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Plant regeneration from calli of melon (*Cucumis melo* L., cv. 'Amarillo Oro')

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Abstract. Callus cultures from cotyledon and hypocotyl explants of a Spanish cultivar of melon ('Amarillo Oro') have been tested for their growth and morphogenic capacity on a series of media with different concentrations of indole-3-acetic acid (IAA) and 6-furfurylaminopurine (kinetin). Melon tissues were able to undergo morphogenesis both via organogenesis and embryogenesis, depending on culture conditions and explant source. Shoot buds were obtained at high rates in cotyledon explants. In response to 1.5 mg/l IAA and 6.0 mg/l kinetin, more than 90% of the calli produced well-developed shoots. Hypocotyls failed to form shoots but formed somatic embryos on auxin containing media while cotyledon explants usually gave abundant shoots but only rarely formed embryos. It was possible to maintain organogenic callus lines for at least 12 months under defined conditions. Plants were recovered from adventitious shoots produced both in cotyledon-derived calli and from organogenic cell lines.

Introduction

Somaclonal variation has potential application to plant breeding, and improved genotypes of several economically important species have been already obtained [7, 8]. We are interested in applying this methodology to the species *Cucumis melo.* In order to achieve this objective it is first necessary to establish reproducible and efficient procedures for regenerating plants from cultured explants or, better yet, from subcultured calli.

In melon, the production of calli [1, 2, 3] and suspension cultures [11, 12] have been described. More recently we have reported methods suitable for obtaining callus from protoplasts of melon [9], and the formation of somatic embryos from these calli (in preparation). In this paper we report the results of a study on the morphogenic responses in explant-derived calli of an economically important cultivar of melon. In addition, methods of obtaining cell lines which retain their morphogenic potential and of plant regeneration from explant-derived calli as well as from the organogenic cell lines, are described.

Material and methods

Plant material

Seeds of *Cucumis melo* L. cv. 'Amarillo Oro' were surface sterilized by immersion in 12.5% commercial bleach (a solution of sodium hypochlorite equivalent to 50 g/l of active chlorine) for 20 min followed by three rinses with sterile distilled water, and sown aseptically on Murashige and Skoog [10] salt solution supplemented with 1% sucrose and 0.8% Oxoid Technical No. 3 agar (MG medium) in 20 x 195 mm test tubes.

Explant culture and experimental design

Hypocotyl, as well as cotyledons from 11–13-days-old seedlings were used as explant sources. Hypocotyls were cut into 1 cm pieces and vertically inserted 1–2 mm into the culture media. Cotyledons were divided perpendicularly to the longitudinal axis into two equal parts, and portions of 1 mm width along the edges were removed from each explant before they were placed onto the culture media with the back side toward the medium. From each seedling, five hypocotyl segments and the four cotyledon halves were used as explants and put into separate containers. Routinely 30–50 replicates were used per treatment.

The different media were prepared by supplementing a basal medium consisting of M & S salts, 3% sucrose, 100 mg/l myo-inositol, 1 mg/l thiamine-HCl and 0.8% agar (MB3 medium), with indole-3-acetic acid (IAA) and 6-furfurylaminopurine (kinetin). A factorial design was performed to test levels of 0.0, 1.5, 3.0, 4.5 and 6.0 mg/l of each growth substance. The pH was adjusted to 5.7 before autoclaving at 115°C for 25 min. Both the germinating seeds and the explants were incubated in a 16 h photoperiod (1300 lux, provided by cool fluorescent tubes Gro-lux, Sylvania) at 27 ± 2°C day and 24 ± 2°C night temperatures. Callus growth from explants was estimated as final fresh weight, and the morphogenic response of the calli as percentage of calli giving developed shoots, roots and somatic embryos, after 30 days of incubation.

Morphogenic cell lines

These lines were obtained from cotyledon-derived calli growing on one of the three following media, consisting of MB3 medium supplemented with 6.0 mg/l kinetin plus IAA at 0.0 mg/l (IK-0060 medium), 1.5 mg/l (IK-1560 medium) or 3.0 mg/l (IK-3060 medium) and subculturing the organized zones of these calli on the same media every 2–3 weeks.

