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狗枣猕猴桃叶片离体培养的器官、体细胞胚形成与植株再生

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摘 要: 以狗枣猕猴桃试管苗的叶片为外植体, 接种于含 3%蔗糖和 0.2% Gelrite 的 BW 培养基上, 外加 2,4- D (0, 0.1, 1 和 10 μmol/L) 与玉米素 (0, 1 和 10 μmol/L)的 12 种激素组合,置于 25 ℃,光周期为 16/8 h,光照强度为 4000 lx 的条件下培养。 在含 1 或 10 μmol/L 2,4- D 与 1 或 10 μmol/L 玉米素组合的 BW 培养基上,产生了体细胞胚,并分化出小植株。随着玉米素浓度的增加,每个外植体上的胚再生频率和体细胞胚的数量也随之增加。同时以叶片为外植体产生的狗枣猕猴桃试管苗的愈伤组织表层产生了不定芽,并抽长成枝。发枝率随着玉米素浓度的增加而增加,并受高浓度的 2,4- D 所抑制。枝芽转接到含 1 μmol/L NAA 的 BW 培养基上生根,长成小植株。

关键词: 狗枣猕猴桃; 不定芽; 体细胞胚; 2,4- D; 玉米素
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Organogenesis, somatic embryogenesis and plantlet regeneration from leaf explants of *Actinidia kolomikta* cultured *in vitro*

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Abstract: Leaf explants from *Actinidia kolomikta* were cultured on BW media containing 30 g/L sucrose and 2 g/L Gelrite and supplemented with different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) and Zeatin. Adventitious buds were generated from calli derived from leaf explants and developed into shoots. Shoot regeneration rate and the number of shoots regenerated increased with the increase in Zeatin concentration and decreased with the increase in 2,4-D concentration. Embryogenesis was achieved in the BW medium added with 1 μmol/L each of 2,4-D and Zeatin. Subculture of the embryogenic callus led to secondary embryogenesis with a high frequency of 66.7%. In rooting culture of shoots, roots formed directly from the basal portion of shoots on the medium containing 2,4-D or α-Naphthaleneacetic acid (NAA). Plants were successfully acclimatized to the field conditions with a survival rate of 100%.

Key words: *Actinidia kolomikta*; Adventitious buds; Somatic embryos; 2, 4-D; Zeatin.

Actinidia kolomikta, a species indigenous to northeast Asian, is highly cold-resistant and bears attractive edible fruit with unique fragrance. Vitamin C content in fruit of this species is two and half times more than Hayward (*A. deliciosa*)a world famous variety. The plant can tolerate a freezing temperature of -40 ℃ and is resistant to diseases. Fruits can be preserved or processed as jam, jellies and juice . Tissue culture for *Actinidia* genus has already been reported. At first, adventitious buds were regenerated *in vitro* from stem and root explants on MS medium containing Zeatin [1]. Somatic embryogenesis in *A. chinensis* from endosperm was first reported by Gui et al [2]. Plantlets of *A. chinensis* were later successfully regenerated via protoplast culture [3]. In addition to *A. chinensis*, plantlets of *A. polygama* were obtained through culture of internodes, petioles and leaves [4]. Adventitious buds of *A. arguta* and *A. kolomikta* were generated *in vitro* from the internodes of current-season shoots and grew into plantlets[5-6]. Somatic embryos were induced from *in vitro*-cultured roots of *A. kolomikta* on BW medium containing Zeatin and 2, 4-D, with a lower

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frequency and slow growth[7]. Establishment of an efficient *in vitro* propagation system for lines of *A. kolomikta* with superior traits is necessary for the domestication of this species. Tissue culture and somatic embryogenesis provide an alternate method for rapid and mass propagation of this species. In this paper we report the establishment of an efficient shoot regeneration protocol by organogenesis and embryogenesis, which could be used as a tool for genetic improvement in *A. kolomikta*.

1 Materials and methods

1.1 Plant material and culture conditions

In the primary culture, 7 mm nodal segments with one bud were excised from *A. kolomikta*. Plantlets were *in vitro* regenerated and cultured as previously reported [6]. After 3 weeks of culture, young leaves were excised from the shoot and used as explants.

