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ORIGINAL ARTICLE

Enhancing Growth and Morpho-physiological Traits of Tissue-cultured Explants of Persian Walnut through Manipulation of *In vitro* Lighting Spectra

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K E Y W O R D S

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Morpho-physiological;
Tissue culture

A B S T R A C T

There are numerous challenges associated with the large-scale production of walnut *In vitro*-plantlets. It is imperative to develop new environmental control systems for its *In vitro* propagation. Additionally, there is a lack of knowledge regarding the impacts of lighting systems on the morpho-physiological traits and biomass accumulation in walnut tissue-cultured explants. In this study, walnut nodal shoots were subjected to eight different light spectra, including white, blue, red, green, far-red, blue-red combination, red-far-red combination, and a fluorescent lamp serving as a control, over a period of 28 days. The results indicated that combined spectra treatments, such as blue-red and red-far-red, led to improved biomass accumulation (total fresh and dry weight) compared to other monochromatic light spectral treatments. Furthermore, Light-emitting diode (LED) treatments had a discernible impact on the morpho-physiological traits of walnut *In vitro*-explants. Specifically, white light spectra enhanced Specific leaf area (SLA), while the green light spectra increased leaf water content (LWC) when compared to other light treatments. Additionally, the application of far-red light elevated leaf mass area (LMA) and water content per unit leaf area (LWCA). The findings of this study demonstrate that the quality, morphological, and growth characteristics of *In vitro* explants of walnut can be enhanced by utilizing specific light spectra.

Introduction

Persian walnut (*Juglans regia* L.) is one of the most important nuts cultivated in the world (Mahmoudian *et al.*, 2021), which is appreciated by consumers due to its therapeutic benefits and high nutritional content (Habibi *et al.*, 2022). Its *in vitro* propagation is of vital importance for the mass propagation of cultivars with desirable features that are high-quality, disease-free, and uniformly multiplied (Vahdati *et al.*, 2006). Plant tissue culture provides the ability to propagate uniformly genetic and disease-free plant material with the desired traits (Deb and Imchen, 2010; Khodadadi *et al.*, 2016). This technology allows us to control the growth of plants by setting various parameters that affect plant growth (Loyola-Vargas and Ochoa-Alejo, 2018). Propagation *In vitro* has some challenges such as optimization of growing and propagation media and acclimatization of the produced plantlets to the *ex vitro* environments (Aliniaeifard *et al.*, 2020; Asayesh *et al.*, 2021; Asayesh *et al.*, 2017; Hazarika, 2006; Kadleček *et al.*,

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2001; Kozai *et al.*, 2005; Vahdati and Aliniaeifard, 2017; Vahdati *et al.*, 2017).

Persian walnut is the most developed and cultivated species of walnut in horticulture, but they are highly recalcitrant to tissue culture (Sadat-Hosseini *et al.*, 2022; Vahdati *et al.*, 2020 and 2022; Yegizbayeva *et al.*, 2021). There is extensive data on the effect of light quality on herbaceous species *In vitro*, while less information is available on woody species (Stefano and Rosario, 2003). The lighting of tissue culture media is usually provided by fluorescent lamps, but a significant part of the spectral output emitted by this light source is not used by tissue culture plants (Saeedi *et al.*, 2023). Recently, light-emitting diodes (LED) have been suggested as a flexible and low-consumption light energy for a multitude of plant tissue culture purposes (Dutta Gupta and Jatothu, 2013). Plant photosynthesis requires light as an energy source, in addition to morphological and signaling effects (Davarzani et al., 2023). LEDs provide the possibility of personalizing the quality of the light spectra suitable for the needs of tissue culture plants (Bello-Bello et al., 2017). The emission spectra of these light sources align with the absorption spectra of plant photoreceptors; however, the majority of studies have primarily concentrated on assessing the impact of blue and red lights. The effects of different types of multi-chrome lights, or other spectra such as far-red and green lights in tissue culture media have not been comprehensively studied. The success in regeneration and improving the growth and morpho-physiological characteristics of tissue culture explants largely depends on the spectra quality and photon efficiency of the light sources in the growth chambers (Gupta and Agarwal, 2017). The changes made in the growth and development of walnut tissue culture plants were mainly through changes in microenvironmental parameters, including the composition of the culture medium, plant growth regulators, temperature of the growth room, the amount of CO_2, and different chemical treatments (Kozai and Xiao, 2008; Sáez *et al.*, 2012; Sáez *et al.*, 2015).

