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Effect of Light Conditions on *In Vitro* Adventitious Organogenesis of Cucumber Cultivars

Jorge Fonseca Miguel*

Abstract

The response on callus and shoot formation under different light incubation conditions was evaluated in cucumber (*Cucumis sativus* L.). Four-day-old cotyledon explants from the inbred line 'Wisconsin 2843' and the commercial cultivars 'Marketer' and 'Negrito' were employed. A four-week culture was conducted on MS-derived shoot induction medium containing 0.5 mg L-1 IAA and 2.5 mg L-1 BAP, under an 8-h dark/16-h light regime, or by a one- or two-week dark pre-incubation followed by the same photoperiod. Significant differences were obtained for the regeneration of shoots in all cultivars. The response in both frequency and number of shoots under continuous photoperiod was at least 3-6 fold higher than with dark pre-incubation. The highest genotypes response was obtained by 'Negrito' and 'Marketer' with identical values. All explants formed callus, and in two of the three cultivars, the response on callus extension was not significantly affected by incubation conditions. The results clearly show that shoot induction under continuous photoperiod regime was beneficial for adventitious shoot regeneration in cucumber.

Keywords: Dark pre-incubation; Photoperiod; Morphogenesis; Shoot regeneration; *Cucumis sativus* L.; Cucurbitaceae

Abbreviations: BAP: 6-benzylaminopurine; IAA: Indole-3-acetic acid; KIN: Kinetin; MS: Murashige and Skoog [1] medium

Introduction

Cucumber (*Cucumis sativus* L.) belongs to the economically important family Cucurbitaceae along with melon, watermelon and squash. World production of cucumber, including gherkins, ranked in 2020 third among vegetable crops with 91,258,272 tonnes, and a harvested area of 2,261,318 hectares (FAOSTAT, http://www.fao.org/faostat/en/#data/QC). Crop improvement of cucumber for traits that confer resistance/tolerance to major biotic/abiotic stresses is difficult through conventional breeding due to its narrow genetic base, low genetic variability, and various crossing barriers with related species [2-4]. Besides, conventional approaches are labor-intensive, time-consuming, and costly. Genetic engineering and plant transformation techniques have the potential to overcome these constraints [4]. Despite the number of studies on genetic transformation of cucumber, its efficiency is still far from ideal [5,6]. The main limitations in obtaining transgenic cucumber plants are the low morphogenetic response, inadequate selection methods, and the high rate of non-transgenic "escape" plants [6]. By improving the efficiency of regeneration systems, we are addressing a part of the problem.

Regeneration via organogenesis and somatic embryogenesis has

Affiliation:

Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universidad Politécnica de Valencia-C.S.I.C., Valencia, Spain

***Corresponding author:**
Jorge Fonseca Miguel, Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universidad Politécnica de Valencia-C.S.I.C., Ingeniero Fausto Elio s/n, 46022 Valencia, Spain.

E-mail: jfonseca@doctor.upv.es

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been reported in cucumber. Commonly used explants are cotyledons, hypocotyls and leaves [4,7-11]. Regeneration from protoplasts and suspension cultures has also been described [12-15]. However, regeneration in this species is still not optimal [5], and is highly genotype-dependent [4,16,17]. An efficient and reproducible regeneration protocol is essential for a successful tissue culture-based genetic transformation of cucumber.

In *in vitro* plant regeneration, in addition to the culture factors that are usually considered, such as genotype, explant type, age of the donor plants, number and duration of subcultures, medium composition and growth regulators, the choice of appropriate incubation conditions, including light, temperature and humidity regimes, are essential to optimize regenerative responses.

Light, in particular, is a crucial environmental factor that, besides providing energy for photosynthesis, triggers and modulates complex developmental and regulatory processes [18-20]. Plants can sense many parameters of environmental light, such as quality (spectral composition), light intensity, direction, and duration (including day length), and use this information to optimize growth and development during their whole life cycle [21-24]. To sense and respond to environmental light conditions, plants are equipped with several classes of photoreceptors, other than photosynthetic pigments, including phytochromes, cryptochromes, phototropins, zeitlupe family members, and UVR-8, that can monitor specific ranges of the light spectrum (from UV-B to far-red), albeit with overlapping action spectra [22,25-27]. Plants have constantly to adapt to a varying light environment [26]. Despite their remarkable plasticity, light fluctuations can have a critical impact on plant competition and survival [28,29]. The effect of light is most visible during seedling development. The patterns of seedling development under light (photomorphogenesis) differ from those under darkness (skotomorphogenesis or etiolation) regarding gene expression, differentiation, and organ morphology [30]. Photomorphogenesis is characterized by short hypocotyls, open and expanded cotyledons, cell-type differentiation, chloroplast development, anthocyanin accumulation, and the expression of a large set of light-inducible genes encoded by the chloroplast and the nucleus. On the other hand, skotomorphogenesis is typically distinguished by long hypocotyls, closed and unexpanded cotyledons, closed apical hooks, and the development of etioplasts [21,31-34]. The interaction between environmental signals (light) and endogenous cues (gibberellin plant hormones, among others) determines the choice of one of the two processes [33]. The optimal lighting conditions depend on species, cultivars, plant growth stages, specific secondary metabolites, and other environmental parameters, such as nutrients, temperature, and CO_2 levels [35]. The specific effects of light in a particular species can differ substantially between organs or cell types, even between nearby cells, as well as throughout development [30]. In Arabidopsis seedling, it was estimated that approximately 1/3 of the genes whose expression is regulated by light, where 3/5 are up-regulated and 2/5 are down-regulated [36], revealing, in particular, the crucial role of light and its complexity in the early stage of plant development. Light signaling pathways are interconnected with many other pathways to modulate plant physiology and development [29].

