

## Genotypical differences in callus induction and regeneration of plantlets produced from asparagus (*Asparagus officinalis* L.) anther cultures

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### Summary

It was the aim of this study to determine genotypical differences for callus induction and differentiation of asparagus (*Asparagus officinalis* L.) anther cultures. At the uninucleate stage, anthers of three asparagus cultivars, „Huchels Alpha“, „Record“ and „Schwetzinger Meisterschuss“, were cultured on Murashige and Skoog medium (MS) supplemented with 3% sucrose, 0.1 mg L<sup>-1</sup> naphthalene acetic acid (NAA), 0.5 mg L<sup>-1</sup> benzyladenine (BA) and 0.5 mg L<sup>-1</sup> 2,4-Dichloro-phenoxyacetic acid (2,4-D). The percentage of responding anthers ranged from 40% to 66%. The cultivar „Record“ showed the highest percentage of responding anthers, „Huchels Alpha“ the lowest. The calli were transferred to the following media for differentiation: MS medium supplemented with 3% sucrose, 0.3 mg L<sup>-1</sup> BA and 0.2 mg L<sup>-1</sup> NAA (medium A) and MS medium supplemented with 3% sucrose, 0.1 mg L<sup>-1</sup> NAA, 0.1 mg L<sup>-1</sup> kinetin and 0.65 mg L<sup>-1</sup> ancymidol (medium B). The cultivar 'Record' produced the highest number of embryogenic calli on medium A. This cultivar showed in both growth media the highest number of embryos per dish, embryos per callus, plantlets per dish and number of plantlets per callus.

Key words: *androgenesis*, *anther culture*, *Asparagus officinalis* L., *embryogenic callus*

### Zusammenfassung

Ziel der vorliegenden Untersuchungen war es, genotypische Unterschiede hinsichtlich der Induktion von Kalli und der Differenzierung von Antherenkulturen von Spargel (*Asparagus officinalis* L.) zu bestimmen. Im einkernigen Stadium wurden Antheren der drei Spargelsorten „Huchels Alpha“, „Record“ und „Schwetzinger Meisterschuss“ auf Murashige und Skoog Nährmedium (MS), zusammen mit 3% Saccharose, 0,1 mg L<sup>-1</sup> Naphtalen-Essigsäure (NAA), 0,5 mg L<sup>-1</sup> 6-Benzyladenin (BA) und 0,5 mg L<sup>-1</sup> 2,4-Dichlorophenoxyessigsäure (2,4-D), kultiviert. Die Anzahl reagierender Antheren variierte zwischen 40% und 66%. Hierbei zeigte die Sorte „Record“ die höchste und die Sorte „Huchels Alpha“ den niedrigsten Anteil reagierender Antheren. Die Kalli wurden anschließend auf Nährmedien mit folgender Zusammensetzung übertragen: A) MS Nährmedium, zusammen mit 3% Saccharose, 0,3 mg L<sup>-1</sup> BA, 0,2 mg L<sup>-1</sup> NAA; B) MS Nährmedium mit 3%

Saccharose, 0,1 mg L<sup>-1</sup> NAA, 0,1 mg L<sup>-1</sup> Kinetin und 0,65 mg L<sup>-1</sup> Ancymidol. Die Sorte „Rekord“ produzierte die höchste Anzahl embryogener Kalli auf dem Nährmedium A. Für die Sorte „Rekord“ wurden ebenfalls auf beiden Nährmedien die jeweils höchste Anzahl an Embryonen pro Petrischale, Embryonen pro Kallus, Jungpflanzen pro Petrischale und Jungpflanzen pro Kallus bestimmt.

Schlüsselwörter: *Androgenese*, *Antherenkultur*, *Asparagus officinalis* L., *embryogener Kallus*

