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Some Morphological and Anatomical Aspects of Date Palm *(Phoenix dactylifera L.)* Somatic Embryogenesis in Tissue Culture

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ABSTRACT

This study was carried out to investigate the morphological and anatomical aspects of somatic embryogenesis in date palm. Lateral bud and shoot tip explants excised from young offshoots were cultured on MS medium with 2,4-D. Somatic embryogenesis was induced by transferring the calli produced on the same medium without hormones. Microtome sectioning of paraffin-embedded specimens was carried out using the callus tissue and its differentiated structures. The sections were stained with safranin and fast green. Observation of three-celled proembryos with the longitudinal and oblique division of the top cell, which in later stages results in wedge-like cell(s), supports the ASTERAD type of embryogenesis in date palm. Polyembryonic structures were raised from the embryonic callus formed in different regions of both the proembryos and germinating embryos and the secondary embryos formed directly from primary embryos.

Keywords: Embryogenesis, *Phoenix dactylifera,* Somatic polyembryogenesis.

INTRODUCTION

In spite of the great potential of tissue culture techniques for plants, there are some difficulties in the propagation and breeding of woody plants like date palm. An extensive effort has been carried out over the last two decades to establish an effective micropropagation system (Rynolds and Murashige, 1970; El-Hennawy and Wally, 1980; Zaid and Tisserat, 1983; Gaber and Tisserat, 1985; Litz, 1985; Sharma *et al.*, 1986; Omar, 1988; Omar and Novak, 1990). Somatic embryogenesis has been accomplished in several palms, including date palm, but only a small percentage of the somatic embryos produce vigorous plantlets (Litz, 1985). Increasing the potential for embryogenesis, providing synchronized cultures and increasing the conversion rate of the embryos to plantlets are very important factors in improving somatic embryogenesis efficiency (Fransz and Schel, 1991; Goebel-Tourand *et al.,* 1993).

Studying the morphological, biochemical and microscopic aspects of embryogenesis at different stages in tissue culture can be a guide for increasing the effectiveness of the system, in addition to increasing our knowledge about *in vitro* associated phenomena (Zaid and Tisserat, 1983; Rodriguez *et al.,* 1990; Cellarova *et al.,* 1992). In a few histological studies on date palm somatic embryogenesis, similarity in somatic and zygotic embryogenesis has been mentioned (Nazeri *et al.,* 1993; Tisserat and Demason, 1980), although the different investigators have not reached the same conclusion about the developmental pattern of zygotic embryos in *Phoenix.* The potential for obtaining somatic embryos from callus tissue produced from lateral buds in Estameran and Kabkab cultivars has been reported (Majidi

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et al., 1991; Nazeri *et al.,* l993). This study has been carried out to investigate the morphological and anatomical aspects of somatic embryogenesis in the above cultivars.

MATERIAL AND METHODS

Tissue Culture

Three to four years old offshoots of date palm cv. Estameran and cv. Kabkab served as source of the material. Leafy lateral buds and shoot tips were collected after removing the leaves and fiber sheaths acropetaly with help of a knife. Excised lateral buds and tips were kept for 24 hr in an antioxidant solution containing 150 and 100 mg/l citric acid and ascorbic acid, respectively. The explants were then sterilized for 15 min. using 9% calcium hypochlorite as surface disinfectant followed by washing with sterile distilled water 3 times under aseptic conditions. The tip was trimmed to about 2 centimeter length and was cut into 10 small segments. The lateral bud also was cut into four segments and along with the tip segments were initially cultured on the nutrient medium comprising MS inorganic salts; thiamin-HCI, 0.1 mg/l; myo-inositol, 100 mg/l; 2,4-dichlorophenoxy acetic acid (2,4-D), 100 mg/l; 3% sucrose; 0.3% activated charcoal and 0.8% agar. The cultures were placed in a dark growth chamber at 28 C for two mounths. At eight-week intervals, they were subcultured in order to obtain callus with granular appearance. For inducing morphogenesis, the same growth medium without hormones was used. These cultures were kept at 28 C and a photoperiod of 16/8 with light intensity of 2000 lux.

