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Tissue Culture Note

Adventitious bud formation through nodule induction by thidiazuron in cultured leaf segments of the Japanese persimmon (*Diospyros kaki* Thunb.)

Tomosaburo Yokoyama, Yuji Moriyasu*, Yasutake Sugawara

Graduate School of Science and Engineering, Saitama University, Saitama 338-8570, Japan
* E-mail: moriyasu@mail.saitama-u.ac.jp Tel: +81-48-858-3411 Fax: +81-48-858-3408

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Abstract Multiple meristematic nodules were induced on leaf segments from the Japanese persimmon (*Diospyros kaki* Thunb.) 'Fuyu' that were cultured on a solidified half-strength Murashige and Skoog's medium containing 2% (w/v) sucrose and 1 μM thidiazuron (TDZ). The nodules 1 to 3 mm in diameter were formed in the cut ends and abaxial side of the leaf segments two weeks after inoculation. The nodule changed from pale green to dense green and multiplied to form many daughter nodules. Adventitious buds were thereafter formed on the nodules, suggesting that the meristematic nodule is an intermediate structure for bud differentiation. The adventitious buds grew poorly on the medium with TDZ, but resumed growth and developed into shoots after transfer to the medium containing 2% (w/v) sucrose and 10 μM zeatin. The meristematic nodules survived more than one year on the medium containing 2% (w/v) sucrose and 1 μM TDZ without transfer, and did not lose their ability to form adventitious buds for more than 5 years when transferred regularly to a fresh medium. These results suggest that the meristematic nodule is a promising material for propagation and long-term conservation of this plant.

Key words: Adventitious bud, cytokinin, *Diospyros kaki*, meristematic nodule, persimmon, thidiazuron (TDZ), zeatin.

The Japanese persimmon is an important fruit tree in Japan and other countries including China, US, New Zealand, Italy, and Spain. The tree has been propagated by slow and costly grafting techniques for many years.

Application of tissue culture techniques is considered important for more efficient propagation of the tree. Adventitious buds and roots were formed on the callus induced from hypocotyls of the Japanese persimmon, 'Tsurunoko', on the MS medium (Murashige and Skoog 1962) supplemented with sucrose, yeast extract, 1-naphthaleneacetic acid (NAA), and kinetin (Yokoyama and Takeuchi 1977). These adventitious buds were able to develop into plantlets after transfer onto the medium free of these phytohormones. Adventitious buds were also induced on the callus induced from twigs of a mature tree 'Fuyu' on the MS medium containing sucrose, NAA and kinetin, although these buds did not develop into plantlets (Yokoyama and Takeuchi 1981). The formation of adventitious buds were also observed on calluses formed from leaf primordia and immature leaves (Choi et al. 2001; Tamura et al. 1992; Tao et al. 1988; Tao and Sugiura 1992). Although these studies have provided useful information about propagation of the Japanese persimmon, further improvements in plant tissue culture methodology are required for practical use.

Thidiazuron (*N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea, TDZ) is a urea-type cytokinin and shows one of the strongest activities among cytokinins (Mok et al. 1982, 1987). It has been reported that TDZ is very efficient for adventitious shoot production in many woody plants (Kerns and Meyer 1986; van Nieuwkerk et al. 1986).

In this study, we examined the morphological changes that occur in leaf segments of the Japanese persimmon cultured with TDZ.

Seeds were collected from mature fruits of the Japanese persimmon (*Diospyros kaki* Thunb.) 'Fuyu' in late autumn and stored dry at 4°C. They were germinated and grown on vermiculite at 23±2°C under continuous light (80 μmol m^{-2}s^{-1}) from fluorescent lamps. Mature leaves (approximately 12 cm×6 cm in size) were sterilized with 0.12% (w/v) sodium hypochlorite, and kept on MS medium containing 3% (w/v) sucrose and solidified with 0.2% (w/v) Gelrite (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 2 days to detect any contamination. Non-contaminated leaves were cut into pieces (5 mm×5 mm), and each piece was placed on 10 ml of basal culture medium (half-strength MS medium, 2% (w/v) sucrose, 0.2% (w/v) Gelrite, pH 5.8) containing

Abbreviations: MS medium, Murashige and Skoog's medium; NAA, 1-naphthaleneacetic acid; TDZ, thidiazuron (*N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea)

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1 and 10 μM of TDZ with its adaxial side up in a test tube (24 mm×150 mm). After the top of the test tubes was covered with a sheet of aluminum foil, the leaf segments were cultured at 23 ± 2°C under a continuous light. Each experimental culture condition was independently repeated 3 times, each using 10 leaf segments per medium condition.