### Introduction

*Asparagus officinalis* L.) is a dioecious, perennial crop. Since asparagus male plants produce 25% more yield than female ones (Sneep, 1953), all-male hybrids (Mm) are produced by crossing super-male (MM) with female (mm) genotypes. Super-males can be obtained by two methods: self-pollination of hermaphroditic flowers on male plants, where only 25% of the progeny are super-male (MM) and by anther culture techniques, respectively. The first approach is limited by genetic and environmental factors, because male plants do not constantly produce hermaphroditic flowers (Feng & Wolyn, 1993). The reason why male asparagus cultivars are superior to female plants in crop yield is most likely that no photosynthate is required for seed production and they do not produce seedlings which compete with established plants for water and nutrients (Aneja et al., 1999). Therefore, plant breeding aims at producing homozygous clones. Traditional breeding of such pure lines, particularly that of open pollinating plants like for instance asparagus is time consuming and labor intensive. A consistent method to produce double-haploid plants is by anther and ovule cultures, which provide homozygous plants for classical and molecular genetic studies (Cao et al., 1995; Shalaby, 1996). Androgenetic calli or embryoids from one or a few microspores are produced from *in vitro* anther cultures of asparagus after 4-6 weeks to the development on a suitable medium (Doré, 1990). Studies with view to produce super-male asparagus plants via anther cultures were reported, but the results were inconsistent (Doré, 1974; Falavigna et al., 1983; Feng & Wolyn, 1991; Hondelmann & Wilberg, 1973; Inagaki et al., 1980; Rotondo et al., 1983). Anther cultures are a powerful technique that after 20 years of its development gained a distinct

impact on the release of asparagus cultivars (Veilleux, 1994). Among the different factors affecting androgenesis, the genotype has probably the most striking influence (Shalaby et al., 2003; Tsay et al., 1982). So, the frequency of anther producing callus depended on the cultivar and ranged from 0.7 to 26.5% (Qiao & Falavigna, 1990; Peilai et al., 1999). This result proved that the genotype is a key factor influencing the *in vitro* androgenesis of asparagus. It was the aim of this experiment to study the effect of three asparagus cultivars widely grown on production fields in Germany on the frequency of callus induction and callus differentiation.

### Materials and methods

The asparagus genotypes used as anther donor plants were „Record“, „Huchels Alpha“ and „Schwetzinger Meisterschuss“.

The developmental stage of the microspores was determined by squashing anthers in 0.5% aceto-carmine. Flower buds of 1.5-2 mm containing microspores at the uninucleate stage, just before mitosis, were collected in August from field-grown plants and exposed to cold temperatures (4° C) for 48 hours. Then the buds were sterilized in 10% calcium hypochlorite for 10 minutes, afterwards rinsed three times with sterile distilled water and finally placed on a sterilized paper towel to absorb excessive surface water. From each genotype, about 250 (10 x 25) anthers without filament were carefully dissected from the flower buds before being placed onto the callus induction medium. This MS medium was that of Murashige and Skoog (1962) and which was supplemented with 3% sucrose, 0.1 mg L<sup>-1</sup> NAA (naphthalene acetic acid), 0.5 mg L<sup>-1</sup> BA (6-benzyl-adenine) and 0.5 mg L<sup>-1</sup> 2,4-D (2,4-dichlorophenoxyacetic acid) (Doré, 1974). Agar (8 g L<sup>-1</sup>) was added before the pH of the medium was adjusted to 5.8 with 0.1 N NaOH and 0.1 N HCl. The medium was autoclaved at 121° C and 1.1 kg cm<sup>-2</sup> for 15 minutes. Every petri-dish (7 x 1 cm) contained 15 ml media. 25 anthers were placed in each petri-dish. This experiment was designed in completely randomized design with ten replicates. The dishes were then sealed with parafilm and incubated at 32° C in darkness for four weeks (Feng & Wolyn 1991). Afterwards, the anthers were incubated at 25° C with a photoperiod of 16 hours for in total 4 weeks. Then the number of calli was determined and the number of calli per 100 anthers was calculated.

The calli were transferred to two kinds of differentiation medium: MS medium supplemented with 3% sucrose, 0.3 mg L<sup>-1</sup> BA and 0.2 mg L<sup>-1</sup> NAA (medium A) and MS medium supplemented with 3% sucrose, 0.1 mg L<sup>-1</sup> NAA, 0.1 mg L<sup>-1</sup> kinetin and 0.65 mg L<sup>-1</sup> ancymidol (medium B), respectively.

After four weeks the number of embryogenic calli and the number of embryos per callus were determined.

