Regeneration In Vitro from the Hypocotyl of Cucumis Species Produces Almost Exclusively Diploid Shoots, and Does Not Require Light

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Abstract. Hypocotyl explants of three cultivars of melon (Cucumis melo L.) (cv. Revigal, Topmark and Kirkagac), and a cucumber (C. sativus L. cv. Taoz) rapidly directly regenerated multiple shoots on Murashige and Skoog medium augmented with 4.4 µM benzyladenine. Regeneration from the hypocotyl resulted in nearly 100% diploid shoots, whereas regeneration from the cotyledons resulted in 40% to 70% polyplody regenerants. Regeneration from cotyledon explants of melon cv. Revigal required light, whereas regeneration from hypocotyl explants of melon cv. Revigal occurred in both light and darkness. Direct regeneration also occurred from the hypocotyl of cucumber cv. Taoz in both light and darkness, even though cotyledonal explants did not regenerate buds or shoots under the same conditions. This is the first report of regeneration from the Cucumis genus producing a fully diploid plant population.

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Curuk et al., 1994; Guis et al., 2000). Consequently, in our studies of a new regeneration pathway from the hypocotyl, we tested our new regenerant populations for ploidy, and we report for the first time regeneration of near 100% diploid populations from the genus Cucumis.

Additionally, we determined the light requirement for regeneration from hypocotyl and cotyledon explants, as light is known to affect in vitro morphogenesis (Lercari et al., 1986), and show that photoregulation of regeneration responses differ between cotyledon and hypocotyl explants.

Materials and Methods

Seed germination. Melon seeds (Cucumis melo L.) of cultivars Revigal (an Israeli ‘Galia’ type) (Hazera Seeds, Israel) and Topmark (a U.S. Western Chipper cantaloupe type) (Asgrow Seed Co., Kalamazoo, Mich.) with seed coats removed, were surface sterilized for 20 min in 1% sodium hypochlorite, with two drops of Tween 20 (polyoxyethylene-sorbitan) per 100 mL. After washing with sterile distilled water, seeds germinated on Murashige and Skoog (1962) (MS) medium with 3% sucrose, MS vitamins and 8 g·L−1 Agar (Sigma A1296) (MS0 medium), in 90 × 15 mm petri dishes (25 mL medium per dish), in a growth room at 25 ± 1 °C, 16-h photoperiod, 30–40 µmol·m−2·s−1 cool-white fluorescent light. These growth conditions were used for all experiments, except where noted. Seeds of the Turkish inodorus winter melon type cultivar Kirkagac 637 (Cagdas Seed Co., Turkey) with seed coats removed, were sterilized for 5 min in 70% ethanol, washed with three times sterile distilled water and 45 min in 1% sodium hypochlorite, with Tween 20 as above. Seeds were washed three times with sterile distilled water, then for 15 min. in 500 mg·L−1 each carbenicillin and claforlan, and germinated on MS0 medium, in a growth room at 28 ± 1 °C, 16-h photoperiod, 100–120 µmol·m−2·s−1 cool-white fluorescent light. All the work with ‘Kirkagac’ was performed in these growth conditions.

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medium with 4.4 µM N6-benzyladenine (BA) (MSBA1 medium), in petri dishes. Cotyledon explants were cut from 4-day-old seedlings, removing the part of the cotyledon adjacent to the apex and placed in petri dishes, with the abaxial side on MSBA1 medium (Gaba et al., 1996), except ‘Kirkagac’ on the regeneration medium of Neidz et al. (1980). After 28–30 d, explant responses were scored.

Shoot elongation. Regenerating areas of hypocotyl or cotyledon explants (or regenerative callus with buds or shoots for ‘Taoz’) were excised after 4 weeks in culture and transferred to 150 × 25 mm tubes with 10 mL of elongation medium (MS medium with 0.44 µM BA and 2.9 µM gibberellic acid), for cultivars Revigal and Taoz. For ‘Topmark’ and ‘Kirkagac’ the elongation medium of Moreno et al. (1985) [MS0 medium with 0.44 µM BA and 0.05 µM α-naphthaleneacetic acid (NAA)] was used. After 3–4 weeks on elongation medium, ‘Revigal’, ‘Kirkagac’ and ‘Taoz’ shoots were excised (‘Revigal’ required a minimum shoot length of 10–15 mm) and transferred to MS0 medium for rooting and further growth. Some ‘Taoz’ explants were maintained on elongation medium for a second round of regeneration after shoot removal. ‘Taoz’ shoots from the second round were excised and rooted. After several transfers each of 3–4 weeks on Moreno et al. (1985) elongation medium, ‘Topmark’ shoots of 10–15 mm length were excised and transferred to MS0 medium with 0.05 µM NAA (Chee, 1991) for rooting.