For light microscopic observation, leaf segments cultured were fixed with formalin/acetic acid/70% ethanol (1 : 1 : 18). After dehydration through ethanol series, they were embedded in Paraplast (Oxford Labware, MO, USA) and cut into 10 μm-thick sections. After stained with safranin or Heidenhain's iron-aluminum-hematoxylin, the sections were observed by light microscopy. The surface structures of leaf segments were visualized by scanning electron microscopy (S-430L, Hitachi, Japan) without fixation.

Leaf segments of the Japanese persimmon were inoculated onto the basal culture medium containing 1 μM TDZ, and cultured at 23°C. One of the most conspicuous morphogenetic events after one week of culture was the formation of many dome-like structures with a pale green color on the cut surface of the leaf segments (Figure 1A). These dome-like structures are referred to as meristematic nodules (or nodules) hereafter according to previous reports (Aitken-Christie et al. 1988; Fortes and Pais 2000; Gautheret 1957; McCown et al. 1988; Steward et al. 1958; Takeuchi 1968). The meristematic nodule had a smooth surface, which was one of the features that distinguish a nodule from callus (Figure 1A).

Two weeks after inoculation, the meristematic nodules, which had become 1 to 3 mm in diameter and a little darker green, were observed on the abaxial side as well as on the cut ends of the leaf segments (Figure 1B). Simultaneously, new nodules were formed on the surface of old nodules. At three weeks after inoculation, the nodules were observed both above and below the medium surface, and the original morphology of a leaf segment was almost completely lost (Figure 1C). We observed complete nodules on a leaf segment at three weeks by scanning electron microscopy (Figure 1D). Several protuberances, possibly daughter nodules were observed on the surface of the nodule (Figure 1D, arrows).

We observed the transverse sections of leaf segments having nodules (Figure 1E, F). A nodule consisted of a surface cell layer, which resembles an epidermal cell layer (Figure 1E, arrowheads), and inner parenchyma. Nodules were always observed on the abaxial side of leaf segments, while no significant morphological changes were observed on the adaxial side. In the interior of a mature nodule, vascular bundles were seen (Figure 1F, circles).

About one month after inoculation, the number of nodules per leaf segment seemed to be maximal. Simultaneously, adventitious buds were observed on some nodules (Figure 2A), the emergence of which was also confirmed by light microscopy (Figure 2B) and by scanning electron microscopy (Figure 2C). The maximal number of adventitious buds per leaf segment was attained at around 50 days after inoculation.

Even after 2 months, most of the adventitious buds did not grow further, although a few of them developed to shoots (Figure 2D). That is, the growth of the adventitious buds seemed to be retarded on the TDZ-containing medium. However, zeatin was effective to make adventitious buds to resume growing. We separated a leaf segment having lots of nodules into six pieces. They were then put on the basal culture medium containing 10 μM zeatin and cultured for another month (Figure 3). As a result, as many as 66 ± 24 (average ± SD, n=30) shoots per leaf segment (i.e. six pieces of leaf fragments) were obtained.

Pre-culture with TDZ is necessary for the induction of adventitious buds. No adventitious buds were induced when the leaf segments were inoculated directly onto the medium containing zeatin (data not shown). In order to determine the duration of pre-culture with 1 μM TDZ necessary to induce shoot formation, leaf segments were pre-cultured on the basal culture medium containing 1 μM TDZ for various days and then cultured on the basal culture medium containing 10 μM zeatin for one month. The results showed that leaf segments require at least ten days of pre-culture with 1 μM TDZ for efficient shoot formation.

On the basal culture medium containing 10 μM TDZ, multiple nodules and adventitious buds were formed in the same way as on the medium containing 1 μM TDZ. One month after inoculation, however, a green callus was formed on the adaxial surface of leaf segments (Figure 4A). It seemed to have originated from a nodule above the surface of the medium. However, it grew rapidly, and covered most of the surface of the leaf segment one and a half months after inoculation (Figure 4A). After 2 months, a few shoots developed from the callus in addition to meristematic nodules and green callus (Figure 4B).

We have established an efficient method of inducing adventitious shoots on the leaf segments of the Japanese persimmon. It regenerated as many as 66 shoots per leaf segment (5 mm×5 mm), which is more efficient than reported previously (Choi et al. 2001). Although it should be examined how frequently somaclonal variation occurs through culture with TDZ, this protocol may be applicable in agricultural practice.

