African Journal of Biotechnology Vol.11 (70), pp. 13472-13476, 30 August, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1827
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Embryogenesis and plant regeneration from unpollinated ovaries of *Amorphophallus konjac*

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Accepted 1 August, 2012

The system of somatic embryogenesis of *Amorphophallus konjac* had been built through unpollinated ovaries. The embryogenic calli were induced on Murashige and Skoog (MS) basal medium supplemented with 9.0 µM 6- benzylaminopurine (BA), 0.4 µM 2,4- dichlorophenoxyacetic acid (D), 1.0 µM naphthaleneacetic acid (NAA), and the induction rate was 34.0%. The differentiation rate was 35.5% on the medium of MS basal medium supplemented with 6.7 µM 6-BA and 2.2 µM NAA. The obtained plantlets were transferred into rooting medium which was 1/2MS supplementing with 2.7 µM NAA, and the rooting rate was above 95%. All of the media were added 3% (w/v) sucrose and 0.3% (w/v) phytagel, the experimental materials for each step were cultured at 25 ± 2°C with a photoperiod of 12 h and light intensity of 50 µmol m^{-2} s^{-1}.

Key words: *Amorphophallus konjac,* unpollinated ovary, embryogenic calli, plant regeneration.

INTRODUCTION

Amorphophallus konjac is a perennial herbaceous species, mainly distributed throughout Southeast Asia and Africa (Vasques et al., 2008). It is an important glucomannan crop, which has been widely used in food, medicine, chemistry and agriculture industries (Cescutti et al., 2002). Konjac mainly reproduces asexually by corms, but its reproduction coefficient is very low. So the shortage of seed corms is very serious for konjac production. Moreover, the routine konjac corms carry many pathogens, which increase the risk to plant konjac. For the plant tissue culture technology can provide many high quality seed corms, the tissue culture of konjac was extensively studied. Different explants of konjac such as anther, the corm, leaf, root (Zhang, 1998) side-bud, subcutaneous tissue, main-bud, basilar-tissue, budlet (Wu and Xie, 2001), petiole (Hu et al., 2005), shoot tip and bud (Xu et al., 1994) have been reported. Based on the researches reported before, all of plant regeneration of konjac was realized by organogenesis instead of the somatic embryogenesis. The propagation coefficient of konjac for the system of organogenesis was low so that it cannot meet the production requirements. At the same time, the soft rot disease of konjac is mainly diffused by corms and soil, and we have no resistant variety for the soft rot disease in konjac. The system of organogenesis is usually form chimeras so that the system of transgene for konjac is very difficult to be built. The acquisition of embryogenic callus can solve the aforementioned problems**.** There are some successful examples to use the somatic embryogenesis calli to transfer some soft rot antagonistic genes has been reported in some other plants (Ahrenholtz et al., 2000; Marta, 2002; Wang et al., 2002).

The experiments described here, for the first time, were conducted to provide an efficient system for the production of embryoids and plantlets from unfertilized ovaries of *A. konjac*. This efficient regeneration system cannot only provide many seed corms but also speed up

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Abbreviations: NAA, α-Naphthaleneacetic acid; **KT**, kinetin; **2,4-D**, 2,4 dichlorophenoxyacetic acid; **6-BA**, N^6-benzylaminopurine.

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Table 1. Effect of growth regulators on embryogenic calli induction from unpollinated ovary of *Amorphophallus konjac.*

6-BA (μM)	2,4-D (μM)	NAA (μM)	KT (μM)	Embryogenic calli induction rate (%)
9.0	0	2.7	0	0
9.0	0.8	0	0	16.2
9.0	0.4	1.0	0	34.0
0	0.4	0	4.6	13.1

BA, Benzyl aminopurine; 2,4-D, 2,4 dichlorophenoxy acid; NAA, naphthalene acetic acid; KT, kinetin.

Table 2. Differentiation response of embroyid calli of *Amorphophallus konjac* to 6-BA and NAA.