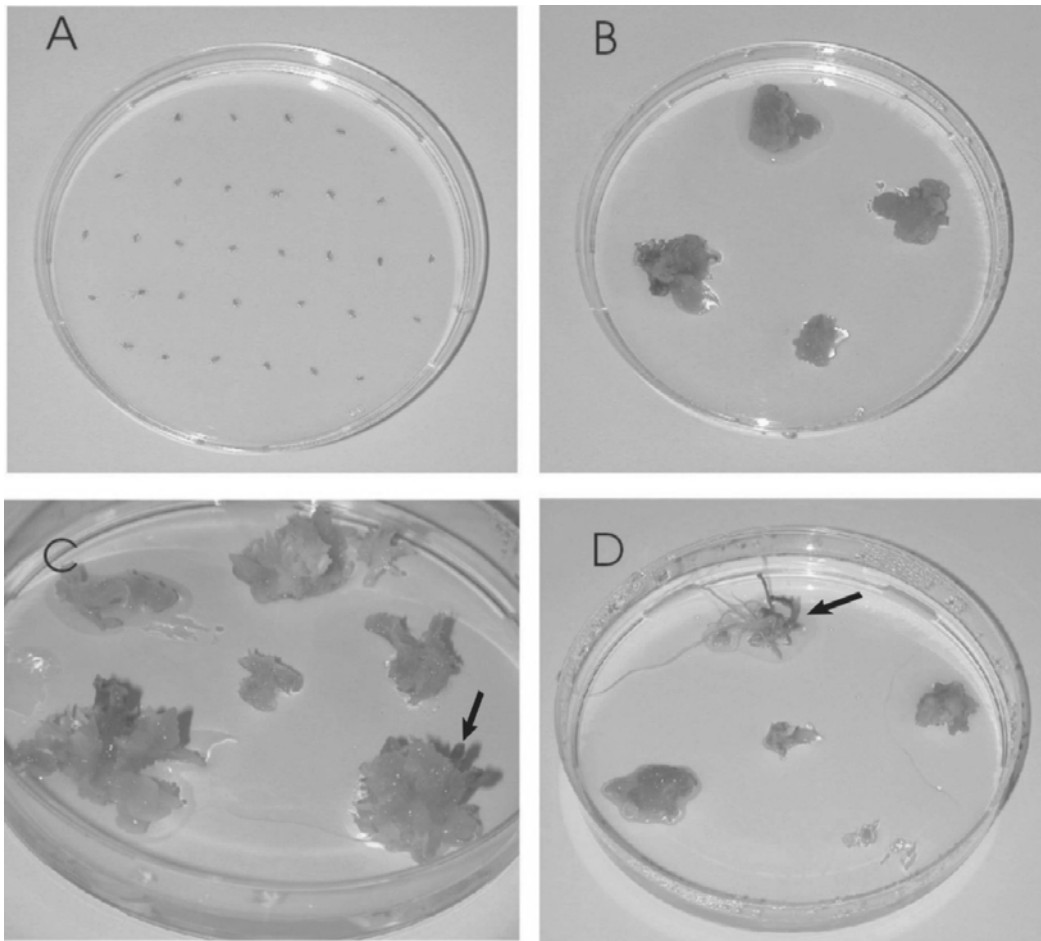
The embryos were transferred to a germination medium (MS hormone free medium) and the number of total plantlets per dish and number of plantlets per callus was determined after four weeks. Then, plantlets were transferred to rooting medium (MS medium supplemented with 0.1 mg L<sup>-1</sup> NAA).

Cytological study: Root tips (1-1.5 cm) from plantlets of the cultivars „Record“ and „Huchels Alpha“ were treated with 0.05% colchicine for two hours and then fixed in 3:1 ethanol:glacial acetic acid for 24 hours, and afterwards washed with distilled water. The roots were hydrolyzed in 1 N HCl for 5 minutes at 60° C. Afterwards, they were stained with aceto-carmine, squashed gently and investigated by employing a light microscope to determine the number of chromosomes. The data was processed by analysis of variance; the Duncan's multiple range test of the SPSS program version 10 was used for the comparison between treatments (SPSS, 1999).

### Results and discussion

#### Callus induction

The data presented in Tab. 1 show that the asparagus genotypes differed significantly in the callus induction rate. The cultivar „Record“ showed the highest percentage of responding anthers, while these values were lower for „Huchels Alpha“ and „Schwetzinger Meisterschuss“ with 40 % and 54 %, respectively. These values are distinctly higher than those reported by Peilai et al. (1999), who found variations of only 7.1% to 26.5% when using the cultivars UC72, UC711, UC157 and MW500, which were cultured on the same medium as those in the presented study. However, Shuxing et al. (1995) showed that 29 to 84% of all anthers formed callus when different plants of the cultivar UC72 were compared. Differences among cultivars and individual plants of the same cultivar can be attributed to the genetic diversity of the F<sub>1</sub> population (Tsay et al., 1982). In experiments of Shalaby et al. (2003) significant differences between asparagus cultivars in callus induction rate and number of embryogenic calli were found. Callus formation, embryo and plantlet development are shown exemplary for the cultivar „Record“ in Fig. 1.



