Intact roots promote shoot regeneration from hypocotyl independent of exogenous plant growth regulators in eggplant in vitro

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Abstract: Eggplant (Solanum melongena) seedlings cultured in vitro were excised at the center of the hypocotyl to generate decapitated seedlings with intact roots. This modification of the complete decapitation method (CDM) developed in vivo by Harada et al. (2005) allowed in vitro culture (CDM in vitro; CDMi). As controls, rootless hypocotyl segment explants (approximately 1 cm) and cotyledon explants were cultured on media supplemented with 4.4 µM 6-benzyladenin (BA) and 0.2 µM thidiazuron (TDZ), respectively. Cotyledon explants had formed calli 2 weeks after excision but did not develop adventitious buds, despite the use of optimal conditions reported previously for a different eggplant cultivar. Calli formed at the cut ends of hypocotyls 1 week after excision in both CDMi and hypocotyl cultures, and adventitious buds regenerated 1 week earlier in CDMi. Six weeks after excision, CDMi yielded 11.4 adventitious buds per explant, but only 4.1 formed in hypocotyl culture. Moreover, shoots longer than 1 cm developed 2 weeks earlier in CDMi than in hypocotyl culture. The number of shoots per explant was 8.1 in CDMi, but only 2.4 in hypocotyl culture 6 weeks after cutting. All shoots that developed were rooted on MS medium in CDMi, but only 71% of shoots formed roots in hypocotyl culture. These results indicate that intact roots are important for explant shoot regeneration and development, and CDMi is a simple and efficient method for obtaining multiple shoots without the need to determine optimal concentrations of plant growth regulators and overcome inhibition of rooting in the obtained shoots.

Keywords: adventitious shoot, complete decapitation method, micropropagation, Solanum melongena

Abbreviations: BA, 6-benzyladenin; CDM, complete decapitation method; CDMi, CDM in vitro; PGR, plant growth regulator; TDZ, thidiazuron.

Introduction

Agrobacterium tumefaciens-mediated genetic transformation is an effective and widely used approach to introduce desirable genes into plants. This transformation method is typically based on in vitro culture method. Therefore, to obtain a number of transgenic plants, a high frequency in vitro regeneration method is required. In vitro regeneration of different explant types, e.g. cotyledon, hypocotyl, leaf and root, from several cultivars of eggplant (Solanum melongena L.) has been reported both via embryogenesis (Matsuoka and Hinata 1979, Rao and Singh 1991, Saito and Nishimura 1994, Sharma and Rajam 1995) and organogenesis (Kamat and Rao 1978, Fassuliotis et al. 1981, Allichio et al. 1982). In both techniques, the combination and concentration of plant growth regulators (PGRs) should be determined through complex and empirical processes. However, in eggplant tissue culture, the optimal PGR conditions for regeneration are reported to differ.
depending on the cultivar, growing conditions of the mother plant, explant type and the morphogenetic response that varies within the same explant (Fassuuliotis et al., 1981, Sharma and Rajam 1995). Similarly, in strawberry culture, adventitious shoot regeneration differed among cultivars and explant types when a range of explant types from seven cultivars were cultured in the same PGR condition (Passey et al. 2003). These reports indicate that decisions regarding optimal PGR concentrations and combinations for in vitro regeneration are difficult, and additional examinations might be required to regenerate plants from an unstudied cultivar in tissue culture using PGRs. In addition, rooting inhibition of shoots derived from explants grown in the presence of thidiazuron (TDZ) (Magioli et al. 1998) or 6-benzyladenin (BA) (Sharma and Rajam 1995) has been reported previously in eggplant culture. To obtain a number of regenerated shoots with high rooting capacity, an efficient in vitro method independent of PGRs for shoot regeneration is demanded.