Plant regeneration

Well developed shoots from both primary calli and permanent morphogenic cell lines were rooted on MB3 medium without growth regulators or supplemented with LAA or NAA (α-naphthaleneacetic acid) at 0.01–0.1 mg/l.

To achieve well developed shoots from the morphogenic cell lines it

was necessary subculturing portions of these calli 2–3 times in a MB3 medium supplemented with 0.1 mg/l BAP (6-benzylaminopurine) and a final passage through a MB3 medium containing 0.1 mg/l IAA.

Results

Callus growth

Media without kinetin but with IAA at zero or low concentrations induced root formation but no growth of calli in both kinds of explants. When the IAA levels were increased to 3.0, 4.5 or 6.0 mg/l in media without kinetin, the hypocotyl explants gave rise to callus growth, while the cotyledons only produced roots. When IAA was omitted, kinetin at 1.5 mg/l and higher induced the growth of organized as well as unorganized tissue in cotyledon explants, and calli were induced on hypocotyl explants by kinetin concentrations greater than 3.0 mg/l.

Generally, the presence of IAA and kinetin together increased the growth in both types of explant; nevertheless, we could not determine the influence of the ratio between the two phytohormones on the measured growth, perhaps because the final fresh weight is the sum of both the unorganized callus tissue and the organized structures, which makes the interpretation of results very difficult.

Shoot development

Hypocotyl explants did not produce adventitious shoots under any conditions tested, while in cotyledon explants the initiation of shoots from the

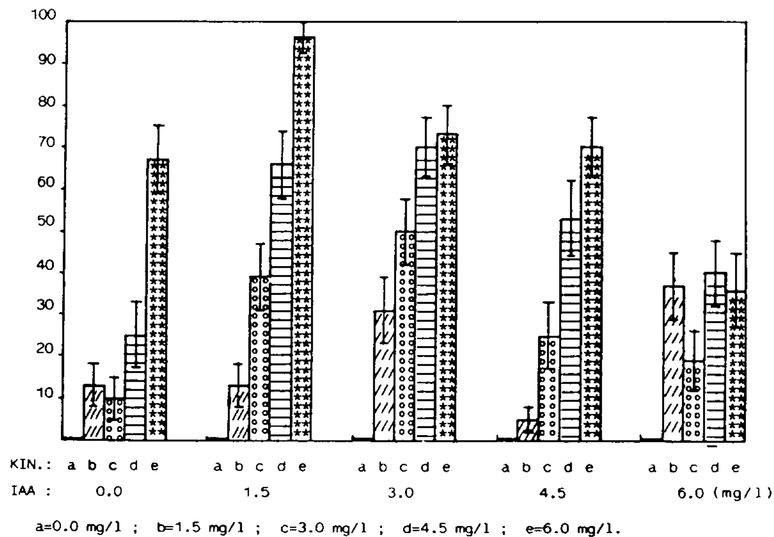

Figure 1. Percentage of calli from cotyledon explants developing shoots.

