Two-Stage System for Micropropagation of Several *Genista* Plants Producing Large Amounts of Phytoestrogens

Maria Łuczkiewicz[a,*] and Arkadiusz Piotrowski[b]

[a] Department of Pharmacognosy, Medical University of Gdańsk, al. Gen. J. Hallera 107, 80-416 Gdańsk-Wrzeszcz, Poland. E-mail: mlucz@farmacja.amg.gda.pl
[b] Department of Biology and Pharmaceutical Botany, Medical University of Gdańsk, al. Gen. J. Hallera 107, 80-416 Gdańsk-Wrzeszcz, Poland

* Author for correspondence and reprint requests

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A two-stage method for *in vitro* propagation of six *Genista* species from shoot tips was developed. Multiple microshoot cultures were obtained by growing the shoot tip explants on Schenk and Hildebrandt medium supplemented with 9.84 μM 6-(γ,γ-dimethylallylamino)-purine and 0.99 μM thidiazuron. The best shoot elongation was achieved on Schenk and Hildebrandt medium containing 4.92 μM indole-3-butyric acid. The rooting of shoots brought best effects (100%) on Schenk and Hildebrandt medium with 2.68 μM 1-naphthaleneacetic acid. HPLC analysis indicated that six-month-old regenerated plants as well as the herb of intact plants produced a rich set of simple flavones (derivatives of luteolin and apigenin) and isoflavones (derivatives of genistein, daidzein, formononetin and biochanin A). Multiple microshoot cultures of all species produced no simple flavones at all. *In vitro* shoots accumulated selectively a rich group of phytoestrogens in the form of aglucones, glucosides and esters (derivatives of genistein and daidzein). Cultures obtained *in vitro* synthesized many times more isoflavones than the intact plants. In all shoots which were micropropagated the dominating compound was genistin (*e.g.* shoots of *G. tinctoria* – ca 3281.4 mg per 100 g dry weight). Possible influence of tissue differentiation on isoflavone content under *in vitro* and *in vivo* conditions is discussed.

Key words: Genista Species, Isoflavones, Multiple Shoot Proliferation

Introduction

The *Genista* genus includes several hundred species of slow-growing, decorative trees and shrubs which contain high concentrations of secondary metabolites of multidirectional pharmacological activity (van Rensen *et al.*, 1993).

Apart from toxic quinolizidine alkaloids, these plants contain a rich set of flavonoids, including several isoflavones which show valuable phytoestrogenic activity (van Rensen *et al.*, 1993; Frische and Steinhart, 1999; Qiang Ren *et al.*, 2001).

Phytoestrogens can be used in menopause symptom treatment and in the prevention of tumours related to the estrogenic balance within the body (Brandi, 1997, 1999; Danzo, 1998; Dixon and Ferreira, 2002). It is also believed that, given their considerable affinity to estrogen receptors β, they might in future be used to treat breast cancer, endometrial cancer and prostate hypertrophy (Dixon and Steele, 1999; Mueller and Korach, 2001; Radzikowski *et al.*, 2004).

The biotechnological research carried out so far on six selected *Genista* species (*G. tinctoria* L., *G. sagittalis* L., *G. radiata* L., *G. monospessulana* L., *G. aethnensis* D.C. and *G. germanica* L.) showed that callus cultures of these plants selectively synthesized a rich set of phytoestrogens, derivatives of genistein and daidzein, which by far exceeded the amounts accumulated in the intact plants (Łuczkiewicz and Głód, 2003). At the same time, the levels of genistein and its derivatives in the calli were much higher than in soy products, which have traditionally been considered to be the best source of these compounds (Federici *et al.*, 2003).

In view of the above, and given the fact that biosynthesis of toxic quinolizidine alkaloids in the

Abbreviations: BAP, 6-benzylaminopurine; DW, dry weight; 2iP, 6-(γ,γ-dimethylallylamino)-purine; GA_3, gibberellic acid; HPLC, high performance liquid chromatography; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; KIN, kinetin; NAA, 1-naphthaleneacetic acid; SH medium, Schenk and Hildebrandt medium (Schenk and Hildebrandt, 1972); SH_0 medium, Schenk and Hildebrandt medium without growth regulators; TDZ, thidiazuron; ZEA, zeatin.

