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Tissue Culture Note

Thidiazuron-induced rapid shoot regeneration via embryo-like structure formation from shoot tip-derived callus culture of sugarcane

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Abstract Rapid shoot regeneration system from callus cultures of a sugarcane (*Saccharum officinarum* L.) cultivar NiF8 was established. Apical meristematic tissues harvested from young sugarcane shoots were cultured on modified Murashige and Skoog medium containing 2 mg l^{-1} of 2,4-dichlorophenoxyacetic acid for callus induction. The sugarcane callus was then transferred onto media with different concentrations of thidiazuron (TDZ) (0.5, 1, 2 and 3 mg l^{-1}) with or without 0.1 mg l^{-1} of 1-naphthaleneacetic acid (NAA) for shoot regeneration. The highest regeneration frequency (80.0%) was observed after three weeks of culture on medium containing 1 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA. Histological observation showed that differentiation of proembryoid-like structure with pro-vascular strands were observed 3 days after transfer onto the optimum medium followed by formation of apical meristematic tissue and leaf-like structures after 5 to 7 days, suggesting that TDZ induced rapid shoot regeneration via a process similar to somatic embryogenesis. This study demonstrated a rapid, reproducible and efficient regeneration procedure of sugarcane, which is suitable for biotechnological application including genetic transformation.

Key words: Callus, embryo-like structure, regeneration, sugarcane, TDZ.

Since sugarcane (*Saccharum officinarum* L.) is one of the most important crops in the world, any efforts to increase its production are of great importance. Sugarcane provides up to 60% of the world sugar supplies (Grivet and Arruda 2001) and is utilized as a source of renewable energy (De Oliveira et al. 2005; Patzek and Pimentel 2005). To increase the productivity of sugarcane cultivars, biotechnologies such as cell and tissue culture and genetic transformation techniques are greatly important (Lakshmanan et al. 2005). So far, various plant regeneration methods have been developed in sugarcane, such as those through direct shoot organogenesis (Gill et al. 2006; Khan et al. 2009; Lakshmanan et al. 2006) and somatic embryogenesis (Ahloowalia and Maretzki 1983; Chengalrayan and Gallo-Meagher 2001; Chengalrayan et al. 2005; Desai et al. 2004; Franklin et al. 2006; Gallo-Meagher et al. 2000; Garcia et al. 2007; Ho and Vasil 1983; Lakshmanan et al. 2006). Among them, Gallo-Meagher and co-authors demonstrated rapid plant regeneration through embryogenic callus formation by using TDZ (Chengalrayan and Gallo-Meagher 2001; Gallo-Meagher et al. 2000). Although the TDZ-mediated regeneration procedure has a significant potential for biotechnological applications such as genetic transformation, it is still unclear whether the procedure is effective for a wide range of sugarcane cultivars or is specific to the cultivar CP84-1198 used in their studies. In addition, the developmental mode of the rapid regeneration process has not been explored. Therefore, the objectives of the present study were 1) to confirm the applicability of TDZ-mediated rapid regeneration method to a Japanese leading sugarcane cultivar, NiF8, and 2) to observe the histological changes of callus at early stages after the TDZ treatment.

Plants of sugarcane (*Saccharum* hybrid sp.) cv. NiF8 grown in the research field of Osaka Prefecture University were used as materials. Shoot tissues containing apical meristems (100 mm long×10 mm diameter) were harvested and surface-sterilized with

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid; TDZ, thidiazuron

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sodium hypochlorite solution (1% active chlorine) containing 0.1% (v/v) Tween 20 for 15 minutes, followed by rinsing with 70% (v/v) ethanol for 2 minutes. The sterilized tissues were rinsed three times with sterile distilled water and cut into small segments (10 to 15 mm) to serve as explants for *in vitro* culture. The explants were cultured for callus induction on modified Murashige and Skoog (MS) medium (Murashige and Skoog 1962) which had their nitrogen components replaced by those of N6 medium (Chu et al. 1975) and was supplemented with 30 g l^{-1} sucrose, 500 mg l^{-1} casamino acid, 2 mg l^{-1} 2,4-D and 10 g l^{-1} agar (pH 5.8). The cultures were incubated at 25ºC in the dark.