6-BA (μM)	NAA (μM)	Differentiation rate (%)
4.5	0.5	0
4.5	1.1	16.9
4.5	2.2	7.1
6.7	0.5	14.2
6.7	1.1	23.3
6.7	2.2	35.5
11	0.5	23.9
11	1.1	15.3
11	2.2	32.3

BA, Benzyl aminopurine; NAA, naphthalene acetic acid.

breeding for disease resistance of *A. konjac*.

MATERIALS AND METHODS

Plant materials

A. konjac with flower buds were planted in the greenhouse of Life Science College of Wuhan University in China. When the pollens were found in a single-nuclear medium-term, the konjac inflorescences were cut off for the further treatment. Then the female inflorescence were selected and sterilized by treating in 75% ethyl alcohol for 30 s at first, then in 0.1% (w/v) mercuric chloride ($HgCl_2$) for 6 to 8 min with occasional shake. These were finally washed thoroughly at least three times with sterile distilled water.

Culture conditions

Explants cultured in 100 ml triangular flask containing 20 ml of MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose and 0.3% phytagel (sigma). The pH of the medium was adjusted to 5.8 with 0.1 M HCl or 0.1 M NaOH, then the medium autoclaved at 121°C for 20 min. All plant hormones were added before autoclaving. The cultures were incubated under 12 h photoperiod (cool-white fluorescent light, irradiance of 50 μmol m^{-2} s^{-1}) at temperature of 25 ± 2°C and relative humidity of 60%.

Embryogenic calli induction and plant regeneration

Young unpollinated ovaries were separated from female inflorescence of konjac one by one after sterilization. 180 to 200 initial explants were used for each hormone combination. Medium with varying concentrations of different plant growth regulators were tested for callus induction and regeneration of plants (Table 1). The embryo calluses then were transferred to the differentiation medium to growth (Table 2). When the adventitious buds grow up to 3 to 5 cm, they could be transferred to rooting medium for 1/2MS supplementing with 0.54 μM NAA, 3% sucrose and 0.3% phytagel (Wu et al., 2008). Each treatment was repeated three times.

Cytology analysis

The embryoid calli were taken for histological examination. The paraffin sections of the induction process were made for histological observations by use of the method described by Hu et al. (2005). The specimens were fixed in the *Formaldehyde-acetic acid* (FAA) solution (50% ethanol, 5:5:90 (v/v/v)) for 36 h and dehydrated in a set of increasing ethanol solutions (30, 50, 75, 95, 100% (v/v)) twice for 30 min in each step. Then the ethanol was replaced by gradual dilution with trichloromethane solution. The specimens were infiltrated by moving them into a vessel that contained the paraffin wax and kept in 56°C for at least 24 h and finally embedded in the paraffin wax. Sections (10 μm) were cut using a rotary microtome (AO 820, Scientific Instruments), and mounted on glass slides. The sections were de-waxed in xylene for 5 to 10 min, stained with Heindenhain's haematoxylin and then covered by the coverslips with a drop of neutral balsam before examination under a Nikon ANTI-MOULD microscope (15×40).

RESULTS AND DISCUSSION

Embryogenic calli induction

The explants of unpollinated ovary were collected from the spathe of *A. konjac* (Figure1a) when the ovary wall was light yellow and about 2 mm in size (Figure 1b). During the first 30 days, the ovary explants enlarged from0.3 to 0.5 cm, and the ovary wall changed from yellowish to green. At this time, the ovaries were removed with giving a bottom wound and continued to put into the induced medium (Table 1). After 30 days, ovary explants were cultured in the MS supplemented with 9.0 μM BA and 2.7 μM NAA without calli, whereas they responded with enlargement and ovary turned yellow, hygrophanous. The incompact, yellowish calli were observed on the medium with 9.0 μM 6-BA and 0.8 μM 2,4-D or 9.0 μM 6-BA, 0.4 μM 2,4-D and 1.0 μM NAA (Figure 1c and d), but on the medium with 9.0 μM 6-BA, 0.4 μM 2,4-D and 1.0 μM NAA more calli could be produced, the callus induction rate was 34.0%. On

