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Impact of Light on *In Vitro* Plant Regeneration

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ABSTRACT

Light is a vital environmental factor, serving not only as the primary driving force behind photosynthesis but also triggering and modulating intricate developmental and physiological processes essential for plant growth and development. It regulates various aspects of plant biology, including adaptive responses, developmental transitions, and cellular-level processes, and serves as the primary cue for the entrainment of circadian rhythms. Light also plays a fundamental role in in vitro plant regeneration, influencing cellular processes such as differentiation and morphogenesis, driving overall growth and development, and determining regeneration efficiency and success. Optimizing specific light conditions is critical for enhancing regeneration systems and their biotechnological applications, including pathogen elimination, germplasm conservation, large-scale micropropagation, genome editing, and genetic transformation. This review examines the impact of light on two key processes of in vitro plant regeneration: somatic embryogenesis and organogenesis. Furthermore, it addresses the effects of dark pre-incubation on regeneration outcomes and explores the underlying mechanisms driving light responses.

Keywords

Light regimes, Light parameters, Light-mediated mechanisms and effects, Dark incubation, *In vitro* morphogenesis, Somatic embryogenesis, Organogenesis.

Abbreviations

DI: Dark Incubation, DPI: Dark Pre-incubation, EOD: End-of-Day, IVO: *In Vitro* Organogenesis, LEDs: Light-emitting Diodes, ORG: Organogenesis, PPFD: Photosynthetic Photon Flux Density, ROS: Reactive Oxygen Species, SE: Somatic Embryogenesis.

Introduction

In *in vitro* plant regeneration, intrinsic elements such as genotype, explant type, cultivation steps, growth regulators, and other medium-related factors must be considered alongside incubation conditions like temperature, humidity, and light to optimize regeneration efficiency [1,2].

Light stands out as an essential environmental factor that not only serves as the primary energy source for photosynthesis but also plays a critical role in inducing and regulating intricate developmental and regulatory processes in plants [3-5]. It modulates various facets of plant biology, encompassing adaptive responses (such as phototropism, shade avoidance, and photoprotective pigment production), governing key developmental transitions (including seed germination, de-etiolation, reproductive phase initiation, and programmed tissue senescence), and modulating cellular processes (like chloroplast movement and stomatal aperture adjustment) [2,6-11]. Moreover, light serves as a primary cue for the entrainment of circadian rhythms [12,13], which are essential for coordinating various physiological processes.

Plants detect and respond to various characteristics of light, such as its quality (spectral composition), intensity, direction, and duration (including photoperiod), which are crucial for optimizing growth and development throughout their life cycle [14-17]. In controlled *in vitro* systems, light plays an equally critical role. They have evolved diverse photoreceptor systems, distinct from photosynthetic pigments, such as phytochromes, cryptochromes, phototropins, ZTL/FKF1/LKP2 group proteins, and UVR-8, which detect specific light wavelengths from UV-B to far-red, yet with overlapping action spectra [2,6,7,15,18].

While plants exhibit remarkable adaptability to fluctuating light conditions, these environmental variations can significantly influence their competitive abilities and survival prospects [6,19,20]. The impact of light is especially pronounced throughout seedling ontogeny. Photomorphogenesis, triggered by light, and skotomorphogenesis, occurring in darkness, represent distinct developmental pathways with differences in gene expression, organ morphology, and differentiation [21]. Photomorphogenic seedlings exhibit short hypocotyls, expanded cotyledons, chloroplast development, synthesis and accumulation of anthocyanins, cell-type differentiation, and the activation of many light-responsive genes in both the chloroplast and nucleus; in contrast, skotomorphogenesis is characterized by elongated hypocotyls, folded and immature cotyledons, folded apical hooks, and the formation of etioplasts [14,22-25]. The interplay between light cues and internal signals (such as gibberellins and other hormones) dictates whether plants undergo photomorphogenesis or skotomorphogenesis [24].

Optimal light requirements vary based on species, cultivar, developmental phase, and particular secondary metabolites, alongside other abiotic factors such as nutrient availability, temperature, and carbon dioxide content [26], which influence *in vitro* plant regeneration, somatic embryogenesis, and *o*rganogenesis. Light's influence on a given species can vary significantly among different organs or cellular categories, even among adjacent cells, and across various developmental stages [21].

