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Hormone Crosstalk and Endogenous Factors Involved in the Mechanism of Adventitious Root Formation in Olive Cuttings

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ABSTRACT

Olive (*Olea europaea* L.) is commonly propagated through semi-hardwood cuttings to ensure true-to-type plant production. However, root formation in some commercial cultivars remains challenging. The formation of adventitious roots (AR) involves complex anatomical, physiological, biochemical, and molecular mechanisms in response to wounding, detachment, or stress, which are not yet fully understood in olive cuttings. Hormonal signaling pathways, including those orchestrated by auxin, ethylene, cytokinin, abscisic acid, gibberellic acid, jasmonic acid, brassinosteroids, and strigolactones, play significant roles in AR formation, with auxin acting as the primary initiator. Hormone crosstalk further complicates this regulation, as the influence of each phytohormone, mediated by many differentially expressed genes, can be either positive or negative. Endogenous phenolic compounds, which interact with auxin, also have a significant impact on AR formation through their concentration and temporal fluctuations. These compounds can act as synergists, enhancers, or inhibitors. Additionally, polyamines function as growth regulators and interact with phytohormones, with exogenous putrescine known to promote rooting in various olive cultivars. Carbohydrates provide the energy necessary for gene expression and hormone signaling related to AR formation in plants. The role of oxidative enzymes and oxidizing agents can also be understood in light of these factors. Moreover, new approaches, such as the use of biostimulants and biotechnology tools like beneficial bacteria and mycorrhizal fungi, have been reported to support root system development. This review highlights the roles of these factors in the rhizogenesis of olive cuttings and provides insights into improving rooting efficiency in difficult-to-root cultivars.

Abbreviations: 1-aminocyclopropane-1-carboxylate (ACC), Arginine decarboxylase (ADC), Adventitious roots (ARs), Arbuscular mycorrhizae (AM), Alternative oxidase(AOX), Wuschel-related homeobox (WOX), Auxin response factor (ARF), Auxin homeostasis (AUX/IAA), Lateral organ boundaries-domain (LBD), PIN-FORMED (PIN), Diamine oxidases (DAO), Difluromethylarginine (DFMA), α-difluoromethylornithine (DFMO), Hydrogen peroxide (H_2O_2), Ornithine decarboxylase (ODC), Indole-3-acetic acid (IAA), IAA-oxidase (IAAox), Indole-3-butyric acid (IBA), Naphthalene acetic acid (NAA), Ethylene (ETH), Abscisic acid (ABA), Nitric oxide (ABA), Polyphenol oxidase (PPO), Peroxidases (POX), Salicylic acid (SA), Salicylhydroxamic acid (SHAM), Jasmonic acid (JA), Brassinolide (BR), Small Auxin Up RNA (SAUR)

Introduction

General overview

Vegetative propagation is the main method for the propagation of major horticultural crops, including fruit trees (Moncousin, 1991; Waite et al., 2015; Love et al., 2017; Rajan and Singh, 2021; Ak et al., 2021; Faramarzi et al., 2022). Several techniques have been developed for vegetative propagation, including division, suckers, layering, and tissue culture or micropropagation (Love et al., 2017; Moradnezhad et al., 2017). However, the most widely employed technique globally is the use of stem cuttings (Ak et al., 2021), which rely on the cutting's ability to produce adventitious roots (ARs). Therefore, the successful clonal propagation of many important horticultural species, such as olive, is dependent on AR formation (Rajan and Singh, 2021).

The process of AR formation in stem cuttings varies not only among different species but also within the same species across different cultivars and even within the same cultivar at different physiological stages of the mother plants (Kasim et al., 2009; Denaxa et al., 2010; Denaxa et al., 2012; Porfírio et al., 2016; Rajan and Singh, 2021). Consequently, there are significant variations in rooting ability among olive cultivars, leading to their classification into three groups: a) easy-to-root, b) moderate-to-root, and c) hard-to-root cultivars (Wiesman and Lavee, 1995). Poor rooting presents a major challenge in the commercial propagation of olive trees, as many valuable cultivars are recalcitrant to rooting through cuttings.

The process of AR formation involves various anatomical, physiological, biochemical, and molecular changes, whose complex interplay and underlying mechanisms remain poorly understood (Legué et al., 2014; Ballester et al., 1999). Among these factors, auxin has been identified as the primary hormone involved in root formation in olive cuttings (Wiesman and Lavee, 1995; Love et al., 2017). Additionally, other hormones, phenolic compounds, polyamines, carbohydrates, nutrients, and oxidative enzymes function either as promoters or inhibitors of rhizogenesis. Environmental factors such as temperature, humidity, light conditions, irradiance, and rooting substrates also play significant roles in influencing and regulating AR formation (Agulló-Antón et al., 2011; Ruedell et al., 2013; De Almeida et al., 2017).

It is worth noting that much of the current understanding of AR formation is derived from studies on model plant species like *Arabidopsis* and *Nicotiana* (Porfírio et al., 2016). Recent advances have been made in identifying transcription factors and hormone-signaling genes associated with the regulation of ARs in woody species, using *Populus* spp. as a model organism (Legué et al., 2014; Zhang et al., 2023). However, in woody perennials such as *Olea europaea*, the anatomy, biochemical basis, genetic control, and the impact of exogenous factors on rhizogenesis remain largely unknown. This review aims to elucidate the effects of plant hormone signaling and crosstalk, the molecular mechanisms involved, and the endogenous compounds that influence AR formation in *O. europaea* cuttings.

Key regulatory systems related to ARs formation in olive cuttings

This section will explore the significance of key regulatory systems involved in AR formation in olive cuttings, describing their roles within the intricate regulatory system.

