PLANT TISSUE CULTURE

In vitro callus induction from adult tissues of peach (*Prunus persica* L. Batsch)

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Abstract We describe an efficient protocol for callus induction from adult tissues of *Prunus persica* (L.) Batsch. Three different commercial peach genotypes, Early May®, Zise May®, and UFO-3®, plus three other genotypes from hybrid crosses performed in February 2006, PS108, PS208, and PS708, were used in the study. Thirteen explant treatments were tested using nine different plant parts. Murashige and Skoog and woody plant medium salts were assayed with several concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (KN), and thidiazuron, and two different photoperiods were tested, a 16-h photoperiod or continuous darkness. In terms of the quantitative response, two parameters were assessed: the number of d to callus induction and relative callus growth recorded after 30 d. Woody plant medium supplemented with 2,4-D and KN significantly increased the rates of callus induction in the majority of treatments. And no significant differences among the *P. persica* genotypes were found. The explants derived from stem and calyx produced up to 85 and 96% callus induction, respectively. The protocol described here could be used for efficient callus induction in a range of *Prunus* spp.

Keywords *Prunus* · Nectarine · WPM · 2,4-D · TDZ · KN

Introduction

Callus is an amorphous and dedifferentiated tissue composed of disorganized cells. It may be produced naturally in response to insect or microorganism attack or stress (George 1993). Several *in vitro* biotechnological techniques have been developed, all of which require a reliable protocol to produce a responsive cell mass. Unorganized cells of *Prunus* spp. have traditionally been cultured for protoplast fusion of different individuals, somatic hybridization (Hidano and Niizeki 1988), to obtain haploids (Peixe *et al.* 2004), or for induction of tolerance to low temperatures (Arora and Wisniewski 1995). Callus induction has also been used for genetic transformation (Scorza *et al.* 1994) and adventitious regeneration (Gentile *et al.* 2002), which is the initial phase in a transformation protocol.

Peach is one of the most widely consumed fruits in the world, but its recalcitrance in many biotechnological processes has hindered the advance of *in vitro* techniques. For most purposes, *in vitro* callus establishment is important as an intermediate step in peach biotechnology. Most of the advances made in peach have used embryo-derived explants. The main disadvantage of developing a protocol from seed-derived material is that each genotype is unique and not a clone of the parent (Abbott *et al.* 2008). Development of these biotechnological tools from mature tissues is important for the improvement of desirable commercial cultivars that have been selected for beneficial features, which may not be present in naturally produced seeds. Only a few authors have developed somatic regeneration protocols using adult peach material (Gentile *et al.* 2002; Pérez-Jiménez *et al.* 2012).

Many factors affect callus induction as well as its growth and development *in vitro*, namely, the type of explant tissue, quality and type of light and photoperiod conditions, plant growth regulators, culture media, gelling agent, pH, temperature, and many others. Plant growth regulators particularly influence callus induction; a phase in which auxins play a major role by inducing callus proliferation and development (Paris *et al.* 2004). Different types of callus are frequently developed for different purposes. A white and globular callus

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has been used for embryogenesis (López-Pérez *et al.* 2005; Hammerschlag *et al.* 1985), while green and nodular calli are used for organogenesis (Gentile *et al.* 2002; Pérez-Jiménez *et al.* 2012). Thus, a thorough study of all the factors involved is important for determining the choice of protocol for the type of callus required.

This work aimed to develop an efficient and reproducible protocol for obtaining callus from adult material in peach, by comparing different types of explant, culture media, growth regulators, and culture conditions. The results will be useful to develop further studies, to regenerate plants, protoplast cultures, and to obtain metabolites from *in vitro* cultures.

Materials and Methods

Plant material. Plant tissues were collected from 4-yr-old peach (*Prunus persica* L. Batsch) trees grown at the Torreblanca experimental field station of the Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario, Murcia, Spain. Three commercial varieties Early May® (nectarine), UFO-3® (flat peach), and Zise May® (peach) and another three preselected varieties from a peach breeding program using hybrid crosses PS108 (Early May® × N-292), PS208 (Early May® × UFO-3®), and PS708 (Zise May® × Early May®) were used as explant sources. The plants were watered daily using a drip irrigation system with 700 m^{3}ha^{-1}/mo at the time of sampling. The samples for callus induction experiments were taken from young branches producing their first shoots after flowering in April 2010.

