Turkish Journal of Agriculture and Forestry

Volume 33 | Number 2 Article 8

1-1-2009

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Turk J Agric For
33 (2009) 165-172

doi:10.3906/tar-0806-29

Regeneration and Histological Analysis of Regeneration in Bottle Gourd (*Lagenaria siceraria* (Molina) Stand.)

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Received: 19.06.2008

Abstract: Two different types of explant (proximal and flamingo-bill) from Emphasis seedlings, a hybrid cultivar of bottle gourd (*Lagenaria siceraria* (Molina) Stand.), germinated under dark and light conditions were cultured on 9 regeneration MS media containing various combinations of BA (0, 1.0, and 2.0 mg l^{-1}) and IAA (0, 0.25, and 0.5 mg l^{-1}). Comparison of the explant types showed that the flamingo-bill type explant had better shoot formation than did the proximal explant. The MS medium containing 1 mg l^{-1} of BA was optimal for shoot formation capacity when flamingo-bill type explants germinated in the dark (44%) and light (36%) were used. Histological analysis showed that explant cell division began after 3 days in regeneration medium and formation of primordium was observed in the tissues in culture between days 5 and 7. Differentiation of meristimatic structures was first observed after 9 days and development completed after 9-12 days in the culture.

Key Words: Bottle Gourd, *Lagenaria siceraria*, regeneration, histology, explant type

Su Kabağında (*Lagenaria siceraria* (Molina) Stand.) Rejenerasyon ve Rejenerasyonun Histolojik Analizi

Özet: Bu çalışmada, hibrit su kabağı (*Lagenaria siceraria* (Molina) Stand.) çeşidi olan Emphasis'in hem ışık hem de karanlık koşullarda çimlendirilmiş olan bitkilerinden alınan 2 eksplant tipi (proksimal ve flamingo-bill), BA (0.0, 1.0 ve 2.0 mg l^{-1}) ve IAA (0.0, 0.25 ve 0.5 mg l^{-1})'in 9 farklı kombinasyonunu içeren MS rejenerasyon ortamlarında kültüre alınmıştır. Eksplant tiplerinin karşılaştırıldığı çalışma sonuçları, sürgün oluşumu üzerine flamingo-bill tipi eksplantının proksimal eksplanta nazaran daha iyi sonuç verdiğini ortaya koymuştur. Karanlık ve aydınlık koşullarda çimlendirilmiş olan flamingo-bill eksplantlarında optimum sürgün oluşum kapasitesi, 1 mg l^{-1} BA içeren MS ortamından % 44 ve % 36 olarak saptanmıştır. Histolojik analizler, eksplantlardaki hücre bölünmesinin rejenerasyon ortamına alındıktan 3 gün sonra başladığını, primordiyum oluşumunun ise 5 ila 7. günler arasında gözlendiği belirlemiştir. Meristematik yapının farklılaşması ilk olarak 9. günde gözlenmiş, 9 ve 12. günler arasında ise oluşum tamamlanmıştır.

Anahtar Sözcükler: Su kabağı, *Lagenaria siceraria*, rejenerasyon, histoloji, eksplant tipi

Introduction

The origin of bottle gourd (*Lagenaria siceraria* (Molina) Stand.), which belongs to *Cucurbitaceae*, is generally considered to be Africa (Robinson and Decker-Walters, 1997). Though the mature fruit of bottle gourd is not a highly desirable food, as it has little flesh, and an offensive smell and bitter taste, this species increases young plant vigor, possesses excellent tolerance to low

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soil temperature and soil-borne pathogens such as *Fusarium oxysporum* f. sp. *niveum*, and is exclusively used as rootstock for watermelon and other cucurbit crops (Han et al., 2004). At present, vegetable production via grafted seedlings is a common practice, especially for cucumber, melon, watermelon, tomato, pepper, and eggplant in greenhouses or plastic houses in Japan, Korea, and China, as well as in some other Asian and European countries (Lee, 1994, 2003). Vegetable species of the family *Cucurbitaceae* accounted for 31% of total vegetable production in Turkey (FAO, 2005). Turkey, with 4 million tons of watermelon production per year, is the second largest vegetable producing country after China (FAO, 2005). Emphasis, a hybrid bottle gourd, is a widely used and important commercial rootstock for watermelon cultivation.

