TÜBİTAK

Research Article

Turk J Agric For
34 (2010) 309-317
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doi:10.3906/tar-0905-8

Regeneration and histological analysis of snake melon (*Cucumis melo* var. *flexuosus* (L.) Naudin) by direct organogenesis

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Received: 06.05.2009

Abstract: In vitro morphogenesis of *Cucumis melo* var. *flexuosus* (L.) Naudin was studied by direct organogenesis. Induction of adventitious buds was obtained from distal and proximal parts of the cotyledon incubated on Murashige and Skoog (1962) medium containing different concentrations of 6-benzyladenine (BA) (0.0, 0.5, 1.0, and 2.0 mg L^{-1}) and indole-3-acetic acid (IAA) (0.0, 0.1, and 0.5 mg L^{-1}). The best organogenesis was obtained from the proximal part of the cotyledon on medium containing 0.5 mg L^{-1} BA and 0.5 mg L^{-1} IAA (88%). This concentration was used for histological analysis. Less regeneration was obtained on media containing 1.0 mg L^{-1} BA (75%) or 1.0 mg L^{-1} BA and 0.1 mg L^{-1} IAA (60%). Plant recovery was much greater from proximal rather than distal explants. A callus formed on almost every explant on media supplemented with BA. Histological analyses showed that the formation of early shoot apical meristems was observed in 14-day-old tissues and meristematic structures were seen in 17-day-old tissues. The first shoot was formed after 21 days. By this time, the surface was covered with leaf primordia and leaves, mostly without accompanying buds.

Key words: Cell division, cucurbits, in vitro, regeneration, tissue culture

Acur'un (*Cucumis melo* var. *flexuosus* (L.) Naudin) direk organogenezis yoluyla rejenerasyonu ve histolojik analizi

Özet: In vitro koşullarda direkt organogenezis yöntemi kullanılarak acur *Cucumis melo* var. *flexuosus* (L.) Naudin'in morfogenezisi üzerine çalışılmıştır. Benzil adenin (BA) (0.0, 0.5, 1.0, 2.0 mg L^{-1}) ve indol asetik asit (IAA) (0.0, 0.1, 0.5 mg L^{-1})'nın farklı konsantrasyonlarını içeren Murashige ve Skoog (1962) ortamında gelişen kotiledon eksplantlarının sap kısmına yakın ve orta kısımlarından yan tomurcukların oluşumu başlamıştır. En yüksek organogenezis, 0.5 mg L^{-1} BA ve 0.5 mg L^{-1} IAA (% 88) içeren ortama dikilen kotiledonun sap kısmına yakın yerlerden elde edilmiştir. Bu hormon konsantrasyonu histolojik analizler için de kullanılmıştır. 1.0 mg L^{-1} BA (% 75) ve 1.0 mg L^{-1} BA ve 0.1 mg L^{-1} IAA (% 60) içeren ortamdan rejenerasyon daha az oranda olmuştur. Bitki gelişimi sap kısmına yakın eksplantlarında orta

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eksplantlara oranla daha iyi gerçekleşmiştir. BA içeren ortamlardaki hemen hemen tüm eksplantlarda kallus oluşmuştur. Histolojik analizler sonucunda, apikal meristem sürgününün 14 günlük dokularda, meristematik yapıların da 17 günlük dokularda görülmeye başlandığı tespit edilmiştir. İlk sürgün 21'inci günde oluşmuştur. Bu süre içerisinde eksplantın yüzeyi yaprak primordiyumu ve göz içermeyen yapraklarla kaplanmıştır.

Anahtar sözcükler: Doku kültürü, hücre bölünmesi, in vitro, kabakgiller, rejenerasyon

Introduction

The origin of snake melon (*Cucumis melo* var. *flexuosus)* is known to lie in southeastern Anatolia, Azerbaijan, Iraq, Palestine, and Central Asia. There are many local genotypes in Anatolia with round, green, hairy, or furrowed appearances (Beşirli and Yanmaz 1995). Fruits are very elongated and not sweet, and are eaten immature, as cucumbers. Snake melon was segregated from the 5 other cultivated varieties of *C. melo* as var. *flexuosus*, according to a simplified taxonomic model of this species (Munger and Robinson 1991). Molecular data suggest that *C. melo* var. *flexuosus* is more related to the *inodorus* and *cantalupensis* varieties, in contrast to morphological analyses that indicated that it belonged to a nonsweet clade (Stepansky et al. 1999). Similar, less elongated melons, var. *adzhur* and var. *chate*, have also been reported as ancient vegetable crops (Pangalo 1929; Hammer et al. 1986). Today snake melon is grown mostly in home and market gardens; hence, the annual production or area sown in Turkey is not accurately known. However, breeding of this plant is not restricted to Turkey. *Cucumis melo* var. *flexuosus* is a very important vegetable throughout northern Africa, the Middle East, and India (Walters and Thieret 1993).

