

IN VITRO PROPAGATION OF *INULA ROYLEANA* DC.

ANNA STOJAKOWSKA, JANUSZ MALARZ

Department of Phytochemistry, Institute of Pharmacology
Polish Academy of Sciences
Śmętna 12, 31-343 Kraków, Poland
e-mail: stoja@if-pan.krakow.pl

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ABSTRACT

A micropropagation method, through axillary shoot proliferation, was elaborated for *Inula royleana* DC. (Asteraceae), a medicinal plant native of Himalaya. Primary explants (cotyledonary node explants) and secondary explants (node explants of in vitro regenerated shoots) of the plant, inoculated on MS medium supplemented with 0.1 μM NAA and 5.0 μM kinetin, regenerated 3.4 ± 1.2 and 5.1 ± 1.9 axillary shoots per explant, respectively. The regenerated shoots were easily rooting and adapting to growth in soil.

KEY WORDS: *Inula royleana*, axillary shoots, micropropagation, Asteraceae.

INTRODUCTION

Inula royleana DC. (Asteraceae, Inuleae) is a perennial herb, native of the Western Himalaya and Cashmere. Roots of the plant were reported to contain sesquiterpene lactones of eudesmane type (Bohlmann et al. 1978; Qurishi M. et al. 1980), abietane diterpenes (Edwards et al. 1962; Bhat et al. 1975) and diterpene alkaloids (Khaleque et al. 1959; Hegnauer R. 1964). The compounds possess a wide array of biological activities e.g. insecticidal (Jennings et al. 1986), insect repellent (Ulubelen et al. 2001), antimicrobial (Yang et al. 2001), antiinflammatory (Dirsch et al. 2000) and antiproliferative against different cancer cell lines (Lawrence et al. 2001; Konishi et al. 2002). Moreover, vasodepressor effect of some abietanes was reported (Kolak et al. 2001; Ulubelen et al. 2002).

Genus *Inula* comprises numerous species of reputed medicinal value (*I. helenium* L., *I. racemosa* Hooker fil., *I. viscosa* (L.) Aiton, *I. britannica* L.) (Blaschek et al. 1998). A number of hybrids is also known from nature. To our knowledge, no reports are available on micropropagation of *Inula* sp., except for that by Thiem et al. (2003).

The aim of the present study was to establish a genetically uniform population of *I. royleana* plants to study their secondary metabolism. A preliminary HPLC analysis of chloroform extracts from roots of in vitro grown plants, raised from seeds delivered by several botanical gardens and commercial growers, showed remarkable quantitative differences in their secondary metabolite content. Axillary shoot proliferation, a method of micropropagation which minimizes risk of genetic variation in regenerants, could be used to propagate highly productive clones.

MATERIAL AND METHODS

Plant material

Seeds of *Inula royleana* DC. were delivered by the Botanical Garden of the University in Padova (Italy). The seeds were surface sterilized with a 15% solution of sodium hypochlorite, rinsed with sterile water and germinated on a hormone free MS (Murashige and Skoog 1962) medium, solidified with 0.8% agar, at 25°C and continuous light (ca. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, cool white fluorescent tubes). Cotyledonary nodes of the aseptic seedlings were used as primary explants. Plants regenerated in vitro were used as a source of secondary explants (nodal explants).

Culture media and conditions

Solidified MS medium, containing 3% sucrose, with pH adjusted to 5.8, before autoclaving (20 min at 121°C) was used as a basal medium in all axillary shoot multiplication experiments. For rooting, MS medium with concentration of macronutrients reduced by half was also applied. All cultures were kept at 25°C, under continuous light (40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, cool white fluorescent tubes).