In vitro culture establishment. Branches and flowers in the balloon stage from the selected genotypes were disinfected in a solution of 2% (*v/v*) sodium hypochlorite and 0.1% (*v/v*) Tween 20 for 2 h. After disinfection, the plant material was transferred to a laminar flow cabinet to separate the plant organs. Six sections of vegetative organs (fully expanded leaf blade, petiole, juvenile leaf, stem, terminal buds, and axillary buds) and three reproductive organs (calyx, petals, and stamens) were selected and cultured as follows: leaf explant with the abaxial leaf surface facing the medium, with and without midvein; leaf explant with the adaxial leaf surface facing the medium, with and without midvein; approximately 1 cm long stem explants plated in an upright or inverted position; juvenile leaf; terminal bud; axillary bud; petiole; calyx; petals; and stamens (Fig. 1).

Culture media. The explants were cultivated on four different media: Murashige and Skoog medium (MS; Murashige and Skoog 1962) supplemented with 1.2 mgl^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 mgl^{-1} of kinetin (KN, MS-DK); MS supplemented with 1.2 mgl^{-1} of 2,4-D and 1 mgl^{-1} thidiazuron (TDZ; MS-DT); woody plant medium (WPM; Lloyd and McCown 1980) with 1.2 mgl^{-1} of 2,4-D and 1 mgl^{-1} of KN (WPM-DK); and WPM supplemented with 1.2 mgl^{-1} of 2,4-D and 1 mgl^{-1} TDZ (WPM-DT). All media contained 3% (*w/v*) sucrose and 0.7% (*w/v*) of plant propagation agar (Pronadisa®), in Petri dishes (12 mm ø). The pH was adjusted to 5.7 with KOH (0.1 N) prior to autoclaving for 16 min at 1.1 kgcm^{-2} (122°C).

Culture conditions. The cultures were incubated in a climatic chamber at 25±1°C with two different light regimes. Half of the plates (selected randomly) with the same type and number of explants were kept under 16 h light (45 μmolm^{-2} s^{-1}, GRO-LUX, Sylvania, Surrey, UK) photoperiod and the other half in constant (24 h) darkness.

Experiment design and data collection. Data were collected from 28,080 explants. Four media were tested with 15 explants for each of the 13 types, 6 genotypes, and 2 different light conditions with three replications. During the study, the following parameters were measured:

Figure 1. Source of explants used for *in vitro* callus induction in peach.
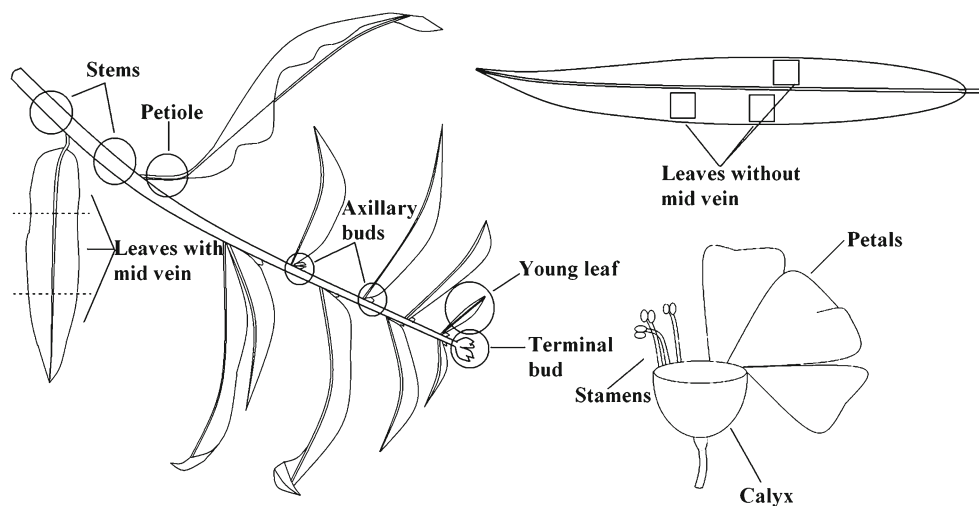
percentage of explants in which callus was induced, d to the appearance of callus, color and type of callus produced, and callus relative growth (CRG)—which refers to the percentage of explant covered by callus. Data were recorded each day to know exactly when callus started to appear, and at 30 d, CRG was recorded, as well as the number of explants and which part of the explant that the callus growth originated. No subcultures were performed during the experiment. Significance was determined by analysis of variance and the least significance ($P \leq 0.05$) differences among mean values were estimated using Duncan's new multiple range test.

