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ORIGINAL ARTICLE

Stock plant etiolation reduces rooting of sub-terminal olive cuttings by reducing total sugars, IAA, indole/phenol ratio, and IAA/GA ratio

Mohamed Abdel-Aziz Abdel-Mohsen[1] · **Ahmed AbdelHady Rashedy**[1]

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Abstract

Adventitious root formation is the key to successful propagation of olives through cuttings. Etiolation, as an eco-friendly practice, can modulate the plant's endogenous phytohormone, which plays an important role in stimulation of rooting of cuttings. Therefore, this study was aimed to investigate the effect of etiolation on the rooting behavior of both 'Manzanillo' and 'Picual' olive cuttings (terminal and sub-terminal) treated with IBA (0, 4000 ppm). The results indicated that sub-terminal cuttings in both cultivars were more effective in olive propagation than terminal cuttings as they recorded increased leaves number, C/N ratio, total carbohydrates, total indoles, IAA, IAA/GA ratio, and total phenols. Etiolation of both olive cultivars for both terminal and sub-terminal cuttings significantly decreased rooting percent, roots number, IAA, and IAA/GA ratio; while, it increased GA and GA/IAA. Moreover, etiolation decreased total sugars, IAA, IAA/GA ratio, and indole/phenol ratio in both sub-terminal cuttings. Terminal 'Manzanillo' cuttings achieved a higher rooting percent than terminal 'Picual' cuttings by 5.83% and 202.2% as mean of both seasons for untreated and IBA-treated cuttings, which was accompanied by significant increase in vascular bundle%, total phenol, phenol/indole ratio, IAA, and IAA/GA ratio by 21.48%, 49.2%, 44.72%, 12.9%, and 22.9%, respectively. Also, terminal 'Manzanillo' cuttings recorded lower GA by 7.98% and GA/IAA ratio by 18.21% and pith by 6.13% than terminal 'Picual' cuttings. This study proved that olive plants need exposure to full sunlight to propagate easily by cuttings since etiolation had negative effects on IBA-treated cuttings.

Keywords C/N ratio · GA · IBA · IAA · *Olea europaea* · Phenol · Anatomy

Introduction

Olive tree (*Olea europaea* L.) belongs to the Oleaceae family, and is considered as one of the most economically important evergreen fruit trees, especially in the Mediterranean basin. The global production of olive oils and table olives was 3,010,000 tons and 2,661,000 tons, respectively, for the 2020/2021 olive crop year (IOC 2022). The total production in Egypt is 932,927 tons from 100,826 ha (FAO 2022).

Olive fruits are the source of nutrients such as Fe, Ca, and Na along with polyphenols, fats, and vitamin E (Visioli et al. 1998). Ferreira et al. (2007) found that antioxidants present in olive fruits play an important role in enhancing human immune system, reducing the onset of asthma, treating diabetes, preventing cancer, delaying body aging, and overcoming the negative effects of high blood pressure (Tektonidis et al. 2015; Schwingshackl et al. 2017; Przychodzen et al. 2019; Romana-Souza et al. 2020).

Olive trees are propagated through leafy cuttings, which is one of the most practical methods in horticultural nurseries due to its mass production within a short period of time, simplicity, low cost, and practicability (Vidal et al. 2003; Hussein et al. 2017; Rashedy et al. 2021). However, olive propagation via cuttings faces a serious setback due to weaker regeneration capacity and low rooting rates (Fabbri et al. 2004). The rooting response of different cuttings depends on endogenous and exogenous factors such as genotype, mother tree age, cuttings collection time, cuttings type,

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Ahmed AbdelHady Rashedy
Ahmed.rasheedy@agr.cu.edu.eg; ahmed.rashedy@gmail.com

Mohamed Abdel-Aziz Abdel-Mohsen
mohamed.abdulmohsen@agr.cu.edu.eg;
mael.shazly@gmail.com

[1] Pomology Department, Faculty of Agriculture, Cairo University, Giza, Egypt

pre- and post-planting care (Frey et al. 2006; Hechmi et al. 2013; Rashedy et al. 2021).

Regarding the type of cuttings, rooting percentage of Sparkleberry (*Vaccinium arboreum*) becomes greater when using softwood cuttings than hardwood and semi-hardwood cuttings (Bowerman et al. 2013). While, semi-hardwood cuttings of Shantung Maple (*Acer truncatum*) were rooted better than softwood, hardwood stem-tip, and second-flush softwood cuttings. In contrast, neither terminal nor sub-terminal cuttings of Shantung Maple affect rooting percentage (Brock and Griffin 2014). Regarding the endogenous components, the highest phenolic compounds were recorded in hard-to-root “Konsrvalia” olive cultivar, while the lowest phenolic compounds were recorded in easy-to-root “Roghani” olive cultivar (Aslmoshtaghib and Reza-Shahsavar 2010). Auxin has been confirmed as a limiting agent for root initiation (Aslmoshtaghib and Reza-Shahsavar 2010). It has been widely used for root formation in woody stem cuttings (El-Sharony et al. 2018; Rashedy et al. 2021). In contrast, the concentration of endogenous IAA in hard-to-root olive ‘Nabali’ cultivar is significantly higher than that of easy-to-root ‘Raseei’ cultivar (Ayoub and Qrunfleh 2006).

To protect the environment and support sustainable agriculture, the European Union is forcing member countries to withdraw, change, or modify the use of synthetic plant chemicals such as IBA (Pacholczak et al. 2017). Therefore, phytohormonal stimulation methods are widely used in nursery practice (Pacholczak et al. 2005; El-Sharony et al. 2018; Rashedy et al. 2021), which may be achieved through natural root stimulators (Rashedy 2022) or by etiolation practices.

Etiolation is a partial or complete absence of light as a pretreatment of stack plants for a period of time before preparing their cuttings (Lu et al. 2017). Also, this term is used by plant propagators when forcing the growth of stock plant under heavy shade (Hartmann et al. 2011). A lack of light or etiolation can stimulate the rooting of some species that are difficult to propagate vegetatively (Iliev et al. 2022). Etiolation caused partial rejuvenation of shoots which increases rooting efficiency (Liu et al. 2018; Pacholczak et al. 2005). In ‘Zhongningsheng’ walnut, combination treatment of etiolation and 3-indolebutyric acid (IBA) rejuvenated mature shoots and stimulated their rooting (Liu et al. 2018). Also, etiolation of ‘Jingxiang 2’ walnut cultivar increased zeatin, GA, IAA, and ethylene; whereas, it decreased chlorophyll, sugar, and ABA content (Wang et al. 2020). In Brown Turkey fig variety, combined treatment of etiolation, and girdling and 1000 ppm of IBA was effective in increasing the rooting percentage and their characteristics (Kahlon and Kaur 2020) Furthermore, in red pitaya, cuttings collected from etiolated plants (grown at 80% shade level) should eliminate the need for a commercial rooting auxin (Almeida et al. 2016). On the contrary, short de-etiolation period of VC801 avocado rootstock for branches stimulated adventitious root formation (Duman et al. 2020). Therefore, the hypothesis of this study was that, could stock plant etiolation stimulate rooting of ‘Manzanillo’ and ‘Picual’ cuttings? Also, what are the physio-biochemical effects of stock plant etiolation on their cutting rooting ability?

Materials and methods

This study was carried out in the nursery of Pomology Department, Faculty of Agriculture, Cairo University, Egypt (30° 01′ 04″ N 31° 12′ 30″ E) during two seasons (2020–2021). The aim of this study was to investigate the effect of etiolation through two shading levels (0%, 60%) on rooting of both terminal and sub-terminal ‘Picual’ and ‘Manzanillo’ olive cuttings either with or without IBA application, which were laid out in total 8 treatments and were doubled to 16 treatments after IBA application with 60 cuttings per treatment in each olive cultivar.

Plant preparation

Through May each year, three 30-year-old stock trees of each of the ‘Picual’ and ‘Manzanillo’ olive cultivars were selected to be covered with black shade cloth at a level of 60% shade, along with other three trees of each cultivar uncovered as control (0% shading). Two months later, two types of cuttings (terminal and sub-terminal) were prepared of the etiolated and non-etiolated trees of both cultivars. The first type of cuttings were terminal cuttings (softwood) with 15 cm long with 5 nodes, 2 ml wide, and 4 terminal leaves with a terminal bud. The second type of prepared cuttings were sub-terminal (semi-hardwood) cuttings which contain 4 leaves, 7 ml width, 15 cm long without a terminal bud.

All previous collected cuttings (etiolated and non-etiolated) were divided into two equal groups, one treated with IBA and the other groupe untreated. Just before planting, the base of IBA-treated cuttings was immersed in freshly prepared IBA at 4000 ppm and the other untreated group was immeresed in water at 0 ppm IBA. Thereafter, the cuttings planted in perforated boxes contain a mixure of sand: peat moss in a ratio of 4:1 under polyethylene as a tunnel until November, and irrigated once or twice a month in economical and simple shaded polyethylene tunnels (10 m × 1 m × 1 m) which was estabished under saran green house.