The impact of light on the regulation of plant growth and development has been extensively characterized, but there has been scarce of information on the effect of light on the growth of tissue-cultured walnut plants. The present study is planned for the development of LED-based lighting systems for physiological experiments and the growth of walnut tissue culture explants. Therefore, growth and morpho-physiological characteristics of micro-propagated explants of walnut cv. 'Chandler' in response to *In vitro* medium light quality was studied. Also, achieving a protocol for the improvement of growth and proliferation of *in vitro* walnut plantlets with little cost and without the use of expensive hormones, which is the main goal of commercial tissue culture labs was planned in this study.

Material and Methods

Lighting treatments Nodal shoots of walnut (cv. Chandler) with a length of 20 ± 2 mm which was in the establishment phase were selected. The DKW medium (50 mL) with sucrose (3%), BAP (1 mg L^{-1}), IBA (0.01 mg L^{-1}), and solidified with Carrageenan (7 g L^{-1}) was used (Driver and Kuniyuki, 1984). Each treatment included six jars and two explants per jars. The pH of medium was adjusted to 5.7 before autoclaving for 20 min at 121°C and a pressure of 1 atmosphere. To apply LED spectra treatments, *In vitro* explants of walnut nodal shoots were placed in a tissue culture growth chamber (Phytotron) equipped with 24W LED units. In each growth rack, there was one LED treatment and the spectra did not overlap with each other. Tissue-cultured walnut nodal shoots samples were exposed for 28 days under different LED spectra treatments including white spectra (400-700 nm), blue (peak wavelength 460 nm), red (peak wavelength 660 nm), far-red (peak wavelength 740 nm), green (peak wavelength 530 nm), the combination of red and blue light (in the ratio of 70:30), the combination of red and far-red light (in the ratio of 70:30) and the fluorescent lamp as a control.,

under a fixed light intensity of 80 µmol $m^{-2}.s^{-1}$ and the photoperiod was 16/8 h of light/dark at a fixed temperature of 25°C.

Measurement of growth characteristics

Growth characteristics, including total fresh and dry weight, were evaluated using a digital scale. For evaluating total dry weight, the samples were placed in an oven at a temperature of 70°C for 48 h. Leaf area was measured by Digimizer software (v.5.4.9).

Morpho-physiological characteristics

Morpho-physiological characteristics of walnut tissue culture explants such as specific leaf area (SLA), water content per unit leaf area (LWCA), leaf mass area (LMA), and leaf water content (LWC) were calculated using the following equations:

1- SLA= (Leaf dry matter weight / leaf area)

2- LMA= (Leaf area / leaf dry weight)

3- LWC= (Leaf fresh weight / leaf dry weight - leaf fresh weight)

4- LWCA= (Leaf area / dry leaf weight - fresh leaf weight)

Statistical analysis

The experiments were conducted as a completely randomized design (CRD) with 8 treatments and 6 replications. The data was analyzed using SAS 9.4 software. Mean separations were determined using Duncan's multiple range tests with a significance level of $p \leq 0.05$.

Results

Growth and biomass analysis

The growth rate of walnut explants under light spectra treatments can be presented in Fig. 1. combined spectral treatments increased the growth and biomass production in walnut explants. Two combined LED treatments including blue and red spectra as well as red and far-red spectral combinations increased total fresh and dry weight in *In vitro* explants of walnut compared to monochromatic spectral treatments. Therefore, the combined LED treatments yielded the highest total fresh weight of the plantlet, while the combination treatment with a combined blue and red spectra yielded the highest total dry weight (Fig. 2 a and b).

Treatments that included the red spectra in addition to blue and far red had a considerable impact on the number of nodes. The number of nodes on explant of walnut tissue-grown plantlets was enhanced by both the red and far-red combined spectral treatment as well as the blue and red combined spectral treatment (Fig. 2 c). Additionally, the lowest number of nodes was observed in far red and fluorescence treatments, respectively.