Plant hormone crosstalk and molecular mechanisms

Plant hormones play a pivotal role as regulators in the formation of AR. However, our understanding of the molecular mechanisms and hormone crosstalk involved in AR formation in olive cuttings remains limited. A series of molecular and biochemical events, along with hormone crosstalk, are involved in ARs formation in response to wounding, detachment, or stress (Devi et al., 2021). At the molecular level, a complex network of interactions between various phytohormone-related genes associated with AR formation has been documented (Li, 2021). Signal transduction pathways and hormone crosstalk among auxin (IAA), ethylene (ETH), cytokinin (CK), abscisic acid (ABA), gibberellic acid (GA), jasmonic acid (JA), brassinolide (BR), and strigolactones (SLs) have been linked to AR formation (Li, 2021; Zhang et al., 2019; Zhang et al., 2023; Tian et al., 2024). In olive cuttings, while auxin is well-known for initiating root formation, an interplay between IAA, ETH, CK, JA, and SLs has been reported to contribute to rhizogenesis (Druege, 2020; Li, 2021).

Previous research has shown that ethylene can stimulate auxin biosynthesis (Růžička et al., 2007). In another study, SLs were found to inhibit AR formation in olive cuttings (Özbilen and Sezer, 2022). The application of the SL biosynthesis inhibitor TIS108 enhanced AR formation in the hard-to-root cultivar 'Domat,' suggesting a promising strategy for improving rooting success in olive cuttings. Transcriptomic studies of differentially expressed genes (DEGs) related to hormone signaling pathways have revealed the dynamic regulation of hormone levels during AR formation (Zhang et al., 2023; Tian et al., 2024). For instance, auxin, as the primary contributor to AR formation, activates various gene families, including auxin biosynthesis, homeostasis, transport, and response genes (Li, 2021). In *Populus*, AR formation was found to be mainly controlled by the auxin signaling pathway, although genes associated with ETH, ABA, CK, GA, JA, and BR signaling pathways also play significant roles (Zhang et al., 2023). This study demonstrated that the up- or downregulation of phytohormone-related genes, particularly in the early stages of AR growth and development, is a key factor in the process.

The influence of each phytohormone on AR formation, mediated by DEGs linked to hormone signaling, can be either positive or negative. Although auxin is a key hormone in root induction in olives (Velada et al., 2020; Li, 2021), in *Arabidopsis thaliana*, ETH and BR have been found to stimulate AR initiation, while CK, GA, JA, and ABA act as inhibitors (Bai et al., 2020b; Lakehal and Bellini, 2019). The interactions between hormones can either facilitate or inhibit AR formation. CK provides a good example of this complexity. While some studies suggest that CK positively affects AR formation due to its role in cell division and primordia formation, a high auxin-to-cytokinin ratio is crucial for root organogenesis (Bollmark et al., 1988; Jing and Strader, 2019; Neogy et al., 2021). Thus, cytokinin signaling is activated to regulate CK concentrations appropriately (Li, 2021).

Auxins have been linked to the induction of oxidative stress, acting as an alternative to hydrogen peroxide (H_2O_2) treatment. When an olive semi-hardwood cutting is taken from the mother plant, high levels of reactive oxygen species (ROS) are generated in response to detachment, leading to increased peroxidase (POX) and polyphenol oxidase (PPO) activity near the wound site (Gaspar et al., 1997; Porfírio et al., 2016; Aslmoshtaghi and Shahsavar, 2016; Zhang et al., 2019). The activity of alternative oxidase 2 (AOX2) has also been observed in olive cuttings, where it is encoded under stress conditions (Santos Macedo et al., 2009; Hedayati et al., 2015). In olive, *OeAOX2* has been identified as a functional marker associated with AR formation (Hedayati et al., 2015). Santos Macedo et al. (2012) demonstrated reduced root formation in olive cuttings when AOX activity was inhibited by salicylhydroxamic acid (SHAM).

In response to wounding, an accumulation of Ca^{2+} ions can alter plasma membrane potential, allowing the exit of H_2O_2 and superoxide anions (O_2^-) from the cell (Camello-Almaraz et al., 2006). Velada et al. (2020) observed that exogenous indole-3-butyric acid (IBA) induces AR formation in *in vitro* olive explants, associated with the expression of *OeAOX1* genes in IBA-treated explants. Devi et al. (2021) reported that wound-induced signaling triggers the expression of WUSCHEL-related homeobox (WOX) transcription factors, which correspond to the emergence of founder cells. This wound signal produces high levels of ROS, which upregulate *WOX11* in olive cuttings, functioning alongside IAA and CK signaling to promote de novo root primordia formation. As a result, ETH and JA biosynthesis is induced at the wound site, with these hormones entering cells through plasma membrane receptors and triggering IAA biosynthesis (Devi et al., 2021). WOX genes also enhance two Lateral Organ Boundaries-Domain (LBD) genes, *LBD16* and *LBD29*, which correspond to root founder cells in *Populus* species (Li, 2021).

The role of ethylene (ETH) in indole-3-acetic acid (IAA) biosynthesis and transport has been reported by Bai et al. (2020a) in apple rootstock. While the effect of ETH on adventitious root (AR) formation in 'Memecik' olive cuttings was not significantly observed, the application of ETH at doses below 50 mg L^{-1} was recommended to increase root diameter (Karalti and Dalkiliç, 2020). Furthermore, an earlier study by Bartolini and Del Ministro (1981) demonstrated that applying ETH synthesis inhibitors, such as ACC (1-aminocyclopropane 1-carboxylic acid) and AVG (2-aminoethoxyvinylglycine), to olive cuttings led to a decrease in AR formation.

