Electronic Journal of Biotechnology ISSN: 0717-3458
Electron. J. Biotechnol.
http://www.ejbiotechnology.info
DOI: 10.2225/vol15-issue4-fulltext-4 *SHORT COMMUNICATION*

Callus induction and plant regeneration of *Ulex europaeus*

Ingrid Ramírez[1] ✉ · Fernando Dorta[1] · Álvaro Cuadros-Inostroza[2] · Hugo Peña-Cortés[1]

1 Universidad Técnica Federico Santa María, Centro de Biotecnología "D. Alkalay L.", Laboratorio de Biología Molecular, Valparaíso, Chile

2 Max Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany

✉ Corresponding author: ingrid.ramirez@usm.cl
Received December 9, 2011 / Accepted May 15, 2012
Published online: July 15, 2012

Abstract A callus induction and plant regeneration protocol was developed from leaf and thorn explants for the plant *Ulex europaeus*. Explants were incubated on 2% sucrose half-strength Murashige and Skoog Medium (MS) with various combinations of plant growth regulators and antioxidants. The best frequency of callus and shoot formation was obtained with 2,4-dichlorophenoxyacetic acid (2,4-D) 1 mg/l x kinetin (Kin) 0.2 mg/l (DK Medium; callus induction) and zeatin (Z) 1 mg/l (DK medium; shoot induction). Both media were supplemented with ascorbic acid 200 mg/l to prevent browning and death of the explants. The regenerated shoots transferred to rooting medium (half-strength MS Medium, 2% sucrose) showed rapid growth and development of roots (100%). Rooted plantlets were successfully transferred to soil in pots containing a 3:1 mixture of soil and vermiculite.

Keywords: antioxidants, callus induction, Fabaceae, gorse, plant regeneration, *Ulex europaeus*

INTRODUCTION

Ulex europaeus (common gorse) is a plant of the Fabaceae-Pea family native to Western Europe and North Africa (Clements et al. 2001). Gorse is a perennial evergreen, leguminous, dense spiny shrub that can grow up to 7 m high (0.6-2 m on average) (Clements et al. 2001; Rees and Hill, 2001). In its region of origin, it is a non-aggressive invader of disturbed areas that is recognized as useful for wildlife protection, soil stabilization (nitrogen-fixer) and revegetation and is also used as an ornamental plant (Clements et al. 2001).

Gorse is an invasive weed in many countries around the world, mainly in Chile, Australia, Hawaii and New Zealand (Clements et al. 2001; Rees and Hill, 2001; Leary et al. 2006). It invades dry and disturbed areas such as beaches, cleared timber lands, and overgrazed pastures (Rees and Hill, 2001; Leary et al. 2006). Gorse spreads readily forming dense impenetrable thickets, difficult to control once established (Rees and Hill, 2001; Leary et al. 2006).

U. europaeus produces biogenic volatile organic compounds (VOC) that make the plant particularly flammable (Boissard et al. 2001). That makes this shrub, in addition to rapid production of biomass and ability to fix nitrogen, a useful energy crop on derelict land (Lawson, 1987; Buddenhagen et al. 2009). In New Zealand for example, 4-5 years-old stands have shown biomass accumulation rates approaching 15t ha^{-1} yr^{-1} with nitrogen fixation rates of 100-200 kg ha^{-1} yr^{-1} (Magesan et al. 2012). However, the control of the invasiveness of wild environments still remains a problem (Buddenhagen et al. 2009).

Plant cell and tissue culture is a potentially useful technique for micropropagation and improvement of plants with biotechnological purposes (Verpoorte et al. 2002; Debnath et al. 2006). Traditional methods like vegetative reproduction or seeds germination have been mainly employed to multiplicate gorse (Clements et al. 2001). Searching the literature, no information was found describing regeneration system or micropropagation through tissue culture. Thus, this study describes the first successful *in vitro* regeneration system of *U. europaeus*, including plant rooting and transferring to soil pots for further development and growth in the greenhouse.