Axillary shoot regeneration from cotyledonary node explants

The primary explants of *I. royleana* were inoculated on culture media containing either 0.1 μM or 0.25 μM of α -naphthaleneacetic acid (NAA) together with 0.25-5.0 μM and 0.5-10.0 μM of kinetin (Kn), respectively (Table 1). Control explants were maintained on hormone-free medium. Ten explants were used per treatment and every

TABLE 1. Axillary shoot regeneration in cotyledonary node explants of *I. royleana*, inoculated on MS medium supplemented with NAA and Kn, after six weeks of culture.

NAA [μM]	Kn [μM]	% response	Multiplication rate \pm SD	Roots	Callus
0.0	0.0	100	1.1 \pm 0.3	+	–
0.1	0.25	100	1.7 \pm 0.9	+	+
0.1	0.5	90	1.6 \pm 0.5	+	+
0.1	2.5	90	1.9 \pm 0.6	–	+
0.1	5.0	100	3.4 \pm 1.2	–	–
0.25	0.5	100	1.2 \pm 0.4	+	–
0.25	2.5	100	1.4 \pm 0.7	+	+
0.25	5.0	100	2.0 \pm 0.5	–	+
0.25	10.0	100	2.6 \pm 0.7	–	–

TABLE 2. Axillary shoot multiplication in nodal explants of *I. royleana*, inoculated on MS medium supplemented with NAA and Kn, after six weeks of culture.

NAA [μM]	Kn [μM]	% response	Multiplication rate \pm SD	Roots	Callus
0.1	2.5	94	3.3 \pm 1.3	+	–
0.1	5.0	94	5.1 \pm 1.9	+	–
0.25	2.5	94	4.1 \pm 1.5	+	–
0.25	5.0	97	5.1 \pm 2.0	+	+
0.25	10.0	100	5.2 \pm 1.7	+	+

experiment was repeated. The multiplication rate, expressed as a number of shoots regenerated from one explant within six weeks and a frequency of response, i.e. percentage of explants showing either callus or organ formation, were calculated for each treatment.

Axillary shoot multiplication from secondary explants

Nodal explants from regenerated plants were inoculated on the media supplemented with either 0.1 μM NAA together with 2.5 μM and 5.0 μM Kn or 0.25 μM NAA together with 2.5 μM , 5.0 μM and 10.0 μM Kn (Table 2). Thirty explants per treatment were used and every experiment was repeated. Multiplication rate and a frequency of response were calculated for each treatment.

Rooting of regenerated shoots and introduction of regenerated plants to growth in soil

Shoots regenerated in vitro were inoculated either on hormone free MS medium or on MS medium supplemented with 1 μM indolebutyric acid (IBA). Two weeks after inoculation, 70% of shoots developed roots on the medium without hormones, whereas nearly all shoots were rooted on the medium containing IBA. However, irrespectively of the medium used, five weeks after the transfer to the rooting medium, all shoots regenerated roots and adapted easily to the growth in soil under greenhouse conditions.

RESULTS AND DISCUSSION

The medium supplemented with 0.1 μM NAA and 5.0 μM Kn proved to be the most favourable for axillary shoot proliferation in both cotyledonary node and secondary nodal explants of *I. royleana* (Table 1 and 2). The multiplication rate for cotyledonary nodes, after six week culture, was 3.4 \pm 1.2, with 100% frequency. Neither callus nor root development were observed. The multiplication rate for secondary explants reached 5.1 \pm 1.9, with similar frequency (94%) (Fig. 1). In some shoots spontaneous rootning

occurred and fully regenerated plantlets could be obtained in the end of a six week culture period. No rhizogenesis was observed independently of shoot regeneration. Axillary shoots, obtained in proliferation step, were further successfully rooted, if necessary, either on the hormone free MS medium with half strength concentration of macronutrients (Fig. 2) or on the MS medium containing 0.1 μM IBA. An addition of IBA speeded up the rooting process, but had no effect on percentage of shoots rooted after four weeks on rooting medium and percentage of shoots adapted to growth in soil.

