Ana Carolina Simões Pedrosa

Genetic variability analysis of Tamarillo (*Solanum betaceum* (Cav.)) and optimization of micropropagation conditions

Tese de Mestrado em Biotecnologia Vegetal
orientada por Professor Dr. Jorge Manuel Leal Canhoto e Professor Dr. Jorge Ferreira
Departamento de Ciências da Vida da Universidade de Coimbra

29 de Julho de 2016

Universidade de Coimbra

Ana Carolina Simões Pedrosa

Genetic variability analysis of Tamarillo (*Solanum betaceum* (Cav.)) and optimization of micropropagation conditions.

University of Coimbra

2016

Acknowledgements

Em primeiro lugar gostaria de agradecer ao meu orientador, Professor Doutor Jorge Canhoto, por me ter integrado no seu grupo de investigação, por todas as ideias, pela confiança depositada e pelo apoio. Agradeço de igual forma, ao meu coorientador, Professor Doutor Jorge Ferreira, e à Filipa, por todo o esclarecimento de dúvidas e apoio.

À Sandra Correia pela disponibilidade que teve comigo, pela troca de ideias, constante e pelo apoio, ao longo deste ano.

À professora Justina Franco, pelo indispensável auxílio e pelo conhecimento transmitido a nível experimental e pessoal.

Aos meus pais e irmão, pelos ensinamentos que me transmitiram, por todo o apoio e por me terem proporcionado esta oportunidade. Sem vocês não seria possível.

À minha "Estina", por me ter ensinado os verdadeiros valores de humildade e de sacrifício. Que um dia seja tão forte como tu e que transmita o mesmo aos meus. Obrigada

À minha Carolina, pela amizade e apoio incondicional, por todas as partilhas diárias, pelo companheirismo, pela força de espírito que possui, por tudo e por nada, "my person".

À minha família adoptiva, Isabel, Fernando e Afonso, por todo o amor.

Aos meus amigos, em especial à Filipa Cerveira, Rafaela, João, Emanuel, Eduardo, Anita, Filipa Borges.

À Sara, pela amizade, por toda a motivação transmitida e por acreditar sempre que sou capaz de mais e melhor.

À Íris, pela sua energia contagiante, pela garra que possui e me transmitiu, pelo seu positivismo, por partilhar "breakdowns" comigo, por ter um coração gigante e por toda a ajuda nos pormenores da tese. Aguardo ansiosamente que o futuro te brinde com "coisas" maravilhosas.

Ao João Martins e à Patrícia, pela constante ajuda e boa disposição.

Ao Professor Xavier, por todo o apoio prestado.

Aos meus colegas de mestrado, Ana Teresa, Maria João, Pedro, Danielly, Xavier, Bruno e Filomena.

Ao André, não só por toda a transmissão de conhecimentos, mas também pela vivacidade com que os transmitiu. Por toda a paciência e pelo fascínio sentido quando traçou algumas das experiências. Que pessoas como tu elevem a ciência a outro nível e sejam reconhecidas.

À malta do crossfit, principalmente à Margarida, por aturar o meu mau humor.

Ao meu padrinho, meu confidente, que me fez acreditar que as coisas não acontecem por acaso e tudo na vida tem um propósito. Que me deu forças para concluir mais uma etapa de vida, mesmo não estando presente. Sei que estarias orgulhoso da pessoa que me tornei e de tudo o que alcancei, desde que a tua ausência se fez sentir. Obrigada por todos os ensinamentos e pela força que me transmitiste.

Contents

Acknowledgements iii

Abbreviations vii

Abstract.. viii

Resumo x

1.Introduction 1

1.1 Context of work 2

1.2 *Solanum betaceum* Cav. (tamarillo).. 3

1.2.1 Origin, botanical, morphological and structural characterization 3

1.2.2 Area of distribution 4

1.2.3 Fruit characterization and postharvest factors that affect fruit quality.. 5

1.2.4 Environmental requirements 8

1.2.5 Nutritional value and health benefits 9

1.3 Potential improvements through breeding strategies.. 10

1.3.1 Tamarillo propagation methods 10

1.3.2 Micropropagation of tamarillo 10

1.4 Molecular analysis: Genetic assessment studies in *Solanum betaceum* (Cav.) 14

1.5 CMF as an improving factor to *in vitro* culture.. 15

1.6 Aims.. 18

2. Material and methods 19

2.1 Physical and morphological analysis of tamarillo fruits.. 20

2.1.1 Plant Material and origin of fruits.. 20

2.1.2 Harvest and pre-sample preparation.. 21

2.1.3 Parameters evaluated.. 22

2.2 Genetic assessment studies through the use of molecular markers 25

2.2.1 Plant Material and DNA extraction.. 25

2.2.2 Random amplified polymorphic DNA (RAPD) 25
2.2.3 Diversity estimates 26
2.3 Culture conditions improvement through the use of CMF 27
2.3.1 Production of microfibrillated cellulose.. 27
2.3.2 Production of CMF films for absorption and releasement assays.................. 29
2.3.3 Production of CMF films for diffusion assays 30
2.3.4 Ehrlich reaction 30
2.4 CMF as support to *in vitro* culture... 31
2.5 Subculture of non-embryogenic callus (NEC) in CMF films.............................. 31
2.6 Statistical analyses 32
3. Results 33
3.1 Physical and morphological analyses of tamarillo fruits 34
3.2 Genetic assessment studies in tamarillo using of molecular markers................... 39
3.2.1 Random amplified polymorphic DNA (RAPD) 39
3.3 Culture conditions improvement through the use of CMF 43
3.3.1 Absorption, CMF- IAA releasement and diffusion....................................... 43
3.4 CMF as support to *in vitro* culture... 44
3.5 Subculture of NEC in CMF films 45
4. Discussion.. 48
4.1 Physical and morphological analysis of tamarillo fruits...................................... 49
4.2 Genetic assessment studies in *S. betaceum* through the use of molecular markers.
.... 52
4.3 Culture conditions improvement through the use of CMF 53
4.4 Use of CMF as an alternative support to *in vitro* culture...................................... 54
5. Conclusions and Future Perspectives 56
6. References 60

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
bp	Base pairs
BAP	6-benzylaminopurine
CMF/MFC	Cellulose microfibrillated / Microfibrillated cellulose
DNA	Deoxyribonucleic acid
dNTP's	Deoxyribonucleotides (datp, dctp,dgtp and dttp)
IAA	Indole-3-acetic acid
$MgCl_2$	Magnesium chlorid
MS	Murashige and Skoog culture medium
NEC	Non-embryogenic callus
NFC	Nanofibrillated cellulose
OPC	Operon Technologies Kit C, sequences of arbitrary primers
PCR	Polymerase chain reaction
PGRs	Plant growth regulators
RAPD	Random amplified polymorphic DNA
SE	Somatic embryogenesis
TCA	Trichloroacetic acid
Taq polymerase	Enzyme originally isolated from the bacteria Thermusaquaticus

Abstract

Tamarillo (*Solanum betaceum* (Cav.)), Solanaceae, also known as tree tomato or "tomate de la Paz" is an Andean small tree cultivated for its appetizing and juicy fruits, having an important role for international export in New Zealand. Tamarillo fruit is becoming increasingly relevant to our market and to answer consumer's requirements physical, morphological and chemical profiles were accessed for red (C1, C3, PC, PM, TS, TC, TCQ), golden-yellow (C5 and C9) and orange (C7) cultivars and compared to a standard red cultivar (TCOL). Fruit quality was determined through a series of parameters, such as firmness, weight, caliber (fruit diameter and length), moisture content, SSC (soluble solid content), titratable acidity (TA) and its linked acids (malic and citric). Related quality factors such as peduncle and calyx were measured as well.

Regarding consumer's preferences, it was assumed that weight, firmness and sweetness were preponderant factors for fruit evaluation. In weight measurements TC variety presented the highest values (71.0 g), whereas C5 variety revealed the maximum values for firmness (84.1%), exceeding the standards (77.3%) and PC produced the sweetest fruits.

Since the information available is scarce on the characterization of genetic resources and breeding of this neglected crop, a more detail study was carried out and the genetic diversity of 16 tamarillo genotypes (4 adult trees - C1, C3, C5 and C7 and 12 hybrids) through the use of molecular markers (RAPDs), was tested. Twenty OPC primers were tested and only 4 (OPC 6, OPC 11, OPC 13 and OPC 15) exhibited polymorphism, scoring a total number of 48 polymorphic bands. The results showed clear RAPD banding patterns and OPC 11, 13 and 15 revealed the highest percentage of polymorphism (50%). To study the genetic similarity among the population, similarity index by Jaccard's coefficient was generated using UPGMA (Unweighted Pair-Group

Method with Arithmetical Averages). Similarity index ranged from 23.5% to 89.5%. Regarding only adult genotypes, C1 and C9, shared more traits with all samples, respectively, 58.63% and 61.58%. To support similarity indices values, a dendogram of hierarchical analysis was generated by MEGA 7 software.

Tamarillo propagation can be performed either by classical methods or through *in vitro* techniques such as somatic embryogenesis, being a significant biotechnological tool for protocols optimization. In this work, it was tried to improve *in vitro* culture conditions through the use of a bio-based material, *i.e.*, cellulose microfibrillated (CMF). Its use as a substitute to the standard filters reveled ineffective efforts, since calluses developed in CMF suffered a reducer mass improvement. Contrarily, as a complement to *in vitro* propagation CMF displayed positive outcomes, once shoots height and nodal segments were superior in comparison with the standard.

Overall, taking into account the several varieties analyzed for its physical, morphological and chemical evaluation, there are good prospects for the selection of tamarillo for quality improvement, although breeding programs and production strategies are required. In terms of genetic assessment studies using molecular markers, RAPD was suitable for an initial approach to tamarillo characterization. Lastly, the first approach of using environmental friendly and sustainable materials, such as CMF, did not improved meaningfully *in vitro* culture conditions. Although, the results obtained suggest that this material could have potential for other applications in Plant Biotechnology.

Keywords: CMF, fruit, genotypes, *in vitro* culture, RAPD, tamarillo

Resumo

Tamarillo (*Solanum betaceum* Cav.), uma solanácea, também designado como árvore tomate ou “tomate de la Paz” é uma árvore de porte pequeno da região dos Andes cultivada pelos seus frutos apetitosos e suculentos, possuindo distinta importância para a Nova Zelândia, em termos de mercado de exportação. Os seus frutos têm ganho uma crescente relevância no nosso mercado e, de forma a responder às necessidades do consumidor, perfis físicos, morfológicos e químicos foram delineados para as variedades vermelhas (C1, C3, PC, PM, TS, TC, TCQ), amarela (C5 e C9) e laranja (C7). De forma a completar esta informação, uma referência pertence à variedade vermelha (TCOL) foi usada como termos de comparação.

A inerente qualidade dos frutos foi determinada através de uma série de parâmetros, tais como firmeza, peso, calibre (diâmetro e comprimento do fruto), matéria seca, TSS (teor de sólidos solúveis), acidez titulável (TA) e os ácidos orgânicos inerentes (ácido málico e cítrico). Fatores indiretamente relacionados com a qualidade, especificamente o pedúnculo e o cálice foram, também, avaliados.