Development in darkness. Petri dishes containing hypocotyl or cotyledon explants of melon ‘Revigal’ or cucumber ‘Taoz’ were wrapped in two layers of aluminum foil, and placed on the same shelf in the growth room as those plates containing explants exposed to the standard environment, on MSBA1 medium. Explant responses were counted at a given developmental stage of the cotyledon, after removal of the area adjacent to the seedling apex, were used in these experiments, treated as above. Samples were observed weekly by stereomicroscope. Regeneration was scored after 4 weeks.

Flow cytometry. Flow cytometry was used for the analysis of ploidy. In vitro-grown leaves of melon and cucumber from rooted plants were chopped with a razor blade into an ice-cold neutral buffer (De Laat and Blaas, 1984), stained with DAPI, filtered through a 40-µm mesh, and the fluorescence of the nuclei measured with a PAS II Flowcytometer (Partec GmbH, Munster, Germany). Analyses were performed by Plant Cytometry Services, Schijndel, The Netherlands.

Statistics. Results were analysed using a Chi-square test under the hypothesis of no association (Sokal and Rohlf, 1981), with Yates’ correction when required. There were 5–6 explants per petri dish, 5–10 petri dishes per treatment, with 2–4 repeats of each experiment.

Results and Discussion

Ploidy of regenerants. Regeneration from both hypocotyl (Curuk et al., 2002) and cotyledons (Gaba et al., 1999) of melon was by direct organogenesis. Regeneration from the hypocotyl resulted in 100% diploid regenerated shoots from ‘Kirkagac’ and ‘Revigal’, and those from ‘Topmark’ were 96% diploid (Fig. 1). Overall, 99% of the regenerants from the hypocotyl explants were diploid (Fig. 1). However, regeneration from the cotyledons of the three melon cultivars resulted in 43% to 70% polyploid regenerants (Fig. 1). All but one of the polyploid plants were tetraploid, the exception being an octaploid plant of ‘Revigal’. Shoots of different ploidy were recovered from individual cotyledon explants of ‘Revigal’. Regenerating ‘Topmark’ cotyledons (but not hypocotyls) were much afflicted by hyperhydration during the elongation phase, and most were lost. There was no cultivar effect on the ploidy of regenerants from the hypocotyl or cotyledon.

Regenerants from the hypocotyl of cucumber ‘Taoz’ were 100% diploid (32 regenerants in all), whether regenerated from tissue incubated in darkness (n = 9) or light (for one or two regenerative cycles) (n = 20), or from callus derived from hypocotyl explants (n = 3). There was no effect of light on ploidy of regenerants from the hypocotyl of cucumber.

This is the first report of highly diploid regenerated populations in the genus Cucumis. Regeneration of mixed diploid/polyploid populations of Cucumis is noted from a variety of explants, and by a variety of regeneration pathways, as first noted by Ezura et al. (1992). Expanding on the analysis of Ezura et al. (1992), tetraploid melon plants are regenerated from cotyledons (Adelberg et al., 1993, 1994; Ezura et al., 1992; Gius et al., 2000), leaves (Yadav et al., 1996; Gius et al., 2000), and callus derived from hypocotyl, cotyledon or leaves (Boudaillah and Branchard, 1986; Moreno and Roig, 1990). In these reports, the percentage of polyploid melon plants regenerated was 30% to 80%, whether by somatic embryogenesis (Ezura et al., 1992; Gius et al., 2000), direct organogenesis (Adelberg et al., 1993, 1994; Ezura et al., 1992; Yadav et al., 1996) or regeneration from callus (Moreno and Roig, 1990). Regeneration of tetraploid plants from cucumber callus (Kim et al., 1988) and directly from cucumber cotyledons (Colijn-Hooymans et al., 1994) is reported. Tetraploid plants are regenerated at similar frequencies in other species, such as tomato (Lycopersicon esculentum Mill.) (Compton and Veilleux, 1991) and Arabidopsis (Morrisey and Altmann, 1994).