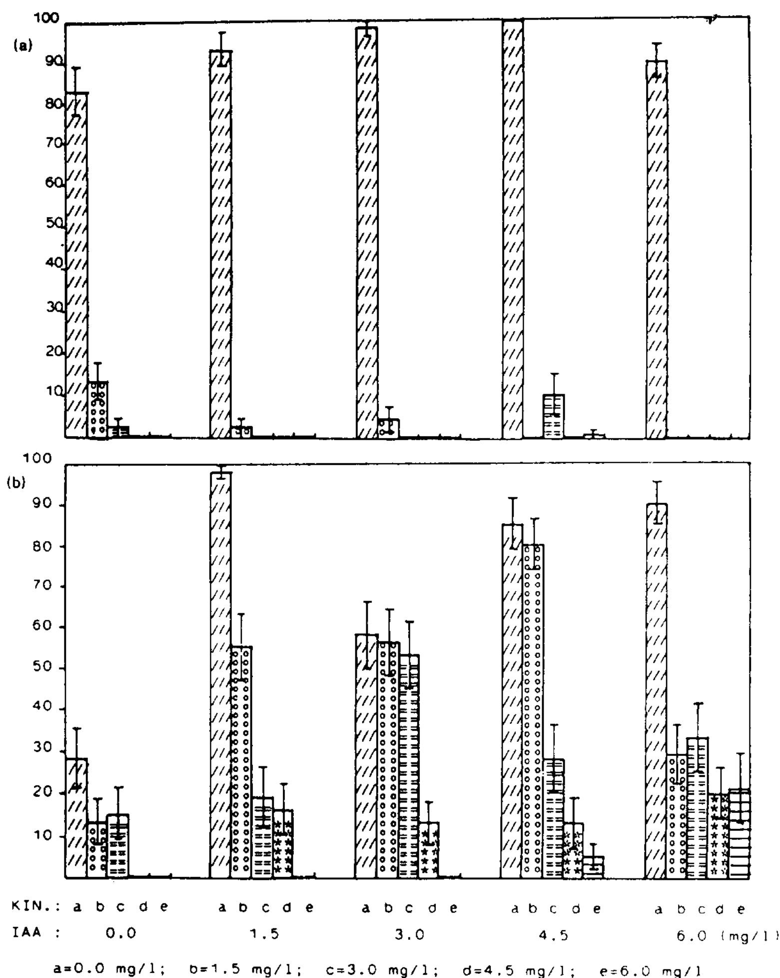

Figure 2. Percentage of primary calli forming roots, from (a) hypocotyl and (b) cotyledon explants.

calli formed was common (Figures 1 and 3a). Kinetin was essential for shoot formation. With 0.0–4.5 mg/l IAA, explant response increased quantitatively to increasing levels of kinetin. Even in the absence of exogenous auxin, but with 6.0 mg/l kinetin (IK-0060 medium), 67 ± 9% of the calli responded. Maximum explant response (96 ± 4%) was obtained on medium containing 1.5 mg/l IAA and 6.0 mg/l kinetin (IK-1560 medium).

Time required for appearance of shoots was about 20 days but final reading was made after 30 days in culture to allow their development. The number of adventitious shoots per callus was difficult to quantify because usually a number of shoot-buds, from a few to innumerable, were formed in the same

callus together with one or some well developed shoots. We could not observe a clear relationship between medium composition and the number of well developed shoots (usually 1–3) or the number or size of callus zones with shoot-buds.

Root formation

Roots were formed only in the absence of kinetin in hypocotyl explants, and rhizogenesis was not increased by the addition of IAA because of the high response already reached in the phytohormone free medium (Figure 2a). In cotyledon explants, the frequency of rhizogenesis (% of calli giving roots) was maximal at the lower levels of kinetin. For a given level of IAA, root formation decreased as kinetin concentration increased (Figure 2b).

Embryogenesis

In cotyledon derived primary calli the formation of somatic embryos occurred sporadically and at low frequencies. In hypocotyl derived calli the embryos appeared only in media without kinetin and with high IAA concentrations (82 ± 5% of the calli growing on medium with 3 mg/l IAA; 48 ± 7% on medium with 4.5 mg/l IAA and 20 ± 6% on medium with 6 mg/l IAA). These embryos did not undergo further development and, therefore, did not become whole plants.

Morphogenic cell lines

Cotyledon explants grown on media IK-0060, IK-1560 and IK-3060 gave rise to organogenic calli that could be maintained through successive transfers to the same media. The success in maintaining these lines depended upon which callus tissues were transferred. Thus, when pieces without organized structures were used as inocula, the morphogenic potential was lost in a few passages, while if organized portions of calli were chosen, the cell lines retained (and even increased) the organogenic capacity.

The length of the interval between transfers influenced morphogenic properties. Periods longer than three weeks caused the appearance of unorganized zones in the calli, so the most suitable interval for transferring the calli was 15–20 days. Calli of thess morphogenic lines were completely green and compact, producing large number of shoot buds (Figure 3b), but lacking the well developed shoots that usually appeared on the primary cotyledon derived calli.

By using this method, morphogenic cell lines without apparent loss of organogenic potential are still being maintained after the eighteenth subculture (about one year) in our laboratory.

Plant regeneration

Whole plants could be obtained from the well developed shoots appearing on the cotyledon primary calli grown on any of the media referred to in Figure 1.