Tendo em conta as preferências do consumidor, foi assumido que o peso, a firmeza e o teor de açúcar foram fatores preponderantes para avaliação dos frutos. Nas avaliações referentes ao peso, a variedade TC apresentou os valores mais elevados (71,0 g), enquanto em termos de firmeza, a variedade C5 destacou-se (84,1%), tendo assim excedido os valores de referência (77,3%). Tendo em conta uma palatibilidade menos acídica, a variedade PC apresentou os melhores índices.

Uma vez que existe pouca informação disponível a cerca da caracterização dos recursos genéticos e melhoramento desta cultura, um estudo mais detalhado foi solicitado. Assim, a diversidade genética de 16 genótipos de tamarilho, 4 correspondendo a árvores adultas e 12 a híbridos, foi realizada, usando marcadores moleculares (RAPD). Vinte

primers foram testados, mas apenas 4 demonstraram polimorfismo, gerando 48 bandas polimórficas. Os resultados demonstraram bandas nítidas, em que os *primers* OPC 11, 13 e 15 exibiram a maior percentagem de polimorfismo (50%). De forma a verificar a similaridade genética dentro da população, o índice de similaridade de Jaccard foi gerado através de UPGMA. Posto isto, o índice de similaridade oscilou de 23,5% a 89,5%, quando os valores de todas as amostras foram cruzados. Tendo em conta, apenas, os genótipos das árvores adultas, C1 e C9 destacaram-se por partilhar mais características com todas as amostras, tendo sido de 58,6% e 61,5%, respetivamente. De forma a suportar esta análise, um dendrograma com classificação hierárquica de todas as amostras, foi gerado através do programa MEGA 7 software.

A propagação de tamarilho pode ser realizada através de técnicas clássicas ou através de técnicas *in vitro*, tais como a embriogénese somática, sendo esta um recurso biotecnológico com elevada relevância em vista para otimização de protocolos. Neste trabalho, visou-se melhorar as condições da cultura *in vitro* através do uso de materiais de base biológica e renovável, ou seja, celulose microfibrilada (CMF). O seu uso sob forma de substituto dos filtros convencionalmente utilizados evidenciou ser ineficaz, uma vez que, os calos desenvolvidos sobre a película de CMF apresentaram um crescimento mais reduzido. Pelo contrário, como um complemento à propagação *in vitro* o uso de CMF revelou resultados positivos, uma vez que foi demonstrado um crescimento superior dos rebentos e um maior número de segmentos nodais, quando comparados com os rebentos dos controlos.

De forma geral, tendo em conta as várias variedades analisadas através da sua avaliação física, morfológica e química existem boas perspetivas para a seleção do tamarilho. Apesar disto, programas de melhoramento e estratégias de produção são necessárias. Em termos de estudos genéticos através do uso de marcadores moleculares,

RAPD demonstrou ser uma boa abordagem inicial para a caracterização do tamarilho. Por fim, a primeira abordagem do uso de materiais ambientalmente sustentáveis, como CMF não melhorou claramente as condições de cultura *in vitro*. Apesar disto, os resultados obtidos sugerem que este material, possivelmente, demonstra potencial para outras aplicações a nível de Biotecnologia vegetal.

Palavras-chave: CMF, cultura *in vitro*, fruto, genótipos, RAPD, tamarilho

1.Introduction

1.1 Context of work

In the last 15 years several lines of research have carried out at the Laboratory of Plant Biotechnology of the CEF (Centre for Functional Ecology) trying to understand the biology of tamarillo and developing new approaches to improve this species. Hence, protocols for micropropagation of this species were developed, the process of somatic embryogenesis induction was deeply investigated, a protocol for protoplast isolation has been developed, methods to induce tetraploidy were established and assays of hybridization are being carried out. However, as achievements on the understanding and breeding of this species develops, new questions arise that need to be answered. Firstly, due to economic importance that relies on tamarillo fruits, a broad morphologic and physical characterization was conducted. Secondly, this research outlined genetic characterization of tamarillo trees, obtained formerly in our lab, to test the occurrence of both molecular genomic variation and genetic conservation, among hybrids and adult plants. Finally, we aimed to obtain improved protocols for *in vitro* propagation using sustainable materials (CMF).

1.2 *Solanum betaceum* Cav. (tamarillo)

1.2.1 Origin, botanical, morphological and structural characterization

Tamarillo, (*Solanum betaceum* Cav., Solanaceae), also known as tree tomato or "tomate de la Paz" (Argentine, Bolivia, France) (Correia & Canhoto, 2012), is a small tree cultivated for its appetizing and juicy fruits (Fig.1B) (Acosta-Quezada *et al.*, 2011). This species was first described in 1801 by Canavilles as *Solanum betaceum*, however, in 1845 was formerly integrated in *Cyphomandra* genus by Sendtner (Guimarães *et al.*, 1996). Nevertheless, in 1995, the genus changed once more to *Solanum*, after intense morphological, taxonomic, phylogenetic and ethnobotanical works carried out by Bohs and his associates (Acosta-Quezada *et al.*, 2011).

In botanical terms, tamarillo is a small perennial tree, with a unique short upper body with branches at a height of 1 – 1.5 m forming a large spreading crown (Lim, 2013), characterized to have a modular growth pattern, in which three or four large deciduous leaves emerge (Fig.1A), with terminal inflorescences (Schotsmans, 2011; Correia & Canhoto, 2012). The leaves, simple, lobed or pinnately compound are often large, a little bit succulent, having 30 to 40 cm length and 20 to 35 cm width, and connect to the stem through a robust petiole (4 – 8 cm long), and exhibit a particular fragrant smell (Bohs, 1989; Prohens & Nuez, 2000; Lim, 2013).

The inflorescence has a set of over 50 pale pink-lavender hermaphroditic flowers with alternating distribution (Schotsmans 2011) with 1.3 – 1.5 cm across (Lim, 2013). Here upon, each fragrant flower has five pointed lobes, a purplish green calyx and five yellow stamens (Morton, 1987). Typically its blossom is undisrupted and the peak occurs from late summer until autumn, nonetheless, exceptions can occur (Correia & Canhoto,

2012). Pollination is primarily autogamic what might be the cause of the low genetic diversity observed in natural populations (Lewis & Considine, 1999).

Figure 1. *Solanum betaceum* (A) Tamarillo tree growing at the Botanical Garden of the University of Coimbra. (B) Fruits from four trees from JBUC, C1: Tamarillo red variety; C5-C9: Tamarillo yellow varieties.

1.2.2 Area of distribution

The precise origin of tamarillo is unclear (Popenoe *et al.*, 1989), but the species is widely found in the Andean regions of Peru, Chile, Ecuador and Bolivia, which seems to indicate that its center of diversity is located in this area. Following the discoveries times, it spread to other tropical and subtropical zones, like Central America (Mexico and West Indies) and Brazil. It attained Europe in the 19th century, (Azores and Madeira islands). Following introduction in UK and further dispersion to the British colonies (India, Hong Kong, Sri Lanka, Australia and New Zealand), the species attained an almost global

distribution (Bohs, 1989). Nowadays, tamarillo is growing in several areas of the globe, namely Brazil, USA, Australia, Southern Europe (Spain, Italy and Portugal), among others, but New Zealand is the production and exportation leading-edge country followed by Colombia (Acosta-Quezada *et al*., 2011).

In our country, this plant is essentially grown as an ornamental species. In the Atlantic islands, commercial exploitation efforts have been made due to the appealing price that the fruit can reach in markets (10 – 15 €/kg), encouraging continent producers to realize the great potential of tamarillo. Nonetheless, large-scale cultivation, has been hampered by spring and autumn frosts that severely can affect plant development and reproduction (Lopes *et al.*, 2000). Beyond this, a tree could produce around 15 – 20 kg of fruits per year during 6 – 10 years (Duarte & Alvarado, 1997).

Although tamarillo displays an extensive variation for fruit characters, only some cultivars have been commercially exploited. In Europe and in the USA, the red and purple cultivars are the preferred by consumers due to its attractive color, flavor and nutritional properties, although showing a more acidic taste than the yellow cultivar, this one being more used as preserves (Carnevali, 1974).

This species, according to the Global Facilitation Unit for Underutilized Species (http://underutilized-species.org/), integrates the category of NUCs (neglected or underutilized crop), *i.e.*, species that has potential for agricultural use but for several unknown reasons, it has not been properly explored.

1.2.3 Fruit characterization and postharvest factors that affect fruit quality

The elliptic fruits are typically found in groups of 3 to 12 units (Fig. 2), commonly ranging from 3 to 5 cm in width and 5 to 10 cm in length. Nonetheless, according to

Prohens & Nuez (2000), round and elongated forms are also currently found. Fruit ripening, occurs between October and April, usually 21 to 26 weeks after flowering.

Figure 2. Branch with elliptic tamarillo fruits at an early stage of development.

Due to the long period of fruit production several harvests are needed to collect all the production. The epicarp is smooth, tough and can be dark red, orange, yellow or a mixture of the previous colors. The juicy mesocarp displays the same variation in color as the skin and has a particular acidic flavor (pH = 3.2 – 3.8) (Prohens & Nuez, 2000; Correia & Canhoto, 2012). Each fruit contains numerous small, nearly flat, thin, hard and bitter seeds (Fig.3) with 3 – 4 mm long by 3.5 – 4 mm wide (Lim, 2013). All the fruit parts are edible, but the seeds and predominantly the epicarp should be removed, prior to

consumption since both give origin to an unpleasant and bitter taste (Guimarães *et al.*, 1996).

Figure 3. Tamarillo fruit with typical seeds and reddish-yellow mesocarp evidenced.

To be accurate and to perform sensorial quality evaluation it is important that the fruits should be collected at its physiological maturity and state of ripeness. According to (Mwithiga *et al.*, 2007), parameters indicative of fruit quality, such as firmness, juice yield, sugar and vitamin concentration, the external and internal fruit color are influenced by the ripeness level. Hence, firmness may be used to predict the internal fruit quality, once its decline is correlated to an increase in juice yield. During ripening, the soluble solid content (SSC) of tamarillo seems to increase to 10 – 12 °BRIX (usually the values lie between 10.0 and 13.5 °BRIX), while the Titratable acidity (TA) lightly decays (typically range between 1.0 and 2.4%), causing an increase in the SSC/TA ratio and consequently a superior sensory flavor rating. As ripening progresses, changes occur, also in the stems, due to an enhanced water loss and chlorophyll degradation which cause a change in color from green to yellow (Pongjaruvat, 2008). Tamarillo seems to be a nonclimateric fruit, since it does not exhibit adequate self-stimulated increase in ethylene production and a consequent respiratory increase as part of its ripening behavior (Pratt &

Reid, 1976). Nevertheless, its harvesting seems to affect its quality, since they continue to ripen and become softer and juicier, suggesting that harvesting should be done at a mature stage. Studies previously carried out to improve post harvesting ripeness showed that application of ethylene or ethephon ($C_2H_6ClO_3P$) was responsible for a decrease in the risk of crop failure and an earlier delivery to the consumer, thereby enhancing the marketability of tamarillo (Prohens & Nuez, 1996). Concerning other postharvest handling factors that can affect the quality, temperatures below 7 °C will slow softening, weight loss, TA reduction and color change. On the other hand, very low temperatures (0 – 2 °C) increase the risk of chilling injury and more discoloration in the calyx and stem (Schotsmans 2011). In the case of tamarillo, the moisture content using AOAC methods, ranges between 81.0 and 87.8 g per 100 g of fresh weight) (Prohens & Nuez, 2000).