A polyethelene sheet is placed under the perforated boxes to save irrigation water and allow the water to evaporate to cover the cutting surface as alternative methods for the fog irrigation system. Finally, the surface of plastic tunnel is covered by 40% saran shade net to reduce excessive solar radiation. (Hussain et al. 2020; Rashedy et al. 2021).

Plant measurements

Two month after etiolation treatments at the planting time, the following biochemical analysis, endogenous phytohormone, and anatomical studies were measured; while morphological studies were recorded four month after cuttings planting.

Biochemical analysis

Total chlorophyll content

At the time of planting, leaves sample were collected from etiolated and non-etiolated cuttings to determine total chlorophyll content (mg/g FW) according to the method of Lichtenthaler and Wellburn (1983). Leaf samples (0.25 g FW) were cut into small pieces and then immersed in 20 ml acetone (80%) for 3 days at 5 °C; then, the absorbances at 663 and 646 wavelengths were measured using a spectrophotometer. Total chlorophyll content was calculated by the following equations:

$$\text{Chlorophyll a} = (12.25 \times \text{A663}) - (2.798 \times \text{A646}),$$

$$\text{Chlorophyll b} = (21.5 \times \text{A646}) - (5.1 \times \text{A663}),$$

$$\text{Total Chlorophyll} = \text{Chlorophyll a} + \text{Chlorophyll b}.$$

Total sugars content

Samples from the cuttings base were collected with a sharp knife to determine total soluble sugars (mg/g FW) based on the phenol sulfuric acid method (Smith et al. 1983). In breif, 0.5 g of samples were extracted with ethanol (70%), and then 1 ml of the extract was placed into the test tube. Add 1 ml of phenol (5%) and then add 5 ml of concentrated H_2SO_4 (98%) and shake the tubes carefully. This mixure was allowed to cool at room temperature and then read by spectophotometer at 490 nm.

Total carbohydrates content

Total carbohydrates were determined according to Herbert et al. (1971) as follows: a dry leaf sample (0.2 g) was added to 10 ml H_2SO_4 (1 N), and placed in a tube overnight in an oven at 100 °C. Then, total sugars were colorimetrically determined according to Smith et al. (1983) as mentioned above in the determination of total sugars.

Nitrogen content

For nitrogen determination, 0.2 g of dried leaf samples was digested with a mixture of sulfuric acid and perchloric, and distilled using the steps of the modified micro-Kjeldahl method and the total nitrogen content was calculated according to Jones et al. (1991).

C/N ratio

C/N ratio was determined by dividing the total carbohydrates by the total nitrogen.

Endogenous phytohormone substances

Total phenols (mg/g FW)

The total phenolic content was determined through Folin–Ciocalteu method according to Sharma et al. (2019). Briefly, 0.5 g fresh weight of cuttings base was extracted in 20 ml of 80% methanol in the dark. One ml of this extract was mixed with folin (1 ml), then mixed with Na_2CO_3 (5 ml of 20%) and distilled water (3 ml) and the final volume was adjusted to 10 ml. This mixture was allowed to stand in the dark (1 h) before absorbance at 765 nm which was determined through spectrophotometer. Total phenolic content was expressed as gallic acid (mg/g FW).

Total indoles (mg/g FW)

Total indole content at the base of cuttings base was determined by the P-dimethyl amino benzaldhyde method (Larsen et al. 1962). Half a gram of fresh weight was extracted in methanol (20 ml of 80%) in the dark for three days; then, 1 ml of this extract was mixed with 4 ml P-dimethyl amino benzaldhyde (a mixure of 50 ml of HCL + 50 ml of 95% ethanol + 1 g P-dimethyl amino benzaldhyde). This mixture was allowed to stand in the dark (90 min at 30 °C). After that, the absorbance was determined at 530 nm by spectrophotometer. Total indole content was expressed as mg of indole acetic acid equivalent per g fresh weight. Then, indole/phenol ratio and phenol/indole ratio were calculated.

IAA and GA content (µg/g FW)

For endogenous plant hormones, freeze-dried plant samples (5 g FW) were ground into a mortar and pestle to obtain a fine powder. This powder was extracted with 80% methanol (15 ml/g FW) three times which supplemented with the antioxidant (2,6-di-tert-butyl-P-crosol) in the dark at 4 °C. After centrifugation of the extract at 4000 rpm, the supernatant was transferred into aluminum foil-coated flasks and the residue was extracted two times. The supernatants were then collected under vacuum at 35 °C, and the total volume was reduced to 10 ml. The pH of aqueous extract was adopted to 8.6 and then extracted three times with an equal volume of pure ethyl acetate. The composite alkaline ethyl acetate extract was dehydrated over Na_2SO_4 and then filtered. The filtrate was evaporated under vacuum to dryness at 35 °C and re-dissolved in 1 ml methanol (100%). After methylation, the methanol extract was used (Fales et al. 1973) to determine indole acetic acid and gibberellic acid. Quantification of IAA and GA was performed using ATI Unicam Gas Liquid Chromatography, 610 Series which was equipped with a flame ionization as a detector (Vogel 1975). Phytohormone fractionations were conducted using a coiled glass column packed with 1% OV-17. Gas flow rates were 30 ml/min for nitrogen, 30 ml/min for hydrogen, and 330 ml/min for air. Peak determination and quantification of plant hormones were performed using exogenous authentic hormones and Microsoft software program was used to calculate the concentrations of the selected peaks. Then, GA/IAA ratio and IAA/GA ratio were calculated.

Anatomical study

In the second season, after a significant and huge difference between rooting percentage of terminal non-shaded cuttings in both olive cultivars, microscopic examination was performed on the third node from apical of terminal cuttings buds to determine the anatomical structure related to rooting behavior in terminal olive cuttings. Samples (1 cm^2) were taken from the stem and immediately fixed with F.A.A. (85 ml ethanol (70%), 10 ml formalin, and 5 ml glacial acetic acid) for at least 48 h. The selected materials were washed in ethanol (50%), dehydrated in butanol series, and immersed in paraffin wax at a melting point (56 °C). After that, it was cut with a rotary microtome up to 20 µm thick and then stained twice with crystal violet-erythrosine, cleared in xylene and mounted in Canada balsam (Nassar and El-Sahhar 1998). The scanned sections were optically read to reveal the anatomical features of the responses observed with a light microscope (LEICA DM750) and a LEICA ICC50 HD using the Leica Application Suite software. Then, the percentage of stem components (periderm, cortex, vascular bundles, and pith) was calculated.

Morphological parameters

Four months after planting of cuttings (November), morphological parameters including Rooting %, number of roots per cuttings, root length (cm), and number of new leaves were determined.

Statistical analysis

The experiment design was a randomized complete block design with one factor for arrange treatments with three replicates for each treatment. Significance differences between treatments were analyzed using ANOVA by the statistical package software MSTAT-C (Freed et al. 1990) LSD values were calculated at 0.05 according to Snedecor and Cochran (1989). The presented data are the mean ± standard error values of independent replications ($n = 3$).

Results

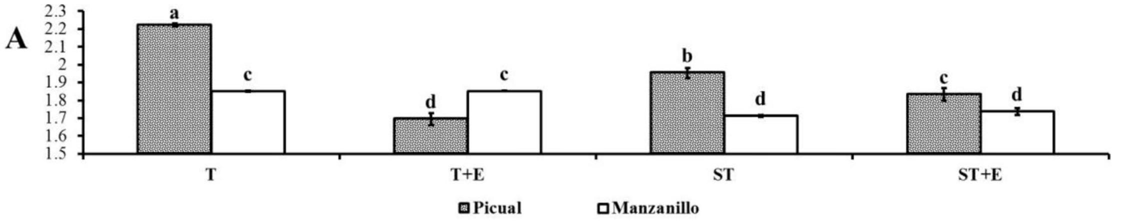
Biochemical analysis

Generally, all ‘Picual’ cuttings, even etiolated or non-etiolated, had a higher significant total chlorophyll content compared with similar ‘Manzanillo’ cuttings type except for terminal ‘Picual’ etiolated cuttings (Fig. 1A). Also, in both non-etiolated olive cultivars, terminal cuttings had a higher significant chlorophyll content by 13.71% and 7.99% for ‘Picual’ and ‘Manzanillo’ cultivars compared with similar sub-teminal cuttings. Etiolation treatment led to a significant decrease in total chlorophyll content in both terminal and sub-terminal ‘Picual’ olive cuttings cultivar compared with similar non-etiolated cuttings by 23.67% and 6.19%, respectively.

