MICROPROPAGATION

In vitro regeneration of wild chervil (*Anthriscus sylvestris* L.)

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Abstract *Anthriscus sylvestris* (L.) Hoffm. (*Apiaceae*) is a common wild plant that accumulates the lignan deoxypodophyllotoxin. Deoxypodophyllotoxin can be hydroxylated at the C-7 position in recombinant organisms yielding podophyllotoxin, which is used as a semi-synthetic precursor for the anticancer drugs, etoposide phosphate and teniposide. As *in vitro* regeneration of *A. sylvestris* has not yet been reported, development of a regeneration protocol for *A. sylvestris* would be useful as a micropropagation tool and for metabolic engineering of the plant. Calli were induced from hypocotyl explants and transferred to shoot induction medium containing zeatin riboside. Regenerated shoots were obtained within 6 mo and were transferred onto growth regulator-free root induction medium containing 1% sucrose. Regenerated plants transferred to soil and acclimatized in a greenhouse. Plants were transferred to the field with a 100% survival rate. Regenerated plants flowered and were fully fertile. This is the first report of complete regeneration of *A. sylvestris* via shoot organogenesis from callus.

Keywords Organogenesis · Micropropagation · Regeneration · Tissue culture · Deoxypodophyllotoxin

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Introduction

Anthriscus sylvestris (L.) Hoffm. (*Apiaceae*) is a common wild plant, native to Europe, North America, Africa, Asia, and New Zealand (Hulten and Fries 1986; Magnusson 2006; Jeong *et al*. 2007) that accumulates the lignan deoxypodophyllotoxin, especially in the roots. The dried root of *A. sylvestris* is used in Korean and Chinese traditional medicine for the treatment of various diseases (Hendrawati *et al*. 2011).

Deoxypodophyllotoxin is structurally closely related to the lignan podophyllotoxin, which is obtained from *Podophyllum* species and is used for the synthesis of the anticancer drugs, etoposide phosphate and teniposide. Podophyllotoxin is obtained from *Podophyllum* species, but the supply of podophyllotoxin from this source is likely to become a major bottleneck because *Podophyllum* species have been listed on the endangered species list in India (Nayar *et al*. 1990). An alternative and more sustainable source of podophyllotoxin may be obtained by the biotechnological hydroxylation of deoxypodophyllotoxin at the C-7 position. This conversion has already been achieved in *E. coli* DH5α transfected with the human cytochrome P450 (Vasilev *et al*. 2006).

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Although gene transfer via *Agrobacterium* can facilitate efforts to engineer secondary metabolic pathways (Birch 1997; Gómez-Galera et al. 2007), this approach has not yet been evaluated for *A. sylvestris*.

Plant regeneration from tissue cultures of many plant species has been reported, but some groups, families, and genera are still regarded as recalcitrant (Ochatt et al. 2010). While there are no reports on regeneration of *A. sylvestris*, regeneration of the following members of the Apiaceae family has been documented: *Daucus carota* (Steward et al. 1958), *Apium graveolens* (Catlin et al. 1988), *Thapsia garganica* (Jäger et al. 1993), *Pimpinella anisum* (Salem and Charlwood 1995), *Ammi majus* (Purohit et al. 1995), *Carum carvi* (Krens et al. 1997), *Coriandrum sativum* (Wang and Kumar 2004), and *Dorem ammoniacum* (Irvani et al. 2010).

Regeneration from callus- and suspension-cultured cells of many crop plants occurs both by organogenesis (Flick et al. 1983) and in vitro embryogenesis (Brown et al. 1995). Using information on the initiation of *A. sylvestris* calli and suspension cultures (Koulman et al. 2003), cells from callus tissue were used to establish a regeneration protocol for *A. sylvestris*. Dormancy problems from *A. sylvestris* seeds presented an additional concern (Baskin et al. 2000).

The main objective of our study was to establish a regeneration protocol for *A. sylvestris*. This regeneration protocol can subsequently be used either as a micropropagation tool to bypass the dormancy in the seeds or for metabolic engineering of *A. sylvestris*. Different concentrations and combinations of auxins and cytokinins were used to induce shoots from callus tissue and to induce root formation from the regenerated shoots.

