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Influence of growth regulators and cultivar on callus and embryo induction in anther cultures of asparagus (*Asparagus officinalis* L.)

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Abstract

The influence of growth regulators and cultivar on callus and embryo induction was studied in anther cultures of asparagus (*Asparagus officinalis* L.). The concentration of different growth regulators stimulated significantly the amount of callus produced. The highest callus induction rate of 58.8 % was obtained on Murashige and Skoog (1962) basal medium (MS) supplemented with 3 % sucrose and 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA) and naphthalene acetic acid (NAA) for the cultivar *Hannibal*. The MS medium supplemented with 3 % sucrose, 1.0 mg L^{-1} 2,4-D, 0.1 mg L^{-1} BA and 0.5 mg L^{-1} NAA produced the highest rate of embryogenic calli, total number of plantlets and number of plantlets per callus. Asparagus cultivars cultivated on the same induction medium reacted differently: statistically significant genotypic differences were found for callus induction, number of embryogenic calli and number of embryos per callus. The cultivar *Hannibal* showed the highest rate of callus induction and embryogenic callus. In comparison, the cultivar *Ariane* yielded the highest number of embryos per callus.

Key words: Androgenesis, anther culture, asparagus, growth regulators

Zusammenfassung

Einfluss von Wachstumsreglern und Sorte auf die Induktion von Kallus und Embryogenese in Antherenkulturen von Spargel (*Asparagus officinalis* L.)

Der Einfluss von Wachstumsreglern und Sorte auf die Induktion von Kalli und Embryogenese in Antherenkulturen von Spargel (*Asparagus officinalis* L.) wurde untersucht. Die Konzentration verschiedener Wachstumsregler hatte einen signifikanten Einfluss auf die Kallusbildung. Die höchste Kallusinduktionsrate von 58,8 % wurde bei der Sorte *Hannibal* auf einem Medium mit 3 % Saccharose und 0.5 mg L^{-1} 2,4-Dichlorophenoxyessigsäure (2,4-D), 6-Benzyladenin (BA) und Naphthalen-Essigsäure (NAA) erzielt. MS Medium, das mit 3 % Saccharose, 1,0 mg L^{-1} 2,4-D, 0,1 mg L^{-1} BA und 0,5 mg L^{-1} NAA ergänzt wurde, produzierte die höchste Rate an embryogenen Kalli, die höchste Anzahl an Jungpflanzen, absolut und je Kallus. Antherenkulturen von Sorten, die auf dem gleichen Induktions-Medium angezogen wurden zeigten statistisch signifikante Unterschiede bei den Parametern Kalliinduktion, Anzahl embryogener Kalli und der Anzahl an Embryonen pro Kallus. Die Sorte *Hannibal* zeigte dabei die höchste Kallusinduktionsrate und die höchste Anzahl an embryogenen Kalli. Im Vergleich hierzu wies die Sorte *Ariane* die höchste Anzahl Embryonen pro Kalli auf.

Schlüsselwörter: Androgeneses, Antherenkultur, Spargel, Wachstumsregler

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1 Introduction

Asparagus is a dioecious plant and the nutritional value of this vegetable crop is high. Male plants are superior over female plants in terms of yield and quality (Aneja et al., 1999). A consistent method to produce double-haploid plants is by anther culture, which proved to be a valuable tool to assist asparagus breeding programs and to provide homozygous materials for classical and molecular genetic studies (Cao et al., 1995). Anther culture is a powerful technique that after 20 years of its development gained a distinct impact on the release of asparagus cultivars (Veilleux, 1994). The short time that is required to produce completely homozygous lines is the major advantage of anther cultures (Veilleux, 1994). Super males can be obtained by two methods: self pollination of hermaphroditic flowers on male plants (but then only 25 % of the progeny are super male), or by anther culture, which yields solemnly super males. The major disadvantage of the first approach is, however, that it is limited by genetic and environmental factors, because male plants do not regularly produce hermaphrodite flowers (Wolyn and Feng, 1993). Studies with view to creating super male asparagus plants via anther culture were reported, but the results were inconsistent (Hondelmann and Wilberg, 1973; Doré, 1974; Shuxing, et al., 1995; Qiao and Falavigna, 1990; Feng and Wolyn, 1991). The composition of the induction medium and the concentration of growth regulators (synthetic phytohormones) in the medium proved to be an important factor for embryo formation and plant regeneration from anther cultures and the subsequent regeneration of plantlets (Inagaki et al., 1980; Shuxing, et al., 1995). The addition of 2,4-dichlorophenoxyacetic acid (2,4-D) to the culture medium seems to be necessary for the induction of androgenesis (Requin, 1973). Significant differences in the amount of callus stimulated by varying levels of different growth regulators were found by Zhang et al. (1994). The significance of growth regulators for asparagus anther cultures is well known so that the optimisation of their concentration and composition is of prime interest when using this technique. It was the aim of this work to study the influence of different concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA) and 6-benzyladenine (BA) on callus and embryo induction in anther cultures of asparagus and to investigate genotypical differences in the response to a defined combination of growth regulators.