In seedlings of *Arabidopsis*, for instance, around one-third are light-regulated genes, with 60% upregulated and 40% downregulated, underscoring the complexity and importance of light's role in early plant development [2,27]. The pathways involved in light signaling interact with various other pathways to regulate plant physiology and development [20].

Optimizing light conditions is therefore essential for improving the efficiency of *in vitro* regeneration systems and their core processes, somatic embryogenesis and organogenesis, which is crucial for advancing biotechnological applications and enabling innovative techniques in genomics and crop improvement.

Role of Light in Plant Regeneration: Mechanisms and Metabolic Interactions

Light is a pivotal environmental factor influencing *in vitro* plant regeneration. Beyond its role in photosynthesis, light also serves as a developmental and environmental signal, modulating key pathways and metabolic responses, which influence morphogenesis processes. However, light's effects on regeneration are context-dependent [28], which complicates the establishment of universal light condition guidelines, emphasizing the need for case-specific optimization. Plants possess marked genomic plasticity, which allows them to reprogram cells and express totipotency or pluripotency [29]. During regeneration, specific inherent developmental pathways are activated in atypical contexts through the context-sensitive integration of developmental and environmental signals, influenced by external factors and cues, resulting in varied regeneration strategies and efficiencies [28]. Furthermore, the ability of plant cells to achieve regeneration potential or a totipotent state may rely on their capacity to modify gene expression in response to external signals such as light cues [29].

Light influences plant regeneration by modulating key genes and transcription factors involved in processes such as auxin-driven callus formation, cytokinin-induced shoot development, and SE through complex interactions with hormonal signalling pathways and epigenetic modifications [30-36]. Li et al. [36] explored the epigenetic regulation of key genes involved in plant morphogenesis. They highlighted the complex gene networks and transcription factors that control regeneration processes. For instance, auxin triggers callus formation through ARF-mediated activation of LBDs, while also inducing cellular pluripotency via the WOX11-LBD16 and PLTs-CUC2 pathways. Cytokinin-driven shoot formation is supported by ARR-mediated WUS expression, whereas wounding-induced regeneration relies on WIND1-mediated ESR1 expression. In SE, embryonic regulators such as BBM, AGL15, LEC1, and LEC2 enhance auxin biosynthesis and signaling via YUCCAs and IAA30, forming a positive feedback loop.

In typical development, several key regulators of regeneration are silenced through epigenetic mechanisms to prevent unwanted cellular reprogramming, and a significant challenge lies in deciphering how external stimuli, such as light, can bypass these repressive controls [28,36,37]. Studies have also demonstrated that epigenetic regulation is crucial for *de novo* regeneration [38], suggesting a balance between reprogramming and control mechanisms. Genetic manipulation or ectopic expression of key regulators, such as WUS and WIND1, has been shown to stimulate organ regeneration and SE, providing a basis for screening species with high regeneration profiles [28,37,39-47].

Light triggers diverse concurrent signalling pathways in plants, some of which can lead to oxidative damage through the production of ROS [28,48]. In shoot regeneration, two key photoreceptors CRY1 (blue/UV-A) and PHYA (far-red), exert opposing effects: CRY1 mediates a pronounced suppression of shoot regeneration, while PHYA mitigates initial light-induced inhibition; downstream, the transcription factor HY5 induces anthocyanin accumulation, potentially protecting explants from light-induced damage [28].

Photomorphogenesis represents the typical developmental pathway in plants, but is suppressed in the absence of light, as in dark-grown seedlings where the COP1/SPA complex degrades key regulators; however, upon light exposure, phytochromes inhibit this complex, enabling the accumulation of transcription factors that drive light-mediated growth, though the precise inhibition mechanism remains unclear [49].

Plant morphogenesis is primarily regulated by photoreceptors that respond to light in the blue, red, and far-red regions of the

