Efficient whole plant regeneration of common bean (*Phaseolus vulgaris* L.) using thin-cell-layer culture and silver nitrate

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Abstract

A method was designed to optimize rapid and high frequency direct shoot regeneration (without intermediate callus) of the commercially important common bean, *Phaseolus vulgaris* L., using the transverse thin cell layer (tTCL) method. The pretreatment of seeds with 10 μM TDZ significantly increased bud regeneration frequency on tTCL. A 2-week culture of tTCLs on 10 μM TDZ followed by a reduction in the TDZ concentration (1 μM) was needed to achieve optimal bud induction and further development of the neo-formed buds. An incubation period greater than 2 weeks of tTCLs with 10 μM TDZ concentration resulted in inhibitory effects on the development of shoots and roots. Shoot development was enhanced by 10 μM BAP and 10 μM AgNO₃, leading to 100% well developed shoots. Regenerated plants developed into true-to-type fertile plants. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Transverse thin cell layers; Common bean; Direct shoot regeneration; Thidiazuron; Silver nitrate; Particle bombardment

1. Introduction

The common bean (*Phaseolus vulgaris* L.) is an annual, predominantly self-pollinated legume that constitutes a major source of dietary protein throughout both Latin America and Eastern Africa [1]. However, it is a crop which is very sensitive to drought [2,3], as well as to a vast array of pathogens such as bacteria, virus, fungus and insects, which largely reduce yield. The production of transgenic bean, through genetic engineering, with the introduction of drought resistance or defence response genes should be highly beneficial as it could raise the yield or improve the seed quality of this important legume. However, transformation procedures can only be successful if an efficient protocol of regeneration has previously been established. Therefore, the first major objective of the present study was to develop a reliable and highly efficient regeneration protocol which would enable large-scale production of true-to-type bean plants. Some bean regeneration procedures have been described from shoot apex cultures [4,5], from cotyledonary and primary leaves node explants [6] devoid of axillary meristems [7,8] or from cotyledonary node explants with axillary buds and a portion of the cotyledons [9], from explants consisting of the petiole and part of the blade of juvenile leaves [10] and from embryo-derived callus [11]. The problem underlying these procedures is a low frequency of regeneration. High-frequency induction of direct shoot formation from intact seedlings has been established by Malik and Saxena [12] using thidiazuron (TDZ) and N⁶-benzylaminopurine (BAP). Never-
theless, such a regeneration procedure using intact seedlings is not suitable for transformation since two phenomena are likely to occur: regenerants developed on a voluminous inoculum might escape from the selective conditions and/or, the killing of the non-transformed tissue under selective conditions might block the regeneration process. Hence, our second objective was to achieve a successful direct shoot regeneration using transverse thin cell layers, thus avoiding intact seedling or voluminous inoculum and therefore raising the compatibility of the proposed protocol with transformation procedures.

In the present paper, we report on the morphogenetic potential of *P. vulgaris* cv. Carioca transverse thin cell layers (tTCLs) to regenerate multiple shoots directly (without a callus phase). The TCL method [13–15] has already been used to regenerate other leguminous species such as *Psophocarpus tetragonolobus* [16], soybean [17], and cowpea [18]. Furthermore, we show that the use of thidiazuron (TDZ), a substituted phenylurea derivative with a high cytokinin-like activity, and silver nitrate (AgNO₃) a potent ethylene action inhibitor, results in highly efficient differentiation and development of shoots.

Up to now, regarding genetic transformation attempts with this crop, no *Agrobacterium*-mediated transformation has been successfully achieved except with another species, *Phaseolus acutifolius* A. Gray [19]; there are some cases where stable transgenic plants were obtained by direct gene transfer through particle bombardment [20–23]. In this work, a preliminary assay to assess the feasibility of the combination of this system with transformation by biolistic was also undertaken.