It has been well-documented that PGRs from the auxin family, such as IBA, are effective in promoting root formation by converting to IAA (Epstein and Lavee, 1984; Kurd et al., 2010). Al Hattab et al. (2018) reported that IBA enhanced the rooting capacity of olive cuttings due to the induction of IAA synthesis. A key gene involved in both wound-induced and IBA-induced AR formation is associated with auxin transport and homeostasis (Li, 2021). Specifically, PIN-Formed (PIN) proteins, which function as auxin efflux carriers, play a crucial role in auxin transport, facilitating its migration to the base of the cutting (Li, 2021). Auxin influx carriers, on the other hand, regulate auxin uptake into the cells. In olive cuttings, the expression of two auxin efflux carriers, *OePIN2b* and *OePIN5b*, is upregulated after exogenous IBA treatment, while the expression of *OePIN1a-c*, *OePIN3a-c*, *OePIN6*, and OePIN8 is downregulated (Velada et al., 2020).

A study found that IAA content in the buds and leaves of the easy-to-root olive cultivar 'Raseei' decreased during the rooting process, compared to the hard-to-root ‘Nabali’ cultivar (Ayoub and Qrunfleh, 2006). This suggests that the role of PIN proteins and AUX/IAA activity may be critical during IBA-induced AR formation in olive cuttings (Li, 2021). Other genes involved in auxin signaling include Auxin Response Factor (ARF) and Auxin Homeostasis (AUX/IAA), both of which regulate AR formation during the initiation phase (Li, 2021; Li et al., 2009). In *Populus*, interactions between auxin homeostasis (GH3), ARFs, and Small Auxin Up RNAs (SAURs) have been linked to AR formation within the first 12 hours of hormone signal transduction (Zhang et al., 2023). GH3 genes are known to regulate free IAA levels (Li, 2021), with ARF6 and ARF8 acting as positive regulators of GH3 genes, while ARF17 serves as a negative regulator (Li, 2021).

The role of other hormones, such as ABA and GA, in AR formation in olive cuttings is not well understood. A previous study indicated that ABA and GA levels were lower in ‘Raseei’ cuttings, which exhibited higher rooting ability (Ayoub and Qrunfleh, 2006). This suggests that AR formation in olive cuttings is regulated by a complex interplay of hormone signal transduction and hormone crosstalk.

Figure 1 displays the mechanism of AR formation in olive cuttings.

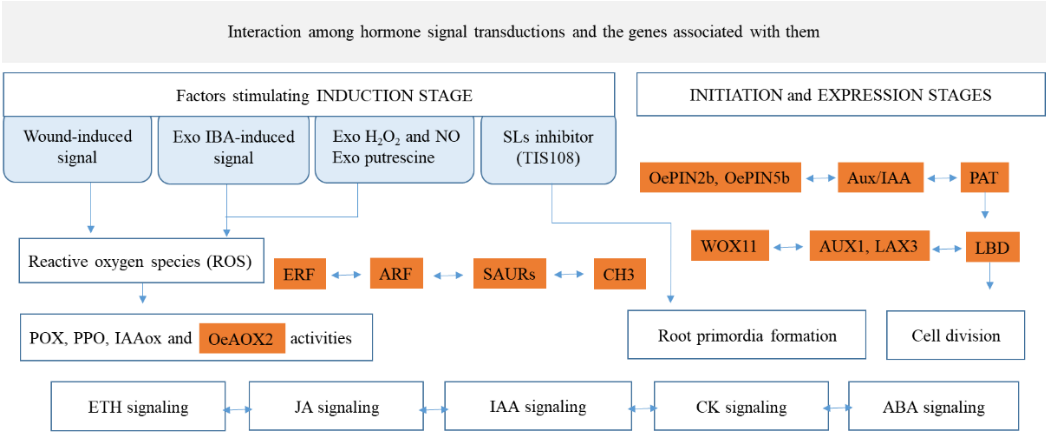


Fig. 1. A proposed model for the hormone signaling pathway in olive cuttings illustrates the factors stimulating the induction phase, including wounding, exogenous indole-3-butyric acid (IBA), exogenous hydrogen peroxide (H_2O_2), nitric oxide (NO), and exogenous putrescine. Each factor triggers a signaling pathway by generating reactive oxygen species (ROS), which increases the activities of peroxidase (POX), polyphenol oxidase (PPO), indole-3-acetic acid oxidase (IAAox), and alternative oxidase (OeAOX2). Four hormone signaling pathways are involved in adventitious root (AR) formation, with this study suggesting that ethylene (ETH) signaling is upregulated, leading to the expression of ethylene response factor (ERF) genes. This is followed by the activation of jasmonic acid (JA), indole-3-acetic acid (IAA), and cytokinin (CK) signaling pathways. During the initiation phase, ETH, JA, and IAA biosynthesis occurs, with IAA playing a pivotal role in AR formation. The Auxin Response Factor (ARF) and AUX/IAA (Auxin Homeostasis) pathways regulate auxin synthesis and concentration. PIN-formed proteins, such as OePIN2b and OePIN5b, function as auxin efflux carriers, contributing to Polar Auxin Transport (PAT). Wuschel-related homeobox (WOX11) transcription factors (TFs) are regulated during the early stages of AR formation, while Lateral Organ Boundaries-Domain (LBD) TFs are involved in cell division and development. The application of a strigolactone (SL) biosynthesis inhibitor, TIS108, to hard-to-root olive cuttings has been shown to increase AR formation, offering a promising approach for enhancing rooting success in difficult cultivars.