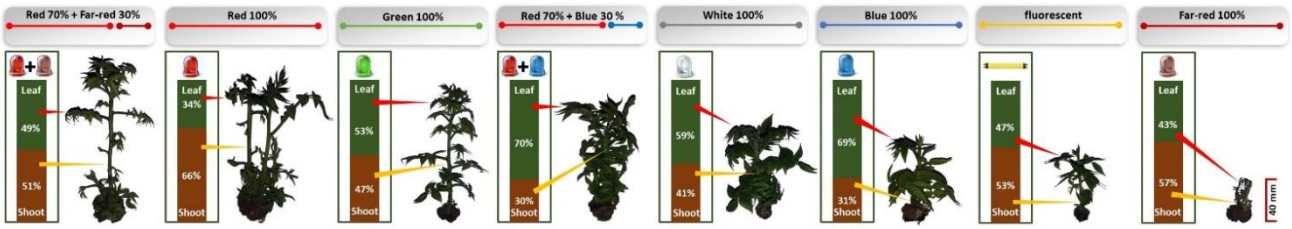

Fig. 1. Growth rate of Persian walnut explants (cv. 'Chandler') after being exposed to various light quality regimes over a period of 28 days

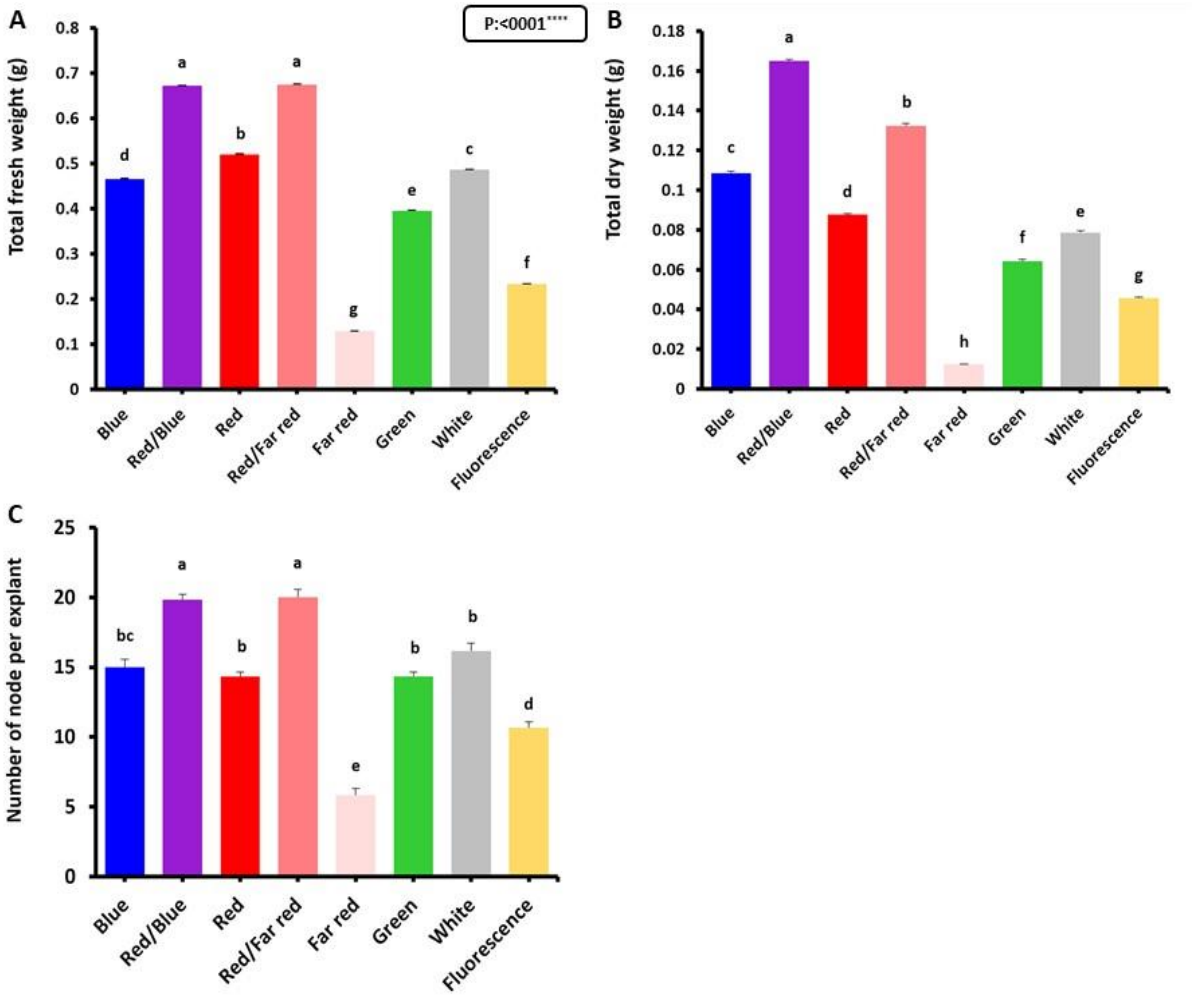

Fig. 2. Growth characteristics of Persian walnut explants (cv. 'Chandler') being exposed for 28 days to different light quality regimes. Columns with the same letters are not significantly different at $p \leq 0.05$