Material and Methods

Plant material. The fruits (ripe mericarps, hereafter called seeds) of *A. sylvestris* L. (Hoffm.) were collected from wild habitat in June 2007 in Groningen, the Netherlands (53°13′34″N and 6°32′43″E). The seeds were surface sterilized with chlorine gas for 3.5 h and subsequently cultured on 25 mL 10 gl^{-1} water agar (Duchefa, Haarlem, The Netherlands) in Petri dishes (94/16, Greiner Bio One, Alphen aan de Rijn, The Netherlands). To overcome dormancy, the seeds were kept in the Petri dishes at 1°C under fluorescent light illumination for at least 3 mo until the seeds germinated. The germinated seedlings were transferred to Gamborg's B5 medium (Gamborg et al. 1968) supplemented with 8 gl^{-1} agar in a sterile container, OS 140 box and ODS filter from Duchefa (Haarlem, The Netherlands) and incubated in a climate room at 22°C/20°C under a 16-h light/8-h dark period (day/night) (60-µmol m^{-2} s^{-1} fluorescent and incandescent light).

Callus induction. After 2 mo, root, hypocotyl and leaf explants from young sterile in vitro-obtained seedlings were excised and cultured on Gamborg's B5 media supplemented with either 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 mg l^{-1} 6-benzylaminopurine (BA) (Koulman et al. 2003) or 2 mg l^{-1} α-naphthaleneacetic acid (NAA) and 0.2 or 1.0 mg l^{-1} zeatin riboside (Z) (Table 1). All media contained 40 gl^{-1} sucrose and 8 gl^{-1} agar (pH 5.7). The calli induced from various explants were subcultured on the same medium every 4 wk.

Plant regeneration from callus cultures. After 3 mo, calli were cut into 400-mg pieces and transferred to Gamborg's B5 medium containing various concentrations of auxins such as 2,4-D, NAA, IBA (Indole-3-butyric acid), indole-3-acetic acid (IAA) and cytokinins such as BA, Z, kinetin (Kin) to induce shoot formation (Table 2). Each treatment consisted of at least five Petri dishes with five calli in each dish. The growth rate and the quality of the calli of each treatment were recorded monthly for at least 6 mo. The growth rate was defined as the ratio of the weight of the calli after 4 wk divided by the initial weight. The quality of calli was given a score from 1 to 5 (5, all five calli were green and proliferating; 3, average; 1, all five calli were brown/dead). The score in Table 2 was the average of at least 6-mo data for both the growth rate and the quality of the calli.

Rooting and acclimatization. Regenerated shoots measuring about 2 cm in length were transferred into rooting medium. The rooting medium contained Gamborg's B5 salts and vitamins supplemented with 10 gl^{-1} sucrose and no growth regulators. After 2 mo, the rooted plantlets were thoroughly washed with tap water to remove the agar and transferred to sterile soil in small pots and covered with

Table 1. Effects of different concentrations of auxins and cytokinins for callus induction from roots, hypocotyls, and leaves explants of *A. sylvestris*

Growth regulators (mg l^{-1})				Callus induction (%) ($\bar{x}\pm SD$)[a]		
Auxins		Cytokinins		Explants		
2,4-D	NAA	BA	Z	Hypocotyls[b]	Roots[c]	Leaves
2.0		1.0		36.1±28.3	28.5±35.7	1.33±1.16
	2.0		1.0	30.5±19.7	61.0±39.3	na[d]
	2.0		0.2	12.5±13.8	75.0±43.3	na[d]

[a] Mean and standard deviation

[b,c] Means in this *column* do not significantly differ from each other ($p < 0.05$)

[d] Mean and standard deviation not available

Table 2. Effects of different concentrations of auxins and cytokinins for shoot regeneration of *A. sylvestris*