Role of phenolic compounds

Endogenous phenolic compounds play a crucial role in the rooting capacity of plants due to their diverse actions at different phases of rhizogenesis (Ahmed et al., 2020; Denaxa et al., 2020). These compounds influence the rooting process through their concentration in the rooting zone during the detachment of cuttings and subsequent alterations in concentration over time (Denaxa et al., 2020; Osterc et al., 2007). Caffeic acid, recognized as an auxin synergist (James and Thurbon, 1981), has been identified as a participant in the formation of adventitious root (ARs) (Wu et al., 2007). However, Izadi et al. (2016) observed no correlation between the content of caffeic acid in leaves and the rooting ability of olive cuttings, suggesting its potentially suppressive impact. Additionally, they reported a positive correlation between the rooting potential of olive cultivars and catechin concentration in the cuttings.

Lavee et al. (1993) proposed that *in vitro* olive callus growth might not require auxin if cinnamic acid is used as a substitute. Chlorogenic acid has also been reported as a co-factor in the AR formation process, exerting a positive influence on rooting (Lavee et al., 1993; Gaspar et al., 1996; De Klerk et al., 2011; Denaxa et al., 2021). Lavee et al. (1993) indicated that chlorogenic acid could potentially substitute for auxin in the *in vitro* growth of olive callus. Nevertheless, Ozkaya and Celik (1997) observed higher concentrations of chlorogenic acid in the hard-to-root 'Domat' olive cultivar compared to the easy-to-root 'Gemlik,' without establishing a definitive correlation between chlorogenic acid and AR formation in cuttings.

Furthermore, Denaxa et al. (2021) found that the application of 0.1 mM chlorogenic acid enhanced rooting performance in 'Arbequina' and 'Kalamata' cuttings, potentially by altering the carbohydrate ratio and content at the base of the cuttings. They also reported that the easy-to-root 'Arbequina' exhibited notably higher levels of chlorogenic acid in the early stages of rhizogenesis compared to the hard-to-root 'Kalamata.' Additionally, they suggested that low concentrations of chlorogenic acid may result in suboptimal rooting performance in certain olive cultivars, such as 'Kalamata.'

Compounds with 2-hydroxy groups in the ortho position (o-diphenols), such as quercetins, are well-documented as IAA-oxidase (IAAox) inhibitors, protecting the IAA pool from degradation and promoting rooting (Curir et al., 1993; Faivre-Rampant et al., 2002; Denaxa et al., 2021; Trobec et al., 2005). Bruun et al. (1992) also suggested that quercetin is involved in polar auxin transport. Denaxa et al. (2020) established a positive correlation between the rooting ability of easy-to-root 'Arbequina' cuttings and their initial quercetin concentration. Osterc et al. (2007) further proposed that when cuttings are detached from the mother plant, the resulting stress enhances the production of some flavonols, such as rutin (quercetin-glycoside), which can promote rooting. Denaxa et al. (2020) confirmed that 'Arbequina' cuttings exhibited higher initial rutin concentrations compared to the recalcitrant-to-root 'Kalamata' cuttings, providing further evidence of the positive effect of rutin on root induction and initiation.

Vanillic acid, an activator of IAAox, plays a dual role depending on its concentration. Volpert et al. (1995) noted that the impact of vanillic acid on rooting or root growth is concentration-dependent (Wu et al., 2007). Izadi et al. (2016) observed a positive correlation between the rooting ability of cuttings from five olive cultivars and the concentration of vanillic acid in the stems, noting that high IAAox activity can inhibit rooting. In such cases, reducing the concentration of co-factors like vanillic acid would be advantageous. Conversely, if high endogenous IAA content is inhibiting rooting, an increase in IAAox activity would reduce the IAA level, thus facilitating the rooting process.

Izadi et al. (2016) also reported a significant negative correlation between the concentrations of naringenin or gallic acid in leaves and the rooting potential of cuttings from various olive cultivars. Table 1 summarizes the role of phenolic compounds in influencing the rooting of cuttings.

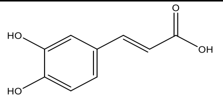
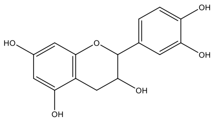
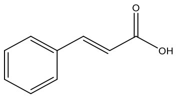
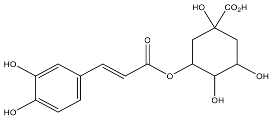
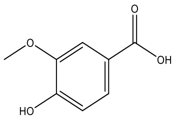
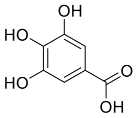
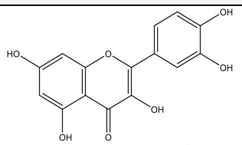
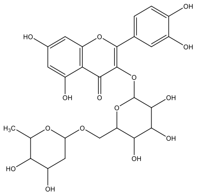
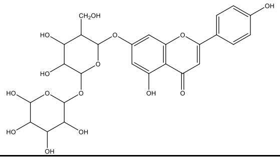
Role of polyamines

Polyamines usually appear in most living organisms (Cohen, 1998), function as growth regulators, and interact with plant hormones (Tonon et al., 2001). Despite ongoing debate concerning their classification (Rademacher, 2015), polyamines play a pivotal role in processes such as organogenesis, embryogenesis, cell signaling, membrane stability, cell division, AR, root growth, protein translation, gene expression, programmed cell death, and responses to biotic and abiotic stresses (Tsafouros and Roussos, 2020; Roussos et al., 2004; Martin-Tanguy, 2001; Couée et al., 2004; Cai et al., 2015; Denaxa et al., 2014; Bartolini et al., 2008). The primary polyamines in plants are putrescine, spermidine, and spermine. However, their precise role in ARs formation in cuttings remains unclear, often due to the focus on tissue polyamine levels and their effects.