This study aimed to determine the influence of different dark/light incubation regimes on *in vitro* adventitious organogenesis, using cotyledons as explants from one inbred line and two commercial cultivars of cucumber.

Materials and Methods

Plant material and regeneration

Cucumber (*Cucumis sativus*) seeds of the inbred line 'Wisconsin 2843' (courtesy of Dr. C.E. Peterson) and of the cultivars 'Marketer' and 'Negrito' (Semillas Fitó S.A.), were the starting material. Obtaining axenic explants and *in vitro* adventitious regeneration were based on the methodology previously described by Miguel [4] in cucumber, with some modifications.

Seeds were surface-sterilized by immersion in a solution of 5% w/v sodium hypochlorite and 0.1 (v/v) 7X-O-matic (Flow Laboratories) for 30 min, and rinsed with sterile distilled water. They were then germinated on MS-derived medium without growth regulators. From 4-day-old axenic seedlings, cotyledons were excised and used as explant source by removing 1-2 mm behind their proximal and distal ends. All culture media were solidified with 0.8% (w/v) agar (Industrial, Pronadisa), and its pH adjusted to 5.7 before autoclaving. Plant material was incubated in a growth chamber at 26 ± 2 ºC under standard 16-h light/8-h dark photoperiod with cool-white fluorescent light at a photon fluence rate of 90 µmol m–2 s–1 (Grolux, Sylvania, fluorescent tubes); in dark pre-incubation, jars were wrapped with aluminum foil to prevent the passage of light. The experimental evaluations are based on observations with a naked eye.

Cotyledon explants were cultivated for 4 weeks on MS-derived Shoot Induction Medium (SIM) containing 0.5 mg L-1 IAA and 2.5 mg L-1 BAP, under the standard photoperiod regime, or by a one- or two-week dark pre-incubation followed by the same photoperiod. Then, Callus Regeneration Frequency (%) (CRF) and Callus Extension Index (CEI) were determined, where CRF (mean ± SE) is the frequency of explants with callus on the cutting zone and, CEI (mean ± SE) correspond to arbitrary values (from 0 to 3) on the extension of callus on the cutting zone, where: 0= absence of callus; 1=traces of callus; 2=callus on less than half; 3=callus on half or more; 4=callus covering the full extension.

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Adventitious buds and shoot primordia obtained were then cultured for 2 weeks on MS-derived Shoot Development and Elongation Medium (SDM) containing 0.2 mg L-1 KIN. Next, Shoot Regeneration Frequency (%) (SRF) and Shoot Number Index (SNI) were evaluated, where SRF (mean ± SE) is the frequency of explants with shoots and, SNI (mean ± SE) correspond to arbitrary values (from 0 to 3) on the number of shoots per explant, where: 0=absence; 1=one shoot; 2=two shoots; 3=three or more shoots. Individualized shoots were then rooted on hormone-free MS medium and were ready for acclimation in 3 to 4 weeks (data not shown).

Data analysis

The experiment was arranged in a 3 × 3 completely randomized factorial design. At least twelve replicate flasks of six explants each were used in each treatment. All statistics were carried out using R version 4.0.4 [37]. Non-linear regression analyses were used to compare treatment means. To perform logistic regression, COM-Poisson regression, and generalized Poisson regression, the R packages 'stats' [37], 'COMPoissonReg' [38], and 'VGAM' [39], were used, respectively. Model fit was evaluated using Akaike Information Criterion [40] and Bayesian Information Criterion [41]. The level of statistical significance was set at $P < 0.05$.