spectrum [50]. Light signals beyond these regions, including UV-A, UV-B, and green light, may also influence developmental processes, although the mechanisms are less understood. This regulation involves photoreceptors such as phytochromes, cryptochromes, phototropins, ZTL/FKF1/LKP2 group proteins, and UVR8 [2,6,7,15,18,51], as well as pigments like carotenoids and chlorophylls that assist in light absorption, photoprotection, and modulate light signalling pathways [52-54]. Moreover, plant morphogenesis is shaped by interactions with hormonal signalling, circadian rhythms, photoperiod, light intensity, and various developmental and environmental cues, demonstrating a complex, integrated regulatory network that extends beyond specific light spectra [55-62]. In plant tissue culture, artificial lighting should provide the specific wavelengths required for both photomorphogenesis and photosynthesis [63]. Precise control of light parameters is essential for optimizing regeneration. However, studies often report conflicting results due to the influence of external and internal factors, as well as the lack of standardized protocols [63]. Since each species has unique light requirements, tailoring light conditions such as spectrum and PPFD is critical to improving regeneration outcomes [50,64]. Light conditions also influence plant morphology, anatomy, and key physiological processes, such as ROS metabolism and antioxidant activity; appropriate adjustments can enhance both *in vitro* and *ex vitro* performance [50,65-67].

Light intensity is another critical factor in plant tissue culture and must be carefully balanced to optimize morphogenesis while preventing stress from either insufficient or excessive light. Sharma et al. [68] reviewed the mechanisms and adaptations of photosystems to high light stress. Insufficient light limits chlorophyll activation, reducing photosynthetic energy, while excessive light can cause photoinhibition, photooxidation, and photoinactivation, as well as photolability, solarization, and photodynamic reactions. Hormones such as abscisic acid, gibberellins, cytokinins, and brassinosteroids are involved in plant adaptation to fluctuating light conditions. Higher light intensities stimulate the synthesis of hormones like abscisic acid and jasmonic acid while reducing the production of auxins and cytokinins. These hormonal fluctuations can significantly impact the efficiency of the regeneration system.

Light parameters influence the efficacy of growth regulators in the culture medium and the endogenous hormonal balance of tissues by modulating their regulation, metabolism, and concentrations [1,63,69,70]. Molecular signals, such as those triggered by explant wounding, induced cell death, and phytohormone interactions, alongside internal and external factors like light conditions, also influence SE and IVO responses [1]. Light also influences both primary and secondary metabolism in plants, including the accumulation of sugars and phenolics like flavonoids and anthocyanins, which can affect *in vitro* regeneration efficiency [71,72]. For instance, the combined application of light and auxin in *Crinum* x *powellii* significantly impacted alkaloid biosynthesis, as well as tissue growth, survival, and morphogenesis; furthermore, a recent study indicated that photoperiod influences both alkaloid production and tissue differentiation in *Narcissus tazetta* [73].

Recent advancements in artificial lighting, particularly LEDs, have transformed *in vitro* plant tissue culture. LEDs offer customizable spectral qualities, energy efficiency, long lifespan, low heat output, and precise light intensity control, providing substantial advantages over traditional systems like fluorescent and high-pressure sodium (HPS) lights [50,74,75]. These benefits enable LEDs to effectively promote *in vitro* culture across diverse plant species, positively influencing shoot ORG, SE, and survival rates [50,76]. However, when transitioning from older lighting systems to LEDs, adjustments are necessary, as light quality can affect the efficacy of growth regulators in the culture medium and the internal hormonal balance of tissues [63].

Light on Somatic Embryogenesis

SE is a complex biological process in which bipolar structures resembling zygotic embryos are derived from non-gametic somatic cells through induced cellular totipotency, either directly or indirectly through a callus phase, potentially leading to the regeneration of complete, genetically identical plants [77-79]. It serves as a valuable tool in various biotechnological applications, including micropropagation, genetic engineering, germplasm conservation, *in vitro* mutagenesis, synthetic seed production, and the generation of clonal plant genotypes for diverse practical and research purposes [50,80,81]. Light plays a critical role in biological systems, and fine-tuning of light parameters is particularly important in *in vitro* culture systems to ensure efficient and optimized SE outcomes [77,82].

