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In vitro propagation of *Achillea asplenifolia* VENT. through multiple shoot regeneration

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Abstract. A method for the micropropagation of *Achillea asplenifolia* VENT. (Asteraceae) is described. Axillary shoots regenerated from nodal explants of adult plants could be stimulated to multiple shoot formation on the basal medium of Murashige and Skoog (1962), supplemented with different cytokinins. The best proliferation (5.33 shoots per culture), together with a healthy appearance of the cultures, was achieved with N-benzyl-9-(2-tetrahydropyranyl)adenine at a concentration of 1 mg/l. Shoots could be easily rooted on hormone-free MS basal medium and subsequently acclimatized to greenhouse and field conditions with 100% survival rate. DNA-fingerprinting, chromosome counts, the proazulene pattern as well as the essential oil analysis proved the true to type character of the micropropagated plants.

Abbreviations: 2iP: 6-(γ,γ-Dimethylallylamino)purine; MS: Murashige and Skoog (1962) medium; BAP: 6-Benzylaminopurine; GA_3: Gibberellic acid; GC: gas chromatography; IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; Kin: Kinetin; PBA: N-Benzyl-9-(2-tetrahydropyranyl)adenine; TLC: thin layer chromatography; WPM: Lloyd and McCown (1981) woody plant medium; Zea: Zeatin

Introduction

The perennial herb *Achillea asplenifolia* VENT. (Asteraceae) belongs to the *A. millefolium* complex whose members are known under the common name yarrow. Due to their anti-inflammatory, spasmolytic, antiseptic and choleretic activity species of this group have been used through the ages as a remedy against various ailments (Chandler et al. 1982; Willuhn 1989; Jurenitsch 1992). The active principles include flavonoids, essential oil and proazulenes - the latter having been shown to be jointly responsible for the anti-inflammatory activity (Della Loggia et al. 1992). They have been found in three taxa belonging to the *A. millefolium* group, namely *A. asplenifolia*, *A. collina*, and *A. roseo-alba* (Kastner et al. 1992b). Each of these three species shows a different, but characteristic composition of monoterpenes/ sesquiterpenes of the essential oil (Kastner et al. 1992b) as well as a typical pattern of the proazulenes (Kastner et al. 1992a).

Due to the fact that the drug is mainly obtained through field collection it can be taken for granted that the plant material which subsequently is used for medical purposes consists of a mixture of different *Achillea* species, including also taxa of inferior pharmaceutical value or even such containing allergenic compounds (Rücker et al. 1991; Zitterl-Eglseer et al. 1991). Thus, efforts are made to achieve a drug supply of controlled origin, e.g. field cultivation; furthermore, the quality of the species *A. collina* concerning the proazulene content was already improved noticeably by means of plant breeding (Langerfeldt 1986).

Propagation of *Achillea* from seeds offers one possibility for cultivation of the plant, but there is evidence that proazulene biosynthesis is only expressed in the plant when the respective gene(s) is (are) dominant in both the parents (for a literature review see Kastner 1991). On the other side, multiplication of a plant of valuable genotype through rhizome cuttings is limited to only few propagules. In vitro techniques therefore could be of advantage: micropropagation of a proazulene-rich *Achillea* plant would provide large amounts of highly uniform plantlets suitable for further cultivation in the field.

To our knowledge only few investigations have been carried out on the in vitro-culture of members of the genus *Achillea*. Cellárová et al. (1982) obtained plantlets of *A. ptarmica* through indirect shoot regeneration from callus tissue. Cell suspension cultures of *A. millefolium* L. were established by Figueiredo and Pais (1991. Within the scope of the present work experiments were carried out to establish suitable conditions for rapid multiplication of *A. asplenifolia* through direct organogenesis.