1.2.4 Environmental requirements

Concerning its agroecology, tamarillo is a subtropical species that flourishes in the tropics and subtropics at elevations between 1.000 and 3.000 meters. The tree has a length of 1 to 5 m depending on the genotype and the soil and environmental conditions. When temperatures are ideal (18 and 22 ºC), the annual precipitation is 600 – 800 mm tamarillo presents a rapid development and the soils are well-drained (Lim, 2013). The species can also thrive in colder climates, in areas with temperatures not lower than 10 ºC and when extreme freezing does not occurs (Correia & Canhoto, 2012). Even tough extreme cold could severely damage tamarillo plants, often the plant has the capacity of recovering. For tree standards the tree can be considered a short lived species usually between 5 to 12 years (Prohens & Nuez, 2000). Fruit production can start one year after planting but better yields are attained by the third year and goes on for seven to eight years (Schotsmans, 2011).

1.2.5 Nutritional value and health benefits

Tamarillo is grown essentially for its edible fruits which have a high nutritional content and a broad spectrum of potential applications (Guimarães *et al.*, 1996). Although yet considered a neglected crop (Acosta-Quezada *et al.*, 2012) the plant is being recognized as a fruit species, due to the quality of its fruits which are poor in calories (28 kcal /100g), rich in protein content (1.5 – 2.5 g/100g), in vitamins, such as, vitamins C and E (30 – 45 mg/100g and 1.86 mg/100g, respectively), B6 and provitamin A (McCane & Widdowson, 1992). Like in other crops of the genus *Solanum*, tamarillo fruits contain several minerals such as calcium, copper, iron, magnesium and potassium (Acosta-quezada *et al.*, 2014) and a reduced carbohydrate content (7.7 g/100g) and lipid content (0.05 – 1.28 g/100g) (McCane & Widdowson, 1992). More recent studies have shown that the fruits are rich in anthocyanins, carotenoids and phenolics (Kou *et al.*, 2009). Osorio *et al*., 2007 using spectroscopic analyses revealed that tamarillo fruits are a rich source of natural pigments with potential antioxidant activity, giving them a remarkable added-value. Acosta-Quezada *et al*., 2014 assessed fruits from purple and yellow/orange cultivars and did not find relevant differences among them, concluding that the anthocyanins present in purple-fleshed cultivars camouflaged the yellow or orange color due to carotenoids. Phenolics are the main antioxidants presents in the tamarillo fruit pulp, although reasonable amounts of ascorbic acid are present, as well (Vasco & Kamal-Eldin, 2008)

1.3 Potential improvements through breeding strategies

1.3.1 Tamarillo propagation methods

Propagation of tamarillo can be achieved from either seeds or cuttings (Prohens & Nuez, 2000), or by grafting onto wild tobacco trees (*Solanum mauritianum*) (Guimarães *et al*., 1996). Seeds germinate easily but plantlets are usually weak and the trees resulting from them possess fewer and higher branches than those obtained through cutting. Moreover, propagation by seeds gives origin to genetically different trees that do not assure a consistent fruit production. Thus, methods of vegetative propagation are usually used to obtain uniform plants (Correia & Canhoto, 2012). In such case, asexual propagation methods are required. These can be the well traditional methods of asexual plant propagation, such as cutting or grafting or the more recent techniques of *in vitro* clonal propagation, usually known as micropropagation and which include axillary shoot proliferation, organogenesis and somatic embryogenesis.

1.3.2 Micropropagation of tamarillo

The first *in vitro* technique applied to the *in vitro* propagation of tamarillo was axillary shoot proliferation (Cohen & Elliot, 1979; Barghchi, 1986). This technique allows not only the study of shoot development but also is a fast and reproducible technique to assure large-scale plant propagation and the genetic uniformity of the obtained plantlets Axillary shoot proliferation has been applied to the *in vitro* cloning of many plant species, is particular those that are difficult to multiply by the traditional methods or when the original plants are unfertile hybrids (Correia *et al.*, 2011).

In vitro organogenesis is the process of forming new organs (meristems, roots, stems), under specific chemical and physic conditions (Thorpe, 1980). It depends on the

application of exogenous hormones, particularly, auxins and cytokinins (Angulo-Bejarano & Paredes-López, 2011), of tissue responsiveness (Sugiyama, 1999), as well as, external factors (incubation temperature or the type and intensity of radiation) (Turk *et al.*, 1994). The formation of plants by organogenesis can be achieved through two distinct processes. The first process is direct, occurring the development of adventitious meristems, which develop into axillary shoots and ultimately form a plant, after rooting. The indirect process is most usual and differs particularly from the first, specifically in the callus formation, from which gems are formed, followed by rooting (Hicks, 1994; Tonon *et al.*, 2001; Canhoto, 2010).

Somatic Embryogenesis (SE) is the process by which the somatic cells, under determined stimuli, underwent a dedifferentiation and a differentiation forming embryogenic cells with the capacity to form embryos and ultimately plants (Yang & Zhang, 2010; Rose *et al.*, 2010). In tamarillo, SE is an asynchronous process during which somatic embryos pass through diverse morphological phases similar to those occurring during zygotic embryo development (globular, heart-shaped, torpedo and cotyledonary) (Correia & Canhoto 2012). The capacity of plant differentiated plant cells to embark into an embryogenic process is a unique developmental process and clearest demonstration of totipotency (Zimmerman, 1993; Canhoto, 2010). Moreover, SE serve as a model to understand the cytological, physiological and genetic mechanisms underlying embryo formation as well as development and maturation (Yang & Zhang, 2010; Rose *et al.*, 2010; Correia SI, 2011)

Somatic embryogenesis in tamarillo was first obtained by Guimarães *et al.*, (1988) from mature zygotic embryos and hypocotyls. Since then, several works have been published showing that different explants can be used to induce somatic embryo formation, such as cotyledons, roots, mature zygotic embryos and leaf segments.

(Canhoto *et al.,* 2005; Lopes *et al.,* 2000). For somatic embryogenesis induction an auxin is usually the trigger effect and 2,4-D or picloram have been used (Canhoto *et al*., 2005; Correia SI, 2011). When either of these auxins is used to induce embryogenesis in leaves, a two-step process occurs (Fig. 4). Thus, following the formation of an embryogenic callus on the auxin-containing medium, somatic embryo formation requires callus transfer to an auxin-free medium (Yang & Zang, 2010). This type of embryogenesis allows that embryogenic callus can be successfully maintained by successive subcultures. Nonetheless, cultures kept under extended periods of time revealed variations at the chromosomal level and in the quantity of DNA (Currais *et al.*, 2013).

The objective of plant cloning through micropropagation is to obtain genetically uniform plants. However, in several species, it has been reported that the plants thus obtained may display characteristics which are not true-to-type. In this context it is important to analyze the genetic diversity of the propagated plantlets to find whether this type of changes occur (Correia & Canhoto, 2012). For this purpose, molecular markers are a useful tool to confirm the uniformity of the regenerants.

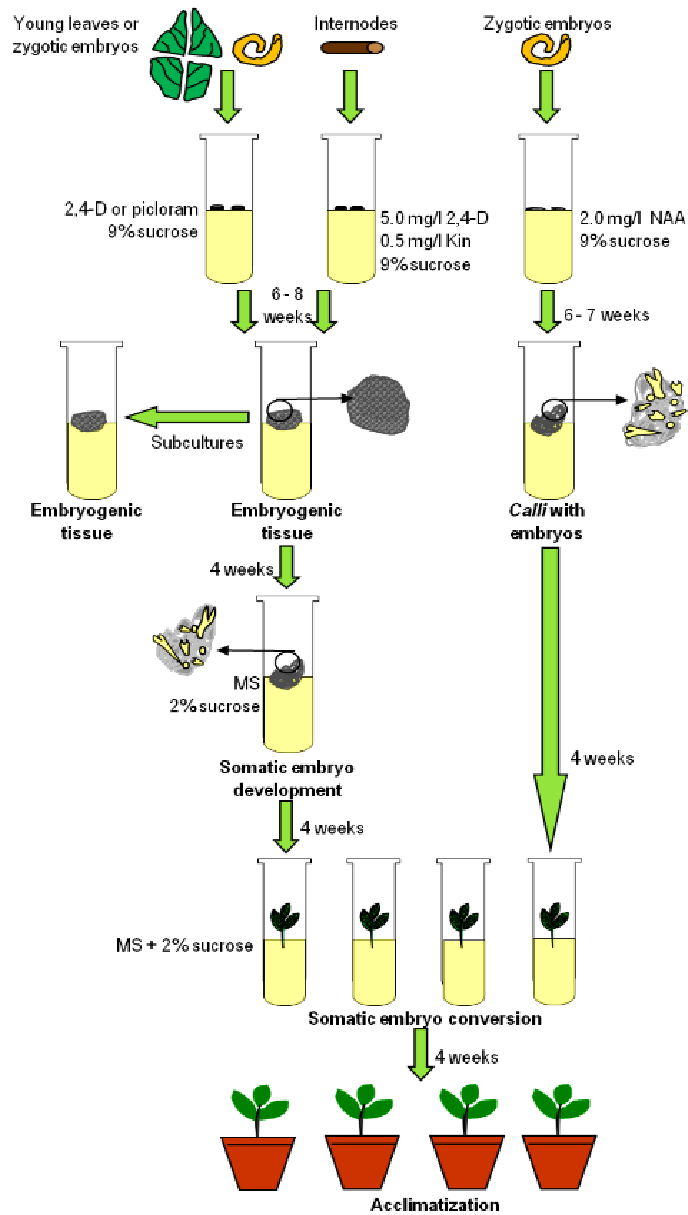


Figure 4. Representation of the protocols for SE induction in tamarillo (Canhoto *et al.*, 2005).

1.4 Molecular analysis: Genetic assessment studies in *Solanum betaceum* (Cav.)

The discovery of PCR (polymerase chain reaction) by Mullis & Faloona (1987) led to the expansion of various types of PCR-based techniques. The major benefits of PCR technique are based in the small amount of DNA required, the fact that a known sequence is not necessary and the high polymorphism that enables to generate many genetic markers within a short time .The advantages can differ depending on the specific technique to implement. Thus, depending on the primers used for amplification, the different PCR-based techniques are of two types: 1) Based in arbitrary or semi-arbitrary primed PCR techniques that developed without prior sequence information (*e.g*., RAPD, AFLP) or 2) site-targeted PCR techniques that developed from known DNA sequences (*e.g*., SSR) (Kumar *et al.*, 2009).

The technique of random amplified polymorphic DNAs (RAPD) technique has been widely used to accomplish plant genetic studies (*e.g*., DNA fingerprinting), since the early nineties when was firstly described. (Williams *et al.*, 1990; Lacerda *et al.*, 2002). The principle of the technique is based on the amplification of random segments of genomic DNA by PCR, using short single primers or arbitrary sequences. The simplicity and flexibility of RAPDs make it appropriate for an expeditious survey of polymorphisms (Williams *et al.*, 1990). Besides, the technique has a relativity low implementation cost (Rafalski, 1991). However, some limitations also occur such as its difficult reproducibility and dominant inheritance. Nevertheless, RAPDs have been used to evaluate genetic diversity in several species of the genus *Solanum* such has *Solanum tuberosum* (Onamu *et al.*, 2015) and *Solanum lycopersicum* (Arias *et al.*, 2010). Regarding the case of *S. betaceum*, no molecular studies based on PCR are available, but Acosta-Quezada *et al*. (2012) used AFLP markers to characterize this species.