Results and Discussion

Callus was induced in all six genotypes of *P. persica* cultivated on four different media supplemented with 2,4-D, KN, or TDZ and two light regimes: 16 h light and constant darkness. Disinfection resulted in effective prevention of contamination and only 1.4% of explants died from the process. No significant genotypic differences were observed for any of the three parameters assessed for the eight different treatments (Table 1). A high percentage of callus formation was demonstrated for all the six genotypes, where the percentage of explants developing callus ranged from almost 80% in PS208 to a maximum of 91% in PS708. Generally, explants started to develop callus from 14 to 16 d after the experiment commenced (Table 1). The amount of callus induced was statistically the same in all the studied genotypes; therefore, further studies in callus induction did not discriminate between peach genotypes.

Thirteen treatments with a variety of explant types were tested for callus induction (Table 2). Statistical differences were observed between groups; however, all the genotypes provided a high level of callus induction for the different explants (above 80%), and all responded in a similar timeframe. The only exception was the stamen explants which

Table 1. Callus induction of six peach genotypes for all four medium and light regimes tested

Cultivar	Explants with callus (%)	Days[z]	CRG[y] (%)
PS708	91.02 a[x]	15.3 a	38.09 a
PS108	84.16 a	15.4 a	38.40 a
PS208	80.96 a	15.8 a	40.28 a
UFO-3®	83.86 a	15.7 a	38.95 a
Early May®	87.12 a	16.4 a	42.36 a
Zise May®	90.04 a	16.1 a	41.29 a

[z] Days of culture required for callus initiation

[y] CRG is the percentage of callus covering the explant

[x] Values in each *column* followed by the same *letter* are not significantly different ($P<0.05$)

Table 2. Callus induction responses of explant types and treatments for six genotypes of peach

Explant	Explants developing callus (%)	Days to callus formation
Ab-Leaf WV	85.65 b[z]	17.2 b
Ab-Leaf WoV	83.37 b	17.3 b
Ad-Leaf WV	85.52 b	16.3 b
Ad-Leaf WoV	96.44 c	16.6 b
Axillary bud	96.50 c	14.8 a
Calyx	98.96 c	14.5 a
Terminal bud	97.19 c	16.3 b
Juvenile leaf	82.37 b	16.2 b
Petals	97.48 c	16.2 b
Petiole	97.69 c	16.3 b
Stamens	0.00 a	–
Stem-up	98.50 c	14.7 a
Stem-in	97.04 c	14.4 a

Ab-Leaf WoV abaxial leaf surface facing the medium without midvein, *Ab-Leaf WV* abaxial leaf surface facing the medium with midvein, *Ad-Leaf WoV* adaxial leaf surface facing the medium without midvein, *Ad-Leaf WV* adaxial leaf surface facing the medium with midvein, *Stem-up* stem placed in an upright position, *Stem-in* stem placed in an inverted position.

[z] Values in each *column* followed by the same *letter* are not significantly different ($P \leq 0.05$)

were completely unresponsive to all media and conditions tested. Due to the small standard deviation, there were three statistical groups: the stamens and the other two in which the differences between both had a small statistical significance. The lack of response from anthers was unexpected since callus induction from anthers has been reported previously in *Prunus* species. Long *et al.* (1994) and Peixe *et al.* (2004) obtained callus from anthers in *Prunus avium* and *Prunus armeniaca*, respectively. Both authors used culture media different to the ones used in these experiments, with the exception of Peixe *et al.* (2004), who also used MS salts, although their best result was obtained with Nitsch and Nitsch medium. As regards the percentage of explants that produced callus, the rates ranged from 82.37 to 98.96%, and the average time when the callus started to develop ranged from 14.4 to 17.3 d, similar to the results obtained in almond by Işikalan *et al.* (2010).