Biomass partitioning

The results showed that the division of biomass into shoot and leaf organs of tissue cultured walnut explants changed due to the use of light treatments. The division of biomass was determined based on dry weight and the percentage of each part relative to the total weight of the plant. Explants treated with monochromatic blue and blue and red combination allocated most of their biomass to leaf organs. Red spectra treatment decreased biomass distribution to leaf and increased biomass distribution to shoot. The highest and lowest contribution of biomass partition to different organs were calculated under the treatment of red, blue and fluorescent compounds, respectively (Fig.3).

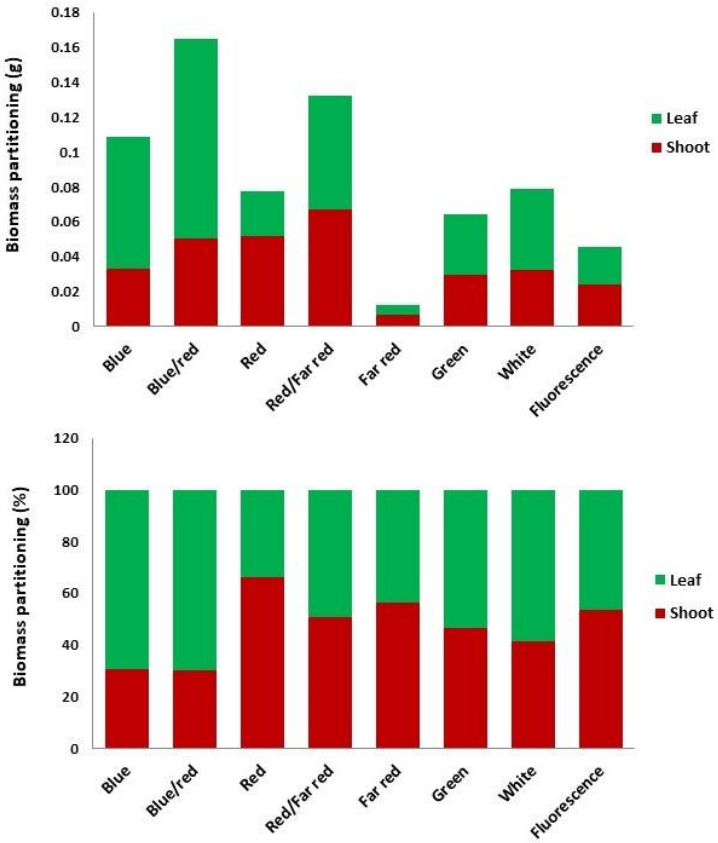

Fig. 3. Biomass division into shoot and leaf of Persian walnut explants (cv. 'Chandler') being exposed for 28 days to different light quality regimes. Columns with the same letters are not significantly different at $p \leq 0.05$

Morpho-physiological analysis

The morpho-physiological parameters of tissue cultured walnut explants were significantly affected by light treatments (P<0001). The highest leaf specific area was observed in explants grown under the white spectra treatment, which was 90.94% higher than the control treatment, and the lowest leaf specific area was observed in the far-red spectra treatment, which was 29.73% lower than the control (Fig. 4 a).

The highest amount of LWC was observed in the explants grown in the shelf with green spectra treatment, which showed a 1.75% increase compared to the fluorescent lamp (control), and the lowest amount of LWC was observed in the combined red and far-red spectra treatment (Fig. 4 b).

The highest amount of LWCA was observed under the treatment of the far-red spectra (6.25% increase compared to the control) and the lowest amount was observed in the treatment of the combined blue and red spectra (Fig. 4 c).

The highest LMA was observed in the explants grown under the far-red spectra treatment, which increased by 33.3% compared to the fluorescent treatment, and the lowest was observed in the green spectra treatment, which showed a 40.47% decrease compared to the fluorescent (Fig. 4 d).