1.5 CMF as an improving factor to *in vitro* culture

In the last few decades there has been an increasing interest in environmental friendly and sustainable materials for several applications. The emergence of bio-based materials has broadly stimulated interest in exploring their physical and mechanical properties concerning its significant applications such as its infrastructure. Several studies were taken to survey the various types of bio-based materials including cellulose and lignin to attend some necessities in terms of engineering applications (Hubbe *et al.*, 2008; Ummartyotin & Manuspiya 2015).

Cellulose is the most abundant biopolymer in the planet, being synthesized in plants, algae and some bacteria (Henriksson & Berglund 2007). This glucose derived polymer is a structural component of plant cell walls, either primary or secondary and has many applications, being the use in papermaking the most common (Siró & Plackett 2010). However, in recent years other applications for cellulose based material have been found in different domains like food, cosmetics, health care, medicine, construction, water treatments and advanced materials with tailor-made properties (*e.g.* electronics) (Dufresne, 2012). In terms of structure, cellulose is an extensive linear-chain polymer generated from repeating β-D-glucopyranose molecules that are linked covalently across acetal groups between equatorial OH group of C4 (nonreducing end) and the C1 carbon atom (reducing end) (β-1,4-glucan) (Klemm *et al.*, 2005). The repeating unit is a homodimer of glucose, known as cellobiose (Abdul Khalil *et al.*, 2014).

Since the eighties various methods have been proposed by Turbak *et al* (1983) and Herrick *et al* (1983) to prepare and isolate fibril materials from wood pulp, through a cyclic mechanical treatment in a high-pressure homogenizer. This process allows wood pulp disintegration and subsequently the fibers are opened into their sub-structural

microfibrils. The more sever the homogenization treatment, the more fibrillated the material will be, *i.e.*, the particle size of fibers can be reduced to the micro-scale (microfibrillar cellulose, CMF) or to the nano-scale (nanofibrillar cellulose, CNF). In addition, some distinct chemical and/or enzymatic pretreatments can be used, in order to reduce the mechanical energy required to fibrillation and to obtain cellulose fibrils with distinct dimensions, branching degree and chemical properties (Abdul Khalil *et al.,* 2014).

Cellulose nanofibrils have diameters in the range of 5 – 30 nm, lengths up to several micrometers and an aspect-ratio usually superior to 100. The cellulose microfibrils are larger than the cellulose nanofibrils, with diameters in the range of 20–100 nm or even superior and an aspect-ratio higher than 50). Bleached kraft pulps or non-woody based material are the most common materials used for the production of CMF and CNF (Henriksson *et al.*, 2007; Dufresne, 2012; Gamelas *et al.*, 2015a).

Appropriate pretreatments of cellulose fibers promote the accessibility of hydroxyl groups, increase the inner surface, alter crystallinity and break cellulose hydrogen bounds and thereafter boost the fibers reactivity. Furthermore, as mentioned, the use of a pretreatment (*e.g.*, chemical or enzymatic), combined with mechanical treatment, can decrease significantly the energy consumption in the overall process which may be the main challenge in CMF and CNF profitable production (Henriksson *et al.*, 2007; Osong *et al.*, 2016). Other difficulties related with scaling-up and reproductibility problems need to be overcame as well (Syverud, 2014). The chemical pre-treatment with NaClO (oxidant) mediated by TEMPO (2, 2, 6, 6-tetramethypiperidine-1-oxyl radical) and NaBr (TEMPO-mediated oxidation) is the most applied (Gamelas *et al.*, 2015), although others can be used, but the enzymatic pretreatment is an alternative. It also improves fibrillation, but in a minor degree (a smaller amount of nanofibrillar material is

obtained), and has the advantage that no surface charges are added to the fibrils surface (Osong *et al.*, 2016). The enzymatic treatment followed by mechanical homogenization was the method used in the present study.

Numerous studies have shown the applications of CMF and CNF as composites reinforcing material or scaffold, as films with barrier properties for packaging, as rheology enhancer, as flocculant, or as matrices for wound dressings or electronic devices (Syverud 2014). In fact, due to its nanometer scale, its high surface energy, water retention value, sustainability, high strength, stiffness and its aptitude to form a nanoporous network, CMF (and CNF) has been explored for the production of many nanocomposites (Lavoine *et al.*, 2014; Kiziltas *et al.*, 2015). When CMF and CNF are used in films, both to increase the mechanical strength and reduce the air permeability, the final quality will depend significantly on the film forming process, the drying method and the storage condition (Syverud & Stenius, 2009), The capacity of CMF and CNF to form a nanoporous network is an advantage for other applications in comparison with classical films (Lavoine *et al.*, 2014; Osong *et al.*, 2016).

1.6 Aims

Through the research conducted at the Laboratory of Plant Biotechnology (CEF), a large number of genotypes of tamarillo have been produced, including tetraploids and hybrids from artificial pollination of different genotypes. To determine its quality for fruit production these plants need to be characterized both morphologically and genetically. Since fruit quality is the major purpose of tamarillo, fruits of tamarillo originated from different trees, including were characterized and their properties compared with fruit from commercial varieties, usually available in the markets and originated from Colombia.

Thus, the second goal of this work was to evaluate the genetic diversity of some of plants, developed in the CEF, in particular the hybrids. For this purpose, RAPD markers were used to determine the genetic diversity of 16 *Solanum betaceum* genotypes.

Finally, as a third goal of this research, it was tried to incorporate new materials in the technology of *in vitro* propagation. For that, CMF / CNF produced by an enzymatic treatment followed by homogenization was tested as a support for tamarillo cultures. In order to achieve this main goal it was required: i) production of CMF films and assessment of its capacity as a barrier and support through chemical quantifications of IAA ii) optimization of micropropagation conditions using CMF as support for plant growth, iii) the use of CMF films to observe the development of non-embryogenic calluses, in order to substitute the standard films.

2. Material and methods

2.1 Physical and morphological analysis of tamarillo fruits

2.1.1 Plant Material and origin of fruits

Tamarillo fruits were collected from trees, located at the Botanical Garden of the University of Coimbra (JBUC). These trees were: 1) an adult tree (PM-red variety), with approximately 17 years, 2) five clones of seedling origin propagated *in vitro* - C1 and C3 from a red variety, C5 and C9 from a yellow variety and C7 from an orange variety, 3) individuals from different regions of Portugal, as seen in figure 5, namely PC, TS, TC, TR and TCQ (all from the red variety) and 4) a red line (TCOL) from Colombia at edible ripeness purchased at a hypermarket (Makro).

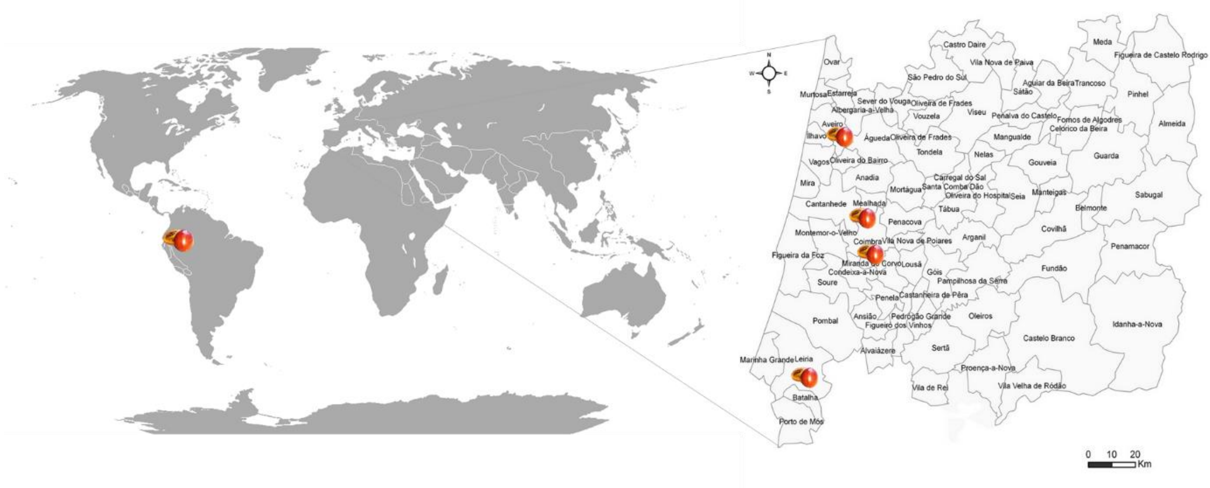


Figure 5. Areas where the trees from which fruits were collected are located. Aveiro (TC), Coimbra- JBUC (PM; PC; C1-C9; TS), Carqueijo (TCQ), Leiria (TR) and Colombia (TCOL).

2.1.2 Harvest and pre-sample preparation

The fruits, showing uniformity of color and firmness, were gathered during November 2015. After harvesting, the fruits were kept at about 4 ºC (never more than 24h) until analysis were carried out at the Laboratory of ESAC (Escola Superior Agrária de Coimbra), as seen in figure 6. The only exception to this procedure occurred with TCOL material which was analyzed in April 2016 due to fact that only at this time it was possible to buy the fruits at a supermarket. All the tests were performed at room temperature (25 °C).

Figure 6. Color variance of tamarillo fruits. Sample standards to perform the several analysis.

2.1.3 Parameters evaluated

2.1.3.1. Firmness

Twelve representative fruits of each origin were arbitrarily selected and placed in plastic boxes (Fig. 7). First, firmness was measured using a digital firmness tester (non-destructive device) with a 25 plunger tip (Agrosta® 100 Field, Agrotechnologie, France). Each fruit was placed on a horizontal surface and then a vertical downward pressure of the probe on one surface and in the completely opposite of the fruit was applied. Thus the device provides readings on a 0 – 100 scale and the measurements of each fruit were recorded.

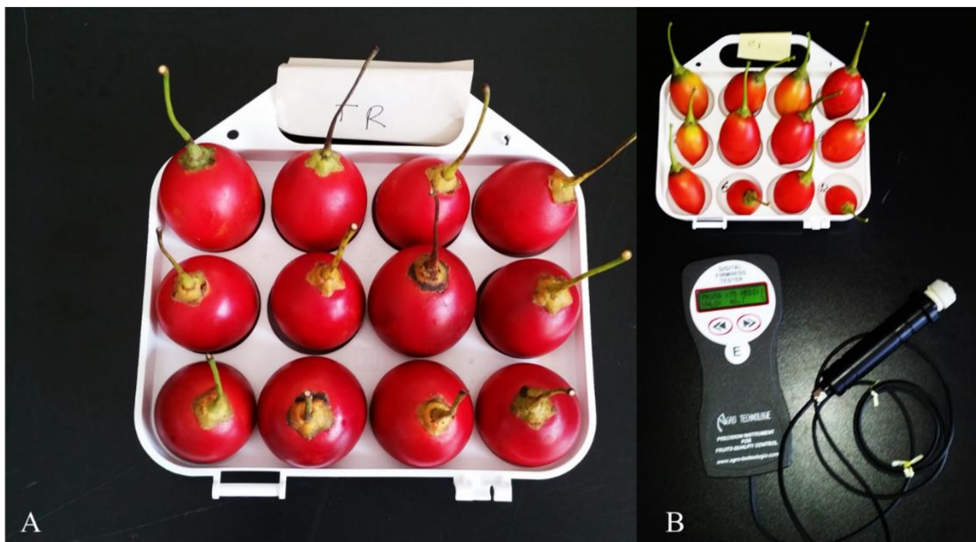


Figure 7. Red tamarillo fruits and firmness device. (A) Twelve samples of TR variety ready to be analyzed. (B) Firmness tester.