Histological Studies

The callus tissue and differentiated structures were sampled at different stages (initial, secondary and differentiated calli) fixed in FAA (Formalin: Acetic acid: Alcohol 90:5:5) for 24 hours then dehydrated using 50, 70, 80, 90% and absolute concentrations of ethanol (Gray, 1958; Johanson, 1940; Sass, 1958). After dehydration, the specimens were embedded in paraffin (MERCK, solidification point about 57-60 C) and cut into 5-10 μm sections. The sections were double-stained with safranin and fast green FCF (Yilun *et al.,* 1992).

RESULTS

During the first 2-3 weeks of culture, the explants grew in length and thickness and callus production occurred after six to eight weeks. The callus tissue was morphologically a mass of transparent and hydrated clear white cells (Figure 1a). Subculturing and propagating these calli in three successive stages, of eight weeks each, resulted in a pearl-type callus which was brittle and white (Figure 1b). One of the aspects noticed within the callus tissue was a mass of cells which included embryogenic meristematic cells with dense cytoplasm in the border areas and vacuolated cells without meristematic activity in the inside. Furthermore, in some parts of this callus, proembryos were densely packed next to each other and separated from each other by a rather thick wall (Figure 2a). The most frequent structures observed in these sections were proembryos at different stages (Figures 2b and 2f. Three-celled proembryos produced as a result of the first longitudinal and oblique division of two-celled proembryos (Figures 2b and 2c) as well as proembryos with suspensor-like structures (Figure 2d), embryo-like bodies with numerous meristematic sites and embryos producing secondary embryos by means of budding were also observed in the callus. Completely differentiated embryos that resembled the mature zygotic embryo (Figure 3a) were not observed in this study.

By transferring the callus tissues to the medium without hormones, the maturation and germination of the embryos took place over several weeks. During the first step of germination, the epicotyl grows and causes cotyledonary sheath. Later, the shoot pole

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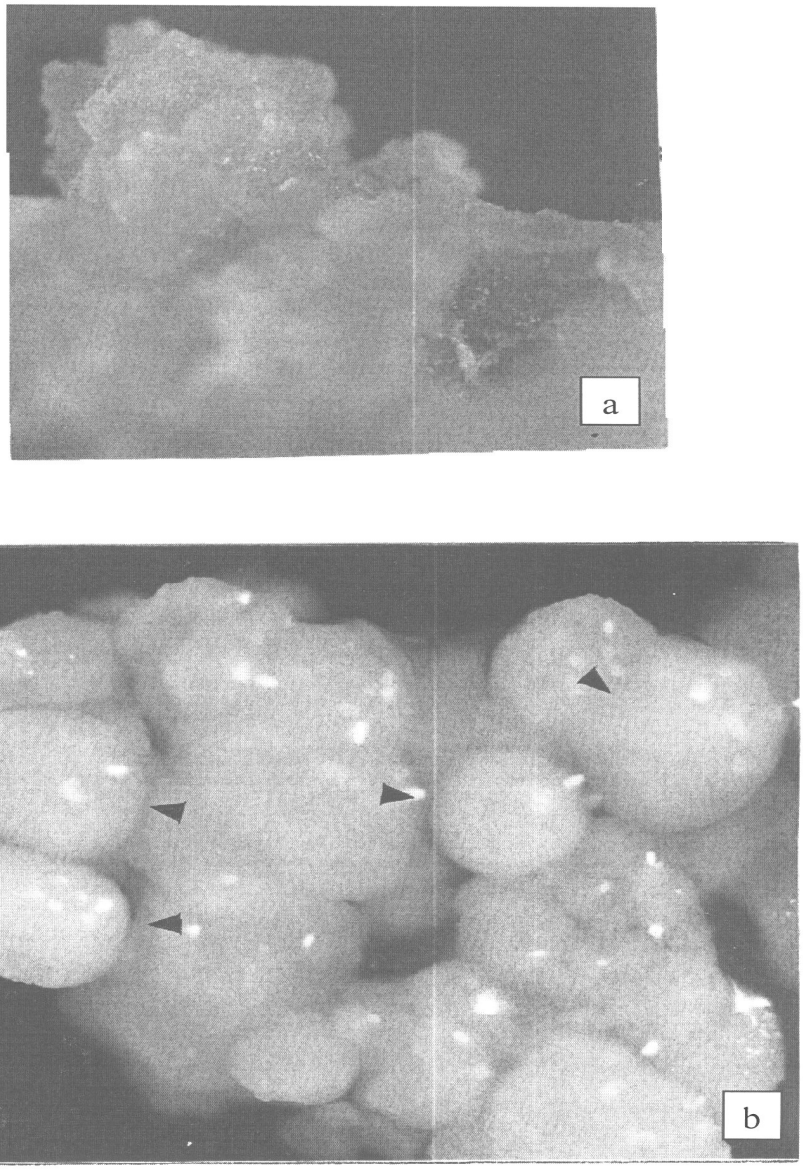