In *Agave tequilana,* SE benefited from red or white light during the induction phase, with wide-spectrum light supporting the expression phase [83]. In *Gossypium hirsutum*, red light during induction significantly improved embryogenic callus formation, elevated expression levels of marker genes associated with SE, and reduced the differentiation period by more than half compared to white light [84]. Red light (at 10 $\mu mol\ m^{-2}\ s^{-1}$) significantly enhanced the total density of somatic embryos from embryogenic calli of carrot [85]. In sugarcane, a 1:4 blue-to-red ratio did not support somatic embryo induction, facilitating only callus formation [50]. In contrast, a 55:45 blue-to-red light ratio optimized germination in Norway spruce, outperforming lower blue light ratios [75]. Conversely, red light alone promoted higher rates of somatic embryo germination and conversion in species such as southern pine and Japanese red pine, compared to blue light [50]. Similarly, red light is often the most effective for promoting both germination and root development in Norway spruce; in pine species, red wavelengths increased germination rates, taproot length, and lateral root formation [75]. In quince, the highest SE rates occurred under red light, with rates declining under red+blue and white light treatments [86]. Furthermore, combinations of red and far-red light stimulated SE in *Doritaenopsis* orchids, while mixed red and blue spectra enhanced embryo development stages in *Peucedanum japonicum* and *Coffea canephora* [50]. In *Phalaenopsis* orchids, both red and red+far-red light spectra, combined with 3 mg L^{-1} thidiazuron (TDZ), effectively induced direct SE in intact protocorms, while green light at the same TDZ level, though less effective, also showed a positive effect [87]. In *Norway spruce*,

exposure to far-red light increased seedling height and dry matter production, compared to red light [75]. These results highlight the critical role of light quality in optimizing SE, with red light proving most effective across various species and phases. Combinations of red with far-red or blue spectra offer context-dependent benefits, enhancing specific stages or parameters of embryogenesis.

Light intensity plays a critical role during *in vitro* culture, influencing callus induction and SE, with preferences ranging from varying light intensities to darkness [71]. In *Malva sylvestris*, light intensity (50, 150, or 250 µmol m^{-2} s^{-1}) and DPI were assessed for their impact on SE. Increased light intensities enhanced embryo proliferation, with at least one day of DPI required for induction. Direct embryogenesis needed 2–6 days of DPI, while longer durations promoted indirect globular embryo formation [88]. In *Aralia elata*, light intensity played a key role in SE, with the highest rates and embryo counts per callus observed at a moderate intensity of 2000 lux (lx) [71]. In *Picea abies*, low-intensity light during the proliferation phase or late maturation had minimal impact on tissue growth, embryo yield, or survival, while during germination, balanced wavelengths and adequate light intensity significantly influenced shoot and root development, as well as embling survival [75]. In *Daucus carota*, red light at 10 µmol m^{-2} s^{-1} significantly enhanced embryo density, whereas lower (1–5 µmol m^{-2} s^{-1}) and higher (20 µmol m^{-2} s^{-1}) intensities had no significant effects. For embryo development, red light up to 20 µmol m^{-2} s^{-1} showed limited influence overall, but blue light at 10 or 20 µmol m^{-2} s^{-1} positively affected development, particularly during the globular and heart-shaped stages [85]. These findings support the species- and stage-specific requirements for light intensity and spectra in SE [71,85], while also emphasizing the beneficial role of DI in certain contexts.

Diverse studies indicate that low light intensity (PPFD) or DI may be more suitable for shoot induction and SE in species like rose, melon, and cucumber; however, lavender showed enhanced callus growth but reduced embryogenesis under darkness, while wall germander exhibited improved embryogenesis without affecting callus growth [88]. These results support that light conditions should be tailored to the specific requirements of each species.

DI or low light intensity may benefit SE in various species [88-90]. Exposure to light can induce phenolic oxidation, leading to tissue darkening and protein inhibition, which may hinder somatic embryo formation. Consequently, darkness is often critical for processes such as induction, callus initiation, maintenance, and maturation in many species [31,90-92]. The optimal duration of darkness depends on several factors, including the species, the phase of the process, and the specifics of the protocol. For example, in *Eucalyptus globulus*, darkness should be maintained until the embryos reach the cotyledonary stage, after which light exposure is recommended [91]. DI also influences SE pathways. In *Malva sylvestris*, at least one day of DPI was required for indirect SE induction, while direct SE required 2–6 days. Prolonged DPI favored the indirect formation of globular embryos [88]. Additionally, darkness during embryo germination may enhance root growth, as observed in *Norway spruce* [75]. Considering the critical role of DI in SE across various species, its optimization is also key to enhancing embryogenesis outcomes.

The photoperiod, along with light quality and intensity, is a key factor in regulating SE. Torné et al. [93] highlighted the critical role of photoperiod, light quality, and EOD phytochrome shifts in influencing SE in *Araujia sericifera*. Using 8- and 16-hour photoperiods and various light treatments (R, FR, R-FR, FR-R) under controlled irradiance, they found that SE levels were comparable under long and short days, with effects achieved after just 8 hours of light. Notably, EOD treatments that reduced Pfr levels inhibited SE after short days but not long days, while FR-R further stimulated SE under long days, even in the presence of polyamine inhibitors, highlighting potential gene expression modulation mechanisms.

