



Effects of some plant growth regulators and light on callus induction and explants browning in date palm (*Phoenix dactylifera* L.) *in vitro* leaves culture

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Abstract

The present study was carried out to evaluate the effects of light and plant growth regulators on callus induction and explant browning in date palm cultivar, Estameran in *in vitro* condition. The explants were pretreated by an antioxidant combination (100 mgL⁻¹ citric acid and 150 mgL⁻¹ ascorbic acid) for 3 days in 5 °C. Then, the leaf explants were cultured in Murashige and Skoog medium (MS) with 3 gL⁻¹ of activated charcoal, 75 mgL⁻¹ citric acid and 75 mgL⁻¹ ascorbic acid, for 14 days. After that, explants cultured in callogenesis medium were supplemented with 12 different levels of plant growth regulators including 2,4-D (0 and 5 mgL⁻¹), TDZ (0 and 5 mgL⁻¹), and BAP at three concentrations (0, 5, and 10 mgL⁻¹), and two incubation conditions (light condition and absolute darkness). The number of explants that induced callus and browning was recorded after six months. Results indicated that 2,4-D and BAP increased browning and this was exacerbated under light condition. Highest callus induction was achieved in medium supplemented with 5 mgL⁻¹ BAP or with 5 mgL⁻¹ TDZ. This experiment showed that low concentrations of cytokinin induced callus in date palm *in vitro* leave culture and callus induction increased in darkness.

Keywords: Adult leaf explants; callogenesis; date palm; light and dark

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Introduction

Date palm (*Phoenix dactylifera*L.) (2n=2x=36) is a dioecious perennial monocotyledon fruit tree that belongs to the family of Arecaceae (Barrow, 1998). Date palm is one of the most economically

important perennial plant in arid areas of the Middle-East and the North Africa (Awad, 2007), where it is widely cultivated for food and many other commercial purposes. Iran is allocated to about 14% the cultivated area and 18% world production for date palm (FAO, 2010).

Date palm propagates either by seeds or offshoots, but resulted seedlings generally differ

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considerably in fruit quality, harvesting time, and production potential. Seed propagation method has been used more often by the fact that date palm produces relatively few offshoots suitable for transplanting in its lifetime (Zaid and De Wet, 2002). Tissue culture propagation method is the most promising technique for production of sufficient plant materials with high quality (Sane et al., 2006).

Review of the literature shows a number of reports published on palm tissue culture (Schroeder, 1970; Reuveni, and Kipnis, 1974; Ammar and Benbadis, 1977; El-Hannawy and Wally, 1978; Reynolds and Murashige, 1979; Tisserat et al., 1979; Aberlenc-Bertossi et al., 1999; Te-chato, 2002). Callogenesis is the first step of plantlet regeneration, which has created unlimited production of clones with elite traits. However, most protocols were proposed by using apical shoot tips which have been most responsive to *in vitro* culture.

The palm leaves have been treated less than shoot tip for *in vitro* micropropagation. Juvenile leaves were used from embryogenic suspension cultures (Fki et al., 2003; Othmani et al., 2009) and leaf segments from seedling's plantlet cultures for callogenesis and rhizogenesis (Gueye et al., 2009). 2,4-D is the most commonly used auxin that have been used to exert a stimulatory effect on *in vitro* regeneration in plants (Lakshmanan, et al., 2002). Zaerr and Mapes (1982) reported that phenoxy auxin such as 2,4-D showed strong effects on callus growth. Victor et al., (1999) indicated that, TDZ effectively induced somatic embryogenesis within a relatively short exposure time. It is possible to speculate a potential dual role for TDZ in induction of somatic embryogenesis. It seems that a cytokinin-like activity is crucial to promotes cell division and differentiation for induction of embryogenic competence. BAP has been reported to enhance multiple shoot formation (Adelberg et al., 1997). Application of BA and NAA has improved callus induction rate in *Eustoma grandiflorum* L. (Rezaee, et al., 2012).