Figure 1. Two different kinds of callus tissue in date palm. Initial callus tissue with transparent and hydrated cells (a). Close-up photo of granular callus showing the embryonic structures of various sizes. Embryoids (arrowheads) (b).

will begin to grow, elongate and open the cotyledonary sheat (Figure 3b).

Some somatic embryo haustoriums in our study, however, were inflated and bladder-like in appearance, in spite of the fact that these haustoriums were not connected with the endosperm (Figure 4a). In some cases, callus formation occurred at the root tip of the germinated embryos (Figure 4b). In sections from some of the healthy embryos at germination, these structures had numerous meristematic sites.

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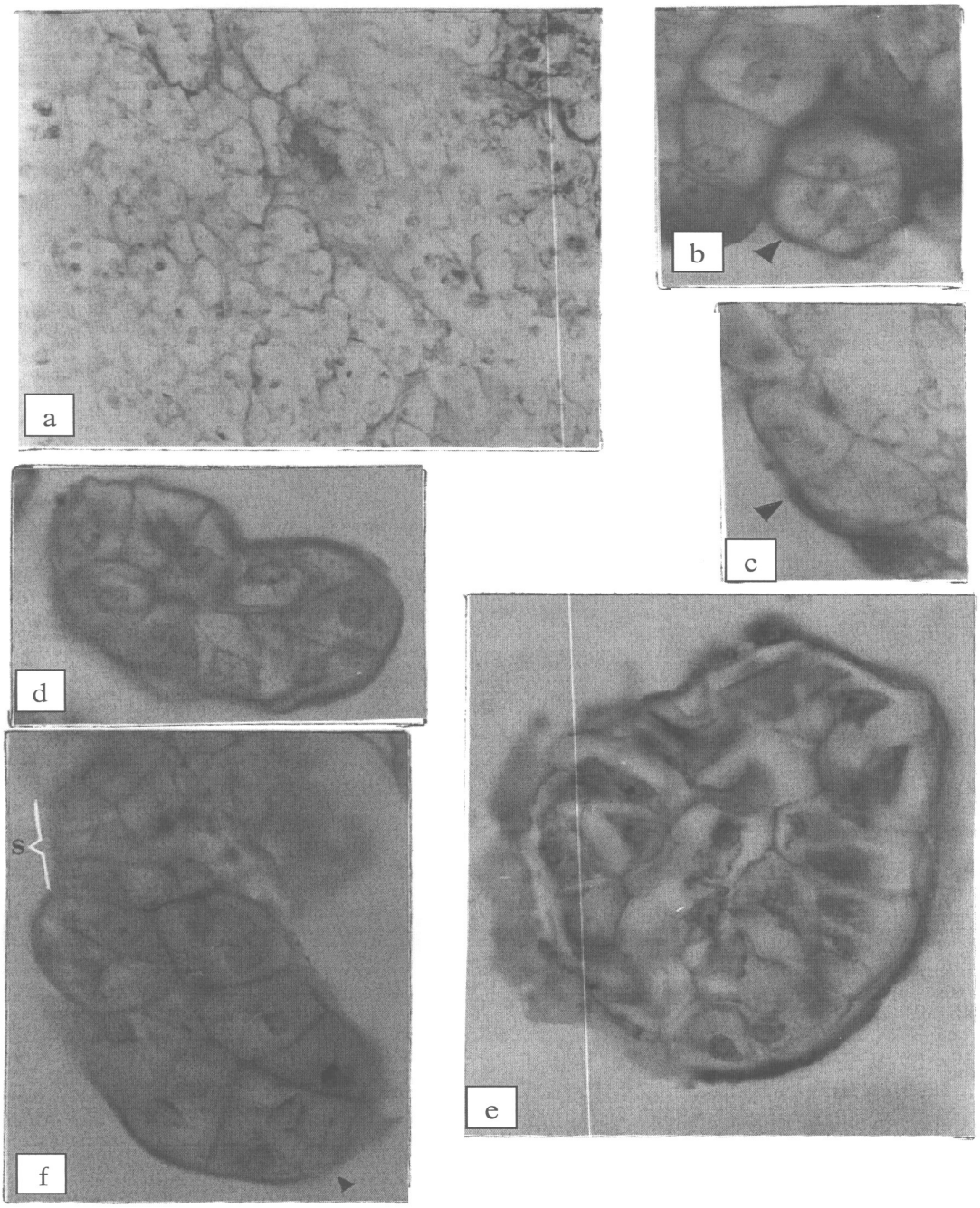