2 Materials and Methods

Two experiments were conducted to study the effect of different growth regulators in anther cultures of the cultivar *Hannibal* on callus and embryo induction and to determine genotypical differences in the response to a defined composition of growth regulators.

In the first experiment, two year old greenhouse-grown plants of the cultivar *Hannibal* were used as anther donor plants. Various kinds of combination of 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA) and 6-benzyladenine (BA) (Table 1) were supplemented to the Murashige and Skoog (1962) basal medium (MS). 30 g L^{-1} sucrose and 8 g L^{-1} agar were added before the pH of the medium was adjusted to 5.8. The experiment was carried out in a completely randomized design with ten replicates.

Table 1:
Concentration of 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA) and naphthalene acetic acid (NAA) in the induction medium of the cultivar *Hannibal*.

Treatment	2,4-D	BA	NAA
	mg L^{-1}		
1	0.1	0.1	0.1
2	0.1	0.5	0.5
3	0.1	1.0	1.0
4	0.5	0.1	0.5
5	0.5	0.5	0.1
6	0.5	0.5	0.5
7	0.5	1.0	1.0
8	1.0	0.5	0.1
9	1.0	0.1	0.5
10	1.0	1.0	1.0

In the second experiment, one year old greenhouse-grown plants of the cultivars *Ariane*, *Hannibal*, *Huchels Alpha* and *Huchels Leistungsauslese* were used as anther donor plants. The induction medium in this experiment was a MS basal medium supplemented with 3 % sucrose and 1 mg L^{-1} 2,4-D, 0.1 mg L^{-1} and 0.5 mg L^{-1} NAA. 8 g L^{-1} agar was added before the pH of the medium was adjusted to 5.8. The experiment was carried out in a completely randomized design with 20 replicates.

2.1 Culture technique and analysis

The developmental stage of the microspores was determined by squashing anthers in 0.5 % aceto-carmine. Anthers with microspores in the late uninucleate stage were chosen for culturing the anthers. For this purpose flower buds of 1.5 - 2 mm length, which contained microspores at the uninucleate stage were collected and stored at 4° C for 48 hours. Then the buds were sterilized for 10 minutes in 10 % calcium hypochlorite, rinsed three times with sterile distilled water and finally placed on a sterilized paper towel to absorb excessive surface water. The anthers were dissected from the flower buds using sterilized fine needles before being placed on the autoclaved (20 min. at 121° C and 1.1 kg cm^{-2}) induction

medium. The dishes were incubated at 32° C in darkness for four weeks according to Feng and Wolyn (1991). Afterwards the anthers were incubated at 25° C with a photoperiod of 16 hours for in total four weeks. Then the number of calli was determined and the number of calli per 100 anthers was calculated.

The calli were transferred to the differentiation medium which was a MS medium supplemented with 3 % sucrose, 0.3 mg L^{-1} BA and 0.2 mg L^{-1} NAA. After four weeks the number of embryogenic calli and the number of embryos per callus were determined.

The embryos were transferred to the germination medium, which was a MS medium supplemented with 3 % sucrose, but hormone free. The number of total plantlets and number of plantlets per callus were determined after another four weeks.