Recent developments in biotechnology have uncovered methods for vegetable crop breeding using transformation, in which heterologous genes are introduced into existing cultivars. In many instances, however, the lack of an efficient regeneration system limits the use of gene transfer technology for vegetable crops (Sarowar et al., 2003).

Therefore, optimization of regeneration and transformation of cv. Emphasis for breeding purposes is necessary. Even though there have been several reports of regeneration and transformation in other species of *Cucurbitaceae*, such as cucumber (*Cucumis sativus*) (Nishibayashi et al., 1996, Mohiuddin et al., 1997), melon (*Cucumis melo*) (Ezura et al., 1990; Oridate et al., 1992; Ficcadenti et al., 1995), watermelon (*Citrullus vulgaris*) (Dong and Jia, 1991; Choi et al., 1994; Dabauza et al., 1997), summer squash (Chee, 1991; Ananthakrishnan et al., 2003), a hybrid of *Cucurbita maxima* × *Cucurbita moschata* (Rahman et al., 1993), and winter squash (*C. maxima*) (Lee et al., 2003), only Han et al. (2004, 2005) have reported a regeneration and transformation protocol for bottle gourd.

Abrie and Staden (2001) also developed a shoot regeneration protocol for 5 *Cucurbitaceae* cultivars. The influences of seed coat, ethanol pretreatment, different seed sources, NaOCl concentration, and treatment time on decontamination were investigated in melon, cucumber, and watermelon varieties.

An efficient plant regeneration system via organogenesis was established from cotyledon explants for bottle gourd by Han et al. (2004). They reported that maximum shoot regeneration was obtained when the proximal parts of cotyledons from 4-day-old seedlings were cultured on MS medium with 3 mg l^{-1} of BA and 0.5 mg l^{-1} of $AgNO_3$, with a 16-h photoperiod. Adventitious shoots were successfully rooted on half-strength MS medium with 0.1 mg l^{-1} of IAA for 2-3 weeks.

The optimization of regeneration protocols and histological studies are essential for gaining an understanding of the regeneration process. The present study sought to optimize an efficient regeneration protocol and histologically analyze regeneration using flamingo-bill (Pozueta-Romero, 2001) and proximal explants of bottle gourd (*Lagenaria siceraria*). To the best of our knowledge this is the first report on the histological analysis of regeneration via adventitious shoot organogenesis in bottle gourd.

Materials and Methods

Plant Material

A hybrid bottle gourd (*Lagenaria siceraria*), cv. Emphasis (Novartis Co. Ltd., Turkey), was used as the explant source.

Methods

Tissue Culture Conditions and Media

Seeds were surface-sterilized with a diluted commercial bleach solution (1.25% sodium hypochlorite) for 15 min and then rinsed with sterile distilled water. Seed coats were removed and washed in ethanol (70%) for 5 min and then sterilized for 20 min with 1.25% sodium hypochlorite plus Tween-20 (2 drops per 100 ml of solution), followed by 3 washings with sterile distilled water. Sterilized seeds were placed on seed germination culture medium supplemented with MS basal salts and vitamins (Murashige and Skoog, 1962), and containing 3% (w/v) sucrose and 0.75% (w/v) agar in petri dishes. pH was adjusted to 5.7 prior to autoclaving at 121 °C for 20 min. Half of the seeds were maintained at 25 ± 1 °C and 75 µmol m^{-2} s^{-1} of cool white fluorescent light, and half were incubated in darkness for 7 days on germination medium. Proximal of cotyledon and flamingo-bill type explants were excised from 7-day-old in vitro-grown seedlings and were incubated on regeneration medium only under light conditions.