During the early stages of rhizogenesis, polyamine accumulation and increased biosynthetic enzyme activity correlate with meristematic activity and lateral root formation, thereby influencing rooting capacity in cuttings. Several studies have suggested that free polyamines are critical for the rooting process, with higher levels associated with greater rooting potential (Tonon et al., 2001; Couée et al., 2004; Bartolini et al., 2008; Li et al., 2009; Neves et al., 2002). Denaxa et al. (2014) demonstrated that olive cuttings from cultivars with higher free polyamine content exhibit better rooting, revealing genotype-dependent differences, such as those between 'Arbequina' (easy-to-root) and 'Kalamata' (hard-to-root). Treatment of 'Kalamata' with IBA and putrescine in autumn, when putrescine levels were lowest, enhanced rooting compared to treatment with IBA alone (Denaxa et al., 2014).

Changes in putrescine levels during ARs formation in olive cuttings highlight different phases of rhizogenesis (Porfírio et al., 2016), with elevated putrescine levels observed during the induction and expression phases (Denaxa et al., 2014). This suggests the onset of differentiation and growth of rooting primordia, as emphasized in studies by Tiburcio et al. (1989), Hausman et al. (1997), and Nag et al. (2001). Polyamine accumulation, especially in bound form, was observed in hard-to-root explants and in explants that failed to root within the first and second day of rooting (Rugini et al., 1997).

Table 1. The role of phenolic compounds affecting ARs of olive cuttings.

Simple Phenols	Structure	Role on AR formation	References
Caffeic acid		Auxin synergist/suppressor	James and Thurbon, (1981); Wu et al. (2007), Izadi et al (2016)
Catechin		Positive correlation	Izadi et al. (2016)
Cinnamic acid		Auxin-like effect	Lavee et al. (1993)
Chlorogenic acid		Co-factor role Auxin-like effect Negative correlation	Lavee et al. (1993); Gaspar et al. (1996); Denaxa et al. (2021); Ozkaya and Celik, (1997)
Vanillic acid		IAAox stimulator positive correlation	Volpert et al. (1995) Izadi et al. (Izadi et al., 2016)
Gallic acid		Negative correlation	Izadi et al. (2016)
Flavonoids	**Structure**	**Role on AR formation**	**Reference**
Quercetin		IAAox inhibitor; Enhancer Polar auxin transport	Denaxa et al. 2021; Faivre-Rampant et al., (2002); Curir et al. (1993)
Rutin		Promote rhizogenesis	Denaxa et al. (2020b)
Naringenin		Negative correlation	Izadi et al. (2016)

Exogenous application of polyamines has been shown to stimulate ARs formation in various olive cultivars. The combined application of putrescine and IBA, either at the base of the cuttings or integrated into the substrate during *in vitro* culture, accelerates rooting, increases rooting capacity, and improves rooting characteristics such as root quantity and length. This effect has been documented in cultivars like 'Chondrolia Chalkidikis', 'Frantoio', 'Moraiolo', 'Frangivento', 'Pendolino', 'Dolce Agogia', and 'Kalamata' (Rugini et al., 1989; Rugini, 1992; Grigoriadou et al., 2002; Denaxa et al., 2014; Porfírio et al., 2016; Sebastiani and Tognetti, 2004). However, the response to polyamine treatments may vary depending on factors such as basal shoot darkening, the type of explant used, and the endogenous polyamine levels within the plant

(Porfírio et al., 2016).

The response to exogenous putrescine treatments has been found to be negatively correlated with total free polyamine content, which is generally low in olive cuttings (Rugini et al., 1993). Furthermore, limited studies suggest that putrescine can regulate the activity of specific enzymes, such as IAA-oxidase (IAA-ox) and peroxidases (POX), in favor of rooting in several species (Rugini et al., 1997; Gaspar et al., 1997; Tsafouros and Roussos, 2020; Hausman et al., 1995; Nag et al., 2001). In olive cuttings, exogenously applied putrescine increases POX activity, resulting from enhanced putrescine degradation via the Δ'-pyrroline pathway. This pathway generates hydrogen peroxide (H_2O_2), which triggers POX activity and ultimately enhances the rooting response (Gaspar et al., 1997; Porfírio et al., 2016).

The presence of spermidine appears to exert mainly inhibitory effects on adventitious rooting, as indicated by its high endogenous levels in cuttings of species with low rooting potential (Jarvis et al., 1983). However, research by Denaxa et al. (2014) found that endogenous spermidine levels were higher in the 'Arbequina' cultivar, which is easy-to-root, compared to the hard-to-root 'Kalamata' cultivar. Spermidine accumulation during ARs formation exhibited varying patterns, similar to putrescine accumulation, particularly during the induction and expression phases of olive cuttings treated with auxin (Denaxa et al., 2014).

The role of spermine in ARs formation in olive cuttings remains unclear due to contradictory results. While higher levels of free spermine may improve the rooting ability of cuttings (Rugini et al., 1997; Jarvis et al., 1983), exogenous application of spermine and spermidine did not enhance rooting in 'Kalamata' cuttings (Rugini et al., 1989; Rugini et al., 1997; Denaxa et al., 2014). Easy-to-root olive cultivars reportedly show lower bound spermine content on the second day after planting compared to recalcitrant cultivars, suggesting that higher free spermine levels may enhance rooting potential (Rugini et al., 1997). Spermine treatment has been shown to increase AOX gene expression (Takahashi et al., 2003), which is associated with rooting (Hedayati et al., 2015), and it has also been found to interact with H_2O_2 (Porfírio et al., 2016). These findings suggest that spermine may serve as a potential rooting marker in olive cuttings (Santos Macedo et al., 2009).