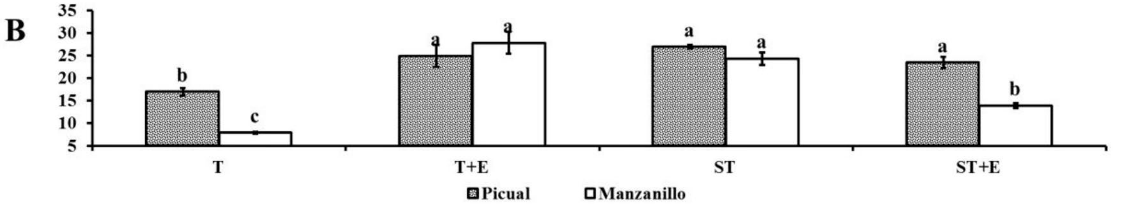
Generally, the non-etiolated sub-terminal cuttings had a more significant total carbohydrates content than terminal cuttings by 58.86% and 211.92% for ‘Picual’ and 'Manzanillo' cultivars, respectively (Fig. 1B). Also, etiolation treatment significantly increased total carbohydrates in terminal cuttings by 47.04% and 256.66% for ‘Picual’ and ‘Manzanillo’ olive cultivar, respectively. In contrast, etiolation reduced total carbohydrates content in sub-terminal cuttings by 12.94% and 42.95% in ‘Picual’ and ‘Manzanillo’ cultivars, respectively with a significant value for ‘Manzanillo’ cultivars.

It is evident that both non-etiolated cuttings type of ‘Picual’ olive cultivar had a higher significant sugar content than that of ‘Manzanillo’ cultivar (Fig. 1C). While, there were no significant differences among all ‘Picual’ cuttings type in sugar contents, non-etiolated sub-terminal ‘Manzanillo’ cuttings had the highest significant sugar content compared to all other ‘Manzanillo’ cuttings. Regarding the effects of etiolation, it can be observed that etiolation had no effect on total sugars content of terminal cuttings of both olive cultivars, while etiolation significantly reduced total sugars content by 10.74% and 14.99 for sub-terminal ‘Picual’ and ‘Manzanillo’ cuttings, respectively.

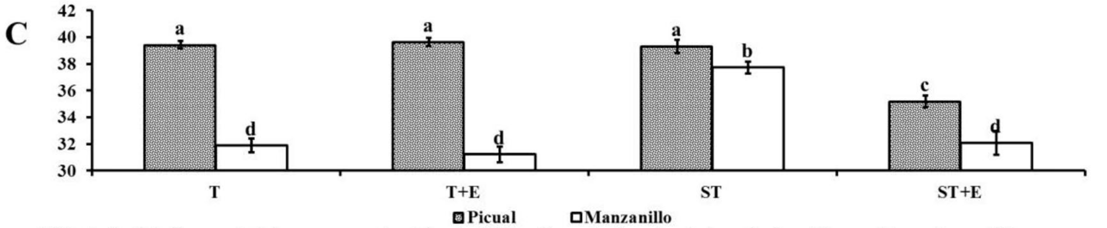
Terminal ‘Picual’ cuttings had a higher significant N% than sub-terminal ‘Picual’, while the opposite trend was



Effect of etiolation on total chlorophyll content (mg/g FW) of terminal and sub-terminal cuttings of two olive cultivars



Effect of etiolation on total carbohydrates content (mg/g DW) of terminal and sub-terminal cuttings of two olive cultivars



Effect of etiolation on total sugars content (mg/g FW) of terminal and sub-terminal cuttings of two olive cultivars

Fig. 1 Variations in total chlorophyll content (**A**), total carbohydrates content (**B**), and total sugars (**C**) content of terminal and sub-terminal cuttings in both 'Picual' and 'Manzanillo' olive cultivars subjected to etiolation treatment during 2021 season. Data were presented by means ($n = 3 \pm$ SE). Letters represent significant differences between treatments at $p < 0.05$ level according to the LSD test. *T* terminal cuttings, *ST* sub-terminal cuttings, *E* stock plant etiolation

found in 'Manzanillo' cv. (Fig. 2A). Also, etiolation led to a significant increase in N% in terminal cuttings in both olive cultivars; meanwhile, it led to a significant decrease in sub-terminal cuttings with a significant value for 'Picual' cv.

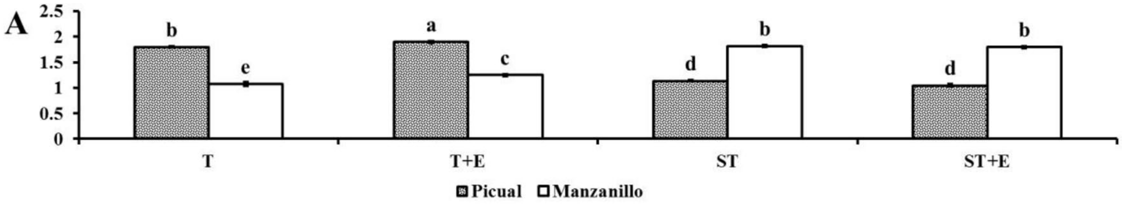
Sub-terminal cuttings contain more significant C/N ratio compared to terminal cuttings in both olive cultivars (Fig. 2B). Also, etiolation significantly increased C/N ratio in terminal cuttings of both olive cultivars; while, it decreased it in sub-terminal cuttings with a significant value for 'Manzanillo' cuttings.

Endogenous phytohormone substances

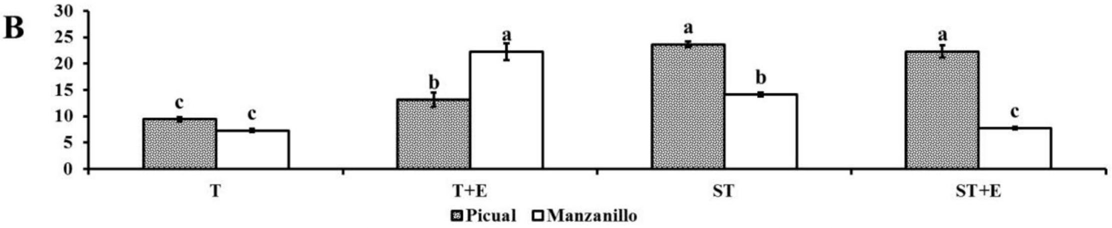
Sub-terminal 'Manzanillo' cuttings had the highest total indoles content compared to all other cutting types in both olive cultivars (Fig. 3A) which recorded total indoles more than sub-terminal 'Picual' cuttings by 76.25%, and the lowest significant total indoles content. Regarding the etiolation effect, it can be noticed that etiolation significantly increased total indoles content by 14.37% and 13.76% in terminal cuttings in both cultivars. Whereas, etiolation significantly reduced total indoles in sub-terminal 'Manzanillo' cuttings by 35.51% compared to non-etiolated ones.

It was evident that non-etiolated 'Manzanillo' cuttings had significantly higher total phenols content by 49.20% and 65.93% for terminal and sub-terminal cuttings, respectively, compared to similar cuttings type of 'Picual' cv. (Fig. 3B). Also, sub-terminal cuttings had a significantly higher total phenols content by 49.27% and 66.00% compared to 'Picual' and 'Manzanillo' terminal cuttings, respectively. Moreover, sub-terminal(non-etiolated)'Manzanillo' cuttings recorded the highest significant total phenols content compared to the other all cuttings type in both olive cultivars. In contrast, etiolation led to a significant decrease in total phenols content in both 'Manzanillo' cuttings types; while, it increased total phenols content in both 'Picual' cuttings types.

Non-etiolated terminal 'Picual' cuttings had the highest significant indole/phenol ratio compared with all other cuttings in both olive cultivars (Fig. 3C). Also, etiolation led to a significant decrease in indole/phenol ratio in sub-terminal cuttings in both olive cultivars and terminal 'Picual' cuttings. Conversely, etiolation increased indole/phenol ratio in



Effect of etiolation on nitrogen leaf % of terminal and sub-terminal cuttings of two olive cultivars



Effect of etiolation on C/N ratio of terminal and sub-terminal cuttings of two olive cultivars

Fig. 2 Variations in nitrogen percent (**A**) and C/N ratio (**B**) of terminal and sub-terminal cuttings in both 'Picual' and 'Manzanillo' olive cultivars either cultivars subjected to etiolation treatment during 2021 season. Data were presented by means ($n = 3 \pm$ SE). Letters represent significant differences between treatments at $p < 0.05$ level according to the LSD test. *T* terminal cuttings, *ST* sub-terminal cuttings, *E* stock plant etiolation

'Manzanillo' terminal cuttings than non-etiolated terminal ones.

Terminal 'Manzanillo' (non-etiolated) cuttings were shown to have a higher phenol/indole ratio than non-etiolated sub-terminal cuttings in contrast to 'Picual' cuttings (Fig. 3D). In both olive cultivars, etiolation resulted in a significant increase in phenol/indole ratio in sub-terminal cuttings, while it led to a significant decrease in total phenols content in terminal 'Manzanillo' cuttings.