In the culture of leaf segments from the Japanese persimmon, meristematic nodules were formed before adventitious buds appeared. The meristematic nodule seemed to produce multiple daughter nodules, and then

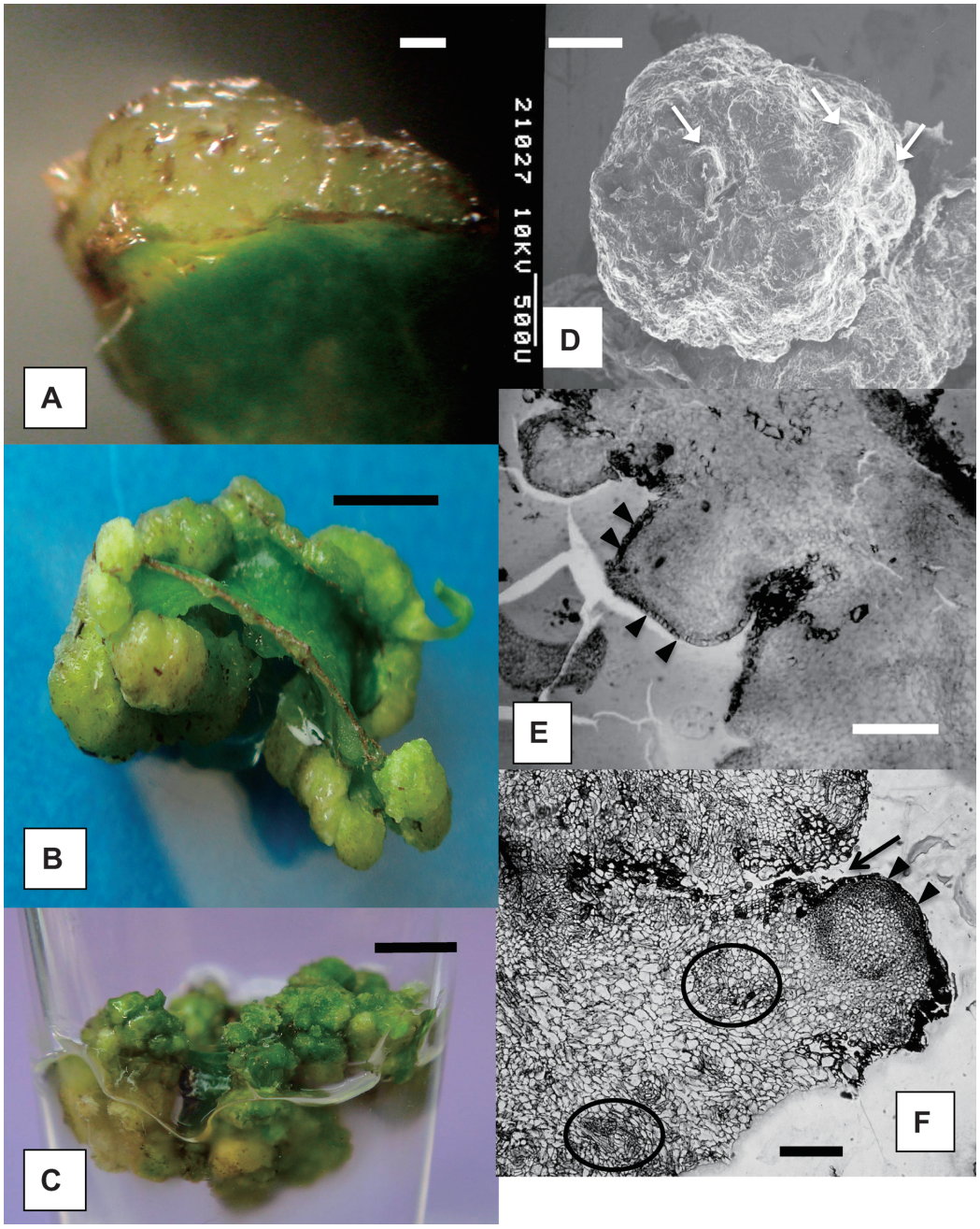

Figure 1. Formation of meristematic nodules on leaf segments of the Japanese persimmon cultured with 1 μM TDZ. (A) Meristematic nodules appeared at the cut end of a leaf segment cultured for one week. Bar, 1 mm. (B) Nodules formed at the cut ends and abaxial side of a leaf segment cultured for two weeks. Bar, 1 mm. (C) Nodules multiplied above and below the surface of the solidified medium after three weeks of culture. Bar, 3 mm. (D) Complete nodule formed after three weeks of culture, observed by scanning electron microscopy. Arrows point at many protuberances on the surface of a nodule. Bar, 0.5 mm. (E) Section of a leaf segment cultured for two weeks. Nodules emerged from the abaxial side of leaf segments. The nodule has a surface cell layer (arrowheads) and parenchymatous cells. Bar, 1 mm. (F) Section of nodules formed after three weeks of culture. Vascular bundles (circles) and a new nodule (arrowheads) are seen in a mature nodule. The arrow points to a cleft between two mature nodules. Bar, 0.2 mm.