2. Materials and methods

2.1. Preparation of seedlings and tTCLs culture

Seeds of *P. vulgaris* L. cv. Carioca (Brazil) were washed thoroughly with distilled water and Tween-20 (10%). They were then sonicated for 5 min and rinsed three times with sterile distilled water. Subsequently they were surface sterilized with 70% ethanol for 2 min followed by sodium hypochloride (6%) for 15 min. After five rinses with sterile distilled water, they were allowed to germinate aseptically on half strength Murashige and Skoog (MS) medium [24] solidified with 0.4% agar–agar (Difco). The effect of the pretreatment of the seedlings by 10 μM thidiazuron (TDZ) on subsequent regeneration expression was assessed by supplying the germination media with this compound. In addition, the effect of light on the same regeneration expression was evaluated by using seedlings germinated in the dark or under a cool fluorescent light (100 μmol m⁻² s⁻¹) at 25 ± 1°C.

Thin cell layers (0.3–0.5 mm thick) were excised transversely (tTCLs) from 2-week-old seedlings at four zones on the plantlet: epicotyl, hypocotyl, cotyledons and roots and were cultured on Petri dishes (10 cm in diameter) with 20 ml MS medium supplemented with Gamborg's vitamin mix [25] (termed MSB₅) and different concentrations of TDZ or BAP (1–10 μM). From this preliminary assay, the responsive zone was determined and used for subsequent study. All culture media were solidified with 0.6% agar–agar and adjusted to pH 5.7 with 0.1 N NaOH or 0.1 N HCl before autoclaving at 121°C for 15 min. The tTCLs were cultured in a 16-h photoperiod (100 μmol m⁻² s⁻¹) at 25 ± 1°C with 60% humidity. Every 7–10 days tTCLs were subcultured on fresh culture medium. In an attempt to improve regeneration efficiency and/or development of regenerated buds, an ethylene action inhibitor, silver nitrate (AgNO₃), was tested.

For each treatment 10–15 seedlings were used (i.e. approximately 150 tTCLs) and each experiment was repeated at least twice. Observation was made every week and treatment effects were quantified on the basis of the number of regenerants obtained on all tTCLs originated from one original seedling. Statistical significance (rejection at *P > 0.05*) of the different treatments used was assessed by the Kruskal–Wallis one-way Anova using the software Statistix© for Windows (version 2.0, Analytical Software).

2.2. Scanning microscope observations

For scanning microscope analysis, tTCLs at different stages of culture were frozen in liquid nitrogen and observed using a scanning electron microscope (JEOL, model JSM-840A, Japan).
2.3. Transformation procedure and gus assay

Explants to be bombarded consisted of the apical and subapical areas of the seedling (1/1.5 cm) prepared from 2-week-old Phaseolus seedlings germinated as described previously. They were inoculated (apex to the top) on MSB₅ medium containing 3 μM TDZ and 2.5% agar–agar and left at 22°C for 24 h before bombardment was undertaken.

The pBI211 plasmid vector was used [26]. This plasmid contains the chimeric gene encoding the GUS reporter gene under the control of the CaMV35S promoter. DNA-coated particles were prepared by resuspending tungsten particles (M10, Biorad) in sterile water and adding DNA to a final concentration of 0.01 mg DNA:mg tungsten with 170 mM spermidine and 18.8 M CaCl₂. The suspension was left on ice for 10 min and then centrifuged. Precipitated DNA-coated particles were resuspended in 100 ml 100% ethanol and mixed by sonication (Tomy Seiko Co. Ltd., Japan). Each Petri plate containing 10–20 explants was bombarded three times (each shot delivering 150–225 mg DNA).

After 48 h, bombarded and control explants were tested for gus gene expression. The histochemical analysis for β-glucuronidase activity was done according to Jefferson and co-workers [27]. Explants were incubated overnight at 37°C in a 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution buffered with 50 mM sodium phosphate buffer at pH 7.

3. Results

3.1. Factors affecting germination and bud regeneration from tTCLs

In order to improve the frequency of bud regeneration in tTCLs several factors were analysed separately or simultaneously.

3.1.1. Germination

The germination percentage of seeds was higher when it occurred in the dark as opposed to light (85 and 50%, respectively). Germinating seedlings were maintained in the dark for 1 week and then were transferred to light where they remained for another week before tTCLs were excised. Furthermore, tTCLs excised on seedlings that had grown in the dark for 1 week neoformed a higher number of buds than those which had not been subjected to dark conditions (data not shown). Thus, seeds were systematically allowed to germinate in the dark for 1 week before being transferred to light for another week.

