UNIVERSIDADE D
COIMBRA

Sandra Isabel Grilo Caeiro

MOLECULAR ANALYSIS OF REGULATORY MECHANISMS INVOLVED IN ALMOND MICROGRAFTS

Dissertação no âmbito do Mestrado em Bioquímica orientada pela Doutora Sandra Isabel Marques Correia e pela Doutora Liliana Maria Bota Marum e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Outubro de 2021

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Results from this work were already presented in national and international conferences:

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2. XXIV Meeting of the Spanish Society of Plant Biology; XVII Spanish Portuguese Congress on Plant Biology; Biologia de Plantas 202, 7th – 9th July, Online

3. The 2nd International Electronic Conference on Plant Sciences—10th Anniversary of Journal Plants (ongoing abstract acceptance)

A technical article was also published in AGROTEC special issue 39, June 2021: 'Caracterização, estabelecimento e preservação *in vitro* de variedades tradicionais e porta-enxertos de amendoeira'.

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List of abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid

ABCB: ATP-binding cassette class B

AC: activated charcoal

ACT11: *ACTIN-11*

ALF4: *ABERRANT LATERAL ROOT FORMATION 4*

AP2/ERF: APETALA2/ETHYLENE RESPONSIVE FACTOR

ARFs: auxin response factors

Aux/IAAs: AUXIN/INDOLE-3-ACETIC ACID

AUX1/LAX: AUXIN TRANSPORTER PROTEIN 1/LIKE-AUXIN TRANSPORTER PROTEIN 1

AVDC: Association of Nursers of the District of Coimbra

AXR 1: AUXIN RESISTANT 1

BAP: 6-benzylaminopurine

bp: base pairs

BSA: Bovine serum albumin

cDNA: complementary DNA

Cq: quantification cycle

CEAT: Centro de Experimentação Agrária de Tavira

DAG: days after grafting

DIC: Differential interface contrast

ESR1: *ENHANCER OF SHOOT REGENERATION*

Fe-EDDHA: ethylenediamine di-o-hydroxyphenylacetic acid

Fe-EDTA: tetraacetic acid

FT: *FLOWERING LOCUS T*

GA_3: gibberellic acid

IAA: indole-3-acetic acid

IAA26: *INDOLE-3-ACETIC ACID INDUCIBLE 26*

IBA: indole-3-butyric acid

IPA: indole-3-pyruvate

NAA: 1-naphthaleneacetic acid

PBS: phosphate-buffered saline

Picloram: 4-amino-3,5,6-trichloropicolinic acid

PIN: PIN-FORMED

PGR: Plant growth regulators

qPCR: quantitative PCR

TAA: TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS

TCA: trichloroacetic acid

TIR1/AFB: TRANSPORT INHIBITOR RESPONSE 1 /AUXIN SIGNALLING F-BOX

Trp: tryptophan

WIND1: *WOUND INDUCED DEDIFFERENTIATION 1*

YUC: YUCCA family of flavin monooxygenase

Abstract

Prunus dulcis (Mill.) D. A. Webb is a Portuguese traditional culture in Trás-os-Montes and Alto Douro and Algarve. However, new orchards are emerging in Beira Interior and Alentejo.

In these orchards, commercial varieties and rootstocks, developed to achieve better adaptation to intensive and irrigated systems, were introduced. In a context where natural resources management is essential, well adapted and resilient traditional varieties selected over the years, might contribute for the search of variability adapted to different scenarios and sustainable options.

The main almond propagation method is grafting, a technique used to promote edaphoclimatic adaptation, and increase production and fruit quality. The molecular mechanisms behind grafting success remain largely unknown and are being studied. In this context, the aim of this work was to study wound healing and auxin-induced regulatory mechanisms involved in scion-rootstock interactions in almond micrografts, using traditional, commercial and non-cultivated varieties. To achieve this main goal, it was necessary to optimize the *in vitro* establishment, multiplication and rooting conditions. *In vitro* establishment and multiplication were successfully achieved for all varieties, with bitter almond and Gama Dura being the most responsive regarding the number of phytomers and multiplication rates. Despite the efforts, i*n vitro* or *ex vitro* rooting, and acclimatization were not achieved.

From the *in vitro* established varieties, different micrografting combinations were defined, and micrografts were established using bitter almond and the commercial GxN15 as rootstocks. Bitter almond based micrografts revealed equal or higher success rates than micrografts established with GxN15 rootstock.

The presence of the endogenous auxin IAA was evaluated through quantification, by Ehrlich reaction, and immunolocalization before and after micrografting in three segments (scion, union and stock). Moreover, gene expression quantification of wound related genes (*WIND1*) and genes involved in auxin response to promote vascular connection between scion and rootstock (*ALF4*, *TIR1* and *IAA26*) was carried out. Results have shown a scion content of 1.292±0.448 μg IAA/ mg FW (Canhota) and 5.505±1.179 μg IAA/ mg FW (bitter almond) and a potential influence of IAA initial levels on micrografting success. A possible earlier wound response at 7 days after grafting was

detected, and the absence of proper auxin response at the graft union when different varieties are joined together. Also, the scion of bitter almond homografts seems to promote a simultaneous wound and auxin response 21 days after grafting, because of the increased gene expression of *WIND1*, *ALF4*, *TIR1* and *IAA26* observed.

The results presented in this work contribute to a better understanding of the role of IAA in almond micrografts and may be a foundation for future studies, even for other *Prunus* species.

Keywords: Auxin quantification; auxin-related genes; grafting; indole-3-acetic acid; *in vitro* culture; wounding.

Resumo

Prunus dulcis (Mill.) D. A. Webb é uma cultura tradicional portuguesa em Trás-os-Montes e Alto Douro e no Algarve. Contudo, novos pomares têm surgido nas regiões da Beira Interior e no Alentejo.

Nestes, estão a ser introduzidas variedades internacionais e porta-enxertos comerciais desenvolvidos para promover uma melhor adaptação da cultura aos regimes intensivos e de regadio. Num contexto em que a gestão de recursos naturais é fundamental, variedades tradicionais resilientes e com boa adaptação, selecionadas ao longo do tempo, podem contribuir para a procura de variabilidade adaptada a diversos cenários e alternativas mais sustentáveis.

Uma das principais formas de propagação da amendoeira é a enxertia, uma técnica usada para promover adaptação a condições edafoclimáticas e aumentar a produção e a qualidade dos frutos. Os mecanismos moleculares subjacentes ao sucesso da enxertia permanecem, em grande parte, desconhecidos, estando a ser analisados. Assim, o principal objetivo deste trabalho consistiu em estudar os mecanismos moleculares de resposta ao ferimento regulados por auxinas e envolvidos na interação entre enxerto e porta-enxerto, em microenxertos de amendoeira com recurso a variedades tradicionais, comerciais e não cultivadas. Para atingir este objetivo tentou-se otimizar as condições de estabelecimento *in vitro*, multiplicação e enraizamento. O estabelecimento e multiplicação *in vitro* foram conseguidos com sucesso para todas as variedades, com as variedades de amêndoa amarga e a Gama Dura a serem mais responsivas em relação ao número de fitómeros e à taxa de multiplicação. Apesar do esforço, o enraizamento *in vitro* ou *ex vitro* e aclimatização não foram conseguidos.

Entre as variedades estabelecidas *in vitro*, diferentes combinações de microenxertias foram definidas, e as microenxertias foram estabelecidas com amêndoa amarga e o porta-enxerto comercial GxN15. Microenxertos com amêndoa brava revelaram taxas de sucesso iguais ou superiores do que as enxertias estabelecidas com o porta-enxerto GxN15.

A presença de IAA endógeno foi avaliada através da quantificação pela reação de Ehrlich e pela imunolocalização desta hormona, antes e depois da realização de microenxertias em três segmentos (enxerto, união e porta-enxerto). Para além disso, foi realizada a quantificação da expressão de genes descritos na resposta ao ferimento

(*WIND1*) e na resposta a auxinas para promoção da ligação dos tecidos vasculares entre o enxerto e porta-enxerto (*ALF4*, *TIR1* e *IAA26*). Os resultados indicaram um conteúdo nos enxertos de 1,292±0,448 μg IAA/ mg FW (Canhota) e 5,505±1,179 μg IAA/ mg FW (amêndoa amarga) e uma possível influência destes níveis no sucesso das microenxertias. Verificou-se também uma possível resposta ao ferimento mais precoce, 7 dias após enxertia, e uma ausência de resposta às auxinas em heteroenxertos. Para além disso, em homoenxertias de amêndoa amarga, parece ocorrer em simultâneo uma resposta ao ferimento e a auxinas, 21 dias após a enxertia, devido ao aumento da expressão de *WIND1*, *ALF4*, *TIR1* e *IAA26* observada em *T2*.

Os resultados apresentados neste trabalho contribuem para uma melhor compreensão do papel do IAA em microenxertos de amendoeira e podem ser a base para futuros estudos, mesmo em outros *Prunus* spp.

Palavras-chave: Ácido indol-3-acético*;* cultura *in vitro*; enxertia; ferimento; genes de resposta a auxinas; quantificação de auxina.

1. Introduction

1.1 Contextualization

Almond - *Prunus dulcis* (Mill.) D.A. Webb – is originated from the mountains of Central Asia but its presence in the Mediterranean region has been known for a long time. For centuries, this species was cultivated in dry to semi-arid regions with concentrated rainfall in the cold season (Aguiar *et al*., 2017; Associação dos Jovens Agricultores de Portugal (AJAP), 2017). Since the period of domestication, 5 000 years ago, until recently, almond was propagated through seeds, bringing to a large genetic and phenotypic diversity. Also, due to the long period it has been cultured, numerous varieties have emerged as result of spontaneous mutations (Neves and Miguel, 2014; Aguiar *et al*., 2017).

The fruit, also known as almond, has several potential health benefits due to its high nutritional value. The increasing demand for dry fruits associated with a healthier lifestyle has led to a higher demand and consumption of almond, including in Portugal, where prices reach 6€/ Kg (Queirós, 2020; Iglesias and Foles, 2021).

The USA is the leading producer with 79% of the world's production, followed by Australia with 7%, and Spain with 6%, whereas Portugal holds about 1% of the world production. However, Spain represents 86% of the total almond growing area, since orchards occur mostly in dryland (Queirós and Sousa, 2017; Lordan *et al*., 2021).

Regarding worldwide productivity, in 2017, Australia recorded a production of 5500 kg/ha, followed by the USA with 4000 kg/ha. In Portugal, the values remain far from these productivities with only 771 kg of kernel/ha (in 2019) in spite of the increasing almond area achieved in the last years (Queirós and Sousa, 2017; Doll *et al*., 2021).

In our country, almond is a traditional culture in the regions of Trás-os-Montes and Alto Douro, and Algarve, but in recent years new orchards have been installed in Beira Interior and Alentejo (Figure 1). In the last five years, almond area from these two last regions increased from 2800 ha to 14000 ha due to water availability and low risk of rains and frosts (Doll *et al*., 2021).

These new orchards are being installed as an intensive culture system, meaning, a higher number of trees per area, allowing mechanization in almond culture. Also, although almond is well adapted to dryland, these new orchards are irrigated to increase production (Santos and Teixeira, 2020).

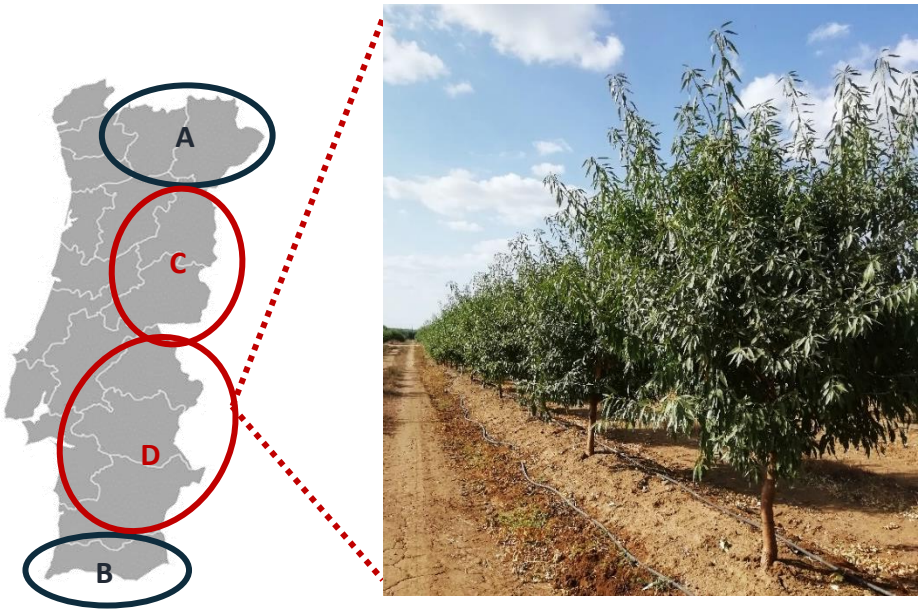

Figure 1. Almond culture in Portugal. Almond is a traditional culture in the regions of Trás-os-Montes and Alto Douro (A), and Algarve (B), but recently new intensive orchards are being installed in Beira Interior (C) and Alentejo (D).

Orchard intensification is associated with the fixation of superior characteristics through vegetative propagation and the consequent loss of genetic diversity and local cultivars (Aguiar *et al*., 2017). The management of natural resources and the introduction of more sustainable practices is fundamental for the new orchards. In the last century an accentuated decrease in overall diversity occurred, since local cultivars have been replaced with ones assuring higher productivities. In many cases, those local cultivars were locally improved over years in terms of production, resistance, and quality traits, presenting valuable germplasm for future breeding programmes (Alonso *et al*., 2011).

In this context, the project CULTIVAR, of the Centre for Functional Ecology of the University of Coimbra, started collaborations between scientific institutions and producers for the valorisation of endogenous resources in the Centre region. In the South, the project INOV-AMENDO-AL, developed at the Alentejo Biotechnology Center for Agriculture and Agro-food (CEBAL), aims to transfer scientific knowledge providing valuable almond plants obtained using biotechnology methodologies.

Through the program CULTIVAR and working in collaboration with researchers from the project INOV-AMENDO-AL this work aims at the characterization and conservation of Portuguese traditional varieties through the development of new methodologies for micropropagation, including micrografting, and achieving a deeper

knowledge of the molecular mechanisms involved in such methodologies, which would ultimately lead to more sustainable options for the producers.

1.2 Almond

Almond (Fig.2) is a naturally self-incompatible deciduous tree (8 to 10 m) with white and light pink flowers and naturally self-incompatible (Costa and Rosa, 2020). The fruit is a drupe, composed of a fleshy hull surrounding a hard shell, which protects the edible seed or kernel (Fig. 2). As the kernel matures, the hull dries and splits opens, allowing the in-shell nut to dry naturally before harvest. Depending on kernel amygdalin content, almond varieties can produce sweet almond or bitter almond (*P. dulcis* var. amara). Kernels of sweet almond consist mainly of lipids, protein, fibre, and high concentrations of vitamin E (Franklin and Mitchell, 2019).

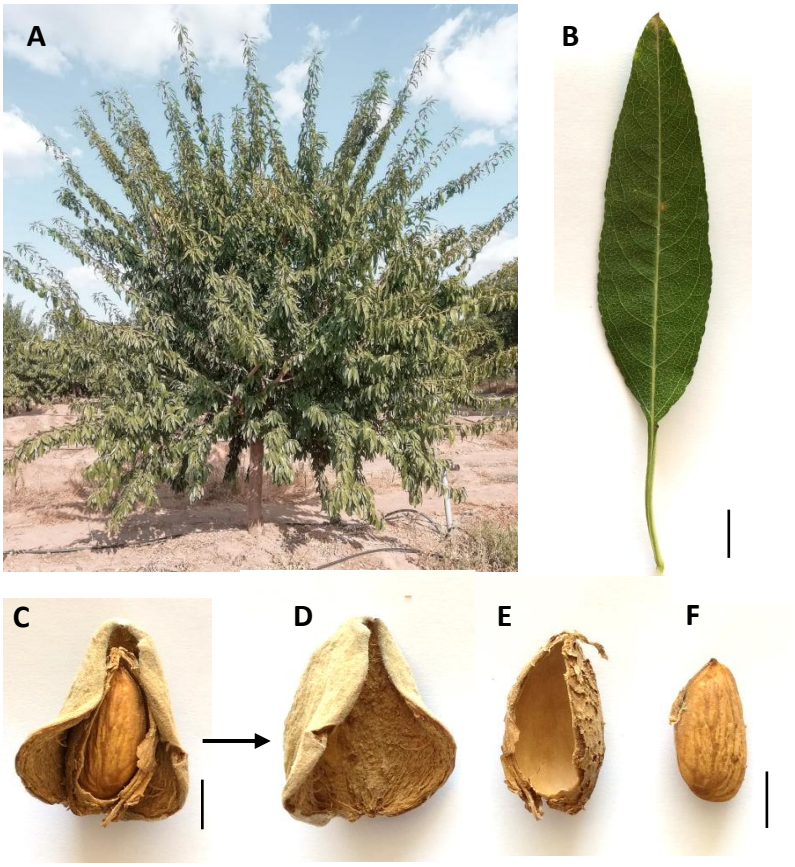

Figure 2. Almond tree (A), leaf (B), dry hull split opened with in-shell nut (C), hull (D), shell (E) and kernel (F). Scale bar - 1 cm.

Among the most important morphological features for fruit production, are the almond and kernel size (Fig. 3), weight, fruit yield (Asaii *et al.*, 1996) and the presence of double kernels, a derogatory feature that makes processing difficult (Queirós and Sousa, 2017). According to the International Board for Plant Genetic (1981), nut and kernel shape can be determined based on the ratio width/length.

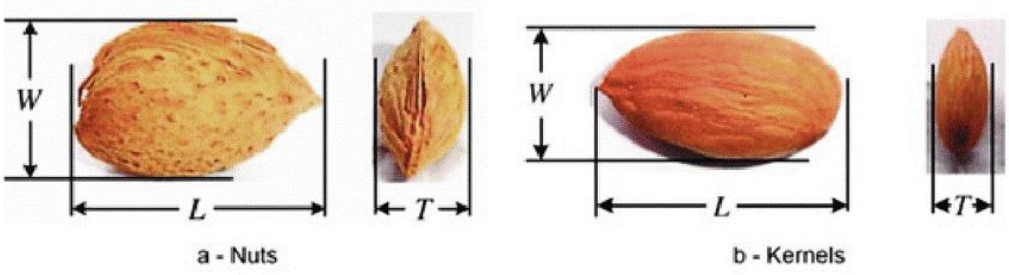

Figure 3. Almond size parameters: width (W), length (L) and thickness (T) (Zheng and Fielke, 2014).

Almond propagation can be achieved through seed germination, grafting or micropropagation (Aguiar *et al.*, 2017). Grafting is the most used technique, and selection of scion and rootstock varieties is extremely important for edaphoclimatic adaptation and ultimately, for economic sustainability (Queirós and Sousa, 2017).

Selection of rootstocks will determine whether the culture is suited for dryland or needs irrigation, the culture intensification degree and adaptation to soil conditions (Queirós and Sousa, 2017). As rootstocks, bitter almond and some other varieties (Marcona, Garrigués, Ferrastar) were used for many years, due to a strong root system, adaptation to dryland and low fertility soils as well as, induced vigour in the scion (Queirós, 2020). Later on, hybrid rootstocks emerged, such as the GF-677, a *Prunus dulcis* x *Prunus persica* hybrid, combining the adaptation to dryland and irrigated soil. Although this rootstock is now the most used, it has some limitations, such as, the difficulty to propagate and sensibility to nematodes. Another widely used hybrid rootstock is the GxN, known as GARNEM (*P. dulcis* var. Garfi x *P. persica* var. Nemared) (Aguiar *et al.*, 2017). More recently, Rootpac® rootstocks were developed to promote different scales of vigour for better adaptation to the new culture systems (Queirós, 2020). In locations where almond culture is more traditional, bitter almond and GF677 are the most commonly used rootstocks, but in the new orchards in Beira Interior and

Alentejo the hybrid rootstocks GF677, GxN15 or Rootpac ® varieties are highly demanded (CEBAL, 2020; Doll *et al.*, 2021).

A huge diversity of traditional varieties with distinctive characteristics and productivity have persisted even though being kept in marginal conditions. In the last years, new varieties have been introduced in the orchards and imposed over the traditional due to greater productivity (Table 1). The new varieties are self-compatible and late blooming a feature crucial to avoid frosts, thus increasing productivity. Some of the varieties introduced into Portuguese orchards were the French varieties Ferraduel, Ferragnès and Lauranne, and the Spanish Guara, Antoñeta®, Glorieta, Francolí and Masbovera. The Spanish varieties Soleta®, Belona and Marinada are even more recent (Queirós, 2020).

Table 1. Source of commercial and Portuguese traditional varieties, flowering time, compatibility, fruit yield and double kernel content.

	Variety	Source	Flowering	Compatibility	Fruit yield	Double Kernel
Commercial	**Ferraduel**	France (INRA)	3rd week Fev 3rd week Mar	Self-incompatible	26-28%	0%
	Antoñeta®	Spain	4th week Fev 3rd week Mar	Autocompatible	35%	0%
	Soleta®	Spain (CITA)	1th week Mar 3rd week Mar	Autocompatible	27-35%	0%
	Lauranne	France (INRA)	4th week Fev 3rd week Mar	Autocompatible	35-38%	<5%
Portuguese traditional	**Rabo de Zorra**	Querença (Algarve)	1st week Fev 1st week Mar	Self-incompatible	23.4%	weak
	Gama Dura	Querença (Algarve)	3rd week Jan 4th week Fev	Self-incompatible	22.9%	strong
	Canhota	Espargal (Algarve)	1st week Fev 1st week Mar	Self-incompatible	24.1%	0%
	Quinta de Valim	CEAT (Algarve)	1st week Fev 1st week Mar	Self-incompatible	25.2%	0%

Adapted from AJAP, 2017; Costa and Rosa, 2020; Gerçekcioğlu and Atasever, 2020; Hernandorena, 2021.

1.3 Grafting

Natural grafting occurs when, under pressure, due to growth or physical restrictions, roots and stems of two different plants come in close contact. This mechanism may have served as the inspiration for grafting used as a horticultural technique for a long time to clonally propagate several commercially important fruit and vegetable crops (Gaut *et al*., 2019; Chilukamarri *et al*., 2021)

Through grafting, two parts, the scion (top plant part) and the rootstock (bottom plant part), from different plants are joined together and grow as a single individual. Scion and rootstock are closely related, usually belonging to the same species or genus (Melnyk, 2016; Sharma and Zheng, 2019).

This technique allows for higher production, fruit quality improvement, adaptation to edaphoclimatic conditions and biotic and abiotic stress tolerance. In fact, different rootstocks can confer distinct salinity and drought tolerances, influence water-use efficiencies, phenology, scion vigour and architecture, size of the tree and mineral element composition and use efficiency (Gautier *et al*., 2018). The mechanisms causing these graft-induced traits remain somewhat unknown but are thought to involve differences in production and transport of hormones or signalling molecules, and in water transport efficiency (Melnyk, 2016).

One of the most famous examples of grafting to promote biotic adaptation occurred with the European grapevine, *Vitis vinifera.* In the 19th century a soil welling insect pest, *Phylloxera*, was introduced to Europe from North America. This pest was devastating for the European vineyards. The solution was grafting European *Vitis* onto American *Vitis* species resistant to *Phylloxera* (Gautier *et al*., 2018).

Another form of grafting, termed *in vitro* grafting, was introduced in the late 90's. *In vitro* grafting is a micrografting technique that uses smaller sized scions and rootstocks, which can be micropropagated *in vitro*, maintaining aseptic conditions during grafting procedure, graft union formation and plant development. In comparison to other plant propagation methods, *in vitro* culture and micropropagation of plants can be used to maintain genetic uniformity, while producing and selecting high quality plants with better disease resistance and stress tolerance capacities at any time of the year (Hussain *et al.,*2012; Chilukamarri *et al*., 2021). Even though in field grafting a rootstock with a well-

established root system is required, in the case of *in vitro* grafting it is not necessary. After successful *in vitro* grafting, micrografts can be cultured on rooting medium for root initiation (Chilukamarri *et al*., 2021).

Ghorbel *et al.* (1998) reported the first *in vitro* grafting method in almond. In this study, apical buds from *in vitro* shoots and meristems from mature trees were grafted onto the same rootstock. Using apical buds from *in vitro* shoots resulted in higher grafting success than using meristems from mature trees.

The environmental issues of agrichemicals application for protection/propagation of field grown trees and the scarcity of natural resources, such as water and land, are strong incentives to use plant tissue culture-based micrografting techniques as large-scale propagation of high-quality fruit trees (Chilukamarri *et al*., 2021).

1.3.1 Graft union formation

After cutting, the scion's plant vasculature is brought in contact with the stock, and plant responses are rapidly activated (Fig. 4). Mechanical and hormonal signals such as, changes in transport dynamics or damaged cells detection may be critical for the initiation of graft formation (Melnyk, 2015). Severing the vascular tissues will presumably cause the accumulation of auxin and sugars above the cut site, and depletion of these substances below, since these substances are transported from growing leaves and photosynthetic sources, respectively, to the roots (Stitt, 1996; Friml and Palme, 2002; Melnyk, 2016).

Ruptured cells collapse and cell wall components, such as polysaccharides, are deposited at the cut site causing opposing tissues to adhere, and strengthening the graft junction (Pina *et al*., 2012). Then, cells at the cutting edges expand to fill the gap. The cambium is a vascular meristem that during secondary growth divides to produce new xylem and phloem cells. During grafting, when the cambia from the scion and the rootstock are in close contact, through cell division, a mass of undifferentiated parenchyma cells, the *callus*, is formed and fills the gaps to allow contact between opposing tissues (Aloni *et al*., 2010; Melnyk *et al*., 2015; Nanda and Melnyk, 2017; Gautier *et al*., 2019). Plasmodesmata, small membrane channels, develop providing membrane and cytosolic continuity between the adjoining cells of the graft. These channels have

been considered crucial to the success of grafts (Kollmann *et al.*, 1985; Gautier *et al.*, 2018). *Callus* will gradually differentiate into vascular cambium, and this secondary meristem will give rise to the vascular tissues, phloem and xylem. The content and nature of *callus* cells play an important role in triggering responses for a strong and successful union (Pina and Errea, 2005). Reconnection of vascular tissues is important for the transport of water, nutrients and signalling molecules as well as to provide physical strength to the graft union. Phloem is formed when vascular cambium-derived cells differentiate and enucleate being composed of living cells transporting nutrients and macromolecules. Xylem is formed when cambium cells differentiate and undergo programmed cell death to transport water and minerals (Hartmann *et al.*, 2002; Melnyk *et al.*, 2015). In *Arabidopsis thaliana*, tissues adhere 1-2 days after grafting, phloem connections form 3-4 days after grafting and xylem connections form 6-8 days after grafting, consistent with phloem forming before xylem in the primary vascular development of young organs (Melnyk *et al.*, 2015).