The production of tetraploid plants is due to endo-reduplication in the cells of the explant prior to regeneration; polyploid plants regenerate from polyploid cells in the explant (Colijn-Hooymans et al., 1994; Adelberg et al., 1994; Adelberg, 1998). Polysomaty, different tissues/organs having different ploidy levels due to differences in age and rates of development, occurs throughout cucumber and Arabidopsis plants, the level of ploidy increasing with time and age (Colijn-Hooymans et al., 1994; Galbraith et al., 1991; Gilissen et al., 1993). Polysomaty is already present in cucumber seed (Gilissen et al., 1993). Changes in the ploidy level of the explant due to maturity or aging (Adelberg et al., 1994; Colijn-Hooymans et al., 1994; Guis et al., 2000) are seen in the composition of the regenerant population. Recently Guis et al. (2000) found that only 15% of plants regenerating by organogenesis from melon leaf explants are tetraploid, demonstrating again that source tissue is an important determinant of ploidy of regenerants. Regeneration of a tomato population with only 3% tetraploids was reported by Pozueta-Romero et al. (2001), using seedling explants consisting of a cotyledon and a shoot, with the apex and other cotyledon removed. The ‘flamingo bill’ explants of Pozueta-Romero et al. (2001) regenerated shoots directly and rapidly from the top of the hypocotyl as reported here; with histology similar to that of Curuk et al. (2002). The population of young, rapidly dividing cells in the proximal...
there was a single well-developed protuberance near the junction of the cotyledon and the hypocotyl. A single small shoot was visible after 2 weeks in darkness. After 3 weeks in darkness, in many samples there were several shoots growing (Fig. 2A). Nearly 90% of explants regenerated shoots after 4 weeks in darkness (Fig. 3). The proportion of hypocotyl explants regenerating after 4 weeks in light was similar (Fig. 3). There was no effect of light on production of shoots from hypocotyl explants (Fig. 3).

Melon—regeneration from hypocotyl explants in darkness. The development of regeneration from the hypocotyl in darkness contrasted strongly with the development of the regenerating part of cotyledon explants. The morphology and anatomy of the development of regeneration of proximal cotyledon explants of Cucumis melo L. cv. Galia on the same medium (MSBA1) in the light has been previously described by Gaba et al. (1999). In contrast, development was greatly slowed in darkness. After 1 week in culture, the proximal cotyledon explant was yellow. A white ridge developed at the proximal edge of the explant. In light-grown samples this ridge was yellow (Gaba et al., 1999). After 2 weeks in culture, the explant enlarged and swelled notably on the proximal part, with no sign of the prolific development found in light-grown cotyledon samples (Gaba et al., 1999). After a month in culture there was little further development, and the explants were gently rippled, without features of regeneration. However, 11% of cotyledon proximal explants developed a leaf or single protuberance (probably a leaf primordium—see Gaba et al., 1999) in darkness (Fig. 2B), usually adjacent to the most proximal edge (Fig. 3). Illumination strongly enhanced primordium/leaf production from the cotyledons (Fig. 3). No shoots regenerated from cotyledon explants in darkness (Fig. 3). Regeneration from cotyledon sections in the light was at a similar level to that in hypocotyl sections. However, as noted previously (Gaba et al., 1999), the regeneration from cotyledons after 4 weeks was only of protuberances and leaves, and there were no visible shoots (Fig. 3).

Regeneration from cucumber. Development of regeneration from the cucumber ‘Taoz’ hypocotyl explants in light was slower than with melon ‘Revigal’ (Curuk et al., 2002), protuberances first being observed after 6 d in culture on MSBA1 medium, 60% of explants having protuberances after 8 d, and 95% having protuberances, leaves or shoots after 10 d. Cotyledon or hypocotyl segments (not including the regenerative area from the proximal region of the hypocotyl demonstrated here) of cucumber ‘Taoz’ did not regenerate directly shoots, buds, or primordia in light or darkness (data not shown). Callus was regenerated frequently from cotyledon segments (67%), but infrequently from hypocotyl segments (16%). Callus from cotyledon segments was rarely regenerative, and only in the light (2/149 explants).

In contrast, hypocotyl explants with a cotyledon fragment attached were very regenerative...
Regeneration from hypocotyl explants of ‘Revigal’ melon and ‘Taoz’ cucumber in darkness was a novel response in cucurbits, and is unusual. Cotyledon explants of both melon and cucumber (Gambley and Dodd, 1991) require light for regeneration, as demonstrated here, in the only other studies of the organogenic response to light of Cucumis explants in vitro.

However, production of axillary shoots induced by cytokinin from the area around the shoot apex in cucumber is not light dependant (Gambley and Dodd, 1991). Dark pretreatments to the seedlings from which the explants were taken stimulate subsequent light-dependant organogenesis in watermelon (Compton, 1999). Light is required for the regeneration of some species, whereas others do not require light, or light may even be inhibitory to regeneration (Lercari et al., 1986).

We demonstrated here novel physiological attributes of a new direct regeneration pathway in Cucumis. The hypocotyl regeneration response was found in three melon genotypes as well as cucumber. The hypocotyl regeneration pathway, widespread in Cucumis, is of interest for biotechnology, as it can potentially avoid the production of tetraploid transformants in melon.

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