IN VITRO CULTURE OF FOUR MEDICINAL ASTERACEAE SPECIES FOR
Agrobacterium rhizogenes TRANSFORMATION

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Abstract

Hairy roots produced by infection with Agrobacterium rhizogenes are phenotypically altered and show an overproduction of secondary compounds. Four Asteraceae species were tested: Artemisia annua (with proven antimalarial activity), Calendula officinalis (antiseptic), Mikania glomerata (a Brazilian plant used for respiratory disease treatment) and Helianthus annuus (sunflower with tonic and anti-neuralgic activities). Seeds of Artemisia annua, Calendula officinalis and Helianthus annuus were surface disinfected with 25% NaOCl solution for 15 min., and Mikania glomerata stems were surface disinfected with 25% NaOCl solution for 30 min. Sterile and viable explants were maintained in complete Murashige & Skoog’s culture medium and subcultured after 28 days. Once established in culture, the explants of all species were inoculated with Agrobacterium rhizogenes strains 8196 and 15834. Only Artemisia annua showed a positive hairy root response. These roots were excised and cultured “in vitro”. In order to confirm the transgenic character of the hairy roots a Southern Blot hybridization was carried out. The culture protocols established for the other three species were used to obtain explants for further transformation tests.

1. Introduction

Medicinal plants are known since remote times and about 64% of the current world population uses at least one plant species as medication. For this work four Asteraceae species were chosen due to their applications in cosmetic and medical industry. These species are artemisia (Artemisia annua), calendula (Calendula officinalis), guaco (Mikania glomerata) and sunflower (Helianthus annuus). Artemisia produces artemisinin, used in malaria treatment and has been used for centuries against fever (Klayman, 1985). Calendula is used as antiseptic and anti-inflammatory. Guaco, indigenous to South America, has antiseptic activity and is used in respiratory disease treatment. Its efficiency was tested by the Brazilian Medication Center (CEME - Central de Medicamentos) as part of the Brazilian Medicinal Plants Research Program. The sunflower is known as febrifuge and for its tonic effects.

The objective of this work was to establish culture protocols for in vitro cultivation of the four medicinal Asteraceae species mentioned as a first step. Once established in culture, the explants will be inoculated with Agrobacterium rhizogenes for transformation tests. Hairy-root response and confirmation of the transgenic character of the hairy-root obtained will be the second step of our work.
2. Materials and Methods

In this work *A. annua*, *C. officinalis* and *H. annuus* seeds and *M. glomerata* stems obtained from field plants were used. *A. annua*, *C. officinalis* and *H. annuus* seeds were washed with water and detergent, and disinfected with 10, 20 and 25% sodium hypochlorite (NaOCl 5-6%, Merck) solutions. Disinfecting efficiency of each treatment was tested in three different time lengths 5, 10 and 15 minutes under agitation. The seeds were pre-treated with 1% Benlate (fungicide, benzimidazol chemical group) for 15 minutes and 70% alcohol for 30 seconds. Once disinfected, the seeds were rinsed three times with autoclaved distilled water to eliminate residues and inoculated in complete Murashige & Skoog’s (1962) culture medium. *M. glomerata* stems were removed from field plants, washed with water and detergent and disinfected with 15, 20 and 25% sodium hypochlorite (NaOCl 5-6%, Merck) solution. Each treatment was tested for disinfecting efficiency in three different time lengths 10, 20 and 30 minutes under agitation. The stems were pre-treated with 1% Benlate (fungicide, benzimidazol chemical group) for 20 minutes and 70% alcohol for 60 seconds. After disinfecting procedures the stems were rinsed three times with autoclaved distilled water and inoculated in complete Murashige & Skoog’s (1962) culture medium. Cultures of each species were incubated in controlled growth room conditions and subcultured monthly, until they were used in the transformation experiments with *Agrobacterium* strains.

In vitro explants inoculations with *Agrobacterium rhizogenes* were carried out with two wild strains (8196 and 15834). The *A. rhizogenes* cultures were maintained in AT culture medium (Sambrook et al., 1989) at 5°C and subcultured every 30 days. Before inoculation the bacterial colonies were grown for 48 hours at 25±2°C in darkness. After inoculation with *A. rhizogenes* the explants were maintained at 25±2°C, under incandescent white light in a 16 hour photoperiod regime. The hairy roots were excised from explants after 33 days and isolated in a half Murashige & Skoog’s (1962) medium supplemented with cefotaxime (100 μg/ml) to eliminate bacteria.