2.1.3.2. Biometric tests

The following measurements were taken: 1) Fruit and peduncle length, 2) Fruit diameter 3) Peduncle thickness near fruit calyx, 4) Thickness at the middle of the peduncle 5) Fruit weight.

Fruit and peduncle length were measured with a metric tape. The first one, from the fruit pedicel to the base of the calyx, whereas the second one, from the sepal's insertion up to the pedicel tip. Measurements 3 and 4, listed above, were conducted using a caliper (Electronic Digital Caliper. Mod. DC-515, 0 – 150 mm), as seen in figure 8. Fruit weight was measured using a scale.

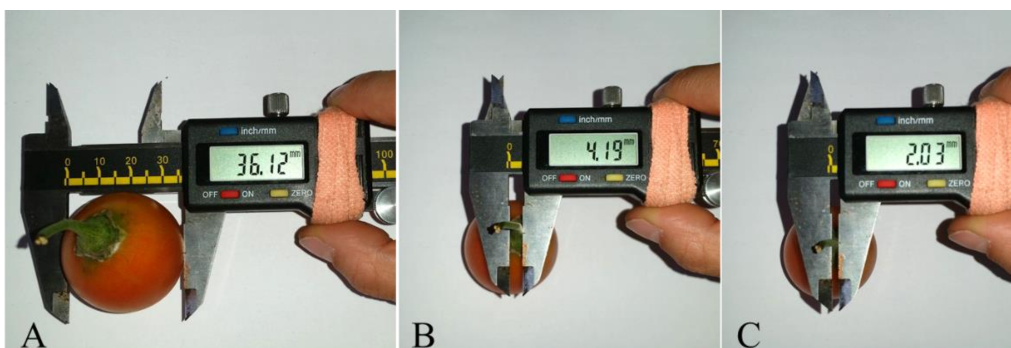


Figure 8. Fruit and peduncle measurements (A) Equatorial diameter measurement. (B) Peduncle thickness near fruit calyx. (C) Thickness at the middle of the peduncle.

2.1.3.3 Physicochemical and sensorial analysis

Following peduncle removal, fruits (12 per origin) were cut in four quarters. Two opposite quarters were used to produce a paste with a domestic blender and the remaining were set aside in order to determine moisture. A portion of the paste was used to perform solid soluble content (SSC) using a digital refractometer (ATAGO®, Pocket Palm Refractometer, PAL-1, Brix 0.0 to 53.0%), whereas the remaining was used to perform titratable acidity (TA). For this last purpose, the paste was filtrated (Fig. 9), and 5 ml of juice were collected and mixed with 25 ml of distilled water and 2 drops of phenolphthalein followed by a titration with NaOH (0.1 M). TA determination and record of the results were taken in accordance with NP EN 12147 (1999). To access moisture content, a known fresh weight of sample was oven-dried at 60 °C, during 24 hours.

Figure 9. Experimental setup for titratable acidity evaluation.

2.2 Genetic assessment studies through the use of molecular markers

2.2.1 Plant Material and DNA extraction

Young leaf tissue of adult trees (C1, C5, C7, and C9) and hybrids (H1 – H12) were used to perform total genomic DNA extraction. Leaf tissue were grounded, using a mortar and a pestle, into a fine powder in liquid nitrogen. Genomic DNA was performed using NucleoSpin® Plant II, Macherey-Nagel.

The yield of DNA was settled using a NanoValue Plus™ Spectrophotometer at 260 nm. Its purity was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm.

2.2.2 Random amplified polymorphic DNA (RAPD)

A total of 20 arbitrary decamer primers (Operon Technologies) were tested for RAPD amplification (Table 1). The most polymorphic OPC were selected and repeated three times to insure the reproducibility of the banding patterns. PCR reactions were carried out as master mixes for each primer and the final volume for this reaction was 20 µl, containing 4 µl of 5x GoTaq® buffer (Promega), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 1U of GoTaq® DNA polymerase (Promega), 0.2 µM of the primer and 25 ng of genomic DNA. The DNA amplification was performed on a Thermal cycler (Bio Rad) using the subsequent profile: initial denaturation (2 minutes, 95 °C), followed by 35 cycles of 1 minute at 95 °C (denaturation), 1 minute at 35 °C (annealing), and an extension step of 1 minute at 72 °C. At the end of the cycles, a final extension was taken at 72 °C for 5 minutes. The PCR reactions products were separated by electrophoresis in agarose gels (2% w/v) in 1x TBE buffer, stained with Midori green DNA stain (3 µl/100 ml) for

the DNA fragment visualization, and visualized and documented through Gel Doc XR+ with Image Lab™Software (Bio-Rad). As a standard, 400 ng of a DNA size ladder (HyperLadder™ II, Bioline) was loaded in the gel along with the PCR products.

Table 1 – Primers used and their sequences (Operon Technologies Kit C - OPC).

Primer	Sequence 5'-3'	Primer	Sequence 5'-3'
OPC-1	TTCGAGCCAG	**OPC-11**	AAAGCTGCGG
OPC-2	GTGAGGCGTC	**OPC-12**	TGTCATCCCC
OPC-3	GGGGGTCTTT	**OPC-13**	AAGCCTCGTC
OPC-4	CCGCATCTAC	**OPC-14**	TGCGTGCTTG
OPC-5	GATGACCGCC	**OPC-15**	GACGGATCAG
OPC-6	GAACGGACTC	**OPC-16**	CACACTCCAG
OPC-7	GTCCCGACGA	**OPC-17**	TTCCCCCCAG
OPC-8	TGGACCGGTG	**OPC-18**	TGAGTGGGTG
OPC-9	CTCACCGTCC	**OPC-19**	GTTGCCAGCC
OPC-10	TGTCTGGGTG	**OPC-20**	ACTTCGCCAC

2.2.3 Diversity estimates

The fragments obtained from the 4 RAPDs (OPC) that showed the most polymorphic bands for all 16 genotypes of *S. betaceum* were scored as 1 (present) and 0 (absent), resulting in a binary matrix for cluster analysis. To transform similarity coefficients, Unweighted Pair-Group Method with Arithmetical Averages (UPGMA) with the Jaccard's coefficient to compare the variables, was applied. To support this analysis, MEGA version 7 software was used and a dendrogram was generated, as well.

2.3 Culture conditions improvement through the use of CMF

2.3.1 Production of microfibrillated cellulose

Cellulose microfibrillated (CMF) was obtained by a combination of enzymatic and homogenization treatments using eucalypt bleached kraft pulp. The enzyme used was a commercial cellulase named Serzym 50 (SZM 50, 150 g/t) obtained from genetically modified *Trichoderma reesei*. For this purpose 30 g of pulp (dry basis) was mixed with 2 l of deionized water .The resulting pulp was placed in a pulp disintegrator (British Pulp Evaluation Apparatus), at 5000 rpm during a few minutes, in order to expose the fibers and to form a homogenous suspension required for the following treatments (Fig. 10A). Next, the water excess was removed and the pulp was beaten at 5000 rpm in a PFI-mill by HAM-JERN, Hamar, Norway, to make the cellulose more easily accessible for the enzymatic treatment (Fig. 10B).

To perform the enzymatic treatment, demineralized water (pH = 6.3) was used to adjust/dilute the pulp to 4.5% consistency. The diluted pulp was incubated at 43 °C and then filtered on a Büchner funnel, in order to achieve a higher volume of solid to add later the enzyme. Right before to start the enzymatic treatment, the enzyme was prepared at 1% and then was dispersed in the previously prepared pulp (pH = 5.5). The enzymatic treatment was performed in a pre-heated water bath (Fig. 10C). The mixture was incubated in a glass beaker with powerful stirring device at 43 ºC for 30 minutes. The resulting pulp was finally passed several times through a high pressure homogenizer (GEA Panther NS3006L): one time at 500 bar, one time at 750 bar, one time at 1000, one time at 1100 and finally, one more time at 1200-1300 bar (Fig. 10D). Homogenization was performed at room temperature and the resulting CMF attained a consistency of 0.885. The fibrillation yield, determined by centrifugation, was 17%, meaning that only 17% of the sample had small size particles (at nano or micro scale). These two

experiments were performed at the Chemical Engineering Department, whereas the production of the CMF/CNF was carried out at RAIZ (Instituto de Investigação da Floresta e Papel).

Figure 10. CMF procedure concerning the several steps of the process: (A) exposure of fibers to pulp disintegrator (B) pulp beating (C) enzymatic treatment (D) homogenization.

2.3.2 Production of CMF films for absorption and releasement assays

To obtain the cellulose films, 90% of CMF suspensions were stirred with 10% of deionized water (pH = 5.7) in a final volume of 15 ml. Following stirring, the solution was placed in Petri dishes and incubated at 60 °C, overnight (CMF_w). Simultaneously, a similar method was used to prepare microcellulose films containing a final concentration of 50 µg/ml of IAA (CMF_{IAA}).

2.3.2.1 IAA absorption and releasement by CMF

The absorption of IAA by CMF was tested by placing CMF_w films on a 25 ml of IAA solution (50 µg/ml) for a period of 72 h under dark conditions and periodically removing 1 ml of solution at 9, 12, 15, 18, 24, 48 and 72 hours. The retrieved liquid samples were then assayed for IAA concentration following Ehrlich reaction and the quantity of IAA in each collection point was determined. The decreasing IAA levels were assumed to be proportional to IAA absorbed by the CMF. Additionally, to study the inverse process (CMF-IAA releasement) a similar experimental design was used with CMF_{IAA} in deionized water (pH = 5.7) and the IAA levels were assayed at different collection points (0, 2, 4, 8 and 24 hours). In this case the increase of IAA in water is proportional to films permeability to this compound. Both assays were made in triplicate and a control was used with only IAA solution to evaluate IAA natural hydrolysis.

2.3.3 Production of CMF films for diffusion assays

For the diffusion experiments, 15 ml of pure microcellulose films were prepared (CMF_p). After correcting pH (5.8) and stirring for a few minutes the resulting liquid suspension was placed in Petri dishes and incubated at 60 °C, overnight.

2.3.3.1 IAA diffusion by CMF

The diffusion of IAA by CMF was tested by placing CMF_p films in Petri dishes (d = 5.4 cm) containing 25 ml of deionized water and an agar cube containing IAA (1.5 cm^3; 250 µg/ml) was placed on the surface of the films. After, 1 ml of water solution was then removed at 0, 2, 4, 6, 8 and 24 hours and assayed for IAA concentration by Ehrlich reaction. The quantity of IAA was determined for the overall volume of solution. The results are presented as µg of IAA per hour. A control consisting of only water and CMF_p was used.