Figrue 2. A photomicrograph showing proembryos that are densely placed next to each other. Note the thickened walls separatng them, (450 X) (a). Three-celled proembryos (arrowhead). Note the longitudinal and oblique division of the top cell, (1130 X) (b, c). Individual somatic proembryos at different stages. Apparently, proembryo at pre-globular (d) and globular stage (e), (1130X) (e). A well-developed proembryo with suspensor (s). Note the posterior wedge-like cell (arrowhead) as a result of the longitudinal and oblique division of the top cell at the two celled stage, (1130 X) (f).

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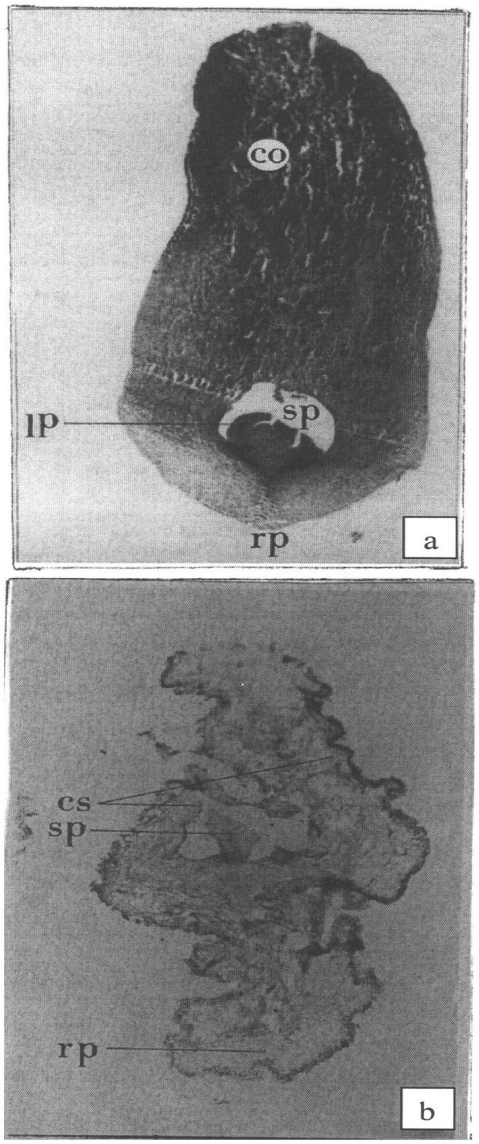

Figure 3. Perfectly mature embryo of date palm seed. Shoot pole (sp); root pole (rp), cotyledon (co), leaf primordium (lp), (60 X) (a). Opening the cotyledonary sheath (cs) and the appearance of the shoot pole (sp) and root pole rp), (27 X) (b).