Several studies have reported the role of polyamine breakdown products in promoting root growth across various species (Hausman et al., 1997; Kevers et al., 1997; Tsafouros and Roussos, 2020). In olive plants, inhibition of putrescine synthesis, which is involved in root growth, by using α-difluoromethylornithine (DFMO) and difluromethylarginine (DFMA) can block rooting by inhibiting the activity of ornithine decarboxylase (ODC) and arginine decarboxylase (ADC). However, the impact of DFMO and DFMA on rooting can be reversed by applying putrescine to the rooting substrate or by treating the explants with H_2O_2, a putrescine breakdown product. Additionally, aminoguanidine, which inhibits diamine oxidase (DAO) activity, has been found to promote rooting in olive plants when applied alone or with putrescine, enhancing the rooting potential in olive explants (Rugini et al., 1997).

Figure 2 summarizes the roles of polyamines in ARs formation.

Role of carbohydrates

The formation of ARs requires a substantial amount of energy (Del Rio et al., 1991; Denaxa et al., 2012; Denaxa et al., 2021; Aslmoshtaghi and Shahsavar, 2016) and is therefore closely associated with the levels of soluble sugars and storage carbohydrates (Bartolini et al., 2004). Carbohydrates serve multiple functions, including providing energy (Haissig, 1989) and structural materials (Wiesman and Lavee, 1995; Sivaci, 2006), and play a critical role in promoting rooting by influencing gene expression (Gibson, 2005), glycosylating DNA, affecting transcription (Veierskov, 1988), and interacting with plant hormone signaling systems (Eveland and Jackson, 2012; Gibson, 2004). The presence of sugars can affect both the conjugation and transport of auxin (Ljung et al., 2015), suggesting that variations in sugar concentrations may be related to changes in auxin content (Sairanen et al., 2012). Carbohydrates influence auxin metabolism and enhance the effect of indole-3-butyric acid (IBA) during the rooting process (Del Rio et al., 1991; Wiesman and Lavee, 1995). Additionally, auxin application has been found to increase carbohydrate concentrations in the rooting zone, either by boosting the activity of hydrolyzing enzymes or stimulating carbohydrate mobilization. Elevated carbohydrate levels provide the necessary energy for cell division and differentiation, underscoring the importance of the auxin-carbohydrate relationship in optimizing conditions in the rooting zone (Haissig, 1989; Husen and Pal, 2007; Denaxa et al., 2012).

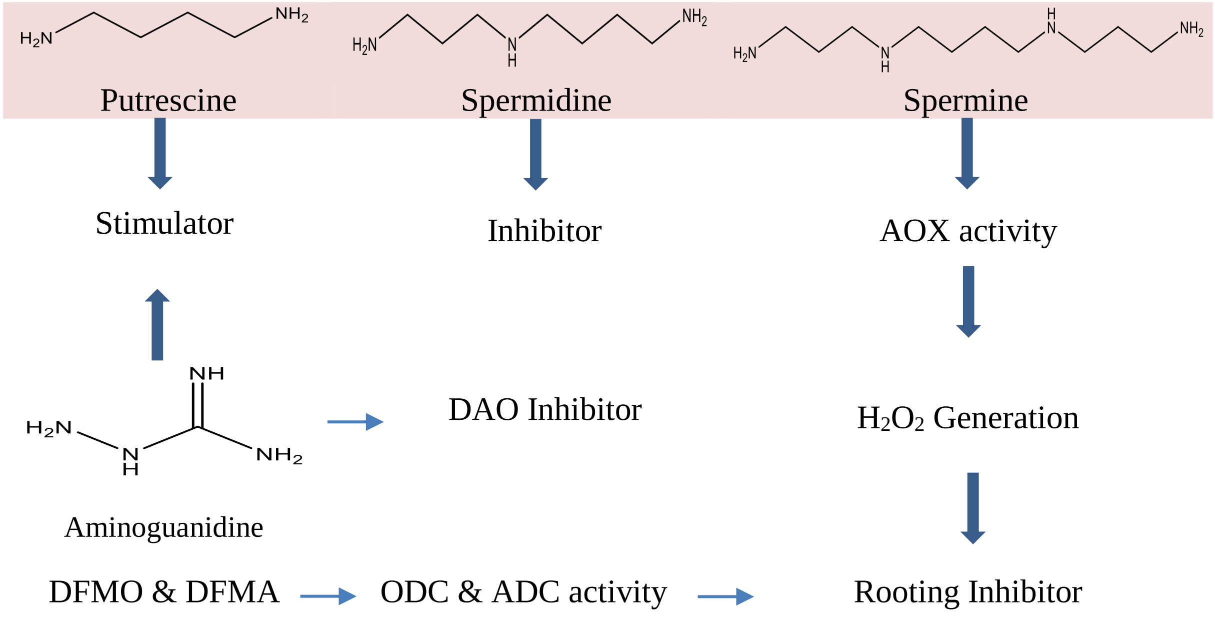


Fig. 2. Roles of putrescine, spermidine and spermine in ARs formation of olive cuttings. Abbreviations: ADC (Arginine decarboxylase), AOX (Alternative oxidase), DAO (Diamine oxidases), DFMA (Difluromethylarginine), DFMO (α-Difluoromethylornithine), H_2O_2 (Hydrogen peroxide), ODC (Ornithine decarboxylase).

