Large-scale Micropropagation of Aloe vera

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Additional index words. Aloe, plant tissue culture, disinfection, organogenesis, plant micropropagation

Abstract. A protocol for large-scale Aloe vera (L.) Burm. f. production was established using micropropagation of apical buds. The effects of two chlorine-based disinfectants were evaluated on the survival of the explants in different treatments in a semisolidified Murashige and Skoog (MS) medium in the presence of 6-benzylaminopurine (6-BAP; 2 mg L–1). During 120 days, 136 green apical shoots bearing axillary buds were multiplied four times at 30-day intervals in the same MS medium, reincubating seven to nine explants per flask each time. The elongation and rooting processes were carried out in the same MS medium without 6-BAP. A total of 40,495 Aloe vera microplants were obtained, a yield of 300 microplants per apical bud at a rate of 1:5.3 in every multiplication period of 30 d in the culture medium taking into account the rates of explant contamination, necrosis, and green apical shoot (GAS) development. Shoot proliferation. After 30 d in the culture medium, the best 136 green apical shoot explants bearing axillary buds (GAS) were selected, longitudinally divided, and transferred from the test tubes to 350-mL glass flasks containing 50 mL of the same medium and cultivated for an additional 90 d under the same experimental conditions with replication at 30-d intervals. Seven to nine explants were transferred in each replication. Elongation and root induction. Developed Aloe plantlets were transferred to culture medium without 6-BAP to induce elongation and rooting for a 30-d period.

Aloe vera (L.) Burm. f., belonging to the family Asphodelaceae (Souza and Lorenzi, 2005), is one of a few Aloe species that has been explored by pharmaceutical and cosmetics industries (Mapp and McCarthy, 1970; Morton, 1961). Biological activities are ascribed to the pulp gel of Aloe vera such as antivirus, antibacterial, antifungal, anticancer, wound healing, and many others (Reynolds and Dweck, 1999), the medicinal principles being phenolics and polysaccharide compounds (Okamura et al., 1996; Park et al., 1998). Those qualities have prompted industrial and commercial increase in the production of Aloe vera throughout the world. The main constraint in this production chain is to obtain healthy primary culture for a sustainable supply of the plant materials. The common practice for the reproduction of Aloe is conventional vegetative propagation. However, this practice should be avoided because it can also allow the propagation of diseases frequently present in the mother plants, because it happens during the conventional vegetative propagation of other plant species such as banana, potato, strawberry, and sugarcane. This problem can be minimized using in vitro propagation of stock materials (Crocomo and Oliveira, 1995; Debergh and Read, 1991) with the objective of the production of not only healthy, but also standardized sizes of plantlets to be used in the subsequent controlled stages of the process suitable for the clonal production of Aloe vera. In this context, this article describes a complete micropropagation system involving disinfection, in vitro multiplication, rooting, and hardening followed by ex vitro acclimatization procedures used to attain that objective, thus producing thousands of Aloe vera plantlets.

Materials and Methods

Plant material sources. Aloe vera plants were obtained from the field collection of the Department of Plant Production of the Escola Superior de Agricultura “Luiz de Queiroz,” University of São Paulo, Piracicaba, SP, Brazil.

Surface disinfection treatments. Four hundred eighty apical buds explants, each ≈1 cm², were isolated from young lateral shoots bearing six to nine leaves and disinfected using three different disinfection treatments (T1, T2, and T3) as shown in Table 1. In each treatment, the final step (Step 7) was conducted in an air sterile chamber followed by removal of the external tissue layers of the explants up to ≈1 cm² and inoculated into the shoot proliferation medium (SPM) based on semisolidified Murashige and Skoog medium (Murashige and Skoog, 1962) with 2 mg L–1 glycine, 100 mg L–1 myo-inositol, 30 g L–1 sucrose, and 2 mg L–1 6-benzylaminopurine (6-BAP); the pH was adjusted to 5.8 before adding 2.3 g L–1 Phytagel® (SIGMA-ALDRICH Inc., St Louis, MO). In each treatment, 160 buds were individually inoculated in 12.5 cm diameter × 8 cm long test tubes containing 12.5 mL of the SPM medium and incubated in culture room at 25 °C ± 2 under light (50 μmol m–2 s–1) with a photoperiod of 16/8 h. The effect of the disinfection treatments was evaluated after 30 d in the culture medium taking into account the rates of explant contamination, necrosis, and green apical shoot (GAS) development.

Shoot proliferation. After 30 d in the culture medium, the best 136 green apical shoot explants bearing axillary buds (GAS) were selected, longitudinally divided, and transferred from the test tubes to 350-mL glass flasks containing 50 mL of the same medium and cultivated for an additional 90 d under the same experimental conditions with replication at 30-d intervals. Seven to nine explants were transferred in each replication.