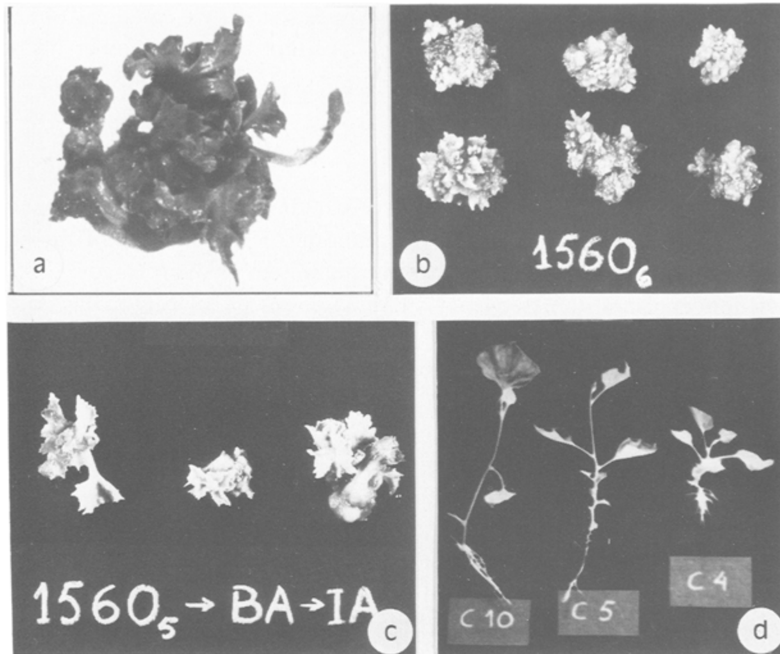

Figure 3. (a) Cotyledon derived primary callus showing shoots and shoot buds. (b) Calli of morphogenic cell line growing on IK-1560 medium after the 6th subculture. (c) Shoot development in calli growing on MB3 medium with 0.1 mg/l IAA after subculturing morphogenic cell lines through MB3 medium supplemented with 0.1 mg/l BAP. (d) Regenerated plantlets of melon after rooting in medium without growth regulators.

The excised shoots were rooted on media lacking growth regulators or containing low auxin concentrations (see Material and Methods), and gave 1–3 plants from each organogenic callus. However, the number of plants could be increased by subculturing organogenic calli on a medium with 0.1 mg/l BAP, where some of the shoot buds became well developed shoots that could be excised and rooted.

Plant regeneration could be also achieved from the morphogenic cell lines but, in this case, not directly because of the absence of well developed shoots in the calli. It was necessary to carry out 2–3 passages through different media to promote its development. Thus, on transferring organogenic calli to MB3 medium supplemented with 0.1 mg/l BAP they grew more slowly and compactly, forming shoots of about 1–3 mm in length. A further subculture of these calli on MB3 medium with 0.1 mg/l IAA promoted the development of 0–5 shoots per callus with well formed leaves (Figure 3c). These shoots could be excised and rooted (Figure 3d).

Discussion

Shoot formation at high frequency in callus cultures of melon has been obtained using the methods and conditions described.

An important factor affecting the morphogenic response was the type of explant: hypocotyl segments were unable to form shoots in any of the media tested while cotyledon halves gave shoots at a high frequency.

It should be also pointed out that melon can regenerate shoots in more than 50% of the calli in media with different hormonal combinations. This means that, for melon, there exists a wide range of growth regulators concentrations where the response is possible, even in media without exogenous auxin.

As with pumpkin [5], melon is able to undergo morphogenesis via organogenesis and via embryogenesis, depending upon the culture conditions employed and the type of explant. Under the conditions used here the embryogenesis was much less frequent than the organogenesis. Recently, we have attained somatic embryogenesis at high rates in cells from shaked cultures of callus in liquid medium as well as from protoplast-derived cells (in preparation).

The morphogenic potential of the calli is not lost with successive subculturing. Jelaska [6], working with pumpkin reported similar results. However, for melon this only happens when organogenic structures are chosen as inocula for each subculture, as described for tomato calli [4]. All these facts seem to indicate a clonal transmission of the morphogenic trait.

The conditions and methods here reported can be used to assess somaclonal variation in melon, since whole plants can be obtained from the well developed shoots. Further studies are needed to determine if useful variation for plant improvement exists in the plants regenerated in vitro.

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