The object of this study was to evaluate the induction of callogenesis in a high quality genotype of date palm (Estameran) originated from Khuzestan region of Iran (semidry cultivar with 16-18% moisture content, and 70% sugar in

their fruits), through adult leaves. Due to the advantages mentioned, in this study effects of plant growth regulator on callogenesis and embryogenesis and organogenesis of adult leaf explants were studied. Callogenesis occurred in dark conditions (Othmani et al., 2009) or in alternation of dark and light (Zaid et al., 2011). In past studies on date palm tissue culture the effect of light was not evaluated; therefore this study is the first study which reports the effect of light factor on induction of callus and explant browning.

Materials and Methods

The experiment was conducted as a factorial split plot based in a completely randomized design (CRD) with three replications. The first factor was 2 concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D); the second factor was 6-Benzyladeninepurine (BAP) with 3 levels; the third factor was thidiazuron (TDZ) with 3 levels of (a total of 12 levels of growth regulator treatments), and the fourth factor was the presence or absence of light in the sub-plots.

Explants preparation and sterilization

The youngest leaves, adjacent to apex of offshoots were sampled from adult date palm plants (*Phoenix dactylifera* L.) of Estameran genotype that was growing in nursery plant, Ramin University of Agriculture and Natural Resources, Khuzestan (Fig. 1 A). Primary foliage (20-30 number) from each alternate leaf were removed and sectioned into 5-6 cm segments. To prevent browning of explants, the leaf segments were transferred to a beaker containing an antioxidant solution (100 mg^l⁻¹ citric acid and 150 mg^l⁻¹ ascorbic acid), covered with aluminum covering and maintained for 3 days at 5 °C (Fig. 1 B). Then the samples were cut into 1.5-2 cm explants by using a sharp sterilized blade after elimination of the narrow part of top and bottom of each explants. Explants were surface sterilized by soaking in 5.25% sodium hypochlorite (NaClO) for 10 min and rinsed three times with sterile distilled water.



Fig. 1. Different stages of culture from adult leaf explants of *Phoenix dactylifera* (CV. Estameran). A: The last and youngest adult leaves were sampled from explants; B: Antioxidant pre-treatment included 100 mg l^{-1} citric acid and 150 mg l^{-1} ascorbic acid, for 3 days at 5° C; C: Initiation of culture in MS medium plus the 30 g l^{-1} sucrose, 8 g l^{-1} Agar-Agar, 3 g l^{-1} activated charcoal, 75 mg l^{-1} citric acid, 75 mg l^{-1} ascorbic acid

Table 1

Treatments of plant growth regulators (mg l^{-1}) for adult leaf explants of *Phoenix dactylifera*

Treatment number	1	2	3	4	5	6	7	8	9	10	11	12
2,4-D (mg l^{-1})	0	0	0	0	0	0	5	5	5	5	5	5
TDZ (mg l^{-1})	0	0	0	5	5	5	0	0	0	5	5	5
BAP (mg l^{-1})	0	5	10	0	5	10	0	5	10	0	5	10

Initiation stage

The initiation stage was composed of MS (Murashige and Skoog, 1962) medium, supplemented with 30 g l^{-1} sucrose, 8 g l^{-1} Agar-Agar, 3 g l^{-1} activated charcoal, 75 mg l^{-1} citric acid and 75 mg l^{-1} ascorbic acid (Khan and BI BI, 2012; Othmani et al., 2009). The pH of the medium was adjusted to 5.7 and then sterilized in autoclave at 121° C for 15 minutes. Leave segments were cultured in Petri dishes (80 mm diameter \times 15 mm high and 20 ml media volume) aseptically (Fig.1 C). Cultures were incubated at dark condition, to reduce phenolic components of explants for 14 days.

The explants were cultured on MS medium supplemented with 30 g l^{-1} sucrose, 8 g l^{-1} Agar-Agar, 3 g l^{-1} activated charcoal and 12 different combinations of plant growth regulators (Table 1). The plant growth regulators included 2,4-D at 2

concentrations (0 and 5 mg l^{-1}), BAP at 3 concentrations (0, 5 and 10 mg l^{-1}), and TDZ at 2 concentrations (0 and 5 mg l^{-1}). After an initial period of 14 days of incubation in dark, three replications of the cultures were incubated in a controlled growth chamber at 25 \pm 1° C with a 16 h photoperiod under low light intensity (3000 lux) provided by fluorescent tubes and three replications remained in the dark for 6 months. The subculture was conducted every four weeks, until callus induction event was recorded.