2.3.4 Ehrlich reaction

The IAA content in the CMF films was first assayed using the colorimetric method described by Anthony & Street (1969). Accordingly, Ehrlich's reagent reacts with the indol group of IAA in an acid medium, under optimized conditions for improved specificity. The reagent was prepared by dissolving 2 g of p–dimethylaminobenzaldehyde in 100 ml HCl 2.5 N. The reaction mixture was composed of 1 ml of sample, 2 ml of trichloroacetic acid (TCA) 100% (w/v) and 2 ml of Ehrlich's reagent added in order. A blank solution of water was prepared simultaneously. After an incubation period of 30 min, the absorbance was measured at 530 nm in a Jenway 7305 spectrometer. A calibration curve was prepared using buffered solutions of IAA with concentrations between 2 and 50 µg/ml. The results are presented in µg of IAA per ml.

2.4 CMF as support to *in vitro* culture

Tamarillo plants previously propagated from established *in vitro* red lines were selected. After a selection of different genotypes, the shoots were established in a MS (Murashige & Skoog, 1962) medium enriched with 1% of CMF , supplemented with 3% sucrose and 0.2 mg/l of 6-benzylaminopurine (BAP). The pH was adjusted to 5.8 and the mixture was then placed in glass flasks and 6 g/l of Agar (Panreac, Spain) was added before autoclaving at 121 °C, for 20 minutes. Controls were made containing only MS medium, with the same proportions as described previously. The shoots (1.5−2.0 cm in length) were used as explants sources and kept in a growth chamber at 25 ºC, with a 16 h photoperiod, for 3 months.

2.5 Subculture of non-embryogenic callus (NEC) in CMF films

Films were previously prepared according to the method described in section 2.3.3, but were subjected to autoclaving at 121 ºC, for 20 minutes.

Controls were used as a standard (Filters Fiorini, 47mm, France) and were autoclaved under the same conditions formerly described. The calluses used in cell suspension cultures were formerly induced and characterized (Correia, 2011). The non-embryogenic callus used were originated from young leafs (in a medium supplemented with picloram-line B).

In this experiment, calluses lines were grown on solid and semi-solid basal MS medium supplemented with 9% (w/v) sucrose, 5 mg/l picloram (pH = 5.8) and 0.6% (w/v) and 0.2 % (w/v) of agar, respectively. Thereafter, all the media were autoclaved at 121 ºC, for 20 minutes. As a support, the pure CMF films and the cellulose standard filters were placed above the former prepared mediums, being kept under dark conditions, at 25

ºC for a period of 7 weeks. Mass increment results were recorded as the fresh mass of calluses developed under different conditions, using a standard reference ratio of 50 mg of calluses for 20 ml of solid and semi-solid medium. Volume and dry mass (performed at 60 ºC during 5 days) were assessed, as well.

For cytological observations, small pieces (1 mm) of NEC grown in CMF films and NEC grown in standards filters were placed on a microscope slide, squashed in acetocarmine and observed with a Nikon Eclipse E400 microscope equipped with a Nikon digital camera (model Sight DS-U1) using the Act-2U software.

2.6 Statistical analyses

The Brown–Forsythe test ($p<0.05$) was used to test the homogeneity of variances. Evidencing homogeneity of variances, the data was evaluated through one-way analysis of variance (ANOVA) and, where necessary, the means were compared using the Tukey test ($p<0.05$). Conversely, in non-homogenous variances, the non-parametric Kruskal-Wallis one-way analysis of variance was used and, in this circumstance, the means were compared by Dunn's multiple comparison test ($p<0.05$).

3. Results

3.1 Physical and morphological analyses of tamarillo fruits

The red cultivars of tamarillo are usually preferred by the consumers. They are also the best characterized in terms of fruit quality. Being tamarillo a type of fruit that is usually imported and being the information about the properties of the fruits produced in Portugal scarce, we have decided to evaluate several parameters of fruit quality and compared them with those of commercial fruits available in supermarkets. Thus several genotypes of the red (C1, C3, PC, PM, TS, TC, TCQ, TCOL), golden-yellow (C5 and C9) and orange (C7) fruits were analyzed. Parameters tested were firmness, weight, caliber (fruit diameter and length), moisture content, SSC (soluble solid content), titratable acidity (TA) and its linked acids (malic and citric).

Regarding firmness (Fig. 11A), C5 showed the highest values (84.09 ± 7.15%), revealing significant differences when compared with C1 (72.38 ± 8.84%), C3 (70.82 ± 11.70%) and TCQ (58.51 ± 18.89%) genotypes. The remaining genotypes do not present significant differences in comparison with the highest value, varying from 75.03 ± 11.98% (C9) until 82.69 ± 6.73% (TR).

Colombian tamarillo fruits (Fig 11B) displayed the highest weight (107.57 ± 11.88 g), but this value was not statistically significantly different when compared to PC (47.73 ± 3.88 g), TS (47.99 ± 7.40 g), TC (71.00 ± 8.24 g), TR (59.85 ± 7.47 g). Furthermore, PM (31.94 ± 3.45 g), C3 (32.01 ± 3.47 g), C7 (32.01 ± 3.47 g) represent the fruits with lowest weight, while C1 (34.38 ± 8.45 g), C5 (32.99 ± 5.20 g) and C9 (35.21 ± 4.48 g) are intermediate. In terms of fruit diameter, TCOL also presents the highest values (55.26 ± 1.96 mm), but according to statistical analysis (Fig. 11C) this value is not significantly different from PC (38.87 ± 1.11 mm), TC (45.63 ± 1.33 mm), TR (44.43 ± 1.66 mm). All other varieties have lower diameters, ranging between 30.10 ± 1.46 mm (C3) and 38.88 ± 2.32 mm (TS), while TCQ presents an intermediate value (41.39 ± 2.11 mm).

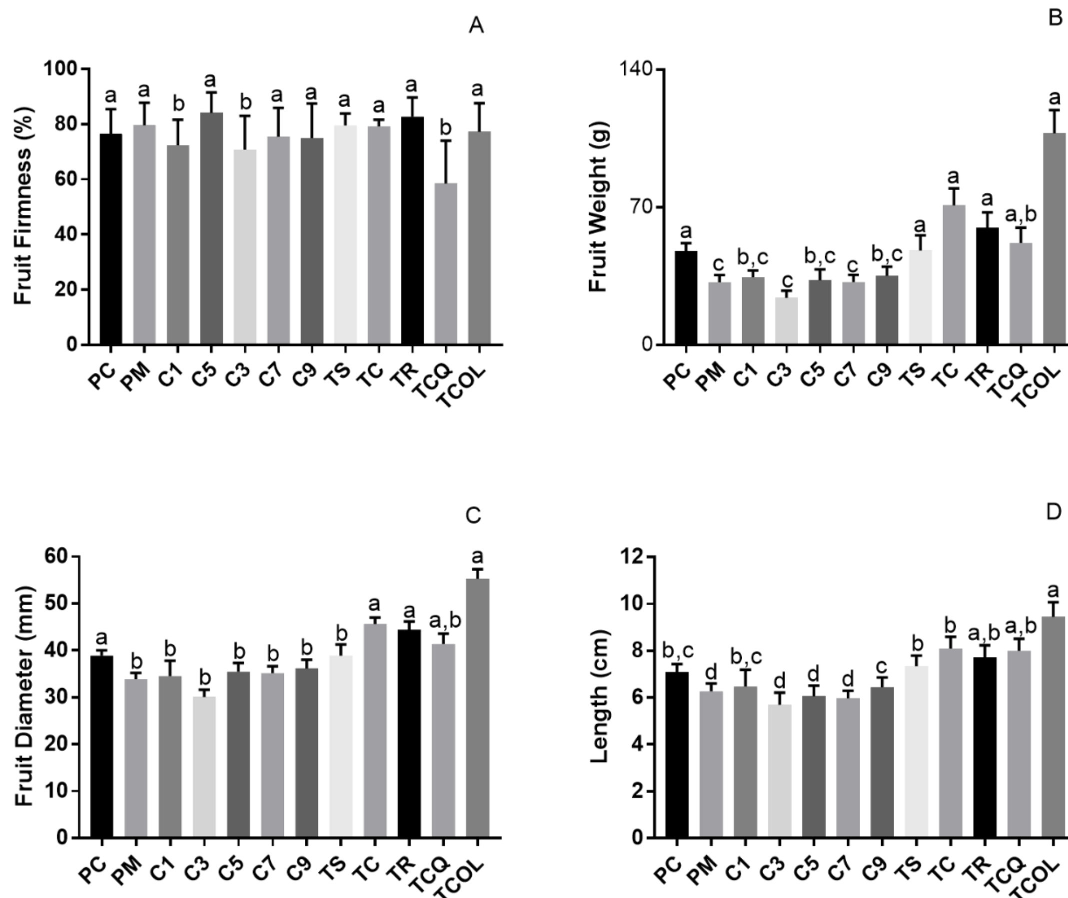


Figure 11. Physical and morphological analysis of tamarillo fruits. Graphics on the top: (A) Fruit firmness, expressed in % of all varieties (PC-TCOL) (B) Fruit weight, expressed in grams (g) of all varieties (PC-TCOL). Graphics on the bottom: (C) Fruit diameter, expressed in millimeters (mm) from all samples analyzed (PC- TCOL) (D) Length, expressed in centimeters (cm) from all samples (PC-TCOL). Values are presented as mean ± SD (n = 12). Values indicated by different letters, in the same row, are statistically significant by Dunn's multiple comparison test ($p<0.05$).

Fruits of TCOL also showed the highest value for fruit length (9.44 ± 0.61 cm), but no differences were observed in comparison with TR (7.70 ± 0.51 cm) and TCQ (8.00 ± 0.49 cm). TS and TC have intermediate values, while the remaining samples tend to have lower lengths, being C3 (5.69 ± 0.5 cm) the variety with lowest length fruits, as seen in figure 11D.

To perform complementary studies of the fruits, evaluation of peduncles within each variety was made (Fig. 12). Additionally, is important to refer that TCOL variety

due to shipping conditions came without peduncle, for this reason, peduncle length and medium peduncle thickness assessments were compromised and were not measured.