The aim of this study was to establish an efficient in vitro method of eggplant culture for adventitious shoot regeneration without PGRs and inhibition of rooting from the regenerated shoot. Recently, the complete decapitation method (CDM) was developed for mass propagation in tomato plants grown in an open field (Harada et al. 2005), and the efficiency of the method was improved by Johkan et al. (2008a, b, c). In this method the main and lateral stems are excised, which enables adventitious shoot regeneration from the cut ends of stems in vivo. Tezuka et al. (2011) hypothesized that the promotive effect of CDM on shoot regeneration was due to utilization of endogenous cytokinin synthesized after decapitation. It is generally believed that endogenous cytokinins in higher plants are mainly biosynthesized in the root system and transported via the xylem to the aboveground parts (Davies 2004). From these reports, in vitro culture of decapitated seedlings composed of an intact root attached to the remaining shoot axis, i.e., applying CDM to in vitro culture (CDM in vitro; CDMi), has potential to be an efficient in vitro method independent of PGRs for mass propagation of eggplant. Pozueta-Romero et al. (2001) developed a similar method independent of PGRs for tomato and bell pepper. In this method, one cotyledon and the apical and axillary meristems are excised and the resulting seedlings, resembling a flamingo bill, are used as explants. Amutha et al. (2009) applied this method to several major dicotyledonous clades. However, this method has the possibility of adventitious bud initiation because complete excision of the axillary meristem near the remaining cotyledon is difficult. In Agrobacterium tumefaciens-mediated transformation by CDMi, we thought that a transformant obtained from an axillary meristem could be a chimera, because the fate of most axillary meristems had been determined at the time of the excision. To reduce the risk of chimera, seedlings should be decapitated at the center of the hypocotyl for complete excision of all meristems. However, the regeneration capacity of seedlings with no leaves and cotyledons were obscured. Therefore, in the present study, we investigated the possible application of the CDM to eggplant culture in vitro at the center of hypocotyl, and estimated the efficiency of intact roots on shoot regeneration and rooting of the regenerated shoots compared with rootless hypocotyl and cotyledon segment cultures using PGRs.

Materials and Methods

Plant material

Seeds of eggplant (Solanum melongena L. cv. Shisui; Takii & Co., Ltd., Kyoto, Japan) were thoroughly washed in running tapwater, and subsequently surface-sterilized for 10 min in 10% sodium hypochlorite solution containing 1–2 drops of Tween 20. After three rinses with sterile distilled water, the seeds were sown on MS basal medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. The medium pH was adjusted to 5.7–5.8 prior to the addition of agar, then the medium was autoclaved for 15 min at 121°C. Seedlings were cultured under 16 h photoperiod with fluorescent light at 70 μmol m−2 s−1 photosynthetic photon flux (PPF) at 25°C. The seedlings grown in vitro (14–16 days after germination) were used as the source of explants.

Effect of CDMi on adventitious bud and shoot regeneration compared with tissue cultures supplemented with plant growth regulators

The eggplant seedlings grown on MS medium were transferred to fresh MS medium and decapitated at the center of the hypocotyl with the roots remaining intact (CDM). As a control tissue culture, hypocotyl and cotyledon explants were used. Hypocotyl explants, approximately 1 cm long, were placed in a vertical position on MS medium supplemented with 4.4 μM BA. Cotyledon explants, approximately 10 mm × 5 mm, were placed with the abaxial side facing downward on MS medium supplemented with 0.2 μM TDZ. The PGR concentration in cotyledon culture was determined by previous report (Magioli et al. 1998) and the PGR condition in hypocotyl culture was selected by our preliminary experiments on the basis of previous reports (Kamat and Rao 1978, 1981, Sharma and Rajam 1995).
Matsuoka and Hinata 1979). These explants were cultured under a 16 h photoperiod with fluorescent light at 70 μmol m⁻² s⁻¹ photosynthetic photon flux (PPF) at 25°C. The percentage of surviving explants, the percentage of surviving explants with callus and adventitious bud formation, and the number of adventitious buds per explant were determined every week after excision. The regenerated shoots from the cut surface were harvested individually when they had grown to approximately 1 cm in length, and the number of harvested shoots was counted every week after excision.

**Rooting**

The regenerated shoots, once approximately 1 cm in length, were excised individually and cultured on MS basal medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar under a 16 h photoperiod with fluorescent light at 70 μmol m⁻² s⁻¹ photosynthetic photon flux (PPF) at 25°C. The percentage of shoots that formed roots and the number of roots per shoot were determined 4 weeks after excision.

**Results and Discussion**

**Effect of CDMi on adventitious bud and shoot regeneration compared with tissue cultures supplemented with plant growth regulators**

All cotyledon explants formed callus at 2 weeks after excision (WAE) and survived until 6 WAE. However, no explants formed adventitious buds and shoots from the callus despite the use of an optimal PGR condition reported for other cultivars (Fig. 1A, B). This result indicates that the concentration and combination of PGRs for shoot regeneration is highly cultivar-dependent in tissue culture of eggplant, and
extensive experimentation is required to regenerate adventitious buds from an unstudied cultivar with protocols using PGRs.