Statistical analysis

Analysis of variance (ANOVA) was done using the SAS (1988) statistical software package version 9.2. The mean comparison was done by Duncan's test at 1% and 5% levels of probability.

Table 2

Analysis of variance for plant growth regulators and light factor on callogenesis induction and browning in adult leaf explants of date palm

Source of variations	d.f.	mean squares	
		browning	callogenesis
PGRs	27	3.686 **	2.660 **
light or dark	1	6.125 **	4.500 **
Error (A)	4	2.666 *	2.586 *
2,4-D	1	55.125 **	12.500 **
TDZ	1	0.125	0.5
BAP	2	2.625 **	1.500 *
light × 2,4-D	1	1.125	0.5
light × TDZ	1	0.125	0.5
light × BAP	2	0.875	1.500 *
2,4-D × TDZ	1	3.125 *	0.5
2,4-D × BAP	2	1.125	0.5
TDZ × BAP	2	0.875	6.500 **
light × 2,4-D × TDZ	1	3.125 *	0.5
light × 2,4-D × TDZ	2	0.375	0.5
light × TDZ × BAP	2	0.125	0.5
2,4-D × TDZ × BAP	2	3.875 **	9.500 **
light × 2,4-D × TDZ × BAP	2	0.125	0.5
BAP			
Error (B)	44	0.484	0.401
C.V		50.6	84.4

* ** -0.05 and 0.01 levels of significance

Results

The first symptoms of callus induction appeared after 10 weeks from the start of the experiment (Fig. III A); callusing and browning development continued for 6 months of culture initiation.

Effect of plant growth regulators and light on browning in adult leaf explants

Effect of light on browning was significant (Table 2, Fig. II) and the minimum browning occurred in dark condition without plant growth regulators. The maximum browning occurred in light condition with plant growth regulators in concentrations of 5 mg l⁻¹ TDZ and 5 mg l⁻¹ TDZ + 5 mg l⁻¹ BAP (Fig. IV B), and in dark condition with 5 mg l⁻¹ TDZ (Fig. IV C), respectively. Plant growth regulators (especially 2,4-D) increased browning and this phenomenon was exacerbated in light condition, 16/8 hours. The results showed that the concentrations of PGRs (2,4-D, TDZ and BAP) showed significant effect on browning (P≤0.05)

(Table 2). The comparison of the effects of PGRs concentrations (Fig.III) showed that the absence of 2,4-D caused less browning in explants, especially if this absence was accompanied by application of 10 (mg l⁻¹) of BAP or 5 (mg l⁻¹) of TDZ (Fig. IV A). Increase in concentration of BAP, increased browning (Fig. IV D). This result confirms that light has intensified the destructive effects of 2,4-D.

Effect of plant growth regulators and light on callogenesis in adult leaf explants

According to the data that presented in Fig. V, the callogenesis in dark condition was more than the light condition (P≤0.01). The data presented in Table 1 and Fig. VI indicate that plant growth regulators (2,4-D, TDZ, and BAP) were effective on callogenesis (P≤0.01). The highest regeneration ratio was observed in medium supplemented with 5 (mg l⁻¹) of BAP or with 5 (mg l⁻¹) of TDZ (Fig.VII B) and the callus induction was less in other treatments. The calluses were not formed in the treatment supplemented with 5 (mg

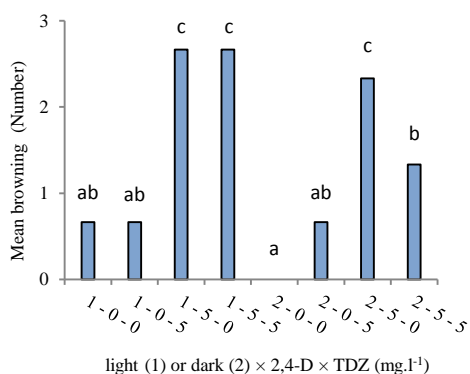


Fig. II. The comparison of effect of light and concentrations of 2,4-D and TDZ (mg l⁻¹) on browning in adult leaf explants of date palm cv. Estameran; Means followed by the same letter in the same column are not significantly different as indicated by Duncan's Multiple Range Test at P = 0.05.