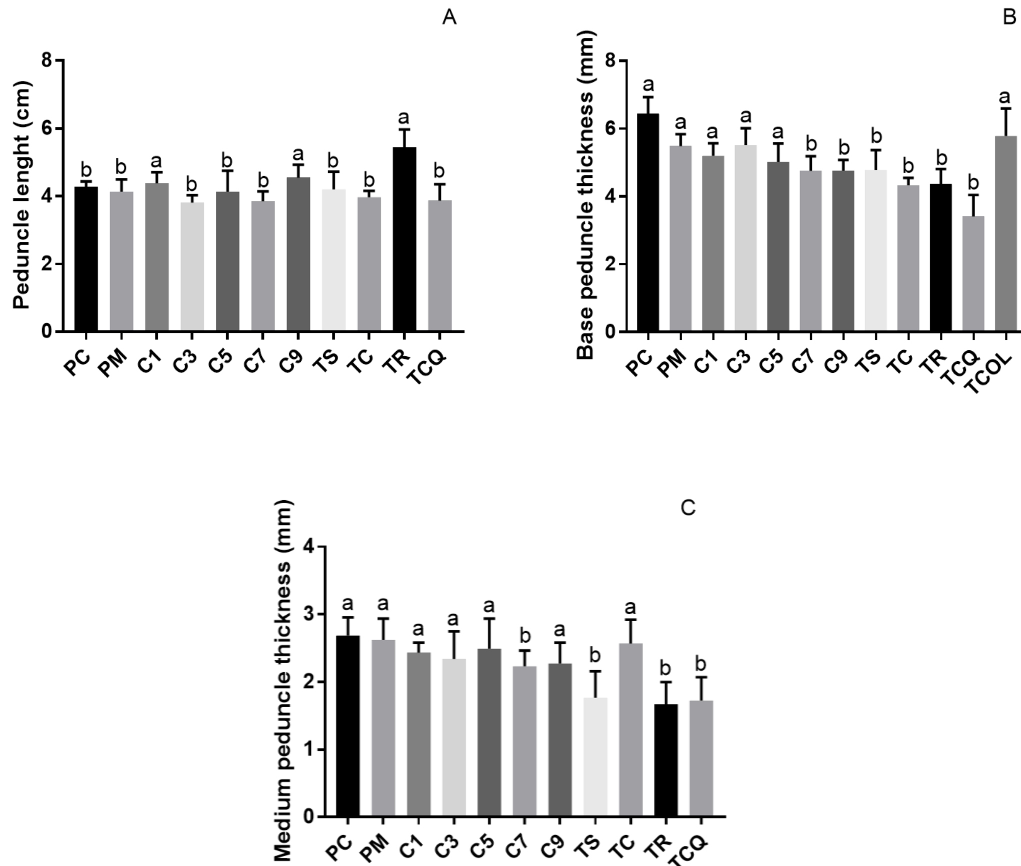


Figure 12. Physical and morphological analysis of tamarillo peduncles. From the left to the right (A) peduncle length expressed in centimeters (cm) from all samples analyzed (PC- TCQ), (B) Base peduncle thickness assay, expressed in millimeters (mm). Samples: PC- TCOL (C) Medium peduncle thickness, expressed in millimeters (mm). Samples: PC- TCQ. Base peduncle thickness values are presented as mean ± SD (n = 12) whereas the values of the remaining evaluations are presented as mean ± SD (n = 11). Different letters are statistically significant by Dunn's multiple comparison test ($p<0.05$).

In view for peduncle length (Fig. 12A), TR samples demonstrated the longest peduncles (5.45 ± 0.49 cm), not revealing significant differences among C1 (4.38 ± 0.31 cm) and C9 (4.55 ± 0.36 cm). Although, comparing with all other samples there were significant differences corresponding to smaller values presented, ranging from 3.80 ± 0.21 cm (C3) until 4.66 ± 1.11 cm (PC).

Considering base peduncle thickness assessment (Fig. 12B), all varieties were able to assess. Whereupon, PC presented the highest values (6.44 ± 0.47 mm), despite not being significantly different of PM, C1, C3, C5 and TCOL. Conversely, C7 (4.75 ± 0.41 mm), C9 (4.76 ± 0.29 mm), TS (4.78 ± 0.56 mm), TC (4.33 ± 0.21 mm), TR (4.37 ± 0.43 mm) and TCQ (3.41 ± 0.61 mm) presented significant differences with the remaining preceded samples.

In terms of medium peduncle thickness assessment (Fig. 12C), almost all samples have not demonstrated significant differences (PC, PM, C1, C3, C5, C9 and TC) wherein the highest value belonged to PC (2.69 ± 0.26 mm). Inversely, the C7 (2.23 ± 0.22 mm), TS (1.77 ± 0.37 mm), TR (1.67 ± 0.32 mm), and TCQ (1.73 ± 0.33 mm) presented significant differences when compared with the remaining.

Table 2. Physical and chemical characteristics of red, orange and golden-yellow varieties of tamarillo fruits from the central region of Portugal (PC-TCQ) and Colombia (TCOL). The results are expressed as mean ± SD (n = 2). Columns from the left to the right: Moisture content expressed as (%), SSC (°Brix), TA (%), Citric and malic acid (%). Statistical comparison are not displayed, since the number of replicates was limited.

	Moisture content	**SSC**	**TA**	**Citric acid**	**Malic acid**
PC	53.93 ± 0.13	11.15 ± 0.25	0.74 ± 0.51	0.48 ± 0.03	0.07 ± 0.03
PM	58.35 ± 1.03	10.55 ± 0.15	0.78 ± 0.15	0.50 ± 0.01	0.05 ± 0.01
C1	46.69 ± 0.05	10.40 ± 0.00	1.71 ± 0.25	1.10 ± 0.02	0.12 ± 0.02
C3	50.72 ± 0.20	11.05 ± 0.15	1.54 ± 0.31	0.98 ± 0.02	0.10 ± 0.02
C5	46.71 ± 0,56	10.20 ± 0.20	1.65 ± 0.83	1.06 ± 0.05	0.11 ± 0.06
C7	48.60 ± 0.33	10.40 ± 0.00	1.80 ± 0.14	1.16 ± 0.01	0.12 ± 0.01
C9	60.07 ± 0.60	8.35 ± 0.05	1.43 ± 0.04	0.92 ± 0.00	0.10 ± 0.00
TS	58.55 ± 1.82	10.10 ± 0.30	1.02 ± 0.19	0.65 ± 0.01	0.07 ± 0.01
TC	59.66 ± 2.72	11.05 ± 0.15	1.57 ± 0.27	1.00 ± 0.02	0.11 ± 0.02
TR	50.67 ± 0.98	10.35 ± 0.15	1.29 ± 0.17	0.82 ± 0.01	0.09 ± 0.01
TCQ	58.31 ± 0.18	10.35 ± 0.05	1.31 ± 0.05	1.11 ± 0.01	0.12 ± 0.01
TCOL	54.86 ± 0.38	13.05 ± 0.05	1.04 ± 0.15	0.79 ± 0.02	0.08 ± 0.02

A seen in the first column of table 2, the edible part of the fruit (moisture content) in all varieties represented an average value of 54%, however, it must be stressed that seeds were removed to perform this analysis. In general, red varieties (Table 2) presented a superior moisture content (54.64 ± 4.25%) in comparison to the yellow-golden ones (53.39 ± 6.68%). The soluble solid content (SSC), expressed in °Brix, ranged between 8.35 ± 0.05 ºBrix (C9) and 11.15 ± 0.25 ºBrix (PC), as minimum and maximum, respectively (second column of table 2) . In general, all varieties presented an SSC nearby 11 ºBrix, whereas the red verities presented a medium value of 10.89 ± 0.84 ºBrix against 9.23 ± 0.93 ºBrix of the yellow-gold variety. The orange variety samples displayed a medium value of 10.4 ±0.00 ºBrix.

Titratable acidity (TA) varied widely in some varieties analyzed. As can be observed, in the third column of the table 2, PC variety displayed the lowest TA content (0.74 ± 0.51%) and consequently, the lowest values for the most presented organic acid in tamarillo fruits (citric acid). Inversely, C7 presented the highest values for TA content (1.80 ± 0.14%), citric (1.16 ± 0.01%) and malic acid (0.12 ± 0.01%), as seen in the fourth and fifth column of table 2).

3.2 Genetic assessment studies in tamarillo using of molecular markers

3.2.1 Random amplified polymorphic DNA (RAPD)

RAPD patterns were reproducible and clear for scoring. From the 20 RAPDs (OPC) assessed, only 4 exhibited polymorphic profiles. As an example, figure 13 exemplifies an agarose gel of the amplified products from OPC-15 RAPD marker.

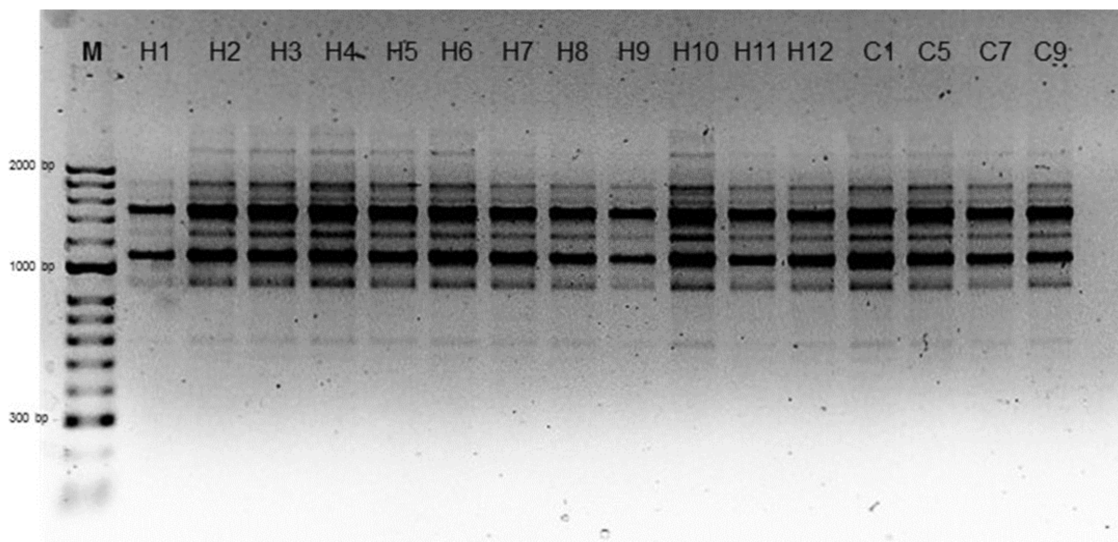


Figure 13. RAPD patterns (OPC-15) of genomic DNA from different genotypes. (M):2000 bp DNA ladder. Samples from H1-C9.The agarose gel was visualized and recorded through GelDoc XR.

Considering the products generated through the RAPDs (OPC - 6, OPC-11, OPC - 13 and OPC - 15) to DNA amplification from 16 samples of tamarillo, a total number of 48 bands was generated, from which 22 were polymorphic, representing 45.83% of total polymorphism, as indicated in table 3.

The higher percentage of polymorphic bands was generated by OPC 11 and OPC 13 having been of 50% (Table 3), whereas OPC 6 only presented 5 polymorphic profiles in a total number of 12 (41.67%).

Table 3. Resume of the results obtained with the 4 OPC primers used in RAPD analysis of *S.betaceum*.

Primer: OPC	Sequence 5'-3'	Number of Bands (nº)	Range Size (bp)	Polymorphic bands number (%)
OPC 6	GAACGGACTC	12	115-2025	5 (41.67%)
OPC 11	AAAGCTGCGG	18	500-2000	9 (50%)
OPC 13	AAGCCTCGTC	8	240-3100	4 (50%)
OPC15	GACGGATCAG	10	240-2730	4 (40%)
Total	-	48	115-3100	22 (45.83%)

A similarity matrix was obtained using Jaccard's coefficient and converted to similarities, as can be seen in the next page (Table 4). The similarity matrix was then used in cluster analysis, and a dendrogram was constructed using the MEGA version 7 software.