3.1.2. Determination of the seedling’s responsive zone

Transverse TCLs were excised from 2-week-old seedlings and cultured in MSB₅ medium supplemented with TDZ or BAP. After 2-week culture, proliferating tTCLs showing shoot organogenesis were obtained with tTCLs excised exclusively at the epicotyl, more specifically from a zone between the primary leaves internode and the apex of the seedling (approximately 5–10 responsive tTCLs per seedling). On tTCLs excised at the cotyledonal nodes, the frequency was <1%. Transverse TCLs from the hypocotyl, cotyledons and roots turned into dark brown calli and did not regenerate. Therefore, all subsequent treatments were effected on tTCLs excised at the epicotyl zone.

3.1.3. Induction medium

The optimal induction medium for bud proliferation on tTCLs was assessed using TDZ or BAP at a concentration range of 0–10 μM supplemented with MSB₅ medium. After 6-week culture, the number of buds neoformed was scored. Analysis of data by Kruskal–Wallis one-way Anova was undertaken and the result indicates that the differential response of tTCLs regarding the growth regulator used was highly significant (P = 0.0001). Maximal bud induction was obtained with a concentration of 10 μM for both growth regulators but the average number of buds obtained on TDZ supplemented medium was 46.2 (per initial seedling) as opposed to 25.3 buds obtained on BAP supplemented medium (Table 1). Therefore, tTCLs bud primary induction medium for subsequent experiments consisted of MSB₅ medium supplemented with 10 μM TDZ.

3.1.4. Pretreatment of seeds

The impact of seed pretreatment with TDZ on bud regeneration frequency was assessed. Seeds were allowed to germinate for 2 weeks on germination medium supplemented with 0 or 10 μM
Table 1
Effect of induction medium on the regeneration of buds in *P. vulgaris* L. cv. Carioca

<table>
<thead>
<tr>
<th>Concentrationa (µM)</th>
<th>Averageb number of bud primordia per original seedling</th>
<th>TDZ</th>
<th>BAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>3.5 (1–9)</td>
<td>28.5 (15–40)</td>
<td>3.5 (1–9)</td>
</tr>
<tr>
<td>3</td>
<td>9 (5–12)</td>
<td>38.8 (33–45)</td>
<td>14.4 (5–25)</td>
</tr>
<tr>
<td>5</td>
<td>14.4 (5–25)</td>
<td>42.5 (31–57)</td>
<td>25.3 (18–33)</td>
</tr>
<tr>
<td>10</td>
<td>25.3 (18–33)</td>
<td>46.2 (38–55)</td>
<td>46.2 (38–55)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TDZ</th>
<th>BAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pret.</td>
<td>Pret.</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>31 (14–54)</td>
</tr>
<tr>
<td>3</td>
<td>28.3 (20–53)</td>
</tr>
<tr>
<td>4</td>
<td>52.3 (23–80)</td>
</tr>
</tbody>
</table>

a Concentration of TDZ or BAP supplemented to MSB5 medium.
b Average of three experiments each using 10 seedlings (total of ~150 tTCLs excised at the seedlings responsive zone). Data scored after 6 weeks culture. Figures in parentheses represent the range observed.

TDZ. Following this time, tTCLs were excised and cultured on primary induction medium (MSB5 + 10 µM TDZ). After 6 weeks culture the number of neoformed buds was scored. Analysis of data indicate that the differences found are significant (*P* = 0.0005). The seed pretreatment raised by ~48% the average number of buds obtained (per initial seedling).

3.2. Optimal procedure for maximal bud neoformation on tTCLs

A seed pretreatment with 10 µM TDZ followed by tTCL culture on primary induction medium (MSB5 + 10 µM TDZ) was found to be optimal for bud induction without callus formation. However, it was observed that a prolonged exposure to this TDZ concentration had an inhibitory effect on further development of shoots and roots. Therefore, the optimal duration of tTCL culture on primary induction medium was assessed. Following 0-, 1-, 2-, 3- and 4-week culture on this high TDZ medium, tTCLs were transferred to a secondary medium consisting of MSB5 medium supplemented with a lower concentration of TDZ (1 µM) or to MSB5 medium without any growth regulator (Table 2). Statistical analysis of data indicate that the differences between the duration of tTCL culture on primary induction medium were significant (*P* = 0.0002). Also, extremely significant differences were found between the secondary media tested (*P* = 0.0000). Hence, optimal bud regeneration and avoidance of TDZ inhibitory effects was achieved by a three-step procedure: a seed pretreatment with 10 µM TDZ and culture in the dark for 1 week, excision and culture of tTCLs on a primary induction medium consisting of MSB5 + 10 µM TDZ for 2 weeks followed by a transfer of proliferating tTCLs to a secondary medium of reduced TDZ concentration (MSB5 + 1 µM TDZ) (Table 2).