Of the 13 explant sources and treatments tested, significant differences were also observed in CRG, which measured the percentage of explant covered by callus after 30 d of *in vitro* culture (Table 3). Of the floral tissues, the calyx surface showed the highest CRG (70–96%). Whereas for the vegetative tissues, the stem treatments (explants plated in either the upright or inverted positions) and the petiole gave the highest CRG. Stem explants plated in the upright position showed surface callus formation covering between

Table 3. Influence of medium, photoperiod, and source of explant on callus relative growth (CRG) of six genotypes of peach

Media	MS-DT			MS-DK			WPM-DT			WPM-DK			Average[z]	
Photoperiod (h of light/24 h)	0	16	Average	0	16	Average	0	16	Average	0	16	Average	0	16
Ab-Leaf WV	32.06	15.64	23.85	10.81	11.58	11.19	15.89	13.08	14.49	61.31	27.11	44.21	30.01 b	16.85 b
Ab-Leaf WoV	40.17	8.33	24.25	16.39	20.39	18.39	15.00	10.33	12.67	85.28	21.78	53.53	39.21 c	15.21 b
Ad-Leaf WV	41.53	6.67	24.10	17.03	25.83	21.43	35.17	9.67	22.42	56.28	24.88	40.58	37.50 c	16.76 b
Ad-Leaf WoV	38.72	24.22	31.47	31.39	26.83	29.11	45.00	22.33	33.67	74.89	36.92	55.90	47.50 e	27.58 e
Axillary bud	35.33	53.06	44.19	44.72	37.10	40.91	36.31	29.06	32.68	83.78	35.78	59.78	50.03 f	38.75 d
Calyx	80.83	88.00	84.42	96.00	91.50	93.75	78.50	72.17	75.33	85.83	70.00	77.92	85.29 j	80.42 f
Terminal bud	61.67	52.50	57.08	34.00	25.38	29.69	36.75	43.69	40.22	83.13	41.25	62.19	53.89 g	40.71 d
Juvenile leaf	29.33	19.00	24.17	23.96	13.61	18.78	43.47	20.29	31.88	52.50	43.33	47.92	37.32 d	24.06 c
Petals	22.50	26.50	24.50	17.67	23.50	20.58	19.00	21.67	20.33	41.67	31.67	36.67	25.21 b, c	25.83 c
Petiole	52.67	42.33	47.50	60.67	60.50	60.58	48.33	55.33	51.83	71.67	57.50	64.58	58.33 h	53.92 e
Stamens	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 a	0.00 a
Stem-up	78.56	55.67	67.11	71.31	30.17	50.74	67.89	60.17	64.03	85.56	49.44	67.50	75.83 i	48.86 e
Stem-in	52.39	73.67	63.03	48.78	25.17	36.97	61.36	48.33	54.85	57.72	64.50	61.11	55.06 h	52.92 e
Total[y]	43.52	35.81	39.67 c	36.36	30.12	33.24 a	38.67	31.24	34.95 b	64.58	38.78	51.68 d	45.78	33.99

CRG is the percentage of the explant covered by callus

Ab-Leaf WV abaxial leaf surface facing the medium with midvein, *Ab-Leaf WoV* abaxial leaf surface facing the medium without midvein, *Ad-Leaf WV* adaxial leaf surface facing the medium with midvein, *Ad-Leaf WoV* adaxial leaf surface facing the medium without midvein, *Stem-up* stem plated in upright position, *Stem-in* stem plated in inverted position

[z] Values in each *column* followed by the same *letter* are not significantly different ($P \le 0.05$)

[y] Values in this *row* followed by the same *letter* are not significantly different ($P \le 0.05$)

30.17 and 85.56% of the tissue (similar to the stem-in inverted position), while the petiole showed a CRG of 42.33 and 71.67%. It has been found that the explant source strongly influences the callus induction process. Previous reports have observed the same effect in other species, such as maize (Green and Phillips 1975) and sugarcane (Guiderdoni and Demarly 1988). Also, Declerck and Korban (1996) reported similar findings in peach, as did Ansley *et al.* (2000) for almond explants. In this study, explants consisting of leaves without a midvein presented a higher percentage of callus development than leaves with midvein. It could be due to the fact that leaves without midvein have a greater surface area of wounded tissue, which is known to be more conducive to callus growth.