DISCUSSION

The review of literature indicates that, among different scientists, there is disagreement on the pattern of embryogenesis in the date palm family. The first zygotic division results in two cells, one top and other basal. The respective contribution of these two cells to the formation of the proper embryo is a matter of dispute. In Phoenix, the basal cell plays a minor role in the formation of the embryo proper. This pattern is classified as Asterad according to Johanson (Johri, 1984). By observing the linear proembryos in the four-cell stage, Tisserat and DeMason (1980) deduced that none had the Asterad or Onagard patterns suggested for the palm family. A linear proembryo with four cells is classified as Solanad or Chenopodiad in which the first division of the top cell is transverse (Johri, 1984). However, the observation made in our studies, in relation to the three cell proembryos with the first longitudinal and oblique division of the top cell, supports the Asterad interpretation of embryogenesis in date palms. The manner of the first division of the top cell results in a posterior wedge-like cell in later stages, if this embryogenesis has a good genetic stability and is little affected by environmental conditions. A distinct example of genetic instability is the varied shapes of the haustorium in the somatic embryos. It has been mentioned in the literature that the zygotic embryo haustorium is inflated and has a bladder-like shape due to the spatial configuration of the site where it comes in contact with endosperm, but the haustorium of somatic embryos are round and finger like (Mendoza *et al.,* 1993). Some haustoriums in our study, however, were inflated and bladder-like.

Comparing the number of proembryos and of germinated ones it becomes clear that the high percentage of proembryos lose the potential to reach maturity. If proembryos remain on a medium supplemented with 2, 4-D, this may cause them to lose their potential for maturation (Tautorus *et al.,* 1991). On the other hand, if germinating embryos remain on the same medium this may stimulate callus formation in their tissues and organs. At this time, the root which remains inside the medium will be more exposed to callus formation. Embryonic callus formation does not include only the root tip, but may include cotyledon and the haustorium.

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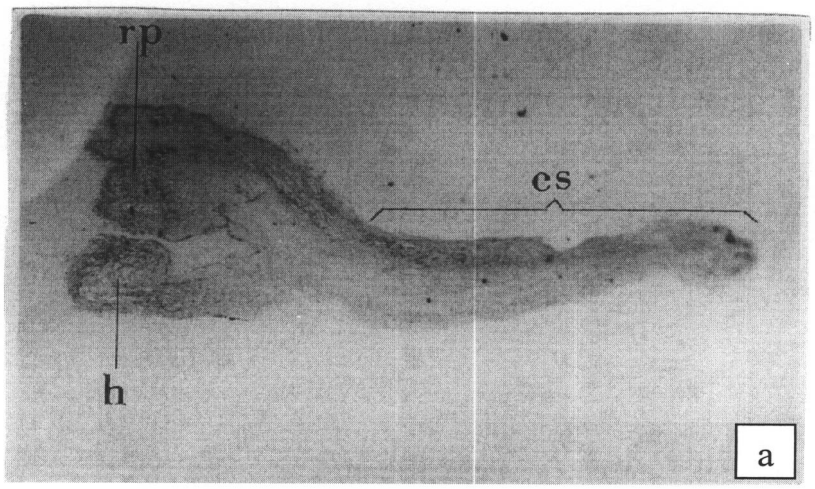