Flamingo-bill type explants were cut from seedlings as follows: the hypocotyl was cut off close to the cotyledon, which was then cut in half, discarding the distal parts. Afterwards, the explant was placed on its hypocotyl stub and forced with a scalpel blade between the cotyledon remnants. The proximal part of 1 cotyledon and the apical meristem were then removed (Pozueta-Romero, 2001). Flamingo-bill type and proximal cotyledon explants were cultured abaxial side down on regeneration media containing MS basal medium that contained different concentrations of BA and IAA (Table). The explants that formed shoots were scored after 4 weeks on the regeneration medium.

Data Analyses

The experiments were implemented and analyzed according to a completely randomized study design (5 replicates and 5 explants in each replicate). All values expressed as percentages were transformed (arcsine (P = original percentage value) $^{0.5}$) according to Barlett, and were subjected to analysis of variance (ANOVA). Tukey's honestly significant difference (HSD value) test was used to compare the treatments at the $P \leq 0.05$ level, unless otherwise noted. The original data are presented.

Histological Analysis

Flamingo-bill type explants grown under light conditions were cultured on regeneration medium containing MS basal salts and vitamins supplemented with 1 mg l^{-1} BA. The explants were observed under a light microscope (Olympus, SZ-PT). They were taken from regeneration medium after 3, 5, 7, 9, or 12 days in culture and fixed in FPA (formaldehyde propionic acetate) solution for at least 24 h. Explants were then dehydrated in 70%, 85%, 95%, and 100% Johansen alcohol solutions, each for 2 h. When the samples were in 95% Johansen solution, they were placed in a vacuum to eliminate bubbles from the explants and solution. Later, explants were transferred to TBA (tert-butyl alcohol) overnight, and then to TBA-2 and TBA-3 solution, each for 3 h. Explants were embedded in liquid paraffin for 2-3 days. Samples in paraffin blocks were kept in an oven and then placed on ice covered by glass to freeze immediately. Sections about 8 µm thick were cut with a rotary microtome from the samples with paraffin and observed under a microscope (Nikon Eclipse E200) after staining with hematoxylin.

Results

The explants (flamingo-bill and proximal cotyledon) taken from seedlings that were germinated under light conditions showed maximum regeneration and shoot formation capacity (27%) on the medium containing 1 mg l^{-1} of BA. The media containing 1 mg l^{-1} of BA + 0.25 mg l^{-1} of IAA (16%), 2 mg l^{-1} of BA (15%), 2 mg l^{-1} of BA + 0.5 mg l^{-1} of IAA (12%), 1 mg l^{-1} of BA + 0.5 mg l^{-1} of IAA (11%), and 2 mg l^{-1} of BA + 0.25 mg l^{-1} of IAA (11%) resulted in low shoot formation capacity ($P < 0.05$) (Table).

Table. Shoot formation (%) of bottle gourd explants (average of flamingo-bill and cotyledon) germinated under dark and light conditions on MS media supplemented with different concentrations of BA and IAA.

Media	Concentrations (mg l^{-1}) of		Explants taken from seedlings grown in light	Explants taken from seedlings grown in light
	BA	IAA		
1	0	0	0c	0b
2	0	0.25	0c	0b
3	0	0.5	0c	0b
4	1	0	27a	24a
5	1	0.25	16b	6b
6	1	0.5	11b	6b
7	2	0	15b	10b
8	2	0.25	11b	10b
9	2	0.5	12b	2b
	HSD (5%)		10.6	10.11

Similar results were obtained from the explants taken from seedlings that were germinated under dark conditions. Maximum shoot formation was obtained with medium that contained 1 mg l^{-1} of BA (24%), whereas the media containing 2 mg l^{-1} of BA (10%) and 2 mg l^{-1} of BA + 0.25 mg l^{-1} of IAA (10%) resulted in low shoot formation capacity ($P < 0.05$) (Table).

