アスパラガスの葯からのカルス形成に及ぼす培養前処理の効果について

誌名	神戸大学農学部研究報告 = The science reports of Faculty of Agriculture, Kobe University
ISSN	04522370
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巻/号	22巻1号
掲載ページ	p. 25-30
発行年月	1996年1月

神大農研報 (Sci. Rcpt. Fac. Agr. Kobe Univ.) 22 : 25 − 30 , 1996

Effect of Low Temperature Pretreatment before Anther Culture on Callus Formation from Asparagus (*Asparagus officinalis L.*) Anthers

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(Received for publication August 10, 1995)

Abstract

In order to induce higher frequencies of calli and embryos from anthers of asparagus, anthers or flower buds were pre-treated with low temperature and distilled water immersion before in vitro culture.

(1) Flower buds harvested from cv.'Mary Washington 500W'with low temperature (5℃) pretreatment for 3 days gave a higher frequency of anthers inducing calli than those without the pretreatment. Calli induced in the pretreatment were emerged on the side of browning anther wall, and ones induced in control completely covered the anther wall.

(2) Anthers and flower buds were immersed in distilled water for 3 days before anther culture. Anthers with the pretreatment decolored and died several days after anther culture. On the other hand,flower buds with immersion pretreatment at 5℃ provided calli and dome like body (DBL) , which emerged from the burst wall of browning anthers. The DBL, of which surface was smooth, had high ability of shoot differentiation.

The optimal period for the pretreatment of water immersion at 5℃ on callus induction was

Introduction

Several reports have described that in some plants including tobacco the percentage of anthers forming calli or embryos more increased when anthers stored for a few days at a low temperature were provided for anther culture[2,8,10]. Appropriate temperature and duration for the pretreatment differ from plant species, but the effect of low temperature pretreatment in anther culture has been confirmed in many plant species, and which has been supposed that microspores in anther were transferred to the de-differentiated pathway from normal development by low temperature pretreatment.

As direct embryogenesis from asparagus anthers has not been succeeded, it is essential for callus formation from anthers to supplement the nutrient medium with plant growth substances[1,4,9,11] Pretreatment of anthers or flower buds prior to in vitro culture might be expected to disturb the normal development of young microspores after meiosis to mature pollens, induce calli from microspores(n), and inhibit callus proliferation from anther tissue(2n).[3,7] We carried out the following experiments in order to investigate effects of pretreatment before anther culture on callus formation.

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Materials and Methods

Experiment 1. Effect of low temperature pretreatment of flower buds on callus induction

Approximately 2mm-long flower buds with anthers at tetrad to early uninucleate stage of microspores in *Asparagus officinalis L.*cv. 'Mary Washington 500W' were harvested at the experimental farm of Faculty of Agriculture, Kobe University. They were surface-sterilized with 70% ethanol for 30 seconds followed by rinsing three times with sterile distilled water, and then placed on the moisturized double layer filter paper in a petri dish of 9 cm in diameter at 5℃ for 3 days. The petri dish

was covered with aluminum foil to prevent the flower buds from desiccation. After the pretreatment,the flower buds were surface-sterilized again for 60 seconds with 70% ethanol followed by 1% sodium hypochlorite for 10 minutes, and then rinsed 3 times with sterile distilled water. Anthers isolated aseptically were cultured on Murashige and Skoog (MS) basal medium supplemented with 30g/l glucose, 0.1mg/l BA and 0.3mg/l NAA at 28℃ in dark condition. The medium was adjusted to pH 5.5-5.7, and solidified with 2.5g/l Gelrite. The investigation on callus formation was carried out 28 days after incubation.

Experiment 2. Effect of water immersion pretreatment of anthers and flower buds on callus formation

From the result of experiment 1, positive effect of pretreatment on callus formation was expected, and therefore four kinds of immersion pretreatment in water in combination with low (5℃) and high (28℃) temperature were provided (Table 2). The variety, the developmental stage of anthers, and the method of sterilization of flower buds in this experiment were same as those in experiment 1. Before anther culture,flower buds and anthers in water immersion pretreatment were placed in 9 cm-diameter petri dishes which contained sterile distilled water for 3 days. After the pretreatment,anthers isolated aseptically were cultured on Murashige and Skoog (MS) basal medium supplemented with 30g/l glucose, 0.1mg/l BA and 0.3mg/l NAA at 28 ℃ in dark condition. The medium was adjusted to pH 5.5-5.7, and solidified with 2.5g/l Gelrite. Ten anthers per test tube were incubated with at least10 replications per treatment.

