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BRIEF COMMUNICATION

Dark preincubation improves shoot organogenesis from *Rhodiola crenulata* leaf explants

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Abstract

An efficient *in vitro* plant regeneration system has been developed using dark preincubated leaf explants of *Rhodiola crenulata*, a traditional Tibetan medicinal plant. The leaf explants, preincubated in the dark for 5 d, developed an average of 9.1 shoots per explant on a medium containing 15 μM N^6-benzyladenine (BA) and 2.5 μM gibberellic acid (GA_3). The biochemical mechanism underlying dark-induced shoot organogenesis was investigated by measuring polyphenol oxidase (PPO) activity. Dark preincubation significantly reduced PPO activity in leaf explants during the initial period of shoot organogenesis and reduced browning compared to explants cultured in the light. Up to 88.4 % of the regenerated shoots formed roots and developed into complete plantlets on a medium containing 5 μM indoleacetic acid (IAA) within 25 d. Approximately 82 % of the regenerated plantlets survived transplantation and grew vigorously in the greenhouse.

Additional key words: auxin, cytokinin, gibberellic acid, polyphenol oxidase.

Rhodiola crenulata H. Ohba is a perennial herbaceous plant from the *Crassulaceae* family that mainly grows in Tibet (Du and Xie 1993). It has been used as one of the traditional herbal remedies for centuries. Extracts from *R. crenulata* roots have been shown to have adaptogenic, antioxidant, and anticancer effects (Cui *et al.* 2003, Wang and Wang 1992, Du and Xie 1994). Several medicinally active constituents of *R. crenulata*, such as salidroside and tyrosol, have also been identified (Wang and Wang 1992). Overharvesting of *R. crenulata* plants caused a serious reduction in its native populations. A major constraint in conventional propagation of *R. crenulata* through seeds is the high mortality of seedlings in the early stages of growth. Further, vegetative propagation from the rhizome is undesirable due to its destructive nature. *In vitro* propagation techniques offer a powerful tool for mass-multiplication and germplasm conservation of many threatened plant species (Liu *et al.* 2004, Guo *et al.* 2007, Arora *et al.* 2011, Mingozzi *et al.* 2011, Mohamed *et al.* 2011). *In vitro* regeneration has been reported for *R. crenulata* (Yan *et al.* 2005, Yin *et al.* 2005, Liu *et al.* 2006) but tissue browning and low rates of shoot organogenesis often resulted in low efficiency. Dark preincubation has been shown to improve shoot regeneration from several explant types and species including *Rosa hybrida* leaf explants (Ibrahim and Debergh 2001) and *Echinacea purpurea* root explants (Zobayed *et al.* 2003). The exact role of darkness is not completely understood but it is likely that dark preincubation reduces phenolic oxidation induced by light.

Rhodiola crenulata plants were collected from Linzhi,

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Abbreviations: BA - N^6-benzyladenine; GA_3 - gibberellic acid; IAA - indoleacetic acid; PPO - polyphenol oxidase.
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Tibet (N29°36.690′, E94°39.230′). The leaves from 3-year-old plants were surface sterilized by dipping in 70 % ethanol for 30 s, then immersing in 5.4 % sodium hypochlorite for 20 min followed by three rinses in sterile-distilled water. Leaf explants (approximately 0.5 × 0.5 cm) were excised using a sharp razor and after 10 d dark preincubation at 25 °C inoculated onto solidified (0.6 % agar) Murashige and Skoog (1962; MS) medium supplemented with varying concentrations of N^6-benzyladenine (BA; 2.5, 5, 10, 15, and 20 μM) alone or in combination with gibberellic acid (GA_3; 0.5, 1, 2.5, and 5 μM). For the determination of the optimal duration of dark treatment, the leaf explants were incubated in the dark for 0, 5, 10, 15, and 20 d before transferring to a 16-h photoperiod (cool-white tubes, irradiance of 30 to 40 μmol m^{-2} s^{-1}). All media were adjusted to pH 5.8 and 0.6 % agar was added prior to autoclaving at 121 °C for 18 min. The number of shoots per leaf explant was recorded after 35 d of culture. In order to investigate the mechanism of the effect of dark preincubation, we compared the activity of polyphenol oxidase (PPO) after every 5 d. MS medium supplemented with 5 μM IAA was used for rooting and after 35 d the plantlets were transferred into the pots with soil and grown in a greenhouse to maturity.