Under light conditions the medium that contained 1 mg l^{-1} of BA was optimal for shoot formation capacity (44%); lower shoot formation capacity was obtained with the media containing 1 mg l^{-1} of BA + 0.5 mg l^{-1} of IAA (26%), 1 mg l^{-1} of BA + 0.25 mg l^{-1} of IAA (24%), 2 mg l^{-1} of BA + 0.5 mg l^{-1} of IAA (18%), 2 mg l^{-1} of BA + 0.25 mg l^{-1} of IAA (14%), and 2 mg l^{-1} of BA + 0.5 mg l^{-1} of IAA (12%) for the flamingo-bill type explant (Figure 1). For the proximal cotyledon explant type, maximum shoot formation capacity was obtained with the medium containing 2 mg l^{-1} of BA + 0.5 mg l^{-1} of IAA (12%). Low shoot formation capacity was obtained with the media that contained 1 mg l^{-1} of BA (10%), 2 mg l^{-1} of BA + 0.25 mg l^{-1} of IAA (8%), 1 mg l^{-1} of BA + 0.25 mg l^{-1} of IAA, 2 mg l^{-1} of BA (6%), and 2 mg l^{-1} of BA + 0.5 mg l^{-1} of IAA (4%) (Figure 1).

Under dark conditions the medium containing 1 mg l^{-1} of BA had the most efficient shoot formation capacity (36%); lower shoot formation capacity was obtained with the media containing 2 mg l^{-1} of BA + 0.25 mg l^{-1} of IAA (14%), 1 mg l^{-1} of BA + 0.25 mg l^{-1} of IAA (10%), and 1 mg l^{-1} of BA + 0.5 mg l^{-1} of IAA (10%) for flamingo-bill explants (Figure 2). The shoot formation capacity of the proximal cotyledon type was satisfactory with the medium containing 1 mg l^{-1} of BA (12%), and was lower on media containing 1 mg l^{-1} of BA + 0.25 mg l^{-1} of IAA (10%), and 2 mg l^{-1} of BA + 0.25 mg l^{-1} of IAA (6%) (Figure 2).

Proximal cotyledon explants from seedlings germinated under light conditions were cultured on regeneration medium containing 1 mg l^{-1} of BA. Cell division was observed adjacent to the cut edge of the explants after 3 days in culture (Figure 3A and B). In addition to cell division, swelling was also observed (Figure 3C). After 7 days in culture young protuberances clustered in groups of 3-5 adjacent to the cut edge of the explant were observed (Figure 3D). Protuberant structures became obvious after 7-12 days in culture (Figure 3D and E). Primordium formation occurred via periclinal and anticlinal division of apical cells. Formation of shoot buds was observed after 9 days. Old tissue and shoot formation were clearly seen in 12-day-old tissues (Figure 3E and F).

Flamingo-bill explants from seeds germinated under light conditions were cultured on regeneration medium containing 1 mg l^{-1} of BA. Cell division increased around the apical meristimatic area after 3 days in culture (Figure 4A and B). Swelling occurred on tissues located on the medium. Protuberant structures of leaves and buds became obvious after 5-7 days in culture (Figure 4C and D). White callus formation began from the point at which the explants connected with the hypocotyls. First, buds were observed among the calluses. The formation of shoot buds was noted after 7 days. Old tissue and shoot formation were visible in 9-day-old tissues (Figure 3D and E). It was clear that shoot formation occurred earlier in the flamingo-bill type explants than in the proximal type explants.

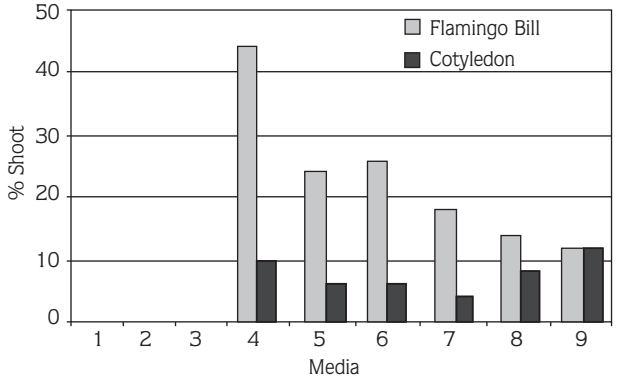

Figure 1. Shoot formation rate of flamingo-bill and proximal explants germinated under light conditions.