Experiment 3. Effect of the duration of pretreatment on callus formation

From experiment 2, water immersion pretreatment of flower buds at 5℃ resulted in good callus formation. We further tried to obtain more optimal duration of pretreatment for the best callus formation. Times prepared for the pretreatment were 1,3,4,5 and 6 days. The materials and methods in Experiment 3 were same as those in Experiment 1 except for the duration of the pretreatment.

Results and Discussion

1.Effect of low temperature pretreatment of flower buds on callus formation

Low temperature pretreatment stimulated callus formation at a higher frequency (82%) than control (60%) (Table 1) . Calli induced after the pretreatment were friable and emerged gradually out of the side of anther wall. On the other hand, calli in control were induced rapidly on the surface of anther wall so that they covered entirely the anther wall. With the pretreatment, 74% of incubated anthers turned brown, compared to 20% of them in control. Browning of incubated anthers was considered to inhibit the supply of nutrients in the medium through the anther tissue to microspores.

In this experiment, however, browning of the surface of anthers had a tendency to inhibit callus formation from anther wall. Anthers without pretreatment, that is anthers incubated on the callus inducing medium in the light condition just after harvesting flower buds, remained green for long time. The anthers, therefore, were considered to have a high cell division activity, and consequently it was supposed that callus induction from anther wall was superior to that from microspores. To the contrary, anther cultures with no addition of plant hormones such as tobacco anther cultures have been observed to induce embryos without callus formation from anther wall. Because of its short longevity (about 7 days), the ability of cell division in anther wall was not seemed to be retained at high level. Asparagus anther cultures which need plant hormones such as cytokinin and auxin for inducing calli would require to inhibit callus formation from anther wall and stimulate de-differentiation of microspores with the pretreatment of anthers or flower buds.

2.Effect of water immersion pretreatment of anthers and flower buds on callus formation

When anthers immersed at 5℃ or 28℃ for 3 days were incubated, most of them discolored and eventually died. Immersion pretreatment of anthers in water was supposed to give a severe stress to normal aerobic metabolism of anther tissue. To the contrary, flower buds used for pretreatment were kept tightly shut, so that anthers within the flower bud were aseptically maintained. The anthers, therefore, might be influenced indirectly. Immersion pretreatment of flower buds

at 5℃ resulted in browning of anthers at the frequency of 89% and callus formation at 19% (Table2). Calli were emerged from the inside of splitanther wall, and approximately two third of them were smooth and like dome (Fig.1). The dome like body (DLB) was morphologically different from friable calli, and was suggested to have a high possibility of embryogenesis or shoot differentiation (Fig.2 and 3). Low temperature pretreatment without immersion resulted in friable calli and no DLB formation, and therefore pretreatment with immersion in water was considered to affect DLB formation. The frequency of anthers inducing callus in this experiment was greatly below that in Experiment 1. This is supposed to be owing to cultivated conditions of plants from which flower buds were harvested; that is the flower buds were harvested early spring in the greenhouse (minimum temperature of 7-10℃) with shallow cultural soil. The age, genotype, and in vitro culture environments (light, temperature and nutrients) of plantlets were considered to affect the response to anthers inducing calli in vitro[6]. Culture condition and physiological state of plants suitable for de-differentiation of anthers and the subsequent re-differentiation were different among plant species or plant genotypes. In this experiment the plants from which anthers were harvested were cultivated in the greenhouse under the unfavorable condition, that is shallow soil and not much reserve in storage roots, and which was supposed to affect the microspores in anthers. Heberle-Bors and Reinert reported that in anther culture of tobacco plant the unfavorable cultural conditions promoted the occurrence of specialized small type of pollens which were induced to embryogenesis[5]. Therefore, the unfavorable cultural condition might also be one of the factors for embryogenesis in asparagus anther culture.

3.Effect of the duration of pretreatment on callus formation

In immersion pretreatment at 5℃, the effect of the pretreatment duration on callus formation was as follows; with 1 and 3 days pretreatments, 29% and 79% anthers induced calli, respectively. Hyperhydricity of both petals and anthers, and no callus formation were observed with 5 and 6 days pretreatments. Three days pretreatment stimulated browning of anthers (85%) and induction of DLB (2%) (Table 3).