MATERIALS AND METHODS

Seed germination

Seeds of *U. europaeus* were collected from a wild population in Colaco, Chile. Because of the seed coat's hardness, seeds were soaked in concentrated sulphuric acid for two hours and then rinsed with distilled water to improve the rate of germination (Sixtus et al. 2003). Surface sterilization was carried out with ethanol 70% for 30 sec followed by 10% commercial bleach for 10 min and then rinsed thrice with sterile distilled water. Seeds were subsequently placed in sterile vessels containing Murashige and Skoog Medium (MS medium; Murashige and Skoog, 1962) supplemented with 2% sucrose and 0.8% agar and maintained in a growth chamber at 18-22ºC with fluorescent light (cool-white light; 200 µmol m^{-2} s^{-1}) and 16 hrs light and 8 hrs dark photoperiod until germination.

In vitro plant growth

Germinated seeds were transferred to vessels containing one of the following media: MS medium supplemented with 1% or 2% sucrose or half-strength MS medium supplemented with 1% or 2% sucrose. All cultures were maintained in a growth chamber at similar conditions as those used for germination. The growth and rooting obtained were evaluated (see Results and Discussion) to select the media used for the preparation of the explants for the callus induction and regeneration assays.

Callus induction

Leaf and thorn explants, from 4-6 weeks old plants, were placed on half-strength MS medium supplemented with 2% sucrose and 0.8% agar in Petri plates. Two combinations of plant growth regulators were used for callus induction: α-naphthaleneacetic acid (NAA) 5 mg/l x benzyladenine (BA) 0.1 mg/l (NBA Medium) and 2,4-dichlorophenoxyacetic acid (2,4-D) 1 mg/l x kinetin (Kin) 0.2 mg/l (DK Medium). The plant hormone combinations were tested either alone or supplemented with the following antioxidants: ascorbic acid 200 mg/l, citric acid 100 mg/l, PVPP 0.5% (w/v) or PVPP 2% (w/v).

Fifteen explants were tested in each Petri dish. Five replicates were used per every type of callus induction media and each experiment was repeated twice.

The Petri dishes were maintained ten days in a growth chamber at 22ºC with fluorescent light (cool-white light; 200 µmol m^{-2} s^{-1}) and 16 hrs light and 8 hrs dark photoperiod. Callus production was evaluated weekly for every type of media. The frequency of explants with viable callus (percentage) was recorded at three and seven weeks.

Shoot formation and plant regeneration

After ten days in the medium for callus induction the explants were transferred to Petri plates containing half-strength MS medium supplemented with 2% sucrose, 0.8% agar and one of the following combinations of plant hormones for shoot induction: zeatin riboside (ZR) 2 mg/l x gibberellic acid (GA_3) 0.02 mg/l x α-naphthaleneacetic acid (NAA) 0.02 mg/l (ZR Medium; explants from medium NBA) or zeatin (Z) 1 mg/l (Z Medium; explants from DK medium). The media were used either alone or supplemented with the same antioxidants used for each explant in the induction of callus (ascorbic acid 200 mg/l, citric acid 100 mg/l, PVPP 0.5% (w/v) or PVPP 2% (w/v)). All the explants were maintained in a growth chamber at similar conditions as those used for callus induction. Until the excision of the

shoots, the media were changed every 10 days. Shoot formation was monitored weekly until seven weeks.

Rooting

The generated shoots (5-10 mm) were excised and individually transferred to sterile vessels with half-strength MS medium supplemented with 2% sucrose for rooting. Rooted plants were transferred to pots containing 3:1 mixture of soil and vermiculite for further acclimation and growth in the greenhouse (16 hrs photoperiod, 16-24ºC and 80-90% humidity).

Statistical analysis

Data were imported into R environment software (http://www.r-project.org) in order to carry out statistical analysis. The following approach was used to determine significant differences between the different treatments and their control. Firstly, a one-way ANOVA was performed to identify significant differences among the treatment-control groups ($p < 0.05$). Secondly, if the previous test was significant, then Tukey's honesty significance differences method was applied to compare simultaneously the means of every treatment against the control group and establish their significance ($p < 0.05$). Letters (a, b, c, d) were used to indicate statistic differences between means. All data were presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Seed germination and growth of plants

In order to maximize germination of *U. europaeus*, seeds were scarificated with sulphuric acid for two hours before sterilization and incubation in MS medium. With this treatment, germination rates at two weeks (60-65%, Figure 1a) similar to those described by Sixtus et al. 2003 were obtained.