In both CDMi and hypocotyl culture, the apical cut end of hypocotyl explants became enlarged within a few days after excision, and all explants formed callus at 1 WAE and survived until 6 WAE (Fig. 2 A). Although calli formed concurrently in both treatments, adventitious buds regenerated from the callus 1 week earlier in CDMi than hypocotyl culture (Fig. 1 A, B and Fig. 2). In CDMi, the intact roots of decapitated seedlings drastically developed within 2 WAE compared with those of 4-weeks-old seedlings grown in vitro without decapitation (Fig. 3). In hypocotyl culture, callus formed at both the apical and basal end of hypocotyl explants. However, adventitious buds formed only from callus at the apical end (Fig. 2 C). Similar polarity in regeneration response was observed in hypocotyl culture of Capsicum frutescens under a 16 h photoperiod (Kumar et al. 2007). At 6 WAE, the number of adventitious buds per explant in CDMi was significantly higher than that in hypocotyl culture, namely 11.4 and 4.1, respectively (Fig. 1 C). CDMi promotes regeneration of adventitious buds from callus on the cut surface compared with hypocotyl culture in eggplant regardless of the absence of PGRs. The shoots that developed from adventitious buds that grew to 1 cm in length were excised individually. Shoot excision was 2 weeks earlier in CDMi than hypocotyl culture (Fig. 1 D). At 6 WAE, the number of shoots obtained per explan in CDMi was significantly higher compared with that in hypocotyl culture, namely 8.1 and 2.4, respectively. This result indicates that the presence of intact roots stimulates shoot development and CDMi is an efficient method in the absence of PGRs for mass propagation because a number of regenerated shoots are obtained without additional examination to determine the optimal PGR condition.

In plant tissue cultures, organ differentiation is regulated by the relative concentration of PGRs in the culture medium; a relatively higher cytokinin concentration induces bud regeneration, whereas a relatively higher auxin concentration induces root regeneration, and intermediate concentrations induce only callus formation (Thorpe 2007). In the present study, cotyledon explants of ‘Shisui’ formed only callus and no adventitious buds developed in the treatment with PGR concentrations optimal for adventitious bud regeneration reported previously for a different cultivar. This difference in adventitious bud regeneration between cultivars might be caused by differences in endogenous hormones levels between the cultivars. On the other hand, CDMi promoted adventitious bud formation from callus on the cut surface of hypocotyls in the absence of PGRs, and the number of adventitious buds in CDMi was higher than that of hypocotyl culture supplemented with exogenous BA at 4.4 µM (Fig. 1 C). This result suggested that the hormonal balance of the explant in
In the present study, the intact roots of decapitated seedlings in CDMI were grown on PGR-free medium, elongation of the shoots was drastically developed within 2 weeks of excision (Table 1). From these results, the well-developed intact roots synthesized cytokinin would be used to regeneration adventitious buds. In fact, the presence of intact roots is important for shoot regeneration.

In addition, shoots longer than 1 cm developed at a higher frequency in CDMI compared with hypocotyl culture supplemented with 4.4 μM BA (Fig. 1 D). In plant tissue culture, media of different composition are used depending on the stage of regeneration, e.g. callus-induction medium, shoot-induction medium, shoot-elongation medium and root-induction medium. In root culture of eggplant, Franklin et al. (2004) reported that elongation of regenerated shoots was not synchronous in cultures of cytokinin-containing media; in some cultures few shoots elongated, whereas others remained diminutive. When root explants cultured on initial media were subcultured on PGR-free medium, elongation of the shoots was promoted (Franklin et al. 2004). This report and our present results indicate that the intact root system of explants in CDMI may regulate the levels of biosynthesis and transport of cytokinin, and optimize the endogenous hormone balance depending on the stage of regeneration, i.e. shoot induction or shoot elongation. In eggplant tissue culture, optimal concentrations and combinations of PGRs in the culture medium are reported to differ markedly depending on the cultivar and explant type (Fassuliotis et al. 1981, Allichio et al. 1982). In the present study, cotyledon explants of ‘Shisui’ formed no adventitious buds under the optimal PGR condition reported for a different cultivar. In contrast, explants with an intact root system in CDMI may self-regulate the endogenous hormone balance to regenerate adventitious shoots and elongate the regenerated shoots, so CDMI is an efficient and simple method for production of multiple shoots without need for extensive subculturing.

### Rooting

The excised shoots were placed on MS basal medium for rooting. All excised shoots in CDMI had formed roots at 4 WAE, whereas 63% of the control shoots had formed roots (Table 1).

