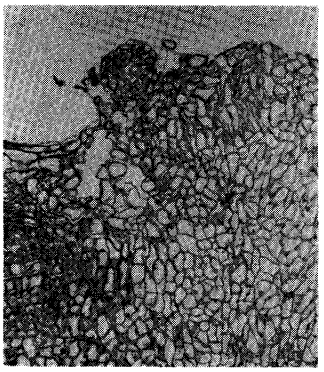

Fig. 3. Transversal section of the cultured leaf segment on SF-medium supplemented with 1 mg/*l* BA and 0.3 mg/*l* IAA.

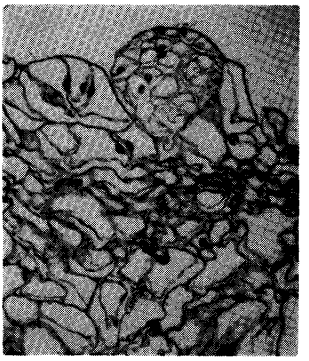

Fig. 4. Palisade of the cultured leaf segment with a bud meristem.

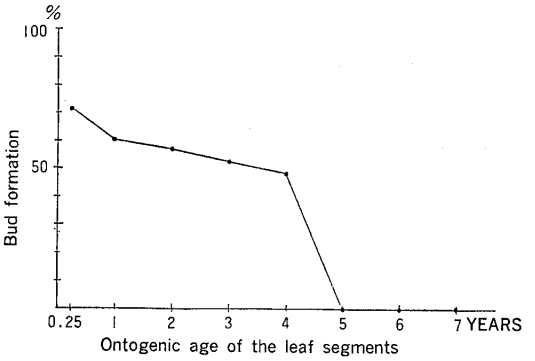

Fig. 5. Relationship between the rate of bud formation and the ontogenic age of the trees from which the leaf segments were removed.

The relationship between bud formation from leaf segments and ontogenic age of the trees

The leaves of 5-8 weeks old (between 5th and 7th from the top of the trees) were collected from 15 trees every early summer from 3 months for 7 years, and the segments of the leaves were cultured on SF-medium. Bud formation occurred on the leaf segments from the trees younger than 4 years old. However, buds were not formed on the leaf segments of the trees older than 5 years old (**Fig. 5**).

Discussion

Bud formation occurred directly in the leaf segments of Japanese persimmon trees younger than 4 years old on the agar medium devised by this work. In previous works, two different callus clones of Japanese persimmon were cultured,[8,9] and the buds were regenerated in the callus culture of immature embryos without any difficulty, on the other hand, bud formation hardly occurred in the callus culture of the twigs of a mature Japanese persimmon tree. These facts suggested that the ontogenic age of explants might be an important factor for the bud formation in the callus culture of woody species. In this experiment, the suitable medium to initiate buds was surveyed in the leaf segments of 1-year-old Japanese persimmon trees. Also, the relationship between the bud formation in the leaf segments and the ontogenic age of the original trees was studied. Although inorganic salts of MS supported the bud formation in the callus cultures,[8,9] a half strength of MS's salts was more suitable for the bud formation in the leaf segments in the combination with inositol, vitamins, amino acids, BA and IAA (SF-medium). The omission of any elements from SF-medium led to a decrease in bud formation. The decreased concentration of mineral salts and enriched organic nutrients was suitable for the direct bud formation without callus growth. Our experimental purpose required the determination of the culture conditions for the direct bud formation in the leaf segments to avoid the influences of the successive subculture of the callus on the masking of the bud and root formation in some deciduous species.[2,9] In other words, the direct bud formation in vitro culture seems to be essential to know the transitional point of the ontogeny from the embryonic to adult character. Bud initials developed only from the palisade parenchyma in this experiment, and the direct bud formation from the palisade was very rare in angiosperm trees.

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≪和文要約≫

カキ葉片上における個体発生的齢と不定芽形成との関係

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カキ（富有）における個体発生的齢と器内培養における不定芽形成との関係を知るために多種の培地を試作し，実生の葉片を培養して，不定芽誘導に適した培地を選定した．この最適培地を用いて，実生苗から発芽初年度より７年間，毎夏，葉の切片を切りとり培養した．その結果，不定芽は４年生より若い苗から分離した葉片上に形成されるが，５年生以上のものには形成されなかった．