adventitious buds. In the culture of poplar callus, the shoots regenerated through a distinctive intermediate structure, which was later termed a nodule, (McCown et al. 1988). Fortes and Pais (2000) investigated the histochemical events occurring during the culture of hop stems. They first observed callus formation by division of cortical cells of the stem followed by nodule development. After a nodule multiplied, many adventitious buds arose from the nodules. Taken together, the meristematic nodule seems to act as an intermediate structure for the formation of adventitious buds.

TDZ seems to induce meristematic nodules more effectively than other phytohormones. We did not succeed in inducing nodules or adventitious buds so

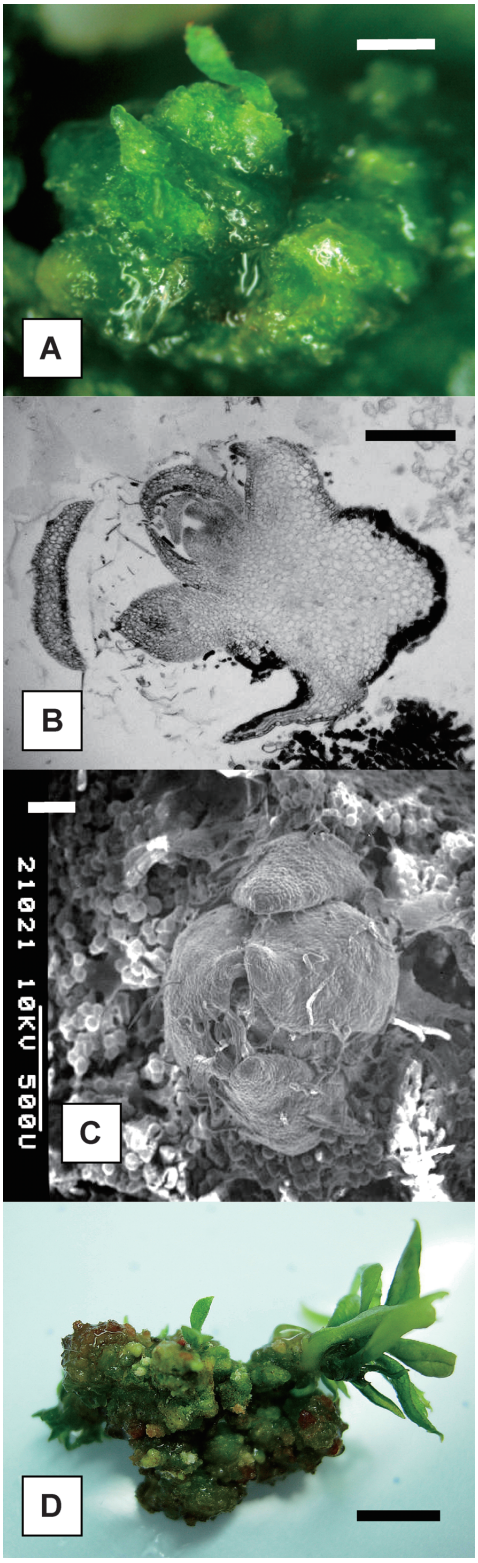

Figure 2. Formation of adventitious buds from meristematic nodules on leaf segments of the Japanese persimmon. (A) Adventitious buds formed on nodules on leaf segments cultured for one month. Bar, 0.5 mm. (B) Section of two adventitious buds emerging from a nodule after two months of culture. Bar, 1 mm. (C) Adventitious bud observed by scanning electron microscopy. Bar, 0.2 mm. (D) Most adventitious buds did not grow but a few developed to shoots. Bar, 3 mm.

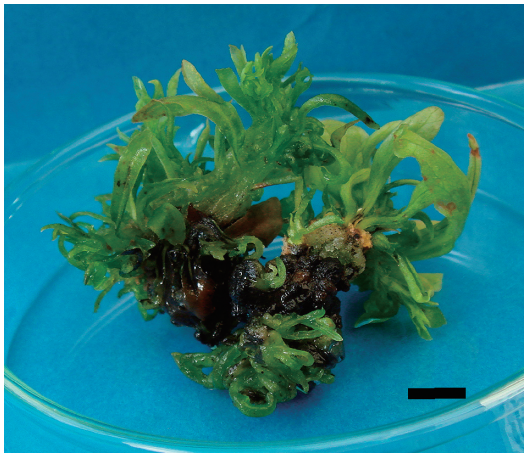

Figure 3. Development of shoots from leaf segments of the Japanese persimmon treated with TDZ followed by zeatin. Leaf segment was cultured on a TDZ-containing medium for one month, and then on a zeatin-containing medium for another month. Bar, 10 mm.

