Rapid Regeneration of Plants from Hypocotyl Protoplasts and Root Segments of Cabbage

C. Lillo¹ and E.A. Shahin

Genetics and Tissue Culture Group, ARCO Plant Cell Research Institute, 6560 Trinity Court, Dublin, CA 94568

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Protoplasts are suitable for use in mutation breeding programs. Furthermore, the prospects of overcoming sexual crossing barriers and transferring cytoplasmic traits, such as cytoplasmic male sterility and herbicide resistance, justifies work with protoplasts. However, an efficient procedure for regeneration of plants is a prerequisite for any further use of protoplasts. Regeneration of plants from roots is of interest as a method for micropropagation and in experiments where Agrobacterium rhizogenes is used for introducing new genes into plants (3). We report here a method for rapid regeneration of plants from cabbage hypocotyl protoplasts and from root segments.

Seeds of Brassica oleracea Group capitata, cv. Ladi, Ladi x Golden cross, and line N10-1 (ARCO Seed Co.), were sterilized in 1% calcium hypochlorite for 45 min, washed in sterile water, and germinated in petri dishes on filter paper soaked with liquid TM-1 nutrient medium (2). After 6 days, hypocotyls were cut into 1- to 3-mm pieces with scissors and placed into a solution for plasmolyzing the cells. This solution contained 10% sucrose, 4.5 μM 2,4-D, 2.2 μM BA, macronutrients, and vitamins (2). The cut hypocotyls were vacuum-infiltrated in the solution for 20 sec and placed in the dark at 4°C for about 5 hr. The solution was pipetted off and fresh solution that also contained 0.1% maceroenzyme R-10 and 0.15% cellulase RS (Yakult Pharmaceutical Industries, Japan), 1% PVP-5, 1% calcium hypochlorite for 45 min, washed in sterile water, and germinated in petri dishes on filter paper soaked with liquid TM-1 nutrient medium (2). After 6 days, hypocotyls were cut into 1- to 3-mm pieces with scissors and placed into a solution for plasmolyzing the cells. This solution contained 10% sucrose, 4.5 μM 2,4-D, 2.2 μM BA, macronutrients, and vitamins (2). The cut hypocotyls were vacuum-infiltrated in the solution for 20 sec and placed in the dark at 4°C for about 5 hr. The solution was pipetted off and fresh solution that also contained 0.1% maceroenzyme R-10 and 0.15% cellulase RS (Yakult Pharmaceutical Industries, Japan), 1% PVP-5, and 10 mg/liter casein hydrolyzate was added. No hormones were added in this step.

About 25-ml enzyme solution was used per 100-100,000 per ml (2 ml per dish) and cultured at 25° in diffuse light (5 μmol·s⁻¹·m⁻² cool-white fluorescent light) 16 hr, darkness 8 hr. After one day many cells had started to divide, and almost all protoplasts had divided within 3 days. They were diluted 1:1 with plating medium every 3 days.

The protoplasts were plated at a density of 50,000–100,000 per ml (2 ml per dish) and cultured at 25° in diffuse light (5 μmol·s⁻¹·m⁻² cool-white fluorescent light) 16 hr, darkness 8 hr. After one day many cells had started to divide, and almost all protoplasts had divided within 3 days. They were diluted 1:1 with plating medium every 3 days.

Table 1. The percentage of B. oleracea Group capitata calli on the shooting media that produced shoots. There were 20 calli for each treatment.

Table 2. The percentage of cabbage cross Ladi x Golden root segments that gave shoots after 3 weeks on different media. Some root segments were pretreated for one week on a medium containing 4.5 μM 2,4-D, 2.2 μM BA, and 0.5 μM NAA. There were 20 segments per treatment.

After one week colonies were formed and 1 ml of 0.8% agarose (Calbiochem, type E) was added to each petri dish. The agarose was solubilized in plating media, except the sugar alcohols (2) were left out. After one week, the minicalli embedded in the agarose medium were transferred to solid medium for further callus formation. At this step the light intensity was increased to 15 μmol·s⁻¹·m⁻². Because agarose had been added, almost all minicalli survived transfer to solid medium. The solid medium used contained 3% sucrose, 0.7% agar (Difco, purified), and extra NH₄NO₃, 2.5 mm; otherwise, the minerals and vitamins included were the same as those in the plating medium.