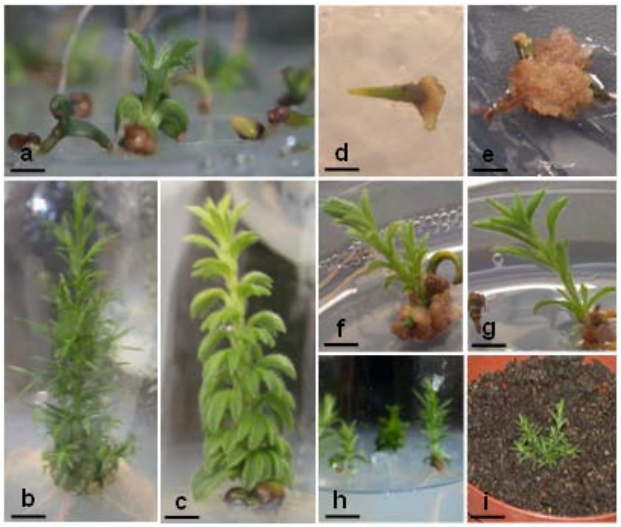

Fig. 1 *In vitro* growth and regeneration of *Ulex europaeus*. (a) *In vitro* germination of *Ulex europaeus* on MS medium, after scarification with concentrated sulphuric acid. Growth of the germinated plants after six weeks transferred to (b) half-strength MS medium supplemented with 2% sucrose or (c) MS medium supplemented with 2% sucrose. Appearance of calli after culture on media supplemented with ascorbic acid 200 mg/l and the following plant growth regulators: (d) NAA 5 mg/l x BA 0.1 mg/l (1 week, callus NBA medium) and ZR 2 mg/l x GA_3 0.02 mg/l x NAA 0.02 mg/l (3 weeks, shoot ZR medium) and (e) 2,4-D 1 mg/l x Kin 0.2 mg/l (1 week, callus DK medium) and Z 1 mg/l (3 weeks, shoot Z medium). Development of shoot of the callus obtained in (e) after 5 weeks (f-g) in the shoot Z medium supplemented with 200 mg/l ascorbic acid . The same shoots of (f-g) after 4 weeks on rooting medium (h) and 6 weeks after transfer to soil pots (i). (bar = 3 (a), 5 (b), 5 (c) mm, 2.5 (d), 3.2 (e), 3 (f), 3 (g), 6 (h), 10 (i) mm).

DOI: 10.2225/vol15-issue4-fulltext-4

Because of the lack of previous protocols on *in vitro* growth of *U. europaeus* in the literature, it was decided to test four different media for plants to obtain the best conditions to grow, propagate and root the germinated plants (see Materials and Methods). The best results were obtained with media containing sucrose 2% (Figure 1b-c). Among them, phenotypes were obtained that are more similar to those obtained for plants grown in soil with the medium with half-strength MS salts. The plants show the typical small leaves and thorns (Figure 1c). Based on that, it was decided to grow and propagate the gorse plants in this medium to obtain the explants required for the regeneration assays.

Callus induction

Initially calli development was tested using leaf and thorn explants obtained from the plants propagated *in vitro* and two combinations of auxin and cytokinin as described in Materials and Methods: NBA medium and DK medium. Calli were observed from the second week of culture on for the two media tested without differences based on the type of explant used. However, the percentage of viable explants with callus at seven weeks was less than ten percent for both media (NBA → ZR media: 3.0 ± 4.8, DK → Z media: 9.0 ± 7.4). There was a high percentage of explants (with or without callus) that became brown and finally died (NBA → ZR media: 82.0 ± 7.9, DK → Z media: 72.0 ± 6.3).