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, 1998).

3 Results and discussion

3.1 Effect of growth regulators on callus and embryo induction

The effect of 2,4-D, NAA and BA on callus induction and regeneration of plantlets from anther cultures of the variety *Hannibal* is presented in table 2. The data shows that there were significant differences in the amount of callus stimulated, the number of embryogenic callus and the number of plantlets per dish and per callus for different concentrations and combinations of growth regulators. In combinations of growth regulators with concentrations of ≤ 0.5 mg L^{-1} no plantlets were produced. Also if 0.1 mg L^{-1} 2,4-D was applied with 0.1 mg L^{-1} and/or 0.5 mg L^{-1} BA and NAA no embryogenic callus was obtained (Table 2). These results indicate that a critical concentration of growth regulators needs to be supplied for a successful growth of asparagus anther cultures. The media supplemented with 0.5 mg L^{-1} 2,4-D, BA and NAA and 0.5 mg L^{-1} 2,4-D, 1.0 mg L^{-1} BA and 1.0 mg L^{-1} NAA yielded the highest number of calli per dish. In comparison, Zhang et al. (1994) found that 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA produced the highest rate in callus induction from isolated asparagus microspores. In another study, Yan et al. (1992) found a callus induction rate of 40.5 % and a embryogenic callus rate of 55 % on MS medium supplemented with 0.5 mg L^{-1} BA and 0.2 mg L^{-1} 3-indolybutric acid (IBA).

The highest percentage of embryogenic calli with 78.8 % and the highest number of plantlets per callus with 10.5 were obtained in the medium supplemented with 1.0 mg L^{-1} 2,4-D, 0.1 mg L^{-1} BA and 0.5 mg L^{-1} NAA (Table 2). Torrey and Peirce (1983) found that the highest rate of anther producing callus of the cultivar *Emerald* was obtained on a modified MS induction medium with 1 mg L^{-1} BA and 5 mg L^{-1} NAA and a differentiation medium with 0.5 mg L^{-1} BA and 1 mg L^{-1} NAA.

3.2 Genotypic differences in callus and embryo induction

The data presented in table 3 and 4 reveals that significant differences existed between asparagus cultivars in callus induction, number of embryogenic calli, total number of embryos and per callus and the total number of plantlets. The number of plantlets per callus was, however, not significantly different between the cultivars (Table 3). Generally, the cultivar *Hannibal* yielded the highest

Table 2:
Influence of different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA) and naphthalene acetic acid (NAA) on callus induction and differentiation from anther cultures of the cultivar *Hannibal*.

Treatment			Callus induction		Embryogenic callus		No. of plantlets	
2,4-D	BA	NAA	calli per dish[1]	%	No. of calli per dish[2]	%	Per dish[2]	Per callus
	mg L^{-1}							
0.1	0.1	0.1	2.3 ab[3]	9.2	0.0 a	0.0	0.0 a	0.0 a
0.1	0.5	0.5	1.0 a	4.0	0.0 a	0.0	0.0 a	0.0 a
0.1	1.0	1.0	4.8 abc	19.2	0.8 a	16.0	0.3 a	0.1 a
0.5	0.1	0.5	11.8 d	47.2	3.3 d	27.7	0.0 a	0.0 a
0.5	0.5	0.1	9.0 bcd	36.0	0.8 a	8.7	0.0 a	0.0 a
0.5	0.5	0.5	14.7 d	58.8	1.0 ab	6.8	0.0 a	0.0 a
0.5	1.0	1.0	14.5 d	58.0	4.3 d	29.3	29.0 c	5.8 c
1.0	0.5	0.1	10.3 cd	41.2	2.8 bc	26.8	3.3 b	0.7 b
1.0	0.1	0.5	8.3 bcd	33.2	6.5 e	78.8	52.3 d	10.5 d
1.0	1.0	1.0	12.8 d	51.2	1.3 ab	9.8	0.6 a	0.1 a

[1] every dish contained 25 anthers; [2] every dish contained 5 calli; [3] different letters indicate significant differences at the 5 % level by the Duncan´s test.

percentage of callus induction and embryogenic callus with 53 % and 52.8 %, respectively. This findings suggest that the induction medium may have been sub-optimum for the other cultivars tested. Deviations of these results, from that of the first experiment for the cultivar *Hannibal* are supposedly due to differences in plant age.