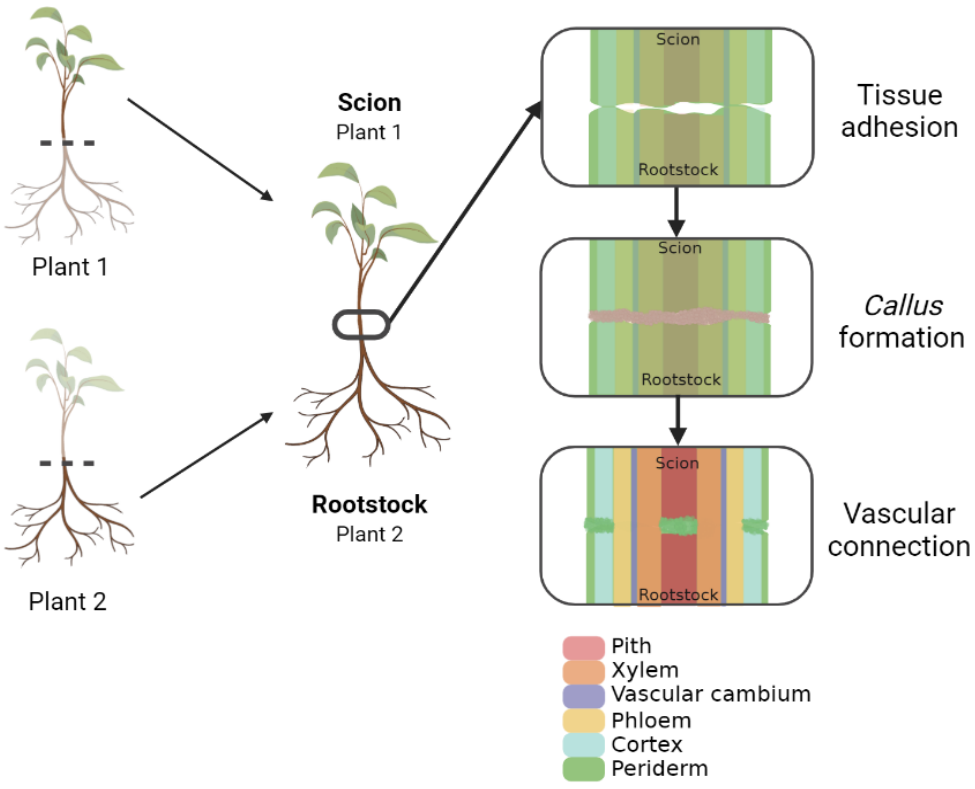

Figure 4. Schematic representation of grafting and graft union formation. After cutting the vasculature, tissues adhere and then through cell dedifferentiation and cell division the *callus* is formed. *Callus* will give rise to vascular cambium, and later on these cells originate the vascular tissues, phloem and xylem. This scheme represents a longitudinal cut section of young woody plant stem. Adapted from Hartmann *et al.*, (2014). Created in Biorender.com.

The juvenile microscions and microrootstocks used in *in vitro* grafting, promote a faster and more successful healing and formation of graft union than the often mature lignified scions and rootstocks used in grafting, due to the better capacity to divide and differentiate, and lower levels of graft-inhibiting toxins and secondary metabolites (Melnyk and Meyerowitz, 2015; Chilukamarri *et al*., 2021).

1.3.2 Long distance communication

Plant physiological responses require many different signalling compounds, some of which need to travel long distances (Chilukamarri *et al*., 2021). In the 1930s, it was discovered that grafting a flowering plant to a non-flowering plant could promote flowering in this last one due to the action of a mobile substance, named florigen, that travels from mature leaves into the shoot apex (ChaÏlakhyan, 1937). Only a few years ago, it was confirmed that part of that signal is the mobile protein FLOWERING LOCUS T (FT - Takada and Goto, 2003; Jaeger and Wigge, 2007). *In vitro* grafting was also used to study the long-distance signalling of FT protein in *Arabidopsis thaliana* (Yoo *et al*., 2013).

In grafting, molecular signals are transported mainly in the phloem, requiring the vascular tissues to be reconnected (Wang *et al*., 2016). Phloem sap profiling demonstrated that many RNAs and proteins are mobile, although not all mobile molecules have signalling functions (Thomas and Frank, 2019). In this way, future studies are required to unveil which mobile molecules may function as signals.

Some authors have reported the movement of nuclear, mitochondrial and choloroplast genomes across the graft junction (Stegemann *et al*., 2012; Fuentes *et al*., 2014). The determination of how molecules are transferred between scion and rootstock might reveal how grafting induces changes in plant traits (Thomas and Frank, 2019). For example, horizontal gene transfer is the asexual transfer of genetic materials from a donor organism to a recipient organism (Keeling and Palmer, 2008). During graft formation, plasmodesmata and the re-establishment of vascular bundles provide transport channels for horizontal gene transfer (Wang *et al*., 2016). Also, protein-coding messenger RNAs (mRNAs) can be transported and act as regulatory signals or produce functional proteins in targeted organs. RNA high-throughput sequencing showed that many transcripts were transferred across the graft union through the vasculature, and

through proteomic analysis in graft tissues, it was possible to conclude that mRNAs may be translated after transfer (Thieme *et al*., 2015). Additionally, it is reported that some RNA-biding proteins are capable of binding mRNAs as chaperones, facilitating transport from source to sink and protecting from degradation (Wang *et al*., 2016). Another potential mechanism of graft inducing traits is by means of epigenetic modifications, such as DNA methylation or structural changes to chromatin, that induce heritable variations in gene expression (Wang *et al*., 2016).

Due to the high speed of graft union formation, *in vitro* grafting is a useful tool recently used for studying the long-distance movement of proteins, hormones, RNAs and secondary metabolites (Melnyk and Meyerowitz, 2015; Chilukamarri *et al*., 2021).

1.3.3 Incompatibility

Grafting may not always be a successful process, leading to the formation of incompatible grafts. Generally, incompatibility increases with taxonomic distance, but plants from the same species or genus are usually compatible (Gautier *et al*., 2018). In *Prunus*, interspecific graft incompatibility has been widely reported (Pina *et al*., 2017).

There are two known types of graft incompatibility, short and long term. Short term incompatibility occurs soon after grafting when tissue attachment is weak or vascular strands fail to reconnect. The plant will survive only a few weeks or months. For most authors, the formation of vascular connections is considered the basic requirement for a successful graft since a failure to form vascular connections will almost always lead to graft failure (Pina and Errea, 2005; Melnyk, 2016). Long term incompatibility can emerge after many months or years, resulting in graft junction breaking or loss of scion vigour. This type of incompatibility might be the result of unobserved symptoms that have been progressing since shortly after grafting (Gautier *et al*., 2018).

The mechanisms associated to incompatibility are not yet clear, but macromolecules present in the phloem sap, such as, proteins, hormones and RNAs, are thought to be important for graft compatibility (Pina and Errea, 2005).

Aloni and co-workers (2008) indicated hormonal imbalances as the main cause for incompatibility in melon (*Cucurbita melo* L.) grafted onto *Cucurbita.* Incompatibility

might have resulted from auxin-induced oxidative stress in the rootstock. They proposed that in compatible grafts, an anti-oxidative mechanism may be activated, reducing oxidative stress in the root.

In vitro grafting can be used to evaluate incompatibility in early stages. In fact, Errea *et al.* (2001) used *in vitro* grafting and grafting to evaluate incompatibility in apricot (*Prunus armeniaca*). In both techniques the accumulation of phenolic compounds was higher in incompatible graft combinations and could be used to detect graft incompatibility at three weeks after grafting. Another study in *P. armeniaca*, using *in vitro* grafting, incompatible grafts showed delayed cambium formation and fewer connections through plasmodesmata (Pina *et al*., 2012).

1.4 Molecular regulation of grafting

Plant hormones, such as, auxins, cytokinins, ethylene, gibberellins and jasmonic acid, are important endogenous factors that regulate every aspect of plant development and responses to biotic and abiotic stresses. Cytokinins are adenine-derived phytohormones involved in cell division, lateral root formation and meristem maintenance (Nanda and Melnyk, 2017). Auxins are plant growth regulators involved in physiological mechanisms, such as, tropisms, apical dominance and embryo and fruit development (Kepinski and Leyser, 2005).

Hormones in plants are usually regulated by feedback loops. A feedback loop between the most abundant auxin, indole-3-acetic acid (IAA), and cytokinins has been described (Jones and Ljung, 2011). In this concept, a decrease in IAA produced in the shoot apex and translocated to the root, stimulates the synthesis and export of cytokinins from the root to the shoot. The increase of cytokinins in the xylem sap would increase the synthesis and translocation of IAA from the shoot apex that will reduce cytokinin levels (Bangerth, 1994).

During grafting, hormonal signalling is involved in graft union formation, scion-rootstock communication and plant growth and development (Aloni *et al*., 2010). In *Prunus*, non-grafted plants show a balance between auxin and cytokinin, however, in grafted plants this balance is disrupted, inducing a higher growth rate in the scion,

possibly due to an increase of cytokinins and decrease of IAA in the shoot (Source *et al*., 2002).

After cutting the plant, the accumulation of cytokinin in the rootstock, and auxin in the scion may play important roles for activating genes associated with wound response and vascular formation during grafting, creating an asymmetry in gene expression (Melnyk and Meyerowitz, 2015).

In the presence of auxins, cytokinins stimulate early stages of vascular differentiation when cell divisions occur in the differentiating tissue, but later on, vascular differentiation occurs in the absence of cytokinin. Cytokinin increases the sensitivity of tissues to auxin stimulation (Aloni, 1995). These two hormones are involved in vascular formation during root development and during the differentiation of vasculature from *callus* (Melnyk and Meyerowitz, 2015). Since these molecules have been proved to be crucial for graft success further studies will provide insights into their role in grafting and scion-rootstock communication.

1.4.1 Factors controlling wound response

Grafting can be considered a modified form of wound healing and *callus* formed at the graft junction might be similar to wound-induced *callus* (Melnyk and Meyerowitz, 2015). *Callus* formation at the graft union is not an absolute requirement for a successful graft and it can occur in both compatible and incompatible grafts since it is a common response to wounding. Even though it is important for many plants, it might be species specific (Moore and Walker, 1981a, b; Melnyk, 2016).

During cellular dedifferentiation, adult somatic cells lose their identity and regain cellular proliferative competence. In *Arabidopsis* cut hypocotyls, the transcription factor *WOUND INDUCED DEDIFFERENTIATION 1 (WIND1)* is activated around the wound and its expression enhances endogenous cytokinin responses that play an important role in graft union growth and development by stimulating cell dedifferentiation and cell division to form wound-induced *callus*. This gene was found to be constitutively expressed in *callus* cells after wounding. WIND1 is a putative APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) family transcription factor (Iwase *et al*., 2011; Melnyk *et al*., 2015). The AP2/ERF family of transcription factors are key regulators in different regulatory

processes, integrating responses to multiple *stimuli* and to several hormones, including in stress responses (Xi *et al*., 2019).

In *Arabidopsis* grafted hypocotyls, *WIND1* is strongly upregulated above the graft junction and later below. The response in grafted rootstocks was promoted by the presence of the scion, since cut but ungrafted rootstocks did not show a *WIND1* response (Melnyk *et al*., 2015).

1.4.2 Role of auxins in grafting

Auxins were the first plant hormones discovered and the group of plant hormones most studied (Masuda and Kamikasa, 2000). This family of plant growth regulators includes natural active auxins, natural inactive auxin precursors, and natural auxin storage forms, such as, indole-3-butyric acid (IBA). Non-endogenous compounds displaying an auxin-like activity are also common, such as 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA) and 4-amino-3,5,6-trichloropicolinic acid (picloram) (Korasick *et al*., 2013).

There are two forms of the predominant auxin, IAA, free and conjugated (Ludwig-Muller, 2011). Free IAA is the active form of auxin, and conjugated IAA is a storage form or an intermediate destined for degradation. Free IAA can be produced through *de novo* biosynthesis or be released from IAA conjugates such as IAA esters, IAA-sugar and IAA-amino acid conjugates by hydrolysis (Jiang *et al*., 2017).

IAA is mainly *de novo* synthesized from the amino acid tryptophan (Trp) by a two-step pathway highly conserved in plants (Fig. 5) that plays an essential role in major developmental processes, such as embryogenesis, seedling growth, root elongation, vascular patterning, gravitropism or flower development. The first step is the removal of the amino group from Trp by the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family of transaminases to generate indole-3-pyruvate (IPA). IPA undergoes oxidative decarboxylation catalyzed by the YUCCA (YUC) family of flavin monooxygenases to produce IAA (Zhao, 2012). However, IAA can also be synthesized in a Trp-independent pathway (Zhao, 2010).

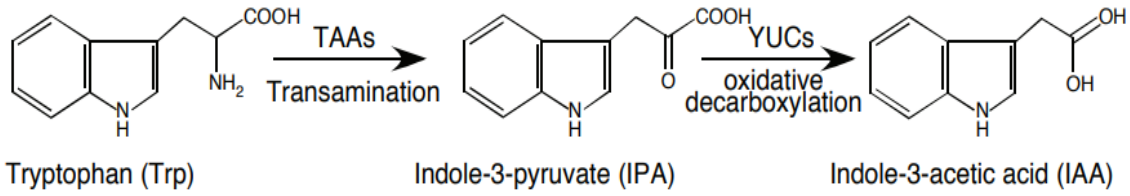

Figure 5. Tryptophan-dependent auxin biosynthesis pathway in plants (Zhao, 2012).

Auxin can be transported in the vasculature through the phloem or by polar transport. Regarding polar transport (Fig. 6), auxin enters the cell by passive diffusion in its protonated form or through the action of an uptake carrier, the AUXIN TRANSPORTER PROTEIN 1/LIKE-AUXIN TRANSPORTER PROTEIN 1 (AUX1/LAX) family of transmembrane proteins. Once it has entered the cell, in exposure to the cytoplasmatic pH, auxin dissociates and requires active transport through the PIN-FORMED (PIN) or ATP-binding cassette class B (ABCB) efflux transporter proteins to exit the cell (Leyeser, 1999; Petrasek and Friml, 2009).

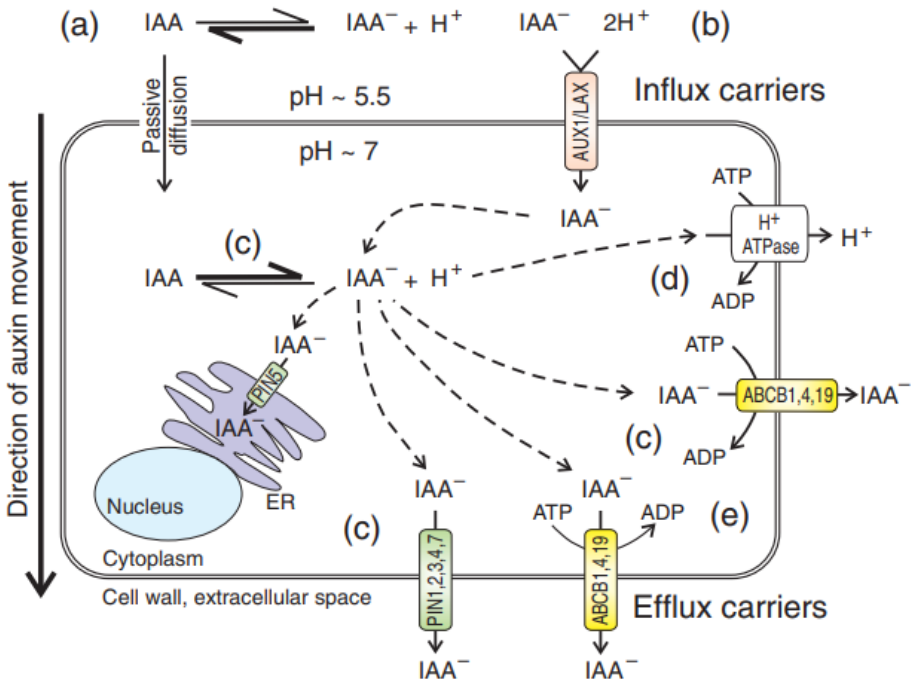

Figure 6. Auxin transport across the plasma membrane. Undissociated IAA enters the cell by passive diffusion (a). Dissociated auxin anions, IAA^-, are transported via AUX1/LAX influx carriers (b). To exit the cell, IAA^-, requires active transport through the PIN or ABCB efflux transporter proteins (Petrasek and Friml, 2009).

Auxin influence on plant growth and development results from the capacity to regulate the differential expression of many genes including the ones coding for the carrier proteins (PIN, ABCB and AUX1/LAX) (Dharmasiri *et al*., 2005). Transcriptional control results from the auxin-enhanced and ubiquitin mediated degradation of a family of transcriptional repression proteins, the AUXIN/INDOLE-3-ACETIC ACID (Aux/IAAs) (Fig. 7) (Woodward and Bartel, 2005). The Aux/IAAs family members, such as the INDOLE-3-ACETIC ACID INDUCIBLE 26 (IAA26) have been identified as short-lived nuclear proteins playing a crucial role in repressing the expression of auxin-responsive genes by binding to auxin response factors (ARFs; Dreher *et al*., 2006). When auxin levels are low, Aux/IAAs are capable of gene repression, but when auxin levels rise, these proteins are ubiquitinated by interacting with TRANSPORT INHIBITOR RESPONSE 1 /AUXIN SIGNALLING F-BOX (TIR1/AFB) receptors and subsequently degraded via the 26S proteasome allowing derepression of ARFs and auxin-inducible gene expression. The TIR1/AFB nuclear auxin receptor is a subunit of the SCF E3-ligase protein complex responsible for ubiquitylation and degradation (Kepinski and Leyser, 2005; Kepinski, 2007; Luo *et al*., 2018).

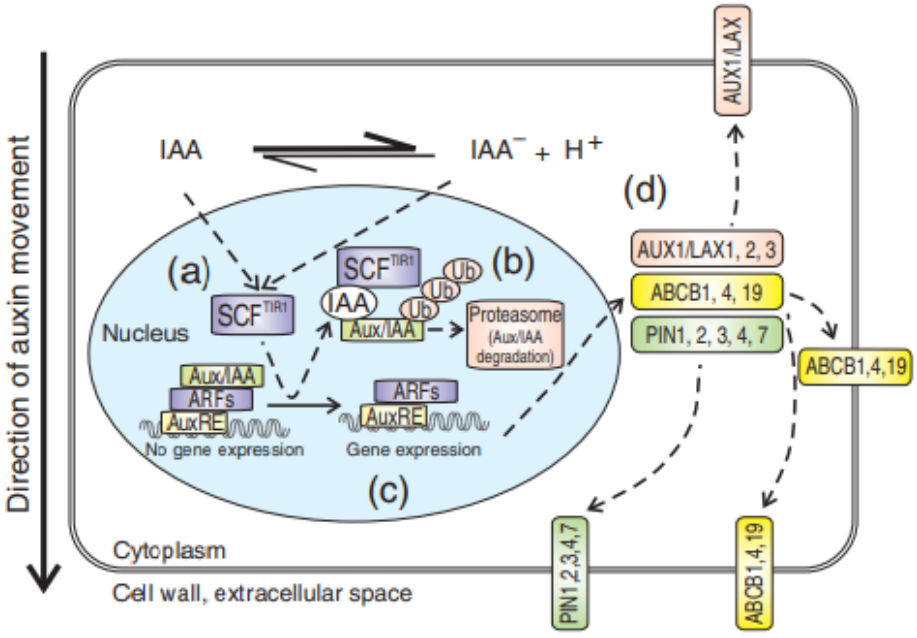

Figure 7. Auxin regulated gene expression. Intracellular auxin binds to the receptor TIR1/AFB (a) leading to the ubiquitylation and proteasome degradation of Aux/IAA transcriptional repressors (b). ARFs can later activate auxin-responsive gene expression, such as the auxin transporters (d) (Petrasek and Friml, 2009).

Auxin is involved in wound response, being transported to the wound site, and triggering vascular tissue regeneration (Mazur *et al*., 2016). This hormone has also been reported to be involved in the development of successful graft unions, inducing the regeneration and connection of vascular tissues (Aloni, 1987). Incised *Arabidopsis* stems showed an asymmetric auxin accumulation due to a block in basipetal auxin transport (Asahina *et al*., 2011). Auxin accumulation can promote the differentiation of *callus* cells into xylem and phloem (Wetmore and Rier, 1963). The reconnection of phloem tissue is controlled by auxin-signalling genes, like the *ABERRANT LATERAL ROOT FORMATION 4* (*ALF4*). This gene is expressed throughout the plant but plays a crucial role in the *callus* and in lateral root formation (DiDonato *et al*., 2004; Sharma and Zheng, 2019). Melnyk *et al*. (2015) recently revealed the requisition of *AUXIN RESISTANT 1* (*AXR 1*) and *ALF4* close to graft junction but only in the tissue below for phloem reconnection, indicating that an asymmetry in auxin response occurs at the graft junction. They also proposed that ALF4 acts in the rootstock to promote transport and perception of scion-derived auxin, which drives vascular formation from the rootstock.

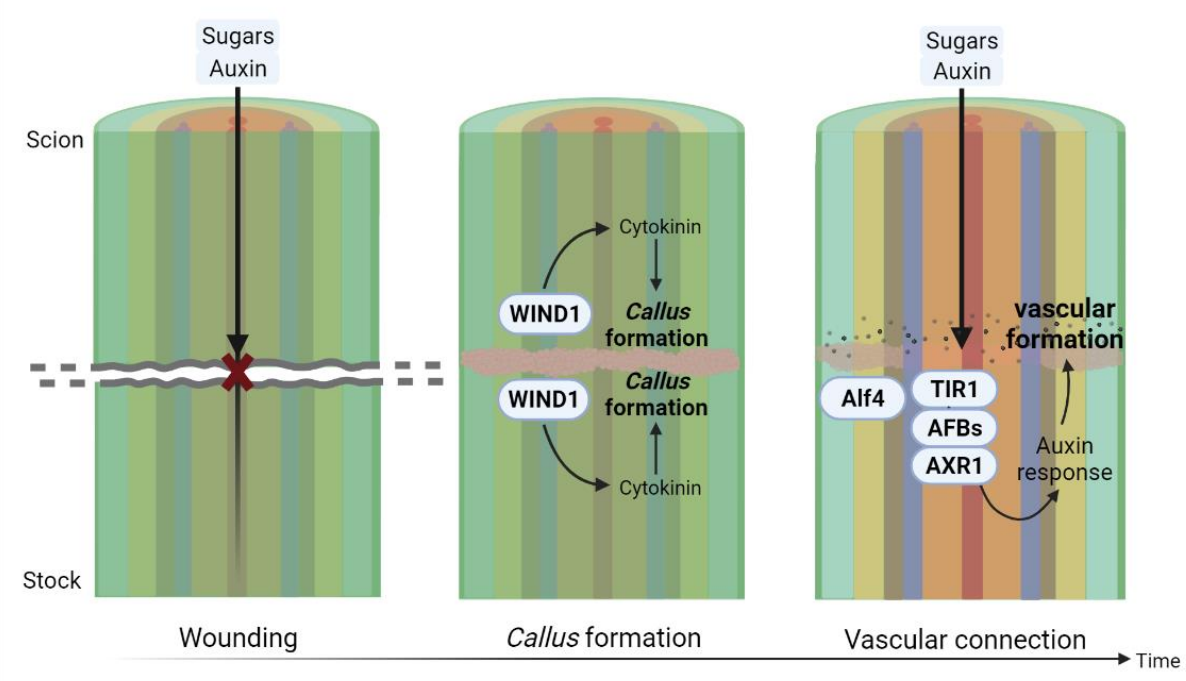

Figure 8. **Schematic diagram of the putative grafting process in *Arabidopsis* hypocotyls**. Upon wounding, WIND1 enhances cytokinin response at the graft junction which induces *callus* formation. At the same time, auxin basipetal transport is affected and auxin accumulates above the graft junction. Below the graft junction, auxin response proteins TIR1, AFBs, AXR1 and ALF4 perceive auxin to promote vascular connection. Adapted from Melnyk (2015). Created in Biorender.com.

Shortly after grafting an asymmetry in gene expression and cell division is promoted. However, the responses lose asymmetry over time and became similar on both sides of the graft junction (Melnyk *et al*., 2015).

Although grafting is widely used, and graft formation comprehensively understood, the specific molecular mechanisms between scion and rootstock that lead to a successful graft union are not yet well understood and most of the knowledge available is from studies in the model species *Arabidopsis thaliana* (Fig. 8), remaining the question to whether this information can be transferred to commercial species such as *Prunus* (Melnyk., 2016; Chilukamarri *et al*., 2021).

1.5 Objectives

In a context where the management of natural resources is essential, and due to the significant almond culture increase in Portugal, there is a need to find sustainable options for almond scions and rootstocks. Conservation and characterization of Portuguese germplasm is fundamental to find solutions for future problems, since they are the result of years of culture and selection of resilient individuals with better adaptation to edaphoclimatic conditions.

Regardless of the relevance of this culture, and of the importance of the selection of the most adequate scions and rootstocks, there is still a lack of information regarding micrografting techniques and scion/rootstock interactions for this species. Thus, the general aim of this work is to understand the scion/rootstock molecular communication regarding wound healing and auxin-induced regulatory mechanisms in almond micrografts.

To achieve the main aim, several objectives for this work are established:

- To achieve the *in vitro* establishment and multiplication of scions and rootstocks;
- To promote rooting of rootstocks;
- To establish different micrograft combinations of scion x rootstock;
- To perform IAA quantification and immunolocalization during micrograft establishment and development;

- To access gene expression quantification of *WIND1*, *ALF4*, *TIR1* and *IAA26* before and after micrografting;
- To access micrograft success rates and correlate with auxin analysis and gene expression quantification.

The results obtained here are a step forward to the understanding of scion and rootstock communication in different almond micrograft combinations and how their communication is associated with graft success.

2. Materials and methods

2.1 Plant material and fruit characterization

Seeds from the Portuguese traditional varieties harvested in September 2020 (Canhota, Gama Dura, Quinta de Valim and Rabo de Zorra), and from the commercial variety Ferraduel were obtained from selected trees and provided by the Centro de Experimentação Agrária de Tavira (CEAT). Commercial varieties (Antoñeta, Lauranne and Soleta) were provided by local producers from Ferreira do Alentejo. Regarding the rootstocks, seeds of bitter almond were provided by the Associação de Viveiristas do Distrito de Coimbra (AVDC) and young plants of Rootpac® were obtained from Agromillora Iberia (Barcelona, Spain) and grown at AVDC. *In vitro* established shoots from the commercial rootstock GxN15 were provided by the company Quality Plant (Coimbra, Portugal).

Morphological characterization of fruits collected from Portuguese traditional varieties was performed. The phenotypic traits evaluated from in-shell fruits were the following: weight, length, width, thickness, almond shape, apex shape and toughness. Almond kernels were also characterized, being recorded: weight, length, width, thickness, apex shape, double kernels (%) and dry kernels (%). For each variety, partition yield was calculated according to the following equation:

$$Partition\ yield\ (\%) = \frac{Kernel\ weight\ (g)}{Kernel\ with\ shell\ weight\ (g)}\ x\ 100\%$$

2.2 In vitro establishment of scions and rootstocks

2.2.1 Seed disinfection

Seeds were removed from the shell and washed with distilled water containing 2-3 drops of detergent for 10 minutes. Then, plant material was disinfected with ethanol 70% (v/v) for 30 seconds, followed by immersion in a solution (1 g/l w/v) of the fungicide Derosal (Bayer) for 20 minutes, ethanol 70% (v/v) for 1 minute, and commercial bleach solution (3.75% active chlorine) for 20 minutes. All steps were performed under stirring. Under aseptic conditions seeds were washed three times with sterile distilled water and left 24h for soaking.

2.2.2 Zygotic embryo germination conditions

The effect of low temperature (4 °C) on germination was evaluated using, commercial almond varieties Antoñeta, Lauranne and Soleta, keeping dry seeds (n=15 seeds/variety) at 4 °C for 3 weeks. As control another group was also kept at room temperature for 3 weeks (Table 2). After seed disinfection, the embryo axis was isolated in aseptic conditions and placed on Petri dishes with MS (Murashige and Skoog, 1962) medium, supplemented with 3% (w/v) of sucrose and 0.25% (w/v) of gelrite, and the pH adjusted to 5.7 with KOH and HCl before autoclaving. In each condition, 3 seeds in 5 replicates were used. Cultures were kept in a growth chamber, at 25 °C, in a 16h light/ 8h dark photoperiod for 4-5 weeks.

In addition, the effect of plant growth regulators (PGR) and explant type on germination was evaluated with the commercial variety Lauranne, using the germination conditions described in Table 2. After seed disinfection the embryo axis or the whole mature zygotic embryo were isolated in aseptic conditions. Embryo axes were placed on Petri dishes with MS medium supplemented with 3% (w/v) of sucrose, 1 mg/l gibberellic acid (GA_3), 0.5 mg/l 6-benzylaminopurine (BAP) and 0.25% (w/v) of gelrite and the pH adjusted to 5.7 before autoclaving. The whole mature zygotic embryos were placed in Petri dishes with filter paper moistened with a solution of 1 mg/l GA_3. In each condition, 3 seeds in 5 replicates were used. Cultures were kept in a growth chamber, at 25 °C, in a 16h light/ 8h dark photoperiod for 4-5 weeks. After that period, germination rate, seedling length, primary root length and the number of secondary roots in the tested varieties was registered.