Results

Frequency and extension of callus

Results are expressed as mean ± SEM (Table 1), with significance defined as $P < 0.05$. Callus was formed within the first two weeks of culture, starting at the cut ends of the primary explant. All explants formed callus (100% of frequency). Two of the three cultivars showed no significant differences on callus extension for the incubation conditions tested. In contrast, 'Negrito' cultivar showed differences between the 2-week dark pre-incubation treatment (2D/2F), the highest response (1.74 ± 0.08), and the other incubation regimes, showing that including a longer pre-incubation favored callus extension. Other scores ranged from 1.44 ± 0.06 to 1.58 ± 0.06. The results lie between the arbitrary values on callus extension of 1- traces of callus, and 2- callus on less than half, at explant cut edges.

Frequency and number of shoots

Data are presented as mean ± SEM (Table 1), with significance set at $P < 0.05$. Shoots formed from callus within 2-4.5 weeks of culture, mainly at the proximal end of the explant. Those obtained in the treatments with pre-incubation in the dark were not etiolated. For both frequency (SRF) and Shoot Number Index (SNI), cultivars followed the same pattern, with a 4-week photoperiod regime (4F) showing significant differences from the other treatments. Its response was at least 3-6 times greater than the dark pre-incubation treatments, which did not differ statistically from each other. With similar values to 'Marketer', 'Negrito' had the highest response, with a frequency of 41.67 ± 5.85 and an index of 0.52 ± 0.09. 'Wisconsin' has a 35% and a 46% lower frequency and index, respectively.

Discussion

The organogenic response of three cucumber cultivars was assessed under different lighting regimes: standard 16L:8D h photoperiod and two periods of dark pre-incubation.

Light is a critical environmental factor that besides being the driving force behind photosynthesis triggers and

Table 1: Effect of incubation conditions on *in vitro* callus and shoot regeneration from cotyledon explants of cucumber (*Cucumis sativus* L.) cultivars. Data are reported as mean ± Standard Error of the Mean (SEM).

Cultivar	Incubation conditions*	Callus regeneration frequency (%)‡	Callus extension index§			Shoot regeneration frequency (%)†			Shoot number index&		
Wisconsin 2843	4F	100	1.45	±	0.06[a]	26.92	±	5.06[a]	0.28	±	0.06[a]
	1D/3F	100	1.58	±	0.06[a]	6.41	±	2.79[b]	0.06	±	0.03[b]
	2D/2F	100	1.46	±	0.06[a]	9.86	±	3.56[b]	0.08	±	0.03[b]
Marketer	4F	100	1.54	±	0.06[a]	40.28	±	5.82[a]	0.49	±	0.09[a]
	1D/3F	100	1.44	±	0.06[a]	11.11	±	3.73[b]	0.08	±	0.03[b]
	2D/2F	100	1.57	±	0.06[a]	8.33	±	3.28[b]	0.08	±	0.03[b]
Negrito	4F	100	1.50	±	0.06[b]	41.67	±	5.85[a]	0.52	±	0.09[a]
	1D/3F	100	1.52	±	0.06[b]	5.63	±	2.76[b]	0.06	±	0.03[b]
	2D/2F	100	1.74	±	0.08[a]	8.33	±	3.28[b]	0.08	±	0.04[b]

*4F: four-week photoperiod regime; t1D/t2F: t1-week dark pre-incubation followed by t2-week photoperiod.
‡,§Data were obtained after 4 weeks of culture on Shoot Induction Medium (SIM).
†,&Data were obtained after 2 weeks of culture on Shoot Development and Elongation Medium (SDM).
§COM-Poisson regression, †logistic regression, and &generalized Poisson regression were used to analyze the data. Mean values within each column followed by different letters are significantly different ($P < 0.05$).

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modulates complex developmental and regulatory processes [18,20,42]. As an environmental cue, light regulates many aspects of plant biology. These include adaptive responses (e.g., phototropism, shade avoidance, and synthesis of photoprotective pigments), developmental transitions (e.g., germination, de-etiolation, flowering time, and senescence), and at the cellular level (e.g., chloroplasts movement, and stomatal opening) [26,27,43-46]. Light is also the main agent that mediates the entrainment of circadian rhythms [47].

In *in vitro* regeneration, explants are often incubated in a growth chamber under a 16L:8D h photoperiod. However, some researchers choose to perform a pre-incubation in the dark.

