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Short communication

Callus induction and plant regeneration from embryonic axes of *Kosteletzkya virginica*

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ABSTRACT

Kosteletzkya virginica, a perennial dicot halophytic species of the Malvaceae, is native to American salt marsh. It was introduced into China as a potential species to improve coastal wetlands and to develop ecologically sound saline agriculture. *K. virginica* adapts excellently to the tidal-flat habitats in China's east coast, with multiple eco-benefits; in particular, its seed oil could be used to produce biodiesel. The purpose of this study was thus to develop a standardized protocol to induce a high frequency of callus and subsequent plantlet regeneration system for a *K. virginica* breeding program with the final objective of applying transgenic techniques to improve seed oil yield. The embryonic axes of *K. virginica* were used as explants for callus induction, shoot induction from the callus and then adventitious root induction from the shoots on nine culture media with different hormone combinations. The best results were achieved on the following media: (1) 93.94% callus induction on MS medium supplemented with 1.0 mg L^{-1} indole-3-acetic acid (IAA), 0.3 mg L^{-1} kinetin, 30 g L^{-1} sucrose and 8 g L^{-1} agar; (2) 65.83% shoot induction on 1/2MS medium supplemented with 0.1 mg L^{-1} IAA, 0.5 mg L^{-1} zeatin, 30 g L^{-1} sucrose and 8 g L^{-1} agar; (3) 96.67% rooting on MS medium containing 30 g L^{-1} sucrose and 8 g L^{-1} agar. The survival rate of plantlets by organogenic regeneration was 85% after being transplanted into potting soil in flowerpots and placed in the greenhouse. This experiment indicates that we established successful callus induction and plant regeneration protocols for *K. virginica*.

1. Introduction

Kosteletzkya virginica, a perennial dicot halophytic species of the Malvaceae, is native to brackish portions of coastal tidal marshes of the mid-Atlantic and southeastern United States (Gallagher, 1985). It has been suggested as a grain-crop for seawater-based agricultural systems, since it produces a high yield of seeds that could be used as a source of oil, food, fodder, health care and industrial use (Islam et al., 1982; Ruan et al., 2004). It was introduced into China as a potential species to improve coastal wetlands and to develop ecologically sound saline agriculture. Over 10 years of experimental research that we conducted in the field indicated that *K. virginica* adapts excellently to the wetland habits of Liaoning, Tianjing, Shandong and Jiangsu Provinces, China, and can be used as an agroecoengineering tool that produces a valuable oil product, especially its seed oil, which can be used to produce biodiesel (Ruan et al., 2008). Although seed yield (957 kg/ha) and oil content (20.64%) of bred fine lines in our fields are both higher than those of unselected mixed lines introduced to China (Ruan et al., 2005), they need further improvement to be able to fully explore their bio-energetic value.

Genetic transformation is an important and effective technique to improve the yield and quality of crops (Zou et al., 1997; Shen et al., 2006), such as transformed *Brassica napus* with a 32.9% increase in seed yield per individual (Luo et al., 2002) and a 40% increase in seed lipid content (Vigeolas et al., 2007). It is a prerequisite for transgenic studies to establish a highly effective callus induction and regeneration system. Somatic embryogenesis and subsequent plant regeneration in the Malvaceae has been reported for several species, such as *Gossypium hirsutum* (Kouakou et al., 2007), *Hibiscus acetosella* (Reynolds and Blackmon, 1991) and *H. cannabinus* (Herath et al., 2004). For *K. virginica*, Cook et al. (1989) reported plant regeneration through organogenesis in

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callus cultures from excised mature embryos or stem sections of aseptically germinated plants, but the survival rate of plantlets transplanted to potting soil was not reported in their study. Hasson and Poljakoff-Mayber (1995) developed callus tissue of *K. virginica* from hypocotyls of young seedlings, but only used it to investigate growth and salt tolerance and not shoot regeneration. It is necessary to establish a high frequency regeneration system for *K. virginica* by optimizing new hormone combinations and creating a novel regeneration procedure.

The purpose of this study was thus to develop a standardized protocol to induce a high frequency of callus and subsequent plantlet regeneration system for a *K. virginica* breeding program with the final objective of applying transgenic techniques to improve seed oil yield. This included establishing and testing procedures and media for the preparation of explants, their sterilization, callus induction, shoot induction from callus and then adventitious root induction from the shoots. Finally, successful acclimatization of plantlets to the greenhouse was an important final goal.

2. Materials and methods

Seeds of *Kosteletzkya virginica* (L.) Presl. from the Halophyte Biotechnology Center (University of Delaware, USA) were planted in the Dafeng site for controlled experiments and assessment in 1993. The Dafeng site is located in the coastal wetlands at Yancheng City, Jiangsu, China. There were about 90,000 individuals in the Dafeng naturalized *K. virginica* populations in 2004. Seeds collected from this population in 2004 were sown in the Dalian site in spring 2005, and the number of individuals was about 20,000. The Dalian site is located in the coastal wetlands of Dalian city, Liaoning, China. Dalian has a maritime climate, with an annual mean temperature of 10.2 °C and an annual mean rainfall of 658.7 mm. It has 191 frost-free days and a total annual radiation of 2764 h.