To quantify PPO activity, 1 g of fresh tissue was ground in liquid nitrogen and extracted with 0.05 g of polyvinylpyrrolidone and 2 cm^3 of buffer (pH 7.2, 0.1 M phosphate, 2 mM EDTA, 4 mM dithiotreitol). The mixture was homogenized at 4 °C and then centrifuged at 10 000 *g* and 4 °C for 25 min. Next, 0.2 cm^3 of the supernatant was combined with 1.5 cm^3 of 0.02 M catecol and 1.5 cm^3 of 0.05 M phosphate buffer (pH 6.0) and its absorbance was detected at 398 nm every 2 min. PPO activity was expressed as the ratio of the change in absorbance to the time interval (Yuan *et al.* 2002).

Of the *R. crenulata* leaf explants on solid MS medium supplemented with varying concentrations of BA alone or with GA_3, significantly more adventitious shoots were observed on explants exposed to 15 μM BA and 2.5 μM GA_3 than to any other combination of growth regulators (5.3 ± 0.4 shoots per explant; 10 d dark preincubation). In addition, significantly more shoots were obtained from leaf explants incubated in the dark for 5 d than for any other darkness duration, with an average of 9.1 ± 0.9 shoots per leaf explant (Fig. 1*A*). *R. crenulata* explants grew more vigorously following the 5 d dark treatment compared to the no dark treated control. As the duration of the dark treatment increased, the number of shoots per leaf explant significantly decreased and was significantly lower than the control after 20 d preincubation under darkness.

Numerous studies on plant tissue culture have demonstrated that a dark preincubation improves adventitious shoot regeneration from various tissues including cotyledons, leaves, petioles, and nodal segments (Ibrahim and Debergh 2001). For example, for *Zizyphus jujube* leaf explants, a 20 d dark preincubation increased adventitious shoot formation 4-fold over the control explants (Gu and Zhang 2005). It is also common for somatic embryogenesis protocols to initially employ a dark incubation period prior to a transfer to light (Zobayed and Saxena 2003, Kim *et al.* 2006). In some plants, tissue browning is a typical feature of plant cultures derived from explants and browning reduces cell growth and inhibits adventitious shoot formation. The enhancement of plant regeneration by dark preincubation is related to the inhibition of browning, however, the mechanism underlying this process is not clear (Jensen *et al.* 1998, Suzuki *et al.* 2004, Qin *et al.* 2005).

The activity of PPO is responsible for many visible browning reactions (Beruto *et al.*1996). PPO is believed to play a key role in browning plant tissues by catalyzing the hydroxylation of monophenols to *o*-diphenols and catalyzing the oxidation of *o*-dihydroxyphenols to *o*-quinones (Vamos-Vigyazo 1981). Quinones can