	Growth regulators (mg l^{-1})							Shoot formation	Growth rate[a]	Quality of calli[b]
	Auxins				Cytokinins					
	2,4-D	NAA	IAA	IBA	BA	Z	Kin		($\bar{x}\pm$SD)[c]	($\bar{x}\pm$SD)[c]
C	2.0				1.0			−	2.92±1.13	3.31±0.69
S3	0.1				2.0			−	1.94±1.37	2.20±0.45
S5		0.1			2.0			−	1.94±0.50	2.67±1.03
S14		0.05			3.0			−	2.01±0.67	2.50±0.97
S15			0.01		0.3			−	1.59±0.40	1.33±0.58
S16				0.1	2.0			−	2.62±1.46	3.24±1.22
S12	0.1					2.0		−	2.56±0.81	3.84±0.60*
S9		0.2				2.0		−	2.80±0.66	4.30±0.56*
S10			0.1			2.0		−	2.33±0.68	3.70±1.08
S11				0.1		2.0		−	2.55±0.64	3.95±0.86*
S17	0.1						2.0	−	2.54±0.80	3.88±1.08
S18		0.2					2.0	−	2.10±2.17	3.38±1.33
S19		0.05					3.0	−	2.40±1.22	3.57±0.79
S20			0.01				0.3	−	1.89±0.61	2.25±0.46
S22		0.1				2.0		−	1.68±0.55	1.00±0.00
S21				0.1			2.0	−	2.39±0.80	3.00±0.86
Z3		0.1				2.0		−	2.71±0.99	3.00±0.97
Z4		1.0				2.0		−	2.60±0.71	4.29±0.75*
Z5		0.05				2.0		−	2.18±0.60	2.42±0.84*
Z6		0.1				1.0		−	2.81±1.06	3.05±1.28
Z7		0.5				1.0		−	3.01±1.05	4.25±0.68*
Z8		0.05				1.0		−	2.30±0.72	3.10±1.09
Z10				1.0		2.0		+	4.39±1.11*	4.55±0.00*
Z11				0.05		2.0		−	3.03±0.88*	4.00±0.97*
Z12				0.1		1.0		−	3.13±0.57*	4.33±0.59*
Z13				0.5		1.0		−	3.67±1.82*	4.41±1.23*
Z14				0.05		1.0		−	3.16±1.10*	4.25±0.93*
Z15			1.0			2.0		−	4.72±1.46*	4.79±0.41*
Z16	1.0					2.0		−	5.58±1.15*	4.68±0.48*

All data were recorded monthly for at least a 6-mo period ($n=30$)

All media were supplemented with 40 gl^{-1} sucrose and 8 gl^{-1} agar

*$p<0.05$ significantly different compared to control (C)

[a] Growth rate: the weight of the calli after 4 wk divided by the initial weight. The initial weight was 2 g per petri dish

[b] Quality of calli: score of the greenish appearance (5, all five calli were green and proliferating; 3, average; 1, all five calli were brown/dead)

[c] Mean and standard deviation

plastic to maintain high humidity. Plantlets were placed in a growth chamber with a 14-h light/10-h dark period, photosynthetic photon flux density 100 μmol m^{-2} s^{-1} at 22°C. After 2 wk, the humidity was reduced by removing the plastic, and 20 plants were transferred to bigger pots and kept for another 2–3 mo. Eventually, the plants were transferred to the field. The regeneration experiment was conducted with three replicates from three different independently obtained calli.

Plant extraction and HPLC analysis. Deoxypodophyllotoxin (DPT) was a gift from Dr. M. Angeles Castro (Salamanca University, Salamanca, Spain). Acetonitrile and methanol were high-performance liquid chromatography (HPLC) grade from Biosolve (Valkenswaard, The Netherlands). Dichloromethane was obtained from Fisher Scientific (Landsmeer, The Netherlands). Samples for HPLC analysis were taken from ten randomly selected individual young regenerated plantlets, which were divided

into an aerial part and a root portion. Plant material was dried at room temperature prior to grinding and extraction. The extraction method was as described by Koulman *et al.* (2003), and the HPLC analysis was as described by Hendrawati *et al.* (2011).

Statistical analysis. The Student's *t* test ($p<0.05$) was used to determine statistical significance compared to the control (SPSS software, version 16.0, 2008).