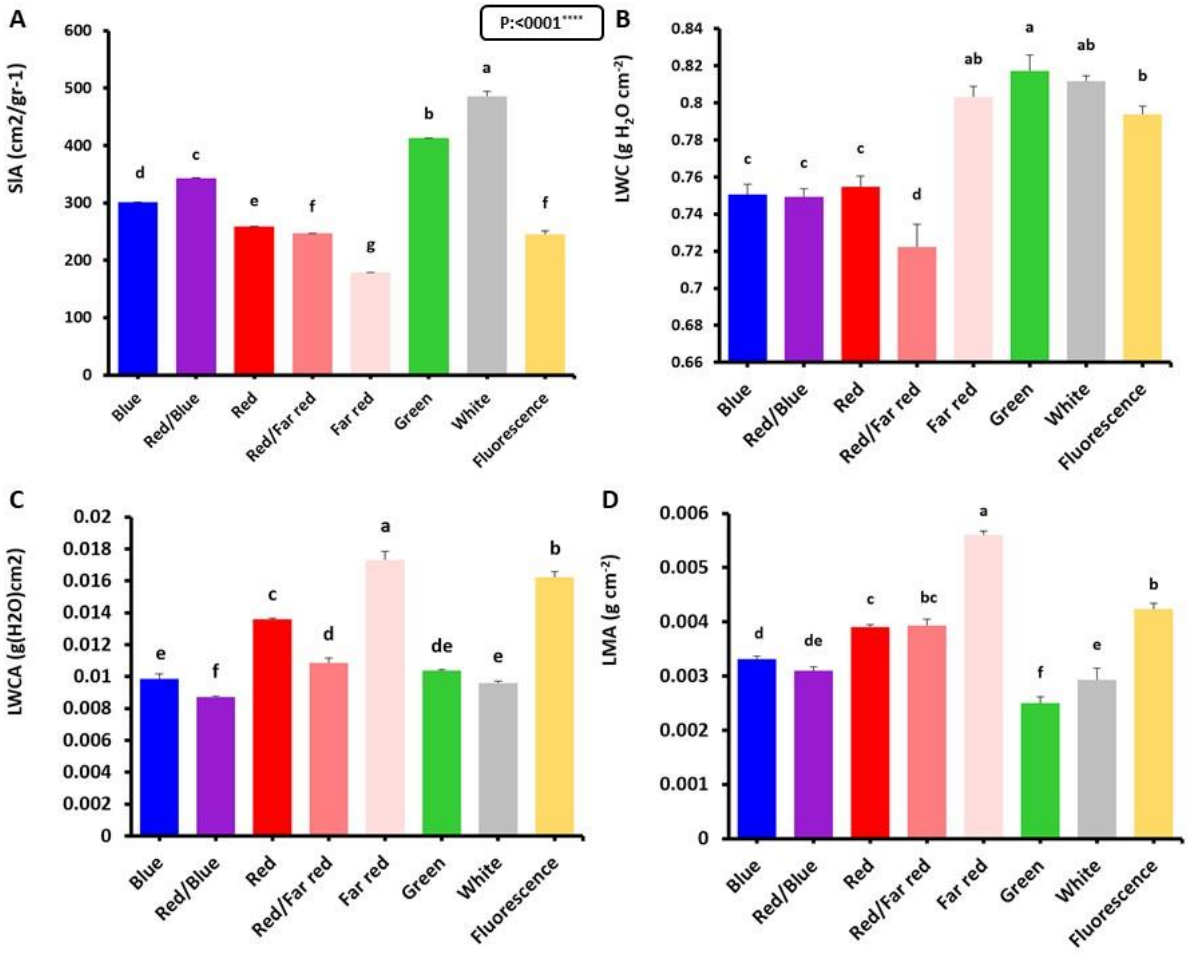

Fig. 4. Morpho-physiological characteristics of Persian walnut explants (cv. 'Chandler') being exposed for 28 days to different light quality regimes. Columns with the same letters are not significantly different at $p \leq 0.05$