On the callus induction medium, two types of callus, a white friable callus and a yellow compact callus, were obtained after 30 days of culture (Figure 1A). The yellow compact callus proliferated well on the callus induction medium without turning brown, while the white friable callus showed slower growth and easily turned brown. When these two types of callus were transferred onto medium containing 1 mg l^{-1} TDZ, the compact callus showed obviously higher (73.0%) shoot regeneration ability than the white friable callus whose regeneration frequency was less than 1% (data not shown). Previous

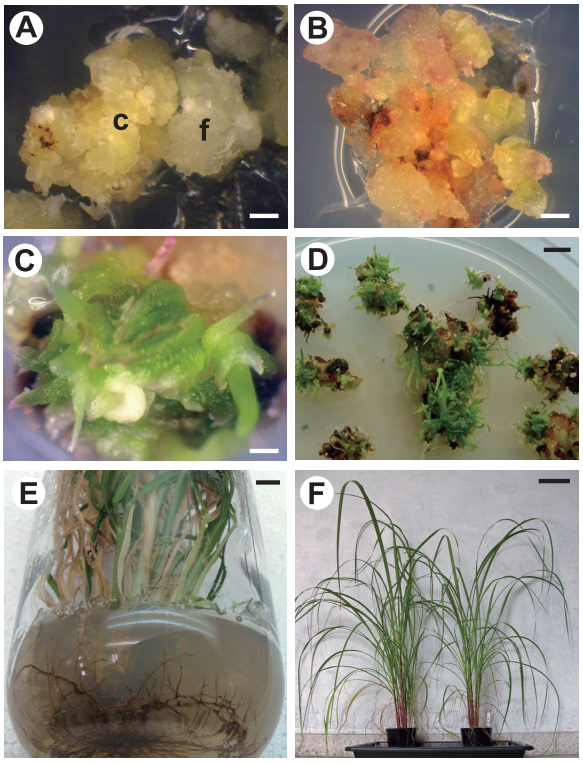

Figure 1. Rapid plant regeneration from sugarcane callus. (A) Yellow compact callus (c) and white friable callus (f) were obtained from apical meristematic tissues of sugarcane cv. NiF8 after 30 days of culture on modified MS medium supplemented with 2 mg l^{-1} 2,4-D. Bar=1 mm. (B) Red spots were shown in yellow compact callus cultured in the presence of 1 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA for 3 days. Bar=1 mm. (C) Shoot formation after 9 days of culture on the regeneration medium. Bar=1 mm. (D) Developed multiple shoots after 21 days of culture on the regeneration medium. Bar=5 mm. (E) *In vitro* rooting of regenerated shoots cultured on modified MS medium without plant growth regulators. Bar=5 mm. (F) Acclimatized sugarcane plants. Bar=50 mm.

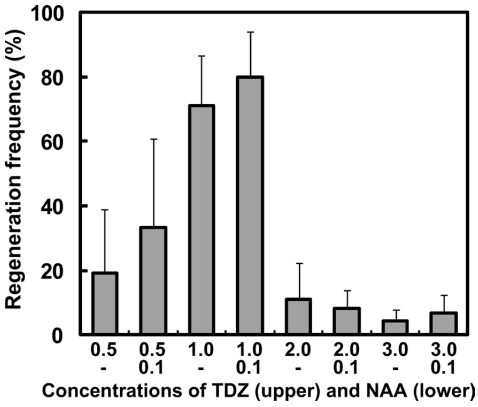

Figure 2. Effects of TDZ and NAA concentrations (mg l^{-1}) on regeneration frequency of sugarcane callus after 3 weeks of culture. Data represent the averages of three independent experiments, and bars indicate the standard deviations.