Table 2. Established conditions for optimization of zygotic embryo development.

	Seed storage temperature	**Explant type**	**Culture conditions**	**Varieties**
Condition 1	Room temperature	Embryo axis	MS medium + 3% w/v sucrose	Antoñeta, Lauranne, Soleta
Condition 2	4 °C	Embryo axis	MS medium + 3% w/v sucrose	Antoñeta, Lauranne, Soleta
Condition 3	Room temperature	Embryo axis	MS medium + 3% w/v sucrose + 1 mg/l GA_3 + 0.5 mg/l BAP	Lauranne
Condition 4	Room temperature	Whole mature zygotic embryo	Filter paper moistened 1 mg/l GA_3	Lauranne

2.2.3 *In vitro* establishment from zygotic embryos

Bitter almond, Portuguese traditional varieties and Ferraduel were established *in vitro* from germination of mature zygotic embryos in condition 3 (Table 2). Seeds were removed from the shell and disinfected as previously described. After soaking, seeds were immersed in commercial bleach solution (3.75% active chlorine) for 20 minutes under stirring and washed three times with sterile distilled water in aseptic conditions.

The embryo axis was isolated and placed on MS medium supplemented with 3% (w/v) of sucrose, 1 mg/l GA_3, 0.5 mg/l BAP and 0.7% (w/v) of agar with pH adjusted to 5.7 before autoclaving. Establishment of bitter almond was achieved in test tubes (15 cm x Ø 22 mm) and for Portuguese traditional varieties and Ferraduel Petri dishes were used. For each variety, 3 seeds in 5 replicates were used. Cultures were kept in a growth chamber, at 25 °C, in a 16h light/ 8h dark photoperiod for 4-5 weeks, and the abovementioned parameters registered.

2.2.4 *In vitro* establishment and multiplication of the rootstock Rootpac®

From 10 young plants of the commercial rootstock Rootpac®, young branches without leaves were used for *in vitro* establishment. Disinfection procedure started by washing the plant material with 2-3 drops of soap under running water. Cut segments of 1.5-2 cm with at least one axillar or apical meristem were immerged and stirred in distilled water with 2-3 drops of Tween 20 for 10 minutes, followed by 0.1% (w/v) fungicide solution (Mancozan, Bayer) for 20 minutes, ethanol 70% (v/v) for 30 seconds, distilled water for 30 seconds and 5% (w/v) calcium hypochlorite for 15 minutes. Under aseptic conditions, segments were washed three times with sterile distilled water.

Small nodal segments (0.5-1 cm), with an axillary or apical meristem, were isolated and placed on test tubes (15 cm x Ø 22 mm) with MS medium supplemented with 3% (w/v) of sucrose, 0.5 mg/l BAP, 50 mg/l citric acid and 0.7% (w/v) of agar with pH adjusted to 5.7 before autoclaving. Segments were dissected from 8 levels (n=10) according to apical distance, to evaluate apical distance influence on shoot development (Fig. 9), Subcultures were made every month for 3 months. In the 4th month, BAP and

citric acid were substituted for 1 mg/l indole-3-butyric acid (IBA). Cultures were kept in a growth chamber, at 25 °C, in a 16h light/ 8h dark photoperiod.

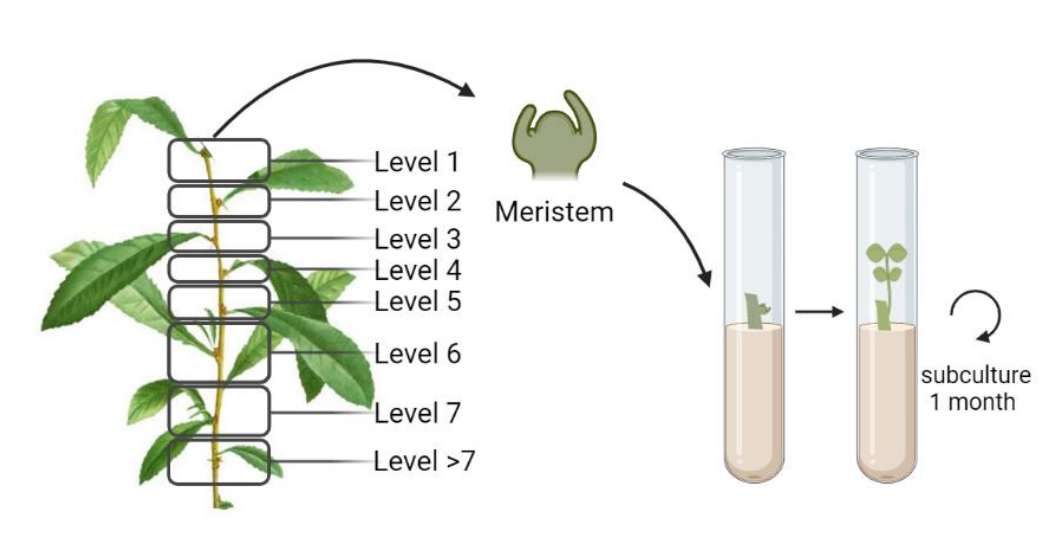

Figure 9. Schematic representation of meristem culture according to apical distance, shoot development and subculture every month. Created in Biorender.com.

2.3 Multiplication

2.3.1 Evaluation of culture media and genotype influence on multiplication

In vitro established bitter almond seedlings were used to evaluate different multiplication media. In sterilized conditions, shoots were cut in various segments with at least one axillar or apical meristem and placed on three different multiplication media previously described (Table 3, Fig. 10). M1 was used as control. All media were supplemented with 3% (w/v) of sucrose, 0.7% (w/v) of agar and the respective PGRs, with pH adjusted to 5.7 before autoclaving. Cultures were kept in the same culture conditions described before. Multiplication began with 6 to 8 genotypes in each medium and genotype influence was evaluated for multiplication on M2. Multiplication was carried out in 3 cycles (T1, T2 and T3) of 1 1 month each, and the number of phytomers was recorded to calculate the multiplication rate according to the following equation (Lopes, 2019):

$$\text{Multiplication rate (\%)} = \frac{Nº\ of\ phytomers\ (t2) - Nº\ of\ phytomers\ (t0)}{Nº\ of\ phytomers\ (t2)} \times 100$$

Table 3. Multiplication media composition

Multiplication media	Composition
M1	MS
M2	MS + 1 mg/l BAP [a]
M3	MS + 1 mg/l BAP + 0.1 mg/l IBA + 0.1 mg/l GA_3 [b]
M4	MS + 1 mg/l BAP + 0.5 IAA [a]

Note: [a] Işikalan *et al.,* 2008; [b] Sánchez *et al*., 2018.

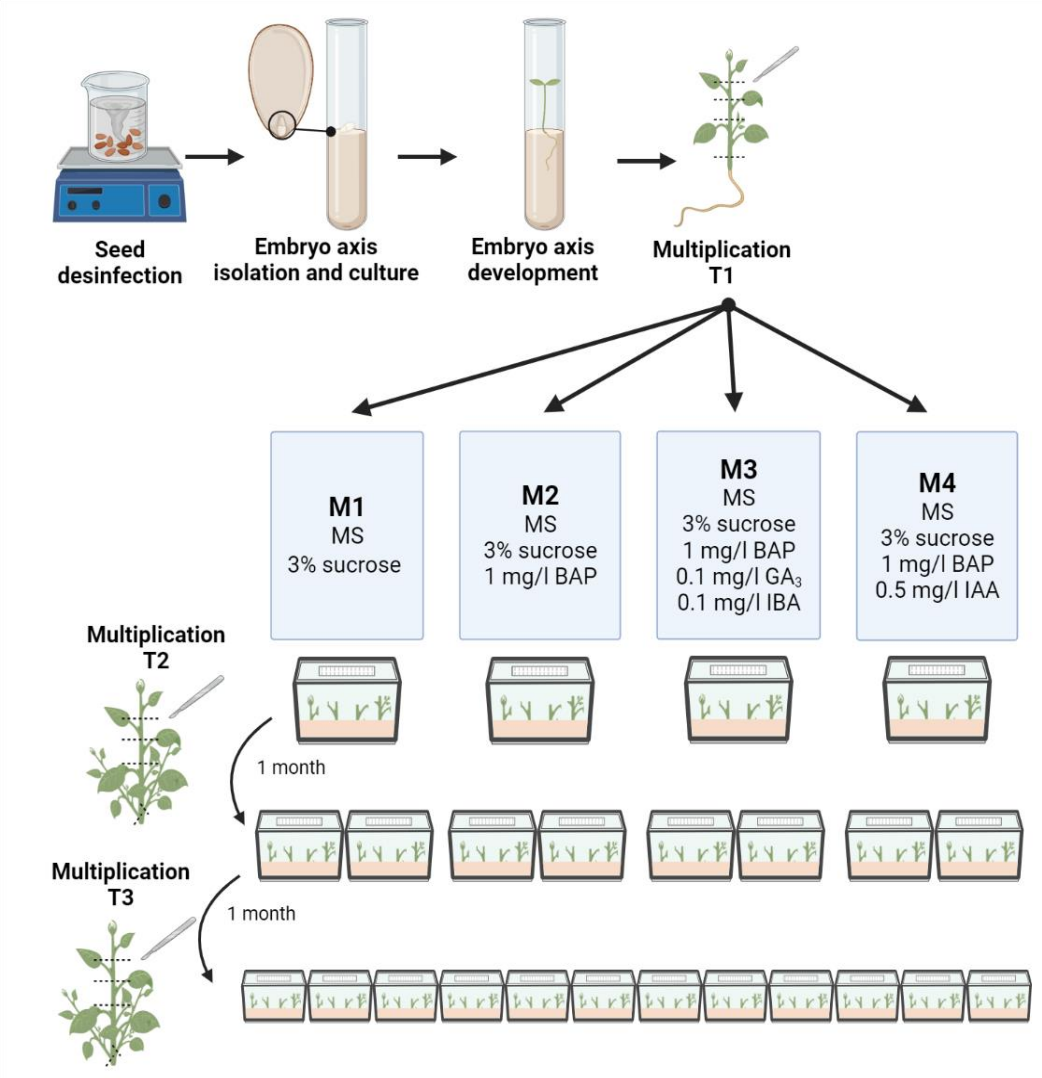

Figure 10. Schematic representation of seed disinfection, embryo axis isolation, culture and development, and multiplication stages for evaluation of multiplication media using bitter almond shoots. Created in Biorender.com.

2.3.2 Shoot multiplication of seedlings and GxN15

To increase the number of shoots of each established variety, in sterilized conditions, seedlings with 1 month and 0.4–4.5 cm were cut in various segments with at least one axillar or apical meristem and placed on MS medium supplemented with 3% (w/v) sucrose, 1 mg/l BAP and 0.7% (w/v) agar and the pH adjusted to 5.7 before autoclaving. Multiplication was carried out in 3 cycles, of 1 month each, with at least 15 genotypes for each variety (Fig. 11), and the number of phytomers was recorded to calculate the multiplication rate according to the abovementioned equation described by Lopes (2019). After multiplication, bitter almond, commercial and traditional varieties were subcultured on MS medium supplemented with 3% (w/v) sucrose, 0.1 mg/l BAP and 0.7% (w/v) agar and the pH adjusted to 5.7 before autoclaving. Cultures were kept in the same conditions described before.

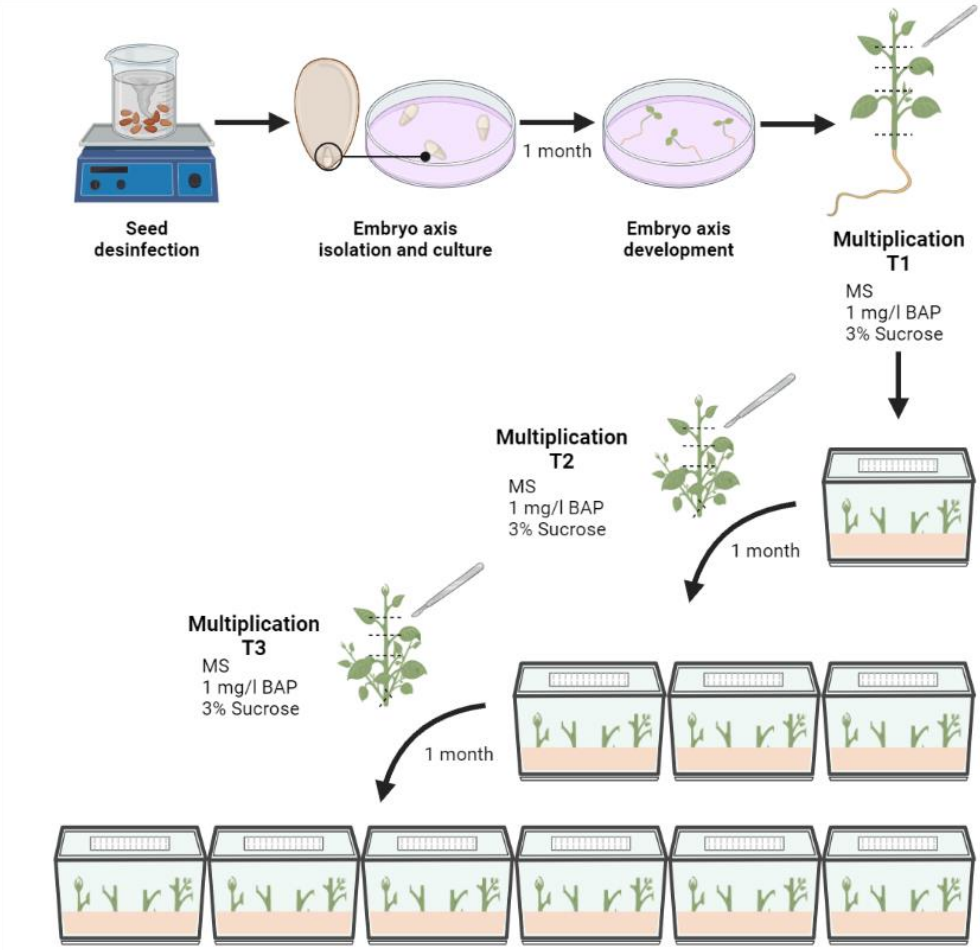

Figure 11. Schematic representation of seed disinfection, embryo axis isolation, culture and development, and multiplication stages for commercial and Portuguese traditional almond varieties. Created in Biorender.com.

For multiplication of GxN15 shoots (donated by Quality Plant, Coimbra, Portugal) the same multiplication procedure was applied, using segments with at least one axillar or apical meristem that were placed on the optimized multiplication medium indicated by the provider. Cultures were kept in the same conditions described before and subcultured every month.

2.4 Rooting

2.4.1 *In vitro* rooting

Since rootstocks provide the radicular system for the grafted plant, it was necessary to induce rooting in the rootstocks of bitter almond and GxN15. In a first assay, rooting was evaluated with shoots of bitter almond obtained from the different multiplication media (Fig. 12). To test the effect of IBA, activated charcoal (AC) and ethylenediamine di-2-hydroxyphenyl acetate ferric (Fe-EDDHA), after removing the *callus* formed at the base, shoots were placed on test tubes (15 cm x Ø 22 mm) with rooting medium supplemented with the different combinations of Fe-EDTA/Fe-EDDHA, AC and IBA (Table 4, Rooting media 2-4), 3% (w/v) sucrose and 0.7% (w/v) agar and the pH adjusted to 5.7 before autoclaving.

Table 4. *In vitro* rooting media composition.

Rooting media	Composition
R1	MS
R2	MS + 3% w/v sucrose + 36.7 mg/l Fe-EDTA + 1 mg/l IBA
R3	MS + 3% w/v sucrose + 36.7 mg/l Fe-EDTA + 1 mg/l IBA + 0.1 g/l AC [a]
R4	Modified MS + 3% w/v sucrose + 280.6 mg/l Fe-EDDHA + 1 mg/l IBA [b]
R5	MS + 3% w/v sucrose + 36.7 mg/l Fe-EDTA + 1 mg/l IBA + 1 g/l AC [a]

Note: [a] Thomas, 2008; [b] Antonopoulou *et al*., 2007.

The medium supplemented with AC had to be shaken and rapidly solidified after autoclaving for uniform distribution of AC particles. For each rooting medium 32 shoots

were used (8 shoots/multiplication medium). Rooting was evaluated after 3 weeks in culture in the same conditions described before.

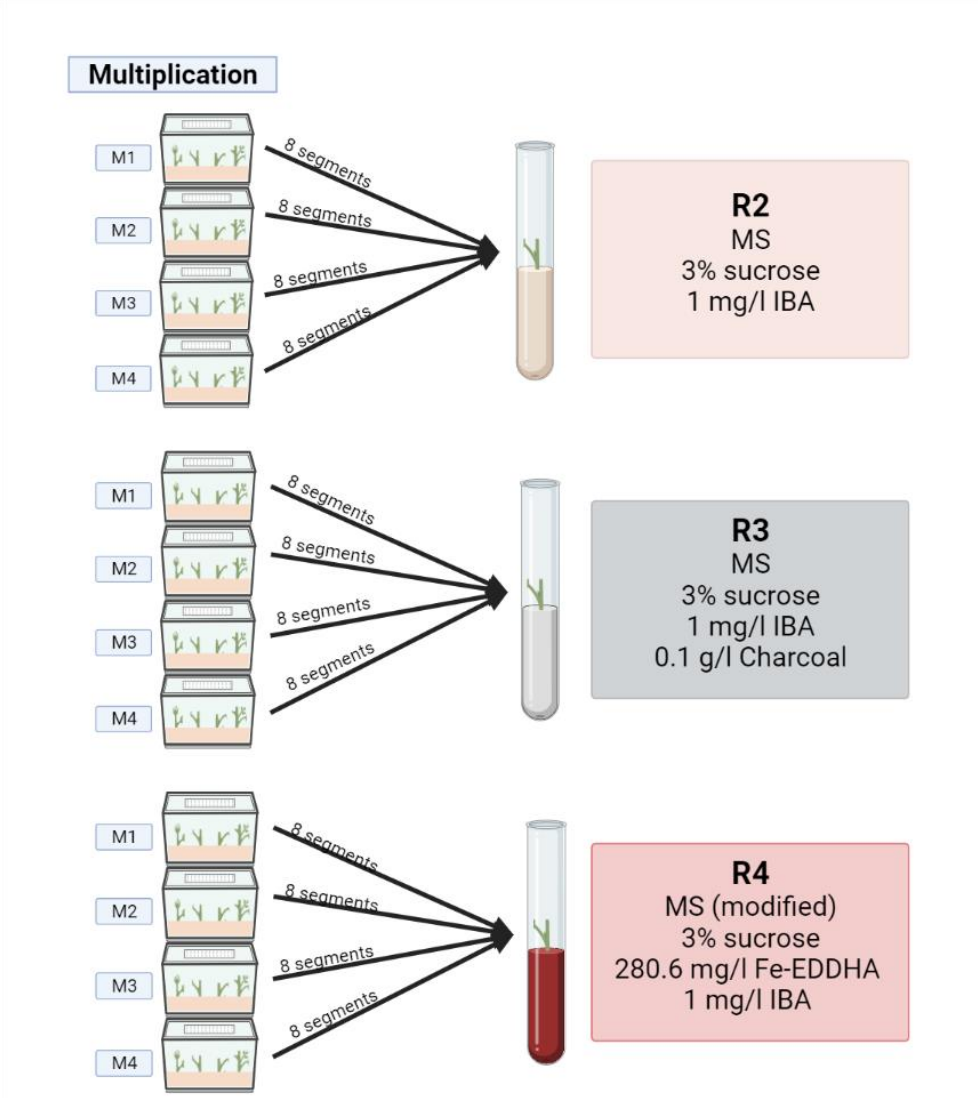

Figure 12. Schematic representation of the first *in vitro* rooting assay. Created in Biorender.com.

A second *in vitro* rooting assay was established (Fig. 13). Shoots from bitter almond in multiplication media 2 and shoots from GxN15 were used. *Callus* was removed from the base and shoots from bitter almond were place on rooting medium 1 and 5 (Table 4), and shoots from GxN15 were placed on rooting medium 5 (Table 4). Plastic containers (125 x 65 x 80 mm Combiness box, with white filters) were used in the rooting assays. Both media were supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar and the pH adjusted to 5.7 before autoclaving. In each rooting medium, 8 shoots

in 3 replicates were cultured. Rooting was evaluated after 6 weeks in culture in the same conditions described before.

2.4.2 *Ex vitro* rooting

Upon the unsuccess of *in vitro* rooting, shoots from the second *in vitro* rooting assay were used to test three *ex vitro* rooting conditions (Fig. 13): (1) direct acclimatization with no treatment, (2) dipping the base of the shoot in a 1 g/l IBA solution for15 minutes and (3) the use of commercial rooting powder (AA powder 0.5%, Rhizopon®). Direct acclimatization was used as control. After each treatment, 3 to 9 shoots were placed on moistened peat:perlite (2:1) substract. This assay was performed on a 35 cm x 22 cm tray with 77 wells covered with a lid that allowed high humidity in the first days. The lid was removed gradually for simultaneous acclimatization. The board was kept in a climatic chamber (FitoClima 10000 HP, Aralab, Portugal) at 25 °C, in a 16h light/ 8h dark photoperiod with a light intensity of 250 µmol $m^{-2}s^{-1}$. Rooting and survival of the plants was evaluated after 1 month.

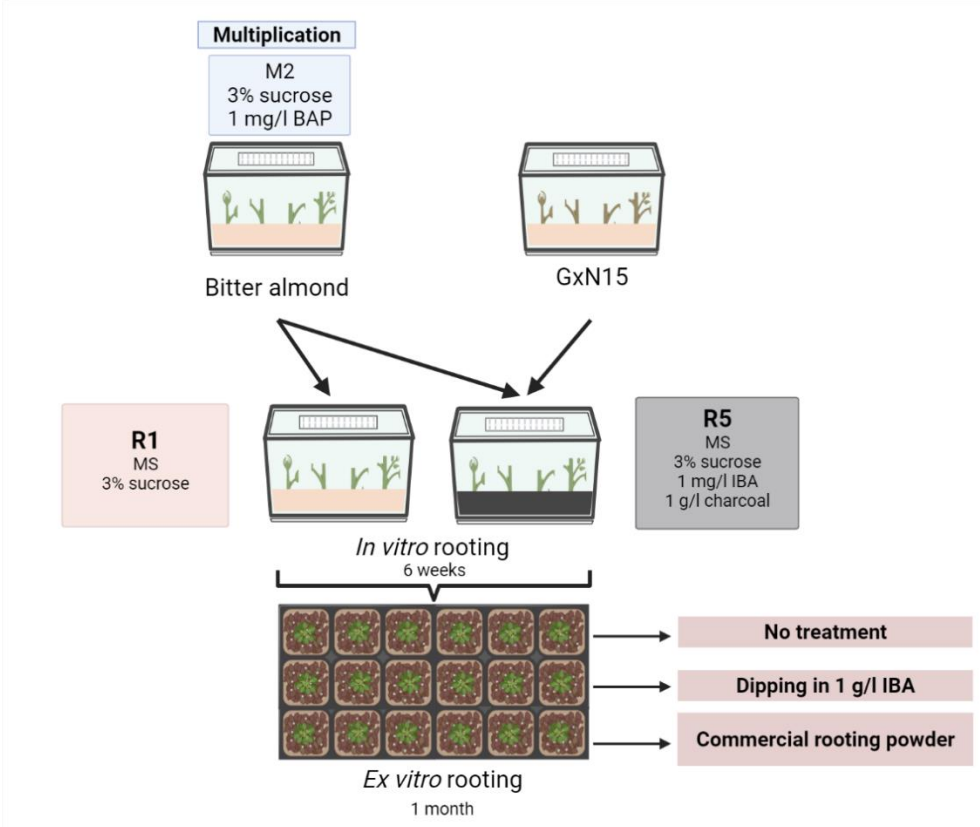

Figure 13. Schematic representation of the second *in vitro* rooting assay and *ex vitro* rooting assay using bitter almond and GxN15. Created in Biorender.com.

2.5 Micrografting and sample collection

2.5.1 Micrografting technique

Micrografting was carried out in sterile conditions in a laminar flow chamber using tweezers and scalpels to manipulate the plant material in a Petri dish. For scion, the top part of the plant, about 1 cm of segment was used in the procedure. The base of the scion was cut in a 'v' shape. For the stock, the upper part of the shoot was removed and in a segment with approximately 1 cm, a slit cut was made. Then, the scion was inserted into the stock in a way so that the micrograft was as vertical and stable as possible (Fig. 14). The micrograft was then placed on culture media and cultures were kept in a growth chamber, at 25 °C, in a 16h light/ 8h dark.

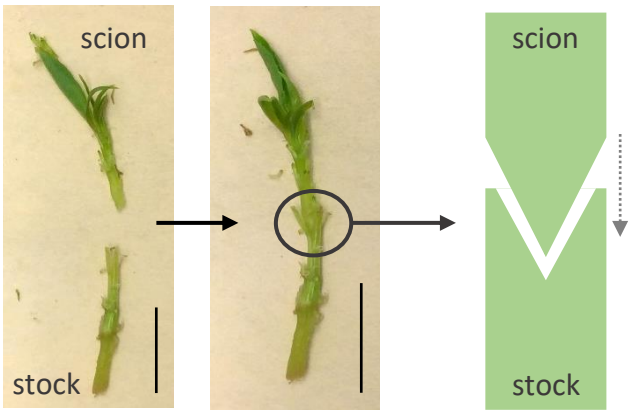

Figure 14. Micrografting method. Scale bar - 1 cm.

2.5.2 Accessing the scion genotype influence on micrografting success rate

Using the varieties Canhota, Ferraduel and Soleta as scions, micrografts were established with bitter almond and GxN15 as stocks. Varieties grafted onto bitter almond were placed on MS, supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar and the pH adjusted to 5.7 before autoclaving. Varieties grafted onto GxN15 were placed on the medium used for their multiplication. For each variety 2-3 genotypes were tested with 9 micrografts each, placed on plastic containers (125 x 65 x 80 mm

Combiness box with white filters). Micrograft success and lateral shoot formation was evaluated at 21 days after grafting.

Micrografts were then transferred to *ex vitro* conditions to promote acclimatization and rooting. Lateral shoots from the stock were removed. Rooting was promoted by dipping the base of the stock on 1 g/l IBA solution for 2 minutes. Micrografts were placed on the same conditions previously described for *ex vitro* rooting. Survival and rooting were evaluated after 15 days.

2.5.3 Establishment of micrografts for sample collection

For sample collection for subsequent analysis, Canhota, Ferraduel and Soleta were grafted onto bitter almond and GxN15. Bitter almond homografts were also established. Due to low availability of large quantities of shoots from each genotype a pool of genotypes was used in each variety. Micrografts were placed on the same type of Combiness boxes previously used, with MS medium supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar and the pH adjusted to 5.7 before autoclaving. For each combination, $16<n<20$ micrografts were established. Micrografting success was evaluated at 0, 3, 7, 10, 13, 17 and 21 days after grafting.

Samples were collected at 0 (T0), 7 (T1) and 21 (T2) days after grafting (Fig. 15). Samples collected at T0 correspond to cut but ungrafted scion and stock. At T1 and T2, micrografts were segmented in scion, union and stock. From each segment fresh weight was recorded, samples were fast frozen in liquid nitrogen and stored at – 80 °C until use. Bitter almond homografts and Canhota x bitter almond were used for IAA quantification and immunolocalization, and gene expression analysis.