Discussion

The success in improving the growth and morphological characteristics of walnut *In vitro*-explants largely depends on the spectra quality of the light sources in the growth rooms. Red and blue LEDs are commonly used to increase the amount of biomass because chlorophyll a and b absorb wavelengths in the blue and red light ranges more effectively (Son and Oh, 2013). LEDs with red spectra generally increase plant growth by increasing fresh and dry weight and height. In addition, blue light prevents cell growth and a higher ratio of blue light to red light will result in a decrease in dry weight (Li *et al.*, 2010; Lin *et al.*, 2013; Poudel *et al.*, 2008). A high rate of blue light causes a decrease in height and biomass, and a high rate of red light causes an increase in height and biomass (Nanya *et al.*, 2012). The quality of light played an important role in differentiating plant cells and improving the growth characteristics of walnut tissue culture plantlets. In confirmation of cases, the highest amount of total fresh weight was observed in two treatments with red-far-red and red-blue combination LEDs. It has been shown that the combination of red-blue light causes a better development of chloroplasts and an increase in dry weight, which is consistent with the results obtained from the combined red-blue spectra treatment that caused an increase in total dry weight (Ouzounis *et al.*, 2016). In several cases, plantlets grown under LED lamps had higher fresh and dry weights compared to explants grown under fluorescent light. *Euphorbia milii* and blueberry tissue culture plantlets were exposed to different combinations of blue and

red LED spectra and caused an increase in fresh and dry weight in these plants (Batista *et al.*, 2016; Hung *et al.*, 2016). Red LED spectra causes an increase in fresh and dry weight in Dendrobium tissue culture plantlets (Lin *et al.*, 2011). Biomass partitioning to stem in response to red light and to leaf in response to blue and white light is a well-documented phenomenon. Research has shown that red light promotes biomass accumulation, growth and photosynthesis, while blue light enhances the assimilation function of the leaf (Tarakanov *et al.*, 2022). Their mutual effects are compensated by the simultaneous presence of blue and red light (Kaiser *et al.*, 2019). It has also been shown that the biomass and yield of various plants can be increased by adding blue light to red light. The effects of light quality on biomass distribution are attributed to the influence of different light spectra on plant development, physiology and photomorphogenesis. Therefore, the specific distribution of biomass in response to different light spectra is a result of the complex interactions between light and plant physiological processes (Moradi *et al.*, 2021). SLA indicates the thinness of the leaf. In general, with the decrease of SLA, the area of receiving light in the plant decreases, but the thickness of the leaf increases. According to the results, the white LED spectra increased the light-receiving surface and also caused explants with thinner leaves compared to other treatments, and the far-red LEDs increased the thickness of the leaves. The spectra of LED treatments with emission spectra corresponding to the absorption spectra of the photoreceptors of plants caused them to create optimal productivity in *In vitro* conditions by influencing the morpho-physiological characteristics. So that the morpho-physiological characteristics were strongly affected by different LED light treatments and these characteristics had better results than the fluorescent treatment as a control.

Conclusions

Under controlled *In vitro* conditions, conscious manipulation of light quality, combined with the advantages of LED technology, leads to improved growth and morpho-physiological characteristics of walnut tissue-cultured plantlets. There is reluctance among many tissue culture laboratories to adopt LED lighting systems instead of conventional ones due to concerns regarding unpredictable and abnormal morphology in *in vitro* conditions. Nevertheless, our research has demonstrated the efficacy of LED lights in promoting robust growth of tissue-cultured walnut nodal shoots. In conclusion, our study findings indicate that the favorable micropropagation of Persian walnut explants heavily relies on the specific light spectra provided.. Exposure to the combined spectra of LED treatments improved the growth characteristics (total fresh and dry weight) and the morpho-physiological characteristics were strongly affected by these light sources.

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References

Aliniaeifard S, Asayesh ZM, Driver J, Vahdati K (2020) Stomatal features and desiccation responses of Persian walnut leaf as caused by *In vitro* stimuli aimed at stomatal closure. Trees. 34(5), 1219-1232.

Arab MM, Marrano A, Abdollahi-Arpanahi R, Leslie CA, Cheng H, Neale DB, Vahdati K (2020) Combining phenotype, genotype, and environment to uncover genetic components underlying water use efficiency in Persian walnut. Journal of Experimental Botany; 71(3), 1107-1127.

Asayesh ZM, Aliniaeifard S, Vahdati K (2021) Stomatal morphology and desiccation response of Persian walnut tissue culture plantlets influenced by the gelling agent of

In vitro culture medium. Journal of Nuts. 12(1), 41-52.

Asayesh ZM, Vahdati K, Aliniaeifard S, Askari N (2017) Enhancement of ex vitro acclimation of walnut plantlets through modification of stomatal characteristics *In vitro*. Scientia Horticulturae. 220, 114-121.

Batista DS, de Castro KM, da Silva AR, Teixeira ML, Sales TA, Soares LI, das Graças Cardoso M, de Oliveira Santos M, Viccini LF, Otoni WC (2016) Light quality affects In vitro growth and essential oil profile in Lippia alba (Verbenaceae). In vitro Cellular & Developmental Biology-Plant. 52, 276-282.