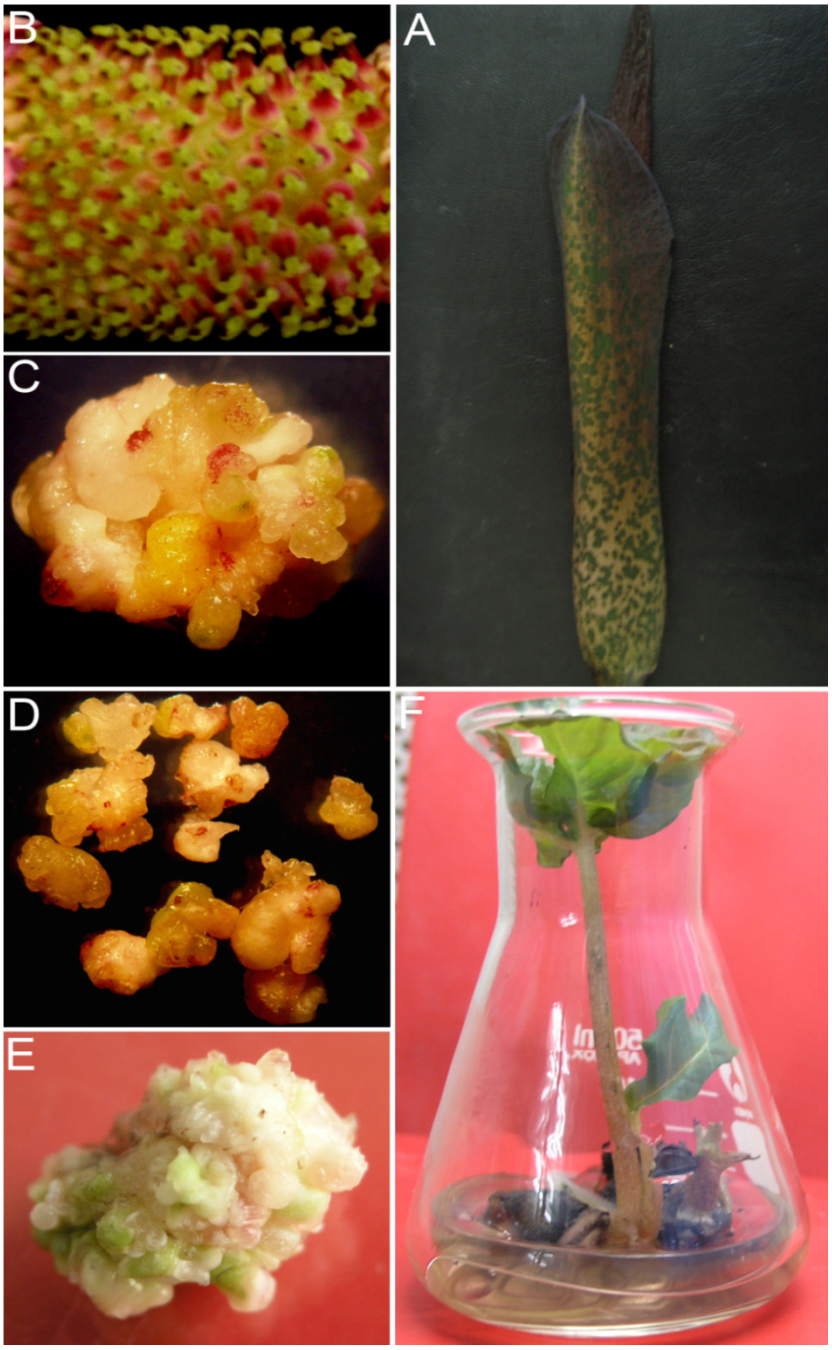

Figure 1. Embryogenic calli and plant regeneration from unpollinated ovary explants of *Amorphophallus konjac*. A. The yong flower buds; B. the unpollinated ovary; C. the embryogenic calli; D. the detached embrygenic calli; E. the bud points differentiated from the embrygenic calli; F. the regeneration of plantlet.