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Material and methods

Plant material. Young plants of a population of *Achillea asplenifolia* were collected in spring (Rust/Burgenland, Austria) and transplanted to the garden of the institute. Five plants of the population were used for the determination of the essential oil content, leaves of the parent plant were taken for DNA-fingerprinting as well as for the analysis of the proazulene pattern and the composition of the essential oil. Nodal cultures were established from the parent plant after surface sterilization on MS basal medium (Murashige and Skoog 1962) supplemented with 1.0 mg/l BAP, 0.1 mg/l IAA, and 0.025 mg/l GA_3. Axillary shoots regenerated from these explants were further multiplied on basal medium with 1.0 mg/l BAP. Two weeks prior to the start of the experiments the shoot explants were transferred to hormone-free WPM medium (Lloyd and McCown 1981) for preconditioning.

General culture conditions. All micropropagation and rooting media were based on the MS formulation with 3% sucrose and 0.8% MERCK agar 1614 throughout. Growth regulators were added in different concentrations and combinations before autoclaving. The pH of the media was adjusted to 5.7 ± 0.1 after dissolution of the agar. Fifteen ml of medium were dispensed into 22x160 mm glass test tubes closed with cellulose Steri-caps and were autoclaved for 15 minutes at 121 °C. All cultures were kept in a growth chamber at 23 ±1 °C under a 16-hour photoperiod of 45 µmol m^{-2} s^{-1} provided by OSRAM-L fluorescent tubes.

Shoot cultures. Single shoots preconditioned on hormone-free WPM medium for two weeks were trimmed to a length of 1-2 cm and placed on the culture medium (one shoot per culture tube). In a first experiment, media supplemented with a single cytokinin were tested, in a second series phytohormones were added in combinations (see Table 1). Twelve cultures were established per treatment. After 5 weeks the average number of shoots per explant as well as the estimated mean length of the shoots were recorded. In addition, the overall appearance of the cultures was scored.

Rooting of shoots. Five week-old multiple shoot cultures were divided into single shoots of which healthy ones (15-20 mm in length) were taken for rooting experiments. Four nutrient media were tested (refer to Table 1); experiments were carried out as described above. Valuation of the results was done after 2 1/2 weeks.

Table 1: Nutrient media used for micropropagation and rooting of *Achillea asplenifolia* shoots (MS basal medium with 3% sucrose, 0.8% agar and pH=5.7±0.1).

medium	hormones (mg/l)	medium	hormones (mg/l)
M1	0.1 Zea	M9	1.0 BAP + 1.0 2iP
M2	1.0 Zea	M10	1.0 BAP + 0.1 Zea
M3	1.0 2iP	M11	1.0 PBA + 0.1 Zea
M4	10 2iP	M12	1.0 2iP + 0.1 Zea
M5	0.1 PBA	M13	1.0 2iP + 1.0 PBA
M6	1.0 PBA	M14	1.0 2iP + 1.0 Kin
M7	1.0 Kin	M15	1.0 Kin + 1.0 BAP
M8	1.0 BAP + 0.025 GA_3	M16	1.0 Kin + 1.0 PBA
R1	-	R3	1.0 IBA
R2	0.1 IBA	R4	1.0 IAA

Acclimatization conditions. Plantlets of 3-5 cm size were potted in Perlite soaked with a solution of MS salts and kept in the growth chamber. During a period of approximately 2 weeks they were gradually exposed to reduced relative humidity by progressively ventilating the vessels. After that the plantlets were transferred to a mixture of soil and peat (2+1) and placed in the greenhouse. After a few weeks of hardening the plants finally were moved to the field.

Cytological studies. Root tips were collected from rooted shoots before transfer to soil and left in a 0.2 % (w/v) colchicine solution for 24 hours in the refrigerator. They were then fixed in a mixture of methanol and acetic acid (3+1) for at least 24 hours. For chromosome counting the root tips were sqashed in a few drops of a solution of 2 % carmine in 45 % acetic acid.