Carbohydrate presence at the bases of cuttings significantly influences rooting, especially in olive cuttings, where initial carbohydrate levels are crucial for ARs development (Del Rio et al., 1991; Wiesman and Lavee, 1995; Ozkaya and Celik, 1997; Denaxa et al., 2010; Denaxa et al., 2012). As ARs develop, there is a concomitant decrease in carbohydrate levels. Haissig (1990) proposed a strong link between carbohydrate concentration during propagation and the regulation of ARs formation, supported by observations that auxin treatments often alter individual carbohydrate concentrations in cutting tissues. Starch accumulation and consumption have been reported as more crucial for root initiation than soluble sugars (Wiesman and Lavee, 1995). Carbohydrate availability and mobilization toward the base are important for ARs formation in olive cuttings. Seasonal variations in carbohydrate concentrations and reproductive cuttings correlate with changes in rooting success, although winter rooting failures and inflorescence impact on rooting cannot be solely attributed to carbohydrate availability or assimilate redirection (Del Rio et al., 1991). Wiesman and Lavee (1995) demonstrated that insufficient starch reserves could lead to a higher failure rate of rooted olive cuttings. However, Denaxa et al. (2012) found that glucose and mannitol, compared to sucrose and starch, had a more significant positive influence on olive cuttings for their rhizogenesis. The allocation and distribution of carbohydrates within the cutting appear to have a greater impact on rooting than their absolute content (Druege, 2009; Druege, 2020; Ruedell et al., 2013). Olive varieties with higher carbohydrate content tend to root more easily (Aslmoshtaghi and Shahsavar, 2016), although this finding contrasts with those of Izadi et al. (2016). Porfirio et al. (2016) found that cuttings with low endogenous carbohydrate reserves showed improved rooting performance when treated with carbohydrates. The type of sugar applied significantly affects root regeneration, particularly in microcuttings, as highlighted by Calamar and De Klerk (2002). Sucrose, for example, serves both as an energy source and as building blocks, and may play a regulatory role (Calamar and De Klerk, 2002). Sucrose is commonly used in tissue culture media due to its role as the primary sugar translocated in the phloem of many plants. Mannitol, fructose, and glucose have also been shown to promote rhizogenesis (Denaxa et al., 2012). The positive effect of exogenous sucrose application on rooting potential in most herbaceous and woody plants has been previously documented (Haissig, 1989), including in ‘Picual’ cuttings with floral buds (Del Rio et al., 1991). The critical role of sucrose application in both the induction and proliferation phases of tissue culture has been emphasized by Devi et al. (2021). Furthermore, plants exhibit varying sucrose concentration requirements due to differences in their ability to absorb and utilize carbon sources (Devi et al., 2021).