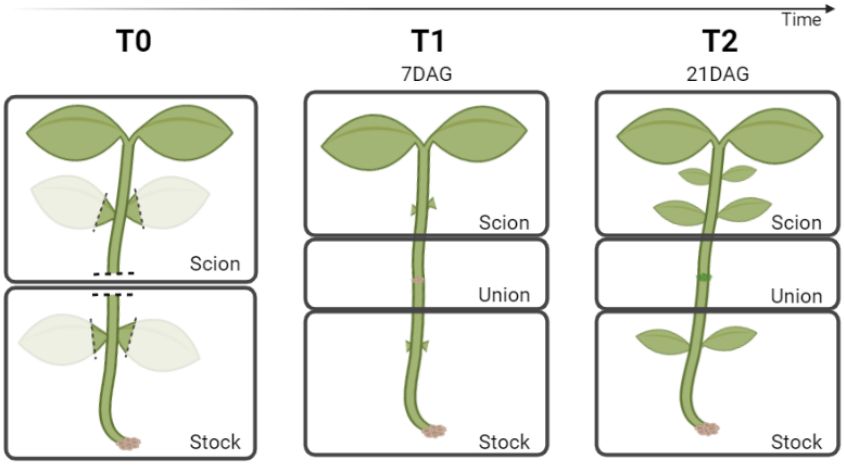

Figure 15. Experimental design for sample collection of micrografts. Samples were collected before micrografting (T0), 7 days after grafting (7DAG) (T1) and 21 days after grafting (21DAG) (T2). Samples collected at T0 correspond to ungrafted scion and stock. Micrografts were segmented in scion, graft union and stock. Created in Biorender.com.

2.6 IAA quantification

The IAA content in the collected samples from bitter almond homografts and Canhota x bitter almond micrografts, at initial (T0) and T2 stages was accessed using the colorimetric method described by Anthony and Street (1969). In this method, Ehrlich's reagent reacts with the indol group of IAA in an acid medium, under optimized conditions for improved specificity. The plant material (3 – 60.5 mg FW) was grounded in a mortar with liquid nitrogen. Then, a volume of Na-phosphate buffer 0.01 M (pH 7.0) 3-fold greater than the fresh weight was added and samples were centrifuged (17 000 rpm; 12 min). After centrifugation, the supernatant was recovered, and Na-phosphate buffer 0.01 M (pH 7.0) was added until the final volume of 1 ml.

The reaction mixture was composed of 1 ml of diluted sample in Na-phosphate buffer 0.01 M, 2 ml of 100% (w/v) trichloroacetic acid (TCA) and 2 ml of Ehrlich's reagent, added in order. A blank solution of Na-phosphate buffer 0.01 M was prepared simultaneously. After an incubation period of 20 min in the dark to avoid IAA degradation, the absorbance was measured at 530 nm in a Jenway 7305 spectrometer. Calibration curves were prepared using buffered solutions of IAA with concentrations between 2 and 250 µg/ml (Appendix I). For each segment 3 replicates were used, and the results obtained in µg of IAA per mg of fresh tissue.

2.7 IAA immunolocalization

IAA immunolocalization was performed in bitter almond homografts and Canhota x bitter almond micrograft sections from T0 (scion and stock) and T2 (scion, union and stock), and whole micrografts from T2. Samples, consisting of segments with maximum 10 mm long, were fixated by complete immersion in a solution of ice-cold 4% (w/v) paraformaldehyde. Vacuum was applied for 20 minutes to help the fixative penetrate the tissues. Samples were left in fixative at 4 °C overnight on an orbital shaker under gentle shaking. For sample dehydration, the fixative was replaced by ice-cold filtered 10% (w/v) sucrose solution in 1x phosphate-buffered saline (PBS) pH 7.4, applied o/n at 4 °C, which was then replaced by a new ice-cold filtered 20% (w/v) sucrose solution in 1xPBS pH 7.4 for a second o/n period at 4 °C. In every solution replacement vacuum was applied for 20 minutes, and samples kept on an orbital shaker under gentle shaking. To prepare samples for cryostat sectioning, sucrose solution was removed, and samples were gently mixed for 2 minutes using forceps on an ice-cold watch glass with Optimum Cutting Temperature compound (O.C.T.) (Tissue-Tek ™, Sakura). Then, samples were placed on molds with O.C.T. and frozen in a beaker containing isopropanol, which in turn was placed in a polystyrene container with liquid nitrogen. Embedding molds were kept at - 80 °C.

Sections of 10 μm were cut in a cryostat (CM3050 S Leica Microsystems, Nussloch, Germany) and collected on coated slides that were then conserved at - 80 °C until use. Each section was surrounded with a hydrophobic marker, and 2% (w/v) driselase in 8.5% (w/v) D-mannitol solution was added and incubated at 37 °C for 30 minutes, to permeabilize the tissues. Samples were pre-treated with blocking solution (10% bovine serum albumin (BSA)) in 1xPBS for 30 minutes to reduce non-specific binding. After washing with 1xPBS for 5 minutes, incubation with IAA primary antibody (Ref.: AS09 421 AGRISERA, Vännäs, Sweden), in a 1:200 dilution in 1% BSA in 1xPBS, occurred overnight in a dark moistened chamber. The secondary antibody, Alexa Fluor® 633 goat anti-rabbit (Molecular Probes, Göttingen, Germany), was diluted 1:200 in 1% BSA in 1xPBS and centrifuged 5 minutes at 10 000 rpm. After washing twice with 1xPBS for 2 minutes, sections were incubated with the secondary antibody for 1h in a dark moistened chamber. Samples were washed twice with 1xPBS for 2 minutes and slides

assembled with Dako Fluorescence Mounting Medium (Agilent). Images were acquired in a Zeiss Axio Observer.Z1 inverted microscope (equipped with a AxioCam HRm and Zen Blue 2012 software (all from Carl Zeiss, Germany)) using a A-Plan 2.5×/0.06 differential interface contrast (DIC) and EC Plan-Neofluar 10x/0.3 Ph1 objectives and R 631/633 nm laser (Colibri 7 LED light source). Images were processed with Fiji Software (version 1.53c).

2.8 Gene expression analysis

2.8.1 Extraction of total RNA

From the bitter almond homografts and Canhota x bitter almond micrograft fragments (scion, union and stock) collected at T0, T1 and T2 total RNA extracts were prepared. All material used in RNA extrations (mortar, pestels and spatulas) was treated and sterilized by autoclaving at 121 °C for 20 minutes twice, to remove potential RNAses. Samples were grinded in liquid nitrogen in a sterilized mortar and turned into a fine powder using a pestle. Samples were kept at a frozen state using liquid nitrogen to avoid RNA degradation and reduce RNAse's activity and transferred into new cold RNAse free 1.5 ml eppendorf tubes. RNA extractions then proceeded as recommended by the kits manufacturer's instructions.

RNA extraction was accessed using two different RNA extraction kits. The first was used to extract total RNA from samples with a fresh weight > 30 mg, and the other to extract total RNA from samples with a fresh weight < 30 mg. For the first extraction, Direct-zol™ RNA MicroPrep (ZYMO RESEARCH, California, USA), was used according to the manufacturer's instructions. For the second extraction RNAqueous®-Micro Kit (Ambion®, Thermo Scientific, Massachusetts, USA), was used according to the manufacturer's instructions. Both procedures included a step of DNase treatment that was performed to eliminate genomic DNA.

The final concentration of RNA of each sample was measured using a spectrophotometer (NanoDrop™, Thermo Scientific, Massachusetts, USA). The concentration values were read at an absorbance peak of 260 nm. RNA purity was

expected with the A260/A280 ratio close to 2.0 and A260/A230 ratio between 2.0 and 2.2.

2.8.2 cDNA synthesis from total RNA samples

To produce cDNA for further analysis, depending on the extract final RNA concentration, 100 ng or 50 ng of total RNA from each sample were used so that cDNA concentration was as equal as possible across all samples. cDNA synthesis was performed using NZY First-Strand cDNA Synthesis Kit (NZYTech, Lda. – Genes and Enzymes, Lisbon, Portugal) according to the manufacturer's instructions.

2.8.3 Quantification of gene expression by quantitative PCR

Gene expression quantification of *WIND1*, *Alf4*, *TIR1* and *IAA26* transcripts was accessed using the NZYSpeedy qPCR Green Master Mix (2x) (NZYTech, Lda. – Genes and Enzymes, Lisbon, Portugal) kit, following the instructions provided. Samples were diluted 50 times. Samples with the mix were pooled in a 96-well qPCR plate and measured in CFX Connect ™ Real-Time System (Bio-Rad Laboratories, Lda., Amadora, Portugal). For reliable quantitative PCR's, a reference gene, *ACTIN-11 (ACT11)*, (Dos Santo Pereira *et al.,* 2014) was also chosen to normalize the data (Udvardi *et al*., 2008).

All primers were designed for *Prunus dulcis* transcript sequences (Table 5), withdrawn from the National Center for Biotechnology Information (NCBI) databases. NCBI Megablast tool was used to search database Nucleotide collection (nr/nt) for highly similar sequences to the ones referred in literature for *Arabidopsis thaliana* (Melnyk *et al*., 2015). Primers were designed using the NCBI primer design tool (product length >100 bp). Before their use in quantitative PCR all primers were tested using samples of cDNA, by Reverse Transcriptase PCR RT-PCR) using NZYTaq II 2x Green Master Mix (NZYTech, Lda. – Genes and Enzymes, Lisbon, Portugal), following the provided protocol, proceeded by an agarose gel electrophoresis to check primer specificity.

Table 5 – Transcripts NCBI accession numbers, primer pairs sequences and respective expected product length, obtained using NCBI Primer Design tool.

	NCBI Accesion number	Forward Primer	Reverse Primer	Product length (bp)
WIND1	XM_034367858.1	GCCGACAACGCAGAGTTTTT	CAACCAGTCATGGCCTAGCA	364
TIR1	XM_034371559.1	TCAAGCTCCTTGTGCTCTCG	GGCTCTCGAGTTCCTTGCAT	414
IAA26	XM_034353103.1	GTGCCCAACAGCTCTCAGAA	GGTTCCACCAGCACAGGAAT	327
Alf4	XM_034359217.1	CACTGGACCAGGCGATCATT	TCCAACTCCAAAGACCTGGC	328
ACT11	XM_034364250.1	GGCATGGGTCAAAAGGATGC	AGGAATAACCACGCTCCGTC	463

The expression values (Cq) obtained were assembled to be analyzed. Data were first normalized using the adequate reference gene to each target gene. The method used to analyze the qPCR data was the relative quantification method, or $2^{-\Delta\Delta CT}$ method, where the ΔΔCT value = (Cq Target – Cq Reference) (Livak and Schmittgen, 2001). Statistical analysis was performed by comparinge expression values at T0, T1 and T2 in each segment, with 3 biological replicates used.

2.9 Statistical analysis

All the statistical analysis was performed using Graph Pad Prism (Version 8.4.3 (686)). Tukey's multiple comparison test at at $p \leq 0.05$ was used to analyse most of the data obtained from the different experiments, except for the zygotic embryo germination when evaluating the effect of cold treatment for which a Welch's parametric test at $p \leq 0.05$ was used.

For gene expression analysis, the normalized data, obtained by applying the $2^{-\Delta\Delta CT}$ method, was statistically analysed using Tukey's multiple comparison test at $p \leq 0.05$ in the same version of Graph Pad Prism.

3. Results

3.1 Morphological characterization of almond fruits

The shell kernels and kernels from Portuguese traditional almonds were characterized according to their phenotypic traits. Therefore, weight, length, width, thickness, shape, apex shape, double kernel, dry kernel and toughness were recorded as described in Tables 6 and 7. The weight of Gama Dura, Canhota and Ferraduel in shell kernels was significantly higher ($p \leq 0.05$) than Bota, Quinta de Valim and Rabo de Zorra. Ferraduel almonds presented the significantly higher length ($p \leq 0.05$). Canhota and Ferraduel presented similar and significantly higher width ($p \leq 0.05$). Canhota variety presented higher thickness values, but no significant differences were observed from Ferraduel and Gama Dura ($p > 0.05$). All the varieties evaluated had hard shell almonds.

Table 6. Characterization of Portuguese traditional varieties almonds with shell. Values indicated with different letters were statistically different at $p \leq 0.05$ using Tukey's test.

Variety	Bota	Quinta de Valim	Rabo de Zorra	Gama Dura	Canhota	Ferraduel
Weight (g)	4.65 ±0.57^{b}	4.48 ±1.01^{b}	4.92 ±0.76^{b}	6.55 ±1.41^{a}	6.40 ±1.41^{a}	6.86±0.72^{a}
Length (mm)	28.6 ±1.63^{d}	30.1 ±1.94^{c}	29.8 ±1.91^{c,d}	31.9 ±2.14^{b}	33.0 ±1.40^{b}	39.0 ±1.62^{a}
Width (mm)	23.7 ±1.52^{c}	25 ±1.51^{b}	25.7 ±1.14^{b}	25.8 ±1.74^{b}	27.8 ±1.83^{a}	27.8 ±1.54^{a}
Thickness (mm)	16.6 ±1.47^{b}	15.8 ±0.78^{c}	16.6 ±0.91^{b}	18.5 ±1.36^{a}	19.2 ±0.75^{a}	18.9 ±0.64^{a}
W/L	0.828 ±0.03^{d}	0.833 ±0.04^{d}	0.865 ±0.05^{b}	0.808 ±0.05^{c}	0.842 ±0.04^{b,d}	0.711 ±0.02^{a}
T/L	0.580 ±0.05^{c}	0.525 ±0.03^{b}	0.560 ±0.03^{c}	0.581 ±0.04^{c}	0.582 ±0.02^{c}	0.485 ±0.02^{a}
Almond shape	Oval	Round	Round	Round	Oval	Elliptic
Apex shape	Obtuse	Obtuse	Obtuse	Round	Obtuse	Obtuse
Toughness	Hard	Hard	Hard	Hard	Hard	Hard

Note: W/L: weight (g)/length (mm); T/L: Thickness (mm)/length (mm).

Ferraduel kernels had a significantly higher weight, than the remain varieties ($p \leq 0.05$). Ferraduel kernel length was the highest, but no significant differences were

observed from Rabo de Zorra ($p>0.05$). Also, Ferraduel presented the highest width and thickness, but no differences were found from the Canhota variety ($p>0.05$). In the varieties Bota and Gama Dura double kernels were found, while dry kernels were observed in Quinta de Valim, Gama Dura and Canhota.

Rabo de Zorra showed the highest partition yield with 28.5%, followed by Bota and Quinta de Valim, with 24.3% and 24.0%, respectively. These varieties had also a higher partition yield when compared to the French variety Ferraduel.

Table 7. Characterization of Portuguese traditional varieties almond kernel. Values indicated with different letters were statistically different at $p \leq 0.05$ using Tukey's test.

Variety	Bota	Quinta de Valim	Rabo de Zorra	Gama Dura	Canhota	Ferraduel
Weight (g)	1.13 ±0.14^{c}	1.08 ±0.16^{c}	1.40 ±0.16^{b}	1.18 ±0.22^{c}	1.38 ±0.13^{b}	1.63 ±0.14^{a}
Length (mm)	22.3 ±1.11^{c}	20.9 ±1.27^{b}	24.2 ±0.96^{a}	22.5 ±1.64^{c}	23.1 ±1.51^{c}	27.6 ±1.38^{a}
Width (mm)	14.6 ±0.91^{c}	14.6 ±1.29^{c}	15.0 ±0.98^{b,c}	15.1 ±1.19^{b,c}	15.7 ±1.02^{a,b}	16.4 ±0.82^{a}
Thickness (mm)	7.9 ±0.93^{c}	8.0 ±0.68^{c}	8.4 ±0.82^{b,c}	8.3 ±0.56^{b,c}	9.0 ±0.68^{a}	8.6 ±0.64^{a,b}
W/L	0.655 ±0.04^{c}	0.698 ±0.05^{b}	0.619 ±0.04^{a}	0.672 ±0.04^{b,c}	0.681 ±0.04^{b,c}	0.595 ±0.03^{a}
T/L	0.354 ±0.05^{c,d}	0.385 ±0.03^{b}	0.347 ±0.03^{d}	0.372 ±0,03^{a,b,c}	0.390 ±0,05^{a,b}	0.314 ±0.03^{d,c}
Apex shape	Acute	Acute	Acute	Obtuse	Acute	Acute
Double kernel (%)	4	0	0	9	0	0
Dry kernels (%)	0	12	0	4	8	0
Partition yield (%)	24.3	24.0	28.5	18.1	21.5	23.8

Note: W/L: weight (g)/length (mm); T/L: Thickness (mm)/length (mm)

In the shell almond and kernel from Portuguese traditional varieties and Ferraduel are presented in figure 16. Dry kernel from Quinta de Valim is presented in the figure 16B (iii). Double kernels, observed for Gama Dura variety are represented in figure 16D (iii).

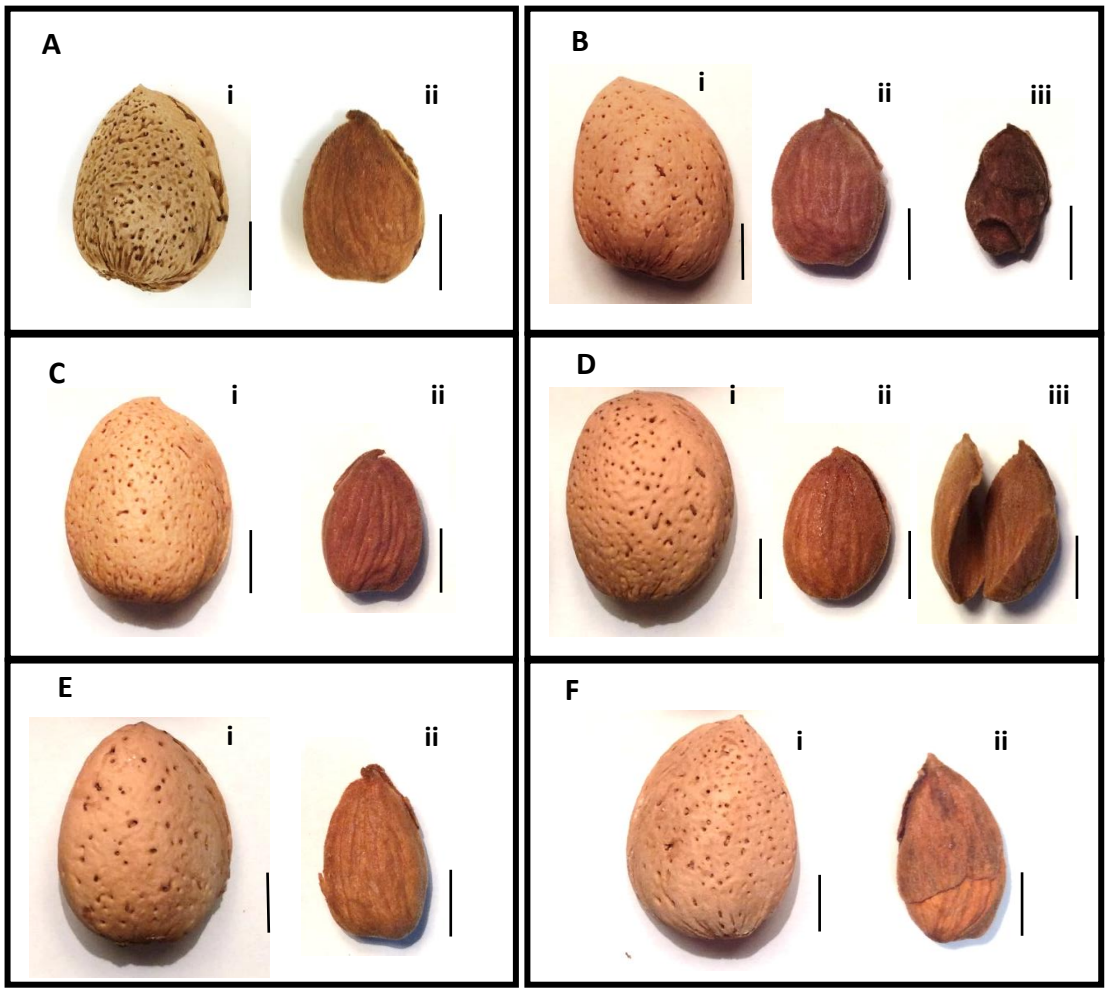

Figure 16. In-shell almond and kernel from Bota (A), Quinta de Valim (B), Rabo de Zorra (C), Gama Dura (D), Canhota (E) and Ferraduel (F). Dry kernel is presented in Quinta de Valim and double kernels in Gama Dura. Scale bar - 1 cm.

3.2 *In vitro* establishment of scions and rootstocks

3.21 Effect of cold treatment, PGRs and explant type on zygotic embryo germination

To evaluate the effect of 3 weeks seed storage at 4 °C in comparison to seed storage at room temperature, seeds from three commercial varieties, Soleta, Antoñeta and Lauranne, were disinfected and the embryo axis isolated and placed on culture medium. After 4 weeks in culture, no significant differences ($p>0.05$) were found for each variety between the two treatments, regarding the germination rate, seedling length, primary root length and number of secondary roots (Fig. 17). Among the varieties

tested, Lauranne seeds showed the lowest germination rates in these conditions, and Antoñeta seedlings had the longest primary roots. Seedling length was similar in all varieties.

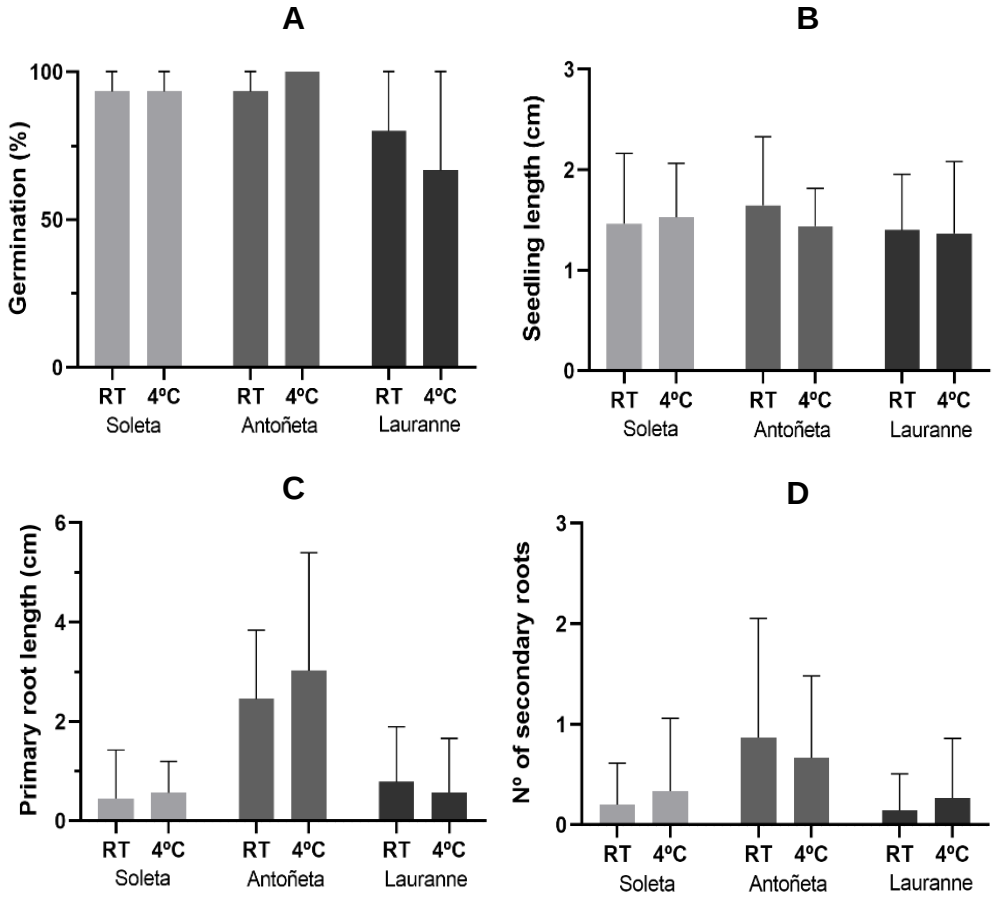

Figure 17. Effect of low temperature treatment on germination. Germination rates (A), seedling length, (B) primary root length (C) and number of secondary roots (D) after 4 weeks of culture. Error bars correspond to standard deviation (SD) values (n=15). No statistically differences were found at $p>0.05$ using Welch's parametric test.

Lauranne variety was also used to test different conditions evaluating the effect of cold treatment, PGRs and explant type on germination (Fig. 18). Although statistical differences were not found, condition 3 presented the highest germination rate and seedling length. Condition 3, with seed storage at room temperature, isolation of embryo axis and addition of PGR to the medium was used for the *in vitro* establishment of traditional and bitter almond seedlings. Primary root length was significantly higher ($p\leq0.05$) when the whole mature zygotic embryo was cultured on filter paper moistened with 1 mg/l GA_3.

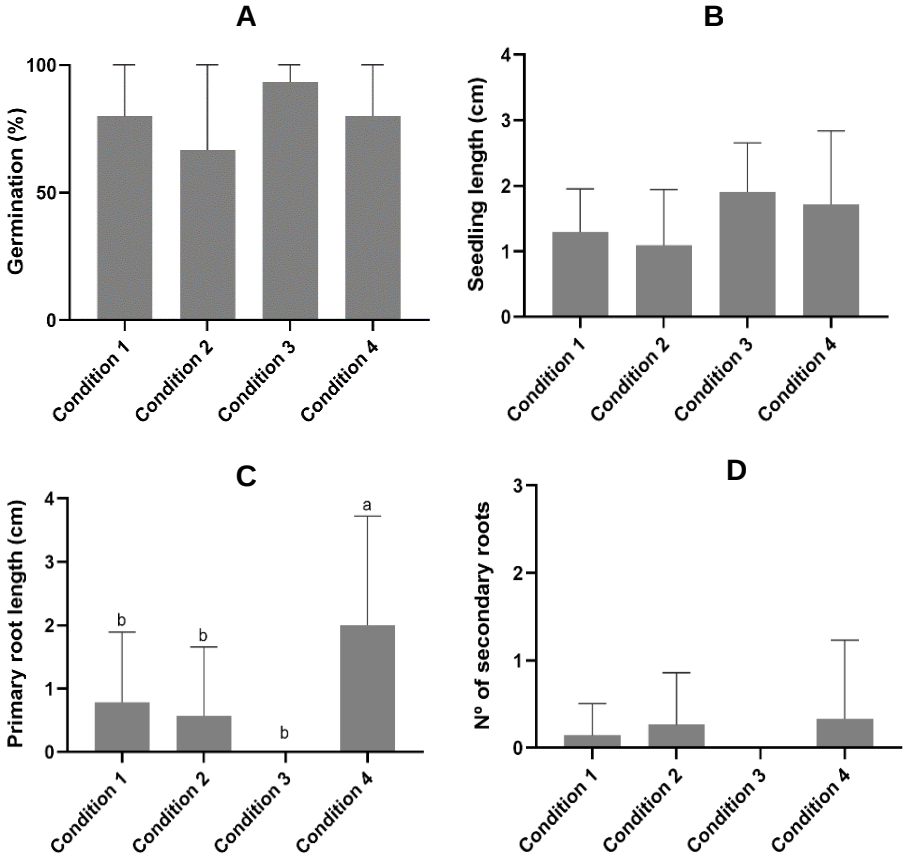

Figure 18. Effect of cold treatment, PGRs and explant type on germination. Germination rates (A), seedling length, (B) primary root length (C) and number of secondary roots (D) after 4 weeks of culture. Conditions 1, 2, 3 and 4 are presented in table 2. Error bars correspond to SD values (n=15). Values indicated with different letters were statistically different at $p \leq 0.05$ using Tukey's test.

3.2.2 *In vitro* establishment from zygotic embryos

Seed storage at room temperature and culture of the embryo axis in medium supplemented with GA_3 (1 mg/l) and BAP (0.5 mg/l) was the condition selected for *in vitro* establishment of the remain varieties. However, bitter almond was not statistically compared since germination occurred in different conditions with seeds stored at 4 °C according to provider's instructions and germination occurring in test tubes. Germination rates, seedling length, primary root length and number of secondary roots were evaluated after 4 weeks of culture (Fig. 19).