As shown in Table 1, the type of hormones used in this first medium were of crucial importance for the later development on the shooting medium. The highest number of shoots eventually were obtained when this medium contained 4.5 μM 2,4-D, 2.2 μM BA, and 0.5 μM NAA, whereas use of zeatin and IAA by this step resulted in no for-
Fig. 1–4. 1) Green nodules on a callus 6 weeks after protoplast isolation. 2) Shoots on a callus 6 weeks after protoplast isolation. 3) Cabbage plant established in soil. 4) Root segments one week on induction medium with 4.5 μM 2,4-D, 2.2 μM BA, and 0.5 μM NAA.

mation of shoots. After 7–10 days, the calli were almost 1 mm in diameter and were transferred on a scalpel to the shooting medium. The light intensity was increased to 25 μmol·s⁻¹·m⁻². Delayed transfer to shooting medium resulted in fewer shoots produced. The shooting medium was as described by Shahin (2) except that it contained 1% sucrose or 0.25% sucrose in combination with 5.4% mannitol. Shoots were obtained both on the zeatin/IAA medium and BA/GA₃ medium. The BA/GA₃ medium with 1% sucrose gave good results with all cultivars.

After 3 weeks on the shooting medium many calli had developed green nodules (Fig. 1) or multiple shoots (Fig. 2) that could be transferred to hormone-free medium. More shoots continued to develop from the nodules during the next couple of weeks. Since multiple shoots were formed on the calli, almost as many plants as wanted could be obtained from only a few calli. All shoots rooted on hormone-free medium and could be transferred to soil (Fig. 3). Few shoots were obtained when a reduced concentration of zeatin (4.3 μM) was used in the shooting medium. We found higher concentrations of zeatin had to be used to get shoots from *Brassica napus* (line C10-2, ARCO Seed Co.), and results were improved with mannitol in the medium. The procedure described has the advantage of regenerating shoots from protoplasts in a short time (6 to 7 weeks). The short regeneration time may reduce the chance of uncontrolled genetic changes during culture.

Root segments from in vitro grown plants and from seedlings were placed on different media and incubated at 25°C and 25 μmol·s⁻¹·m⁻². The hormone combination kinetin and IBA used by Lazerri and Dunwell (1) for regeneration from *B. oleracea* Group *it- alica* roots did not induce shoots on the cabbage roots. When placed directly on the shooting medium, the segments developed green callus at both ends, but almost no shoots developed (Table 2). However, when root segments were first placed on the medium containing 4.5 μM 2,4-D, 2.2 μM BA, and 0.5 μM NAA for one week before transferring them to shooting medium, many of the segments developed multiple shoots within 3 weeks (Table 2; Fig. 4). The procedure also was effective with a rapid cycling *B. oleracea* (CrGC-3, P. Williams, Univ. of Wisconsin), but we did not obtain shoots this way on the *B. napus* line. The shoots rooted
on hormone-free medium, and grew into normal-looking plants after transfer to soil. Since multiple shoots were obtained on each root segment, this may be an efficient micropropagation procedure. This direct root organogenesis observed in *Brassica* may also be useful in isolating mutants with desired horticultural traits that are associated with roots, such as resistance to soil-borne pathogens, tolerance to heavy metals, and other abiotic factors.

**Literature Cited**


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**The Effect of Agar vs. Liquid Medium on Rooting in Tissue-cultured Sweetgum**

Ni Lee¹, Hazel Y. Wetzstein¹, and Harry E. Sommer²

*University of Georgia, Athens, GA 30602*

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It is increasingly evident that the concentration and type of matrix used in nutrient media can profoundly influence the response of tissues cultured in vitro. *Picea abies* shoot cultures exhibited increased dry weight yields (4) and increased shoot growth (9) with decreasing agar concentrations. Singha (5) compared shoot proliferation of crabapple and pear in Phytagar concentrations ranging from 0% to 1.2%. Optimal proliferation and growth occurred in crabapple on 0.3% agar, with higher concentrations decreasing these growth characteristics. In pear, increasing agar concentration also decreased shoot growth, but proliferation was greatest at 0% or higher agar concentrations. Rooting has been promoted without or with lower agar concentrations in grape (1), Norway spruce (9), and apple (10). However, vitrification (glassiness or waterlogging) has been shown to increase with lower agar concentrations (9) and limits the use of liquid culture in some species.