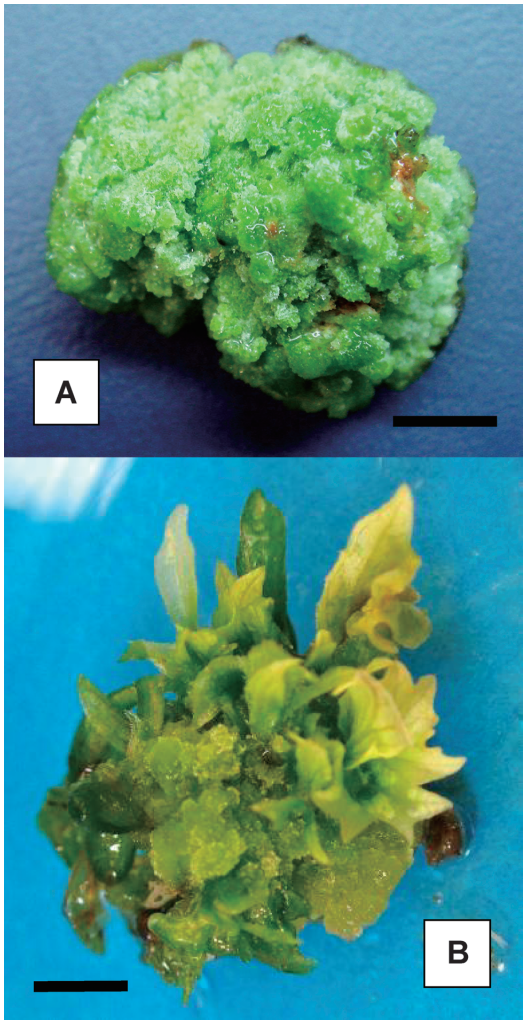

Figure 4. Formation of callus on leaf segments of the Japanese persimmon cultured with 10 μM TDZ (A) Callus formed after a month of culture with 10 μM TDZ. Bar, 5 mm. (B) Callus with a few shoots, nodules, and adventitious buds formed after two months of culture with 10 μM TDZ. Bar, 4 mm.

efficiently using other phytohormones (Yokoyama and Takeuchi 1977, 1981, 1988). There are many reports on tissue culture with TDZ in various plants (Huetteman and Preece, 1993) including sweet potato (Gosukonda et al. 1995), apple (Sarwar and Skirvin 1997), evening primrose (de Gyves et al. 2001), pothos (Qu et al. 2002), hydrangea (Ledbetter and Preece 2004), rhododendron (Tomsone et al. 2004), lentil (Khawar et al. 2004), strawberry (Debnath 2005), begonia (Nhut el al. 2005), henbane (Uranbey 2005), mungbean (Amutha et al. 2006), safflower (Radhika et al. 2006), poplar (Wang et al. 2008), milk vetch (Basalma et al. 2008), and potato (Sajid and Aftab 2009). Most of these reports described the formation of adventitious buds/shoots, but not the formation of meristematic nodules. But it is likely that careful observations clarify the emergence of the nodules before the formation of the buds in these materials. Thus it is reasonable to suppose that the formation of nodules is a common developmental response to TDZ.

TDZ seems to inhibit the growth of adventitious buds although it was important for their induction. In the culture of leaf segments of the Japanese persimmon, most of the adventitious buds induced by TDZ did not grow further unless they were transferred to a medium containing zeatin. This is consistent with other reports on the effect of TDZ (Huetteman and Preece 1993). In hydrangea, low concentrations (0.05 to 0.5 μM) of TDZ induced few shoots that tended to elongate whereas high concentrations (1 to 5 μM) of TDZ induced many tiny shoots that elongated slowly (Ledbetter and Preece 2004). These reports and our result suggest that TDZ inhibits the growth of adventitious buds although it effectively induces the buds.

The meristematic nodules in the Japanese persimmon were able to survive more than one year without transfer to a fresh medium. Furthermore, nodules, transferred regularly to a fresh basal culture medium containing 1 μM TDZ, continued to multiply and form adventitious buds for at least 5 years. This stability of nodules to continue forming adventitious buds is in contrast to that of calluses from hypocotyls of the Japanese persimmon (Yokoyama and Takeuchi 1977). The longevity and stability of nodules will enable long-term preservation of the useful genome of this tree in the form of nodules.

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