The explants were cultured on BW medium, containing 3% sucrose, 0.2% Gelrite, and 12 combinations of 2,4- D (0, 0.1, 1 and 10 μmol/L) and Zeatin (0, 1 and 10 μmol/L). The pH of the medium was adjusted to 5.8. The medium was autoclaved at 121 ℃ for 15 min. The leaf explants were placed on the medium (25 mL) in 100 mL Erlenmeyer flasks, and cultured in a growth chamber at 25 ℃ under a 16 h light (4 000 lx) and 8 h dark photoperiod. After 7 weeks of culture, the calli formed were transferred and subcultured for 7 weeks on BW medium containing 3% sucrose, 0.2% Gelrite and 1 μmol/L Zeatin. The numbers of shoots and embryos formed were counted after 7 weeks of subculture.

In another experiment, the influence on somatic embryo formation of Zeatin concentration in the subculture medium was examined. Here, the leaves obtained as before were cultured on BW medium containing 1μmol/L 2,4- D and 1 μmol/L Zeatin, Since the best results were obtained with this combination of growth regulators. After 7 weeks of culture in these conditions, explants induced were subcultured in BW medium containing 0, 0.1, 1 or 10 μmol/L Zeatin. The number of embryos was counted after 7 weeks of the subculture.

1.2 Rooting of shoots and acclimatization

Shoots about 1 to 2 cm in length were transferred to BW media containing 2,4-D or NAA and 2 g/L Gelrite for rooting. Data on root induction on the shoots were recorded after 35 days of culture. Covers of Erlenmeyer flasks were gradually opened to acclimatize rooting plantlets for 1 weeks. Plantlets were transferred into small pots filled with soil and vermiculite mixture (soil: vermiculite=4:1, V/V) and hardened in greenhouse for 20 days before transplantation to the natural environments.

1.3 Histology

After 20 to 35 days in the primary culture media, calli were fixed in FAA (ethanol: glacial acetic acid: formalin: distilled water =45 :5 :6 :44, *V/V/V/V*), and dehydrated by a passage through an ethanol / butyl-alcohol series, and embedded in paraffin wax. Sections (9 μm in thickness) were cut and stained with Safranin-Fast green. Differentiation states were observed and photos taken under the microscope.

2 Results and discussion

2.1 Shoot regeneration and embryogenesis

Adventitious buds were generated on calli derived from leaf explants and developed into shoots. The formation of adventitious buds was significantly effected by the concentrations of 2,4-D and Zeatin (Table 1). Rate of shoot formation was low on the media without Zeatin. In the tested treatments, both formation rate and number of shoot increased with the increasing concentrations of Zeatin. 2,4-D had no effect on shoot formation in low levels (0 and 0.01 μmol/L). Both the formation rate and the number of shoot were lower in the presence of higher concentrations of 2,4-D. The highest rate of shoot regeneration (72.2%) was achieved on the medium containing 0.1 μmol/L 2,4-D and 10 μmol/L Zeatin. While maximum number of shoot per explant was obtained on the medium with 10 μmol/L Zeatin.

Globular embryos and torpedo -shaped embryos were observed on the outer part of leaf-originating callus. Plantlets developed from the embryos are shown in plates. As observed under light microscope, the cells of somatic embryos were dense, little vacuolated and with a big nucleus.

The embryos were regenerated in a medium con-

Table 1 Effects of 2,4-D and Zeatin concentrations on shoot regeneration and somatic embryogenesis from leaf explants of *A. kolomikta*

2,4-D (μmol/L)	Zeatin (μmol/L)	Number of explants	Shoot formation rate (%)	Number of shoots per explants	Somatic embryo formation rate (%)	Number of somatic embryos per explant
0.0	0	24	0.0	0	0.0	0
0.0	1	24	66.7	2.4±0.4 c	0.0	0
0.0	10	24	68.7	10.0±2.9 a	0.0	0
0.1	0	24	5.5	2.0±0.0 c	0.0	0
0.1	1	24	44.4	3.0±0.9 c	0.0	0
0.1	10	24	72.2	5.3±1.4 b	0.0	0
1.0	0	24	0.0	0	0.0	0
1.0	1	24	33.3	2.3±0.7 c	50.0	1.8±0.5
1.0	10	24	50.0	2.5±0.5 c	37.5	1.6±0.5
10	0	24	0.0	0	0.0	0
10	1	24	18.8	1.0±0.0 c	31.3	1.2±0.2
10	10	24	31.3	1.6±0.3 c	37.5	1.3±0.2