Most snake melon cultivars are quite wild genotypes. Moreover, accessions from different regions (Asia, the Middle East, and Asia Minor) are not closely related (Stepansky et al. 1999). This background makes *C. melo* var. *flexuosus* a good target for a genetic breeding program. Research indicates that early sowing increased the yield but did not increase fruit length (Saglam and Yazgan 1997). Breeding may help in obtaining better fruit characteristics. Since powdery mildew is a major problem for snake melon crops, some research was performed on breeding a resistant variety (Ahmed et al. 1997). Using modern techniques, problems would be solved and the crop upgraded very quickly. Thus, tissue culture seems essential for improving the snake melon crop, just as it has been important for other cucurbit crops (Oridate et al. 1992). Although optimization of regeneration was obtained in commercial Cucurbitaceae species, including muskmelon (*Cucumis melo* L.) (Fang and Grumet 1990; Ezura et al. 1992; Taner and Yanmaz 1999), watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai) (Compton and Gray 1993), summer squash (*Cucurbita pepo* L.) (Ananthakrishnan et al. 2003), winter squash (*Cucurbita maxima* Duch.) (Lee et al. 2003), cucumber (*Cucumis sativus* L.) (Curuk et al. 2003), and bottle gourd (*Lagenaria siceraria* Standl.) (Han et al. 2004), there is no study on the organogenesis of snake melon. An effective regeneration system is essential for gene transfer techniques as well as for breeding programs.

This work presents the results of research on the most effective morphogenic process for *C. melo* var. *flexuosus*. Direct organogenesis was performed to characterize the most suitable hormone concentrations for regeneration. Histological research determined the source of regeneration and associated morphological events. This is the first report on the histological analysis of regeneration in snake melon.

Materials and methods

Plant Material and Preparation

Mature seeds of *Cucumis melo* var. *flexuosus* (Acur Efe 34, May Company, Turkey) were used as an explant source for organogenesis induction. Seed coats were removed and seeds were sterilized with 70% ethanol for 10 min, followed by 20 min in 20% commercial bleach solution (approximately 1% NaOCl) (Yalçın Mendi 2003). Seeds were then rinsed 6 times with sterile distilled water. Seeds were blotted dry and germinated on Murashige and Skoog (MS) (1962) medium with MS vitamins, 3% (w/v) sucrose, and 0.75% (w/v) agar for 3 days. The pH level was adjusted to 5.7 prior to autoclaving at 121 °C for 20 min. Cultures were incubated in a growth room at 25

°C with a 16 h photoperiod under 75 μmol m^{-2} s^{-1} cool white fluorescent light.

Organogenesis

After 3 days, the axes of plant embryos were excised from seedlings and discarded, the borders of the cotyledons were excised, and each cotyledon was divided into 2. Then 2 distal and 2 proximal explants from each cotyledon were cultured on MS medium with different concentrations of BA (0, 0.5, 1.0, and 2.0 mg L^{-1}) and IAA (0, 0.1, and 0.5 mg L^{-1}) (Yalçın Mendi et al. 2009). Each combination of concentrations was replicated 5 times in petri dishes (100 × 15 mm), with 8 proximal or distal segments in each replication. The plantlets were transferred onto the same regeneration medium for subcultures.