With regard to the culture media, taking into account all the treatments, WPM salts were the most successful in all the tested explants except for calyx and stem (inverted position) explants, for which, MS salts produced the higher callus induction rate (Table 3). Zhou *et al.* (2010) also found WPM to be more favorable for callus initiation than MS in a regeneration study in peach rootstocks. WPM-DK was the most successful treatment, inducing substantial callus growth in either of the light conditions tested; 64.58% of the CRG in darkness and 38.78% under a 16-h photoperiod. MS-DT was the second most prolific medium for callus induction, with 43.52 and 35.81% CRG for 0 and 16 h light, respectively. Therefore, the results show that there is a combined effect induced by the culture medium and the plant growth regulators used.

The callus obtained from explants exposed to the 16-h photoperiod had a green and very compact texture in vegetative tissues (Fig. 2*A–C*), whereas in petals (Fig. 2*F*) and calyx (Fig. 2*G*), the calli were mainly pink in color. The
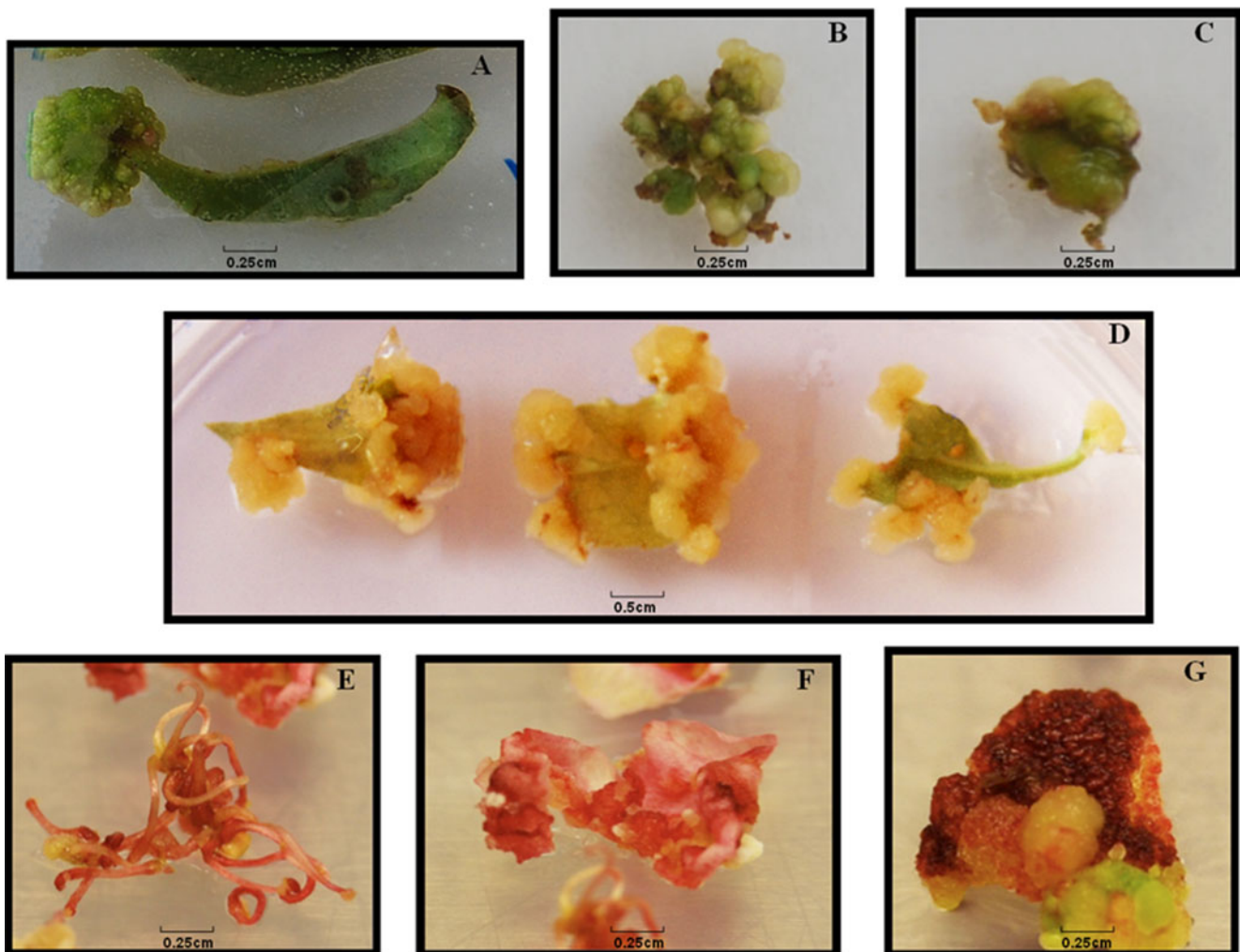