Seeds in this study were collected from the naturalized *K. virginica* populations growing at the Dalian site in autumn 2005.

2.1. Media

In our experiments, basic media were MS medium (Murashige and Skoog, 1962), 1/2 MS, and MSB [MS salts with B5 (Gamborg et al., 1968) vitamins]. MSB contains macro- and micro-elements and iron salt of MS medium and organic components of B5 basic media (Gamborg et al., 1968; Jin et al., 2005) (Table 1). Our preliminary results of media containing new different hormones, which were different from the media in Cook et al.'s experiments, showed that combinations of IAA (indole-3-acetic acid) and KT (kinetin), and IBA (3-indolebutyric acid) and ZT (*trans*-zeatin) could successfully develop callus, achieving a maximum rate of plantlet regeneration of 64%. Based on these initial findings we used the following hormone combinations in this study: IAA + KT or ZT, IBA + ZT, or 2,4-D (2,4-dichlorophenoxyacetic acid) alone (Table 1). Using these combinations, three media for callus induction (M1, M2 and M3), one callus multiplication medium (M4), three shoot regeneration media (M5, M6 and M7) and two root induction media (M8 and M9) were developed. One litre of any medium contained 30 g sucrose and 8 g agar, and the pH was adjusted to 5.8 before high-pressure (0.1 MPa and 121 °C for 20 min) sterilization. Any hormone added to media was filter-sterilized (Whatman Puradisc 25AS filter with 0.22 μm film pore radius).

Table 1
Composition of media.

Medium	Composition of plant growth substances (mg L^{-1})
M1	MS + 2,4-D 0.5
M2	MS + IBA 1.0 + ZT 0.1
M3	MS + IAA 1.0 + KT 0.3
M4	1/2MS + IAA 0.3 + ZT 0.3
M5	MS + IAA 0.1 + ZT 0.3
M6	MS + IAA 0.1 + ZT 0.5
M7	1/2MS + IAA 0.1 + ZT 0.5
M8	MS[a]
M9	MSB[b]

[a] Details in Murashige and Skoog (1962).
[b] Including macro-elements, micro-elements and iron salt of MS basic media, and organic components of B5 basic media. Details in Murashige and Skoog (1962) and Gamborg et al. (1968).

2.2. Embryonic axis explants

To successfully surface-sterilize *K. virginica* seeds, they were dipped in filter-sterilized 75% ethanol for 10 min on the clean bench, rinsed in running sterile deionized water, dipped in concentrated sulphuric acid for 1 h, and washed five times with sterile deionized water. Mature embryos were dissected from seeds by peeling off the testa using sterilized forceps and a scalpel, and embryonic axes were dissected from normal mature embryos (Fig. 1A) by excising the cotyledon and radicle. Thereafter, embryonic axes were dipped in filter-sterilized 75% ethanol to sterilize for 10 s, washed three times with sterile deionized water, and inoculated on callus induction media (Fig. 1B).

2.3. Callus induction and multiplication

Callus was induced on three media held in sterile Petri dishes with a 10.0-cm diameter (8–15 explants per Petri dish): M1, M2 and M3 (Table 1). Fifty-five embryonic axes were inoculated on each medium, with three replications. After 72 h dark incubation, embryonic axes were incubated in a culture case with 45 $\mu mol\ s^{-1}\ m^{-2}$ light, a 14-h photoperiod, 70–80% relative humidity and at 26 °C. During the process of callus induction, the callus and its morphology were observed. After 20 d incubation, we measured the percentage callus induction (induction % = (No. of explants producing callus/No. of explants inoculated) × 100%) and percentage callus browning (browning % = (No. of callus browning/No. of callus produced) × 100%), and transferred the non-callus to M4 medium to multiply for 21 d. During callus proliferation, the color of calli was observed.

We tested the influence of different media on the percentage of callus induction and the percentage of callus browning, using a one-way *ANOVA* followed by Duncan's multiple range test (SPSS 11.0).

2.4. Shoot regeneration

After 21 d multiplication, proliferated calli on M4 medium with a diameter of 1.0–1.5 cm were dissected into smaller, fixed callus masses (about 0.7 cm^3 per piece), and transferred to adventitious bud induction media (M5, M6 and M7). Forty pieces of callus were inoculated on each medium held in sterile flasks with a diameter of 10 cm at the base (three pieces per flask), with three replications. Induction percentage of adventitious buds was determined at 10, 20, and 25 d after incubation. Induction % = (No. of calli producing adventitious buds/No. of calli inoculated) × 100%. We tested the influence of different media on induction percentage of adventitious buds, using a one-way *ANOVA* followed by Duncan's multiple range test (SPSS 11.0).