Essential oil analysis. The total contents of the essential oil was determined by steam destillation according to the Pharmacopoea Austriaca (1990).
For the analysis of the proazulene pattern and the determination of the significant compounds of the essential oil, 100 mg samples of air-dried leaves were sonicated in 1.0 ml methylene chloride for 20 minutes. The proazulene pattern was determined by thin layer chromatography (TLC) on MERCK silica 60 (mobile phase: methylene chloride - acetone (9+1), detection: UV_{254}, EP-reagent [Stahl 1953]). The compounds showed the following hRf values: Achillicin 65, 8α-tigloxy-10-epi-artabsin 75, 8α-angeloxy-10-epi-artabsin 80.
Five µl of the methylene chloride extract were used for GC analysis on a Perkin Elmer 300 instrument equipped with a fused silica capillary column (Macherey & Nagel; SE-54-SF; 50 m x 0.25 mm i.d.) under the following conditions: injector and detector temp. 270 °C; temp. prog.: 5 min at 60 °C, 60 °C-108 °C at 5 °C/min, 5 min at 108 °C, 108 °C-270 °C at 6 °C/min; carrier gas N_2 at 5 ml/min; split 1:10. Data obtained were subjected to a computer-based trend analysis as described by Kastner et al. (1992b).

Results and Discussion

In a previous paper Cellárová et al. (1982) reported on the regeneration of whole plants from callus tissue of *Achillea ptarmica*. However, this species does not belong to the *A. millefolium* complex (Ehrendorfer 1973); moreover, indirect regeneration may lead to altered genotypes due to somaclonal variation (Larkin and Scowcroft 1981). Thus, in our work shoot explants were used with the aim of obtaining direct shoot regeneration and, subsequently, the production of true to type plants.

Preliminary experiments revealed that the addition of auxins to the culture medium impaired shoot regeneration; moreover, callus and root formation strongly increased (data not shown). For this reason only cytokinins, alone or in combination, were used for the establishment of multiple shoot cultures, in one experiment BAP was applied together with GA_3.

In the first series of treatments, media M1-M7, each supplemented with a single cytokinin (refer to Table 1), were tested. All media had a promoting effect on multiple shoot proliferation, even though results differed with type and concentration of hormone used, as shown in Table 2. The best multiplication rates (i.e. number of shoots at the end of the culture period, including the starting explant) were achieved on media M2, M6, and M1, but on media M1 and M2 (0.1 and 1.0 mg/l Zea) leaves were partly brownish and showed malformations. The other media supported lower multiplication rates, and also the general impression of the cultures was unsatisfactory. The use of 2iP (media M3 and M4) resulted in poor multiplication and also in leaf malformation: especially leaves of shoots cultured on nutrient medium M4 were deformed and thickened. Leaf deformation also occurred in the presence of 0.1 mg/l PBA (medium M5). This was an interesting result as a ten-fold increase of the concentration of this cytokinin (medium M6) led to mainly healthy shoots. Finally, on all media a more or less intense formation of roots occurred.

In order to increase multiplication rates and to reduce unnecessary root regeneration, combinations of cytokinins were investigated in further experiments. In one treatment, BAP was supplied together with GA_3 (medium M8). Evaluation of the results after a 5 week culture period did not show any appreciable differences when compared to the first series of experiments. As shown in Table 2, multiplication rates ranged from 2.33 to 4.33. Low multiplication was most often found on media supplemented with 2iP; besides, on these media similar effects could be observed as on medium M3

(1 mg/l 2iP): thin shoots with either brownish or unnatural dark green leaves were typical when 2iP was added to the nutrient medium.

Addition of GA_3 (medium M8) did not show any advantage: the multiplication rate was satisfactory, but in comparison to the other media root regeneration was the highest.

Table 2. Multiplication of shoots on media M1-M16 (refer to Table 1).

medium	multiplication rate	mean length of shoots (mm)
M1	5.08	13.1
M2	5.91	14.5
M3	3.5	15.0
M4	2.7	10.8
M5	3.25	17.9
M6	5.33	17.1
M7	3.92	15.8
M8	4.0	30.0
M9	3.16	22.5
M10	4.33	15.0
M11	3.66	18.9
M12	2.33	23.3
M13	2.58	16.2
M14	3.16	13.6
M15	3.83	19.2
M16	3.08	13.1

After an overall evaluation of the results medium M6 which combined a satisfactory multiplication rate of 5.33 with largely healthy and vigorous shoots was chosen for routine propagation.