Bello-Bello JJ, Perez-Sato JA, Cruz-Cruz CA, Martínez-Estrada E (2017) Light-emitting diodes: Progress in plant micropropagation. InTech. 6(1), 93-103.

Davarzani M, Aliniaeifard S, Mehrjerdi MZ, Roozban MR, Saeedi SA, Gruda NS (2023) Optimizing supplemental light spectrum improves growth and yield of cut roses. Scientific Reports. 13(1), 21381.

Deb C, Imchen T (2010) An efficient In vitro hardening technique of tissue culture raised plants. Biotechnology. 9(1), 79-83.

Driver JA, Kuniyuki AH (1984) *In vitro* propagation of Paradox walnut rootstock. HortScience. 19(4), 507-509.

Dutta Gupta S, Jatothu B (2013) Fundamentals and applications of light-emitting diodes (LEDs) in *In vitro* plant growth and morphogenesis. Plant Biotechnology Reports. 7, 211-220.

Goins GD, Yorio N, Sanwo M, Brown C (1997) Photomorphogenesis, photosynthesis, and seed yield of wheat plants grown under red light-emitting diodes (LEDs) with and without supplemental blue lighting. Journal of Experimental Botany. 48(7), 1407-1413.

Gupta SD, Agarwal A (2017) Light emitting diodes for agriculture. LED supplementary lighting: 27-36.

Habibi A, Hamedpour-Darabi M, Vahdati K (2022) Local cultural values of Persian walnut in Iran. In: Wall J (ed) The Cultural value of trees. 1st edn. Routledge. New York.

Hazarika, B. (2006). Morpho-physiological disorders in *In vitro* culture of plants. Scientia Horticulturae. 108(2), 105-120.

Hung CD, Hong CH, Kim SK, Lee KH, Park JY, Nam MW, Choi DH, Lee HI (2016) LED light for In vitro and ex vitro efficient growth of economically important highbush blueberry (Vaccinium corymbosum L.). Acta Physiologiae Plantarum. 38: 1-9.

Julian LMR, Alexandru F, Georgi C (2020) Micropropagation of valuable walnut genotypes for timber production: new advances and insights. Annals of Silvicultural Research. 44(1), 5-13.

Kadleček P, Tichá I, Haisel D, Čapková V, Schäfer C (2001) Importance of *In vitro* pretreatment for ex vitro acclimatization and growth. Plant Science. 161(4), 695-701.

Kaiser E, Ouzounis T, Giday H, Schipper R, Heuvelink E, Marcelis LF (2019) Adding blue to red supplemental light increases biomass and yield of greenhouse-grown tomatoes, but only to an optimum. Frontiers in Plant Science. 9, 2002.

Khodadadi F, Tohidfar M, Mohayeji M, Dandekar AM, Leslie CA, Kluepfel D, Butterfield T, Vahdati K (2016) Induction of polyphenol oxidase in walnut and its relationship to the pathogenic response to bacterial blight. Journal of the American Society for Horticultural Science. 141(2), 119-124.

Kozai T, Xiao Y (2008) A commercialized photoautotrophic micropropagation system. Plant Tissue Culture Engineering. Focus on Biotechnology, 6: 355-371, Springer, Dordrecht.

Kozai T, Xiao Y, Nguyen QT, Afreen F, Zobayed SM (2005) Photoautotrophic (sugar-free

medium) micropropagation systems for large-scale commercialization. Propagation of Ornamental Plants. 5(1), 23-34.

Li H, Xu Z, Tang C (2010) Effect of light-emitting diodes on growth and morphogenesis of upland cotton (*Gossypium hirsutum* L.) plantlets *In vitro*. Plant Cell, Tissue and Organ Culture (PCTOC). 103, 155-163.

Lin KH, Huang MY, Huang WD, Hsu MH, Yang ZW, Yang CM (2013) The effects of red, blue, and white light-emitting diodes on the growth, development, and edible quality of hydroponically grown lettuce (*Lactuca sativa* L. var. capitata). Scientia Horticulturae. 150, 86-91.

Lin Y, Li J, Li B, He T, Chun Z (2011) Effects of light quality on growth and development of protocorm-like bodies of Dendrobium officinale In vitro. Plant Cell, Tissue and Organ Culture (PCTOC). 105, 329-335.