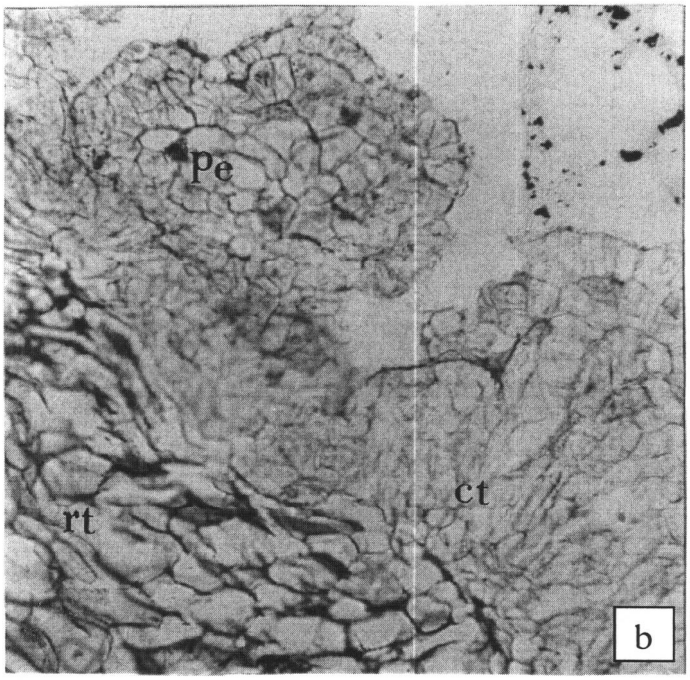

Figure 4. Longitudinal section of a germinating somatic embryo in which the haustorium (h) is bladder-like in appearence. Cotyledonary sheath (cs), root pole (rp), (22 X) (a). Callus formation at the polar end of a root. Root tissue (t), callus tissue (ct), proembryo (pe) (160 X) (b).

Dedifferentiation of the root will cause a lack of root formation. Lack of roots and the means of nutrient transport to the growing points of the germinating embryo weakens the aerial parts. Slow germination of the date palm mature embryos seems to be due to the lack of endosperm tissue and the existence of a small haustorium as mentioned in literature (Litz, 1985).

Embryonic callus formation may cause the development of secondary embryos and they could also be formed directly from the pri-

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mary embryos. These two phenomena may cause the formation of polyembryonic structures under *in vitro* conditions, which in some cases are not separable. Of course, under natural conditions, some of the ovaries in the date palm may have polyembryos (Litz, 1985) but the origin of additional ones has not yet been studied. Observation of differentiated structures with numerous meristematic sites, embryonic callus formation and secondary embryos due to the budding of primary embryos in the granular callus indicates that the orginal phenomena of polyembryonic structures will occur at the first stages of culture and, therefore, it cannot easily be controlled. However, with hormonal control, it is possible to decrease it. The effect of Abscisic acid (ABA) on the embryogenesis has been discussed in several reports. Reduction of neomorphism, prevention of secondary somatic embryogenesis and improvement of growth and development of individual somatic embryos are several effects of this hormone (Nadal *et al.,* 1990; Nickle and Yeung, 1994; Tautorus *et al.,* 1991). Hence, it is recommended that experiments considering the effects of ABA on the *in vitro* embryogenesis of date palm be conducted.

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جنبه های مورفولوژیکی و آناتومیکی جنین‌زائی غیر جنسی در کشت بافت خرما

د. داودی، ا. مجیدی، و ص. خوشکام

چکیده

این مطالعه به منظور بررسی برخی خصوصیات مورفولوژیکی و آناتومیکی جنین‌زائی غیرجنسی در خرما انجام شد. ریزنمونه‌های آغازین جوانه و منطقه مریستم انتهایی از پاجوش‌های دو تا سه ساله پس از ضد عفونی سطحی روی محیط کشت پایه MS با ۱۰۰ میلی گرم تو، فور- دی در لیتر کشت داده شدند. پس از القاء جنین‌زائی غیرجنسی به کالوس‌های حاصله و انتقال به محیط کشت فاقد هورمون، قطعات کالوس و ساختارهای تمایز یافته آنها با استفاده از تکنیک قالب‌گیری پارافینی جهت تهیه برش‌های میکروسکوپی قالب‌گیری شدند. برش‌های میکروتومی با رنگ‌های SAFRANIN و FAST GREEN تحت رنگ‌آمیزی مضاعف قرار گرفتند. مشاهده پیش جنین‌های سه سلولی با تقسیم طولی و اریب سلول راسی که در مراحل بعد منجر به ایجاد سلول گوه مانند در پیش جنین می‌شود تاییدی بر مدل "ASTERAD" در جنین‌زائی خرما می‌باشد. تولیدمستقیم جنین‌های ثانویه روی جنین‌های اولیه، تشکیل کالوس جنین‌زا درمناطق مختلف

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پیش جنین‌ها وجنین‌های درحال جوانه‌زنی منشا تولیدساختارهای چندچنینی درکشت بافت خرما هستند.

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