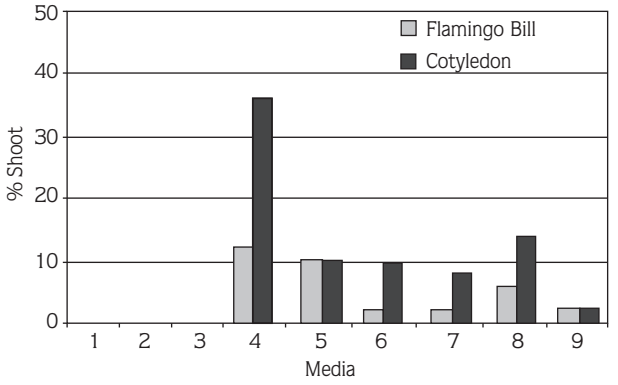

Figure 2. Shoot formation rate of flamingo-bill and proximal explants germinated under dark conditions.

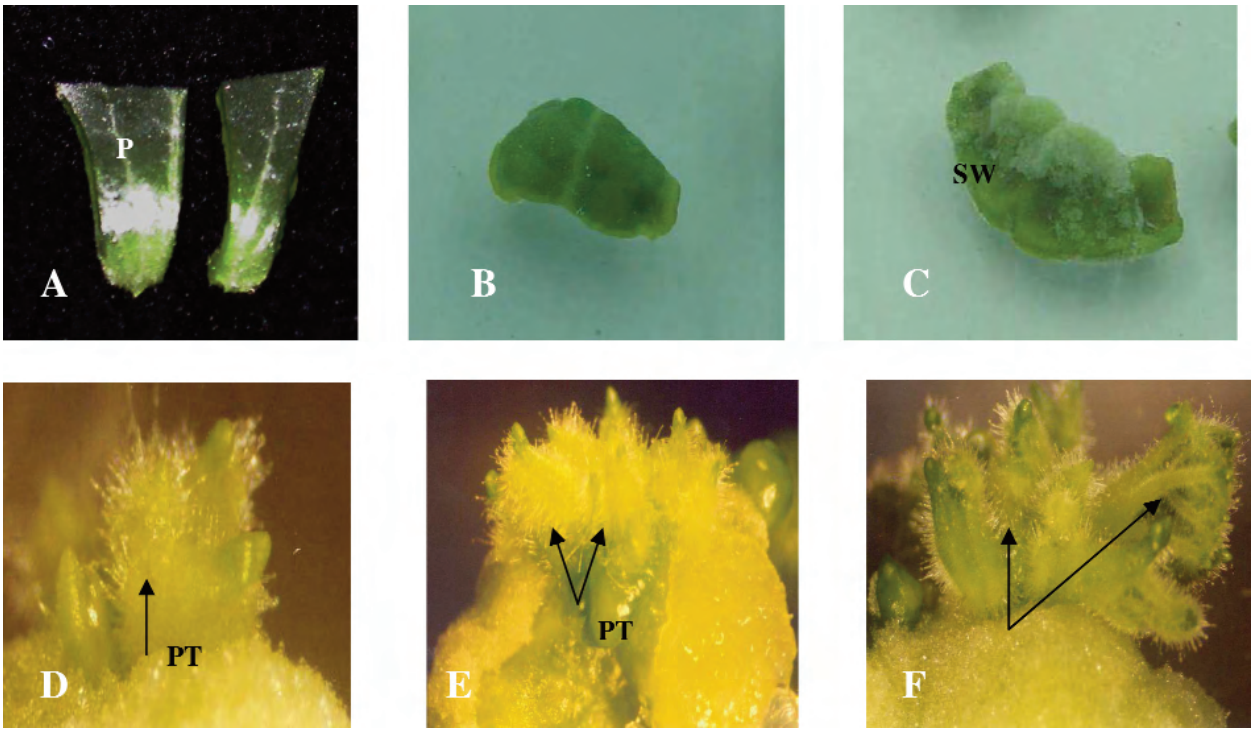

Figure 3. Morphological structures of proximal cotyledon explant. A: Day 0; B: day 3; C: day 5; D: day 7; E: day 9; F: day 12. P: Proximal; SW: swelling; PT: protuberance of leaf; S: shoot.