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tested biomasses was restrained (Łuczkiewicz *et al.*, 2004a), it can be concluded that callus cultures of *Genista* plants could be used in the future to develop a rich, alternative source of phytoestrogens for the pharmaceutical industry. Unlike the intact plants, the callus cultures did not synthesize simple flavones, derivatives of luteolin and apigenin, with a different isoflavone production profile (Łuczkiewicz and Głód, 2003). The question was whether the changes in flavonoid accumulation in the calli of *Genista* plants were due to growing the plants *in vitro* (elements of abiotic stress) (Barz and Mackenbock, 1994) or due to the changed morphological status of the biomasses. To find an answer to this questions *in vitro* shoot cultures from six selected *Genista* plants were developed and their flavonoid production profiles were compared with those of original plants and the previously cultivated callus cultures. Based on these results, it will be possible to choose the type of *in vitro* plant material for further modifications in order to optimize the biosynthesis of isoflavones. Thanks to the diversity of the *in vitro* biomasses developed it will be possible to carry out research onto the mechanisms of biosynthesis of phytoestrogens *in vitro*.

Moreover, a method for effective micropropagation of the researched species was developed. *Genista* plants are currently propagated by air layering. In order to overcome low rate of *in vivo* propagation Curir *et al.* (1990) and Ruffoni *et al.* (1999) proposed for the first time for *Genista monosperma* Lam. the method of micropropagation through somatic embryogenesis. This procedure, when applied by the authors of this paper to the other *Genista* species, yielded unsatisfactory results (low survival and development of primary, globular embryos, depending on the species). A decision was made to develop an universal, reproducible method of micropropagation for these species. Compared with the traditional method, it would allow fast production of significant amounts of plant material rich in pharmacologically active compounds. The intention was to achieve this by proliferating axillary shoots from shoot tip explants on media supplemented with variable compositions of growth regulators, identifying at the same time the effect of the particular auxins and cytokinins on the production of primordial cultures, the elongation of shoots and the rooting of *Genista* plants.

As the biotechnological research involved six species, it was possible to carry out comparative analyses of the species and to draw general conclusions about micropropagation of *Genista* genus.

In this paper we report for the first time the micropropagation method for six *Genista* species. The effect of cell differentiation and morphogenetic status of plant material on isoflavone and flavone accumulation is discussed for the first time, too.

Materials and Methods

Plant material

Seeds of *Genista tinctoria* L., *Genista radiata* L., *Genista aethnensis* D.C. (Botanic Gardens, Glasgow), *Genista sagittalis* L., *Genista germanica* L. and *Genista monospessulana* L. (Botanischer Garten der Technischen Universität Braunschweig), obtained in 2000, were used for *in vitro* studies. Samples of these seeds are deposited at the herbarium of the Medicinal Plant Garden of the Medical University of Gdańsk.

In order to confirm the identity of the plant material all seeds were subjected to DNA isolation with modified CTAB protocol (Doyle and Doyle, 1987) with additional purification by phenol/chloroform extraction. Nuclear rDNA ITS1 & 2 region was amplified using the primers ITS4 and ITS5 of White *et al.* (1990) (*G. tinctoria, G. radiata, G. aethnensis, G. monospessulana*). Non-coding cpDNA was amplified using the universal primers c and d described by Taberlet *et al.* (1991) for *trnL*(UAA) intron (*G. sagittalis*). Both standards of purified PCR product were sequenced using big-dye terminator chemistry (Applied Biosystems, Foster City, CA, U.S.A.) with ABI 377 automated sequencers following the protocol provided by the manufacturer. The Phred/Pharp/Consed software suite was used to call the bases from the ABI trace data, to assign quality values, and to edit/view sequences (Ewing and Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998). Resulting sequences were aligned together with the Gen Bank sequences corresponding with the species from botanical identification. ClustalW (Thompson *et al.*, 1994) and BioEdit (Hall, 1999) were used to align the sequences and to view the alignment, respectively. Next, partial ITS1, ITS2 and *trnL*(UAA) sequences were separately searched against the nt database using standard nucleotide-

nucleotide BLAST [blastn] with default parameters.

The seeds were surface sterilized in an 0.1% aqueous solution of $HgCl_2$ for 45 min, rinsed 3 times with sterile double distilled water and placed in petri dishes, on sterile filter paper soaked in gibberellic acid (GA_3) (28.9 μM) and kinetin (KIN) (46 μM). The dishes were sealed with parafilm and kept in the dark at 25 ± 2 °C and after a week seedlings were obtained. The seedlings were transferred on Schenk and Hildebrandt medium (SH_0 medium) (Schenk and Hildebrandt, 1972), supplemented with 3% w/v of sucrose and solidified with 0.7% w/v of agar. The plant material was incubated in baby food jars, in a growth chamber at 25 ± 2 °C with continuous light (intensity 88 ± 8 μmol m^{-2} s^{-1}) supplied by fluorescent TLD Philips lamps. The shoot tips from 4-week-old sterile plants were used to initiate *in vitro* propagation of *Genista* plants.