3.3. Mode of regeneration

Buds neoformed directly on the surface of the responsive tTCL without callus formation as can be observed from scanning electron microscopy.

Table 2
Regenerative response of *P. vulgaris* L. cv. Carioca tTCL as modulated by the pretreatment of seeds, the duration of tTCLs exposure to 10 µM TDZ and the subsequent culture medium (with or without 1 µM TDZ)

<table>
<thead>
<tr>
<th>No. weeks on TDZ (10 µMa)</th>
<th>Averageb number of regenerated bud primordia per original seedling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSB5</td>
</tr>
<tr>
<td></td>
<td>No pret.c</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>6.3 (1–14)</td>
</tr>
<tr>
<td>2</td>
<td>11 (9–15)</td>
</tr>
<tr>
<td>3</td>
<td>23.3 (19–30)</td>
</tr>
</tbody>
</table>

a Medium in which excised tTCLs were initially cultured.
b Average of three experiments each using 10 seedlings (total of ~150 tTCLs excised at the seedlings responsive zone). Data scored after 6 weeks of culture. Figures in parentheses represent the range observed.
c Seeds were germinated in normal germination medium.
d Seeds were germinated in germination medium supplemented with 10 µM TDZ.
Table 3
Effect of silver nitrate (AgNO₃) and N⁶-benzylaminopurine (BAP) on shoot development of *P. vulgaris* L. cv. Carioca

<table>
<thead>
<tr>
<th>Shoot developing media</th>
<th>% of developed shoots</th>
<th>Range of shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSB₅</td>
<td>41.8</td>
<td>0.3–0.6</td>
</tr>
<tr>
<td>MSB₅+BAP (10 μM)</td>
<td>63.8</td>
<td>1.3–1.7</td>
</tr>
<tr>
<td>MSB₅+BAP (10 μM) + AgNO₃ (10 μM)</td>
<td>100</td>
<td>1.8–2.9</td>
</tr>
<tr>
<td>MSB₅+AgNO₃ (10 μM)</td>
<td>51.3</td>
<td>0.8–1.1</td>
</tr>
</tbody>
</table>

*Average percentage of shoots which developed from initial buds on 6-week-old tTCLs (N=265). Data scored after 2 weeks culture.

(Fig. 1A–C). No callus occurred on proliferating tTCLs (Fig. 1D). Further buds originated at the base of neoformed shoots leading to a high density of buds on tTCLs (Fig. 1C).

3.4. Effect of AgNO₃ on shoot development

Full development of shoots was obtained when bud primordia were transferred to MSB₅ medium containing 10 or 20 μM BAP which allowed full leaf development as opposed to TDZ under the same conditions (data not shown). However, only a small number developed. Based on previous results [28], an inhibitor of ethylene action (AgNO₃) was added to a medium with or without BAP. Analysis of data indicate that the combination of BAP and AgNO₃ at equimolar concentrations (10 μM) significantly raised the number of developed shoots. An average of 100% developed shoots were scored with a length range of 1.8–2.9 cm indicating that these conditions were optimal for shoot development (Table 3, Fig. 1E). Other concentrations of AgNO₃ (1 μM, 50 μM) tested in combination or not with BAP had no effect (data not shown).

In preliminary assays for shoot growth, we have observed that with increasing shoot/bud density, shoot elongation and rooting decreased. To minimize such an effect, clusters of no more than five shoots were isolated from original tTCL explants and transferred to a medium containing 1 μM BAP, 10 μM AgNO₃ and 3 μM GA₃ to promote further elongation. Shoots were subcultured in fresh medium every 7–10 days. After 1 week, several shoots attained 4 cm length. They then were transferred into glass jars (200 ml) containing rooting medium.