**Fig. 1:** Plantlet development from anther cultures of the cultivar “Record”. **A:** Excised anthers. **B:** Callus formation after 8 weeks. **C:** Embryo formation after 12 weeks. **D:** regenerated plantlets after 16 weeks

**Tab. 1:** Genotypical differences in callus induction of asparagus anther cultures

Cultivar	Number of calli per dish <sup>1</sup>	Anthers forming calli (%)
Record	16.5 a	66
Huchels Alpha	10.0 c	40
S. Meisterschuss	13.5 b	54

<sup>1</sup>Numbers with different characters indicate statistically different means at the 5% level of the Duncan test. Every dish contained 25 anthers.

#### *Callus differentiation*

The influence of the genotype on the number of embryogenic calli and the percentage of embryogenic calli is shown in Tab. 2. The data reveal that the growth medium had an impact on the number of embryogenic calli. Again the cultivar „Record“ had the highest rate of embryogenic calli. The medium A was favorable with view to a higher rate of embryogenic callus than medium B. This could possibly be due to the presence of BA (Shuxing et al., 1995). The highest percentage of calli (86% and 46.6%) that yielded embryos was determined for the cultivar „Record“ in both growth media (Tab. 2). In

studies of Qiao and Falavigna (1990) about 20% of the asparagus calli were not regenerated or produced abnormal shoots and roots. This rate was distinctly higher for „Huchels Alpha“ and „Schwetzinger Meisterschuss“ (Tab. 2). In comparison, Peilai et al. (1999) found that only 20% from the calli formed shoot apices or plantlets. Also, Shalaby et al. (2003) reported that the percentage of embryogenic calli ranged from 19.7% to 52.8% in dependence on the genotype. Wolyn and Feng (1993) revealed that genotype, incubation temperature and sampling date strongly affected the number of embryogenic calli in asparagus anther cultures.

**Tab. 2:** Genotypical differences in the number of embryogenic calli produced from asparagus anther cultures in relation to the differentiation medium

Cultivar	Medium A		Medium B	
	Number of embryogenic calli per dish	Embryogenic calli (%)	Number of embryogenic calli per dish <sup>1</sup>	Embryogenic calli (%)
Record	4.3 a	86.0	2.5 a	46.6
Huchels Alpha	2.5 b	50.0	2.0 a	40.0
S. Meisterschuss	2.0 b	40.0	0.6 b	12.0

<sup>1</sup>Numbers with different characters indicate statistically different means at the 5% level of the Duncan test.

Every dish contained 25 anthers

**Tab. 3:** Genotypical differences in the number of embryos, embryos per callus, plantlets and plantlets per callus differentiated from asparagus anther culture in relation to the growth medium

Cultivar	Medium A				Medium B			
	Number of embryos per dish <sup>1</sup>	Number of embryos per callus	Number of plantlets per dish	Number of plantlets per callus	Number of embryos per dish	Number of embryos per callus	Number of plantlets per dish	Number of plantlets per callus
Record	37.0 a	7.4 a	29.0 a	5.8 a	42.5 a	8.5 a	26.5 a	5.3 a
Huchels Alpha	31.0 b	6.2 b	9.5 b	1.9 b	31.5 b	6.3 b	21.5 b	4.3 b
S. Meisterschuss	27.5 c	5.5 c	2.5 c	0.5 c	18.5 c	3.7 c	0.0 c	0.0 c

<sup>1</sup>Numbers with different characters indicate statistically different means at the 5% levels by the Duncan's test. Every dish contained 25 anthers

#### Regeneration of plantlets

Data presented in Tab. 3 show that the influence of the genotype on the number of embryo-like structures and plantlets was similar in both growth media. Again the cultivar „Record“ yielded the highest number of embryos, embryos per callus, plantlets and number of plantlets per callus. In this study, the number of plantlets per callus for the cultivar „Huchels Alpha“ was much higher than that determined by Shalaby et al. (2003), who used the same cultivar, but another induction medium. Feng and Wolyn (1991) tested three media for embryo maturation and found that 15% to 50% of the embryos matured and germinated. In another study, Shuxing et al. (1995) found that the highest frequency of shoot formation was obtained on the MS medium containing 0.1mg L<sup>-1</sup> BA as shoot formation proved to be sensitive to BA.