Histological Analysis

For histological analysis, the proximal cotyledon explants were cultured on an MS regeneration medium containing 0.5 mg L^{-1} BA and 0.5 mg L^{-1} IAA. Explants were observed by microscope and samples were taken after 4, 7, 10, 14, and 17 days in culture and fixed in formaldehyde propionic acetate (FPA) solution for at least 24 h. Explants were then dehydrated in 70%, 85%, 95%, and 100% Johansen alcohol solutions for 2 h each. Finally, the samples were vacuum-infiltrated with 95% Johansen solution. Later, explants were transferred to tert-butyl alcohol (TBA) overnight, and to TBA-2 and TBA-3 solutions for 3 h, respectively. Explants were embedded in liquid paraffin for 2-3 days. Samples in paraffin blocks were kept in the oven and then placed on ice covered by glass to freeze immediately. Samples with paraffin were stuck on to a wood block; sections were cut in thicknesses of 8 μM by a rotary microtome and observed by microscope after staining with hematoxylin (Eti and Stösser 1987).

Statistical Analysis

ANOVA and Duncan's multiple range test with a 95% confidence interval ($P < 0.05$) were used to compare the means of all treatments.

Results

Organogenesis

The response of explants cultivated on MS media with various BA and IAA contents was as follows. The proximal parts of the cotyledons exhibited better shoot regeneration than the distal parts (Figure 1). After 4 months, no regeneration was observed from the following media: MS without hormones (control), MS with 0.5 mg L^{-1} IAA, and MS with 1.0 mg L^{-1} IAA. Although some explants cultured on medium with low IAA concentrations formed some weak calli, their size was restricted to the beginning stage of growth and ceased expanding after a few days. Callus color was pale green with a tight structure. Generally, these explants showed little growth, but kept their green color. Some of the explants, however, regenerated roots (Figure 1). According to Moreno et al. (1985) and Kathal et al. (1988), this was observed as a normal reaction to different IAA hormone concentrations in *C. melo*.

Callus formation was also observed regularly with media containing BA and IAA, or containing only BA. One month after establishing the culture, clear differences in response were visible. The best callus formation was obtained from the media containing 1 mg L^{-1} BA for proximal explants (100%) and 2 mg L^{-1} BA for distal explants (98%). The media with 2 mg L^{-1} BA and 0.1 mg L^{-1} IAA also showed high callus formation for proximal (98%) and distal (95%) explants. However, after an additional month in culture (with subculture after one month), callus formation reached almost 100% for both types of explant and most hormone concentrations. Control and low-IAA media resulted in very low callus formation. The lowest callus formation was obtained from proximal explants on the media containing 0.1 mg L^{-1} IAA (13%) and the control groups of distal explants (0.3%) (Figure 2).

There was no bud formation observed in the control, 0.5 mg L^{-1} IAA, or 0.1 mg L^{-1} IAA media. A low level of regeneration was obtained with distal and proximal cotyledon explants (8%) on the media containing 0.5 mg L^{-1} BA, in comparison to the regeneration medium (0.5 mg L^{-1} BA and 0.5 mg L^{-1} IAA) with the maximal regeneration of 88%, suggesting that IAA in the media in combination with low BA is essential for efficient regeneration. On the other hand, high BA concentrations (2 mg L^{-1} BA, and 2 mg L^{-1} BA and 0.1 mg L^{-1} IAA) showed a reduced regeneration rate for distal (13%) and proximal (34%) explants (Figure 3).