Elongation and root induction. Developed Aloe plantlets were transferred to culture medium without 6-BAP to induce elongation and rooting for a 30-d period.

Received for publication 18 June 2009. Accepted for publication 6 Aug. 2009.

We thank Prof. Keigo Minami, Department of Plant Production/E.S.A. “Luiz de Queiroz”/University of São Paulo, for supplying selected Aloe vera plantlets. To whom reprint requests should be addressed; e-mail etolivei@esalq.usp.br.

Table 1. Sequential procedures for Aloe vera explant disinfection.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>T2</td>
<td>H</td>
<td>S</td>
<td>S</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>T3</td>
<td>S</td>
<td>S</td>
<td>H</td>
<td>H</td>
<td>S</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>
Medium (Mm), 6 to 8 cm long bearing four to five leaves; and large (Ml), 13 to 16 cm long bearing five to seven leaves (Fig. 1). The microplants were classified as: small (Ms); medium (Mm); large (Ml).

Hardening and acclimatization. The average rate of Aloe microplant production was evaluated following the in vitro proliferation; 38,480 microplants were used in the hardening and acclimatization processes. The microplants were classified as: small (Ms), 3 to 4 cm long bearing two to three leaves; medium (Mm), 6 to 8 cm long bearing four to five leaves; and large (Ml), 13 to 16 cm long bearing five to seven leaves (Fig. 1). The microplants were transferred to two different types of black polyethylene trays labeled B1 (80 cm3/cell) and B2 (40 cm3/cell) with 36 and 64 cells each, respectively. The trays were maintained in two different types of greenhouses with automatically controlled relative humidity and proper irrigation namely: G1, pad-house system using microaspersion irrigation and air exhaustion; and G2, convectional greenhouse using nebulization and forced air ventilation. A mixture of bark pinus, vermiculite, sand, and fertilizers (Terra do Paraiso™; Substratos Agricolas Ltda, Holambra, SP), pH 6.04 and electric conductivity 0.62 μS cm−1, was used as substrate. After a period of 3 months, the rate of microplant survival and their fresh and dry matter contents were determined.

Statistical analysis. The data were submitted to the analysis of variance (5% probability) using the Statistical Analysis Systems (Version 8.0; SAS, Cary, NC). The average data for the rates of contamination, necrosis, and green apical shoot production were compared by least significant difference (LSD) test. "ns" means no significant differences (< 0.05). In relation to the contamination and the GAS rates, the best results were obtained by T3 (13.12% for contamination and 32.50% GAS) and T2 (15% for contamination and 35% for GAS) and T1 (32.81% for contamination and 17.50% for GAS). This was attributable mainly to washing the start material in the disinfectant solutions at the beginning of the process (Step S0 or H0). In regard to necrosis of the explants (Fig. 2), no significant differences were observed among the three treatments: T1, 49.69%; T2, 50.00%; and T3, 54.38%.

The degrees of injury on the explants depend on the type of disinfection processes and the nature of the plant material. The Aloe vera tissue has low lignin content and is very sensitive to disinfectants. This is not likely to be the result of chlorine toxicity, either of the hypochlorite or the sodium dichlorisocyanurate, which is largely used to disinfect "fresh-cut" products (Lund et al., 2005). One can assume that this could be attributed to the alkalinity of the disinfectant solutions. This aggressiveness was observed mainly in the rates of necrosis of the explants.

From 480 inoculated explants, 136 green apical shoots with axillary buds (GAS) were multiplied three times at 30-d intervals in the same SPM medium. The shoots were longitudinally divided and seven to nine explants were reinculted per flask in the same culture medium each time.

After the stage of multiplication, the plantlets were separated and transferred to the previous culture medium without 6-BAP to induce elongation and rooting. At the end of the multiplication and rooting periods, the Tukey test was used for hardening and acclimatizing, comparing microplant sizes × tray types × greenhouse conditions.

Results and Discussion

Disinfection, shoot differentiation, and rooting. The three disinfection treatments were effective in different degrees for disinfecting the Aloe vera apical buds in vitro. The statistical analysis of the effect of the disinfection processes on the rates of contamination, mainly by bacteria, and on the green apical shoots with axillary buds (GAS) production (Fig. 2) showed no significant differences (P > 0.05) between T2 and T3 treatments, both being different from T1 (P < 0.05). In relation to the contamination and the GAS rates, the best results were obtained by T3 (13.12% for contamination and 32.50% GAS) and T2 (15% for contamination and 35% for GAS) and T1 (32.81% for contamination and 17.50% for GAS). This was attributable mainly to washing the start material in the disinfectant solutions at the beginning of the process (Step S0 or H0).

The statistical analysis (Table 2) was performed using 480 explants. Analysis was made using 480 explants.

![Fig. 1](Image)

**Fig. 1.** Effect of the disinfection treatments on the survival of apical shoots of Aloe vera. Analysis was made using 480 explants.