Regarding the GA content of olive cuttings, the results showed that terminal cuttings of both cultivars were significantly higher in GA content by 9.5% and 1.65% for 'Picual' and 'Manzanillo' cultivars, respectively, compared to the sub-terminal cuttings with a significant value for 'Picual' cultivar (Fig. 4A). Moreover, all 'Picual' cuttings have a higher significant GA compared with 'Manzanillo' except for terminal etiolated ones. Furthermore, etiolation treatment resulted in a significant increase in GA in all treated cuttings by 28.01% and 48.48% for terminal cuttings and 43.44% and 43.60% for sub-terminal 'Picual' and 'Manzanillo' cultivars, respectively.

The results showed that, in general, 'Manzanillo' cuttings had significantly higher IAA content by 10.9% for terminal cuttings and 3.87% for sub-terminal cuttings than those of similar 'Picual' cuttings (Fig. 4B). Also, sub-terminal cuttings had significantly higher IAA content compared to terminal ones by 24.50% and 16.52% for 'Picual' and 'Manzanillo' cultivars, respectively. Moreover, etiolation treatments significantly reduced IAA in all types of cuttings from 15.40% and 6.12% for terminal cuttings to 22.30% and 11.26% for sub-terminal cuttings in 'Picual' and 'Manzanillo' cultivars, respectively.

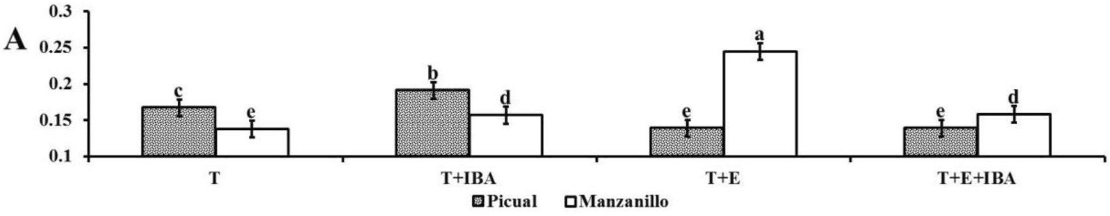
Furthermore, sub-terminal cuttings of both cultivars had the highest significant IAA/GA ratio and lowest GA/IAA ratio (Fig. 4C, D). In both terminal and sub-terminal cuttings of both olive cultivars, etiolation treatments recorded the highest significant increase in GA/IAA ratio, while it significantly decreased IAA/GA ratio.

Anatomy study

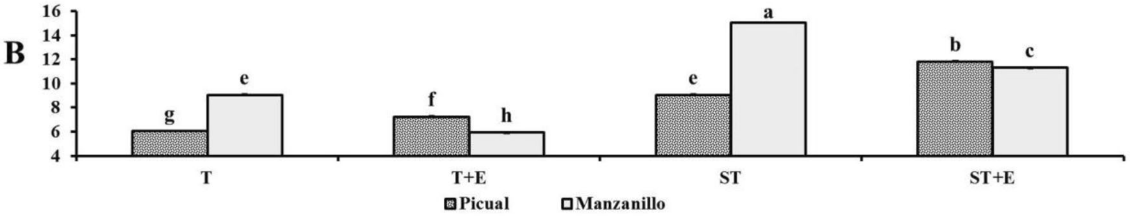
Anatomical features of both terminal cuttings of easy-to-root 'Manzanillo' and hard-to-root 'Picual' olive cultivars are shown in Fig. 5A–C. In general, the stem structure typically showed a herbaceous dicotyledons stem structure which is characterized by a noticeable thick cortex and pith. There was no significant difference between cortex and periderm percentage in both olive cultivars. Pith is the largest anatomical structure and over 60% of cross section was significantly much higher in 'Picual' than in 'Manzanillo' cultivars by 6.4%. Moreover, 'Manzanillo' cultivar had significantly higher vascular bundle by 17.2% than 'Picual' cultivars.

Morphological measurements

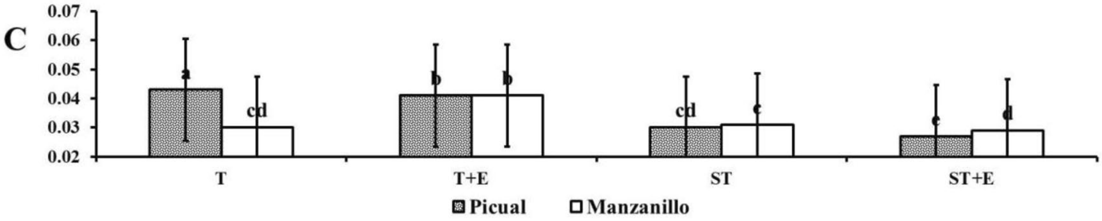
'Manzanillo' olive cuttings recorded a significantly higher rooting percent in both terminal and sub-terminal cuttings whether or not treated with IBA, whether or not etiolated, compared to similar cuttings type of 'Picual' cultivars (Fig. 6A, B). Also, sub-terminal cutting treated with IBA



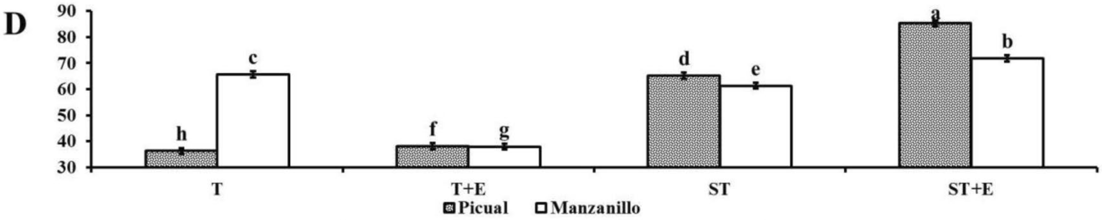
Effect of etiolation on total indoles content (mg g^{-1} FW) of terminal and sub-terminal cuttings of two olive



Effect of etiolation on total phenols content (mg g^{-1} FW) of terminal and sub-terminal cuttings of two olive cultivars



Effect of etiolation on indole/phenol ratio of terminal and sub-terminal cuttings of two olive cultivars



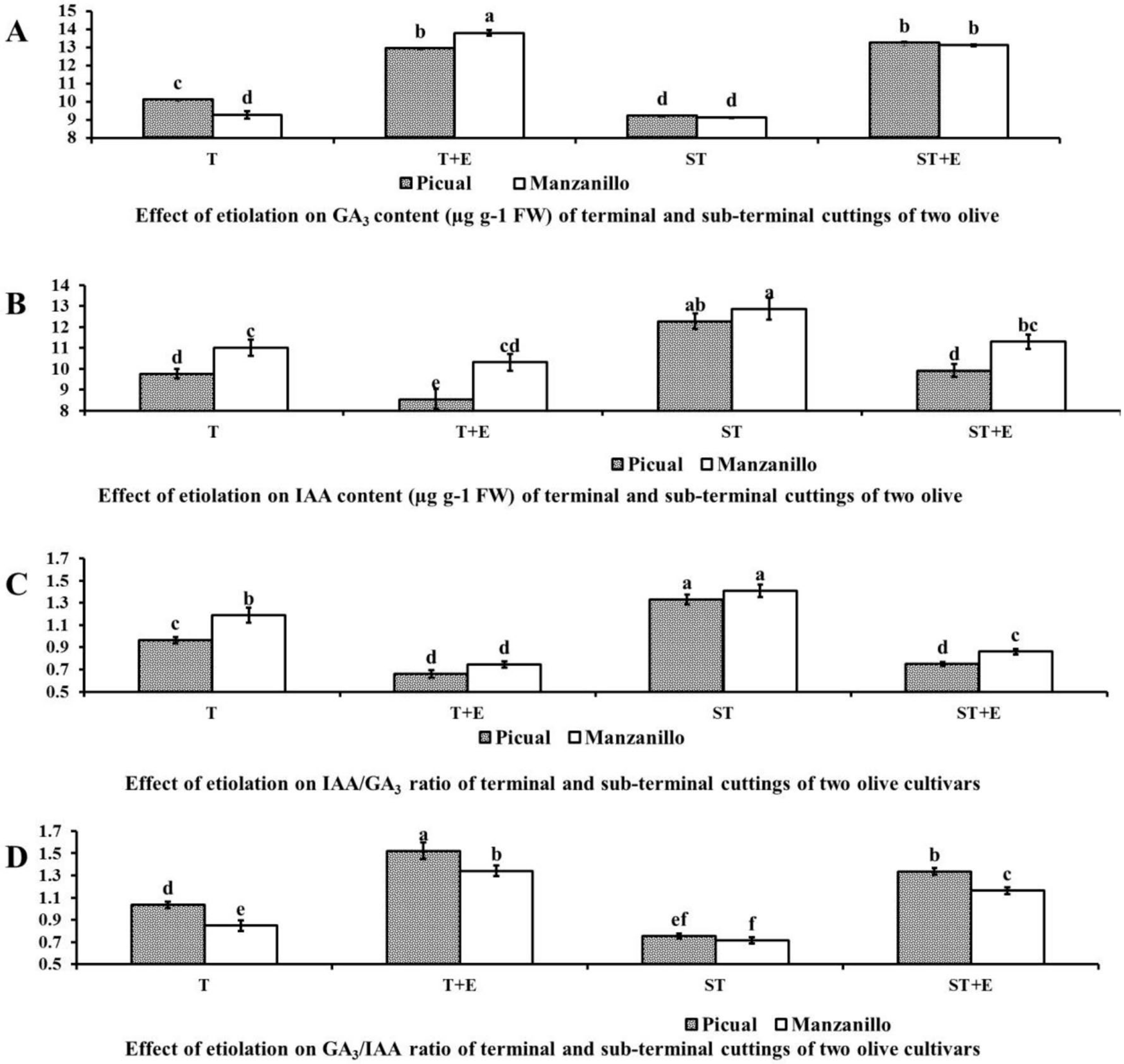
Effect of etiolation on phenol/indole ratio of terminal and sub-terminal cuttings of two olive cultivars