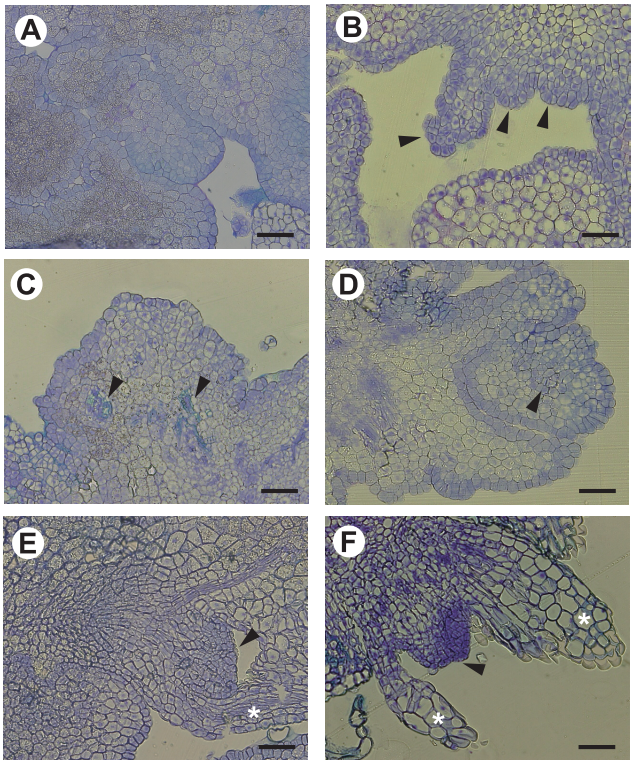

Figure 3. Histological observations of explants at different stages of regeneration. (A) Section of compact callus on the day of transfer onto callus induction medium. (B) Differentiation of proembryoid-like structures (arrowheads) comprising small densely cytoplasmic cells (day 0). (C), (D) Differentiation of pro-vascular strands (arrowheads) within the globular proembryoid after 3 days of the culture on the regeneration medium containing 1 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA. (E), (F) Well developed embryoid-like structures formed meristematic tissues (arrowheads) and coleoptiles (asterisks) after 5 (E) and 7 (F) days on the regeneration medium. Bar=50 μm.

studies also reported the difference in shoot regeneration ability between compact and friable calluses (Chengalrayan and Gallo-Meagher 2001; Ho and Vasil 1983; Matsuoka et al. 1998). When the calluses were transferred to modified MS medium without plant growth regulators, low frequency (approx. 20%) of shoot regeneration was observed.

Effects of different concentrations of TDZ (0.5, 1, 2 and 3 mg l^{-1}) and NAA (0.1 mg l^{-1}) on shoot regeneration from yellow compact callus were examined using the modified MS medium described above containing 30 g l^{-1} sucrose, 300 mg l^{-1} casamino acid and 10 g l^{-1} agar (pH 5.8). Fifteen compact callus clusters (Ho and Vasil 1983) each corresponding to 0.1 g fresh weight were placed on the shoot regeneration media and incubated at 25°C under cool white fluorescent light at 60 μmol m^{-2} s^{-1} with a 12 h photoperiod. The experiment was arranged in a completely randomized block design with 3 replicates per treatment. Data were collected weekly for 3 weeks and analyzed by one way analysis of variance (ANOVA) followed by Scheff's F-test ($P<0.05$). As a result, TDZ concentration clearly affected the shoot regeneration frequency. Among the four concentrations tested, 1 mg l^{-1} gave the highest shoot regeneration frequency (73.0%) after three weeks of culture (Figure 2). At this TDZ concentration, co-application of 0.1 mg l^{-1} NAA increased the regeneration frequency (80.0%) but not significantly ($P<0.05$). Gallo-Meagher et al. (2000) also reported a rapid and high frequency (~100%) regeneration of shoots from embryo-like structure on medium containing 5–10 μM (approx. 1.1 to 2.2 mg l^{-1}) TDZ. These results suggest that TDZ might be effective for inducing shoots from compact callus in a broad range of sugarcane varieties, while the variation in their regeneration frequencies might be attributed to the starting material used for callus induction. As material tissues for callus induction, Gallo-Meagher et al. (2000) used pre-emergent immature inflorescence tissues, whereas we used shoot meristematic tissues, which is generally easier to obtain.