Additional factors associated with olive cutting adventitious rooting

Oxidative enzymes, including PPO, POX, and IAAox, are usually associated with the formation of ARs in various plant species (Denaxa et al., 2019; Macedo et al., 2013; Tchinda et al., 2013). POX is a well-established marker for the rooting process, significantly influencing root initiation (Hartmann and Kester, 1975; Metaxas et al., 2004). Similarly, PPO is implicated in rhizogenesis, potentially oxidizing auxins during the growth and development of root primordia (Qaddoury and Amssa, 2003; Tchinda et al., 2013). Additionally, PPO is involved in regulating the synthesis of phenolic precursors essential for lignin biosynthesis during root differentiation (Aslmoshtaghi and Shahsavar, 2016). Güneş (2000) demonstrated a strong correlation between IAAox activity and AR formation, as this enzyme regulates IAA levels. Vatulescu et al. (2004) suggested that high levels of IAA might inhibit rooting if IAAox activity declines, whereas lower IAA levels generally support rooting. A complex regulatory network of plant hormones has been shown to be related to AR formation (Zhang et al., 2023). The application of salicylhydroxamic acid (SHAM), an AOX inhibitor, can impede AR development in semi-hardwood cuttings and *in vitro* cultured microshoots of various olive cultivars (Porfirio et al., 2016). The inhibitory effect of SHAM on root formation may be due to (a) AOX inhibition, reducing phenolic substrates available for PPO due to the downregulation of phenylpropanoid biosynthetic pathways, (b) an increase in IAAox activity, leading to lower levels of free IAA, or (c) interference with the conversion of IBA to IAA in the presence of SHAM. Salicylates, a type of hydroxybenzoate in plants, are also recognized as phytohormones (Raskin, 1992). In both woody and herbaceous plants, the application of SA combined with auxin has been found to significantly promote rooting in vivo (İsfendiyaroğlu and Özeker, 2008). However, SA inhibited rooting of *in vitro* apple stems during the first 24-96 hours (Klerk et al., 1997). İsfendiyaroğlu and Özeker (2008) observed no root formation in the hard-to-root 'Domat' leafy cuttings when treated with 5 g L^{-1} IBA and varying concentrations of SA (2.5 to 10 g L^{-1}), with no significant effect observed. Furthermore, both pre- and post-application of SA significantly inhibited rhizogenesis compared to simultaneous application. Numerous studies have indicated that H_2O_2 plays a role in the formation of lateral roots and ARs in various plant species, including annuals and woody perennials (Roussos, 2023). H_2O_2 interacts with plant growth regulators and can affect physiological and molecular responses by participating in signaling pathways of plant hormones such as CK, JA, IAA, and ETH (Li et al., 2017; Gong et al., 2022). Thus, H_2O_2 may enhance auxin signaling required for improved AR formation (Roussos, 2023). Additionally, H_2O_2 appears to interact with AOX and nitric oxide (NO) (Porfirio et al., 2016; Roussos, 2023). In olive, a 3.5% w/v H_2O_2 treatment combined with 4000 mg g^{-1} IBA slightly increased rooting percentages in 'Frantoio' and 'Gentile di Larino' cuttings and improved rooting quality, such as the number of roots (Sebastiani and Tognetti, 2004). Rugini et al. (1997) reported that H_2O_2 treatments led to a higher rooting percentage, increased the number of roots per cutting or explant, and accelerated root development in olive cuttings and *in vitro* explants. Nutrients, along with organic molecules such as polyamines, phenolic compounds, and carbohydrates, act as structural components, co-factors for enzymes, and signaling molecules (Roussos, 2023), thereby influencing AR formation. Biostimulants have also been found effective in enhancing vegetative cutting propagation, though optimal usage rates vary by species (Wise et al., 2020). Eid et al. (2018) found that natural extracts from algae, garlic, and licorice were more effective than yeast in promoting rooting in 'Picual' olive cuttings. Mohammed (2021) reported enhanced AR formation in 'Sorani' hardwood cuttings using 6 and 9 g L^{-1} licorice extract, and 9 g L^{-1} willow extract. Recent research by Rashedy (2022) revealed that humic, ginger, and licorice extracts were among the most effective natural root stimulators for 'Coratina' hardwood cuttings. Ethanol or vinegar solvents were also found to significantly enhance the efficiency of natural extracts, compared to water, in promoting AR formation (Rashedy, 2022). These findings underscore the potential of natural extracts as sustainable alternatives to synthetic growth regulators for promoting rooting in semi-hardwood and hardwood olive cuttings. Auxin-producing bacteria reportedly aid AR formation (Centeno and Gomez-del-Campo, 2008; Montero-Calasanz et al., 2013; Montero-Calasanz et al., 2014). Commercial products such as Auxym oligo, Roots, and Myco+AA demonstrated higher rooting percentages when used on 'Picual', 'Manzanilla', and 'Picudo' cultivars, compared to untreated control samples. However, natural auxin sources like germinating seeds, fungi, and algal extracts did not significantly increase rooting percentages in the 'Cornicabra' cultivar, with the exception of 'Terrabal OrganicoTM'. This compound, derived from a soluble fraction of macerated cereal seed extract, may cause toxicity with prolonged treatment (Centeno and Gomez-del-Campo, 2008). *Agrobacterium rhizogenes* has also reportedly impacted *in vitro* rooting induction in olive explants (Rugini, 1992). Montero-Calasanz et al. (2013) evaluated the rooting efficiency of semi-hardwood cuttings from 'Arbequina', 'Hojiblanca', and 'Picual' olive cultivars using various plant growth-promoting rhizobacteria, finding that all bacterial strains induced ARs, similarly or more effectively than IBA-treated cuttings under nursery conditions. The response varied by bacterial strain and inoculation method, with *Pantoea* sp. AG9 being the most effective due to its ability to synthesize the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Montero-Calasanz et al. (2014) identified a new bacterial species, *Chryseobacterium oleae* (type strain CT348T), isolated from the ecto-rhizosphere of an organically farmed 'Arbequina' olive tree. This strain produced several polyamines, including sym-homospermidine, which facilitates AR formation, as well as putrescine, spermine, spermidine, and cadaverine. The *Chryseobacterium* genus is associated with plant growth-promoting activities (Montero-Calasanz et al., 2013; Dardanelli et al., 2010). Arbuscular mycorrhizae (AM) are commonly used in micropropagation to enhance plantlet performance and reduce acclimatization duration (Porfirio et al., 2016). Introducing AM fungi results in an extensively branched root system with larger ARs (Kapoor et al., 2008), improving survival rates of difficult-to-root plants (Azcón-Aguilar and Barea, 1997). While certain AM fungi are effective on various olive cultivars (Porfírio et al., 2016), the effectiveness of mycorrhizae depends on the plant genotype and the specific AM fungal species or strain used (Calvente et al., 2004). Olive trees form AM associations with obligatory plant symbionts from the *Glomales* order (BE and Barea, 1986). These symbiotic relationships are crucial for mitigating transplantation stress during the acclimatization of micropropagated plants (Kapoor et al., 2008). Therefore, incorporating AM fungi into micropropagation programs can enhance the cultivation of important olive genotypes.

Conclusions

The formation of adventitious root is intricately regulated by the interaction of phytohormones and a complex array of biochemical factors, all of which must act in coordination to achieve optimal rooting responses. While auxin and auxin-based regulators are key players in initiating adventitious root formation, recent research has increasingly recognized the significant roles of phenolic compounds, polyamines, and carbohydrates in this process. However, a comprehensive understanding of their mechanisms, synergies, interactions with other molecules, and their potential influence on adventitious root formation in olive cuttings remains incomplete. The existing literature lacks extensive quantification of the endogenous levels of compounds affecting adventitious root formation in olive cuttings. Future research should investigate underexplored factors and processes, including the roles of sugars and nutrients, the complex interplay between hormones and these elements, microorganism-assisted approaches, and the involvement of small auxin up RNAs as auxin-related genes in adventitious root formation. Additionally, examining the differential expression of alternative oxidase genes in olive cultivars with varying rooting capacities is of paramount importance. Research should aim to establish a foundational knowledge base for adventitious root in olives, paralleling the progress made in model plants. Such knowledge will be instrumental in developing innovative breeding and propagation technologies that are scientifically grounded and capable of addressing future ecological and economic challenges.

Conflict of Interest

The authors indicate no conflict of interest in this work.

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