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Micropropagation of *Chamomilla recutita* (L.) Rauschert: A shock treatment model with growth regulators

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Abstract

A protocol for in vitro clonal propagation of a Brazilian variety of chamomile (*Chamomilla recutita* (L.) Rauschert) is described. Nodal segments were submersed in liquid MS basal medium containing 1.0–4.0 mg l^{-1} GA_3 or thidiazuron (TDZ) or 0.5–4.0 mg l^{-1} 2,4-D for 2 h. After that, they were transferred to solid MS basal media without growth regulators and maintained for 2 months. This process avoided the callus development which was undesirable for shoot multiplication. The best treatment for shoot proliferation and plant rooting contained 1.0–2.0 mg l^{-1} gibberellin (GA_3) or 0.5 mg l^{-1} 2,4-D. Plants treated with 0.5 mg l^{-1} 2,4-D were subcultured for half-strength nitrogen solid MS medium and they were acclimatized two months later. The hardening process in the greenhouse was difficult and time consuming, however, 54% of plants survived and were successfully adapted to field conditions.

Keywords: Tissue culture; Growth regulators; Medicinal plant; Chamomile

1. Introduction

Herbal medicine practice has had a remarkable increase in the last decade (Calixto, 2000). Some plants are consumed mainly due to their well-known therapeutic actions, despite the existence of scientific support. Additionally, herbal medicines can proceed from non-standardised protocols to handle preparations based on plant material. This being the fact, active ingredients can vary quantitatively and qualitatively, depending on the plant species, its origin, and its harvesting period (Currier et al., 2000). Techniques on tissue culture of medicinal plants have been directed to the production of special metabolic, genetic transformation, germplasm conservation or development of protocols for plant micropropagation (Rout et al., 2000; Lima et al., 2001). Standardised in vitro culture has been considered a promising tool to obtain homogeneous plant material to serve as appropriate source of drugs.

Chamomilla recutita (L.) Rauschert (Asteraceae) is a widespread European plant known as chamomile. Medicinal teas prepared from the flowering plant are used in folk medicine to treat gastrointestinal diseases and its active ingredients are indicated as containing carminative, antispasmodic or sedative activities (Corrêa Júnior, 1998). The essential oil present in the flower heads of the plant is responsible for its major pharmacological effects (WHO, 1999). Any report about micropropagation of *C. recutita*, a Brazilian variety is available. Therefore, the present investigation was undertaken to develop an effective method for in vitro multiplication, which could result in high adaptation to field conditions.

2. Material and methods

2.1. Establishment of in vitro cultures

Seeds (Isla® Pak) were used as source of plant material. Seeds' surface were sterilised in 50% commercial bleach solution for 30 min, rinsed three times for 5 min in distilled water, dipped in 70% ethanol and rinsed three times for 5 min in sterile distilled water. The disinfected seeds were inoculated on glass tubes (10 cm × 1.5 cm) containing sterilised water and paper filtering as a support. Seeds were maintained in a growth

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room at 25 ± 2 °C under a 16/8 h light/dark photoperiod (*Silvania* daylight fluorescent lamps, 23 μmol m^{-2} s^{-1}).

2.2. Culture treatments with growth regulators

Initially, 1.0 cm length nodal segments containing 2–4 buds were excised from in vitro culture seedlings and placed in glass bottles (500 ml) containing 50 ml of MS (Murashige and Skoog, 1962) added to 3% sucrose and solidified with 0.8% agar–agar. In order to obtain at least 30 shoots for each treatment, plantlets were subcultured in the same kind of culture medium until enough shoots were available to establish the experiments. Preliminary tests were conducted to examine growth regulators effects on the development of *C. recutita*. Thus, nodal segments were transferred to solid MS media supplemented with either thidiazuron (TDZ; 0.1, 0.5 and 1.0 mg l^{-1}), indole-3-acetic acid (IAA; 0.1, 0.5 and 1.0 mg l^{-1}), kinetin (Kin; 0.5 and 1.0 mg l^{-1}) or gibberellin (GA_3; 0.5 and 1.0 mg l^{-1}).