Light conditions are crucial for regulating a variety of biological processes during SE. For example, Yu et al. [84] showed that red light significantly improved embryogenic callus formation in *Gossypium hirsutum* and notably reduced the differentiation period, with increases in polyamine levels (specifically spermidine and spermine), the lowest levels of putrescine, higher concentrations of endogenous auxin, and balanced levels of antioxidative enzymes. Elevated expression of marker genes associated with SE was also observed in response to red light. These findings demonstrate that light influences both metabolic regulation and gene expression, resulting in enhanced SE.

The lack of standardized definitions for light quality, combined with variability in spectra and intensity across different light sources, complicates determining the exact effects of light on SE [94]. This process is shaped by numerous factors, including genetic background, explant type, cell density, stressors that trigger cellular reprogramming, carbohydrate sources, dissolved oxygen levels, and plant growth regulators—particularly auxins and cytokinins— and their interactions, as well as gene expression and associated products, all within cultural and environmental contexts, including those related to light [94]. Recent findings highlight that SE involves a complex interplay of transcription factors, hormone signalling pathways, and epigenetic control mechanisms [31].

Light on *in Vitro* Organogenesis

IVO is a regenerative process in which new organs, such as shoots and roots, and ultimately whole plants, are derived from various plant tissues or cells, either directly or indirectly through a callus phase, in response to specific stimuli, with the potential to produce genetically identical plants [28,95]. It is a fundamental tool in plant biotechnology, enabling diverse applications, from rapid production of large-scale, disease-free, and genetically identical plants, to crop improvement through modern techniques [81]. Light plays a crucial role in IVO by influencing the initiation and development of organs and whole-plant formation, making it essential for optimizing regeneration systems and their applications in biotechnology [2,28,50].

Research on light quality shows that specific wavelengths are beneficial for IVO processes. Red and blue spectra, whether in mixed or monochromatic forms, often promote shoot formation and regeneration, while the addition of far-red light to blue or red has been found to further enhance regeneration in various species [50]. However, in *Lachenalia* sp. cultivars, various light spectra (white, blue, red) and darkness were tested, with white and blue light promoting the highest shoot and bud formation, followed by darkness and red light [72]. In *Begonia x erythrophylla*, white or red light, in contrast to far-red, blue light, or darkness, significantly enhanced shoot regeneration [96]. This highlights the species-specific light requirements for IVO. However, certain light conditions can inhibit organogenesis. For example, far-red light has been reported to suppress meristem development, an effect that is reversible, whereas blue light can permanently reduce shoot formation, likely mediated by an/independent blue spectrum photoreceptor, possibly cryptochrome, rather than directly through phytochrome [96]. In some scenarios, light can hinder not only shoot formation but also root regeneration [28]. This variability underscores the importance of light quality in developing optimized protocols. In a recent review, Cavallaro et al. [63] emphasize that shoot response is highly sensitive to light spectrum quality, highlighting its critical role in successful *in vitro* regeneration. Light quality effects vary among plant species and explants, significantly influencing regeneration outcomes, likely due to plant genetics and light receptor interactions [50].

Studies have highlighted species-specific responses to light intensity and photoperiod. For instance, Banu et al. [97] observed significant variation in optimal light intensities for shoot formation across species, identifying 1000 lx for Stevia rebaudiana, 3000 lx for Solanum tuberosum, and 1500 lx for Bacopa monnieri. Light intensity also affected shoot initiation time, and post-regeneration light conditions influenced root formation. In contrast, a review by Gupta and Agarwal [50] reported that high PPFD promoted shoot elongation in several plant species. A 16-hour light/8-hour dark photoperiod generally supports shoot and root regeneration across different species [31]. However, the impact of photoperiod length may vary according to environmental factors, such as temperature, which can influence IVO and response type; additionally, components of the medium, such as glucose—which has proven more effective than sucrose as a carbon source under specific incubation conditions—interact with environmental factors to enhance shoot ORG [98].