3.5. Rooting and transplantation of the neoformed shoots

Rooting was obtained on MSB₅ containing 1 μM NAA and 10 μM AgNO₃ (Fig. 1F) on 80% of the regenerated plants. After 2–3 weeks, they were transferred to the greenhouse in 8-cm pots containing a mixture of peat and vermiculite (50/50, v/v) (Fig. 1G). Roots were previously washed in order to eliminate any remaining culture medium. Maximal humidity was assured during the first week by placing an inverted glass beaker over each plant before transfer to field conditions. The survival rate of rooted plantlets transferred to soil was 95% and these were fertile with normal pod development.

3.6. Expression of β-glucuronidase following bombardment

In order to determine the capacity of explants to be transformed, gene transfer was undertaken using a biolistic approach. Explants of 1/1.5 cm consisting of the seedling zone shown to be responsive to bud regeneration were bombarded with tungsten beads containing a binary vector with the *uidA* (GUS) gene under the transcriptional control of the CaMV35S promoter. Two days after the bombardment, explants were incubated overnight at 37°C with X-gluc and the strong blue staining was observed (Fig. 1H). The bombarded explants tested were excised to tTCLs and these remained viable (data not shown).

4. Discussion

The lack of ability to regenerate common bean with high frequency has been the main reason of the failure to produce transgenic plants efficiently. Transfer of genes into beans by the *Agrobacterium* system has been attempted, but no regeneration of transformed plants was achieved [29]. Stable transgenic bean plants were obtained using an electrical acceleration device for the delivery of DNA to embryos [20]. However, the tissue culture protocol was time-consuming and with a low regeneration frequency (an average of two shoots/explant harvested) leading to a low frequency of transgenic plants (0.03%). More recently, stable transformants by bombardment of DNA were attained by
Fig. 1. A–G. Direct regeneration of *P. vulgaris* (cv. Carioca) plants from transverse TCLs. A, B, C, Scanning microscope observation of tTCLs. (A) Newly formed buds, *b*, arose directly without callus formation after 10 days culture. Bar = 100 µm. (B) Clusters of shoot buds continue to proliferate after 2 weeks culture, bar = 100 µm, and after 3 weeks culture, newly formed buds (arrows, C) arose at the base of existing ones (*b*). Bar = 1 mm. (D) Four-week-old tTCLs presenting multiple buds (arrows). Bar = 1 mm. (E) Cluster of shoots on developing medium at 6-week stage. Bar = 1 mm. (F) Well-developed shoot cultured separately on rooting medium at 9-week stage. Bar = 5 mm. (G) Regenerated *P. vulgaris* plant at 11-week stage. Bar = 3 cm. (H) Gus expression on 2-week-old *P. vulgaris* seedling explants comprising the responsive zone of bud regeneration, after particle bombardment with the pBI211 vector carrying the *uid A* gene under the control of the 35SCaMV promoter, *pl*, primary leaves, *a*, apex. Bar = 1 mm.
other researchers [22,21], but in one case, one bombarded embryonic axis gave rise to one transformed plant (six plants transformed out of the 319 bombarded embryonic axes) since no multiple shoot induction was possible [22] and in the other, only 9% regenerated shoots were obtained after the bombardment of 3079 embryonic axes, with an average transformation frequency of 0.9% [21].

In this study, we utilised an efficient and reproducible method (Fig. 2) to obtain a high number of well-developed common bean regenerants. The TLC system which has been successfully used for the regeneration of different plants, including recalcitrant leguminous [16–18], ligneous plants [30], dicotyledonous [13,31] and monocotyledonous species [32–36], is perfectly adapted for the regeneration of *P. vulgaris*. It is of interest to note that as opposed to the findings of Malik and Saxena [12] using intact seedlings of *P. vulgaris*, the tTCLs system permits high frequency shoot neoformation and plant regeneration without the need of morphological integrity. A recent work, using more classical, voluminous explants such as individual cotyledons or hypocotyl segments from eucalyptus plants [37], presented a callus phase and a relatively high time interval for bud differentiation, 7–8 weeks instead of 2 weeks with tTCLs.