Loyola-Vargas VM, Ochoa-Alejo N (2018) An introduction to plant tissue culture: advances and perspectives. Plant Cell Culture Protocols. 1815, 3-13.

Mahmoudian M, Rahemi M, Karimi S, Yazdani N, Tajdini Z, Sarikhani S, Vahdati K (2021) Role of kaolin on drought tolerance and nut quality of Persian walnut. Journal of the Saudi Society of Agricultural Sciences. 20(6): 409-16.

Moradi S, Kafi M, Aliniaeifard S, Salami SA, Shokrpour M, Pedersen C, Moosavi-Nezhad M, Wróbel J, Kalaji HM (2021) Blue light improves photosynthetic performance and biomass partitioning toward harvestable organs in saffron (*Crocus sativus* L.). Cells. 10(8), 1994.

Nanya K, Ishigami Y, Hikosaka S, Goto E (2012) Effects of blue and red light on stem elongation and flowering of tomato seedlings. In VII International Symposium on Light in Horticultural Systems. 956, 261-266.

OuzounisT, Heuvelink E, Ji Y, Schouten H, Visser R, Marcelis L (2016) Blue and red LED lighting effects on plant biomass, stomatal conductance, and metabolite content in nine tomato genotypes. In VIII International Symposium on Light in Horticulture. 1134, 251-258.

Poudel PR, Kataoka I, Mochioka R (2008) Effect of red-and blue-light-emitting diodes on growth and morphogenesis of grapes. Plant Cell, Tissue and Organ Culture. 92, 147-153.

Sadat-Hosseini M, Arab MM, Soltani M, Eftekhari M, Soleimani A, Vahdati K (2022) Predictive modeling of Persian walnut (Juglans regia L.) *In vitro* proliferation media using machine learning approaches: a comparative study of ANN, KNN and GEP models. Plant Methods. 18(1), 48.

Saeedi SA, Vahdati K, Sarikhani S, Daylami SD, Davarzani M, Gruda NS, Aliniaeifard S (2023) Growth, photosynthetic function, and stomatal characteristics of Persian walnut explants *In vitro* under different light spectra. Frontiers in Plant Science. 14,1292045

Sáez PL, Bravo LA, Latsague MI, Sánchez ME, Ríos DG (2012) Increased light intensity during *In vitro* culture improves water loss control and photosynthetic performance of *Castanea sativa* grown in ventilated vessels. Scientia Horticulturae. 138, 7-16.

Sáez PL, Bravo LA, Latsague MI, Toneatti MJ, Coopman RE, Alvarez CE, Sánchez-Olate M, Ríos DG (2015) Influence of *In vitro* growth conditions on the photosynthesis and survival of *Castanea sativa* plantlets during ex vitro transfer. Plant Growth Regulation. 75, 625-639.

Son KH, Oh MM (2013) Leaf shape, growth, and antioxidant phenolic compounds of two lettuce cultivars grown under various

combinations of blue and red light-emitting diodes. HortScience. 48(8), 988-995.

Stefano M, Rosario M (2003) Effects of light quality on micropropagation of woody species. Micropropagation of woody trees and fruits. 75, 3-35.

Tarakanov IG, Tovstyko DA, Lomakin MP, Shmakov AS, Sleptsov NN, Shmarev AN, Litvinskiy VA, Ivlev AA (2022) Effects of light spectral quality on photosynthetic activity, biomass production, and carbon isotope fractionation in lettuce, *Lactuca sativa* L., plants. Plants. 11(3), 441.

Vahdati K, Aliniaeifard S (2017) Investigation of physiological components involved in low water conservation capacity of *In vitro* walnut plants. Scientia Horticulturae. 224, 1-7.

Vahdati K, Asayesh ZM, Aliniaeifard S, Leslie C (2017) Improvement of ex vitro desiccation through elevation of CO2 concentration in the atmosphere of culture vessels during *In vitro* growth. HortScience. 52(7), 1006-1012.

Vahdati K, Sadeghi-Majd R, Sestras AF, Licea-Moreno RJ, Peixe A, Sestras RE (2022) Clonal Propagation of Walnuts (*Juglans* spp.): A Review on Evolution from Traditional Techniques to Application of Biotechnology. Plants. 11(22), 3040.

Yegizbayeva TK, García-García S, Yausheva TV, Kairova M, Apushev AK, Oleichenko SN, Licea-Moreno RJ (2021) Unraveling factors affecting micropropagation of four Persian walnut varieties. Agronomy. 11(7), 1417.