Note: Values followed by the same letters are not significant difference at 5% probability level (Duncan's multiple range test). The same as below.

taining 1 or 10 μmol/L 2,4-D combined with 1 or 10 μmol/L Zeatin. The frequency of somatic embryogenesis was highest in the medium containing 1 μmol/L 2,4-D combined with 1 μmol/L Zeatin (Table 1). However, the number of somatic embryos was not significantly different when the medium contained 2,4-D and Zeatin at the different concentrations.

Zeatin in the subculture medium promoted somatic embryo formation by increasing both the formation rate and the number of somatic embryos regenerated from an explant (Table 2). In the medium without Zeatin, the somatic embryos stopped growing and rapidly senesced. It seems that the formation of somatic embryos in *A. kolomikta* is increased by transplanting the calli into the medium supplemented with Zeatin. This fact indicates that the regenerated somatic embryos require exogenous growth regulators for their development.

Table 2 Effect of Zeatin concentration in subculture medium on secondary embryogenesis from the embryogenic calli induced on the medium with 1 μmol/L 2,4-D and 1 μmol/L Zeatin

Zeatin (μmol/L)	Number of embryogenic calli	Rate of somatic embryo formation (%)	Number of somatic embryos per explant
0.0	12	33.3	1.3±0.2 b
0.1	12	41.7	1.2±0.1 b
1.0	12	58.3	1.7±0.2 a
10	12	66.7	1.9±0.1 a

Formation of organogenesis and embryogenesis differ with species and varieties[8-9]. *A. chinensis* and *A. kolomikta* were found to beable to form adventitious buds and somatic embryos[3,6-7]. However, with the same combinations of growth regulators, *A. arguta* produced only adventitious shoots[5]. In the present experiment, both adventitious buds and somatic embryos were observed from leaf explants of *A. kolomikta*. Explant types had also a significant effect on embryogenesis. Liu et al[7] reported that embryogenesis frequency from root segments was not higher than 15%. In this study, a maximum rate of 66.7% in somatic embryo formation was obtained from leaf explants.

In the primary culture, growth regulators were one of important factors to affect somatic embryos formation of *A. kolomikta*. In many experiments, the combination of 2,4-D and Zeatin was proved to be effective in inducing somatic embryos. In this study, the effective range of 2,4-D and Zeatin was 1 to 10 μmol/L. Liu et al[6-7] used IBA (Indolebutyric acid), CPPU[N-(2-Chloro-4-pyridyl)-N'-phenylurea], NAA and BA (N^6-Benzyladenine) in their earlier experiments for induction of somatic embryogenesis in *A. Kolomikta* but their efforts were not successful.

In this research, we found two ways of plant regeneration: somatic embryo and adventitious buds formation from leaf explants of *A. kolomikta*. Studies on the regulation of somatic embryos formation will enrich our knowledge about morphogenesis of *A. kolomikta*. Moreover establishment of efficient somatic embryos regeneration system will provide an effective path to genetic transformation.

2.2 Rooting of shoots, acclimatization and transplantion of plantlets to the field

Healthy shoot were selected and transferred to the rooting medium containing 2,4-D or NAA (Table 3). Roots appeared on the shoots after 20 days of culture (Plate A~F). Type and concentration of growth regulators significantly affected root formation (Table 3).

Without growth regulators, root formation rate was only 9.6% with 1.4 roots per plantlet. On the media containing 0.1 μmol/L 2,4-D or NAA, the root formation rate was 29.2 % and 62.5 % respectively, and the average root number was 2.4 and 5.5 respectively. Rooting frequency reached 100% on the medium containing 1 μmol/L 2,4-D or NAA. Average root number in NAA treatments was higher than in 2,4-D treatments. NAA was thus more effective than 2,4-D in root induction of *A. kolomikta*.