Results

Seed germination. Following chlorine gas sterilization (Fig. 1*a*), the seeds did not germinate immediately or at the same time prior to cold stratification at 1°C. The sterilized seeds started to germinate after a 3-mo cold stratification (Fig. 1*b*) with an average germination rate of 39%. *In vitro* sterile seedlings were successfully obtained for callus induction (Fig. 1*c*).

Callus induction. Calli were induced from leaf, hypocotyl (Fig. 1*d*) and root explants from *in vitro*-grown seedlings. Several media were evaluated for callus induction in addition to the one reported previously for *A. sylvestris* (Koulman *et al.* 2003) (Table 1). The best callus formation was obtained from roots with 75% induction using Gamborg's B5 medium supplemented with 2.0 mg l^{-1} NAA and 0.2 mg l^{-1} Z (Table 1). The best callus induction from hypocotyl explants was 36% using Gamborg's B5 medium supplemented with 2.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} BA (Table 1). Calli were formed, on average, after 2 mo and one to two additional months were required to obtain sustained growth (Fig. 1*e*) before transferring to shoot induction media. Calli were either green, friable, or compact.

Shoot formation. In general, media supplemented with Z yielded better callus quality, and their growth rate was 2–5.6-fold compared to media supplemented with Kin or BA (Table 2).

The growth rate of calli in the shoot induction media with BA was 1.9–2.6, and the quality score was 1.3–3.2. Use of media containing Kin gave a similar growth rate between 1.7 and 2.5 but with better callus quality (1.0–3.9). However, none of these media led to shoot induction after 6 mo. The quality of calli was improved using media with Z (from 3.0 up to 4.8). The best media (Z15 and Z16), yielding the fastest callus growth (4.7- and 5.6-fold) and the
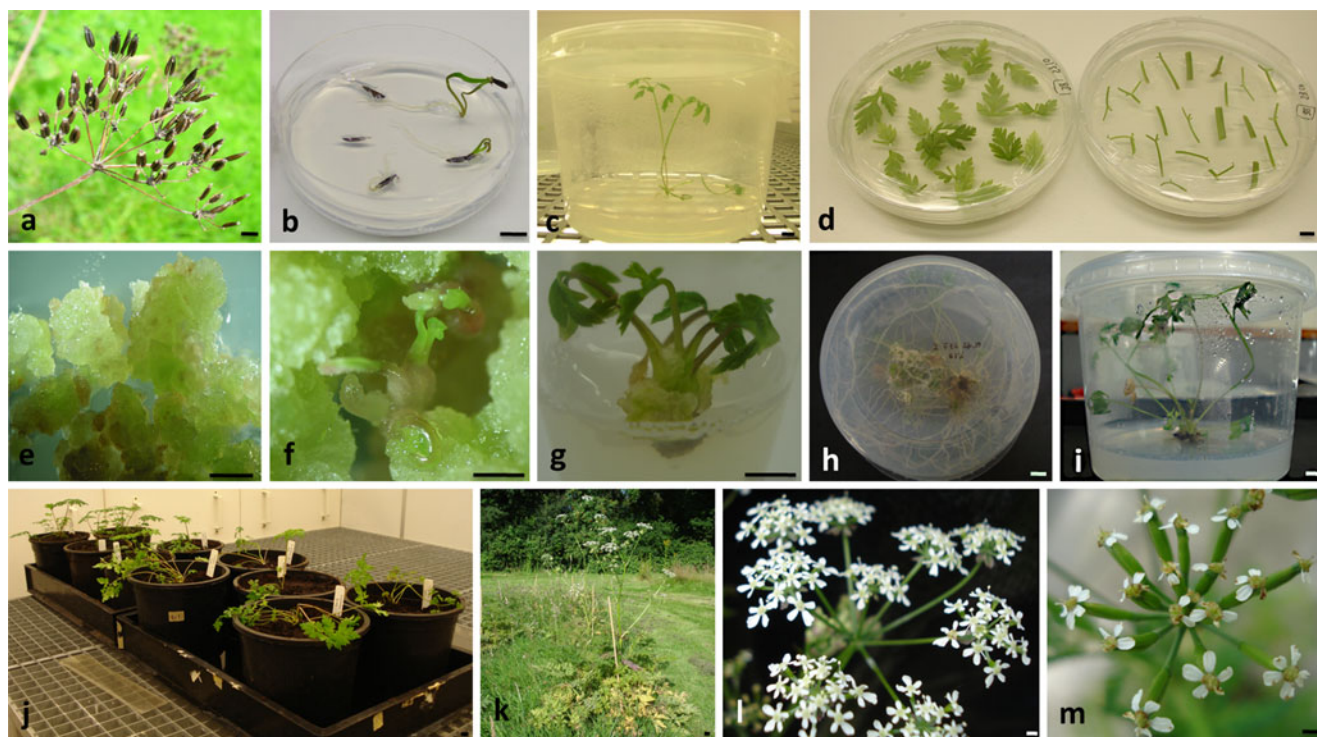