the combination medium of 0.4 µM 2,4-D and 4.6 µM KT the yellowish, dense calli were formed. Histological examination showed that the embryogenic calli could be obtained on medium with both 9.0 µM 6-BA, 0.4 µM 2,4-D, 1.0 µM NAA and 9.0 µM 6-BA, 0.8 µM 2,4-D. However, the medium supplemented with lower NAA could produce more calli. 2,4-D-derived calli, developed embryo-like structures and this increased the ratio of embryogenic calli (Palmer and Kller, 2001). Based on the data, in this study, three combinations of the medium were added low concentration of 2,4-D except one combination.

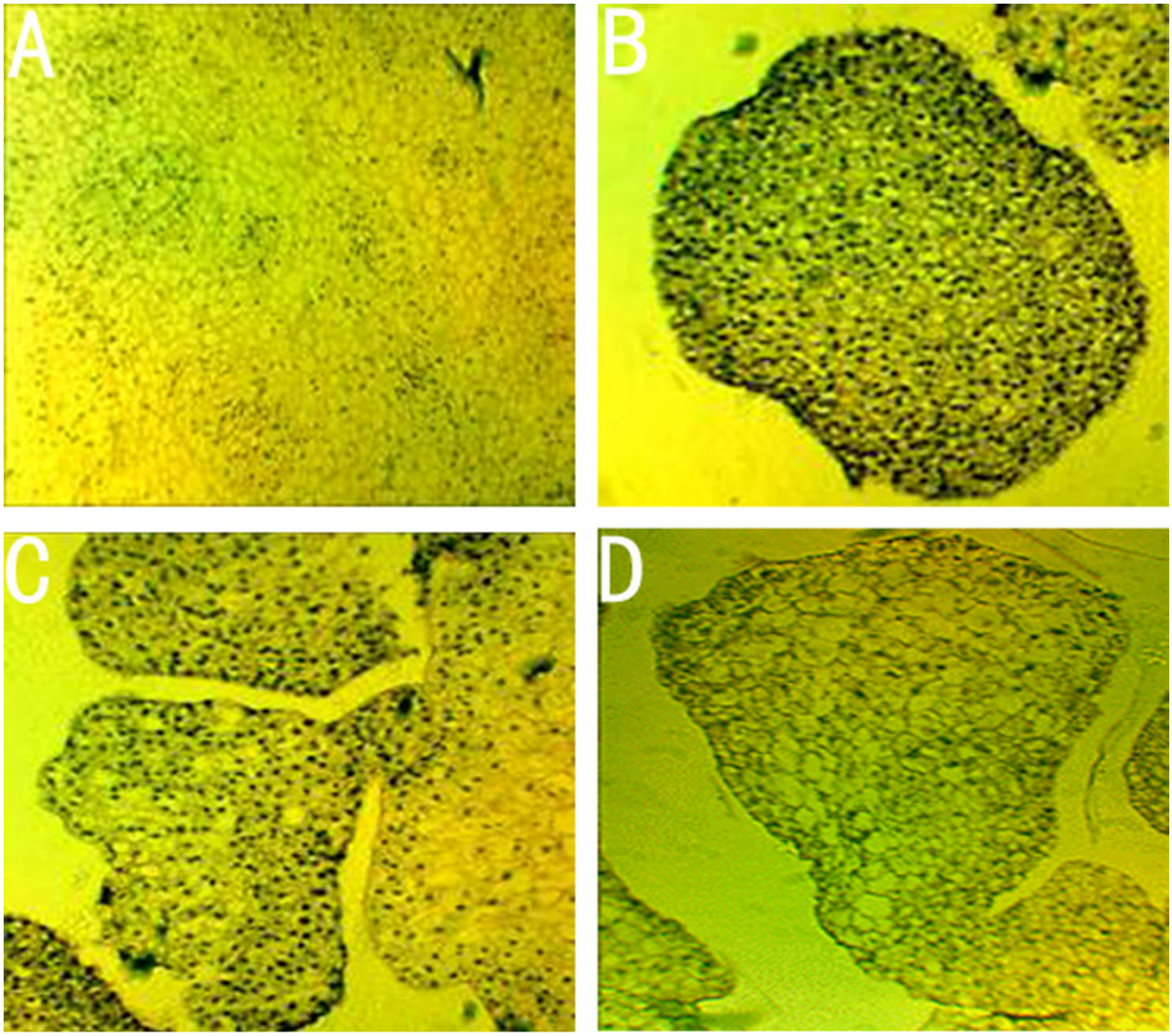

Figure 2. The process of embryogenic calli by paraffin sections. A. Embryonic cell; B. global embryo; C. heart-shaped embryo; D. torpedo embryo.

Cytology analysis

As the growth of the somatic embryogenesis calli they developed and formed global embryo, heart-shaped embryo and torpedo embryo (Figure 2a, b, c and d). The observation of paraffin sections clearly showed the developmental process of calli.

Plant regeneration

The embryogenic calli were transferred to the differentiation culture medium. Differentiation media were prepared with different concentrations of NAA and 6-BA. Many researches showed that NAA alone did not induce organs formation which occurred only in combination with BA (Nakano et al., 1994). MS with 6-BA and NAA had been used for calli differentiation in many plants (Tang et al., 2010). So the orthogonal test was used in the calli differentiation with 6-BA and NAA which was 4.5 µM, 6.7 µM and 11 µM 6-BA combinated with 0.5 µM, 1.1 µM and 2.2 µM NAA (Table 2). Tukey HSD is used in data analysis. These different concentrations of 6-BA and NAA were used to study the effect on calli differentiation. About 30 days, some embryos calli began differentiation (Figure 1e). Among these combinations including 4.5 µM 6-BA and three different concentrations NAA, there were only bud points which could not form a complete plant, and the bud differentiation was at a low level. On the medium substitute for 11 µM 6-BA, the differentiation rate