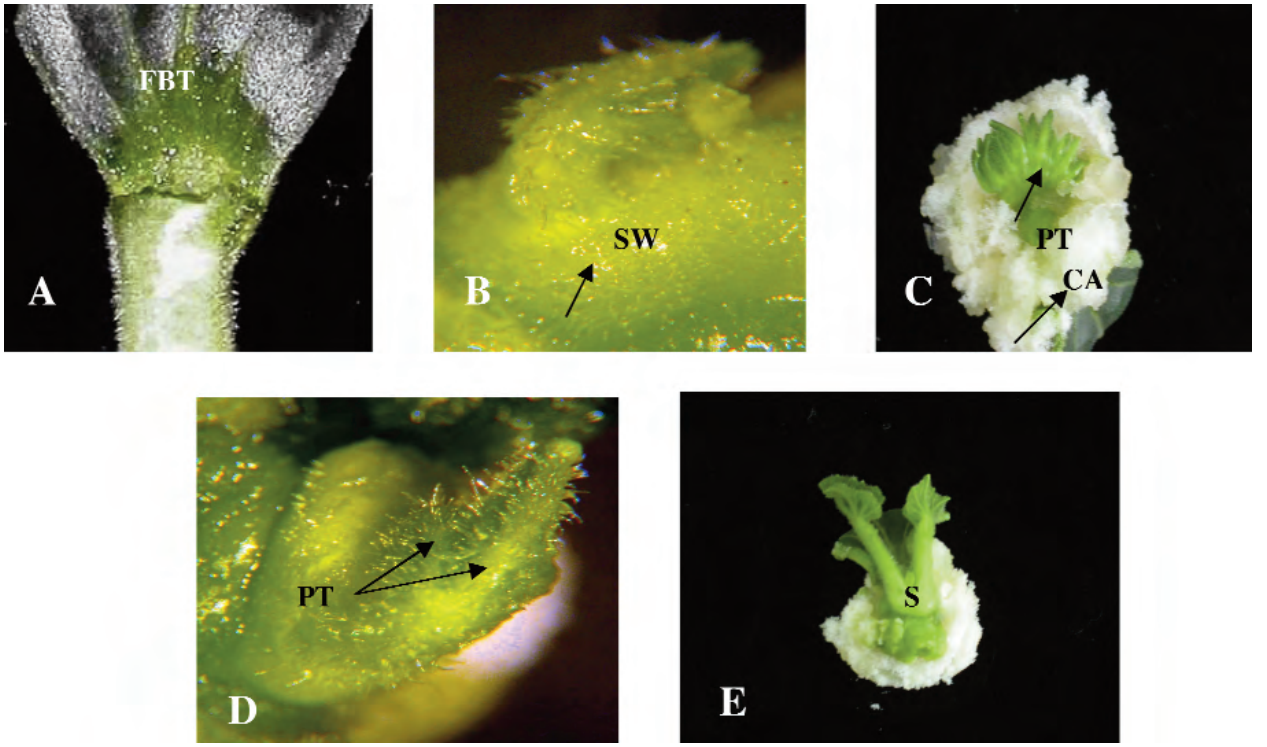

Figure 4. Morphological structures of flamingo-bill explant. A: Day 0; B: day 3; C: day 5; D: day 7; E: day 9. FBT: Flamingo-bill type explant; CA: white callus, SW: swelling; PT: protuberance of leaf and bud; S: shoot.

Histological analysis of flamingo-bill explant regeneration showed that initiation of cell division occurred in the parenchymatous area of the cells, dividing periclinally in this area (Figure 5A and B). Parenchymatous cells grew and enlarged after 5 and 7 days in culture (Figure 5C-E). This enlargement caused the explants to swell. These areas were the starting points of bud formation. Cells around the buds continued dividing. Promeristems formed after 7 days and developed into meristimatic structures between 9 and 12 days on regeneration medium (Figure 5F-H).