Culture media and procedure used for micropropagation

The shoot tips of all *Genista* plants from SH_0 media were transferred to a shoot multiplication SH medium, with growth regulators as indicated in Table I, and cultured for 5 weeks. For shoot elongation explants containing multiple shoots together with shoot primordia were transferred to SH media with various auxins (Table II). After 5 weeks shoots that were 1 cm or longer were excised from the basal biomass and cultured on various rooting media (Table III) for 4 weeks. In the experiments reported here, the pH value of the media was adjusted to 5.8 and 0.7% w/v agar was added prior to autoclaving. The cultures were maintained at 25 ± 2 °C under continuous light (intensity 88 ± 8 μmol m^{-2} s^{-1}).

All experiments concerning shoot multiplication and rooting were repeated 10 times. The results were analysed with t-Student's test for comparison of the mean and to assess the differences.

Rooted shoots (4-week-old) were washed in tap water and transferred into plastic pots containing a sterile soil mixture with 10% perlite. To maintain humidity, the plantlets were covered with glass caps gradually opened during a 3 week acclimatization period. They were watered every 5 d with tap water. The potted plants were grown in the culture room under standard conditions. Acclimatized plants were transferred to the greenhouse (for 4 weeks) and then to outdoor conditions at the Medicinal Plant Garden for 6 months.

Flavonoid extraction and quantitative analyses of phytoestrogens by HPLC were performed as described by Łuczkiewicz *et al.* (2004b).

Results and Discussion

The experiments described here led to the development of a micropropagation method for the six selected species of *Genista* genus. All of the species tested reacted in a very similar way to the change of the growth regulators in the modified SH media. Therefore in order to avoid multiplying similar results for each of the species *G. tinctoria*, which produced the highest amount of isoflavones of phytoestrogenic activity (Łuczkiewicz and Głód, 2003) was chosen to show the effect of growth regulators on shoot propagation (Tables I and II) and rooting (Table III) of *Genista* plants. Detailed results for the particular species are available from the authors of this paper.

After the optimum conditions for *in vitro* propagation of *Genista* plants were developed, the biosynthesis of isoflavones in the *in vitro* shoot cultures of all six species was compared (Table IV). The optimum SH medium was used and the results were compared with those of respective original herbs and regenerated plants.

In vitro shoot production

Shoot tips from aseptically established plants on SH_0 medium were used as explants to develop *in vitro* shoot cultures. Modified SH medium had previously been used to establish callus cultures of the six *Genista* species (Łuczkiewicz and Głód, 2003). In order to test the effect of growth regulators on axillary shoots proliferation, shoot tips and nodal segments were transferred to SH medium supplemented with different cytokinins applied alone or in combination with indole-3-acetic acid (IAA, Table I). Unlike the other auxins, this one induced least callus formation in *Genista* plant explants (Łuczkiewicz and Głód, 2003).

In order to achieve shoot proliferation in *Genista* plants *in vitro* it is necessary to use media supplemented with cytokinins. When SH medium containing only IAA was used, only 3% of the explants of *G. tinctoria* formed axillary shoots (Table I). The amount of shoots per explant (ca 1.0) also indicates that this was a spontaneous process, possibly related to the presence of endogenous cy-

Table I. Effect of different growth regulator treatments on shoot proliferation in shoot tips of *Genista tinctoria.*