was lower compared with the 6.7 µM 6-BA. The maximum Differentiation rate (35.5%) was observed in the medium with 6.7 µM BA and 2.2 µM NAA followed by 6.7 µM BA and 1.1 µM NAA (32.3%). The rooting medium included 1/2MS (half strength MS macronutrients, micronutrients, malysite and vitamins) supplementing with 2.7 µM NAA and 3% sucrose (Wu et al., 2008). The plantlet was formatted (Figure 1f).

In this investigation, the regeneration plant of konjac was obtained by somatic embryogenesis. The system of embryogenic callus for konjac was first reported. It was possible that the unpollinated ovary was used as explants, which cells were undifferentiated. While the regeneration plant of konjac was obtained by the organogenesis, which the differentiation of tissues was used as explant. In this study, the rate of embryogenic callus induction was only 34% which was relatively low. The ratio of hormone-swap and the carbon source was further adjusted in the next step. For example, the Pumpkin embryogenic callus induction rate was increased by adjusting the carbon and hormones (Urbanek et al., 2004). The plantlet formation efficiency increased from 12.44 to 23.99% by changing sucrose to trehalose of *Oncidium* 'Gower Ramsey' (Fang et al., 2006). The system of the konjac somatic embryogenesis can be useful not only for germchits or seed corms production, but also for germplasm conservation, mutant screening, genetic transformation and so on.

ACKNOWLEDGMENT

This work was financially supported by the National Natural Science Funds [Grant No.30971829].

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