Fig. 3 Variations in total indole (**A**), total phenol (**B**), indole/phenol ratio (**C**), and phenol/indole (**D**) ratio of terminal and sub-terminal cuttings in both 'Picual' and 'Manzanillo' olive cultivars subjected to etiolation treatment during 2021season. Data were presented in means ($n=3 \pm$ SE). Letters represent significant differences between treatments at $p<0.05$ level according to the LSD test. *T* terminal cuttings, *ST* sub-terminal cuttings, *E* stock plant etiolation

recorded a significantly higher rooting percent compared to terminal cuttings in both olive cultivars. In addition, terminal 'Manzanillo' cuttings can be successfully used for olive propagation. Moreover, etiolation treatments significantly reduced rooting percent of both cultivars in both cuttings type even treated with IBA.

It is evident from Fig. 6C, D that 'Manzanillo' terminal cuttings had a significantly higher root length whether or not they were treated with IBA compared to all types of 'Picual' cuttings, except for sub-terminal cuttings that were treated with IBA in the second season. Moreover, etiolation significantly reduced root length of both 'Picual' type cuttings compared to IBA-treated 'Picual' cuttings. Whereas, for 'Manzanillo' cuttings, etiolation reduced root length in both type of IBA-treated cuttings with significant values for terminal cuttings.

It is clear from Fig. 7A, B that sub-terminal 'Manzanillo' cuttings treated with IBA showed the highest significant number of roots compared to all other cutting types in both olive cultivars. Also, etiolation treatments reduced the number of roots of IBA-treated 'Picual' cuttings. Meanwhile, etiolation increased the number of roots in terminal



Effect of etiolation on GA$_3$ content (µg g-1 FW) of terminal and sub-terminal cuttings of two olive

Effect of etiolation on IAA content (µg g-1 FW) of terminal and sub-terminal cuttings of two olive

Effect of etiolation on IAA/GA$_3$ ratio of terminal and sub-terminal cuttings of two olive cultivars

Effect of etiolation on GA$_3$/IAA ratio of terminal and sub-terminal cuttings of two olive cultivars

Fig. 4 Variations in GA (**A**), IAA (**B**), IAA/GA (**C**), and GA/IAA (**D**) of terminal and sub-terminal cuttings in both 'Picual' and 'Manzanillo' olive cultivars subjected to etiolation treatment during 2021 season. Data were presented by means ($n = 3 \pm$ SE). Letters represent significant differences between treatments at $p < 0.05$ level according to the LSD test. *T* terminal cuttings, *ST* sub-terminal cuttings, *E* stock plant etiolation

cuttings of both cultivars compared to control cuttings (IBA-untreated cuttings). Whereas, for IBA-treated 'Manzanillo' cuttings, etiolation resulted in a significant decrease in number of roots of sub-terminal cuttings, while it led to a slight increase in the number of roots for terminal cuttings.

It was found that terminal 'Manzanillo' cuttings treated with IBA recorded the highest significant root weight compared to the other cuttings types in both olive cultivars (Fig. 7C, D). In 'Manzanillo' olive cultivar, etiolation treatment significantly decreased root weight of IBA-treated terminal cuttings, while it significantly increased root weight of IBA-treated sub-terminal 'Manzanillo' cuttings compared to sub-terminal 'Manzanillo' cuttings either etiolated or IBA-treated only. In 'Picual' olive cultivar, etiolation significantly reduced root weight in all IBA-treated cuttings.

It was observed from Fig. 8A, B that in both olive cultivars, sub-terminal cuttings, whether or not treated with IBA, resulted in a significant increase in number of leaves compared with similar terminal cuttings type except for sub-terminal etiolated 'Picual' cuttings. Also, etiolation treatment alone significantly increased the leaves number in terminal 'Manzanillo' cuttings compared with terminal control (non-treated with IBA). Moreover, in both olive cultivars, etiolation significantly increased the leaves number of sub-terminal cuttings treated with IBA compared to sub-terminal cuttings treated with IBA alone.