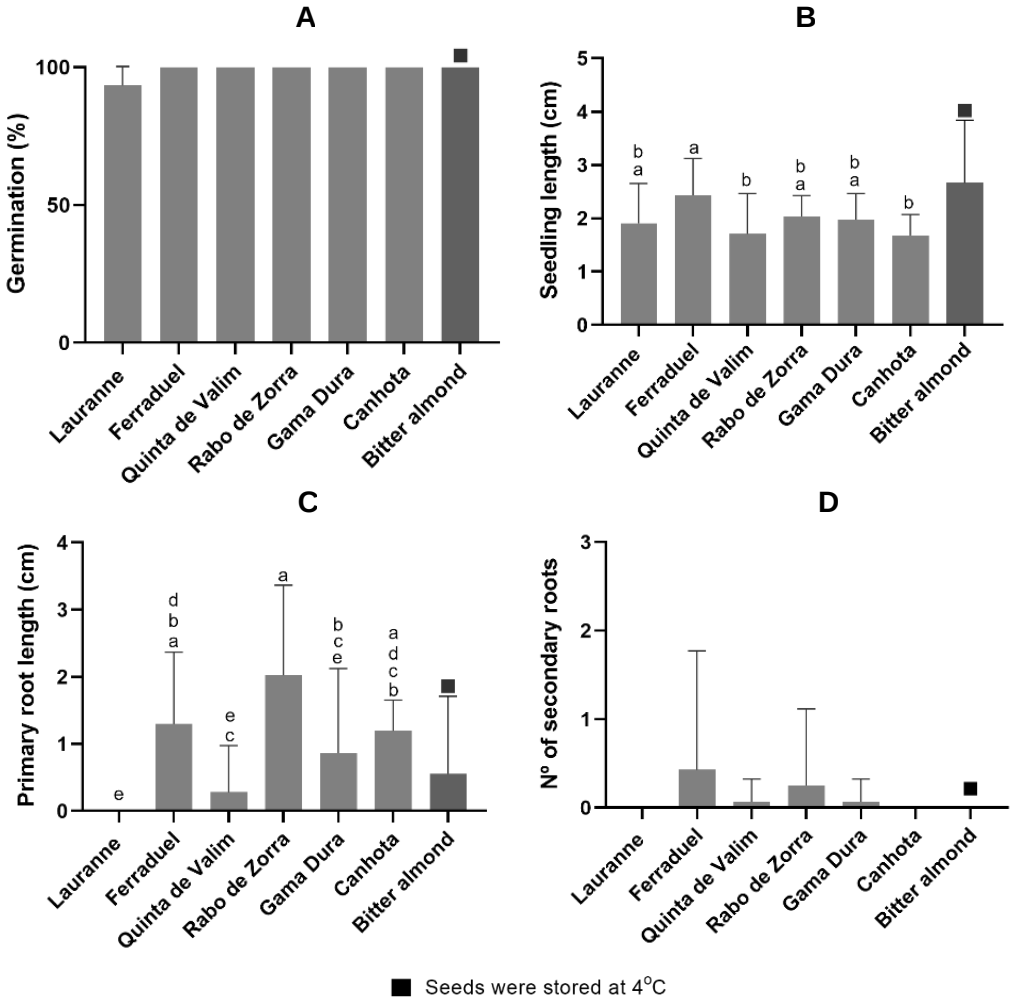

Figure 19. **Establishment of commercial, traditional, and bitter almond varieties.** Germination rates (A), seedling length, (B) primary root length (C) and number of secondary roots (D) after 4 weeks of culture. Note: bitter almond variety seeds were stored at 4 °C and not statistically compared to the other varieties submitted to similar treatment. Error bars correspond to SD values (n=15). Values indicated with different letters were statistically different at p≤0.05 using Tukey's multiple comparison test.

All embryo axis from both traditional varieties and Ferraduel developed in the selected conditions. Ferraduel seedling length was significantly higher (p≤0.05) than from Quinta de Valim and Canhota varieties. However, bitter almond seedlings were the longest. Although Lauranne did not showed primary root formation in this condition, the remain varieties showed different behaviours with the longest root length belonging to Rabo de Zorra (p≤0.05). With Canhota and bitter almond varieties secondary roots were not observed.

Seedling development, of commercial and traditional varieties, from embryo axis inoculation after 4 weeks in culture conditions, of commercial and traditional varieties are presented in Figure 20. During seedling development, some hyperhidricity, resulting from water accumulation in the tissues, was observed since leaves from some seedlings were darker and translucent.

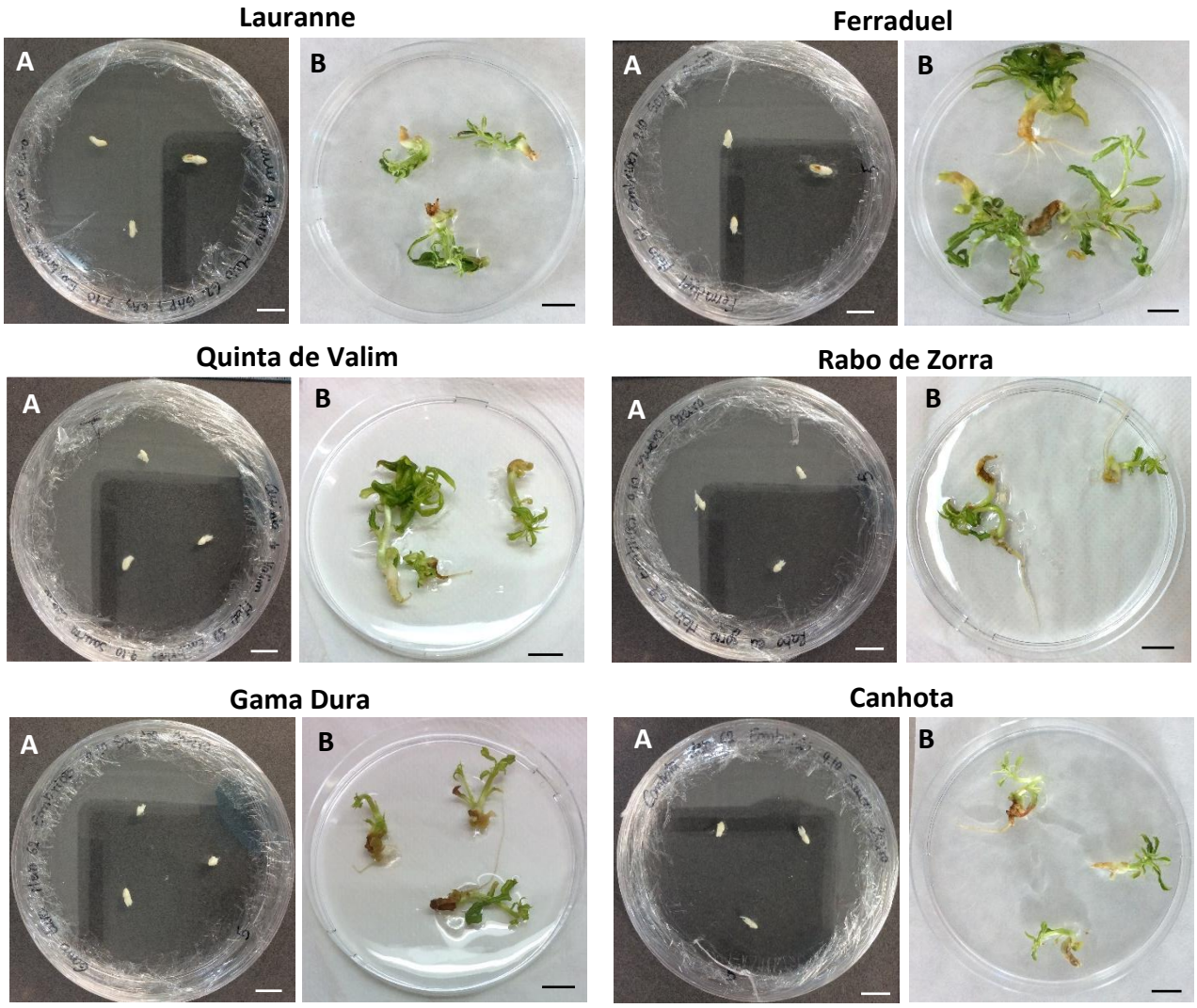

Figure 20. Embryo axis culture (A) and development after 4 weeks (B) of commercial (Lauranne and Ferraduel) and Portuguese traditional varieties (Quinta de Valim, Rabo de Zorra, Gama Dura and Canhota). Scale bar - 1 cm.

During bitter almond seedlings *in vitro* establishment (Fig. 21), after 4 days the embryo axis started to develop, and by the 12th day after culture initiation, shoots were formed. Later on, at 21 days of culture, shoots continued to develop, and primary roots were formed.

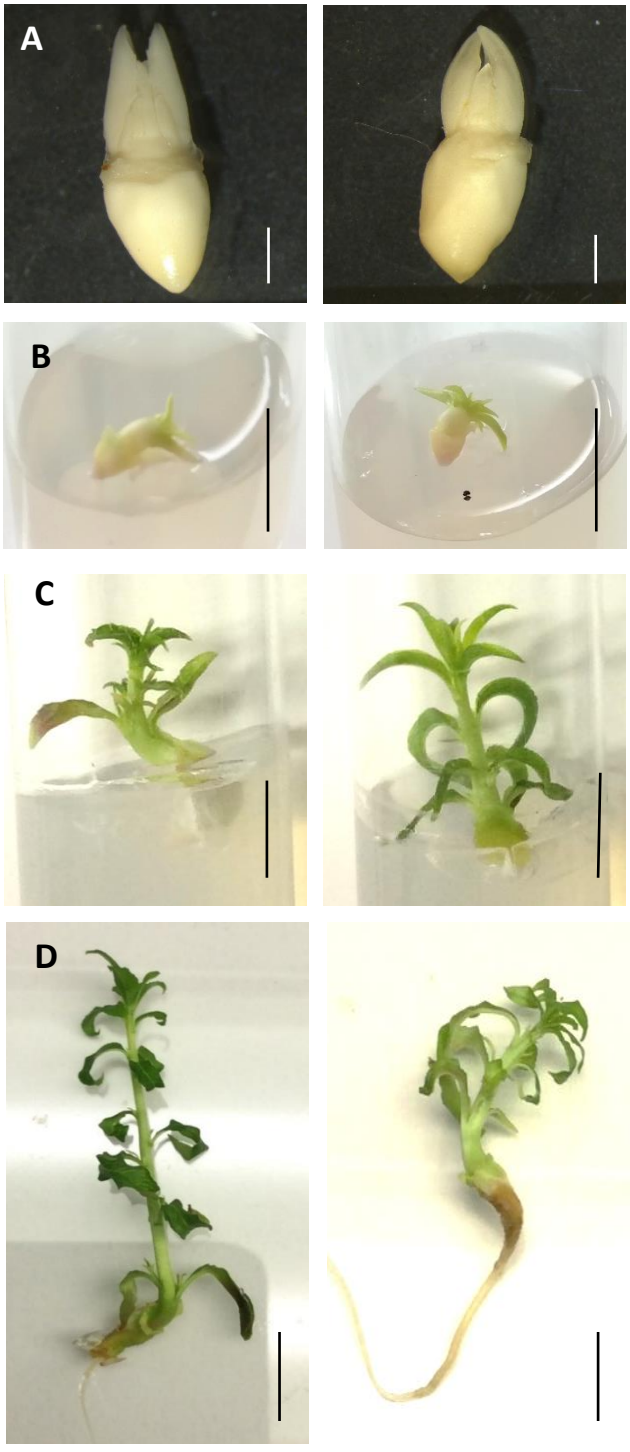

Figure 21. Bitter almond embryo axis (A) and seedling development after 4 (B), 12 (C) and 21 days (D) in culture. Scale bars correspond to 1 mm in A, and to 1 cm in B, C and D.

3.2.3 *In vitro* establishment and multiplication of the rootstock Rootpac®

Upon Rootpac® *in vitro* establishment, apical distance influence on meristem development was evaluated. Initially, for each level, 10 nodes were used. According to the data presented in Table 8, after 1 month of culture, all apical meristems were contaminated, and the remain meristem levels showed contamination equal or superior to 50%, which has made impossible the statistical analysis of the obtained data. Nevertheless, it was possible to observe that from nodes obtained from level 7, no shoot development was observed. Also, during the first 2 months, an increase in shoot formation was observed in all the remaining levels. However, level 3 and >7 reduce shoot formation after 3 months. During 3 months of subcultures in the same medium, level 4 was the only showing a consecutive increase in shoot formation. When transferred to a different medium at 4 months, levels 3, 4 and >7 were the most responsive with an increase in shoot formation. Also, in medium supplemented with IBA a large *callus* formation was observed.

Table 8. Influence of apical distance on meristem shoot development (n=10). Level 1 corresponds to the apical meristem.

Meristem level	MS + 0,5 mg/l BAP + 50 mg/l Citric acid				MS + 1 mg/l IBA
	1 month		2 months	3 months	4 months
	Contamination (%)	Shoot formation (%)			
1	100	-	-	-	-
2	70	10.0	33.3	33.3	0.0
3	70	20.0	60.0	57.1	75.0
4	50	10.0	16.7	28.6	70.6
5	70	0.00	33.3	33.3	33.3
6	70	10.0	33.3	33.3	33.3
7	60	0.00	0.00	0.00	0.00
>7	60	10.0	50.0	25.0	40.0

3.3 Multiplication

3.3.1 Effect of culture media and genotype on multiplication

After *in vitro* establishment, bitter almond seedlings were used to evaluate different multiplication media (Table 3). Seedlings were multiplicated during 3 cycles (T1, T2 and T3), while multiplication rates and the number of phytomers obtained in each medium were recorded in each cycle (Fig. 22).

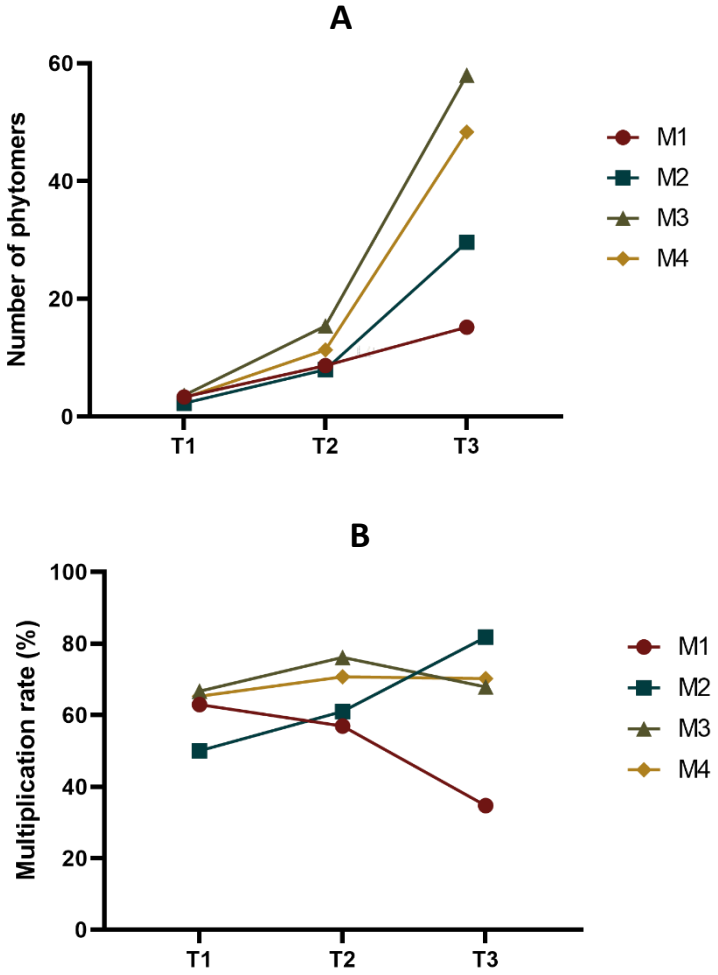

Figure 22. Multiplication rate (%) (A) and number of phytomers (B) obtained after 3 multiplication cycles of bitter almond shoots in different culture media.

When shoots were cultured on medium without PGRs (M1, culture medium), the multiplication rate diminished over time, and the number of phytomers was the lowest after three multiplication cycles. The addition of PGRs led to higher multiplication

rates and number of phytomers. M2 medium, showed the most accentuated increase in shoot multiplication rate after the 3 cycles. Regardless of the decrease in multiplication rate from T2 to T3, the highest number of phytomers was obtained in M3 at T2.

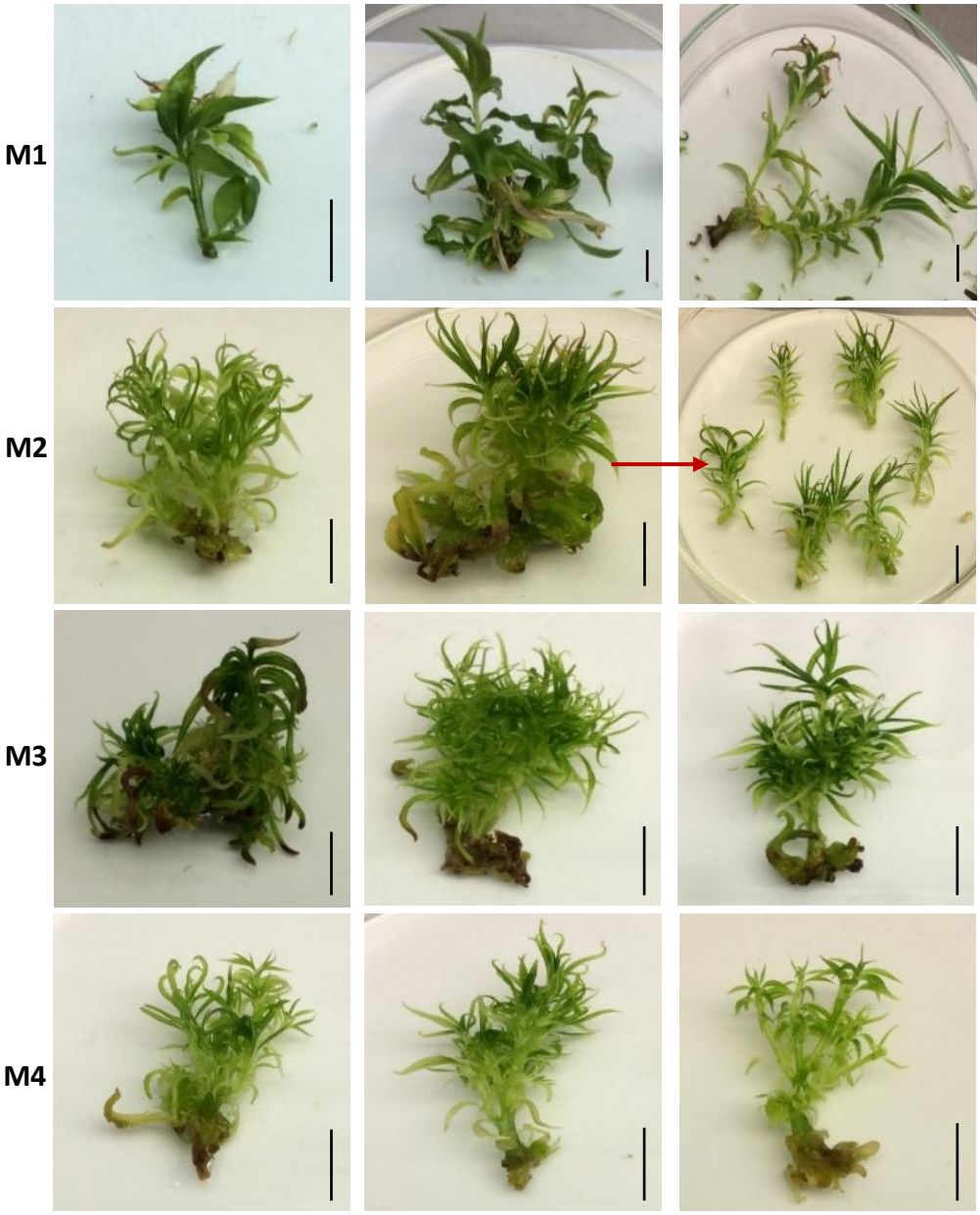

Figure 23. Almond explants in different multiplication media (M1, M2, M3 and M4). In M2, the red arrow indicates various shoots obtained from one explant. Scale bar - 1 cm.

As shown in figure 23, lateral shoots are present in explants from M1, but when growth regulators are added to the culture medium, especially cytokinins, the number of lateral shoots increases. Also, explants from media supplemented with growth

regulators presented hyperhidricity (Fig. 24), while explants from M1 showed less abnormalities.

Figure 24. Leaves from *in vitro* shoots in healthy condition obtained from M0 (left) and signs of hyperhidricity, obtained from M3 (right) condition. Scale bar - 1 cm.

About 15 genotypes of bitter almond were cultured for *in vitro* establishment. From those, genotypes numbered 1, 2, 5, 10, 11, 12 and 13 were contaminated, and although the embryo axis from genotype 9 germinated, it did not allow multiplication showing oxidation signs. During 3 cycles the number of phytomers and multiplication rate were recorded in the remain genotypes of bitter almond to evaluate the influence of genotype on multiplication (Fig. 25). Even though multiplication rates were similar for all genotypes at the end of the 3rd multiplication cycle, the highest number of phytomers were registered for genotypes 15 and 6.

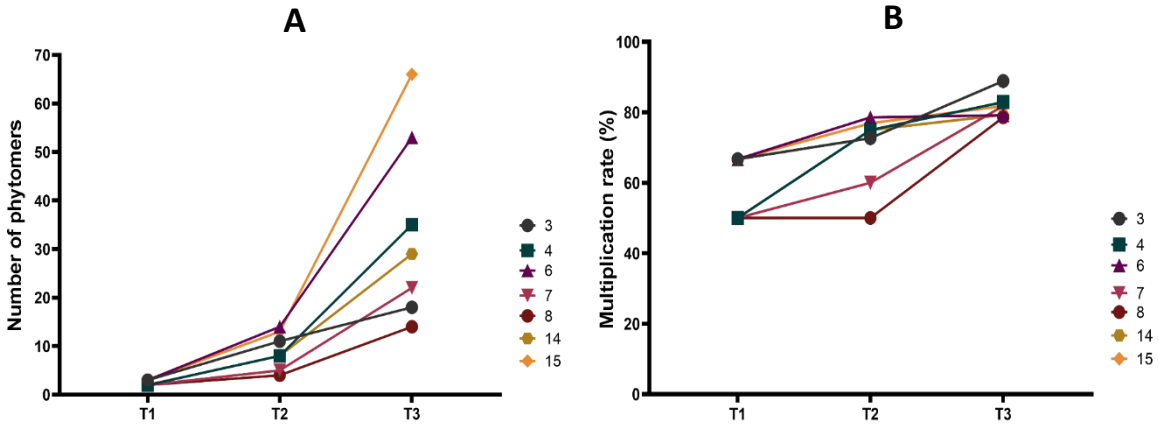

Figure 25. Number of phytomers (A) and multiplication rates (B) obtained throughout the 3 multiplication cycles of bitter almond genotypes.

3.3.2 Multiplication of scions and rootstocks

After the *in vitro* establishment, commercial and Portuguese traditional varieties were multiplicated in M1 medium, and the number of phytomers and multiplication rates were recorded at the end of 3 multiplication cycles (Fig. 26).

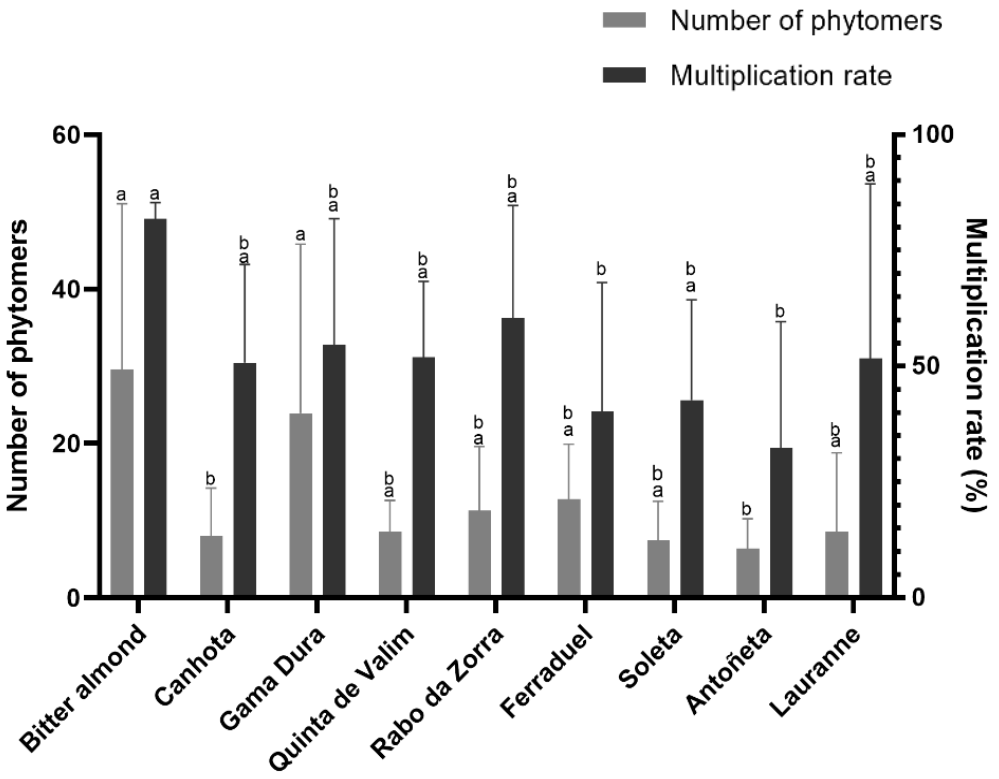

Figure 26. Number of phytomers (left axis) and multiplication rates (right axis) obtained at the end of 3 multiplication cycles of bitter almond, commercial and traditional varieties. Error bars correspond to SD values (n≥15). For each variable values indicated with different letters were statistically different at p ≤ 0.05 using Tukey's multiple comparison test.

Significant differences were found in the number of phytomers and multiplication rates (Fig. 26). Bitter almond and Gama Dura showed a significantly higher number of phytomers than Canhota and Antoñeta (p≤0.05). The highest multiplication rate was obtained with bitter almond and was significantly higher than Ferraduel and Antoñeta (p≤0.05).

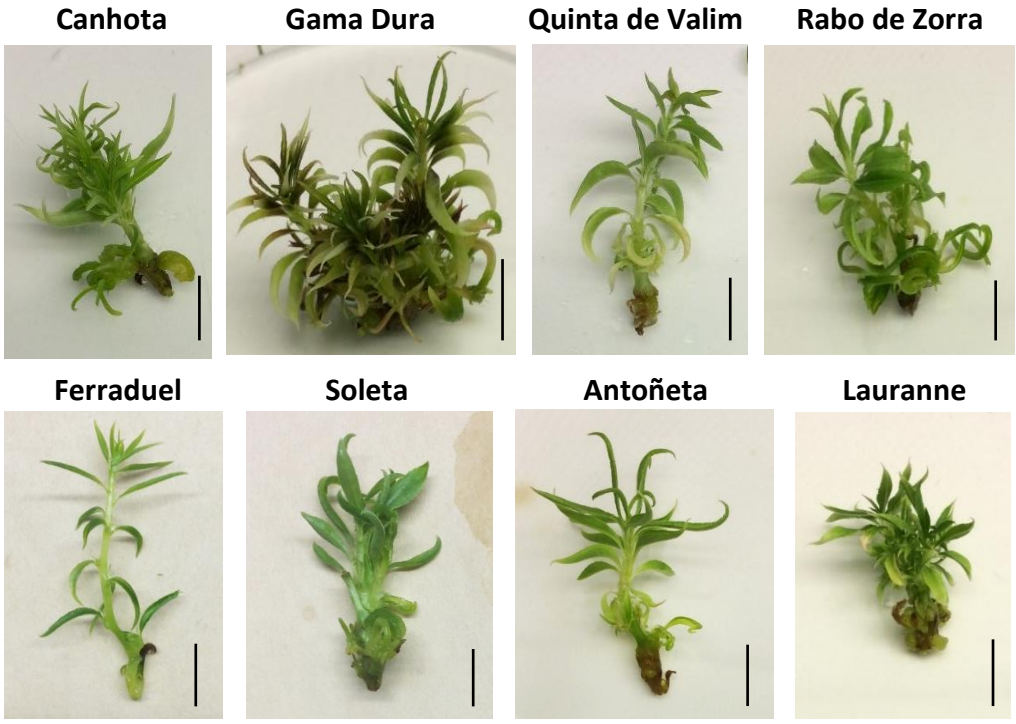

Figure 27. Almond explants of traditional (Canhota, Gama Dura, Quinta de Valim, Rabo de Zorra) and commercial (Ferraduel, Soleta, Antoñeta and Lauranne) varieties obtained in multiplication medium M1 after 3 multiplication cycles. Scale bar - 1 cm.