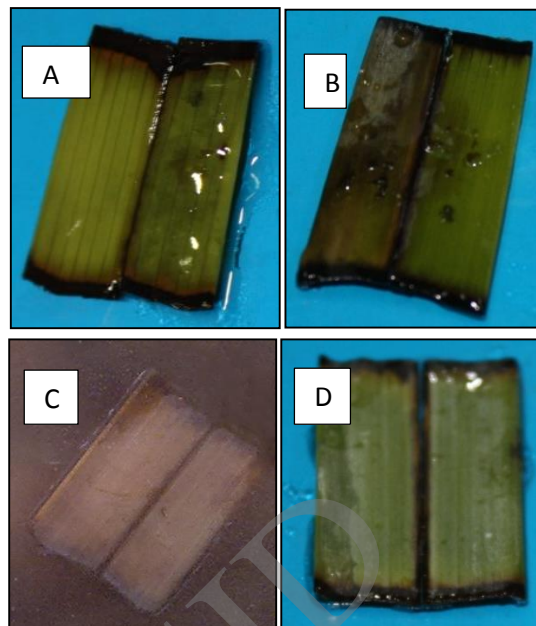


Fig. IV. Effect of plant growth regulators concentrations and light on browning in adult leaf explants of *Phoenix dactylifera* (cv. Estameran); A. Minimum browning in treatment with 5 mg l⁻¹ TDZ at light condition; B. Effect of light on browning increased in treatment with 5 mg l⁻¹ TDZ + 5 mg l⁻¹ BAP; C. Effect of dark condition on browning decreased in treatment with 5 mg l⁻¹ TDZ; D. Death of treatment with 5 mg l⁻¹ 2,4-D + 10 mg l⁻¹ BAP.

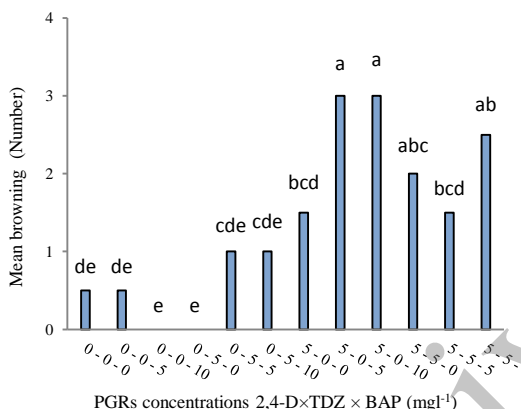


Fig. III. The comparison of effect of plant growth regulators concentrations (2,4-D×TDZ × BAP (mg l⁻¹) on browning in adult leaf explants of date palm cv. Estameran; Means followed by the same letter are not significantly different as indicated by Duncan's Multiple Range Test at P = 0.01.

l⁻¹) of TDZ + 5 (mg l⁻¹) BAP, 5 (mg l⁻¹) 2,4-D + 5 (mg l⁻¹) BAP, 5 (mg l⁻¹) 2,4-D + 10 (mg l⁻¹) BAP.

Discussion

Browning and callus induction

In this research we used antioxidant pre-treatment (100 mg l⁻¹ citric acid and 150 mg l⁻¹ ascorbic acid) for 3 days in 5° C which decreased explant browning. According to Zaid (1987) browning of date palm tissue and the adjacent medium is assumed to be due to phenolic compounds oxidation. Antioxidant pre-treatment

was effective against explant browning. This finding was in agreement with the results of Mustafa et al. (2013), Khan and Bibi (2012), and Othmani et al. (2009).

In studies on young leaf explants of date palm, both embryogenic calli and somatic embryogenesis formed with 10 mg l⁻¹ of 2,4-D (Othmani et al., 2009). The combination of 2,4-D+NAA+2,4,5-T/TDZ+BAP induced direct embryogenesis in culture of cotyledonary nodes of oil palm (Jayanthi et al., 2011). The application of 2,4-D / TDZ (10 / 10 mg l⁻¹) produced somatic embryogenesis by shoot tips culture (Sidky and Eldawyati, 2012). The ratio of auxin and cytokinin (10 / 0, 4 / 0 or equal) is required for organogenesis. In Damask rose thidiazuron

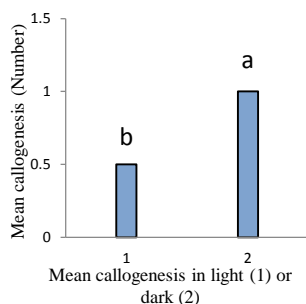


Fig. V. The comparison of effect of light on callogenesis in adult leaf explants of date palm cv. Estameran; Means followed by the same letter in the same column are not significantly different as indicated by Duncan's Multiple Range Test at $P \leq 0.01$.