Plant growth regulators [μM]	Explants forming auxillary shoots (%)	Number of shoots (> 3 mm per explant)	Cultures with multiple shoots (%)	Shoots forming callus (%)
IAA (5.70)	3.0	1.0 ± 0.09	none	100.0
IAA (5.70) + BAP (2.22)	15.0	3.0 ± 0.1	none	100.0
IAA (5.70) + BAP (4.44)	19.0	8.2 ± 0.4	none	100.0
IAA (5.70) + BAP (8.87)	21.0	7.4 ± 0.5	none	73.0
BAP (4.44)	22.0	8.6 ± 0.2	none	5.0
IAA (5.70) + KIN (2.32)	2.0	1.0 ± 0.1	none	100.0
IAA (5.70) + KIN (4.65)	3.0	1.0 ± 0.08	none	100.0
IAA (5.70) + KIN (9.29)	1.0	1.2 ± 0.1	none	100.0
KIN (4.65)	1.0	1.0 ± 0.09	none	4.0
IAA (5.70) + ZEA (2.28)	3.0	1.6 ± 0.09	none	100.0
IAA (5.70) + ZEA (4.56)	5.0	3.0 ± 0.1	none	100.0
IAA (5.70) + ZEA (9.12)	10.0	3.4 ± 0.2	none	97.0
ZEA (4.56)	4.0	2.7 ± 0.1	none	10.0
IAA (5.70) + 2iP (2.46)	34.0	10.6 ± 0.4[a]	11.0	54.0
IAA (5.70) + 2iP (4.92)	59.0	17.8 ± 1.4[a]	34.0	19.0
IAA (5.70) + 2iP (9.84)	97.0	29.7 ± 2.1[a]	68.0	4.0
2iP (4.92)	74.0	19.1 ± 1.3[a]	78.0	2.0
IAA (5.70) + 2iP (9.84) + TDZ (0.99)	100.0	31.0 ± 2.8[a]	67.0	2.0
2iP (9.84) + TDZ (0.99)	100.0	30.8 ± 2.4	91.0	none
TDZ (0.99)	100.1	1.5 ± 0.09	none	none

Shoot cultures were grown on various modified solid SH media for 5 weeks (light intensity 88 ± 8 μmol m^{-2} s^{-1}; temperature ± 25 °C).
Each value represents the mean ± S. D. of ten samples.
[a] Nodal segments.

tokinins. Moreover, all explants formed cauliflower-like callus growing between the elongating shoot tips, which is highly undesirable in the micropropagation process (Marcotrigiano *et al.*, 1996).

Shoot tips on media supplemented only with cytokinins did not die and in varying degrees differentiated into shoot cultures. This process was clearly related to the type and amount of the growth regulator used (Table I). Although the shoot cultures grew in the absence of auxins, the authors eventually did not remove IAA from SH medium in the cytokinin tests [6-(γ,γ-dimethylallylamino)-purine (2iP), 6-benzylaminopurine (BAP), kinetin (KIN), zeatin (ZEA)]. In the absence of the auxin in the medium the callus formation completely stopped, but the shoots obtained were fuzzy in shape and their morphology was not identical with those of the intact plants. Moreover, hyperhydricity was observed in cultures grown on SH medium with high concentrations of BAP and 2iP without IAA. This potentially limitating factor in plant propagation, previously reported by Wysokińska (1993) for *Penstemon serrulatus*, was significantly slowed down when IAA was added to the medium.

Of all the cytokinins tested kinetin and, to a lesser degree, zeatin clearly inhibited the differentiation of explants into shoot cultures (Table I). In case of kinetin, the shoot tips of *G. tinctoria* practically did not form axillary shoots, irrespective of the concentration of the growth regulator in the medium (Table I). Zeatin in SH medium without auxin supplement resulted in only 4% initial cultures of *G. tinctoria* forming axillary shoots (Table I). Moreover, a single explant produced ca 2.7 shoots. Increasing the content of zeatin in the medium to 9.12 μM while supplementing IAA (5.70 μM) improved the results only slightly (ca. 10% explants forming axillary shoots; ca 3.4 shoots per explant – Table I).

BAP, a cytokinin commonly used for *in vitro* cultures of legumes (Thiem, 2003) initiated the formation of shoot cultures in *Genista* plants to a

much higher degree than the previously discussed growth regulators. The concentrations of BAP in the medium had little effect on the percentage of cultures which formed axillary shoots. On the other hand, the number of shoots per explant clearly increased with the growing concentration of BAP in the medium (Table I).

The removal of IAA from the medium, the presence of which resulted in excessive callus growth interfering with the micropropagation process, always resulted in the formation of vitrificating shoots with changed morphology.