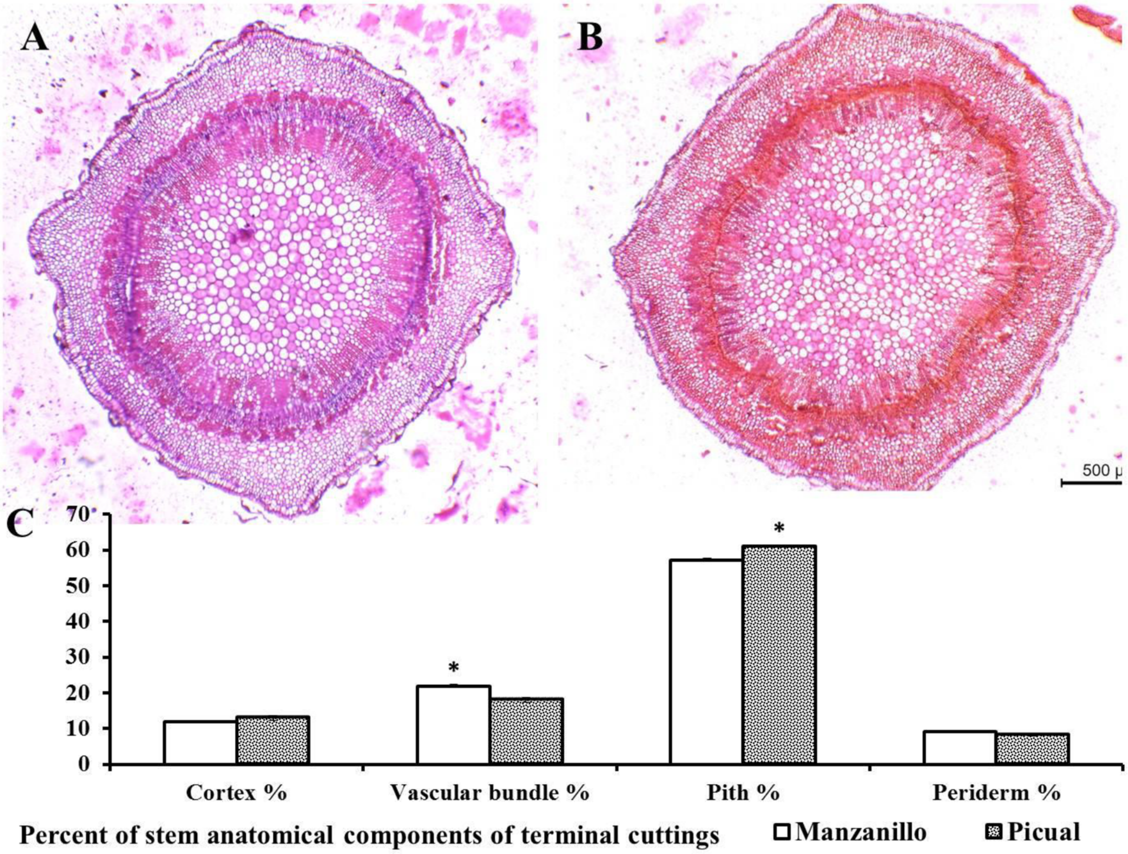


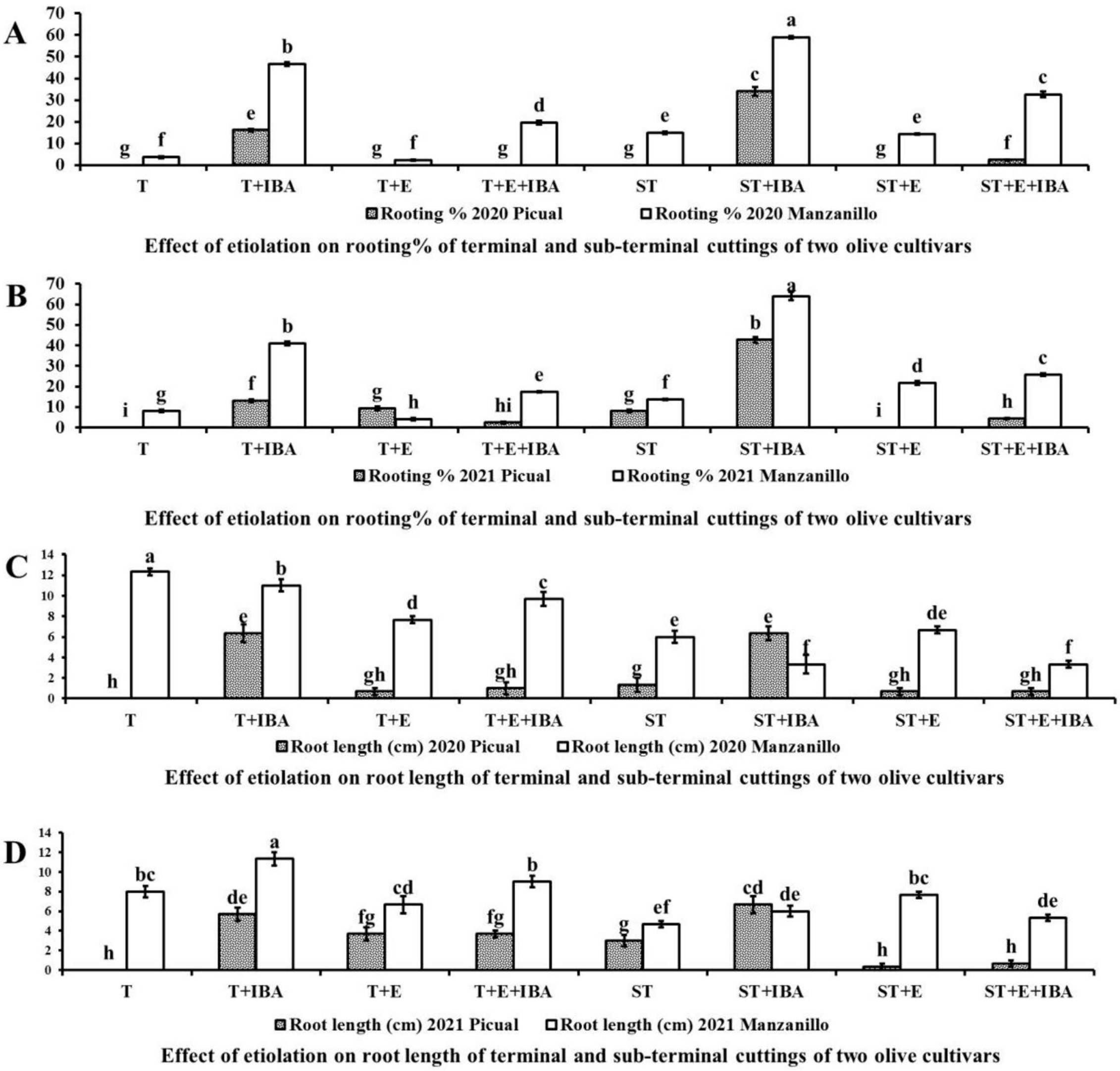
Fig. 5 Variations in stem anatomy of terminal 'Manzanillo' cuttings (**A**) as an example for easy-to-root cuttings and terminal 'Picual' cuttings (**B**) as an example for hard-to-root cuttings in percentage of each stem component (**C**). Data were presented by means ($n = 3 \pm$ SE). Letters represent significant differences between treatments at the $p < 0.05$ level according to the LSD test

Discussion

Regarding the effect of olive cultivar on rooting capacity, it can be noted that 'Manzanillo' cultivar recorded higher rooting percentage compared to 'Picual' cultivar when propagated by terminal or sub-terminal cuttings. In addition, 'Manzanillo' cuttings are more advanced in rooting percent, root length (Fig. 6A–D), number of roots, and roots weight (Fig. 7A–D). This may be due to 'Manzanillo' olive cultivar had higher growth stimulants substances such as total indoles, total phenols (Fig. 3A, B), and IAA (Fig. 4B) than 'Picual' olive cultivar. These results were in line with Izadi et al. (2016), Denaxa et al. (2021), Rashedy et al. (2021) and Martins et al. (2022) as they reported the differences between olive cultivars in rooting ability.

Regarding the effect of cuttings type, the results indicated that sub-terminal cuttings in both olive cultivars recorded the highest rooting percent (Fig. 6A, B), number of leaves (Fig. 8A, B) than terminal cuttings. This may be due to more growth stimulants and nutritional content of sub-terminal cuttings of both olive cultivars than terminal one such as total carbohydrates, sugar content, (Fig. 1B, C), C/N ratio (Fig. 2B), total phenol content (Fig. 3B) along with total indoles in 'Manzanillo' cuttings (Fig. 3A) supporting their ability for propagation. These results go in line with Abdel-Rahman et al. (2020) as they reported that sub-terminal hardwood cuttings of *Conocarpus erectus* contain more significant total carbohydrates content compared with the terminal and middle ones.

Regarding the effect of IBA on rooting of olive cuttings, the results showed that IBA led to a significant increase in rooting percent and number of roots in both cultivars (Figs. 6A, B and 7A, B). Also, IBA increased roots length of 'Picual' (Fig. 6C, D). Moreover, IBA increased roots weight of both types of cuttings in both cultivars except for sub-terminal 'Manzanillo' cuttings (Fig. 7C, D). On the contrary, IBA significantly reduced the number of leaves in sub-terminal cuttings of both olive cultivars (Fig. 8A, B) which may be due to depletion of growth-stimulating substances in root formation rather than leaf production. Therefore, non-rooted 'Picual' cuttings (non-treated with IBA) produced more leaves without rooting due to depletion of growth substances in leaf growth rather than root formation. These results were in agreement with Al-Hamdani and



Effect of etiolation on rooting% of terminal and sub-terminal cuttings of two olive cultivars

Effect of etiolation on rooting% of terminal and sub-terminal cuttings of two olive cultivars

Effect of etiolation on root length of terminal and sub-terminal cuttings of two olive cultivars

Effect of etiolation on root length of terminal and sub-terminal cuttings of two olive cultivars

Fig. 6 Variations in rooting percentage (**A**, **B**) and root length (**C**, **D**) of terminal and sub-terminal cuttings in both 'Picual' and 'Manzanillo' olive subjected to etiolation treatment either treated or non-treated with IBA application during two seasons (2020–2021). Data are mean ± standard error ($n=3$). Letters represent significant differences between treatments at $p<0.05$ level according to the LSD test. *T* terminal cuttings, *ST* sub-terminal cuttings, *E* stock plant etiolation, *IBA* cuttings treated with 4000 ppm before planting,

Mohammed (2017) as they found that treating 'Bashiqa' and 'Menzinelloa' olive cuttings with 3000 ppm IBA increased rooting percent. Also, IBA increased rooting percent of olive cuttings (Rashedy et al. 2021).

Regarding the effect of etiolation on olive cuttings, it can be concluded that etiolation generally reduces rooting percent (Fig. 6A, B), roots length (Fig. 6C, D), and number of roots (Fig. 7A, B) in both cuttings even treated with IBA. In addition, etiolation increased roots weight in both olive cultivars except for sub-terminal 'Manzanillo' (Fig. 7C, D). Etiolation succeeded in increasing the number of leaves of terminal cuttings in both olive cultivars (Fig. 8A, B) but failed in stimulating root formation (Fig. 6A, B). These results were in harmony with Frölech et al. (2020) who found that rooting of Maria da Fé olive cuttings was better with 1000 ppm IBA than etiolation for 110 days.