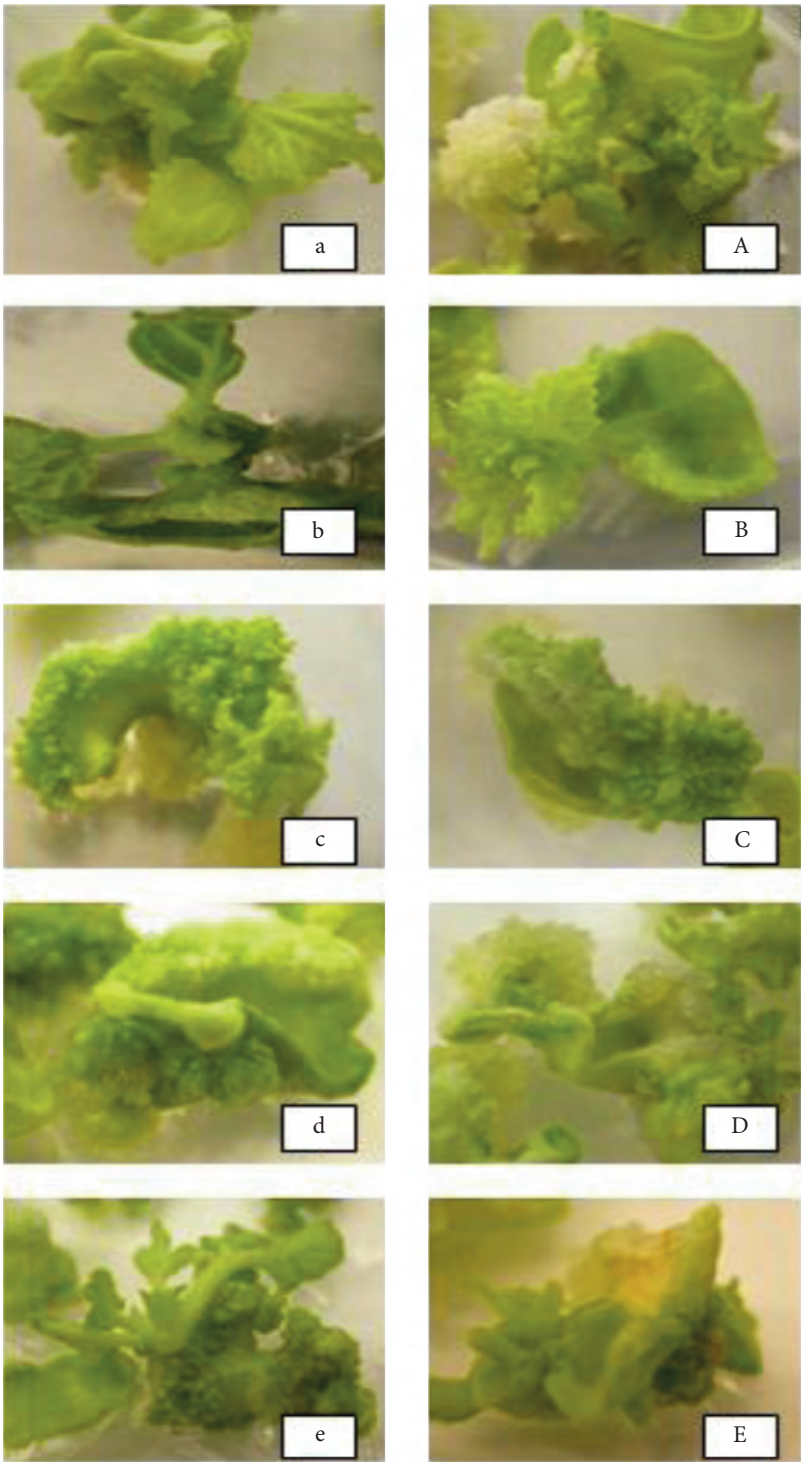

Figure 1. Organogenesis in snake melon. (a) shoot regeneration from distal and (A) proximal cotyledon explants one month after induction on MS medium containing 0.5 mg L^{-1} BA and 0.5 mg L^{-1} IAA; (b) distal and (B) proximal cotyledon explants on medium containing 0.5 mg L^{-1} BA and 0.1 mg L^{-1} IAA; (c) distal and (C) proximal cotyledon explants on medium containing 1.0 mg L^{-1} BA; (d) distal and (D) proximal cotyledon explants on medium containing 1.0 mg L^{-1} BA and 0.1 mg L^{-1} IAA; (e) distal and (E) proximal cotyledon explants on medium containing 1.0 mg L^{-1} BA and 0.5 mg L^{-1} IAA.