For rooting of the micropropagated shoots 4 different media were compared. The results obtained showed no significant difference between the hormone-free medium R1 and the three media supplemented with auxin (see Table 1): each of the media brought about the formation of a well-developed root system. Furtheron, no difference in the functionality of the roots could be observed during acclimatization. Therefore, the routine rooting was carried out on the hormone-free medium.

After rooting the plantlets were acclimatized to greenhouse conditions. This step could be executed without any difficulties and with a 100% survival rate. After a few weeks in the greenhouse, the plants were transplanted to the field where they were shaded during the first week. After that they continued to develop normally.

The genetic uniformity of the in vitro-propagated *A. asplenifolia* plants could be proved through DNA-fingerprinting (Wallner, unpublished results): no deviation in the genomic DNA fingerprints was observed over an observation period of 1 year. The true to type character of the propagated plants was also revealed through chromosome counts: examination of root tip sqashes of 97 plants proved all individuals to be diploid (2n=18). Subsequently, the proazulene pattern of the micropropagated plants was screened qualitatively by TLC. The spectra of these compounds were very uniform and typical throughout for *A. asplenifolia* as described by Kastner et al. (1992a) and revealed the occurrence of achillicin, 8α-tigloxy-10-epi-artabsin, and 8α-angeloxy-10-epi-artabsin.

Due to the low essential oil content of one single plant the parent plant did not supply enough material for analysis: therefore, the determination of the content was carried out with 5 plants of the parent population and four times with 5 propagated plants. The measurement revealed an essential oil content of 0.32 % for the parent plant population and 0.33±0.02 % for the clones, thus confirming that the property of high essential oil content was maintained.

For the valuation of the essential oil composition the amounts of 13 defined mono-/ sesquiterpenes (see Table 3) were recorded. Each species of the *Achillea millefolium* aggregate has a characteristic spectrum of these substances: *A. asplenifolia* typically shows a simple pattern with high amounts of β-pinene, low to middle amounts of β-caryophyllene, and low amounts of α-pinene, 1,8-cineole, *p*-cymene, and sabinene (Kastner et al. 1992b). The trend in the relative amounts also correlates with morphological characteristics and the proazulene pattern, and is representative for the respective *Achillea* species.

In Table 3, the values obtained for the parent plant are compared with the mean values calculated from 175 analyses of cloned individuals. The amounts of the single compounds coincided well in principle; however, the mean values of the compounds of the micropropagated plants showed noticeable standard deviations. These variations may be explained by the fact that the position of the analyzed leaves on the plant was not taken into consideration: Länger et al. (1993) could show that the essential oil composition of leaves of *Salvia officinalis* differs considerably depending on the location of the leaves at the top or at the base of a plant. Similar findings have been described for *Mentha x piperita* where the composition of the essential oil varies with the developmental stage of the leaves (Kokkini 1991). These facts may be the reason for the deviations we found with the in vitro-propagated *Achillea asplenifolia* plants.

Table 3: Contents of 13 selected compounds of the essential oil (A: parent plant; B: mean values ±SD (n=175) of clonally propagated plants).

component	A	B	component	A	B
α-pinene	4.30	4.20±1.51	camphor	<0.1	<0.1
camphene	<0.1	<0.1	terpinene	<0.1	<0.1
sabinene	3.68	3.80±1.17	borneol	<0.1	<0.1
β-pinene	46.47	45.94±7.29	terpineol	<0.1	<0.1
p-cymene	4.33	4.31±1.71	bornyl acetate	<0.1	<0.1
1,8-cineole	9.50	9.52±2.18	β-caryophyllene	13.60	14.22±4.46
thujone	<0.1	<0.1	not identified	18.12	18.01

The results indicate that the individuals of the established clone of *A. asplenifolia* show genetic uniformity with regard to DNA-fingerprints, chromosome number, proazulene pattern, occurrence and amounts of the essential oil compounds significant for the species, as well as a high yield of essential oil.

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