The negative role of etiolation on rooting efficiency of olive cuttings may be illustrated by the effect of etiolation on endogenous plant growth substances. The results indicated that etiolation significantly decreased chlorophyll content and total carbohydrates (Fig. 1A, B) in terminal cuttings of both cultivars as well as total sugars in sub-terminal cuttings of both olive cultivars (Fig. 1C) and C/N ratio in sub-terminal 'Manzanillo' cuttings (Fig. 2A, B). In addition, etiolation significantly decreased indole/phenol ratio in all olive cuttings except for terminal 'Manzanillo' cuttings (Fig. 3C). Also, etiolation significantly decreased phenol/indole ratio in terminal 'Manzanillo' and total phenols in

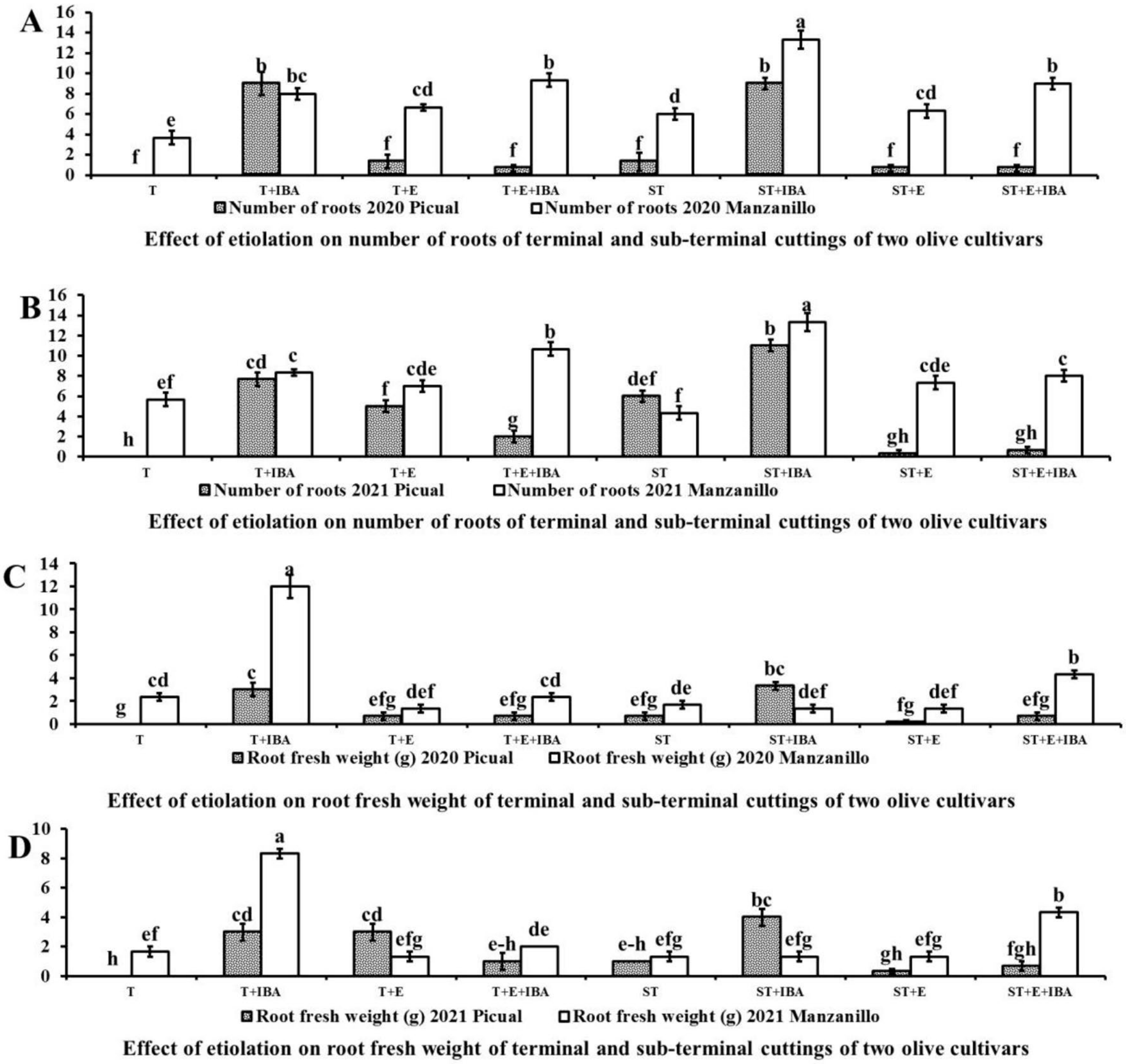


Fig. 7 Variations in root number (**A**, **B**) and root fresh weight (**C**, **D**) of terminal and sub-terminal cuttings in both 'Picual' and 'Manzanillo' olive cultivars subjected to etiolation treatment either treated or non-treated with IBA application during two seasons (2020–2021). Data are mean ± standard error ($n = 3$). Letters represent significant differences between treatments at the $p < 0.05$ level according to the LSD test. *T* terminal cuttings, *ST* sub-terminal cuttings, *E* stock plant etiolation, *IBA* cuttings treated with 4000 ppm before planting

all 'Manzanillo' cuttings (Figs. 1B and 3D, B). In contrast, etiolation significantly increased N% in terminal cuttings (Fig. 2A). Regarding the effect of chlorophyll content on rooting of cuttings, our results indicated that higher rooting percent in sub-terminal 'Manzanillo' cuttings was accompanied by lower chlorophyll content. Also, the highest rooting percent in sub-terminal cuttings were coincided with lower chlorophyll content than terminal cuttings in both cultivars. Moreover, moderate to high chlorophyll content in terminal and sub-terminal 'Picual' cuttings were coincided with moderate to low rooting of cuttings. Although etiolation succeeded in reducing chlorophyll content in 'Picual' cultivars, it failed to increase their rooting efficiency. The previous study showed that cuttings of shaded dogwood plants (*Cornus alba* L.) were associated with decreases in soluble proteins, total soluble sugars, and increases in total chlorophyll, polyphenolic, free amino acids, and free IAA contents (Pacholczak et al. 2005). Also, Barbados cherry stem cuttings treated with IBA 5000 ppm achieved the highest rooting percent which is accompanied by the highest total chlorophyll content (Samim et al. 2021).

For the opposite effect of etiolation on total carbohydrates, it was dependent on the type of cuttings (Fig. 1B), since etiolation significantly increased total carbohydrates in terminal cuttings which is mostly not used in olive propagation due to its difficulty to root, but etiolation decreased carbohydrates in sub-terminal cuttings in both olive cultivars which is the most efficient cutting in olive propagation. In addition, etiolation significantly reduced total sugars content in sub-terminal 'Manzanillo' and 'Picual' cultivars. The

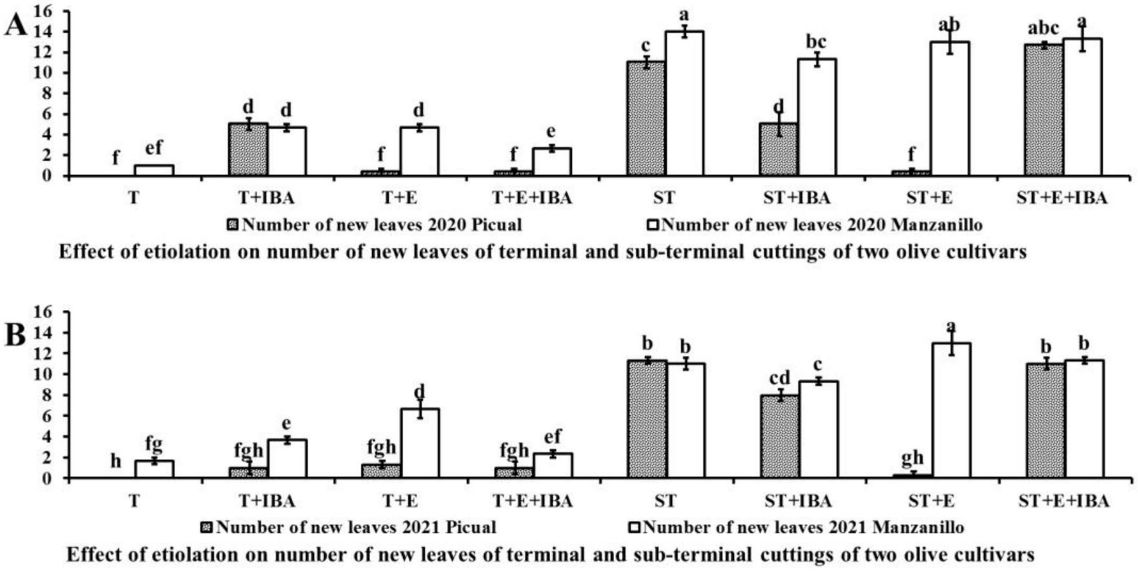


Fig. 8 Variations in leaves number of terminal and sub-terminal cuttings in both 'Picual' and 'Manzanillo' olive cultivars subjected to etiolation treatment either treated or non-treated with IBA application during two seasons (2020–2021). Data are mean ± standard error ($n = 3$). Letters represent significant differences between treatments at $p < 0.05$ level according to the LSD test. *T* terminal cuttings, *ST* sub-terminal cuttings, *E* stock plant etiolation, *IBA* cuttings treated with 4000 ppm before planting,