We have been investigating the differentiation and development of sweetgum (*Liquidambar styraciflua* L.) in vitro. The culture environment has been shown to have a pronounced effect on the anatomy and physiology of cultures (3, 11, 12). The objective of this study was to examine the effects of the use of a liquid vs. an agar rooting medium on total plantlet development, vitrification, and the initiation and extent of root growth from adventitious shoots of sweetgum.

Adventitious shoots were initiated from hypocotyl sections cultured on a modified Risser and White’s agar medium with 5.7 μM IAA and 25 μM 2iP, then multiplied in a modified Blayde’s liquid medium with 54 nm NAA and 2.2 μM BA as previously described (6, 8). Shoots (1 to 2 cm long) were transferred into 25 × 150 mm culture tubes with 20 ml of Risser and White’s medium modified by the use of Brown’s trace elements (7) with no hormones, and with either 0% (liquid medium) or 0.8% Phytagar (GIBCO) (agar medium) incorporated. Shoots in liquid medium were supported on Heller’s rafts (Whatman #1 filter paper). Cultures were maintained stationary in a growth room at 25° ± 2°C, under cool-white fluorescent lamps at 30 μmol·s⁻¹·m⁻² at culture level, with a 14-hr photoperiod. The experiment was a completely randomized design with 30 cultures per treatment. Cultures were rated for percent rooting and number of roots per culture after 3 weeks and continued weekly for 10 weeks, after which shoot and root fresh weights were determined. Dry weights were determined after drying for 48 hr at 70°C.

The growth characteristics of plantlets grown in agar vs. liquid are summarized in Table 1. Shoots were larger (Fig. 1), and shoot fresh weight, but not dry weight, was significantly increased in cultures grown on liquid medium. Water content in leaves of plantlets grown in liquid was higher than in agar. These results concur with preliminary microscopic evaluations that show somewhat thinner-walled, less densely packed cells in leaves differentiated in liquid vs. agar medium. Although shoots of tissue-cultured plantlets were succulent in appearance, no evidence of vitrification was found in terms of excessive turgescence, translucence, or necrosis. Type of medium had a significant effect on shoot root and dry weight (Table 1). Roots in liquid were much longer than those in agar (Fig. 1). Root-shoot ratios were greater for liquid than agar-grown plantlets.

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**Table 1. Growth characteristics of agar- and liquid-grown *Liquidambar styraciflua* L. (sweetgum) plantlets.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Liquid</th>
<th>Agar</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot fresh weight (mg)</td>
<td>92</td>
<td>65</td>
<td>**</td>
</tr>
<tr>
<td>Shoot dry weight (mg)</td>
<td>25</td>
<td>20</td>
<td>NS</td>
</tr>
<tr>
<td>Shoot water content (%)</td>
<td>73</td>
<td>69</td>
<td>**</td>
</tr>
<tr>
<td>Root fresh weight (mg)</td>
<td>72</td>
<td>15</td>
<td>NS</td>
</tr>
<tr>
<td>Root dry weight (mg)</td>
<td>18</td>
<td>4</td>
<td>**</td>
</tr>
<tr>
<td>Root water content (%)</td>
<td>75</td>
<td>78</td>
<td>**</td>
</tr>
<tr>
<td>Root-shoot ratio (dry wt)</td>
<td>0.72</td>
<td>0.18</td>
<td>NS**</td>
</tr>
</tbody>
</table>

**NS**“Non-significant and significant at 1% level, respectively.

The percentage of shoots that rooted was greater on liquid than on agar medium at all time intervals (Fig. 2). About 94% of the liquid cultures had rooted at 6 weeks, compared to only 46% in agar. At 10 weeks, 95% of the liquid cultures vs. 58% in agar had rooted. In addition, the number of primary roots per plantlet was higher in liquid vs. agar culture (Fig. 2). Root initiation stabilized at a lower number in agar medium, whereas root numbers were higher and increased with time on liquid medium (Fig. 2).

Rooting of adventitious shoots in liquid medium was thus superior to rooting in agar for Sweetgum in terms of rooting percentage, rooting rate, and root numbers per plantlet. Although not quantitated, root hair development was also enhanced in liquid medium. Root-shoot ratio was more balanced in liquid than in solid medium. No evidence of vitrification was observed. Descriptions of morphological adaptations of water roots, particularly if subjected to reduced O₂ tension, include adventitious root-

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¹Dept. of Horticulture.
²School of Forest Resources.