In plant cell tissue culture, explant browning and subsequent death is a major problem in the initiation of cultures of woody plants and is generally attributed to phenolic compounds (Anthony et al. 2004). However, it has been found that antioxidants are able to reduce the browning and death rates, increasing the percentages of regeneration of woody plants (Dan, 2008). In an effort to improve the *U. europaeus* callus induction process, the effect of adding antioxidants to the callus/shoot media was tested in this research (see Materials and Methods). Creamy yellow soft smooth calli without signs of browning were obtained in all the media with antioxidants tested (data not shown). A notorious browning and/or death of the calli could only be observed for the control media. Figure 1d-e shows the results obtained in media supplemented with ascorbic acid. Within the two media with this antioxidant, the best callus yields per explant were obtained in the DK medium.

Figure 2 summarizes the results obtained with ascorbic acid, citric acid or PVPP. Calli were developed in all media combinations; however viable explants with callus (percentage) varied significantly ($p < 0.05$) depending on the combination used. DK medium was more responsive to the presence of antioxidants for callus growth than NBA media. The highest callus frequency (40.0%; $p < 0.05$) was obtained with DK medium combined with ascorbic acid. Significant frequency increases also were obtained for citric acid (25.3%) and PVPP 0.5% (18.7%) compared to the control. For the DK medium, the addition of antioxidants also prevented the death of developed callus. As seen in Figure 2a-b, in the DK medium without antioxidants a decrease in the viable explants with callus from 25% to 10% between the third and seventh week of culture was observed. On the other hand, callus induction was less than 10% for all NBA medium combinations and a significant increase was only observed with ascorbic acid.

Shoot formation and plant regeneration

As described in Materials and Methods, after ten days in the callus induction media all the explants were transferred to the shoot induction media supplemented with antioxidants. All the media tested showed production of shoots (Figure 2c), the number of shoots being slightly higher for the Z medium combinations: 3.3-11.3% compared to 2.0-4.7% for ZR media. No clear differences were observed in the frequency of shoots obtained with the different types of explants (data not shown) and antioxidants tested (Figure 2c). Only PVPP 0.5% showed a significant increase shoot frequency (11.3%) compared to the control of Z medium alone (3.3%,Figure 2c).

When the appearance of the obtained shoots was analyzed, the explants from the Z media combined with ascorbic acid showed healthy and faster growing shoots (Figure 1f-g). Many of these explants also showed multiple shoots per callus (Figure 1f). Based on these results and those obtained for the production of calli, it was decided to perform the following rooting and acclimation tests only with the shoots obtained from DK → Z media supplemented with ascorbic acid 200 mg/l.