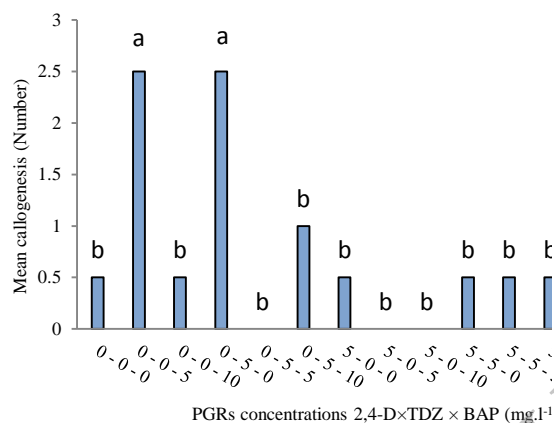


Fig. VI. The comparison of effect of PGRs concentrations (2,4-D x TDZ x BAP (mg l^{-1}), on callogenesis in adult leaf explants of date palm cv. Estameran; Means followed by the same letter in the same column are not significantly different as indicated by Duncan's multiple range test at $P \leq 0.01$.

(TDZ) in combination with benzylaminopurin (BAP) induced significantly higher number of shoots per explants than the most optimum BAP treatments alone (Mamaghani et al., 2010) and in safflower the leaf explants produced callus on MS medium supplemented with 1.0 mg l^{-1} BAP (Ghasempour et al., 2014).

In the present experiment the concentration of $5 \text{ (mg l}^{-1}\text{)}$ of 2,4-D was used (Table 1) because the increase in 2,4-D concentration enhanced somaclonal variation whereas the lower concentrations improved callogenesis, these results are similar with findings of McClintock (1984). Mean comparison showed application of $5 \text{ (mg l}^{-1}\text{)}$ of 2,4-D in comparison with non-

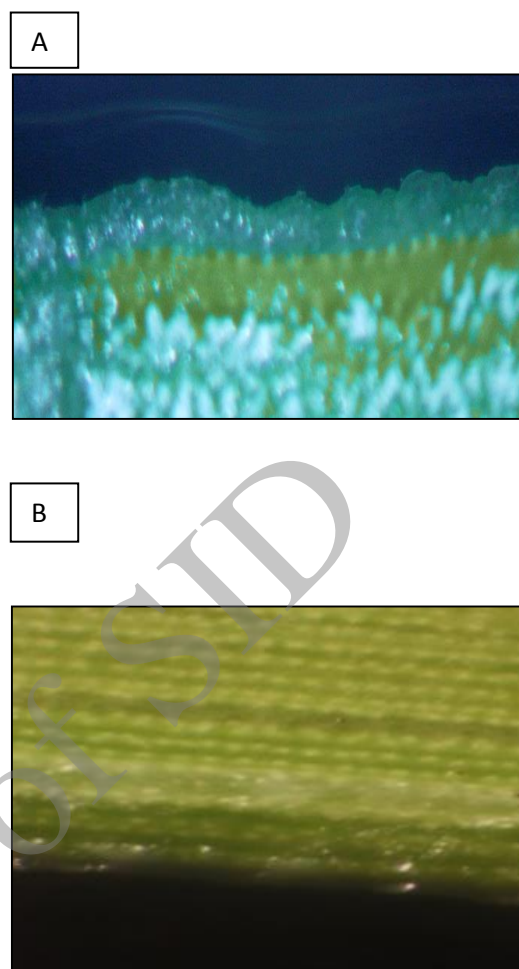


Fig. VII. Effect of plant growth regulators concentrations and light on callogenesis in adult leaf explants of *Phoenix dactylifera* (cv. Estameran); A. callogenesis in edge of leaf explants, the shape of serrated swelling; B. Highest callogenesis in treatment with 5 mg l^{-1} BAP.

application increased callogenesis, but no organogenesis was observed in this research.