Successful rooting of regenerated shoots is a prerequisite for micropropagation. In root explant cultures of eggplant, shoots obtained from media supplemented with TDZ, which promotes shoot formation, did not form roots even after being transferred to root-induction medium containing auxin (Franklin et al. 2004). Similar inhibition of rooting has been reported in shoots derived from explants cultured with TDZ (Magioli et al. 1998) and BA (Sharma and Rajam 1995). Magioli et al. (1998) achieved a 70% rooting efficiency when calli were maintained on PGR-free media before excision of the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rooting (%)</th>
<th>Number of roots/cm shoot</th>
<th>Root length/cm</th>
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</thead>
<tbody>
<tr>
<td>Hypocotyl culture</td>
<td>63</td>
<td>1.6 ± 0.2</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>CDMI</td>
<td>100</td>
<td>2.1 ± 0.2</td>
<td>3.4 ± 0.2</td>
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Data represent the mean ± SE (n = 16).

** indicates a significant difference at P < 0.01 by Student’s t test.

CDMI was more optimal for adventitious bud regeneration than in hypocotyl culture with 4.4 μM BA. It is generally believed that endogenous cytokinins in higher plants are mainly biosynthesized in the root system and transported via the xylem to the aboveground parts where they regulate growth and development (Van Staden and Davey 1979, Noden et al. 1990, Davies 2004). From these reports and our present results, we hypothesize that the promotive effect of CDMI may be attributed to the presence of the intact root system and utilization of endogenous cytokinin that is biosynthesized by the roots for adventitious bud and shoot regeneration.

In many plant species, growth and development are regulated by hormonal interactions, e.g. the shoot apexes repress axillary bud growth and grow dominantly. Auxin, derived from the shoot apex, inhibits the growth of axillary buds, whereas cytokinin, derived from the roots, promotes the growth of axillary buds. Decapitation of *Vicia* plants induces outgrowth of axillary buds, but application of auxin to the stump prevents outgrowth of axillary buds (Thimmann and Skoog 1933, 1934). In addition, decapitation in bean and pea leads to transient increases in cytokinin levels in the xylem sap or shoot, and exogenous auxin application partially suppresses these increases (Bangerth 1994, Li et al. 1995). These reports indicate that decapitation, i.e. removal of the site of auxin biosynthesis, accelerates the biosynthesis of endogenous cytokinin in the intact root system, and subsequently axillary buds develop as a result of cytokinin transport through the xylem. In addition, Wightman and Thimmann (1980) reported that removal of epicotyl and cotyledons increased the length of primary and lateral roots of pea seedlings. In the present study, the intact roots of decapitated seedlings in CDMI drastically developed within 2 WAE compared with those of 4-weeks-old seedlings grown in vitro without decapitation (Fig. 3). From these results, the well-developed intact roots synthesized endogenous cytokinin after excision, and the
shoots for a period of 2 weeks after adventitious bud induction by TDZ. From these observations, we hypothesize that excess exogenous cytokinins are not suitable for micropropagation because they inhibit rooting of regenerated shoots. In the present study, rooting of excised shoots was not inhibited in CDMi, whereas hypocotyl culture in the presence of BA inhibited rooting. Kuroha et al. (2002) reported that endogenous cytokinin negatively regulates adventitious root formation on cucumber hypocotyls. These results support the above hypothesis, and indicate that the intact root system in CDMi regulates the synthesis and transport of endogenous cytokinins depending on the stage of regeneration, i.e., induces them during shoot regeneration and suppresses them during shoot elongation. In contrast, regenerated shoots from hypocotyl culture continued to be supplied with BA from their medium. Therefore, the inhibition of rooting from these shoots might be due to increase in the endogenous BA level.

In the present study, CDMi formed adventitious bud and shoot formation in the absence of PGRs. This promotive effect of CDMi might be attributed to the presence of intact root systems that biosynthesized endogenous cytokinin. The utilization of endogenous cytokinin enabled all excised shoots to develop roots, although exogenous cytokinin treatment inhibited rooting in hypocotyl culture. It is concluded that CDM with intact roots is a simple and efficient method for mass propagation of eggplant in vitro without the need for complicated experimentation to determine the optimal concentration and combination of PGRs required. In addition, CDMi has potential to be an efficient in vitro transformation method.

Acknowledgments

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References

Sharma P, Rajam MV 1995 Genotype, explant and position


Mr. Hideyuki Tanaka is a doctoral student in Osaka Prefecture University. He is interested in the role of endogenous auxin and cytokinin in regeneration via both embryogenesis and organogenesis.