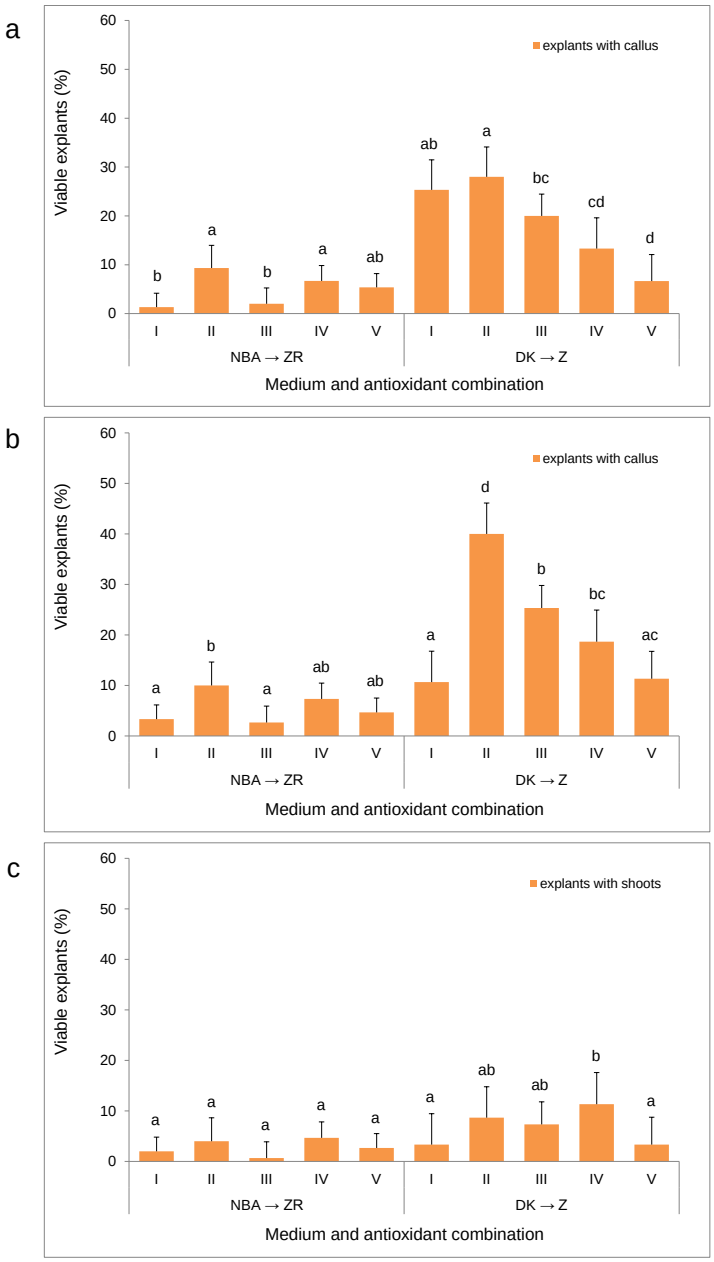

Fig. 2 Effect of various auxin, cytokinin and antioxidants combinations on callus and shoot induction of explants of *Ulex europaeus*. Formation of callus was monitored at three (a) and seven weeks (b) of culture. The shoot induction is shown at seven weeks (c). NBA → ZR: combination of medium with NAA 5 mg/l x BA 0.1 mg/l for callus induction and ZR 2 mg/l x GA_3 0.02 mg/l x NAA 0.02 mg/l for shoot induction. DK → Z: medium with 2,4 -D 1 mg/l x Kin 0.2 mg/l for callus induction and Z 1 mg/l for shoot induction. I: control without antioxidant; II: ascorbic acid 200 mg/l; III: citric acid 100 mg/l; IV: PVPP 0.5%; V: PVPP: 2%. Values represent the mean ± standard deviation of 15 explants of 10 independent Petri dishes for each treatment. Letters (a, b, c, d) indicate statistically significant differences between means (Tukey's HSD test. $p < 0.05$).

Rooting and acclimation

In the seventh week of culture, 5-10 mm long shoots were separated from the leaf and thorn derived calli and transferred to rooting medium (half-strength MS medium supplemented with 2% sucrose). After 4 weeks, 100% of the plants survived and rooted (Figure 1h). Whole plantlets were transferred to

pots (see Materials and Methods) and grown until maturity under greenhouse conditions. The plants showed high survival rates (over 80%, data not shown), grew successfully into normal mature plants (Figure 1i) and set flowers and seeds.

In summary, this study presents the first report of a regeneration protocol for *Ulex europaea*. DK → Z media mixed with ascorbic acid showed to be the most effective combination in producing calli and shoot regeneration. The complete protocol until transfer of rooted plants to soil takes approximately 12 weeks. Further refinement of the protocol could be conducive to achieving increased efficiency and reduce times involved.

It has been described that gorse produces biogenic VOC that mainly include terpenes as isoprene, α-pinene, camphene, β-pinene, limonene, trans-ocimene (Boissard et al. 2001). This regeneration system provides an effective tool to establish diverse strategies which might potentiate the biotechnological use of this plant. The generating capacity of plant material by using the described regeneration protocol will facilitate the isolation and characterization of regulatory components of important metabolic pathways in order to improve the production of desired molecules. The identification of such components could provide new instruments for implementing innovative strategies to increase for instance terpenoid yield or change terpenoid distribution for desired properties such as enhanced flavour, fragrance, colour (Roberts, 2007) or chemical composition for use in bioenergy production (Carroll and Somerville, 2009). It could also provide an environmentally friendly, renewable and alternative supply for secondary metabolite production, for example volatiles with high-value for the pharmaceutical industry (Predieri and Rapparini, 2007; Roberts, 2007).