![Fig. 2](Image)

**Fig. 2.** Classification of Aloe vera microplants by size: (A) Small size (Ms). (B) Medium size (Mm). (C) Large size (Ml).

![Fig. 3](Image)

**Fig. 3.** Microplant survival as a function of their sizes. Ms = small size; Mm = medium size; Ml = large size. Columns followed by at least one same letter did not differ among them by Tukey test (P < 0.05). Each value is the average of 40 replicates.

![Table 2](Table)

**Table 2.** Effect of acclimatization conditions on the increase of fresh matter weight (g) of Aloe vera microplants.

<table>
<thead>
<tr>
<th>Microplants</th>
<th>Greenhouse 1 (G1)</th>
<th>Greenhouse 2 (G2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tray 1 (B1)</td>
<td>Tray 2 (B2)</td>
</tr>
<tr>
<td>Ms</td>
<td>10.49 aA</td>
<td>8.60 aA</td>
</tr>
<tr>
<td>Mn</td>
<td>15.58 aA</td>
<td>12.01 aA</td>
</tr>
<tr>
<td>Ml</td>
<td>32.50 aA</td>
<td>20.55 bA</td>
</tr>
</tbody>
</table>

Averages followed by at least the same letter or symbol did not differ by the Tukey test (P < 0.05). Lower case letters: comparison for trays (constant microplants and greenhouse). Upper case letters: comparison for greenhouses (constant microplants and trays). Symbols (* †): comparison for trays (constant microplants and greenhouse). Upper case letters: comparison for greenhouse conditions (constant tray and greenhouse).

M1 = Small size microplants; Mm = medium size microplants; G1 = pad-house type greenhouse with microaspersion irrigation; G2 = greenhouse with nebulization irrigation and forced air; B1 = 36-cell trays (80 cm3/cell); B2 = 64-cell trays (40 cm3/cell). Each value is the average of 10 replicates.
Table 3. Effect of acclimatization conditions on the increase of dry matter weight (g) of Aloe vera microplants.

<table>
<thead>
<tr>
<th>Microplants</th>
<th>Greenhouse 1 (G1)</th>
<th>Greenhouse 2 (G2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tray 1 (B1)</td>
<td>Tray 2 (B2)</td>
</tr>
<tr>
<td>M_s</td>
<td>0.20 aA*</td>
<td>0.16 aA*</td>
</tr>
<tr>
<td>M_m</td>
<td>0.29 aA*</td>
<td>0.24 aA*†</td>
</tr>
<tr>
<td>M_l</td>
<td>0.60 aA†</td>
<td>0.38 bA†</td>
</tr>
</tbody>
</table>

Averages followed by at least the same letter or symbol did not differ among them by the Tukey test (<i>P</i> < 0.05). Lower case letters: comparison for trays (constant microplants and greenhouse). Upper case letters: comparison for greenhouses (constant microplants and trays). Symbols (* †): comparison in the same column for microplant size (constant tray and greenhouse).

M_s = Small size microplants; M_m = medium size microplants; M_l = large size microplants; G_1 = greenhouse with microaspersion irrigation; G_2 = greenhouse with nebulization irrigation and forced air; B_1 = 36-cell trays (80 cm³/cell); B_2 = 64-cell trays (40 cm³/cell). Each value is the average of 10 replicates.

**Conclusions**

At least 300 microplants were produced from a single apical bud of Aloe vera in a period of 4 months. The best results of the apical shoot disinfection were obtained when the field-harvested Aloe vera stocks were previously rinsed with a solution either of sodium hypochlorite or sodium dichloroisocyanurate.

The Aloe vera plantlets were successfully ex vitro acclimatized in a greenhouse bearing a pad-house system with microaspersion and air exhaustion using 36- (80 cm³/cell) or 64- (40 cm³/cell) cell polyethylene trays containing the proper substrate. Because the cell number in the trays showed no influence on the rate of microplants survival and FW and DW content, the choice of the number of cells is a practical one: using 64-cell trays (40 cm³/cell), there is an economy of 47.37% in greenhouse space and 50% in the amount of substrate used per microplant.

**Literature Cited**

Araújo, P.S., J.M.O.D. Silva, C.A. Nechel, C. Ianssem, A.C. Oltramari, R. Passos, E. Tieto, and R. Passos. 2002. Aloe vera plantlets were successfully ex vitro acclimatized in a greenhouse bearing a pad-house system with microaspersion and air exhaustion using 36- (80 cm³/cell) or 64- (40 cm³/cell) cell polyethylene trays containing the proper substrate. Because the cell number in the trays showed no influence on the rate of microplants survival and FW and DW, the use of 64-cell (40 cm³/cell) trays represents an economy of 47.37% in greenhouse space and 50% in the amount of substrate used per Aloe vera plant.