In order to confirm the transformation, a DNA extraction and molecular characterization by Southern Blot was made according to Dellaporta et al. (1983). DNA was digested with the restriction endonuclease *KpnI* and *EcoRI*, and the incorporated radioactive nucleotide (α-32P)dCTP at #6 fragment of pRi8196 (7.2 kb) was used as probe. DNA was separated by electrophoresis in agarose gel and transferred to nylon membrane during 16 hours. After probe hybridization the membrane was washed and exposed to a RX film in chassis with intensifier, and maintained at -70°C until the signal was obtained.

3. Results

For 7 days after the disinfecting procedures, the treated seeds were screened for any bacterial contamination. Only fungal contamination was observed in treatments where 10 and 20% NaOCl solutions were used for 5, 10 and 15 min. The best disinfecting procedure was the one in which explants were submitted to 25% NaOCl solution for 15 min. This procedure was effective for *C. officinalis* and *A. annua* cultures, but for *H. annuus* it was only effective after fruit tissues were removed. For germination assays, it was used 100 seeds for each specie. *A. annua* and *C. officinalis* seeds showed positive photoblastism, while *H. annuus* seeds showed neutral photoblastism, as it can be seen in Table 1.

Fifteen, 20 and 25% NaOCl solutions for 10 and 20 minutes were ineffective to disinfect *M. glomerata* stems, but after a pre-treatment of explants with fungicide, 25% NaOCl solution for 30 minutes was effective resulting in 90% of disinfected explants.

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The four Asteraceae species were inoculated with *A. rhizogenes* 8196 and 15834 strains. However, only the inoculations in *A. annua* with 8196 strain presented positive hairy root response. The other three species (C. officinalis, H. annuus and M. glomerata) presented a characteristic expansion of the inoculated area, which is an indication of effective transformation, but failed to produce transformed root response. The hairy roots in *A. annua* explants first appeared at 14 days of inoculation, as shown in Table 2.

The molecular characterization of hairy roots was carried out by Southern Blot (Southern, 1975). Figure 1 schematizes the fragment positions after probe hybridization.

4. Discussion

Explants obtained from field plants frequently show high contamination rates, mainly fungal contamination, as it was observed in our experiments, especially in *M. glomerata*. In these cases a fungicide application becomes indispensable to achieve axenic cultures, which allowed the development of 100% axenic cultures for seeds and 90% for stems. Culture was undertaken in complete Murashige & Skoog's (1962) culture medium because it is indicated for many plant species and it allows an appropriate explant growth to an optimal size for *Agrobacterium* transformation. The photoblastism data obtained for seeds germination confirm previous data of Shepherd *et al.* (1992).

Although *A. rhizogenes* 15834 strain is more virulent than 8196 strain, only explants infected with 8196 presented hairy roots development in *A. annua* explants. Phenotypic characteristics such as hairy roots growth pattern, growth speed and lack of geotropism were observed during culture and showed clearly that transformation had occurred. The molecular characterization of hairy roots was necessary only to confirm definitively the passage of the bacterial genetic material to the plant. For this molecular characterization the hairy root clones were cultured for one year and the results confirm the T-DNA integration to the hairy roots genome, unlike the results obtained for the negative controls, where characteristic fragments that can be found in the transformed roots can not be detected.

5. Acknowledgments

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6. References


Table 1 - Seed germination in light and dark conditions (Murashige & Skoog’s medium - for each specie it was used 100 seeds)

<table>
<thead>
<tr>
<th>Species</th>
<th>Light</th>
<th>Dark</th>
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<tr>
<td><em>A. annua</em></td>
<td>90%</td>
<td>5%</td>
</tr>
<tr>
<td><em>C. officinalis</em></td>
<td>70%</td>
<td>5%</td>
</tr>
<tr>
<td><em>H. annuus</em></td>
<td>80%</td>
<td>80%</td>
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Table 2 - Hairy root response in *A. annua* after 14, 21 and 33 days of the *A. rhizogenes* 8196 and 15834 inoculation

<table>
<thead>
<tr>
<th>A. rhizogenes strain</th>
<th>14 days after inoculation</th>
<th>21 days after inoculation</th>
<th>33 days after inoculation</th>
</tr>
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<tbody>
<tr>
<td>15834</td>
<td>no response</td>
<td>no response</td>
<td>No response</td>
</tr>
<tr>
<td>8196</td>
<td>20% of stems</td>
<td>50% of stems</td>
<td>80% of stems</td>
</tr>
</tbody>
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Figure 1 - Southern Blot analysis using a [α-32P]dCTP-labelled 7.2 kb BamHI fragment isolated from pRi8196 as a probe. The homologous sequences related to hybridization are shown in lanes 2, 3 and 4.