Explants from traditional (Canhota, Gama Dura, Quinta de Valim, Rabo de Zorra) and commercial (Ferraduel, Soleta, Antoñeta and Lauranne) varieties in multiplication stage are shown in figure 27. As previously seen for bitter almond multiplication, traditional and commercial varieties explants also showed hyperhidricity, and roots formation was not observed. Subcultures in MS medium supplemented with 0.1 mg/l BAP allowed recovery from hyperhidricity symptoms, while maintaining lateral shoot formation.

3.4 Rooting

3.4.1 *In vitro* rooting

In a first rooting assay, bitter almond shoots from the different multiplication media were cultured on 3 rooting media to evaluate the effect of IBA, charcoal and Fe-EDDHA. After 3 weeks in culture (Fig. 28) only 12.5% of shoots from rooting medium 4 (modified MS + 280.6 mg/l FeEDDHA + 1 mg/l IBA), previously multiplicated in M1 medium, showed root formation. However, wide formation of *callus* was observed.

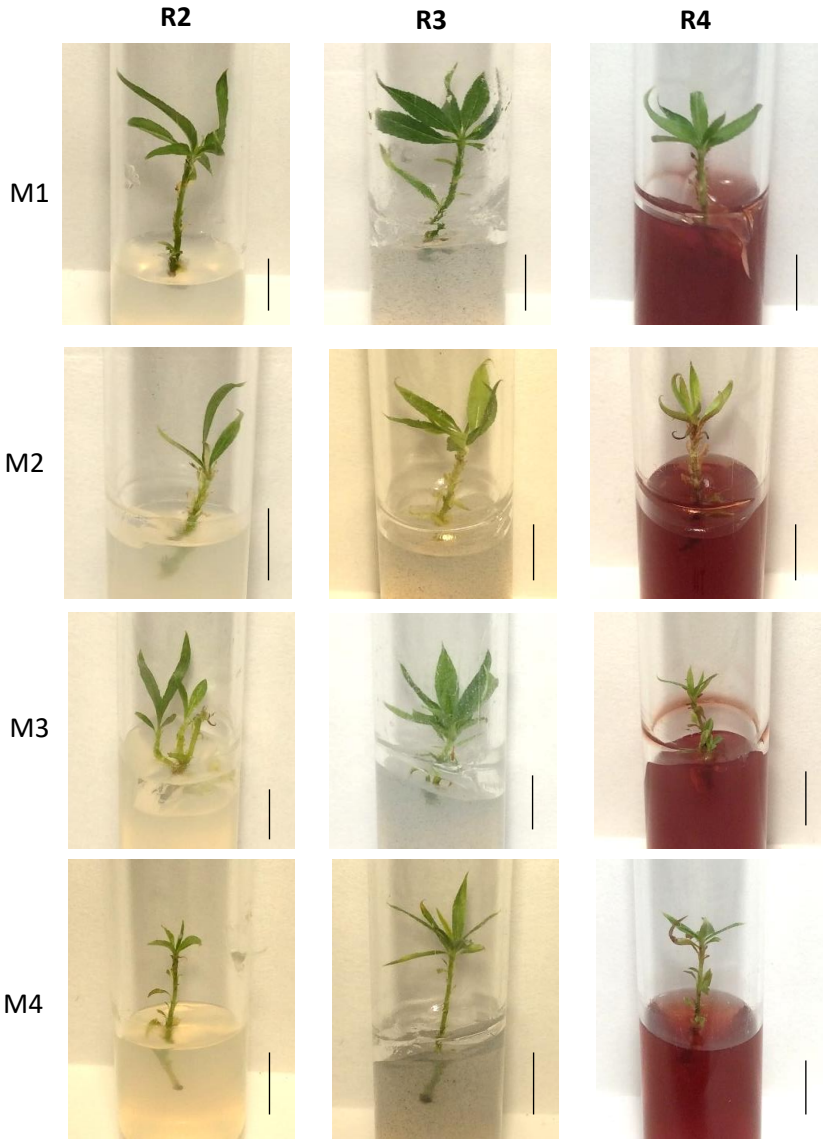

Figure 28. Bitter almond shoots from different multiplication media (M1, M2, M3, M4) placed on different rooting media (R2, R3 and R4) after 3 weeks. Scale bar - 1 cm.

In vitro rooting was not successfully accomplished in the first assay, and so a second *in vitro* rooting assay was established with bitter almond shoots from M1 cultured on rooting medium 1 (MS) and 5 (MS + 36.7 mg/l Fe-EDTA + 1 mg/l IBA + 1 g/l AC), and GxN15 cultured on rooting medium 5. Rooting was evaluated after 6 weeks. For bitter almond no rooting was observed. However, in GxN15 16.6 % of the explants formed potentially functional roots (Fig. 29).

Figure 29. GxN15 with potentially functional roots after 6 weeks in R5. Scale bar - 2 cm.

3.4.2 Ex vitro rooting

Since the second *in vitro* rooting assay was also not successful for bitter almond and showed low success rates for GxN15, *ex vitro* rooting was tested with no treatment, using commercial rooting powder and dipping in IBA solution (1 g/l). Shoots presented low survival in *ex vitro* conditions after 15 days (Fig. 30), resulting in no shoot survival during acclimatization.

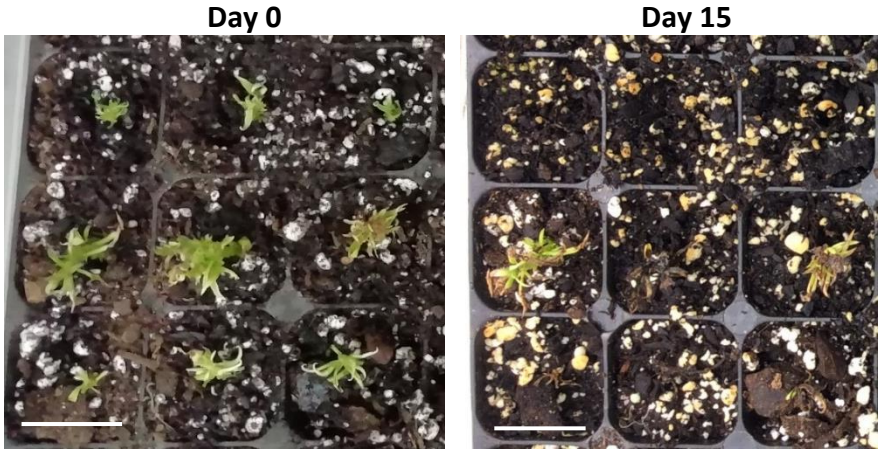

Figure 30. *Ex vitro* rooting tray with bitter almond and GxN15 shoots. Scale bar - 4 cm.

3.5 Micrografting and sample collection

3.5.1 Accessing scion genotype influence on micrografting success rates

Using a traditional variety (Canhota), and two commercial varieties (Ferraduel and Soleta), the influence of scion genotype on micrografting success was evaluated with 6<n<9 micrografts for each combination. After approximately 5 days, scion and stock showed some level of adhesion, and 30 days after micrografting it was possible to determine micrograft success based on micrograft stability, and scion and stock vigour (Table 9). Another parameter evaluated was the percentage of micrografts showing shoot formation from the stock.

The genotype 9 was more successful than genotype 1 from Canhota variety, when both were grafted onto GxN15. Concerning Soleta variety, genotype 4 was more successful when grafted in bitter almond, while genotype 21 was more successful when grafted onto GxN15. However, Ferraduel genotypes, appear to have higher micrograft success when grafted onto bitter almond shoots.

Table 9. Micrograft success (%) and shoot formation from the stock (%) evaluated at 30 days after grafting, for 6<n<9 micrografts for each combination.

		Micrograft success (%)		Shoot formation in the stock (%)	
Variety	**Genotype**	**Bitter almond**	**GxN15**	**Bitter almond**	**GxN15**
Canhota	9	Contaminated	66.7	Contaminated	77.8
	1	77.8	44.4	11.1	100.0
Soleta	4	75.0	44.4	62.5	100.0
	21	33.3	66.7	100.0	100.0
Ferraduel	10	88.9	77.8	66.7	100.0
	1	85.7	Contaminated	57.1	Contaminated
	3	77.8	71.4	88.9	100.0

Figure 31 shows micrograft establishment and development through 3 weeks. At 7 days after grafting (DAG) leaves started to develop in the scion, and by the 15 DAG, in all combinations, stocks showed lateral shoots. On the other hand, micrografts established with GxN15 presented earlier stock lateral shoots formation at one week after establishment. Although in micrografts with bitter almond, formation of lateral

shoots from the stock was observed, in micrografts with GxN15, this formation and development was stronger. Also, some scions grafted onto GxN15, gained a slight reddish colour.

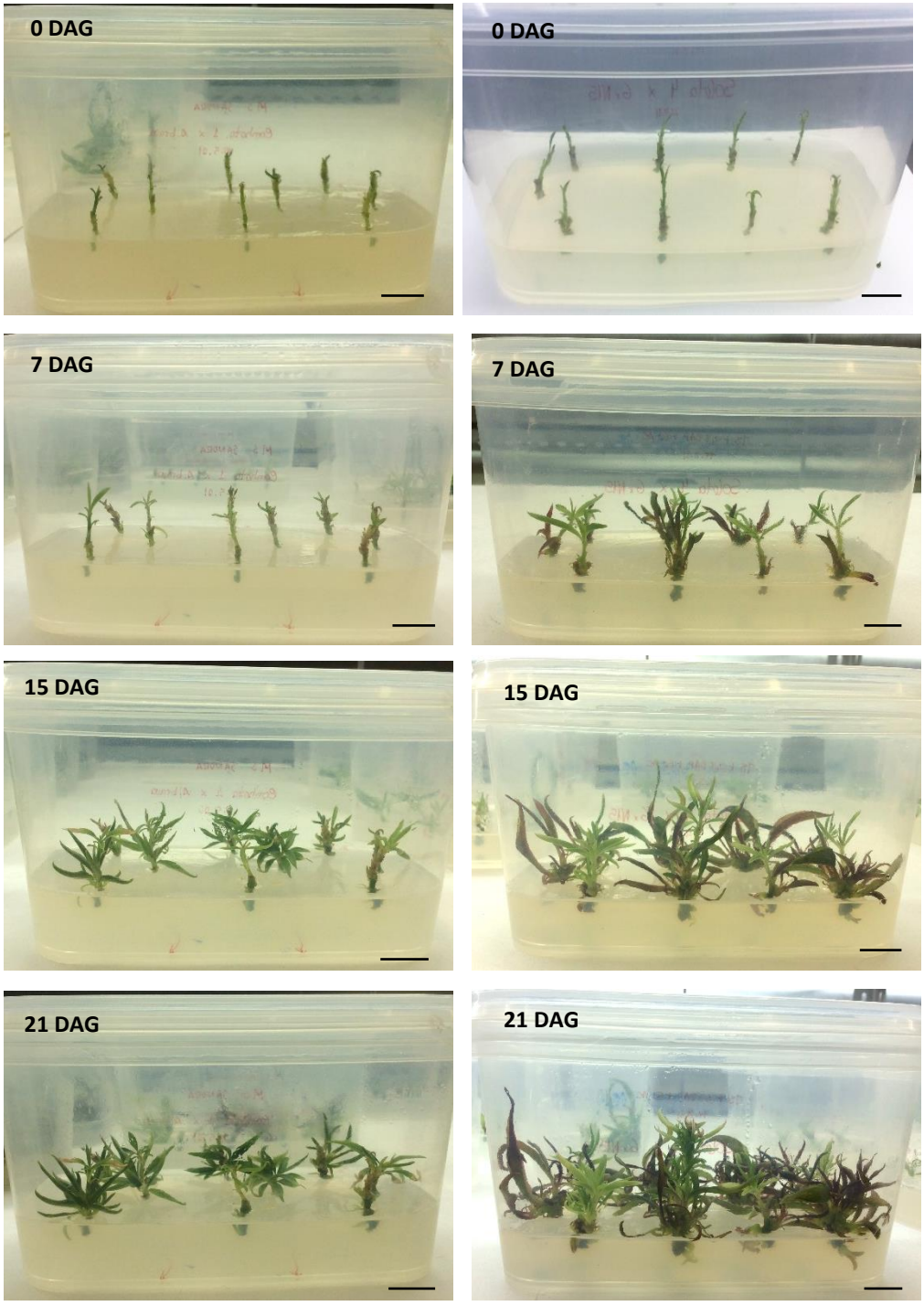

Figure 31. Establishment (0 DAG) and development at 7, 15 and 21 days after grafting (DAG) of Canhota variety (genotype 1) grafted onto bitter almond (left), and Soleta variety (genotype 4) grafted onto GxN15 (right). Scale bar - 1 cm.

Micrografts were then transferred to *ex vitro* conditions for rooting and acclimatization. Rooting was promoted by dipping the shoot base in IBA solution (1 g/l). During this transfer, union break was evaluated (Table 10). After 2 weeks, micrografts did not survive and rooting could not be evaluated.

Table 10. Micrograft union break during transfer to *ex vitro* conditions, for 2<n<5 micrografts for each combination.

		Union break (%)	
Variety	**Genotype**	**Bitter almond**	**GxN15**
Canhota	9	Contaminated	16.7
	1	0.0	60.0
Soleta	4	50.0	16.7
	21	16.7	0.0
Ferraduel	10	25.0	16.7
	1	33.3	Contaminated
	3	0.0	0.0

3.5.2 Establishment of micrografts for sample collection

The establishment of n=10 micrografts for each scion x stock combination and their development until sample collection was followed for 21 days. During this period, observations were registered to determine micrograft success (Fig. 32). Canhota and Ferraduel grafted onto bitter almond showed more success than the same varieties grafted onto GxN15. However, Soleta grafted onto GxN15 showed a similar success than Soleta grafted onto bitter almond. At 21 DAG micrograft success ranged from 90 % (Canhota X bitter almond) to 30 % (Ferraduel X GxN15).

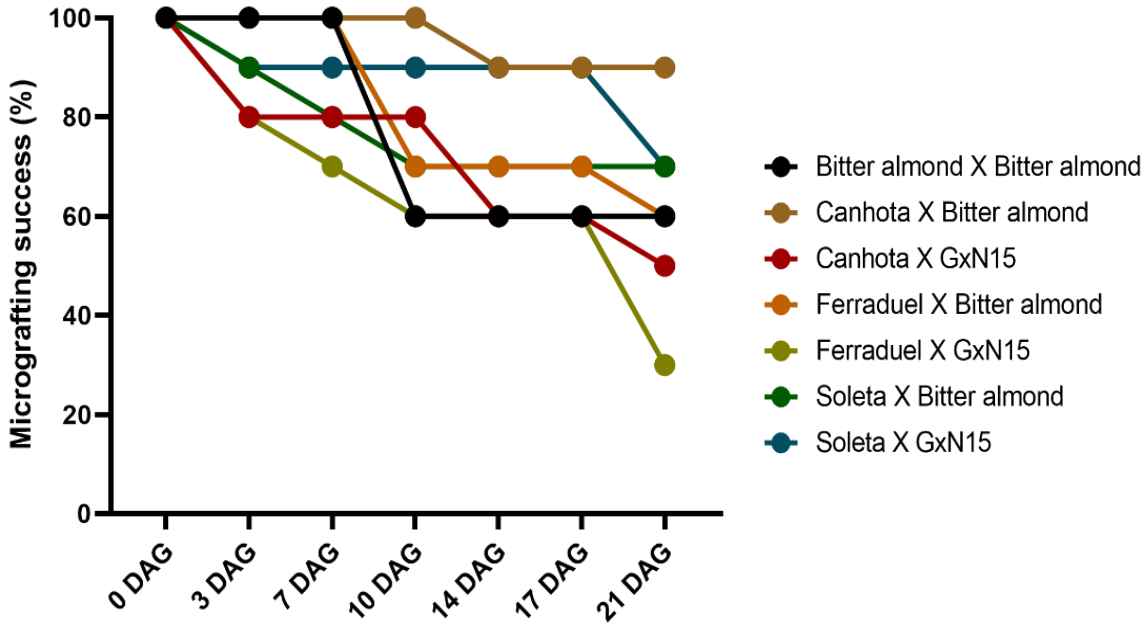

Figure 32. Micrografting success of Canhota, Ferraduel and Soleta micrografted onto bitter almond and GxN15 stocks, and homografts of bitter almond at 0, 3, 7, 10, 14, 17 and 21 days after grafting (DAG) (n=10).

During the development of micrografts established with bitter almond it was possible to observe that leaves and lateral shoots from stocks started to develop 10 DAG. At 21 DAG micrografts were established (Fig. 33). However, in comparison to the micrografts established with bitter almond earlier in the previous assay, these micrografts were a lighter green and presented less shoot formation from the stock. At the graft union it was possible to observe the development of leaves emerging from the 'v' cut parts of the stock.

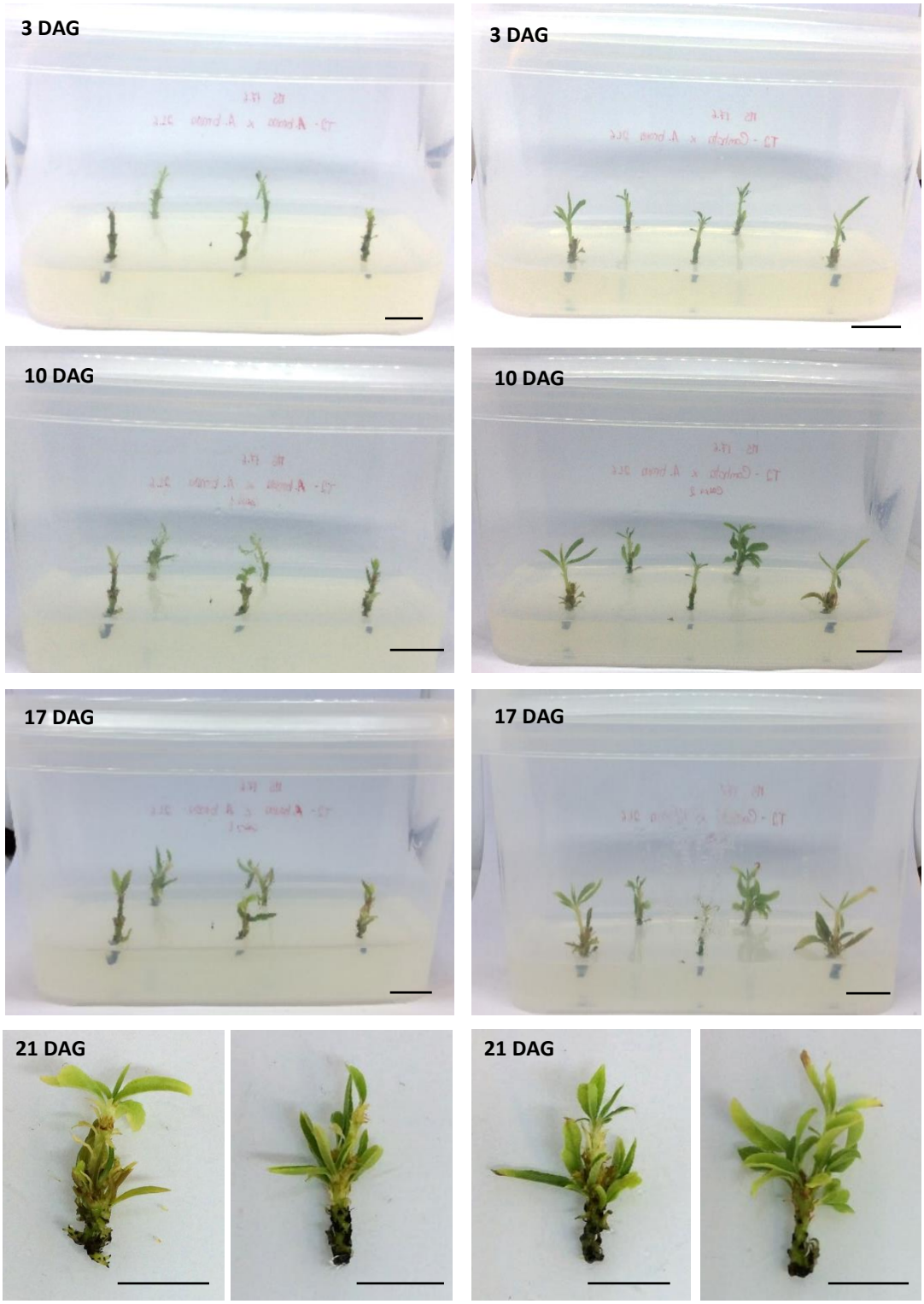

Figure 33. Establishment and development of bitter almond X bitter almond homografts (left) and Canhota X bitter almond micrografts (right) at 3, 10, 17 and 21days after grafting (DAG). Scale bar - 1 cm.

3.6 IAA quantification

Auxin quantification was carried out in micrografts segments at initial (T0) and T2 stages. At T0, samples correspond to the scions and stocks ungrafted. Micrografts collected at T2 were cut in three segments, each belonging to scion, graft union and stock. It was intended to evaluate IAA levels during micrografting in the various micrograft segments. Auxin quantification was carried out using Ehrlich reaction (Anthony and Street, 1969). The results were normalized considering the initial mass of tissue used, being presented in μg of IAA per mg of fresh tissue (Figs. 34 and 35).

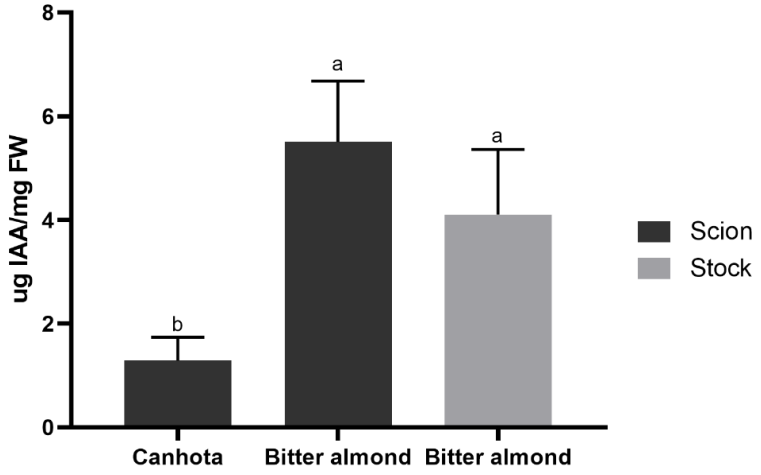

Figure 34. IAA quantification in Canhota and bitter almond varieties ungrafted scions and stocks (T0) using Ehrlich reaction. IAA is presented in μg of IAA per mg of tissue fresh weight (FW). Error bars correspond to SD (n=3). Values indicated with different letters were statistically different at $p \leq 0.05$ using Tukey's multiple comparison test.

Multiple comparisons were made between auxin quantification in scions and stock. Endogenous auxin levels are significantly different ($p \leq 0.05$), with bitter almond scion and stock presenting higher levels of IAA than Canhota scion. In bitter almond, when comparing scions and stock auxin content the scion presented higher levels of IAA (5.51±1.18 μg IAA/fresh tissue) than the stock (4.11±1.25 μg IAA/fresh tissue), but no significant differences were found ($p<0.05$).

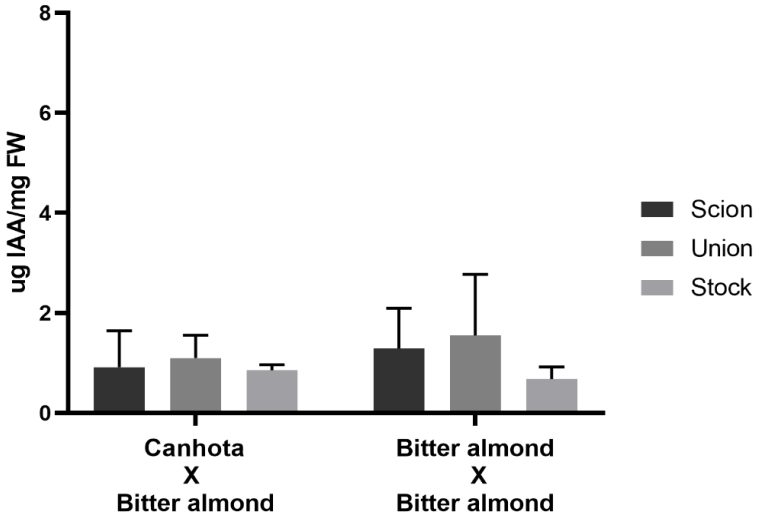

Figure 35. IAA quantification in Canhota grafted onto bitter almond and bitter almond homografts segments at 21 days after grafting (T2) using Ehrlich reaction. IAA is presented in µg of IAA per mg of tissue fresh weight (FW). Error bars correspond to SD (n=3). No significantly differences were registered on both micrograft combinations, at $p \leq 0.05$ using Tukey's multiple comparison test.

For auxin quantification at T2, statistical analyses were made on grouped samples according to micrografts combinations. Between segments of micrografts no statistical differences were found ($p > 0.05$). However, both combinations presented the same potential behaviour, with graft unions presenting the highest auxin level, followed by scion, and then the stock. The highest IAA level was recorded for the union of biter almond x bitter almond (1.55±1.22 µg IAA/fresh tissue). In comparison to IAA levels at T0, scions and stock presented lower IAA contents at T2.

3.7 IAA immunolocalization

To investigate the presence of IAA before and after micrografting with bitter almond, thin cut sections were treated with an anti-IAA primary antibody, and then a secondary antibody was coupled to a marker. Despite the observation of some sections' fragmentation and sample poor fixation, it was possible to find well preserved zones in the cuts. At T0, IAA was detected in Canhota scion and in bitter almond stock (Fig. 36). At T2, IAA was detected in whole micrografts (Fig. 37) and in segments of the bitter almond x bitter almond micrografts (Fig. 38).