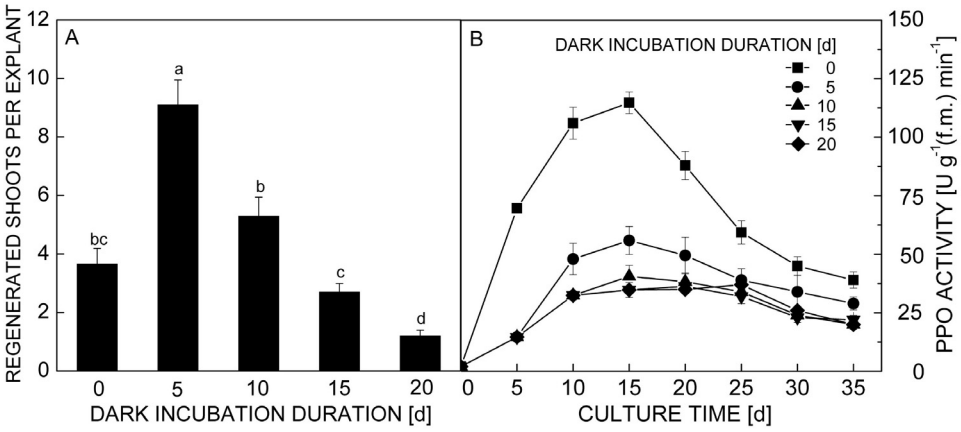

Fig. 1. The effect of the duration of the dark preincubation on number of *R. crenulata* shoots regenerated after 35 d of *in vitro* cultivation (*A*) and on the activity of polyphenol oxidase (PPO) in *R. crenulata* leaves during the *in vitro* cultivation (0 to 35 d) (*B*). Means ± SD, $n = 3$, different letters indicate significantly different means at $P < 0.05$.

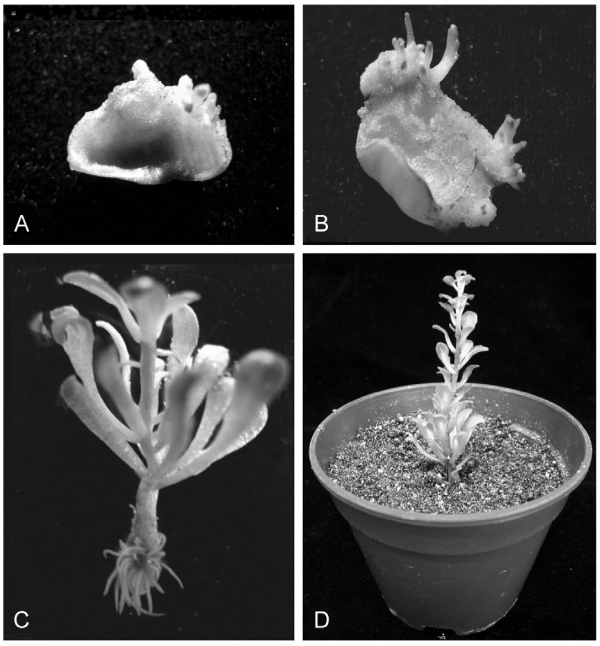

Fig. 2. Plant regeneration from *R. crenulata* leaf explants. Shoot regeneration from a leaf explant cultured on MS medium supplemented with 15 μM BA and 2.5 μM GA_3 for 25 d with 5 d dark preincubation (*A*), shoot regeneration from a leaf explant cultured on the same medium for 35 d with 5 d dark preincubation (*B)*, rooting of the regenerated shoots incubated on MS medium supplemented with 5 μM IAA after 25 d (*C)*, and a micropropagated plant following transfer to soil and grown for 35 d in a greenhouse (*D)*.

polymerize and lead to tissue browning or the formation of black pigments in plants (Mayer and Harel 1991). In this study, over the time course of leaf explants cultured under irradiance, the timing of PPO activity paralleled that of explant browning. The PPO activity was significantly lower in the leaf tissue exposed to all dark preincubations tested compared to the untreated leaf tissue (Fig. 1*B*). These results indicate that PPO activity is related to the browning of the *R. crenulata* leaf explants. This relationship was also observed in Scots pine (*Pinus sylvestris* L.) buds and immature embryos (Laukkanen *et al.* 2000); PPO activity was significantly higher in the browning callus tissue initiated from Scots pine buds than in the non-browning cultures derived from immature embryos (Laukkanen *et al.* 2000). The increased PPO activity in browning tissue indicates that there was a mechanical injury and that environmental stress responses existed in these callus cultures (Kim *et al.* 2001). Therefore, it is likely that the dark preincubation improved shoot regeneration from leaf explants due to the inhibition of browning.

The optimal medium for rooting contained 5 μM IAA in which 88.4 % of the regenerated shoots developed roots within 25 d (Fig. 2). Increasing the IAA concentration above 5 μM decreased the rate of root development, the number of roots per shoot, and the length of the roots. The rooted plantlets were transferred to soil after 35 d and eventually grown to maturity with a survival rate of 81.7 %.

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