The results presented in Table I and Fig. 1 show that the media supplemented with 2iP were the most effective in promoting *G. tinctoria* shoot development. 2iP is a cytokinin of choice in the micropropagation of plants from Ericaceae family (Zimmerman and Broome, 1980) but it had not been successfully used for *in vitro* propagation of legumes. A growing concentration of 2iP in the media significantly stimulated the percentage of *G. tinctoria* shoot tips forming shoot cultures and the number of shoots per explant (Table I). In addition, unlike the other cytokinins, it limited the IAA-related callus formation. Just 2.46 μM supplement of 2iP resulted in only 54% of initial explants forming callus (Table I). Under the same conditions, 34% of shoot tips formed axillary shoots with ca 10.6 shoots per explant (Table I). This was a much better result than in the case of maximum BAP content in the medium (Table I). On SH media with 5.70 μM IAA and 9.84 μM 2iP as many as 97% initial explants of *G. tinctoria* formed shoot cultures, with a significant number of shoots per explants (ca 29.7, Table I). 2iP is the only cytokinin used here which conditioned multiple microshoot formation of *Genista* plants, useful from the economic perspective. All cultures growing on SH media containing 2iP consisted of two different tissues: microshoots and hard compact tissue with many shoot primordia (Fig. 1). A similar type of *in vitro* cultures was obtained for *Penstemon serrulatus* by Wysokińska who explained the phenomenon with a significant concentration of cytokinins in the experimental media (Wysokińska, 1993). In the case of *Genista* plants the result can be attributed both to the use of a specific cytokinin (2iP) and its high concentration. An attempt to remove IAA from the medium, like in all other cultures, inhibited callus formation but it also caused hyperhydracidity and abnormal leaf formation. In order to eliminate IAA, which was not necessary for axillary shoot formation, SH media were supplemented with the basic cytokinin and a small amount of thidiazuron (TDZ) (0.99 μM, Table I). TDZ conditioned the formation of shoot cultures of correct morphology in experiments involving micropropagation of *Cajanus cajana* (Eapen *et al.*, 1998). That substituted plant urea showing cytokinin-like activity, used in minimum concentrations, increased both the number of shoot tips of *G. tinctoria* forming axillary shoots and the number of shoots per explant (Table I). This effect, however, was related to the presence of both 2iP and TDZ in the media, because the attempt to remove 2iP and use SH supplemented only with TDZ resulted in a dramatic decrease of shoots per explant (ca 1.5, Table I). Higher concentrations of TDZ could have slowed that process but the high price of this growth regulator restricts its use for commercial micropropagation. Most importantly, in the cultures formed on SH media supplemented with 2iP and TDZ, despite the absence of IAA, the shoots did not vitrify and their morphology was normal (Fig. 1). In effect, SH medium with 9.84 μM supplement of 2iP and 0.98 μM of TDZ gave best results in the form of 100% of shoot culture formation with ca 30 shoots per explant (Table I).

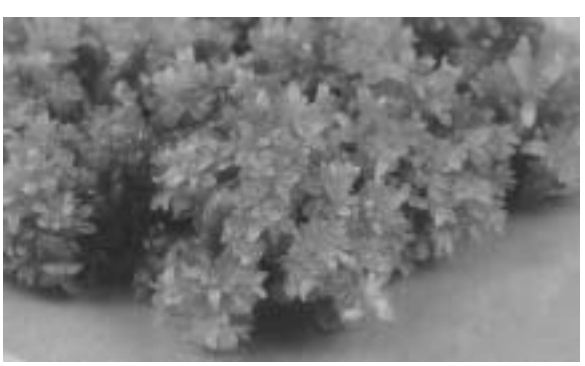

Fig. 1. Multiple microshoot formation of *Genista tinctoria* from shoot tip explants after 5 weeks on SH medium containing 9.84 μM 2iP and 0.99 μM TDZ.

Shoot elongation

In the conditions described above, most shoots were short (under 1 cm) and therefore difficult to isolate and root. The production of such shoots with short internodes is a typical effect of high cytokinin level (Wysokińska, 1993). An attempt to lower the content of the selected cytokinins in

Table II. Effect of IBA and GA_3 on shoot elongation of *Genista tinctoria.*

Plant growth regulators [μM]	Explants responding (%)	Number of shoots per explant	Shoots > 1 cm (%)
2iP (9.84) + TDZ (0.99)	7.0	32.7 ± 2.9[a]	2.0
2iP (9.84) + TDZ (0.99) + IBA (4.92)	99.0	29.5 ± 3.1[a]	57.0
2iP (9.84) + IBA (4.92)	98.0	29.8 ± 2.4[a]	59.0
IBA (4.92)	100.0	21.4 ± 2.1	64.0
2iP (9.84) + TDZ (0.99) + GA_3 (2.89)	15.0	24.0 ± 1.2[a]	27.0
2iP (9.84) + GA_3 (2.89)	12.0	27.6 ± 1.9[a]	24.0
GA_3 (2.89)	14.0	19.7 ± 1.0	31.0