Table 3 Effects of 2,4-D and NAA concentrations on rooting of shoot excised from cultures of *A. kolomikta*

Growth regulators		Number of Explants	Rooting Rate (%)	Number of roots
2,4-D (μmol/L)	NAA (μmol/L)			
0.0	0.0	24	9.6	1.4±0.6 c
0.1	0.0	24	29.2	2.4±0.6 c
1.0	0.0	24	100.0	5.0±0.8 b
0.0	0.1	24	62.5	5.5±0.9 b
0.0	1.0	24	100.0	7.9±0.8 a

After the covers of Erlenmeyer flasks were gradually removed to acclimatize rooted plantlets for one week, the plantlets were transferred into small pots filled with soil and vermiculite mixture and hardened in greenhouse for 20 days and transplanted to the field. After one month, the plants grew healthily in field with 100% survival rate.

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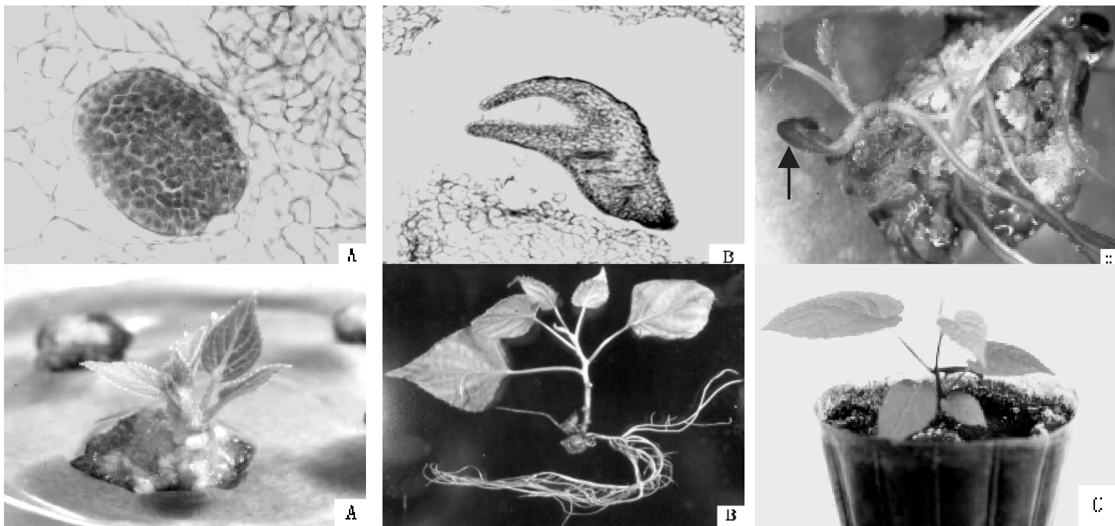
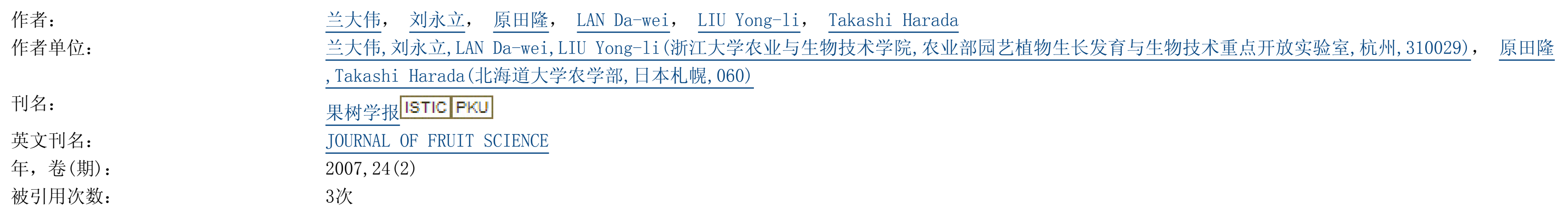


Explanation of plates

A. Globular embryo (appeared at 25 day of culture); B. Torpedo-shaped embryo (appeared at 30 to 35 days); C. Plantlet developed from embryos; D. Shoots formed from calli induced from leave of *A. kolomikta* cultures; E. Roots formed from the basal portion of the shoot after 1 μmol/L NAA treatment; F. The plantlet transfered into a small pot for 20 days



狗枣猕猴桃叶片离体培养的器官、体细胞胚形成与植株再生

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