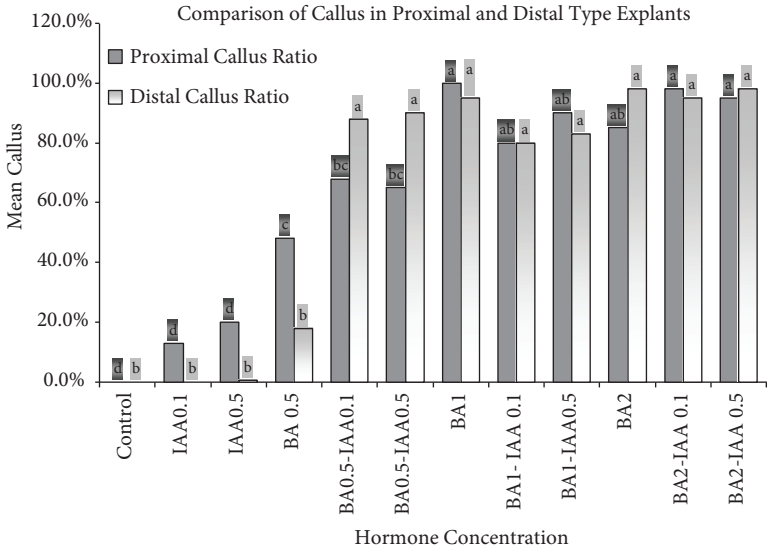

Figure 2. Callus formation of proximal and distal explants at different hormone concentrations (BA and IAA).
A multiple comparison test, Tukey's honestly significantly difference test (Tukey's HSD), was run to identify whether calli of proximal and distal explants at different hormone concentrations were significantly different at the 0.05 level.
In Figure 2, means with the same letter are not significantly different according to Tukey's HSD at 0.05.

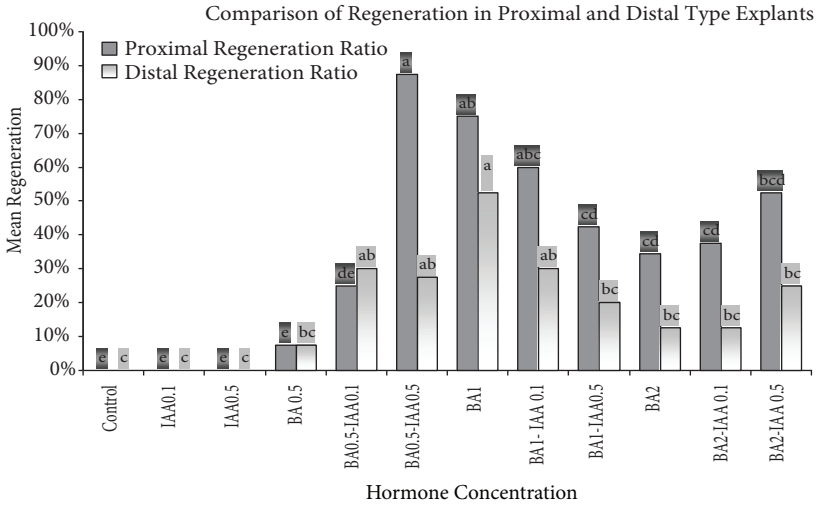

Figure 3. Regeneration of proximal and distal explants at different hormone concentrations (BA and IAA).
A multiple comparison test, Tukey's honestly significantly difference test (Tukey's HSD), was run to identify whether regeneration by proximal and distal explants at different hormone concentrations was significantly different at the 0.05 level. In Figure 3, means with the same letter are not significantly different according to Tukey's HSD at 0.05.

Increased regeneration was achieved by lowering the BA concentration to 0.5 mg L^{-1} with 0.1 mg L^{-1} IAA, with proximal and distal explant shoot formations of 25%-30%. An increase of the IAA concentration to 0.5 mg L^{-1}, with 1 or 2 mg L^{-1} BA, caused regeneration of 43% and 53%, respectively, for proximal explants, and 20% and 25%, respectively, for distal explants. Although the regeneration of explants treated with medium containing 0.5 mg L^{-1} BA and 0.5 mg L^{-1} IAA was highest (88%) for proximal explants, the result for distal cotyledon explants was much lower (28%) (Figure 2). Maintaining the same ratio of cytokinin to auxin (1 mg L^{-1} BA to 0.1 mg L^{-1} IAA) reduced regeneration for proximal explants to 60%, but not for distal explants (30%) (Figure 3).

Histological Analysis

Cytological observations of explants at different regeneration stages showed that epidermal cells were the source of organogenesis. In the early stages of the experiment, no clear histological changes were detectable in 4-day-old tissues (Figure 4A), but cell division and multiplication were detectable in the further growth and development. The first visible changes were cell division in the epidermal and subepidermal layers, on a base of parenchyma cells. These cells started dividing periclinally and anticlinally to produce further epidermal and subepidermal cells. After 4 days in culture, the proximal cotyledon explants increased in size, exhibited swelling, and produced calli on the adaxial surface in contact with the medium in 7-day-old tissues (Figure 4B). Premeristematic cells were visible in the epidermal layer on the adaxial surface in the center of cotyledon explants near the proximal cut edge at 11 days in culture (Figures 4C and D). After 11 days, the explants expanded further in mass, and one to several adventitious buds arose on the callus surface. These cells formed meristematic structures and multicellular protuberances, which developed shoots and leaves within 14-17 days in culture. The protuberances observed after 14 days were neither primordia nor buds, although some meristematic bulges were observed (Figures 4E and F).