A dark pre-incubation has been reported in the micropropagation of different plant species. Regeneration via somatic embryogenesis and organogenesis with 2-3 weeks dark pre-treatment was described in two inbred lines and in an F1 hybrid of cucumber, using cotyledon, leaf, and petiole explants [17]. Regeneration via embryogenesis with 3-4 weeks pre-incubation was reported in cucumber cultivars from explants of petiole [48], cotyledon and hypocotyl [49]. In other cucurbits, 2-3 weeks dark pre-treatment was described in the organogenesis of melon (*Cucumis melo* L.) and African horned cucumber (*C. metuliferus* E. Mey. ex Naudin) [17], as well as in increasing somatic embryo production in melon and squash (*Cucurbita pepo* L.) [50]. In species other than cucurbits, a pre-incubation in the dark has also been reported. A 2-3 weeks pre-treatment favored somatic embryo induction and development in pepper (*Capsicum annum* L.) [50]). A 2-week dark pre-incubation was described in adventitious organogenesis of apple cultivars [51], and in increased frequency of somatic embryogenesis in purple coneflower (*Echinacea purpurea* (L.) Moench) [52]. A 1-week pre-treatment enhanced shoot regeneration in chrysanthemum (*Chrysanthemum morifolium Ramat*) [53]. In other reports, a dark pre-incubation had no positive effect on regeneration. Callus from cotyledon explants of cucumber failed to produce shoot buds in the dark [7]. A pre-incubation had a detrimental impact on gardenia (*Gardenia jasminoides* L.), and no somatic embryos were formed for less than 7 weeks in the dark [50]. In rose (*Rosa hybrida* L.), a pre-treatment of 1 to 10 weeks in darkness failed to induce somatic embryogenesis [50].

In the present investigation, frequency and extension of callus were not influenced by light incubation conditions, except for cv. Negrito, where a 2-week dark pre-incubation enhanced callus extension. The findings on callus formation are in line with some reports. Punja et al. [17], when using different cucumber genotypes, growth regulators combinations, and explant types, the percentage of callus was not affected by pre-incubation in the dark. Likewise Gammoudi et al. [54], when using pepper cotyledon explants, callus frequency was not influenced by a dark pre-treatment. The opposite was observed in hypocotyl explants. Also unlike the present study, in the organogenesis of bael (*Aegle marmelos* (L.) Corr.) using different explants, a dark incubation (1-7 days) favored abundant callus formation, with cotyledon explants and the 3-day treatment giving the best response [55]. In anther culture of *Capsicum annuum* L., both growth regulators combinations and light regimes influenced the frequency and intensity of callus formation [56].

The results on shoot regeneration frequency and shoot number index revealed that all genotypes followed the same pattern for the incubation conditions tested, indicating no interaction between the factors. In both variables, a photoperiod regime responded at least 3-6 times higher than treatments with a dark pre-incubation. The degree of response depended on the genotype. No relation seems to exist between callus extension and shoot formation for the incubation conditions tested. The findings on shoot regeneration are in general agreement with other studies in which a pre-incubation in the dark did not promote regeneration. Gammoudi et al. [54] found that a dark pre-incubation was not effective in regenerating four pepper cultivars. In Petunia hybrida cv. R27, it had a detrimental effect on shoot regeneration frequency [57]. In lavandula (*Lavandula latifolia* Medicus), it was not beneficial on the frequency of bud and shoot regeneration when a high auxin concentration (6.0 or 11.0 µM) was used in the induction medium [58]. Unlike, a dark pre-incubation was essential for optimal frequency of embryos or shoots in cucumber [17]. Likewise, it resulted in optimal shoot frequency and number of shoots per explant in chrysanthemum [53], and in black locus (*Robinia pseudoacacia* L.) [59].

The mechanisms underlying the effects of a dark pre-incubation on *in vitro* morphogenesis are complex and poorly understood. By pre-incubating in the dark, tissues could experience a redirection of resources, a change in the levels of endogenous growth regulators, or an altered sensitivity to growth regulators [52]. Light and dark regimes influenced hormonal balance needed for efficient regeneration [58]. A short light exposure on seedlings grown in the dark reduced the growth rate and altered the ratio of free to conjugated IAA [60]. In the initiation of shoots in light- and dark-grown tobacco callus, the ethylene produced in dark culture was much higher [61]. A link has been established between ethylene, responses to stress, and the ability to regenerate [62]. Light conditions also play a role in the biosynthesis of secondary metabolites [63]. Different secondary compounds are known to modulate *in vitro* plant morphogenesis [64]. For example, some phenolic compounds regulate IAA degradation, phenylpropanoids interact with auxins and act antagonistically to gibberellins, and the role of flavonoids as auxin transport inhibitors [64,65]. Light regimes can also influence the cell cycle [66,67]. Reuveni and Evenor [57] reported a genetic component for regeneration in darkness or light in species of petunia.

Further molecular, genetic and physiological studies are needed to understand the role of light on *in vitro* morphogenesis, how it interacts with other elements, and how its effects are mediated.

Conclusions

In vitro shoot regeneration of one inbred line and two commercial cultivars of cucumber was significantly higher when, in shoot induction, incubation was performed under photoperiod (16L:8D h), unlike when pre-incubation in the dark followed by the same photoperiod was used. Optimized regeneration systems for selected genotypes are required to achieve a workable efficiency to apply biotechnological approaches in this species, such as large-scale micropropagation and the application of culture-based genetic transformation technologies for crop improvement, and a better understanding of its genetic basis.

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Conflicts of Interest

The author declares no conflict of interest in the publication of this work.

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