The examination of the regenerated plantlet root tips by light microscopy revealed that the chromosome number of cultivars 'Record' and „Huchels Alpha“ were haploid (10 chromosomes) in 15% and 10% of the plantlets, while correspondingly 85% and 90% were diploid. This is relevant as anther cultures in asparagus are used to produce homozygous supermale plants that can generate all-male progeny, an important criteria for breeding high yielding cultivars. The results of this study can be summarized as follows: the asparagus cultivar „Record“ produced

12% more calli than „Schwetzinger Meisterschuss“ and 26% more than „Huchels Alpha“, respectively. The same cultivar yielded a higher number of embryogenic calli than other cultivars and the differentiation medium A was favorable with view to a higher rate of embryogenic callus than medium B.

#### References

- Aneja M, Gianfagna TJ, Garrison SA, Duner EF (1999) Rapid sex-typing of asparagus for male hybrid seed production using n-propyl N-(3,4-dichlorophenyl) carbamate (NPC). Hort Science 34 (6): 1090-1094
- Cao MQ, Li Y, Liu F, Jiang T, Liu GS (1995) Application of anther culture and isolated microspore culture to vegetable crops improvement. Acta Hort 392: 27-38
- Doré C (1974) Production de plantes homozygotes males et females a' partir d'anthères d'asperge, cultivées in vitro. CR Acad Sci Paris 278 D: 2135-2138
- Doré C (1990) Asparagus anther culture and field trials of dihaploids and F1 hybrid. Biotechnology in agriculture and forestry 12: 322-345
- Falavigna A, Tacconi MG, Soressi GP (1983) Recent progress in asparagus breeding by anther in vitro culture. Acta Horticulturae 131: 215-222

- Feng XR, Wolyn DJ** (1991) High frequency production of haploid embryos in *Asparagus* anther culture. *Plant Cell Rep* 10 (11): 574-578
- Feng XR, Wolyn DJ** (1993) Development of haploid asparagus embryo from liquid cultures of anther-derived calli is enhanced by ancymidol. *Plant Cell Rep* 12 (5): 281-285
- Hondelmann W, Wilberg B** (1973) Breeding all-male cultivars of asparagus by utilization of anther and tissue culture. *Z. Pflanzenzuechtg* 69: 19-24
- Inagaki N, Harada T, Yakuwa T** (1980) Studies on the anther culture of Horticultural plants. I- Callus formation from the asparagus anther. *J Japan Soc Hort Science* 49 (1): 71-78
- Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol Plant* 15: 473 - 497
- Peilai H, Liming C, Zhenglai Z, Shihua L, Gezhi S, Lin J** (1999) Anther culture and plant regeneration for *Asparagus officinalis* L.. Proceeding of the Eucarpia meeting on leafy vegetables genetics and breeding; 8-11 Jun 1999. Palacky University; Olomouc (Czech Republic) 302 p
- Qiao YM, Falavigna A** (1990) An improved in vitro anther culture method for obtaining doubled-haploid clones of asparagus. *Acta Hort* 271: 145-150
- Rotondo J, Harney P, Tiessen H** (1983) Direct and indirect embryogenesis in microspores of *Asparagus officinalis* L. cv. Viking. *Asparagus Research Newsletter* 1 (2): 8 - 9
- Shalaby TA** (1996) Producing double haploid plants through ovule and anther culture technique in *Cucurbita pepo* L. M.Sc. Thesis, Fac. Aric., Tanta Univ., Egypt
- Shalaby TA, Haneklaus S, Schnug E** (2003) Influence of growth regulators and cultivar on callus and embryo induction in anther cultures of asparagus (*Asparagus officinalis* L.) *Landbauforsch Voelkenrode* 53: 217-221
- Shuxing S, DaoQian Z, ChengHe Z, ShiXing L** (1995) Improved rate of callus and plantlet from anther culture of asparagus (*Asparagus officinalis* L.). *Acta Hort* 402: 299-305
- Sneep J** (1953) The significance of andromonoecy for the breeding of *Asparagus officinalis* L. *Euphytica* 2: 89-95
- SPSS** (1999) Statistical Package for the Social Sciences Incorporation, Chicago, Ill. SPSS base 10.0 applications guide. Chicago: SPSS, XI, 426 p, ISBN 0-13-017901-9
- Tsay HS, Lai PC, Chen LJ** (1982) The development of haploid plants of *Asparagus officinalis* L. through anther culture. *Taiwan Asparagys Res* 1981: 23-26
- Veilluex RE** (1994) Development of new cultivars via anther culture. *Hort Science* 29: 1238-1241
- Wolyn DJ, Feng X** (1993) Genotype, temperature, and sampling date affect embryogenesis in asparagus anther culture. *Hort Science* 28 (3): 216-217