Figure 1. Regeneration of *A. sylvestris* L. (Hoffm.). (*a*) Ripe seeds, (*b*) Germination of the sterile seeds after 3 mo 1°C, (*c*) *In vitro* young seedling, (*d*) Leaves and hypocotyls explants, (*e*) Callus growth after 2 mo induction, (*f*) Shoot regeneration in Z10 media, (*g*) Further developed shoot regeneration, (*h*) *In vitro* rooting of shoots, (*i*) Young regenerated plantlets, (*j*) *Ex vitro* acclimatization of young regenerated plantlets, (*k*) Field planting of the regenerated plants, (*l*) Flowering of the regenerated plants, (*m*) Seed pods of regenerated *A. sylvestris*. *Bars* equal to 0.5 cm in all figures.

best callus quality (4.8 and 4.7), did not induce shoot formation within 6 mo.

Among the media used, only Gamborg's B5 medium supplemented with 2.0 mg l^{-1} Z and 1.0 mg l^{-1} IBA was able to induce shoot formation (Fig. 1*f–g*). The shoot induction period required up to 6 mo, but the number of regenerated shoots was relatively high (>100) once shoot induction was obtained. Shoots were regenerated from three independent calli from hypocotyl explants, taken from different *in vitro* seedlings. Although shoot-like structures were observed after 4 mo, some shoots seemed stressed (purple-whitish color) and died, but some of them survived and eventually regenerated shoots. The elongation of shoots took 2 mo.

Root formation and acclimation. For complete plantlet development, regenerated shoots were rooted on plant growth regulator-free Gamborg's B5 medium supplemented with 10 gl^{-1} sucrose. After 2 mo, roots developed (Fig. 1*h*), and regenerated plants were obtained (Fig. 1*i*). Twenty regenerated plants were transferred to small plastic pots for acclimatization in a growth room for 2 wk before transfer to bigger pots (Fig. 1*j*). After 3 mo, the regenerated plants were planted in the field (Fig. 1*k*) in June 2010 with a 100% survival rate.

Flowering and seed formation (R1). After 2–4 mo in the field, 55% of the regenerated plants (11 out of 20) flowered (Fig. 1*l*), formed seed pods (Fig. 1*m*) and eventually shed seeds. The phenotype of the regenerated plants was similar to wild type except for the flowers, which showed a reduced apical dominance (Fig. 2). The regenerated plants flowered in the branches and at the basal part (Fig. 2*c–f*), whereas wild-type plants flower in the apical part (Fig. 2*a–b*). Seeds (R1) from the regenerated plants were collected, dried, and placed at 1°C for germination. After a 2–3 mo period of cold stratification, seeds from four regenerated plants germinated. Twenty regenerated seedlings (R1) of each regenerated plant (R0) were grown in the greenhouse.

Deoxypodophyllotoxin content. The DPT content of the aerial part of the regenerated plants was between 0.6 and 1.2 µg/mg dry weight (DW). In the root part of the regenerated plant, the DPT content was 0.3–0.7 µg/mg DW. The DPT content of the aerial part of the wild type was 0.3–0.5 µg/mg DW; in the root part 1.4–1.5 µg/mg DW (Hendrawati *et al.* 2011). There was no difference observed on the profile of metabolites of the regenerated plants compared to the wild type.