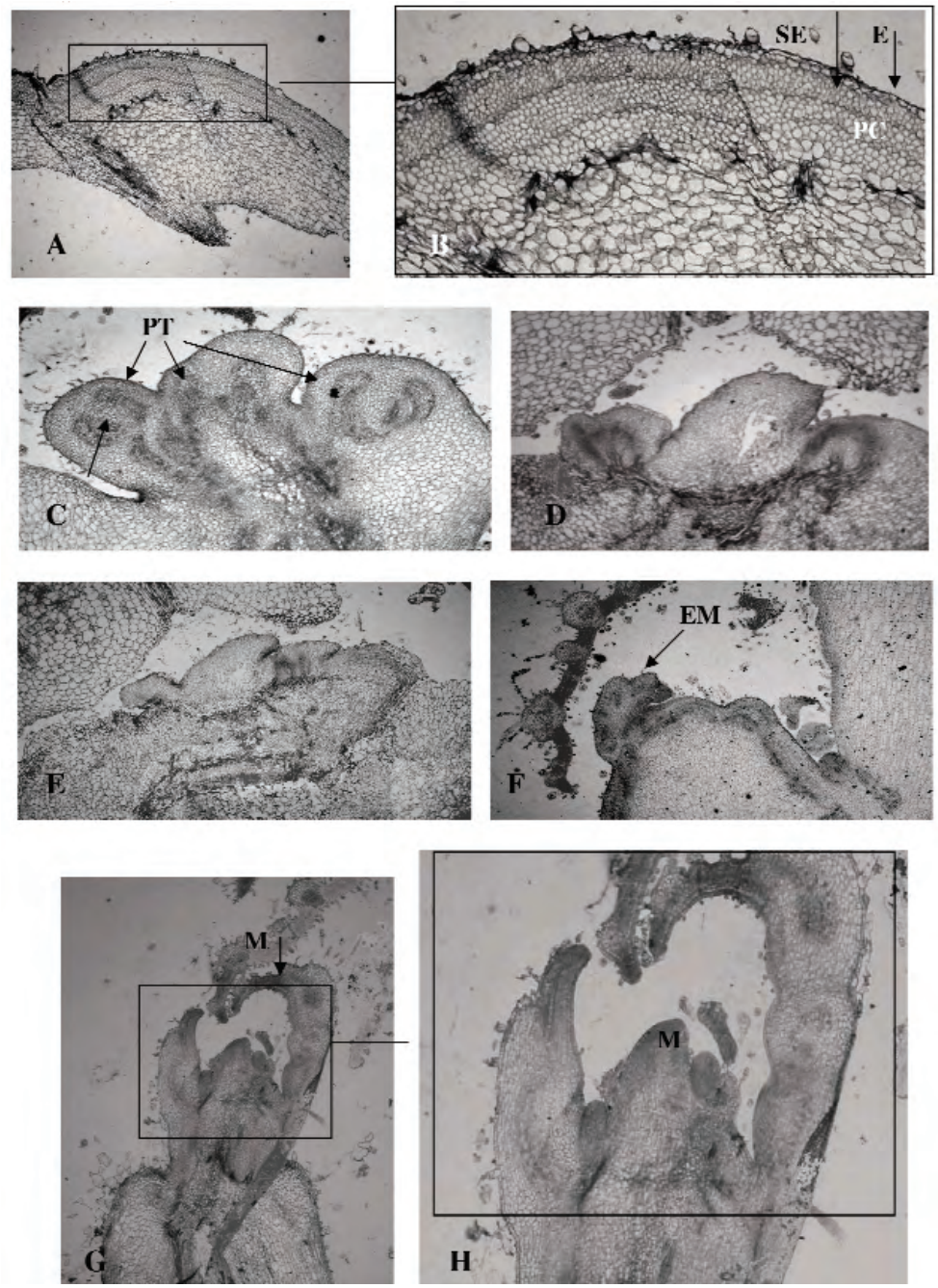

Figure 5. Histological analysis of regeneration in flamingo-bill explants. A: Day 3; B: close up of A; C-D: day 5; E: day 7; F: day 9; G: days 9-12; H: close up of G. E: Epidermis; SE: subepidermis; PC: parenchymal cell; PT: protuberance; EM: early meristimatic tissue; M: meristem.