Meristematic cells were usually thin-walled and more isodiametric in shape than the cells of mature tissues. The regeneration of adventitious shoot meristems originated directly from the epidermal and subepidermal layers of the explants. The formation of early shoot apical meristems was observed in 14-day-old tissues, and meristematic structures were seen in 17-day-old tissues. The first shoot was found after 21 days. By this time, the surface was covered with protrusions and leaves, mostly without accompanying buds (Figures 4G, H, J, and K).

Discussion

The proximal parts of the cotyledons exhibited better shoot regeneration than the distal parts in snake melon. Other researchers also observed that the proximal part of the cotyledon showed a higher frequency of adventitious shoot regeneration in comparison to the distal part. Mohiuddin et al. (1997) indicated that the distal cotyledons of cucumbers are less responsive than the proximal cotyledons. In winter squash, Lee et al. (2003) also found that cells in the proximal cotyledon have the potential for adventitious shoot formation. Moreover, the proximal part of the cotyledon being used as an explant in regeneration studies such as those for melon (Gaba et al. 1999), watermelon (Compton and Gray 1993), winter squash (Lee et al. 2003), cucumber (Mohiuddin et al. 1997; Yalçın Mendi 2003), summer squash (Ananthakrishnan et al. 2003), and bottle gourd (Han et al. 2004) was motivated by the proximal segment's greater vigor.

The regeneration of explants treated with medium containing 0.5 mg L^{-1} BA and 0.5 mg L^{-1} IAA was the highest (88%) for proximal explants; the result for distal cotyledon explants was much lower (28%). Maintaining the same ratio of cytokinin to auxin (1 mg L^{-1} BA to 0.1 mg L^{-1} IAA) reduced regeneration for proximal explants to 60%, but not for distal explants (30%). The combination of these 2 hormones also caused the best shoot formation in another cucurbit (*Citrullus lanatus* cv. 'Sugar Baby'), as Chaturvedi and Bhatnagar (2001) reported that the media supplemented with 1 µM BA and 1 µM IAA resulted in high, direct shoot regeneration, while 3 µM BA and 3 µM IAA caused high, indirect shoot regeneration. On the other hand, for bottle gourd, results suggest that the highest shoot formation ratio was obtained from media containing 3 mg L^{-1} BA (Han et al. 2004). Other research is supported by the results of