Discussion

In general, three phases can be distinguished in regeneration (organogenesis) *in vitro*. The first is dedifferentiation in which the tissue becomes competent to respond to the organogenic stimulus. It is generally initiated by culturing on an auxin-rich callus-inducing medium and may involve a period of callus growth. The second is induction in which cells become determined to form either a root or a shoot. The explants are cultured on a shoot-inducing medium or root-inducing medium that contains a specific auxin/cytokinin ratio combination. The third is the realization in which the explants grow to an organ (De Klerk *et al.* 1997; Ochatt *et al.* 2010).

Initially, seeds of *A. sylvestris* (Fig. 1*a*) were dormant and have underdeveloped embryos. The embryos of the seeds grow to maturity during cold stratification. Germination frequency increased with the length of the cold stratification period. Approximately 30% of the non-sterilized seeds germinated after 8 wk of cold stratification at 1°C and then warm stratification at 15°C/6°C or 20°C/10°C or 25°C/15°C (Baskin *et al.* 2000). The germination increased to 77% with the increased duration of the cold stratification (12 wk). Most *A. sylvestris* embryos completed growth during a 12-wk period of cold stratification at 1°C (Baskin *et al.* 2000).

The surface sterilization treatment with chlorine gas yielded on average 39% germination, which is approxi-
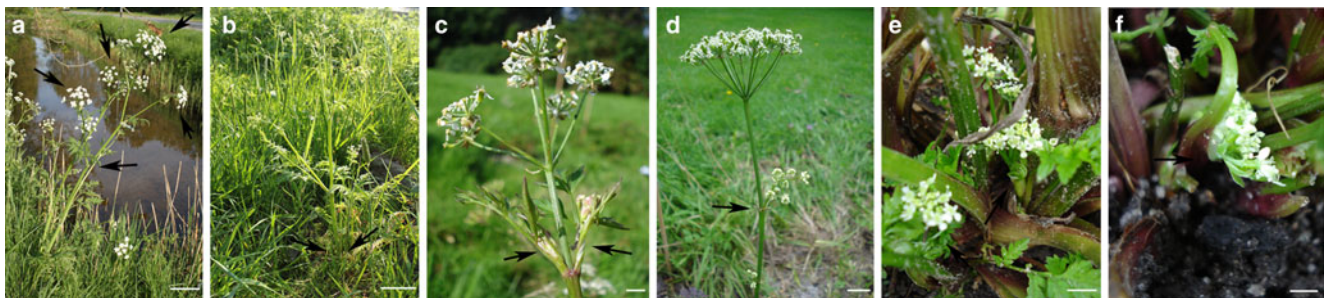

Figure 2. Flowering of *A. sylvestris*. (*a*) Wild type. (*b*) Wild type (no flower at the bottom part). (*c–d*) Reduced apical dominance flowering in the branches of the regenerated plants. (*e–f*) Flowering in the bottom part of the regenerated plants. *Arrows* show the differences. *Bars* equal to 10 cm (*a–b*) and 1 cm (*c–f*).

mately half the germination obtained from the non-surface sterilized seeds (Baskin *et al.* 2000).

Depending on the plant species, only a limited number of cells in an explant show the organogenic response (De Klerk *et al.* 1997). Therefore, different explants from hypocotyls, leaves, and roots from 1- to 2-mo young seedling were used for callus induction. The root explants showed the best callus induction (75%) using Gamborg's B5 medium supplemented with 0.2 mg l^{-1} Z and 2.0 mg l^{-1} NAA. However, use of seedlings to obtain root explants was not practical because 3 mo were needed to obtain *in vitro* seedlings. Hypocotyl explants were preferred because they are renewable, and the 36% callus induction was sufficient for our purpose. The callus medium contained Gamborg's B5 salts and vitamins, supplemented with 2.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} BA (Table 1). Callus induction from leaf explants was low compared to hypocotyl and root explants. Callus tissue was formed on the cut sections after 2 mo, but a further 1–2 mo was required to obtain consistent growth of green friable or compact calli.