The cultivar *Ariane* produced the highest number of embryos per callus with a value of 3.5. In this experiment the response of anthers ranged from 3.8 % for the cultivar *Huchels Alpha* to 53 % for the cultivar *Hannibal*. A comparison of these results with previous experimental data (Shalaby et al., 2003) reveals that anthers from field-grown plants of *Huchels Alpha* cultured on MS medium supplemented with 0.1 mg L^{-1} NAA, 0.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA yielded a higher callus induction with 40 % than the same cultivar in the present study with 3.8 %. This indicates that growth conditions of anther donor plants obviously affect induction and differentiation rates.

Tsay et al. (1982) reported genotypical differences in the response of anther cultures to different induction media that varied between 0 % and 93 % for anthers producing calli and 0 % and 31 % for calli that produced shoots. Inagaki et al. (1980) studied the effect of growth regulators on anther cultures of three asparagus cultivars. The optimum concentration of growth regulators for the cultivar *May Washington* (*MW 500*) was 1 mg L^{-1} BA and 5 mg L^{-1} NAA, whereas for the cultivars *Zuiyo* and *Gruene Krone* these were 0.5 to 1 mg L^{-1} BA and 3 mg L^{-1} NAA. The highest rates of anther producing calli were 72 %, 90 % and 86 % for MW 500, *Zuiyo* and *Gruene Krone*, respectively. Among the different factors affecting androgenesis, genotype has probably the most striking influence (Doré, 1974; Feng and Wolyn, 1991; Shalaby et al., 2003). Previous studies revealed large discrepancies in callus induction rates of asparagus anther cultures (Doré, 1990). Doré (1974) and Qiao and Falavigna (1990) reported callus induction rates from 2 % to 10 %, whereas Inagaki (1980), Shuxing et al. (1995) and Shalaby et al. (2003) reported callus induction rates from 50 % to 86 %. These discrepancies can be explained by the fact that the former authors considered only the pollen calli, whereas in the latter case all calli, somatic and androgenetic ones were included (Doré, 1990).

Anther cultures in asparagus cultivation are used to develop homozygous super male plants that can generate all-male progeny, an important criteria for breeding high yielding cultivars. The results of the presented study reveal that only specific concentrations and combinations of growth regulators are suitable as induction media and though genotypical differences existed for parameters such as the rate of callus induction, embryogenic callus and number of embryos, the most important criteria, the number of plantlets per callus was not affected.

Table 3:
Effect of cultivar on callus induction and embryogenic callus produced from asparagus anther cultures.

Cultivar ranking	Callus induction		Cultivar ranking	Embryogenic callus	
	calli per dish[1]	%		calli per dish[2]	%
Huchels Alpha	0.8 a[3]	3.8	Ariane	0.98 a	19.7
H. Leistungsauslese	1.7 a	8.3	H. Leistungsauslese	1.52 b	30.3
Ariane	3.1 b	15.3	Huchels Alpha	2.33 c	46.7
Hannibal	10.6 c	53.0	Hannibal	2.46 c	52.8

[1] every dish contained 20 anthers; [2] every dish contained 5 calli; [3] different letters indicate significant differences at the 5 % level by the Duncan´s test.

Table 4:
Effect of cultivar on number of embryos and plantlet regeneration from asparagus anther cultures.

Cultivar ranking	Number of embryos		Cultivar ranking	Number of plantlets	
	Total	Per callus		Total	Per callus
Huchels Alpha	13	1.9 a	Huchels Alpha	10	1.4 a
H. Leistungsauslese	25	2.5 ab	Ariane	17	1.4 a
Ariane	42	3.5 b	H. Leistungsauslese	21	2.1 a
Hannibal	285	2.5 ab	Hannibal	121	1.1 a

[1] different letters indicate significant differences at the 5 % level by the Duncan´s test.

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