Shoot cultures were grown on various modified solid SH media for 4 weeks (light intensity 88.8 ± 8 μmol m^{-2} s^{-1}; temperature ± 25 °C).
Each value represents the mean ± S. D. of ten samples.
[a] Cultures with multiple shoots

shoot cultures of *Genista* plants always resulted in lower shoot culture formation from the initial explants and frequent callus formation (Table I). It was therefore decided that a two-stage micropropagation system should be used. After the initiation, multiple microshoot cultures were transferred to the fresh SH medium. To minimize the inhibitory effect of high concentration of cytokinins, instead of 2iP and TDZ, SH medium was supplemented with either IBA (indole-3-butyric acid) or GA_3. Other media were also tested, in which new growth regulators were used together with 2iP and TDZ (Table II). Although GA_3 was successfully used to elongate shoot primordia of plants of Fabaceae family (Thiem, 2003), in case of *Genista* genus very few shoot cultures elongated after its application (Table II). Similarly unsatisfactory effects were achieved when the medium was supplemented with GA_3 together with TDZ and 2iP (Table II). Unlike IAA, IBA did not initiate callus formation and it did not reduce the number of shoots per explant. The use of SH without cytokinins and supplemented only with 4.92 μM of IBA resulted in the elongation of 100% of shoot primordia of *G. tinctoria* and the number of shoots over 1 cm in the entire culture was 64%. This made it possible to discontinue supplementing cytokinins (2iP and TDZ) at elongation stage and significantly reduced the cost of the two-stage process of micropropagating *Genista* plants. Microshoots which developed into normal long shoots were easy to excise from the basal tissue and the other remaining primordia (Fig. 2). With this method over the 6 months period as many as ca 500 shoots could be obtained from a single shoot explant.

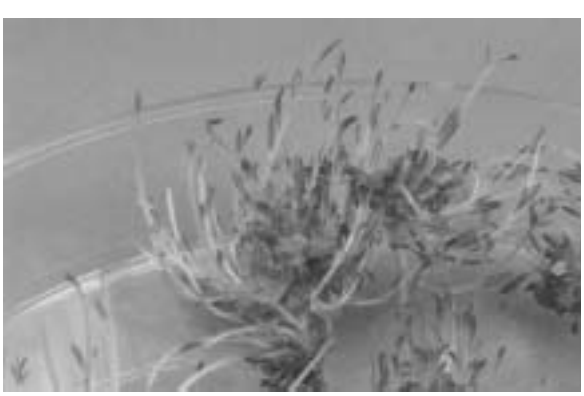

Fig. 2. *Genista tinctoria* shoot elongation on SH medium containing 4.92 μM IBA.

Rooting and acclimatisation

The shoots (more than 1 cm long) obtained from the two-stage micropropagation system were transferred to SH media supplemented with auxins [IAA, IBA, NAA (indole-3-acetic acid)] or without growth regulators (Table III). 100% rooting occurred already on SH_0 medium but the roots obtained in this way were long, thin and without secondary roots.

IBA, the growth regulator frequently used in rooting plants of Fabaceae family (Thiem, 2003), when used in larger concentrations, inhibited rhizogenesis of *G. tinctoria* almost completely (Table III). IAA also inhibited rhizogenesis of *Genista* plants (Table III). Moreover, with only 2.85 μM of IAA in the medium the few forming roots were accompanied by round, pale green callus-like structures, a phenomenon previously reported by Pitta-Alvares and Giulietti (1995) for

Table III. Effect of auxins on *in vitro* rooting of *Genista tinctoria* shoots.

Auxin type	Auxin concentration [μM]	Rooting frequency (%)	Number of roots per explant
No auxins	–	100.0	0.5 ± 0.1
NAA	5.37	87.0	5.4 ± 0.2
	2.68	100.0	13.2 ± 0.9
IBA	4.92	0.0	0.0 ± 0.01
	2.46	5.0	1.2 ± 0.07
IAA	5.70	0.0	0.0 ± 0.0
	2.85	32.0	1.7 ± 0.1

The cultures were grown on various modified solid SH media for 5 weeks (light intensity 88 ± 8 μmol m^{-2} s^{-1}; temperature ± 25 °C).
Each value represents the mean ± S. D. of ten samples.

root cultures grown in the light. Under these conditions survival of young plantlets was lowered. Only the amount of 2.68 μM NAA (1-naphthaleneacetic acid) resulted, as was the case in SH_0 medium, in 100% rhizogenesis of the shoot explants of *G. tinctoria* (Table III), but here, the roots had better morphology than on SH_0 medium. They were thicker, shorter with many laterals and outgrowths and the number of roots per explant (ca. 13.2) was much higher (Table III). That is why the media eventually used to root *Genista* plants were always SH supplemented with 2.68 μM NAA. Rooted shoots (1 month-old) were transferred to sterile soil in pots with controlled humidity and 70–80% of the plants (depending on the species) survived. After growing for half a year in the plant garden, all plants were morphologically normal in comparison to the original plants.

