The influence of explant orientation and contact with the medium on the pathway of shoot regeneration in vitro in epicotyl cuttings of Troyer citrange

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Abstract

The effect of orientation as regards to gravity, and that of contact with the medium of culture, on shoot regeneration at the cut edges of epicotyl explants of Troyer citrange (Citrus sinensis (L.) Osbeck × Poncirus trifoliata (L.) Raf.) have been separated. The shoot regeneration pathway was not affected by the orientation of the explants as regards to gravity, and was determined by explant polarity and the contact with the culture medium. At the apical edge of the explants, the contact with the medium shifted the pathway of shoot regeneration from a direct one to an indirect one, with formation of a callus. This callus formation was cytokinin-dependent, but the change in the pathway of organogenesis was not caused by the increase in cytokinin availability resulting from the contact with the medium. In contact with the media, regeneration at the basal edge of the explants occurred through an indirect pathway after callus formation. No regeneration occurred, at the basal edge, if the contact with the media was prevented. The orientation of the explants as regards to gravity affected shoot formation through the direct pathway of organogenesis. The number of buds differentiated, and that of growing shoots increased when the orientation of the explants departed from the vertical upright position.

Abbreviations: BA-6 – benzylaminopurine; NAA – naphthaleneacetic acid

Introduction

An efficient system of regeneration via organogenesis is the first step for the successful genetic transformation of Citrus rootstocks and cultivars, and for the multiplication and utilization of rootstocks having mono embryonic seeds. Plant regeneration protocols have been developed for different explant sources (Gmitter et al., 1992), but considerable variation has been reported as regards to the capacity of regeneration, the response to hormone addenda and in the pathway of organ regeneration. Some of this variation seemed to arise from differences in genotype (Gmitter et al., 1992; Pérez-Molphe-Balch and Ochoa-Alejo, 1997; Ghorbel et al., 1998; Bordón et al., 2000). In some cases, however, differences in response were reported using the same plant material. Thus, shoot regeneration from epicotyl cuttings of Troyer citrange has been reported to occur by direct morphogenesis, without or with little callus formation (Edriss and Burger, 1984; Moore et al., 1992), or by and indirect process ensuing callus formation (Barlass and Skene, 1982; Moore, 1986). García-Luis et al. (1999) demonstrated that the contact with the culture medium
determined the pathway of shoot regeneration with this plant material. In contact with the medium, the shoots formed at the two edges of the explants in a cytokinin-dependent indirect pathway after callus formation. If the contact with the medium was prevented, shoots formed at the apical edge of the cuttings through a direct pathway of organogenesis. Further work demonstrated that the direct and the indirect pathways of organogenesis differed both in hormone requirements (Moreira-Dias et al., 2000) and in the response to light (Moreira-Dias et al., 2001). In these reports, however, the effect of the contact of the edge of the cuttings with the culture medium was not unequivocally separated from the effect of the orientation of the explants as regards to gravity.

In the present report we demonstrated that the pathway of organogenesis was determined by the contact of the cut edge of the explants with the culture medium, and that was not altered by explant orientation as regards to gravity. Further, we provide evidence that the effect of contact with the medium was not caused by an increase in cytokinin availability or uptake.

**Materials and methods**

**Plant material**

The experiments were performed using 1 cm long epicotyl cuttings obtained from 42-day-old etiolated seedlings of Troyer citrange (*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.). To raise the seedlings we followed the protocol described by Moreira-Dias et al. (2001). The seeds were disinfected for 30 min in a 20% solution of chlorine in water and then rinsed three times with sterilised distilled water. The seed coats were removed, the naked seeds further disinfected for 30 min with a 15% solution of bleach, and rinsed three times with sterilised distilled water. These naked seeds were germinated in the dark at 26 ± 2°C in capped 21×150 mm test tubes containing 10 ml of Murashige and Skoog (1962) mineral medium supplemented with 30 g l⁻¹ sucrose, and solidified with 8 g l⁻¹ agar (Sigma, microbiologically tested). At the end of germination the epicotyls were at least 50 mm long. Therefore, five cuttings could be obtained from each seedling. The five cuttings from the same seedling were allocated to the same treatment. In this way the effect of the parameters studied was not confounded with the effect of explant origin (distance to the cotyledons in the seedling). As reported by Moreira-Dias et al. (2000), regeneration was slightly higher in the explants taken close to the cotyledons. This effect is not presented, and the results are average for the explants.

**Culture media and conditions of incubation**

The explants were cultured in a medium containing the inorganic salts of Murashige and Skoog (1962), supplemented with 100 mg l⁻¹ myo-inositol, 10 mg l⁻¹ thiamine HCl, 10 mg l⁻¹ pyridoxine, 1 mg l⁻¹ nicotinic acid and 30 g l⁻¹ sucrose. The pH of the medium was adjusted to 5.8 before the addition of 8 g l⁻¹ agar (Sigma, microbiologically tested), and the mixture was autoclaved at 105 kPa for 30 min. The growth regulators were filter sterilised and added to the medium after autoclaving. The medium was dispensed as 50 ml aliquots into 84×93 mm culture jars capped with polypropylene caps. In each culture jar were placed ten explants obtained from two seedlings. Six to eight culture jars (60 to 80 explants) served as replicates for each treatment. The incubation was performed at 26±2°C in the light, with a 16/8 h photoperiod and a photon fluence rate of 56 μmol m⁻² s⁻¹ provided by ‘cool white’ OSRAM® (L36 w/21–840) tubes. After 41 days of incubation, the number of developed shoots (longer than 2 mm) was recorded separately for each end of the cutting. The number of non-growing adventitious buds was also counted under the dissecting microscope. The sum of these two figures was the number of buds formed. Callus size was estimated by the area of its projection on the culture medium.

**Experimental design**

The effect of gravity and of contact with the medium on organogenesis

In this experiment, 4.4 μM benzyl adenine (BA) and 0.54 μM naphthalene acetic acid (NAA) were added to the medium. An orthogonal experimental design combining three planting modes with three orientations of the explants as regards to gravity