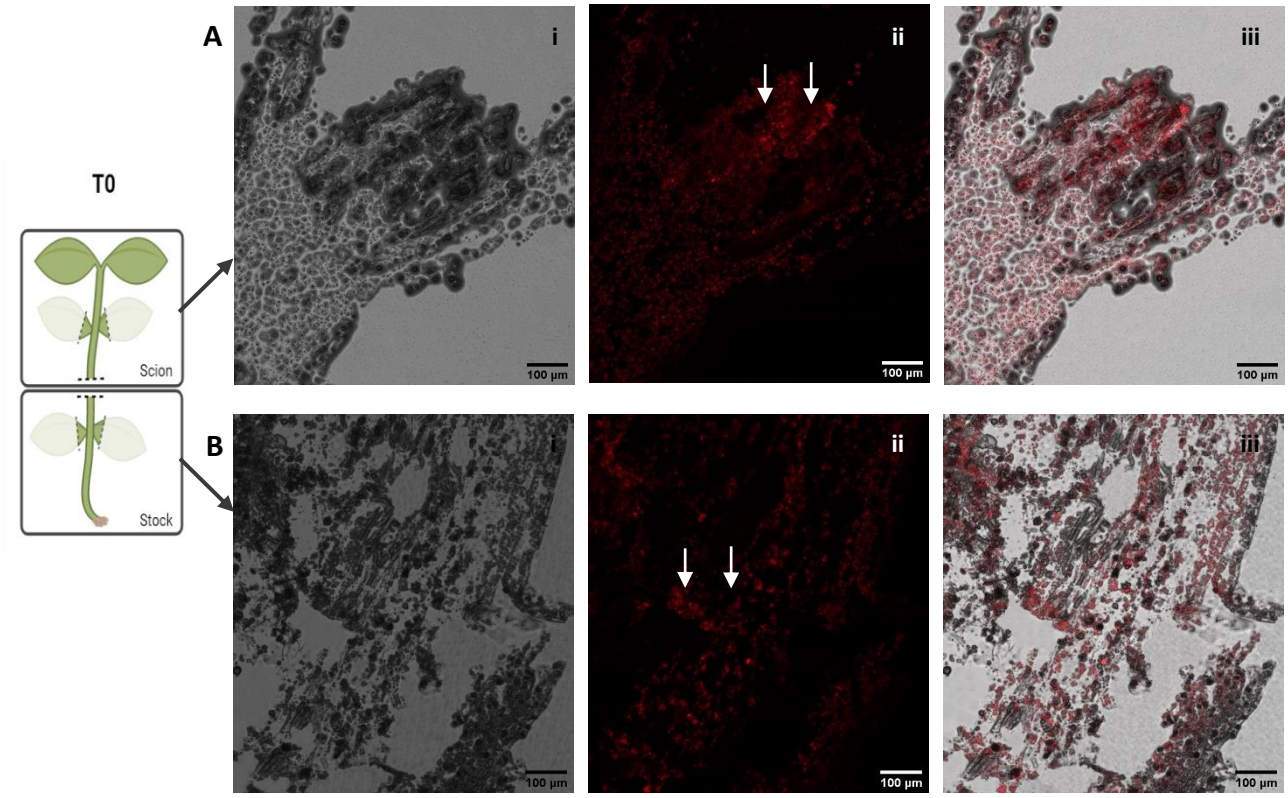

Figure 36. Immunolocalization of IAA in 4% (v/v) paraformaldehyde fixed histological sections (10 µm) of Canhota scion (A) and bitter almond stock (B) at T0. (i) Sections observed under transmission light (right column) were used for histological control. (ii) Sections stained with the anti-IAA antibody and Alexa Fluor® 633 (center column). (iii) Merged images.Arrows indicate the places where IAA has been more intensivelly labelled. Scale bar - 100 µm.

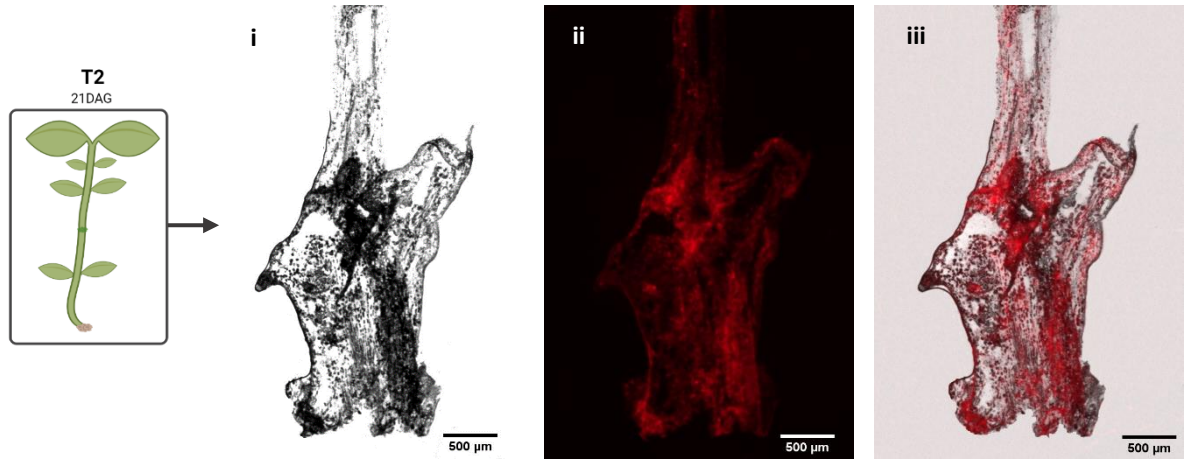

Figure 37. Immunolocalization of IAA in 4% (v/v) paraformaldehyde fixed histological sections (10 µm) of bitter almond x bitter almond whole micrografts at T2. (i) Sections observed under transmission light (right column) were used for histological control. (ii) Sections stained with the anti-IAA antibody and Alexa Fluor® 633 (center column). (iii) Merged images. Scale bar - 500 µm.

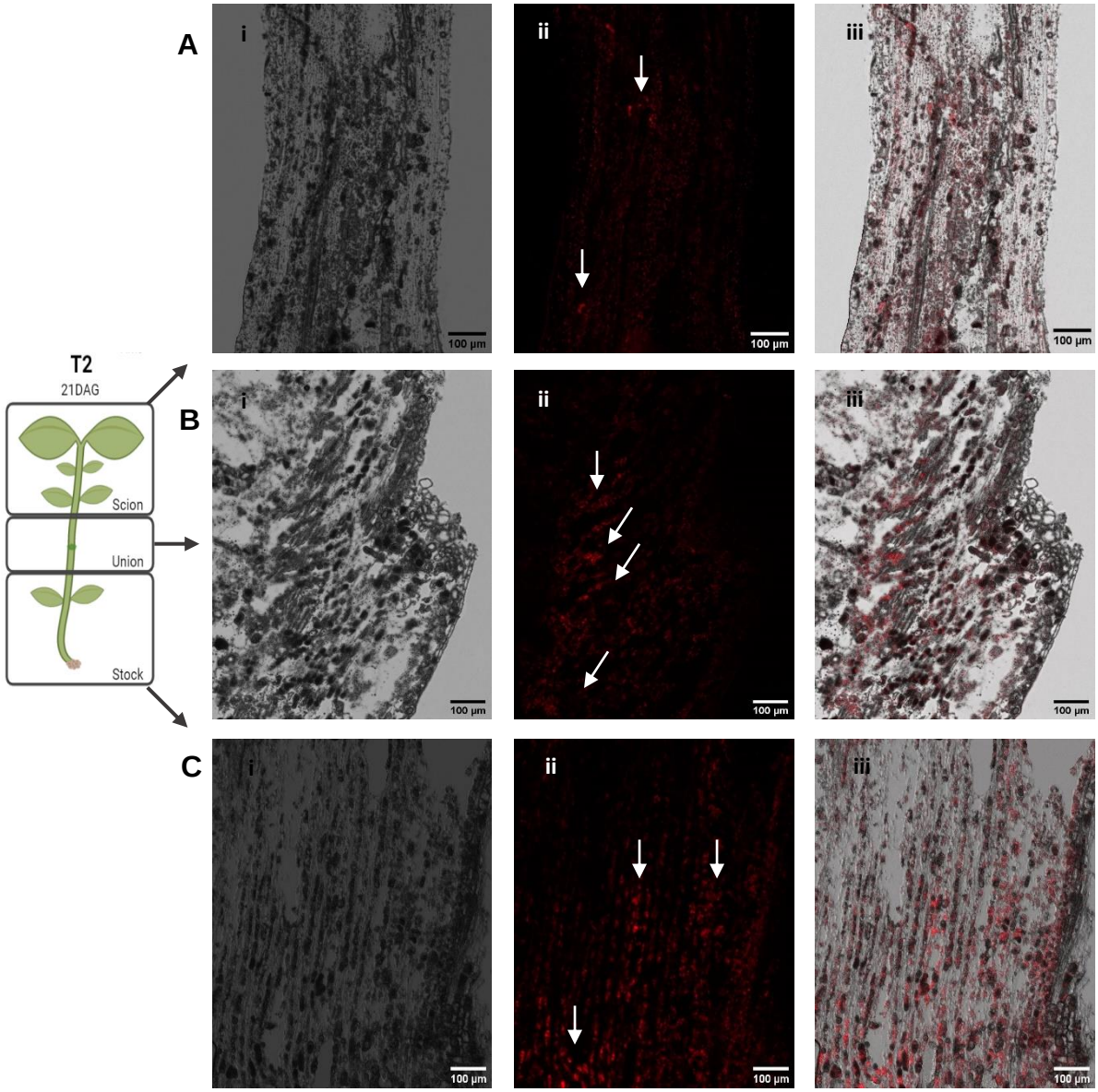

Figure 38. Immunolocalization of IAA in 4% (v/v) paraformaldehyde fixed histological sections (10 µm) of bitter almond x bitter almond micrografts scion (A), graft union (B) and stock (C) at T2. (i) Sections observed under transmission light (right column) were used for histological control. (ii) Sections stained with the anti-IAA antibody and Alexa Fluor® 633 (center column). (iii) Merged images. Arrows indicate the places where IAA has been more intensively labelled Scale bar - 100 µm.

At T0, IAA appears to be slightly more localized in Canhota scion than in the bitter almond stock when considering more preserved tissues (Fig. 36).

The brightfield images of whole micrografts and graft unions revealed possibly connected scion and stock. In whole micrografts images (Fig. 37), it appears that an accumulation of auxin is present at the graft union, and in closer images of graft union segments (Fig. 38B) the accumulation of auxin close to the graft union appears to occur

in the scion part. IAA seems to be strongly present in stocks (Fig. 37), but in closer images (Fig. 38) it is possible to see the less intense fluorescence in tissues closer to the wound, and a more intense fluorescence in tissues far from the wound.

3.8 Gene expression analysis

3.8.1 Extraction of total RNA

Before cDNA synthesis, extracted RNA quality was evaluated. The total RNA content was quantified by spectrophotometric measurements of absorbance at 260 nm and evaluated by using the A260/A280 ratio that should be close to 2.0, and the A260/A230 ratio that should be between 2.0 and 2.2 (Desjardins and Conklin, 2010). Results are presented in Table 11. In most samples, the ratio A260/A280 was lower than expected, but the ratio A260/A230 was much lower in all samples.

Table 11. Evaluation of RNA extracts quantity and purity.

Time	Sample		RNA (ng/µl)	A260/280 ratio	A260/230 ratio
T0	Canhota	Scion	144.0±163.4	1.84±0.09	1.09±0.15
	Bitter almond	Scion	9.70±2.52	1.98±0.16	0.42±0.35
		Stock	52.97±8.52	2.00±0.20	0.75±0.56
T1	Canhota X Bitter almond	Scion	29.37±32.50	1.53±0.27	0.77±0.32
		Union	36.60±28.46	1.79±0.10	0.85±0.31
		Stock	79.15±52.86	1.72±0.09	0.55±0.37
	Bitter almond X Bitter almond	Scion	104.13±100.56	1.72±0.21	0.92±0.41
		Union	19.73±0.75	1.95±0.29	0.49±0.39
		Stock	24.53±4.71	2.37±0.84	0.61±0.67
T2	Canhota X Bitter almond	Scion	9.90±6.95	1.94±0.28	0.13±0.08
		Union	8.40±1.57	1.62±0.50	0.42±0.17
		Stock	13.23±6.67	1.74±0.38	0.55±0.17
	Bitter almond X Bitter almond	Scion	7.60±3.04	2.01±0.05	0.33±0.18
		Union	9.17±1.31	1.82±0.46	0.37±0.29
		Stock	15.50±4.70	1.61±0.25	0.55±0.12

3.8.2 Gene expression quantification during graft union formation

Gene expression quantification was carried out at T0, T1 and T2 with Canhota x bitter almond and bitter almond x bitter almond micrografts. Samples collected at T0 belong to scions and stock cut but ungrafted. Micrografts collected at T1 and T2 were cut in three segments, belonging to scion, graft union and stock.

The primer's specificity test showed only one band with the expected sizes (between 327 bp and 463 bp) for each gene, ensuring primer specificity (Fig. 39).

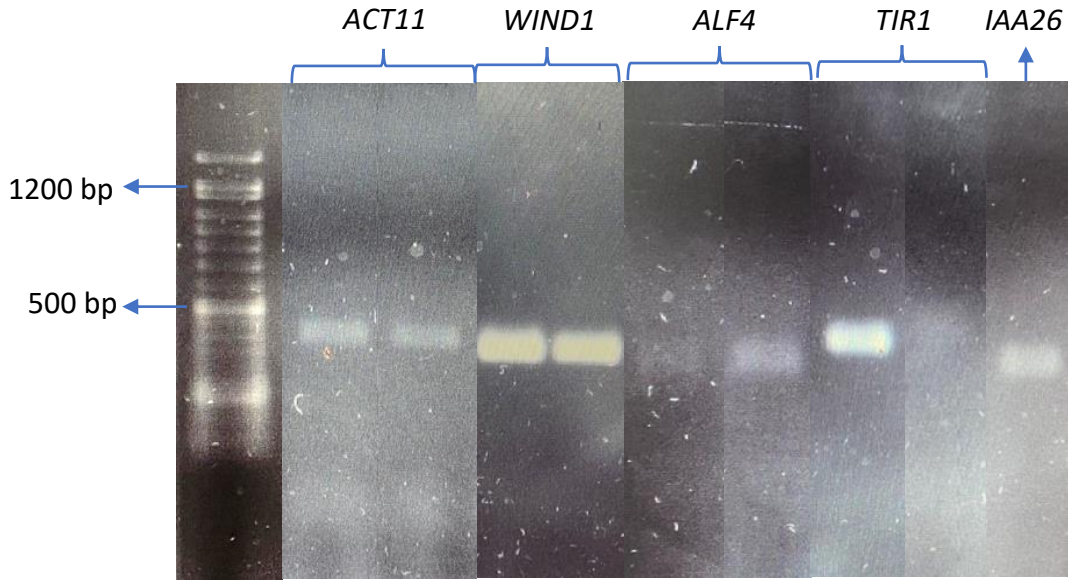

Figure 39. Primer's specificity test for *ACT11*, *WIND1*, *ALF4*, *TIR1* and *IAA26*.
Agarose gel electrophoresis showing only one band with the correct size.

In figure 40, relative gene expression data in micrografts segments from bitter almond x bitter almond and Canhota x bitter almond at T0, T1 and T2, is presented. Cq values obtained are presented in appendix I.

WIND1 gene expression was similar in both scions and stock at T0. In homografts a higher expression was observed at T2 in the scion, while in Canhota x bitter almond it was at T1 at the graft union. *WIND1* presented different expression tendencies at the graft union from the two combinations. In homografts graft union an increase was observed, while in Canhota x bitter almond the expression decreased.

In each homograft segment, *ALF4* presented the same expression tendency as *WIND1* in the respective segment, but for this gene the increase observed in the scion at T2 was significant ($p \leq 0.05$). Also, in graft unions from both combination *ALF4* presented the same expression behaviour as the one observed with *WIND1*.

Regarding *TIR1* expression in stock, a decrease overtime was observed in both combinations. In homografts scion and union, *TIR1* levels at T2 were higher, although in the scion were significantly higher ($p \leq 0.05$). In Canhota x bitter almond *TIR1* levels were not detected at the graft union at T2.

At the graft union *IAA26* presented the same tendency to decrease overtime in both combinations. However, *IAA26* levels in Canhota x bitter almond were higher than in bitter almond homografts. However, scions and stock, showed different dynamics. From T0 to T2, *IAA26* levels in homografts increased in the scion and decreased in the stock, and in Canhota x bitter almond, levels decreased significantly ($p \leq 0.05$) in the scion and increased in the stock.

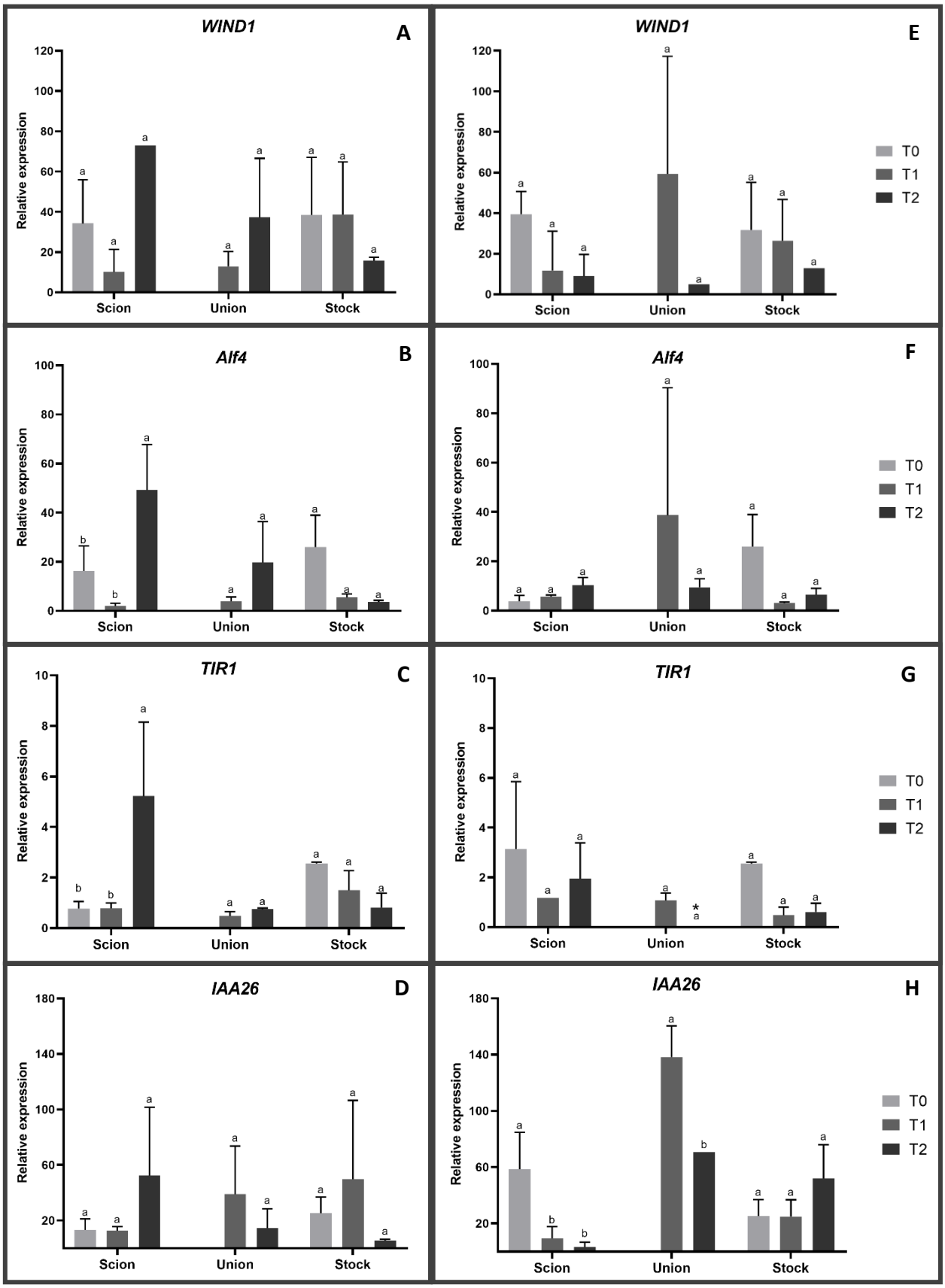

Figure 40. Gene expression quantification in bitter almond homografts (A, B, C, D) and Canhota x bitter almond micrografts (E, F, G, H) segments (scion, union and stock) at T0, T1

and T2. Values indicated with the same letter were not statistically different, at $p \leq 0,05$, using a Tukeys's multiple comparison test. *Indicates no gene expression detected.

4. Discussion

4.1 Morphological characterization of almond fruits

Almonds fruits in which the edible seed is the commercial product that can be consumed raw, roasted and in the confectionery industry (Martínez-Gomez *et al*., 2008). Fruit parameters are highly variable depending on the cultivar (Socias i Company *et al*., 2017). Thus, fruit characterization is very important for commercial purposes and to help producers decide which varieties to use, especially when considering traditional varieties. Among the most relevant fruit characteristics is fruit yield, which is determined by in shell kernel and almond weights.

In comparison to what has been described by Costa and Rosa (2020), Rabo de Zorra, Canhota and Ferraduel in shell kernels presented a higher weight, while Quinta de Valim and Gama Dura in shell kernels weighted less. Regarding kernel weight, all characterized varieties weighted less than what has been described by the previously mentioned authors for the same varieties. Rabo de Zorra presented the highest partition yield with 28.5%, even though it was lower than the partition yield recorded for commercial varieties, such as Antoñeta, Soleta and Lauranne, with 35%, 27-35% and 35-38% respectively (Hernandorena, 2021).

Even though it is a varietal characteristic, the presence of double kernels might change under influence of local culture conditions and variations of climatic conditions (Queirós and Sousa, 2017). This is in accordance with what was registered in Bota and Rabo de Zorra varieties for the presence of double kernels. Dry kernels, also a depreciative feature, were found in Quinta de Valim, Gama Dura and Canhota varieties.

Among the morphologically characterized varieties, Rabo de Zorra appear to be most advantageous, since it had the highest partition yield without the presence of double or dry kernels. On the other hand, Gama Dura presented the lowest partition yield, with the presence of double and dry kernels. Nevertheless, weight and size of in shell kernel and kernel from each variety might change each year (Queirós and Sousa, 2017), and further analysis should be made in successive years.

4.2 *In vitro* establishment of scions and rootstocks

One traditional way to obtain seedlings from almonds is based on the stratification of seeds at low temperatures for several months. I*n vitro* germination allows

seedlings to be obtained in a short period of time, regardless of the time of year (San and Yildirim, 2008).

In the first assay, the effect of a cold treatment on germination was evaluated with Antoñeta, Soleta and Lauranne varieties. The results indicate that the seed storage at low temperature had no significant influence on germination, seedling length, primary root length and the number of secondary roots. According to what the co-authors San and Yildirim (2008) described in almond, germination of the embryo axis can occur with or without cold treatment.

Also, the effect of growth regulators and explant type on germination was evaluated with Lauranne variety. When whole mature zygotic embryos were cultured on filter paper moistened with GA_3 (Table 2, condition 4) the significant longer primary roots ($p \leq 0.05$) might have resulted from the addition of GA_3 or from the influence of the cotyledons present in the initial explant. Despite the described possibility that the cotyledons and seed coat promote inhibition of germination (San and Yildirim, 2008; Sallom *et al.*, 2021), an 80 % germination rate of whole mature zygotic embryos was found. However, by the results showed here and in Sallom *et al.* (2021) isolation of embryo axis promotes higher germination rates.

Generally, a high ratio of auxin to cytokinin favours root regeneration, while a low ratio of auxin to cytokinin stimulates shoot regeneration (Moubayidin *et al.*, 2009). The addition of the cytokinin, BAP, to the medium in condition 3 (MS supplemented with 1 mg/l BAP and 0.5 mg/l GA_3), possibly led to a low ratio of auxin to cytokinin which resulted in the absence of primary root formation in Lauranne.

Even though the effect of PGRs and explant type had no significant influence on germination when different conditions were tested with Lauranne variety ($p > 0.05$), culture of embryo axis in MS medium with BAP and GA_3 allowed a higher germination rate and seedling length, which might be due to the role of GA_3 in dormancy break, seed germination and stem elongation (Bhattacharya, 2019). Condition 3 was used in the successful *in vitro* establishment of Portuguese traditional varieties and bitter almond seedlings. Despite the differences, bitter almond was also successfully established.

Commercial rootstock Rootpac® was *in vitro* established by the culture and development of apical and axillary meristems, a tissue with the ability to form shoots. In Imani and Abdollahi (2006) the establishment of hybrid rootstocks was also achieved by

meristem culture techniques. With the results here presented, apical distance appears to not have influence on shoot development.

4.3 Multiplication

Plant tissue culture is widely used for plant multiplication, where small pieces of tissue, termed explants, can be segmented and produce large numbers of plants in a short period of time under controlled conditions, regardless of the season (Hussain *et al*., 2012). In this work, one of the goals was to obtain large quantities of shoots from the established varieties, while characterizing their multiplication behaviour. Initially, multiplication conditions needed to be optimized, and so bitter almond seedlings were used to test different multiplication media described in the literature for almond.

M3 was described for *P. dulcis* unidentified varieties by Sanchéz *et al*. (2010) while M2 and M4 were described for *P. dulcis* cv. Nonpareil by Işikalan *et al.* (2008). BAP, a synthetic cytokinin that elicits shoot formation, was added to M2, M3 and M4, which resulted in a higher number of phytomers in comparison to the medium without cytokinin.

Upon the results obtained, M2 was selected for multiplication of traditional, commercial and bitter almond varieties. The selection was made on the observation that even though M3 and M4 allowed a higher number of phytomers, explants showed strong signals of hyperhidricity, resulting in a multiplication rate reduction or slowdown. However, despite the occurrence of hyperhidricity in M2 explants, this medium allowed the increase of the multiplication rate overtime.

Hyperhidricity results from metabolic and morphologic disorders that are caused by various culture factors in woody plants *in vitro* propagation. These factors could be the culture vessel, light intensity, poor transpiration, macro and micronutrient composition of the medium and high concentrations of cytokinin (Pérez-Tornero *et al*., 2001). The disorders are manifested mainly in the leaves but can be also present in stems and roots and the two major processes affected are photosynthesis and gas exchange (Ziv, 1991). For these reasons it is important to reduce hyperhidricity and assaying different PGRs with cytokinin activity or reducing BAP concentration throughout subcultures might be effective strategies on that matter, as it was seen in the subcultures

in MS medium supplemented with 0.1 mg/l BAP. This aproach should be further explored in almond varieties micropropagation optimization.

The micropropagation potential of bitter almond genotypes was also evaluated after 3 cycles of multiplication.

At T3, the number of phytomers ranged from 14 to 66 for genotypes 8 and 15, respectively. With this evaluation, it is possible to conclude that the genotypes tested might not have influenced multiplication rates, since even though multiplication increased at different rates for the different genotypes through the 3 multiplication cycles, at T3, multiplication rates came close, ranging from 78.6% to 88.9%. Even though here bitter almond genotypes did not affect the multiplication rates, some authors have reported the influence of genotype on *Prunus persica* (L.) Batsch (Al Ghasheem *et al*., 2018), *Prunus avium* L. (Matt et al., 2005) and *Arachis hypogaea L*. (Banerjee *et al*., 2007) micropropagation.

Significant differences between varieties regarding the number of shoots formed have been reported in almond cultivars (Channuntapipat *et al*., 2003). Multiplication of traditional, commercial and bitter almond revealed significant differences ($p \leq 0.05$), highlighting the micropropagation potential of bitter almond and the traditional variety Gama Dura. During multiplication some signals of hyperhidricity were observed in all varieties, and so, the evaluation of the full micropropagation potential of this varieties need further improvement of culture conditions as previously mentioned.

4.4 Rooting

During multiplication, shoots did not develop roots, and so *in vitro* rooting assays were established to evaluate the potential of IBA, charcoal and Fe-EDDHA on rooting.

MS medium contains iron in the chelated form of ethylenediamine tetraacetic acid iron (Fe-EDTA). This form is not stable, leading to a loss of initial Fe concentration (Dalton *et al*. 1983). Moreover, Fe-EDTA is photochemically degraded leading to the formation of formaldehyde, which is toxic for plant growth (Hangarter and Stasinopoulos, 1991). Using Fe-EDDHA instead of Fe-EDTA as chelating agent in MS medium has been reported to induce rapidly a greater formation of roots in GF-677 (Molassiotis *et al*., 2004; Antonopoulou *et al*., 2007).

AC is a porous material with a fine network of pores, large surface area and volume with a unique adsorption capacity (Baker *et al*., 1992). AC can be added to the medium to improve plant growth and development by adsorption of inhibitory substances, decrease phenolic oxidation and establishment of a darker environment. In several plants, rooting of micropropagated shoots was induced by AC alone or in combination with an auxin (Thomas, 2008).