Discussion

The media containing 1 mg l^{-1} of BA had the best regeneration capacity for seeds germinated under light and dark conditions. The exact mechanism of how dark pretreatment stimulates subsequent light-dependent organogenesis is not completely understood, but it is likely that incubation of plant tissues in darkness preserves light sensitive endogenous plant growth regulators and other compounds (Evans et al., 1981; Hartmann et al., 1997; Compton, 1999). The beneficial effect of dark pretreatment before preparing cotyledon explants for subsequent organ regeneration was also reported in watermelon (Compton, 1999). In comparison, dark pretreatment before preparing cotyledon explants decreased the frequency of adventitious shoot regeneration when compared with light pretreatment in gourd (Han et al., 2004).

In vitro induction of organogenesis depends on the endogenous concentration of plant growth regulators, and their distribution in cultured tissue and interaction with exogenously supplied growth regulators. Good shoot organogenesis (70%) was observed using BAP in combination with GA_3 from hypocotyl derived calluses (Pal et al., 2007). Comparison of explant types in the present study showed that flamingo-bill type explants had better shoot formation than the proximal explants.

Under light conditions the media containing 1 mg l^{-1} of BA and 2 mg l^{-1} of BA + 0.5 mg l^{-1} of IAA were optimal for shoot formation capacity (44% and 12%, respectively) for flamingo-bill type and proximal cotyledon explants, respectively. Although proximal parts of the cotyledons had a higher frequency of adventitious shoot regeneration when compared with the distal parts of gourd (Han et al., 2004), in the present study flamingo-bill type explants had better shoot formation capacity than proximal explants. Han et al. (2004) established an efficient plant regeneration system for bottle gourd from cotyledon explants via organogenesis. They reported that maximum shoot regeneration was obtained from proximal parts of the cotyledons of 4-day-old seedlings cultured on MS medium containing 3 mg l^{-1} of BA (regeneration rate: 21.9%) and 0.5 mg l^{-1} of $AgNO_3$ (regeneration rate: 80.7%). Under dark conditions the medium containing 1 mg l^{-1} of BA resulted in the most efficient shoot formation capacity for flamingo-bill type (36%) and proximal cotyledon (12%) explants.

Explant cell division began after 3 days in regeneration medium and formation of primordium was observed in the tissue samples after 5-7 days in the culture. The formation of meristimatic structures were first observed after 9 days and development was completed after 9-12 days in the culture.

Yalcin-Mendi et al. (2003b) also reported that cell division began in the epidermal and subepidermal layers of melon cotyledon explants. The first meristimatic structures were observed in 10- and 14-day-old explants of Kırkağaç 637 and Ananas varieties, respectively. The anatomy and morphology of bud regeneration was investigated in melon (*Cucumis melo* L.) cv. Galia, which regenerates only in vitro via direct organogenesis from proximal cotyledon explant of 3-day-old in vitro seedlings (Gaba et al., 1999). Gaba et al. (1999) reported that the first sign of development was visible on the adaxial surface adjacent to the proximal cut edge and that protuberances were observed after 10 days. The first regenerated shoot buds and shoots were observed histologically after 15 and 22 days, respectively (Gaba et al., 1999). Yalcin-Mendi et al. (2003a) investigated the morphology and histology of regeneration in watermelon. Histological analysis showed that cell division occurred in the epidermal and subepidermal layers. Protuberant structures were observed in tissues after 7-12 days in culture. Meristematic structures were observed after 12 days in culture, which later developed into buds. Findings in the present study related to cell division, the promeristem and meristem, and shoot formation are similar to those reported by Gaba et al. (1999) and Yalcin-Mendi et al. (2003a).

In summary, we reported an efficient plant regeneration system and the histological basis of regeneration by direct organogenesis using flamingo-bill type explants excised from 7-day-old seedlings following in vitro germination in bottle gourd. Improving the efficiency of regeneration and histologic analysis of regeneration will aid future transformation studies of bottle gourd.

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