After the results of the preliminary tests were obtained, a new experiment was done. Nodal segments were excised from plantlets previously cultured in solid MS media, and submitted to a "shock" of growth regulators in liquid MS media supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D; 0.5, 1.0, 2.0 or 4.0 mg l^{-1}), GA_3 (1.0, 2.0 or 4.0 mg l^{-1}) or TDZ (1.0, 2.0 or 4.0 mg l^{-1}) for 2 h, under shaking. After that, nodal segments were transferred to solid MS medium without adding growth regulators. Cultures were maintained in the growth room under the conditions mentioned above. The number of shoots per explant, shoot height (cm), percentage root formation and percentage callus development were recorded after 2 months. Thirty random explants were used per treatment, and each experiment was performed twice. Data were submitted to ANOVA analysis (analysis of variance) and the means were compared by the Tukey's test at 5% significance.

2.3. Acclimatization

After the two-month period of culture in solid MS medium, vigour was evaluated for each plantlet group obtained from the different hormone treatments. According to the growth parameters indicated in the item above, plantlets that were shocked on liquid MS medium supplemented with 0.5 mg l^{-1} 2,4-D for 2 h were chosen as the experimental group to proceed to the acclimatization protocol. To avoid excessive stress of the plants while transferring them to soil, those plantlets were sub-cultured for additional 2 months in half-strength nitrogen MS medium (1/2 N) containing 1.5% sucrose, and solidified with 0.8% agar, before being acclimatized. Whenever a rooted plantlet appeared, it was removed from the culture medium, the roots were gently washed in tap water, and then transferred to a seed pot containing soil conditioner. They were kept under greenhouse conditions for four weeks before being transferred to outdoor conditions. Plantlets were irrigated every day with the filtered and UV sterilised water.

3. Results

3.1. In vitro establishment and growth regulators on culture

The disinfecting method was 100% successful in eliminating any seed contamination. After 13 days, 24.5% of the seeds germinated, as considered by emission of the first radical.

The preliminary test with growth regulators TDZ, IAA, Kin or GA_3 added to solid MS medium showed no satisfactory results. The nodal segments cultured in those media frequently developed callus, and the few shoots, whenever formed, soon initialized a senescence process, which was evidenced after about 5 weeks of culture.

Except for the shock treatments with 2.0 mg l^{-1} TDZ or with 4.0 mg l^{-1} GA_3, all other concentrations of growth regulators enhanced shoot formation. The greatest shoot number (3.31 per explant) and the highest shoots (4.61 cm in length) were obtained from nodal segments submitted to the shock treatment with 1.0 mg l^{-1} 2,4-D. The increase in 2,4-D concentrations up to 4.0 mg l^{-1}, as well as its lowest concentration (0.5 mg l^{-1}), reduced both the shoot formation and elongation. Nodal segments treated with 1.0 mg l^{-1} GA_3 or 4.0 mg l^{-1} 2,4-D developed three shoots per explant. Yet, the media supplemented with either 1.0–2.0 mg l^{-1} GA_3 or 0.5–4.0 mg l^{-1} 2,4-D did not cause plants to attain statistically different results. Besides, shock treatment with 2.0 mg l^{-1} TDZ produced both the lowest number of shoots (1.93 per explant), and elongation (3.42 cm). Treatment with either 1.0 mg l^{-1} TDZ or with 2.0 mg l^{-1} GA_3 resulted in plant 4.25 cm tall in average, and no statistical difference was found in the number and length of shoots.

C. recutita shoots succeeded to root with all treatments, although 2.0 mg l^{-1} GA_3 resulted in the highest rooting percentage (100%), and no callus was observed after treatment with this growth regulator. The increase in TDZ concentration from 1.0 to 4.0 mg l^{-1} promoted the increase of both the rooting and callus percentage. Conversely, increasing the 2,4-D concentration caused the plantlets to reduce rooting. The percentage of callus formation was the highest with 2.0 mg l^{-1} 2,4-D treatment, as well as 1.0 mg l^{-1} TDZ (data not shown). The ideal interval of time for subcultures was 2 months for all treatments with growth regulators.

The best media tested contained 1.0–2.0 mg l^{-1} GA_3 or 0.5 mg l^{-1} 2,4-D in terms of what was observed during the proliferation and rooting phases (Fig. 1). Ex vitro rooting phase appeared not to be necessary. The complete morphologic results are summarized in Table 1.

3.2. Acclimatization

Plantlets developed in solid MS media without previous shock did not survive acclimatization. Treatment of plantlets with liquid MS media plus 0.5 mg l^{-1} 2,4-D, nevertheless, caused plantlets to overcome such stressing period. A pre-adaptation to autotrophic conditions can be stimulated on in vitro plants (Grattapaglia and Machado, 1998), and an attempt