There are differences in the organogenetic potential between plant families, genera, species, and genotypes, and different genotypes of a species may show widely different responses (George 1996). For example, *Capsicum* species that belong to the *Solanaceae* have been shown to be recalcitrant to differentiation and plant regeneration under *in vitro* conditions compared to *Nicotiana tabacum*, *Lycopersicum esculentum,* and *Solanum tuberosum,* which belong to the same family (Ochoa-Alejo and Ramirez-Malagon 2001). Although plant regeneration, somatic embryogenesis, and genetic transformation have been reported for several *Apiaceae* family members, tissue culture regeneration systems for different genotypes or species may not generally be applicable to all members of the same family.

Among the 28 different media used, only Gamborg's B5 medium supplemented with 2.0 mg l^{-1} Z and 1.0 mg^{-1} IBA led to shoot formation after 6 mo (Table 2). Among three different cytokinins, use of Z led to a better callus quality (score 4.8) and growth rate (up to 5.6). We obtained three independent regenerants from different calluses from hypocotyls of different *in vitro* seedlings.

The regenerated plants were planted in the field in the beginning of June 2010, with a 100% survival rate. In the Netherlands, the flowering season is between April and May. Interestingly, 55% of the regenerated plants flowered between August and October in their first year (Fig. 2).

Daucus carota and *A. sylvestris* are biennial plants from the *Apiaceae* family that flower after exposure to a period of cold (vernalization), generally during the second year of growth (Baskin *et al.* 2000; Punja *et al.* 2007). Carrot roots, transformed by *A. rhizogenes,* converted from biennial to annual without vernalization (Tepfer 1984; Limami *et al.* 1998). A similar phenomenon was observed in endive plants (*Cichorium intybus*) containing Ri T-DNA (Sun *et al.* 1991). Natural genetic transformation by *A. rhizogenes* might provide genes that allow flowering in the absence of a cold treatment (Limami *et al.* 1998). Annualism was correlated with the segregation of a truncated transferred DNA (T-DNA) insertion. Root locus (*rolC*) was the primary promoter of annualism (Limami *et al.* 1998). Expression of *rolC* in transgenic plants mainly leads to a modification of the cytokinin balance and causes developmental, physio-logical, and morphological alterations (Estruch *et al.* 1991). In *A. sylvestris* regenerants, the observed annualism may be caused by the effects of tissue culture as reported for *Chichorium intybus* (Sun *et al.* 1991) and long-term growth in shoot induction media containing high a cytokinin concentration.

The regenerated plants in the field showed wild-type phenotypes except that they flowered on the secondary branches (Fig. 2c–d) and at the base of plants (Fig. 2e–f), as opposed to the apical flowering of the wild-type plants (Fig. 2a–b). This phenomenon may be caused by somaclo-nal variation as a result of tissue culture (Cassells and Curry 2001) and/or cytokinin imbalance from the long-term growth in shoot induction media. R1 seeds were germinated in February 2011 and grown in the field. Recalcitrance and somaclonal variation are the two main problems in plant regeneration via tissue culture. They are complex, multi-factorial phenomena based on genotype, media, and environmental interaction (Cassells and Curry 2001).

Calli of *A. sylvestris* did not produce DPT (Koulman *et al.* 2003) and whole plants were required. Therefore, it is essential to achieve the differentiated form to restore the capability of the cell to produce DPT for metabolic engineering purposes. The DPT content of the aerial part of the regenerated plant was comparable with the root part of the wild-type plants. This is beneficial for the isolation of the bioconversion product for the metabolic engineering purpose. The ability of the regenerated plant to produce secondary metabolites, especially DPT, was not altered.

In conclusion, this is the first report on the regeneration of *A. sylvestris* plants *in vitro*. This may provide a valuable tool in creating new opportunities to produce metaboli-cally engineered *A. sylvestris* (Hendrawati *et al.* 2010). Further optimization is needed to increase the efficiency and frequency of regeneration and to reduce the total regeneration period.

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