Effect of plant growth regulators and light on browning in adult leaf explants

Browning the tissue of the date palm is assumed to be due to the oxidation of phenolic compounds and formation of quinines which are toxic to the tissues (Zaid, 1987). According to Kefeli (1978) the internal growth regulator is influenced by duration, intensity, light quality, and activity of polyphenol oxidase. The internal growth regulator is more effective in light than darkness.

Joshaghani et al (2014) indicated that in Artichoke the amount of produced phenolic component increased when the concentration of 2,4-D increased to 0.75 mg l⁻¹.

In this research it was perceived that the cultures incubated in light condition were weak and yellowish, and died after 6 months; but the ones that were incubated in dark may have secreted less phenolic compounds. Othmani et al., (2009), reported that callusing from young leaf explants date palm occurred after 8 months in dark condition and this was similar to our results.

Effect of plant growth regulators and light on callogenesis in adult leaf explants

Cytokinins are required for callus induction and cell division (Minocha, 1987). The balance between auxin and cytokinin application is needed for callus formation (Rout, 2004). The results of this experiment showed that application of high concentrations of cytokinin, caused browning and death in callus. Vescovi et al. (2012) also found that the use of high levels of BA (9 mg l⁻¹) induced apoptosis of the cells in cultures of *Arabidopsis thaliana*.

This result developed an initial protocol for callus induction and micropropagation of leaf explants. The type of plant growth regulators and conditions of explants incubation were found to have significant effect on callus induction and browning of explants. Callus induction was more in darkness. Treatment with higher levels of cytokinin (especially BAP) decreased browning, but its low concentrations improved callus induction. This successful protocol would provide a suitable technique for callus induction of leaf explants and help to clear up the way for determining best plant growth regulators treatments for organogenesis. It also would facilitate the vegetative propagation, conservation, and genetic engineering of this species.

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تأثیر تعدادی از تنظیم کننده های رشد و نور بر القاء کالوس و قهوه ای شدن ریزنمونه در کشت درون

شیشه ای برگ خرما (*Phoenix dactylifera L.*)

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چکیده

تحقیق حاضر بر روی تأثیر تنظیم کننده های رشد و نور بر القاء کالوس و قهوه ای شدن ریز نمونه در شرایط درون شیشه ای در خرما، رقم استعمران می باشد. ریزنمونه ها به وسیله ترکیبی از آنتی اکسیدانت ها (100 mgL^{-1} اسید سیتریک و 150 mgL^{-1} اسید آسکوربیک) برای ۳ روز در دمای 5°C پیش تیمار شدند. سپس ریز نمونه های برگ در محیط کشت موراشینگ و اسکوگ (MS) با 3 gL^{-1} زغال فعال، 1 mgL^{-1} 75 mgL^{-1} اسید سیتریک و 75 mgL^{-1} اسید آسکوربیک برای ۱۴ روز کشت شدند و پس از آن در محیط کشت با ۱۲ سطح مختلف از تنظیم کننده های رشد شامل 2.4-D (0 و 5 mgL^{-1})، TDZ (0 و 5 mgL^{-1}) و BAP در سه غلظت (0 ، 5 و 10 mgL^{-1}) و شرایط نگهداری (نور و تاریکی) کشت شدند. بعد از ۶ ماه تعداد ریز نمونه هایی که در آنها القاء کالوس رخ داده بود و میزان قهوه ای شدن ثبت شد. نتایج نشان داد که تنظیم کننده های رشد 2.4-D و BAP میزان قهوه ای شدن را افزایش می دهند که این اثر در شرایط وجود نور افزایش می یابد. بالاترین میزان کالوس زایی در محیط کشت حاوی تنظیم کننده های رشد 5 mgL^{-1} BAP یا 5 mgL^{-1} TDZ رخ داد. این تحقیق نشان داد غلظت پایین سایتوکینین کالوس زایی را در کشت درون شیشه ای برگ خرما القاء می کند و القاء کالوس در تاریکی افزایش می یابد.

کلمات کلیدی: ریزنمونه برگ بالغ، کالوس زایی، خرما، نور و تاریکی.