Light conditions play a critical role in influencing a range of physiological, biochemical, metabolic, and morphological aspects during in vitro regeneration. For example, Cavallaro et al. [63], in their review, reported that light treatments enhancing chlorophyll and carotenoid levels—key components of photosystems—are typically associated with increased biomass accumulation and overall shoot development. In the work by Bach et al. [72], different light qualities played distinct roles in Lachenalia sp. organogenesis, with white and blue light enhancing adventitious shoot and bud formation and increasing total phenolics, particularly caffeic acid, while red light restricted organogenesis, promoted shoot elongation, and raised glucose or fructose levels; ferulic acid content varied by light spectrum and genotype, with adventitious root formation associated with low levels of this compound. Light regimes also influence callus formation and oxidative responses, as described by Houllou et al. [99]. Additionally, shoot development from primordia depends on light and an external carbohydrate source, while root initiation can occur under both light and dark conditions [100]. These findings underscore the intricate nature of light-activated signalling and the interplay of diverse mechanisms leading to specific morphogenic outcomes, illustrating various examples in which light influences plant processes during IVO.

Light quality, intensity, and photoperiod are fundamental factors influencing *in vitro* organogenesis. By fine-tuning these parameters, researchers can optimize regeneration protocols, enhancing selective control over the regeneration process. Burritt and Leung [96] highlight light manipulation as a valuable tool in *in vitro* studies and micropropagation, enabling targeted outcomes such as suppressing meristem formation and promoting shoot primordia without transferring explants between culture media. LEDs have demonstrated greater efficacy than traditional fluorescent lighting in promoting organogenesis, with their precise control over wavelength and intensity making them particularly useful for guiding key developmental processes in plant cell and tissue cultures [50]. Specific wavelengths can be adjusted to create tailored light environments for different *in vitro* regeneration stages, an advantage over traditional fluorescent lights, which offer a broader, less tunable spectrum and limited intensity adjustments.

Influence of Dark Pre-incubation

DPI can play a crucial role in the micropropagation of various plant species, influencing both somatic embryogenesis and organogenesis. This technique involves exposing plant tissues to darkness for a specific period before transferring them to standard light conditions. It can significantly impact *in vitro* responses, including callus formation, shoot regeneration, and embryo induction and development [2,89]. However, its effectiveness depends on multiple factors such as species, genotype, tissue type, and specific regeneration systems used.

The positive effects of pre-incubation in darkness have been observed in various plant species, leading to improved tissue culture outcomes. However, its optimal duration can vary considerably. For example, a period of 2-4 weeks has been reported to positively influence regeneration rates in cucurbits, including cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), and squash (*Cucurbita pepo* L.), promoting SE and ORG [89,101-103]. In other species, a 1-week incubation enhanced shoot regeneration in chrysanthemum (*Chrysanthemum morifolium* Ramat) [104]; while 2-3 weeks of darkness has been reported to enhance somatic embryo induction and development in pepper (*Capsicum annuum* L.) [89], and a 2-week period promoted adventitious organogenesis in various apple cultivars, as well as SE in purple coneflower (*Echinacea purpurea* (L.) Moench), and in date palm (*Phoenix dactylifer*a L.) [90,105,106]. Despite these favorable results, the efficacy of DPI cannot be generalized; in some instances, it

has shown no positive effects or even detrimental impacts. For instance, DPI failed to induce shoot bud production in cucumber cotyledon callus [107], while a dramatic decrease in shoot formation was observed in two cultivars and one inbred line of cucumber [2]. Additionally, it negatively affected SE in gardenia (*Gardenia jasminoides* L.) and was also ineffective in inducing SE in rose (*Rosa hybrida* L.) [89]. Furthermore, DPI did not lead to regeneration in various pepper cultivars, and in *Petunia hybrida* it adversely affected the frequency of shoot regeneration [108,109].

In cucumber organogenesis using cotyledon explants, both 1- and 2-week DPI negatively impacted regeneration efficiency, leading to modifications in the *Agrobacterium*-mediated transformation protocol. The co-cultivation step was shifted to a photoperiod regime, which, along with other changes, significantly enhanced transformation efficiency [110].

Regarding the impact of DPI on callogenesis, it did not affect callus formation in cucumber, despite testing several genotypes, explant types, and growth regulators [101], nor in pepper cotyledon explants; however, the opposite was observed in pepper hypocotyl explants [108]. In bael (*Aegle marmelos* (L.) Corr.), a 1- to 7-day DPI of cotyledon explants resulted in abundant callus formation [111]. In *Capsicum annuum* L. anther culture, callogenesis was influenced by both light regimes and growth regulator combinations [112].