Figure 2. Differences between treatments and explants in callus induction of peach after 30 d. (*A*) Calli induced in a young leaf under a 16-h light photoperiod. (*B*) Callus induced in a leaf explant without midvein with the abaxial leaf surface facing the medium under a 16-h light photoperiod. (*C*) Callus induced in a stem explant plated in upright position under a 16-h photoperiod. (*D*) Calli induced in leaves with midvein and the abaxial leaf surface facing the medium, in the dark. (*E*) Stamens with no callus. (*F*) Callus induced in petals under a 16-h photoperiod. (*G*) Callus induced in calyx under a 16-h photoperiod.

stamens (Fig. 2*E*) turned pink, but no callus was formed. The appearance of calli in green tissues varied slightly depending on the explant type, but in general, it was composed of small green globular structures (Fig. 2*A, B*). In young leaf explants (Fig. 2*A*), the presence of callus was found mainly in the petiole or along the cut surface. These results agree with the previous research of Zhou *et al.* (2010) where young leaves of the hybrid *P. persica* × *Prunus davidiana* were tested. In this study, under 16 h light conditions, the callus that developed from buds and stems differed from leaves, being limited by the outer structure of the stems (Fig. 2*C*) and buds, while the callus on leaf tissue was unconstrained and globular. The appearance of all calli induced in the dark was the same, regardless of the explant type (Fig. 2*D*) or the culture media. These calli were white to yellow, smooth, friable, and more voluminous than those obtained under light conditions. In all cultures, irrespective of exposure to light, the callus initially developed from cut tissue surfaces. Declerck and Korban (1996) tested different concentrations of auxins (2,4-D, dicamba) and cytokinins (BA, zeatin, kinetin, TDZ) in callus induction of peach. These authors maintained that in leaf tissues of *P. persica*, cytokinins are more likely to produce chlorophyllous and compact callus cultures, whilst auxins increase callus production, inducing friable callus. To the best of our knowledge, those typologies are produced by the light since, in our study, the same media have been used in both conditions and the results were the same in the 13 peach explants, genotypes, or treatments tested. Both typologies of peach callus we observed have been previously described as being embryogenic (white callus; Svircev *et al.* 1993) or organogenic (green callus; Gentile *et al.* 2002; Zhou *et al.* 2010; Pérez-Jiménez *et al.* 2012). Friable, white callus can be used effectively in cell suspension cultures due to its propensity to crumble (Bhansali *et al.* 1991).

In summary, this study demonstrates that the growth of *P. persica* calli is greatly influenced by the type of explant, the combination of plant growth regulators, culture media, and light conditions. The media composed of WPM supplemented with 2,4-D and KN induced a higher percentage of callus than the other media tested. A 16-h light photoperiod or constant darkness can be applied for callus induction for different purposes. White to yellow and friable callus was obtained under dark conditions and green compact, nodular callus was produced when explants were cultured in the light. The calyx was the most productive explant with regards to callus induction, followed by the vegetative explants, buds, stems, and petioles. No callus was obtained from the anthers or filaments with the conditions used in this study.

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