In this experiments, it might be concluded that three days

Table 1. Effect of low temperature pretreatment bofore culture on callus formation from anthers of asparagus (*Asparagus officinalis* L.)

(incubated for 28 days)

Pretreatment days	No. of anthers incubated	No. of anthers forming callus	% callus formation	No. of anthers which turned brown	% of anthers which turned brown
0	189	113	60	37	20
3	280	230	82	206	74

Table 2. Effects of low temperature treatment prior to incubation of anthers or flower buds on induction of callus and dome like body (DLB)

(Incubated for 28 days)

Materials for pretreatment	Pretreatment	No. of incubated anthers	No. of anthers forming callus	% callus formation	No. of anthers forming DLB	% of anthers forming DLB	No. of anthers which turned brown	% of anthers which turned brown
Flower bud	5℃ in air	130	5	4	0	0	63	48
	5℃ in water	150	28	19	18	12	134	89
Anther	5℃ in air	150	0	0	0	0	77	51
	5℃ in water	100	0	0	0	0	0	0

pretreatment in low temperature (5℃) was suitable for callus formation, and the longer pretreatment reduced the physiological activity of anthers and inhibited callus induction.

Table 3. Effect of the duration of low temperature pretreatment bofore culture on the induction of callus and dome like body (DLB) from anthers of asparagus (incubated for 28 days)

Pretreatment days	No. of incubated anthers	No. of anthers forming callus	% of anthers forming callus	No. of anthers forming DLB	% of anthers forming DLB	No. of anthers which turned brown	% of anthers which turned brown
1	410	118	29	2	0.5	106	26
3	300	236	79	6	2	255	85
4	250	15	6	4	1.6	67	27
5	300	25	8	0	0	46	15
6	70	0	0	0	0	0	0

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アスパラガスの葯からのカルス形成に及ぼす培養前処理の効果について

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要　約

花粉起原のカルスおよび不定胚の誘導を促進する目的で、培養に用いる葯または花蕾を培養前に低温および蒸留水浸漬処理を行った。
結果は以下の通りであった。

(1) 'メリーワシントン500W'から採取した花蕾を5℃のシャーレ内で3日間低温処理した区は、対照区（無処理区）より高いカルス形成率を示した。処理区のカルスは葯壁の側面から徐々に形成されたのに対し、対照区のカルスは葯壁全体を包むように形成された。

(2)花蕾または葯を5℃または28℃で3日間水浸漬前処理した後培養した。その結果、葯を前処理した区は培養後まもなく褪色し、カルス形成は認められなかった。一方、花蕾を5℃で前処理した区では、褐変した葯壁を割るようにカルスが形成され、その中には高い分化能を持ったドーム状の表面のなめらかな細胞塊が認められた。

(3) 花蕾の低温（5℃）水浸漬前処理の最適時間について検討したところ、4日間以上では花蕾に傷害が生じ、3日間の前処理が好適であった。

Fig.1. Callus and dome like body (DLB) formation from anthers of asparagus in dark condition. Arrows indicate white colored dome like body.

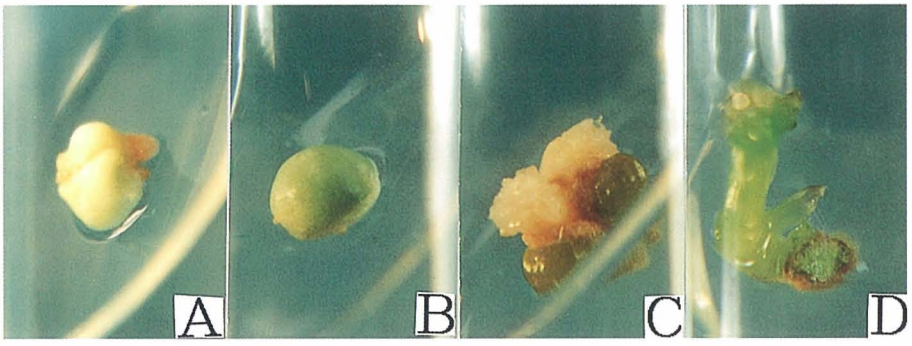

Fig.2. Morphological changes in DLB with time after transfer to the shoot inducing medium in light condition
12days 14days 40days 55days after transfer
A : slightly greening B : green DLB C : differentiation of shoot primordia
D : shoot formation from DLB

Fig.3. Shoot and shoot primordia differentiation from DLB on the shoot inducing medium