Flavonoid accumulation

Phytochemical analysis showed significant differences in flavonoid accumulation between the original plants, regenerated plants and shoots micropropagated *in vitro* (Table IV). This observation holds true for all investigated species. All of the *Genista* herbs, both intact plants and regenerated species, contained simple flavones, derivatives of luteolin and apigenin and a rich isoflavone group dominated by derivatives of genistein, daidzein, formononetin and biochanin A (Table IV). Regardless of the species, in all shoots propagated *in vitro* the simple flavones characteristic for the intact plants were completely absent. The same result was previously observed in callus cultures of *Genista* plants (Łuczkiewicz and Głód, 2003). This means that the absence of flavone production was directly related to transferring the plants to *in vitro* conditions. Both *in vitro* shoots (Table IV) and callus cultures of all *Genista* species (Łuczkiewicz and Głód, 2003) produced much more isoflavones than the intact plants. It can be inferred that certain substrates in callus and shoot cultures were fully used for the production of isoflavones making *in vitro* cultures of *Genista* species a selective source of phytoestrogens. Within the isoflavone group, glucosides and aglucones were produced in higher quantities by calli of *Genista* plants (Łuczkiewicz and Głód, 2003) than by *in vitro* shoots (Table IV). The reverse was true for esters (genistin malonate and genistin acetate) produced in higher quantities by shoot cultures. That is why it can be said that the biosynthesis of these compounds is directly related to the degree of morphogenesis. In regenerated plants both the flavones and the isoflavones were identified in amounts comparable with original plants (Table IV). This demonstrates that the production of flavones unlike isoflavones requires hormone status of the entire plant. Growing shoots alone, without a root system, blocks the biosynthesis of simple flavones. From the biotechnological point of view this is very interesting, since *in vitro* shoots, like the previously described callus cultures of *Genista* plants, became a rich, selective source of isoflavones which have specific phytoestrogenic activity. For example, the main compound identified in the shoots of *G. tinctoria*, was genistin in the amount of 3281 mg per 100 g DW which is almost three times more than in the herb of the original plant (1454 mg per 100 g DW, Table IV).

Summing up, the research led to the development of an effective procedure for *in vitro* propagation of several *Genista* plants. The biological material produced here, already at this stage significantly exceeds *Glycine max* in terms of isoflavone content (Federici *et al.*, 2003). Given numerous reports on failed attempts to achieve increased production of secondary metabolites by *in vitro* cultures of higher plants, the results presented here seem encouraging.

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Table IV. Quantitative analysis of major flavones and isoflavones in herb and shoot cultures of several *Genista* species (flavonoid content in mg per 100 g DW plant material).

	Plant material	Genistein-7-*O*-diglucoside	2′-Hydroxy-genistein-7-*O*-glucoside	Daidzin	Apigenin-7-*O*-glucoside	Luteolin-7-*O*-glucoside
Genista tinctoria	Orginal plant (herb)	437.94 ± 0.1	420.6 ± 0.11	42.8 ± 0.21	169.6 ± 0.15	1097.0 ± 0.1
	In vitro shoots	858.5 ± 0.91	374.3 ± 0.27	74.8 ± 0.12	–	–
	Regenerated plant (herb)	397.2 ± 0.24	417.3 ± 0.19	38.2 ± 0.42	154.8 ± 0.21	997.0 ± 0.19
Genista sagittalis	Orginal plant (herb)	528.7 ± 0.18	21.7 ± 0.17	47.3 ± 0.29	13.1 ± 0.05	52.5 ± 0.05
	In vitro shoots	1002.3 ± 0.34	22.2 ± 0.4	67.5 ± 0.17	–	–
	Regenerated plant (herb)	514.2 ± 0.2	23.6 ± 0.14	48.2 ± 0.36	5.9 ± 0.09	47.6 ± 0.13
Genista germanica	Orginal plant (herb)	102.5 ± 0.11	198.2 ± 0.08	10.4 ± 0.25	488.1 ± 0.12	571.2 ± 0.19
	In vitro shoots	217.6 ± 0.24	47.3 ± 0.14	31.2 ± 0.15	–	–
	Regenerated plant (herb)	98.2 ± 0.17	205.3 ± 0.09	10.1 ± 0.34	477.3 ± 0.18	564.6 ± 1.24
Genista radiata	Orginal plant (herb)	278.0 ± 0.08	458.5 ± 0.09	41.9 ± 0.28	34.9 ± 0.19	46.5 ± 0.15
	In vitro shoots	347.5 ± 0.11	781.0 ± 0.15	46.7 ± 0.21	–	–
	Regenerated plant (herb)	242.3 ± 0.19	432.3 ± 0.16	39.6 ± 0.19	35.2 ± 0.14	48.2 ± 0.20
Genista aethnensis	Orginal plant (herb)	64.2 ± 0.12	123.3 ± 0.24	11.2 ± 0.09	–	6.1 ± 0.08
	In vitro shoots	153.8 ± 0.21	75.4 ± 0.18	27.8 ± 0.14	–	–
	Regenerated plant (herb)	71.2 ± 0.18	119.2 ± 0.11	12.0 ± 0.13	–	2.9 ± 0.07
Genista monospes-sulana	Orginal plant (herb)	13.1 ± 0.01	96.6 ± 0.22	27.8 ± 0.13	14.1 ± 0.17	–
	In vitro shoots	21.4 ± 0.09	87.6 ± 0.15	41.3 ± 0.20	–	–
	Regenerated plant (herb)	14.4 ± 0.02	92.2 ± 0.21	25.5 ± 0.15	10.2 ± 0.09	–