Media containing benzyladenine (BA) (2.22-8.87 μM) together with NAA (0.27-0.54 μM) were preferably used for multiple shoot induction and maintenance in Asteraceae plants (Winand et al. 1986; Akita et al. 1994; Laparra et al. 1997; Stojakowska and Kisiel 1997; Wildi et al. 1998; Liu et al. 1998). However, our preliminary attempts to induce axillary shoot proliferation using MS medium containing 0.25 μM NAA together with 0.25-10.0 μM BA failed either due to low multiplication rate (up to 1.8 \pm 0.5 using 2.5 μM BA) or due to abundant callus formation using 5.0-10.0 μM BA. According to Wildi et al. (1998), application of Kn instead of BA to shoot multiplication was reasonable when shoot length and spontaneous rooting of shoots were taken into consideration. In our cultures also the tendency to indirect organogenesis was diminished using Kn.

Literature data concerning micropropagation of Asteraceae tribe Inuleae are sparse. Meyer and van Staden (1989) described the effects of different combinations of growth regulators on the organogenesis in *Geigeria aspera* Harv. callus explants. Optimum for shoot regeneration was a medium containing 0.1-1.0 μM BA in combination with 0.1-1.0 μM indoleacetic acid (IAA). Thiem et al. (2003) used a combination of BA (2.22 μM) and NAA (0.26 μM) to regenerate shoots in apical explants of *Inula verbascifolia* var. *aschersoniana* (Janka) seedlings. The shoots were rooted either on MS medium supplied with 0.98 μM IBA or on half strength, hormone free MS medium and were subsequently adapted to growth in soil.



Fig. 1. Multiple shoots regenerated from node explant of *Inula royleana* on MS medium supplemented with 0.1 μ M NAA and 5.0 μ M kinetin.

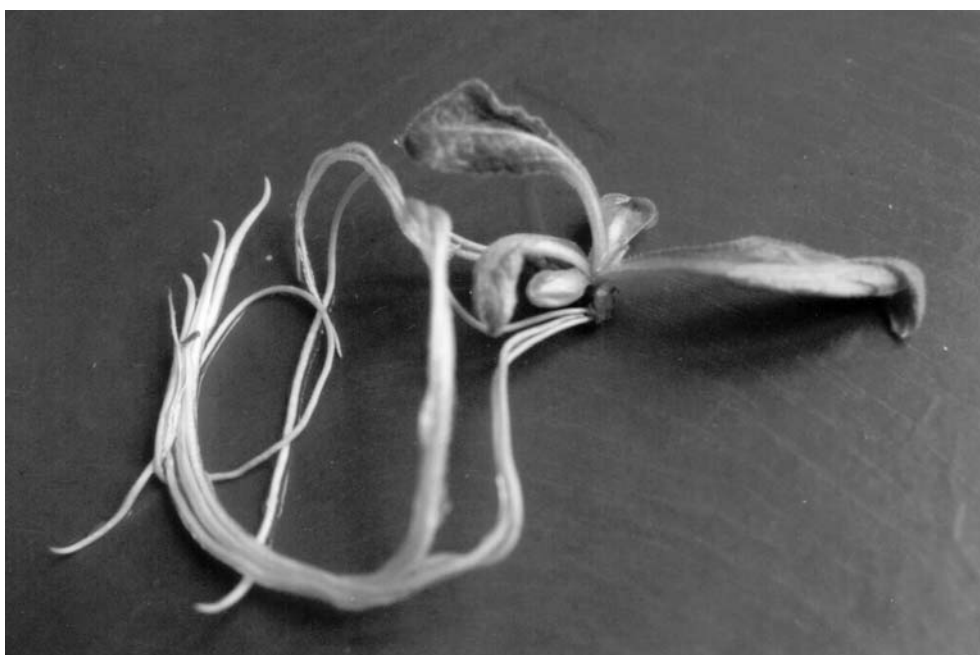


Fig. 2. *Inula royleana* plant rooted on MS medium with reduced content of macronutrients.

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