Our study has described the efficacy of thidiazuron (TDZ) on direct multiple shoot formation from tTCLs. TDZ cytokinin activity has been shown to be comparable or even superior to common adenine-based cytokinins [38,39]. Since then, TDZ has been successfully used in many tissue culture protocols of different grain leguminous known to be somewhat recalcitrant to regeneration procedures such as pea, chickpea and lentil [40], peanut [41], faba bean [6] cowpea [18] and common bean [6,12]. The mechanism by which TDZ induces morphogenetic responses equivalent to amino purine cytokinines is still not fully understood.

In this context, an interesting feature was that 2-week exposure to high TDZ concentration (10 μM) was sufficient to achieve optimal bud induction on *P. vulgaris* tTCLs. Similarly, other authors have demonstrated that a continuous exposure of tissue explants to TDZ is not needed since cultures after exposure to TDZ showed cytokinin autonomy [12,42]. Furthermore, our results demonstrate the inhibitory effect of a prolonged exposure (4 weeks) to a high concentration of TDZ (10 μM) on shoot and root development. This could be due to the fact that TDZ, similar to other synthetic cytokinines, is less susceptible to enzymatic degradation by the plant when compared to the endogenous amino purine cytokinines [43]. Furthermore, it has also been suggested that TDZ may be involved in the synthesis and/or accumulation of cytokinines in tissue cultures [39,42].

In this study, we showed that the addition of 10 μM silver nitrate (AgNO₃) to the medium with BAP (10 μM) enhanced the number of developed shoots per explant as well as the length range of shoots (Table 3). In a recent report, on the regeneration of two eucalyptus species (*Eucalyptus nitens* and *E. globulus*), no significant effect was found with the use of silver ions and although shoot regeneration occurred from callus-derived...
seedling explants, the frequency achieved was not very important (20–35%) [37]. Plant cell tissues and whole plants grown in culture produce ethylene which has been shown to be involved in plant cell differentiation [44]. In our work, the use of AgNO₃, a potent inhibitor of ethylene action [45] with BAP had a synergistic effect on enhanced shoot regeneration in *P. vulgaris*. When BAP was used alone, only 63.8% of the shoots developed (with a length range of 1.3–1.7 cm) whereas when it was applied in combination with AgNO₃, 100% of the shoots developed (1.8–2.9 cm length range). Similar results were obtained on the promotion of morphogenesis on cotyledonary node explant of *P. vulgaris* cultured on MS medium supplemented with 1 mg/l BAP, by the use of ethylene inhibitors [28], and it has also been suggested that the common recalcitrance of cowpea to regeneration could be due to ethylene [46]. Ethylene production could be the main reason of the failure to regenerate efficiently leguminous species.

Preliminary results conducted on gene transfer by biolistic of *P. vulgaris* explants (excised from 2-week-old seedling), originated strong transient expression of the *uidA* (GUS) reporter gene (Fig. 1H), opening the way to devise reproducible transformation procedures for this species using tTCLs. Recent reports have shown that the same bean cultivar (*P. vulgaris* cv. Carioca) had a shoot apex morphology favourable for the efficient delivery of genes by biolistic [23], but shoot apex cultures would regenerate only 6–8 shoots per apex. In our preliminary assay, bombarded explants maintained their capacity to give rise to tTCLs which respond positively to bud regeneration (results not shown). This tTCL method should enable the production of large-scale transformed material. TCLs system (transverse or longitudinal) has already been successfully associated with transformation procedures [47–49]. Recently, high temperature tolerance has been improved in transformed tobacco plants by gene silencing of a chloroplast omega-3 fatty acid desaturase [50]. It should be extremely valuable to test this achievement on bean plants, since drought/high temperatures are major environmental problems involving this crop.

Since we emphasise the necessity of a highly productive regeneration method in order to raise the frequency of transformed plants, the noteworthy aspects of this protocol are: (1) tTCLs are simple to produce and integrity of seedlings is not needed for the induction of multiple bud proliferation, hence they can be submitted to the selective conditions associated with the screening of transformed material; (2) the high frequency of regeneration (an average of 68.5 well developed shoots per seedling) should increase the frequency of fully transformed shoots; (3) shoot apex explants have proven to be suitable to gene delivery by biolistic and tTCLs proliferation capacity was not lost after bombardment.

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