In *Pyrus syriaca* direct regeneration, optimal shoot formation was achieved with a 2–3-week DPI and 2.0 µM TDZ, among other growth regulators tested [113]. In *Lavandula latifolia* Medicus, DPI did not improve bud and shoot regeneration with elevated auxin levels (6.0, 11.0 µM) [114]. Both studies suggest that the effectiveness of pre-incubation depends on its interaction with specific growth regulators and their concentrations.

The impact of DPI on *in vitro* morphogenesis involves intricate, largely undefined processes [2]. This treatment may alter resource allocation, growth regulator levels, and shifts in their sensitivity [106]. Hormonal balance, influenced by light/dark regimes, is key to successful regeneration [114]. A brief exposure to light in dark-grown seedlings slowed growth and shifted the free-to-conjugated Indole-3-acetic acid (IAA) ratio; shoot induction in dark-grown callus significantly increased ethylene production, a factor linked to both stress response and regeneration capacity [115-117]. In a recent study on date palm, somatic embryos significantly accumulated total protein after a 2-week DPI compared to a photoperiod condition [90]. Light regimes also influence the biosynthesis of secondary compounds, some of which may modulate various aspects of *in vitro* plant regeneration [118,119]. For instance, some phenolics, phenylpropanoids, and flavonoids are known to regulate hormone activity, including auxin breakdown, transport, and interactions with other growth regulators [119,120]. Additionally, these regimes may affect cell cycle dynamics [121,122], and research on petunia species has revealed genetic factors that govern regeneration responses under varying light or dark conditions [109].

While dark pre-incubation can be a valuable tool for optimizing *in vitro* plant regeneration protocols, its implementation should be carefully tailored to the specific requirements of each species, genotype, and regeneration system. Further research at various biological levels is crucial to unravel the complex mechanisms underlying the effects of dark pre-incubation and how they are mediated. Such insights will help develop more efficient and reproducible regeneration protocols across diverse plant species and genotypes.

Conclusion

Light plays a pivotal role in *in vitro* regeneration, shaping the outcomes of somatic embryogenesis and organogenesis, influencing regeneration efficiency and success. The precise fine-tuning of light parameters such as quality, intensity, and duration is essential for optimizing regeneration systems across various biotechnological applications, including large-scale micropropagation, germplasm conservation, secondary metabolite production, pathogen eradication, and advanced techniques like genome editing and genetic transformation. The intricate interplay between light, plant metabolism, and other environmental factors, such as temperature and humidity, presents challenges for establishing universal guidelines. These factors, in combination with light, influence photosynthesis, hormone regulation, and cellular responses, adding layers of complexity to regeneration protocols. Species-specific responses, genotypic variability, and tissue- or cell-specific requirements demand tailored approaches. For instance, the benefits of a dark pre-incubation or darkness during certain stages and systems add complexity to uncovering the underlying mechanisms and highlight the need for context-specific protocols.

While some progress has been made in understanding light's regulatory mechanisms in *in vitro* regeneration, research in this area remains limited. Future investigations would benefit from addressing the following aspects:

a. Elucidating the specific roles of photoreceptors in regulating plant regeneration processes.
b. Investigating the interaction between light signals and other environmental factors (e.g., temperature and CO_2 levels) in modulating regeneration responses.
c. Investigating the circadian clock's role in regulating regeneration processes, along with the impact of controlled light cycles and dark incubation.
d. Investigating how light influences endogenous hormones and the signalling pathways that regulate regeneration.
e. Exploring the interaction between light signalling and key transcription factors in regulating gene expression associated with regeneration.
f. Investigating the role of chloroplast signalling in light-induced changes during regeneration.
g. Exploring how light parameters affect gene splicing and its role in regeneration.
h. Exploring how light-induced signalling pathways regulate regeneration at the cellular and tissue level.
i. Investigating light's impact on epigenetic modifications and

gene expression regulation during regeneration.

j. Elucidating the mechanisms and interactions between genetic, molecular, and epigenetic pathways in light-induced plant regeneration processes, and how these components influence key factors such as transcription factors, gene expression, and regulatory networks.

Finally, leveraging advanced LED lighting technologies known for their energy efficiency and customizable light parameters will enhance the precision and efficiency of *in vitro* regeneration, ultimately contributing to the optimization of regeneration outcomes and their diverse biotechnological applications.

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