When the yellow compact callus was transferred onto the optimum regeneration medium containing 1 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA, red spots appeared within 3 days. The colour of red spots intensified with the length of incubation period (Figure 1B), and shoots emerged mostly from the red spotted area after 4 days of culture. After 9 days, a mass of shoots with approx. 5 mm of length was clearly observed (Figure 1C), and numerous shoots longer than 10 mm in length were observed on the calluses (>20 shoots per callus cluster) 21 days after the transfer (Figure 1D). The regeneration process was somewhat shorter than that previously reported by Gallo-Meagher et al. (2000), where shoots longer than 10 mm was obtained after four weeks of regeneration culture.

To examine the initial process of the rapid shoot regeneration on TDZ-containing medium, histological observation of the callus was performed at an early stage after its transfer onto the medium containing 1 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA. The calluses and/or regenerated shoots were collected 0 (untreated) to 7 days after culture and embedded in Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany) after treating with the standard procedures of fixation and dehydration. Sections of about 4 μm thick were cut with Microtome (Yamato Kohki Co. Ltd., Asaka, Japan) and stained with toluidine blue. Microscopic images were captured using a VB-6000 image control system (Keyence, Osaka, Japan) equipped with an IX71 microscope (Olympus, Tokyo, Japan).

At the initial stage (day 0), callus tissues consisted of various sizes of cell clusters, each of which was surrounded by a distinct cell layer, presumably a protoderm (Figure 3A). At this stage, some of the cell clusters gave rise to small clustered cells that resembled proembryoid (Ho and Vasil 1983) comprising small cells with high nucleus to cytoplasm ratio at the peripheral cell layer (Figure 3B; indicated by arrowhead). After 3 days of culture, pro-vascular strands differentiated within the globular proembryoid (Figure 3C, D; indicated by arrowheads). The embryo-like structures formed meristematic tissues and coleoptile-like structures after 5 days of culture (Figure 3E), and the structures continued to develop after 7 days of culture (Figure 3F). However, development of root primordia was not observed at this stage. Nevertheless, the developmental pattern of the proembryoids and embryo-like structures was similar to those of somatic embryogenesis observed in the previous studies (Franklin et al. 2006; Garcia et al. 2007; Ho and Vasil 1983), which applied zeatin with or without gibberellic acid (GA), 6-benzylaminopurine (BA) with NAA, and no growth regulators, respectively. Somatic embryogenesis was also stimulated by TDZ in rice caryopsis culture (Gairi and Rashid 2004), suggesting that TDZ may commonly affect the differentiation of embryogenic calluses in gramineous plants. Thus, it is presumed that the TDZ-stimulated rapid shoot regeneration is achieved through a process similar to somatic embryogenesis and that TDZ stimulated the onset of shoot differentiation of embryo-like structure but not the root primordium in sugarcane cv. NiF8 used in the present study.

The shoots produced on TDZ-containing medium easily produced roots (100% rooting) when they were transferred onto a culture bottle (55 mm diameter×110 mm height) containing the modified MS medium without plant growth regulators (Figure 1E). In previous studies, roots were also induced using medium without plant growth regulators (Desai et al. 2004; Franklin et al. 2006; Garcia et al. 2007; Lakshmanan et al. 2006),

although IBA was used for root induction of TDZ-treated shoots (Chengalrayan and Gallo-Meagher 2001; Chengalrayan et al. 2005; Gallo-Meagher et al. 2000). These i*n vitro* rooted plants were successfully acclimatized (100%) in soil in pots (Figure 1F) and then grown in the field. Since the callus used in the present study could maintain the regeneration competence for more than a year, an efficient and reproducible genetic transformation using the rapid callus regeneration method should be feasible in the near future.

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