REFERENCES

ANTHONY, J.M.; SENARATNA, T.; DIXON, K.W. and SIVASITHAMPARAM, K. (2004). The role of antioxidants for initiation of somatic embryos with *Conostephium pendulum* (Ericaceae). *Plant Cell, Tissue and Organ Culture*, vol. 78, no. 3, p. 247-252. **[CrossRef]**

BOISSARD, C.; CAO, X.-L.; JUAN, C.-Y.; HEWITT, C.N. and GALLAGHER, M. (2001). Seasonal variations in VOC emission rates from gorse (*Ulex europeaus*). *Atmospheric Environment,* vol. 35, no. 5, p. 917-927. **[CrossRef]**

BUDDENHAGEN, C.E.; CHIMERA, C. and CLIFFORD, P. (2009). Assessing biofuel crop invasiveness: A case study. *PLoS One*, vol. 4, no. 4. **[CrossRef]**

CARROLL, A. and SOMERVILLE, C. (2009). Cellulosic Biofuels. *Annual Review of Plant Biology*, vol. 60, p. 165-182. **[CrossRef]**

CLEMENTS, D.; PETERSON, D. and PRASAD, R. (2001). The biology of Canadian weeds. 112. *Ulex europaeus* L. *Canadian Journal of Plant Science*, vol. 81, no. 2, p. 325-337. **[CrossRef]**

DAN, Y. (2008). Biological functions of antioxidants in plants transformation. *In Vitro Cellular and Developmental Biology-Plant*, vol. 44, no. 3, p. 149-161. **[CrossRef]**

DEBNATH, M.; MALIK, C.P. and BISEN, P.S. (2006). Micropropagation: A tool for the production of high quality plant-based medicines. *Current Pharmaceutical Biotechnology*, vol. 7, no. 1, p. 33-49.

LAWSON, G.J. (1987). Hill weed compensatory allowances: Very alternative crops for the uplands. In: BELL, M. and BUNCE, R.G.H. eds. *Agriculture and conservation in the hills and uplands.* Grange-over-Sands, NERC/ ITE, (ITE Symposium, 23), p. 99-106.

LEARY, J.K.; HUE, N.V.; SINGLETON, P.W. and BORTHAKUR, D. (2006). The major features of an infestation by the invasive weed legume gorse (*Ulex europaeus*) on volcanic soils in Hawaii.*Biology and Fertility of Soils*, vol. 42, no. 3, p. 215-223. **[CrossRef]**

MAGESAN, G.N.; WANG, H. and CLINTON, P.W. (2012). Nitrogen cycling in gorse-dominated ecosystems in New Zealand. *New Zealand Journal of Ecology*, vol. 36, no. 1, p. 21-28.

MURASHIGE, T. and SKOOG, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum,* vol. 15, no. 3, p. 473-497. **[CrossRef]**

PREDIERI, S. and RAPPARINI, F. (2007). Terpene emission in tissue culture. *Plant Cell, Tissue and Organ Culture*, vol. 91, no. 2, p. 87-95. **[CrossRef]**

REES, M. and HILL, R.L. (2001). Large-scale disturbances, biological control and the dinamics of gorse populations. *Journal of Applied Ecology*, vol. 38, no. 2, p. 364-377. **[CrossRef]**

ROBERTS, S. (2007). Production and engineering of terpenoids in plant cell culture. *Nature Chemical Biology*, vol. 3, no. 7, p. 387-395. **[CrossRef]**

SIXTUS, C.R.; HILL, G.D. and SCOTT, R.R. (2003). The effect of temperature and scarification method on gorse (Ulex europaeus L.) seed germination. *New Zealand Plant Protection*, vol. 56, p. 201-205.

VERPOORTE, R.; CONTIN, A. and MEMELINK, J. (2002). Biotechnology for the production of plant secondary metabolites. *Phytochemistry Reviews*, vol. 1, no. 1, p. 13-25. **[CrossRef]**

How to reference this article:

RAMIREZ, I.; DORTA, F.; CUADROS-INOSTROZA, A. and PEÑA-CORTÉS, H. (2012). Callus induction and plant regeneration of *Ulex europaeus*. *Electronic Journal of Biotechnology*, vol. 15, no. 4. http://dx.doi.org/10.2225/vol15-issue4-fulltext-4