difference in carbohydrates and total sugars content between the two types of cuttings (Fig. 1B, C) may be due to the etiolation effect on transfer of carbohydrates and total sugars as growth substances from old shoots (sub-terminal cuttings) to a new shoot (terminal cuttings) to stimulate their growth for overcoming etiolation stress as well as terminal shoot did not transfer their production of carbohydrates to stored organs and old shoots (sub-terminal cuttings). Although etiolation succeeded in increasing carbohydrates content in terminal cuttings, it failed to increase their rooting ability which mean that carbohydrates have a supportive role in rooting process which is less than indoles. The rooting ability of terminal 'Manzanillo' cuttings had the lowest carbohydrates content compared to all cuttings in both olive cultivars, but it had high level of total indoles. In this regard, etiolation of 'Jingxiang 2' walnut cultivar decreased sugars content (Wang et al. 2020). Carbohydrates supply and redistribution at the base of cuttings is a limiting factor for root formation (Hartmann et al. 2011). Thus, cuttings must rely on stored or produced carbohydrates. Also, Aslmoshtaghib and Reza-Shahsavar (2010) reported that carbohydrates availability and their transport to the base of the cuttings were the most important factors associated with rooting in olive cuttings. Where higher carbohydrate, total sugars, and C/N ratio through the season positively correlated with increased rooting percent of olive cuttings. Moreover, Denaxa et al. (2012) recorded the highest rooting percent of 'Arbequina' olive cuttings during summer which is correlated with the highest initial concentration of soluble sugars in cuttings. While, it significant reduced during early phases of root formation. Therefore, they concluded that rooting of olive relies more on soluble sugars rather than starch. Veierskov (1989) reported that sugars can positively influence rooting formation coordination due to the fact that glucose and glucose-6-phosphate can glycosylate DNA and alter transcription, which regulates root initiation. On the other hand, there was no correlation between rooting ability of five olive cuttings ('Dusti', 'Rowghani', 'Tokhmekabki', 'Amigdalolia' and 'Konservalia') and their stems and leaves nitrogen or soluble sugars contents (Izadi et al. 2016).

Regarding the effect of C/N ratio on rooting of olive cutting, our results showed that sub-terminal cuttings that are most efficient in rooting had a significantly higher C/N ratio compared to terminal cuttings (Fig. 2B). In this regard, a negative relationship was found between bitter almond rooting and their nitrogen concentration (Kasim et al. 2009). Also, in tetraploid locust cuttings, rooting percent was negatively correlated with high nitrogen level in stock plants (Ling and Zhong 2012). While, Al-Hamdani and Mohammed (2017) found that treating 'Bashiqa' and 'Menzinelloa' olive cuttings with 3000 ppm IBA increased rooting percent as well as nitrogen, carbohydrates and C/N ratio in the leaves of cuttings. Moreover, Gordal Sevillano olive cuttings, which were obtained in March, had a significantly higher in C/N ratio and were accompanied by increasing the rooting percent (Hamid and Al-Imam 2019). Furthermore, Barbados cherry stem cuttings treated with IBA 5000 ppm achieved a high rooting percent which is accompanied by the highest C/N ratio (Samim et al. 2021). Lower C/N ratio in terminal cuttings was coincided with higher carbohydrates in sub-terminal cuttings which may be resulted from transferred and stored carbohydrates in sub-terminal cuttings.

Formation of adventitious roots is a complex process, which has been affected by numerous hormone signaling pathways (Tahir et al. 2021). A great view of the effect of endogenous growth substances on rooting of olive cuttings may be illustrated by comparing the higher rooting % of sub-terminal 'Manzanillo' and the lower rooting of terminal 'Picual' cuttings. Easy-to-root terminal 'Manzanillo' accompanied by increased total indoles content, IAA content, total phenols content, phenol/indole ratio, and IAA/GA ratio, as well as decreased GA content and GA/IAA ratio. In contrast, the lower rooting (terminal 'Picual' cuttings) contained a higher indole/phenol, GA, GA/IAA ratio as well as a lower phenol/indole ratio, phenols, IAA, and IAA/GA ratio. Rooting enhancement of shaded dogwood plant (*Cornus alba* L.) cuttings was associated with decreases in soluble proteins, total soluble sugars, and increases in total chlorophyll, polyphenolic acids and free IAA contents (Pacholczak et al. 2005). Also, etiolation of 'Jingxiang 2' walnut cultivar increased GA content (Wang et al. 2020). Moreover, exogenous GA decreased adventitious roots diameter (Zhang et al. 2021). Also, exogenous GA significantly inhibited grapevine adventitious root formation (Chang et al. 2022). In addition, Quan et al. (2022) reported that endogenous IAA, zeatin, IAA/ABA ratio, and IAA/zeatin ratio were high with low content of GA and abscisic acid in Catalpa bignonioides softwood cuttings which were closely related to rooting. The ratios of IAA/ABA, IAA/GA, and IAA/zeatin reflect endogenous hormonal homeostasis during the formation of adventitious root in Paeonia suffruticosa. Also, higher IAA/zeatin and IAA/ABA stimulate induction and elongation of adventitious root (Wang et al. 2021). In apple rootstock, high nitrate concentration resulted in higher levels of endogenous GA which in turn led to hormonal imbalance and inhibition of adventitious roots (Tahir et al. 2021). Moreover, in in vitro culture of Pinus massoniana lamb, Wang and Yao (2020) reported that in the twentieth generation shoots, endogenous IAA was the highest, which exhibited the strongest rooting capacity. However, in the fortieth generation shoots, endogenous GA concentration was the highest which showed poor rooting ability. Furthermore, adventitious root formation was decreased in apple rootstocks cuttings by increasing the endogenous contents of methyl jasmonate, abscisic acid, zeatin riboside, and reducing GA and IAA contents (Li et al. 2022). Endogenous total phenols, total indoles, IAA, ABA and GA as growth substances have different effects on the rooting behavior of olive cuttings dependent on their concentration and the ratio between them (Hartmann et al. 2011).

It is promising to show the accumulation of total phenols and phenol/indole ratio in high-rooted olive cuttings (sub-terminal 'Manzanillo') compared to low-rooted cuttings (terminal 'Picual'). While, leaf total phenolics, stem caffeic acid, chlorogenic acid, and naringin contents in olive cuttings did not affect the rooting of the cuttings. However, leaf catechin, stem vanillic acid, and total phenolics had positive effects on rooting percent of the cuttings (Izadi et al. 2016). In addition, total phenols content in the basal cuttings were markedly lower than that in middle and tip cuttings (Abdel-Rahman et al. 2020). The effect of phenolic compounds on the formation of the adventitious root is complicated and relies on their composition, concentration, and stage of growth. Also, Denaxa et al. (2021) noted positive effect of phenolic compounds in combination with rutin and chlorogenic acid may protect IAA from degradation during root induction and initiation stages. The important role of phenolic compounds in adventitious root formation in some olive cuttings (Grappolo 541, Ascolano 315, Santa Catalina, and Maria da F´e) resulted from their presence in the cambial region, as phenolic compounds have close relations with enzymes related to auxin metabolism and transport (Martins et al. 2022). For the effect of phenol type on root formation, initial total phenols (chlorogenic acid, tyrosol, rutin, quercetin, luteolin-7-glucoside, luteolin) were positively associated with the rooting ability of 'Arbequina' olive cuttings compared to 'Kalamata' olive cuttings in summer and autumn (Denaxa et al. 2021).

A comparison between terminal 'Manzanillo' and 'Picual' is a second good example for endogenous root factors. Terminal 'Manzanillo' cuttings had lower significant total chlorophyll, total carbohydrates, total sugars, total indole content, and total indole/phenol content than terminal 'Picual' olive cuttings by 16.72%, 53.9%, 19.14%, 17.36 and 30.2%, respectively. However, it recorded a higher rooting content accompanied by a significant increase in vascular bundle, total phenol, total phenol/indole ratio, IAA, and IAA/GA ratio by 17.2%, 49.2%, 44.72%, 12.9%, and 22.9%, as well as a decrease in GA by 7.98% and GA/ IAA ratio by 18.21% which recorded more endogenous substances supported to be more successful for olive rooting % by 5.83% and 202.2% than untreated and IBA-treated terminal 'Picual' cuttings as mean of both seasons.

Conclusion

Sub-terminal cuttings which recorded more total indoles, IAA, IAA/GA ratio, total phenols, total carbohydrates, C/N ratio, and number of leaves were the most effective tools for olive propagation than terminal cuttings in both olive cultivars. Etiolation of both olive cultivars significantly decreased rooting percent of terminal and sub-terminal cuttings as well as number of roots as a result of decreased IAA and IAA/GA ratio; while, it increased GA and GA/IAA ratio of sub-terminal cuttings. In addition, etiolation treatment had negative effects on IBA-treated cuttings.

Author contribution statement Both authors (MA-AA-M and AAHR) contributed to the study conception, study design, material preparation, data collection and data analysis. Also, the first draft of the manuscript was written, read, and approved by both authors.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declared that they have no conflicts of interest.

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