Each value represents the mean ± S.D. of five samples.

Genistin	Genistin malonate	Genistin acetate	Daidzein	Sissotrin	Genistein	Luteolin
1454.2 ± 0.11	303.7 ± 0.91	68.3 ± 0.19	0.3 ± 0.01	1.4 ± 0.08	32.7 ± 0.13	5.4 ± 0.07
3281.4 ± 0.9	528.2 ± 0.72	114.0 ± 0.25	3.17 ± 0.19	–	61.8 ± 0.24	–
1405.1 ± 0.24	342.4 ± 0.54	72.3 ± 0.31	0.2 ± 0.01	2.2 ± 0.1	33.2 ± 0.21	4.4 ± 0.19
1058.2 ± 0.21	405.2 ± 0.19	215.9 ± 0.38	3.1 ± 0.19	1.4 ± 0.09	1633.6 ± 0.1	–
2372.3 ± 0.7	498.3 ± 0.3	274.1 ± 0.24	4.8 ± 0.15	2.3 ± 0.12	153.2 ± 0.15	–
1101.8 ± 0.31	441.6 ± 0.41	232.7 ± 0.28	3.0 ± 0.09	1.3 ± 0.1	989.2 ± 1.4	–
217.8 ± 0.09	533.1 ± 0.2	315.7 ± 0.32	1.1 ± 0.1	1.4 ± 0.07	711.7 ± 0.44	–
2437.1 ± 0.11	731.6 ± 0.15	412.5 ± 0.48	7.4 ± 0.18	5.6 ± 0.11	427.2 ± 0.38	–
248.3 ± 0.24	528.2 ± 0.23	321.3 ± 0.17	1.5 ± 0.09	2.0 ± 0.19	704.5 ± 0.35	–
1194.1 ± 0.1	137.4 ± 0.31	54.6 ± 0.16	3.5 ± 0.1	440.6 ± 0.09	38.8 ± 0.11	2.1 ± 0.09
1298.1 ± 0.1	498.6 ± 0.21	77.2 ± 0.24	5.8 ± 0.14	41.3 ± 0.11	94.7 ± 0.21	–
1104.5 ± 0.19	202.7 ± 0.34	49.7 ± 0.13	3.1 ± 0.11	351.5 ± 0.2	30.5 ± 0.22	1.8 ± 0.4
845.2 ± 0.19	42.7 ± 0.01	1.2 ± 0.09	2.1 ± 0.04	0.5 ± 0.07	3.2 ± 0.09	–
1234.4 ± 0.21	170.7 ± 0.11	2.7 ± 0.08	5.3 ± 0.03	–	34.0 ± 0.2	–
838.8 ± 0.24	58.3 ± 0.09	1.8 ± 0.1	2.2 ± 0.07	0.1 ± 0.01	4.5 ± 0.18	–
74.6 ± 0.16	28.1 ± 0.14	6.8 ± 0.25	3.1 ± 0.09	–	0.9 ± 0.04	–
834.4 ± 0.24	75.9 ± 0.22	17.4 ± 0.19	11.2 ± 1.1	–	7.8 ± 0.09	–
453.5 ± 0.18	27.3 ± 0.19	7.0 ± 0.11	4.0 ± 0.08	–	1.2 ± 0.01	–

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