Despite the *in vitro* rooting success reported with *Prunus* rootstocks by Molassiotis *et al*. (2004) and Antonopoulou *et al*. (2007), here *in vitro* rooting of bitter almond was not successful in any of the conditions tested in the first assay. In the second assay, GxN15, although with a small percentage, presented a 16.6% formation of potentially functional roots when cultured on MS medium supplemented with IBA (1 mg/l) and AC (1 g/l). This rooting recalcitrancy has also been described in the literature has one major problem in *P. dulcis* micropropagation that still needs improvement (Yildirim *et al*., 2010).

In a last attempt to promote root formation, bitter almond and GxN15 were used to test three *ex vitro* rooting conditions. The effect of the auxin IBA was evaluated due to the known roles of this hormone in lateral root development and formation of adventitious roots (Frick and Strader, 2017). The potential of a commercial rooting powder was also evaluated. As no shoot survival was observed, *ex vitro* rooting was not possible to be evaluated. The absent shoot survival observed during acclimatization might have resulted from the small size of the shoots and the presence of hyperhidricity (Rojas-Martínez *et al*., 2010).

4.5 Micrografting

The technical difficulties of micrografting and low success rates often associated (Hussain *et al*., 2014) requires large quantities of micropropagated shoots and stocks, and so the establishment of an optimized micropropagation protocol is of crucial importance. Thus, although the previous mentioned stages of this work were time consuming, they were of critical importance to get enough plant material to be tested in micrografting and molecular analysis stages.

4.5.1 Accessing scion genotype influence on micrografting success rates

According to the results, the influence of genotype on micrograft success appears to be variety dependent, being more relevant for Soleta than for Ferraduel. In Canhota it was not possible to establish this relation due to high incidence of contaminations.

Bitter almond potential was highlighted by the fewer lateral shoot formation in the stock and by the higher success of the majority of the micrografts established with bitter almond.

The stronger shoot formation observed in stocks from micrografts established with GxN15, was probably due to the establishment of these micrografts in culture medium optimized for the micropropagation of the stock. In exception of Canhota genotype 1, the remaining scions grafted onto GxN15 stock showed a potentially stronger graft union, since during transfer to *ex vitro* conditions, these micrografts showed fewer union break than the micrografts established with bitter almond stock. The stronger formation of lateral shoots from the stock close to the micrograft union in micrografts established with GxN15 might have contributed for the strengthening of the graft union.

In previous reports, almond micrografts established in MS medium without growth regulators showed a 50% survival rate, whereas establishment of micrografts in MS medium containing growth regulators resulted in 100% survival (Yildirim *et al*., 2010). Although this could be the cause for the loss of micrografts established with bitter almond during acclimatization, the lack of a root system and the small size could have also been crucial factors, affecting as well micrografts established with GxN15.

Upon the observation of strong lateral shoot formation from the stock in micrografts established with GxN15, the culture medium used in the following assay was MS medium without growth regulators.

It is worth mentioning that these varieties are usually grafted onto commercial rootstocks, such as, GF677, with which grafting can achieve 98.3% success (Atli *et al*., 2014).

4.5.2 Variety influence on micrografting success rate

Some causes of graft failure are incompatibility of graft partners, the use of desiccated or diseased scions, an inefficient grafting technique or bad vascular cambium alignment (Hartmann *et al*., 2011). *In vitro* grafting can also lead to failure, but here the main limitation is the technical skill required for manipulation of small sized plant material and delicate graft unions (Hussain *et al*., 2014).

Using *P. dulcis* cv. Achak, Ghorbel *et al*. (1998) reported 80% micrografting success. More recently, 100% micrografting success was reported for Ferraduel grafted onto bitter almond seedlings placed on MS medium (Yildirim *et al*., 2010). In the present work, Ferraduel grafted onto bitter almond resulted in 60% micrograft success at 21 days after grafting. The difference from the values presented in the literature highlights the importance of the root systems for micrografting success. However, these results also indicate that it is possible to establish *in vitro* micrografts without an established root system, what has previously been described (Chilukamarri *et al*., 2021).

Regardless of the absence of the root system, the traditional variety Canhota grafted onto bitter almond managed to achieve a 90% success rate at 21 days after grafting. Once again, the potential of bitter almond is emphasized due to higher success of Canhota and Ferraduel grafted onto this stock than grafted onto GxN15.

The micrografting technique used here did not used any support to improve micrograft stability, so, in the future, other techniques, such as the addition of gelling agents or the use of a sterilised silicon tube (Ashrafzadeh, 2020) could be tested to improve micrografting success.

4.6 IAA quantification

In previous works (Caeiro, 2015) that involved auxin quantification at the Laboratory of Plant Biotechnology of the Department of Life Sciences of the University of Coimbra (LBV-UC), the colorimetric quantification of endogenous IAA levels by using Ehrlich reaction (Anthony and Street, 1969), was selected as a good presumptive test before investigation with more precise and expensive analyses. In the present work,

Ehrlich reaction was used for the quantification of endogenous IAA levels before and after micrografting.

The significant differences observed between scions at T0 ($p \leq 0.05$) analysed in relation to the micrografting success, might indicate that potentially initial high IAA levels observed in bitter almond scion caused the earlier decrease in micrograft success in bitter almond homografts at 10 days after grafting.

Chen *et al.* (2017) compared IAA concentrations in compatible and incompatible *Litchi chinensis* graft unions and observed a slight decrease until 14 days after grafting, and an increase to the highest level at 30 days after grafting in compatible grafts. In this work, IAA levels decrease from T0 to T2 in scions and stocks from both combinations, so to evaluate if the results here obtained attend the tendency observed in the previously mentioned study, the quantification of IAA should be carried out in more time points after grafting.

Interestingly, according to the indication that IAA might promote graft compatibility (Chen *et al*., 2017) and the requirement of IAA to promote a vascular bridge ate the graft union (Lu, 2000), a tendency in which IAA levels are higher in the graft union followed by scion and then stock, was observed in both combinations. The decrease in auxin content in the stock could be explained by the destabilization of auxin transport from the shoot apex to the base upon severing the vasculature during grafting.

4.7 IAA immunolocalization

Immunolocalization of IAA has been described in investigations of IAA distribution in several plants to directly detect IAA molecules. This technique is based on the detection of specific targets, antigens, in biological samples by binding of specific antibody, which in turn is coupled to a marker. The marker can be coupled by many methods, but the one used in this work consisted in the addition of a secondary probe coupled to a marker (fluorescent dye), to the primary antibody after this last one was bounded to a specific target (Hoppert and Wrede, 2011). Accession of the antigen in tissues was achieved by cryosectioning and permeabilization.

It is important to highlight that immunolocalization images revealed some tissue fragmentation, what could have resulted in secondary antibodies trapped in the

fragments causing misleading fluorescence. This could have been improved by additional washes. Despite that, it was possible to observe IAA staining in some preserved tissues.

Close to the graft junction, the accumulation of auxin in the scion and the depletion in stock tissues, could be the result of transport changes upon wounding. Since auxin transport occurs from the upper part of the plant to the base, upon wounding the stem, during micrografting, auxin accumulates in the scion close to the wound, which in this case is close to the graft union, and a depletion of auxin starts to appear in the stock close the wound site until vascular connection. Auxin accumulation at the graft union here observed, could lead to regeneration and connection of vascular tissues (Aloni, 1987; Chen *et al.* 2017).

4.8 Gene expression quantification during graft union formation

The gene expression quantification by RT-qPCR may have two sources of variation: inherent technical or experimentally induced variation, and true biological variation (Bustin *et al*., 2009). An adequate reference gene selection is important to remove the technical variation as much as possible, to end up with only true biological changes. An inappropriate choice of reference gene can result in loss of accuracy, statistical significance, and power (Derveaux *et al*., 2009).

The reference gene chosen for this work might have not been the most adequate or stable enough throughout the developmental stages evaluated, since a high variability in the results was observed. In addition, other reference genes should be tested and new primer pairs design for a more accurate quantification of relative gene expression.

In micrografts, *callus* formation is one of the first events to occur as a response to wounding during graft union formation (Aloni *et al*., 2010). *WIND1* is a central regulator of wound-induced cellular reprogramming in plants, and the expression of this gene has been reported to induce cell dedifferentiation and proliferation to form *callus* in *Arabidopsis* (Iwase *et al*., 2011a; Iwase *et al*., 2011b). In the present work, Canhota x bitter almond appear to have a faster wound response than bitter almond micrografts, since in the first combination, *WIND1* showed a higher expression at T1 in the graft union, while in bitter almond homografts *WIND1* expression at the graft union only

increased at T2. Expression at the graft union is consistent with the activation of *WIND1* locally at the wound site, and in proliferating *callus* cells (Iwase *et al*., 2011).

In *Arabidopsis*, *WIND1* did not show a response in cut but ungrafted rootstocks (Melnyk *et al*., 2015). Here, these indications were not observed since in cut but ungrafted stocks, *WIND1* was expressed in similar levels in the scions and stocks. The similar levels of *WIND1* in scion and stock at T0 could be associated to stress caused by *in vitro* culture, particularly, mechanical injuries and wounding during subcultures (Desjardins *et al*., 2009).

After grafting, *WIND1* was found to be upregulated above the graft junction and later below (Melnyk *et al*., 2015). Here, homografts revealed a possible upregulation firstly in the stock and later on the scion and graft union, while in Canhota x bitter almond this gene was firstly up regulated at the graft union and then downregulated in all segments.

According to Iwase *et al*. (2017), in addition to *callus* formation, *WIND1* promotes shoot regeneration by upregulating the expression of *ENHANCER OF SHOOT REGENERATION* (*ESR1*). In fact, during micrograft development, shoot formation close to the graft junction was observed. To access the influence of *WIND1* expression at the graft union on shoot regeneration, further studies on *ESR1* levels are required.

At the graft union, overtime, *ALF4* follows the same tendency of gene expression as *WIND1*, increasing in homografts and decreasing in Canhota x bitter almond. This information is according to the formation of *callus* induced by *WIND1 (*Iwase *et al*., 2011b*)* and the described role of *ALF4* in *callus* development (Melnyk *et al*., 2015), thus, gene expression of both genes can be directly related.

According to Melnyk *et al*. (2015) *TIR1* and *ALF4* are required below the graft junction to perceive auxin and promote vascular reconnection. On the contrary, the present results show a decrease from T0 to T2 for both gene in the stock of the two combinations. Nevertheless, the described requirement of *TIR1* and *ALF4*, requires further studies in more restricted stock tissues closer to the graft union, since the graft union fragment analysed incorporates scion and stock tissues. It also appears that *ALF4* and *TIR1* are required in the scion of homografts due to the significant increase registered at T2.

The expression of *TIR1* relates to the expression of *IAA26* in the sense that when auxin levels rise, auxin enhances binding of TIR1/AFB to the domain II of Auxs/IAAs, and

results in ubiquitination and further degradation of these transcriptional repressors (Petrasek and Friml, 2009). The amount of endogenous TIR1 appears to be rate-limiting for a proper auxin response, and when present at sufficiently high levels, TIR1 mediates Aux/IAA degradation (Dos Santos Maraschin *et al*., 2009).

According to the results obtained in this work, due to significantly higher *TIR1* levels, a proper auxin response might have occurred in the scion of bitter almond homografts at T2. The proper auxin response could have required a tighter control by the Aux/IAA, which translates into the simultaneous *IAA26* increased levels. On the contrary, the possible absence of a proper auxin response might have occurred in the scion of Canhota x bitter almond due to the significant decreased in *IAA26* levels. In the remain results, *TIR1* levels might have not been enough for a proper auxin response but could have been sufficient to mediate Aux/IAA degradation, what resulted in a decrease in *IAA26* levels while *TIR1* slightly increased. Comparing *IAA26* levels at the graft union of the two combinations, Canhota x bitter almond presented higher levels, what could be interpreted as a more intense attempt for obstruction of graft union formation by blocking auxin response when two different varieties are grafted.

In summary, homografts appear to have a simultaneous wound response and auxin response at T2, meanwhile in Canhota x bitter almond a possible earlier wound response was observed at T1, and a proper auxin response was not observed in the time points analysed.

5. Concluding remarks and future prospects

Micrografting is a potential important biotechnological tool for the development of efficient *in vitro* protocols of almond propagation. Although almond micrografting protocols have been described, until this moment the establishment of micrografts with Portuguese traditional varieties has not been reported. Also, the molecular mechanisms behind graft union formation and scion-rootstock communication are still scarce. While aiming at the molecular characterization of scion-rootstock interaction in almond micrografts this work allowed the establishment and characterization of traditional and bitter almond varieties multiplication potential, contributing for the valorisation of endogenous resources.

In vitro establishment of almond varieties can be achieved through isolation of the embryo axis from seeds stored at room temperature, and cultured in MS medium supplemented with 1 mg/l GA_3 and 0.5 mg/l BAP. Micropropagation of seedlings can be achieved in MS medium supplemented with 1 mg/l BAP. However, culture conditions might need further improvement since hyperhidricity was present and can affect micropropagation ability. Bitter almond and Gama Dura stood out with greater micropropagation potential.

In the tested conditions rooting and acclimatization was not achieved. Further studies are required to induce rooting, a key step for survival in *ex vitro* conditions.

Regarding the influence of the genotype on micrograft success, it appears to be variety dependent, however, this assay requires the evaluation of a higher number of genotypes. A 90% micrograft success was achieved in Canhota x bitter almond 21 days after grafting. The higher success of micrografts established with bitter almond in comparison to micrografts established with the commercial rootstock GxN15, contributed for the valorisation of traditional and uncultivated varieties. For a greater micrograft success rates, micrografts stability could be improved by testing other techniques, such as the addition of gelling agents or sterilized silicone tubes. Also, evaluation of field grafting success with the same combinations should be performed.

The differences observed in IAA quantification in bitter almond and Canhota scions before micrografting might correlate to the grafting success of micrografts, indicating that higher IAA initial levels could cause an earlier and more accentuated decrease in grafting success. This assay should be improved by increasing the number of

biological replicates. Also, this colorimetric quantification of IAA could be complemented with a more precise method, such as, high performance liquid chromatography (HPLC).

From IAA immunolocalization it appears to accumulate at the graft union in the scion part, contributing to the regeneration and vascular connection since scion and stock appear to be connected. However, IAA immunolocalization requires optimization of tissue fixation and washing steps during IAA immunostaining to avoid misleading fluorescence.

Gene expression quantification revealed a possible earlier wound response and absence of auxin-related response in Canhota x bitter almond micrografts. In scions of bitter almond homografts the increased levels of *WIND1*, *ALF4*, *TIR1* and *IAA26* indicate a possible simultaneous wound and auxin-related responses. Reproduction of this analysis in more time points after micrografting could reveal additional information about the responses occurring after grafting. Due to the downregulation of *TIR1* and *AFL4* in the stock of both combinations here observed, and the described role in the rootstock close to the graft junction, further studies on the expression of these genes will be required. To ensure the reliability of these observations, the results should be improved by testing additional reference genes and by designing new primer pairs.

The results presented in this work contribute to a better understanding of the role of IAA in grafting and how almond micrografts respond to wound healing and auxin to promote vascular reconnection. Although some of the methodologies need further improvement, the results may be a foundation for future studies, even for other *Prunus* spp.

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Appendix I

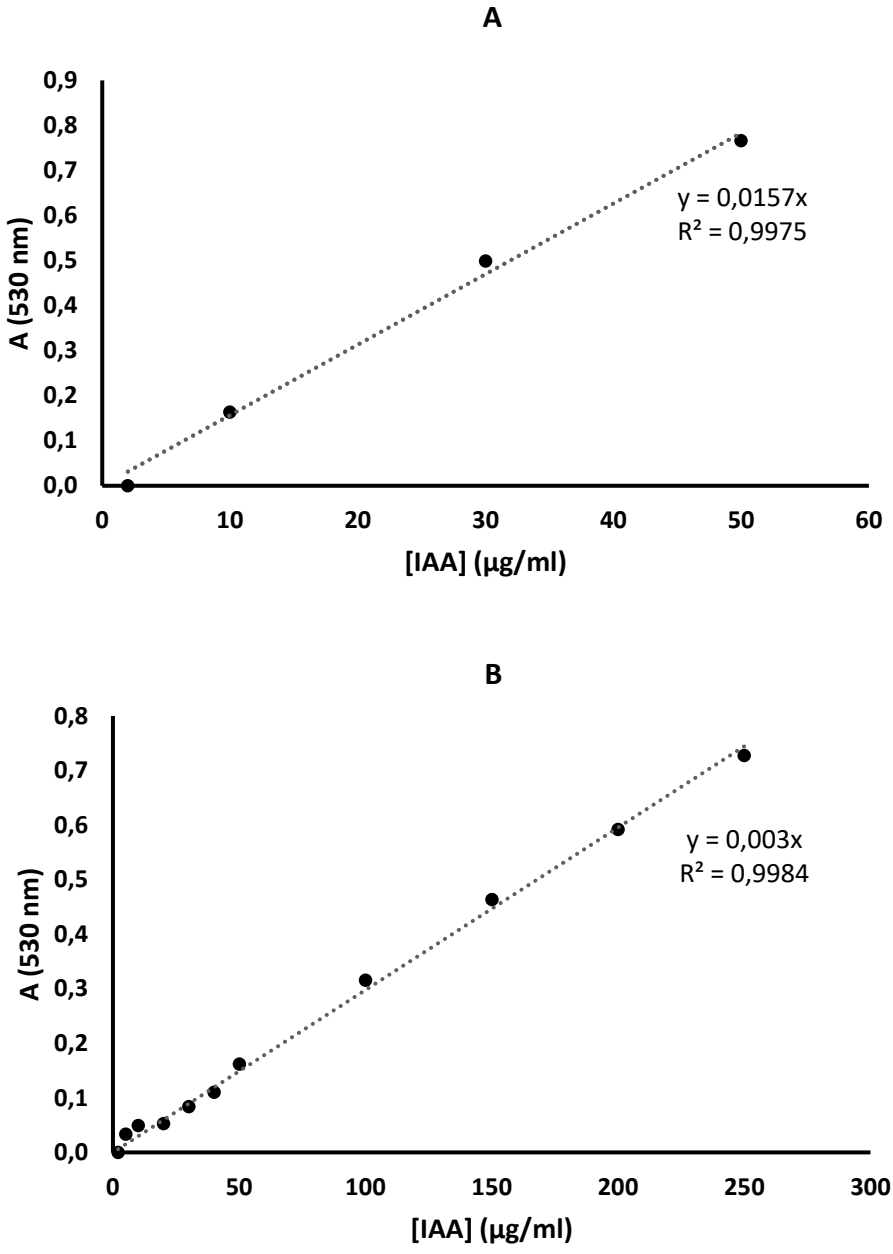

Figure 41. Standard curves for IAA quantification using buffered solution of 2, 10, 30 and 50 µg/ml at T0 (A) and buffered solution of 2, 5, 10, 20, 30, 40, 50, 100, 150, 200, and 250 µg/ml at T2 (B). Absorvance was measured at 530 nm.

Appendix II

Table 12. Cq values obtained from RT-qPCR for *ACT11* and *ALF4* with two technical replicates and three biological replicates used for the measurement of relative expression.

Time	Variety/micrograft	Micrograft segment	*ACT11*			*ALF4*		
			1	2	Mean	1	2	Mean
T0	Canhota	Scion	37,75	35,94	36,84	34,56	33,92	34,24
			33,79	32,98	33,39	33,43	32,49	32,96
			32,32	31,32	31,82	29,57	30,10	29,84
	Bitter almond	Scion	34,54	33,00	33,77	31,08	30,46	30,77
			35,10	36,91	36,00	32,66	32,01	32,34
			35,05	35,68	35,37	30,58	30,57	30,58
	Bitter almond	Stock	36,92	37,31	37,11	30,62	30,12	30,37
			36,73	36,37	36,55	31,50	31,34	31,42
			33,56	33,27	33,41	29,43	29,25	29,34
T1	Canhota x Bitter almond	Scion	34,10	34,90	34,50	32,19	31,46	31,82
		Union	36,07	35,40	35,74	32,95	32,51	32,73
		Stock	35,64	34,70	35,17	34,09	32,58	33,33
		Scion	36,17	37,48	36,82	33,72	34,99	34,36
		Union	35,42	35,07	35,24	33,13	30,84	31,99
		Stock	33,14	33,75	33,44	31,56	32,19	31,88
		Scion	37,54	35,38	36,46	33,94	34,23	34,09
		Union	35,96	32,28	34,12	27,14	27,85	27,50
		Stock	33,14	32,79	32,96	31,20	31,78	31,49
	Bitter almond x Bitter almond	Scion	30,87	31,07	30,97	30,51	31,11	30,81
		Union	32,64	32,50	32,57	30,83	29,26	30,05
		Stock	32,27	31,67	31,97	29,87	29,75	29,81
		Scion	31,40	31,38	31,39	30,15	29,71	29,93
		Union	32,02	31,76	31,89	30,42	30,90	30,66
		Stock	34,55	34,55	34,55	32,38	31,35	31,86
		Scion	*	37,06	37,06	*	*	
		Union	33,03	33,05	33,04	31,01	31,34	31,17
		Stock	37,80	37,53	37,66	35,87	35,31	35,59
T2	Canhota X Bitter almond	Scion	36,33	35,76	36,04	32,40	33,67	33,03
		Union	*	36,46	36,46	33,21	34,07	33,64
		Stock	32,00	31,56	31,78	29,30	29,86	29,58
		Scion	34,27	33,80	34,03	30,59	31,14	30,87
		Union	34,75	35,61	35,18	31,09	32,12	31,60
		Stock	*	*		35,47	36,21	35,84
		Scion	36,37	35,44	35,91	32,01	32,22	32,11
		Union	*	38,42	38,42	37,62	37,05	37,34
		Stock	34,22	34,91	34,57	30,99	32,06	31,52
	Bitter almond X Bitter almond	Scion	*	38,81	38,81	34,14	37,05	35,60
		Union	33,90	36,57	35,24	30,27	30,25	30,26
		Stock	30,77	31,11	30,94	28,88	29,49	29,19
		Scion	34,91	37,52	36,21	30,62	31,44	31,03
		Union	35,30	35,32	35,31	31,29	33,38	32,33
		Stock	30,36	30,51	30,44	28,61	28,03	28,32
		Scion	35,27	36,14	35,70	29,53	29,95	29,74
		Union	*	*		34,06	34,97	34,52
		Stock	*	36,60	36,60	35,67	34,14	34,91
No template control 1			*	*		*	36,97	
No template control 2			36,74	*		38,73	*	

* No Cq detected.

Table 13. Cq values obtained from RT-qPCR for *TIR1* and *IAA26* with two technical replicates and three biological replicates used for the measurement of relative expression.

Time	Variety/micrograft	Micrograft segment	*TIR1*			*IAA26*		
			1	2	Mean	1	2	Mean
T0	Canhota	Scion	*	*		*	37,04	37,04
			30,10	32,00	31,05	26,99	27,24	27,12
			32,14	30,91	31,52	26,29	26,72	26,50
	Bitter almond	Scion	34,27	35,02	34,65	31,28	30,67	30,98
			38,31	35,89	37,10	32,50	32,77	32,63
			37,67	35,94	36,81	31,07	30,73	30,90
	Bitter almond	Stock	36,18	32,42	34,30	29,20	30,22	29,71
			36,71	33,65	35,18	32,58	32,34	32,46
			33,42	30,76	32,09	28,38	28,32	28,35
T1	Canhota x Bitter almond	Scion	34,40	34,15	34,27	30,38	30,75	30,57
		Union	36,14	36,23	36,18	28,51	28,74	28,63
		Stock	35,35	35,47	35,41	27,53	32,30	29,91
		Scion	*	*		39,49	*	39,49
		Union	35,72	34,23	34,97	27,78	28,06	27,92
		Stock	33,80	36,19	34,99	27,53	30,61	29,07
		Scion	*	*		34,68	*	34,68
		Union	33,10	34,43	33,76	27,26	38,91	33,09
		Stock	33,22	36,66	34,94	28,72	29,25	28,99
	Bitter almond x Bitter almond	Scion	31,66	31,55	31,61	27,63	27,50	27,57
		Union	33,40	33,92	33,66	26,04	26,50	26,27
		Stock	31,06	33,03	32,05	25,54	31,79	28,66
		Scion	30,95	32,04	31,49	26,75	28,27	27,51
		Union	33,52	33,65	33,58	28,25	27,65	27,95
		Stock	33,55	33,48	33,52	27,73	28,39	28,06
		Scion	39,73	*	39,73	*	35,98	35,98
		Union	31,18	36,13	33,65	28,54	37,46	33,00
		Stock	36,20	34,11	35,15	33,03	35,72	34,38
T2	Canhota X Bitter almond	Scion	36,25	36,06	36,16	*	33,20	33,20
		Union	*	*		*	*	
		Stock	32,48	34,12	33,30	26,65	*	26,65
		Scion	32,46	37,29	34,88	32,46	34,66	33,56
		Union	38,28	*	38,28	29,03	*	29,03
		Stock	*	*		*	*	
		Scion	*	*		39,00	31,76	35,38
		Union	*	*		*	*	
		Stock	35,63	*	35,63	27,55	29,37	28,46
	Bitter almond X Bitter almond	Scion	36,63	34,32	35,47	29,51	37,24	33,37
		Union	36,68	34,53	35,60	36,69	29,35	33,02
		Stock	32,92	31,48	32,20	30,80	25,82	28,31
		Scion	33,35	*	33,35	34,10	30,07	32,08
		Union	36,25	35,33	35,79	31,58	29,84	30,71
		Stock	30,21	30,10	30,16	30,55	25,67	28,11
		Scion	33,03	35,07	34,05	28,50	30,01	29,26
		Union	*	*		*	*	
		Stock	38,61	*	38,61	*	*	
No template control 1			*	*	*	*	*	
No template control 2			*	*	*	39,02	36,67	37,84

* No Cq detected.

Table 14. Cq values obtained from RT-qPCR for *WIND1* with two technical replicates and three biological replicates used for the measurement of relative expression.

Time	Variety/micrograft	Micrograft segment	*WIND1*		
			1	2	Mean
T0	Canhota	Scion	*	*	
			28,58	27,15	27,86
			27,33	26,85	27,09
	Bitter almond	Scion	29,10	28,71	28,90
			31,78	32,33	32,06
			29,52	29,50	29,51
	Bitter almond	Stock	30,23	29,22	29,73
			32,75	31,98	32,36
			28,05	27,03	27,54
T1	Canhota x Bitter almond	Scion	29,73	29,09	29,41
		Union	28,99	28,53	28,76
		Stock	26,79	26,24	26,52
		Scion	*	38,88	38,88
		Union	30,88	30,01	30,44
		Stock	28,05	28,13	28,09
		Scion	36,61	36,23	36,42
		Union	29,30	29,77	29,54
		Stock	29,38	29,37	29,38
	Bitter almond x Bitter almond	Scion	29,82	29,97	29,89
		Union	28,19	28,14	28,16
		Stock	27,71	27,55	27,63
		Scion	27,19	27,25	27,22
		Union	28,59	28,47	28,53
		Stock	28,65	28,78	28,71
		Scion	37,52	*	37,52
		Union	30,56	29,76	30,16
		Stock	33,38	35,38	34,38
T2	Canhota X Bitter almond	Scion	32,47	36,22	34,35
		Union	*	*	
		Stock	28,15	28,05	28,10
		Scion	32,17	33,64	32,90
		Union	35,34	30,40	32,87
		Stock	38,56	*	38,56
		Scion	31,37	31,62	31,50
		Union	*	*	
		Stock	27,06	27,59	27,32
	Bitter almond X Bitter almond	Scion	32,64	31,66	32,15
		Union	30,23	28,52	29,38
		Stock	27,43	26,27	26,85
		Scion	30,82	29,23	30,02
		Union	31,44	31,09	31,26
		Stock	26,83	26,32	26,58
		Scion	27,78	27,60	27,69
		Union	34,55	*	34,55
		Stock	*	*	
No template control 1			*	*	*
No template control 2			*	*	*

* No Cq detected.