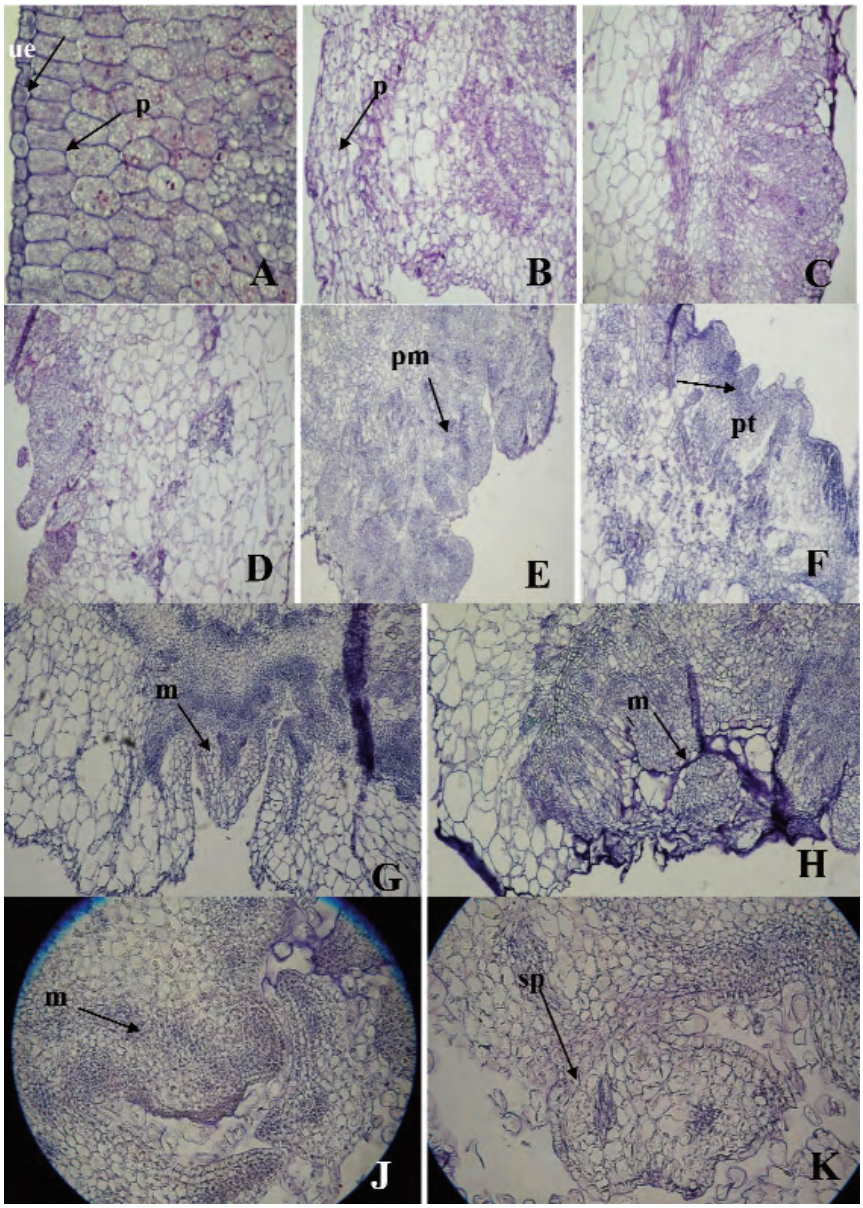

Figure 4. Anatomical structures of explants developed from 3-day-old cotyledons of *Cucumis melo* var. *flexuosus* on the medium containing 0.5 mg L^{-1} BA and 0.5 mg L^{-1} IAA. (A) Transverse section of cotyledon explant prepared from 4-day-old snake melon seedling, 40×; (B) Explant after 7 days in culture, 10×; (C, D) Explant after 11 days in culture, 10×; (E, F) Protuberances and premeristematic structures on an explant after 14 days on regeneration medium, 10×; (G, H) Formation of meristematic tissues after 17 days, 10×; (J, K) Shoot formation after 21 days in culture, 40×. ue: upper epidermis, p: parenchymal cell, pm: premeristem, pt: protuberance, m: meristem, sp: shoot primordium.

Cucurbita moschata Duch. ex Lam. regeneration observations, which also indicated media with 3 mg L^{-1} BA as the best ones (Zhang et al. 2008). Crops from unrelated families, such as *Sesamum indicum* L., may also regenerate better on medium containing a BA and IAA combination, with effective regeneration on medium containing 20 μM BA and 3 μM IAA (Were et al. 2006).

In histological analysis, epidermal cells were the source of organogenesis. Epidermal cells were also found to be the starting point of regeneration by other scientists, such as for melon (Gaba et al. 1999; Chovelon et al. 2008), sunn hemp (Daimon et al. 2002), watermelon (Yalçın Mendi 2003), and pepper (Mezghani et al. 2007). Protuberances and some meristematic bulges were observed after 14 days. Similar results were obtained by Gaba et al. (1999) with melon and Yalçın Mendi (2003) with watermelon. In watermelon, young protuberances often clustered in groups of 3-5 in rough circles, and protuberances were finger-like structures without shoot apical meristems that developed into leaves, as also observed by Gaba et al. (1999). Gaba et al (1999) also mentioned that a group of young protuberances had no shoot buds or shoot apical meristems. The first shoot was found after 21 days. In watermelon, premeristematic and meristematic tissues were seen in 7- and 12-day-old tissues. The first regenerated shoot buds and shoots were observed in 15- and 22-day-old tissues, respectively, in melon. This indicates that the formation of meristematic structures and shoots in snake melon might be earlier than in melon and later than in watermelon.

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