Table 4. Similarity indices (Jaccard's coefficient) of the tested accessions.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	C1	C5	C7	C9
H1	1															
H2	0.421	1														
H3	0.450	0.895	1													
H4	0.412	0.722	0.737	1												
H5	0.500	0.889	0.895	0.722	1											
H6	0.312	0.647	0.579	0.389	0.556	1										
H7	0.444	0.833	0.842	0.765	0.833	0.500	1									
H8	0.562	0.778	0.789	0.611	0.882	0.444	0.824	1								
H9	0.429	0.500	0.526	0.600	0.500	0.235	0.625	0.562	1							
H10	0.421	0.889	0.895	0.824	0.889	0.556	0.833	0.778	0.500	1						
H11	0.333	0.632	0.571	0.556	0.550	0.389	0.667	0.526	0.500	0.632	1					
H12	0.389	0.778	0.700	0.611	0.684	0.444	0.722	0.667	0.562	0.684	0.706	1				
C1	0.450	0.636	0.727	0.500	0.636	0.364	0.667	0.619	0.450	0.636	0.650	0.700	1			
C5	0.250	0.421	0.381	0.333	0.350	0.400	0.444	0.316	0.333	0.350	0.500	0.562	0.450	1		
C7	0.571	0.526	0.550	0.444	0.611	0.353	0.556	0.588	0.571	0.526	0.529	0.588	0.550	0.375	1	
C9	0.467	0.611	0.632	0.625	0.706	0.278	0.750	0.800	0.692	0.611	0.529	0.688	0.550	0.375	0.600	1

According to the similarity index by Jaccard's coefficient (Fig. 14), the lowest similarity found was between H6 and H9 genotypes with a value of 0.235 (23.5%) and the highest was between H2 and H3, H3 and H5, H10 and H3 being 0.895 (89.5%).

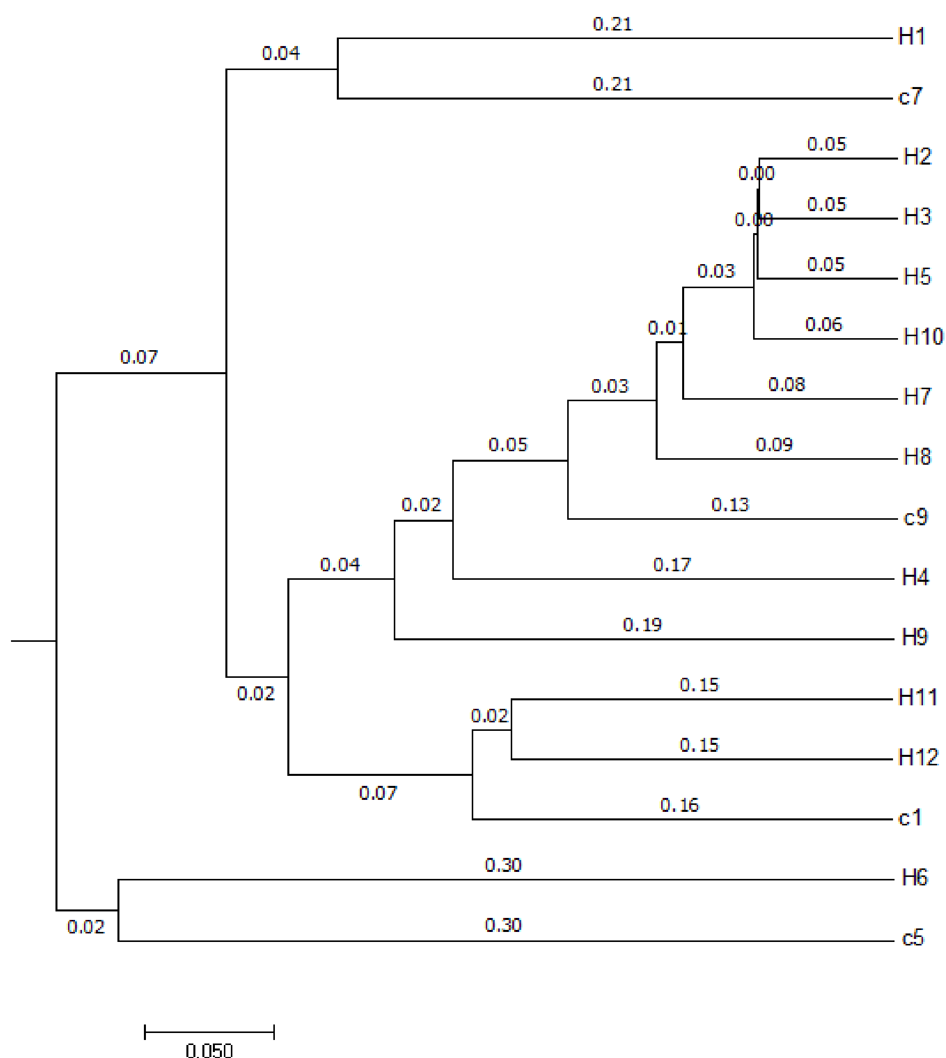


Figure 14. Dendogram of hierarchical analysis. Obtained by UPGMA, based on Jaccard's RAPD fragments, showing the genetic relationship between the 16 accessions of tamarillo.

The resulting dendrogram (Fig. 14) provides a visual representation of similarities in the studied genotypes of *S. betaceum.* As may be observed, it displays two main clusters. In the first one, genotypes C5 and H6 are accommodated, and share a higher

similarity among themselves, being of 40%. The second main cluster it is divided in two sub clusters. As seen in the dendogram, genotype H1 and C7 are isolated in one branch, revealing higher resemblance with each other (44%) than with the remaining samples assessed. The other sub cluster, possess two known genotypes (C1 and C9) and the remaining genotypes (H2, H3, H5, H7, H8, H10, H11 and H12). Finally, the genotypes of the 4 adult trees share, subsequently, 58.63% (C1), 38.67% (C5), 53.4% (C7) and 61.58% (C9) of similarity among all samples.

3.3 Culture conditions improvement through the use of CMF

3.3.1 Absorption, CMF- IAA releasement and diffusion

For IAA absorption, the concentration of the aqueous solution remained constant within the range of experimental errors (data not shown). In terms of release from CMF_{IAA} to a buffered water solution (pH = 5.8), there was steep increase in the first 2 hours, followed by a plateau until the end of the 24 hour experiment (Fig. 15, dark green curve). During the first 2 hours, the average IAA release rate was calculated taking into account

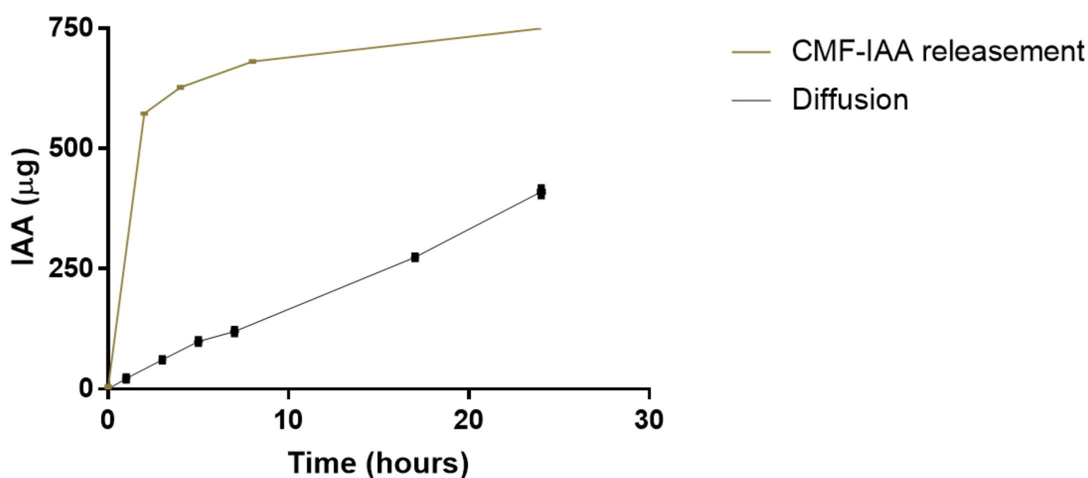


Figure 15. IAA quantification (µg) by Ehrlich reaction in CMF-IAA releasement and diffusion assays. Results are presented as mean ± SD (n = 3)

slope of the quantity versus time plot present as 286.46 µg / h. In the diffusion experiment, an approximate linear relation between IAA quantity and time was observed with an average rate of 16.91 µg / h (Fig. 15, black curve).

3.4 CMF as support to *in vitro* culture

In vitro propagation of tamarillo using CMF as a support for plant growth did not showed significant differences in terms of shoot height (Fig. 16A). Nonetheless, generally, shoot height was higher in a media supplemented with cellulose (4.14 ± 0.43 cm).

Some significant differences were observable, as seen in figure 16, in number of nodes and in adventitious roots. On one hand, the number of nodes in a medium containing CMF was significantly higher (4.2 ± 0.35) when compared with the control. On the other hand, the number of adventitious roots was significantly lower in the CMF medium (1.3 ± 0.19). In terms of secondary roots, despite there were not visible significant differences, CMF presented lower values in comparison with the control (9.8 ± 1.57 against 14.5 ± 1.9).

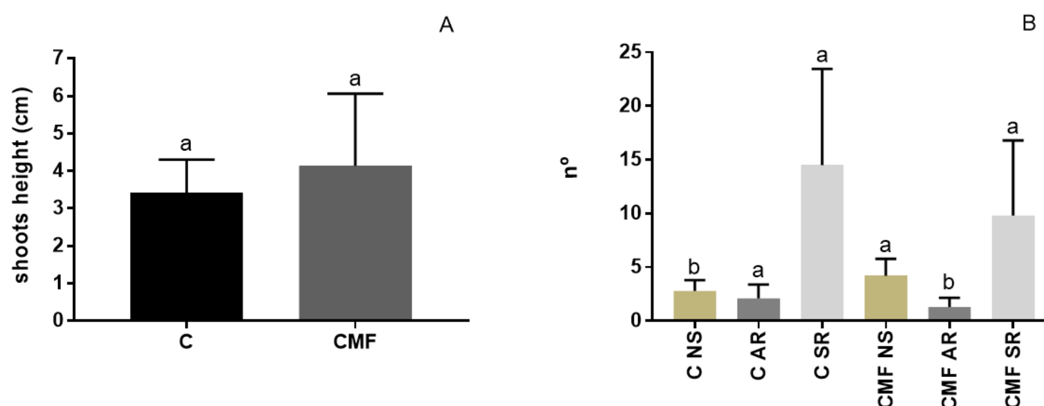


Figure 16. Shoot growth evaluation using CMF as a complement to *in vitro* culture. (A) Shoots height assessment between C (control) and CMF, after 3 months (B) Nodes (NS), Adventitious roots (AR) and Secondary roots evaluation, after 3 months. Results are presented as mean ± SD (n = 20). Different letters are statistically different by Tukey test ($p<0.05$).

3.5 Subculture of NEC in CMF films

The semi-solid media with CMF films was not measurable, once the calluses did not remain on the film surface. For this reason, only solid medium supported with CMF films was showed.

In terms of subculture of non-embryogenic calluses using CMF films as support, its mass increment revealed no significant differences (Fig. 17A), although CMF presented a lower growth (1.3 ± 0.24 g vs 2.29 ± 0.37 g). Inversely, CMF presented a significant and superior volume (Fig. 17C), having an average value of 2.83 ± 0.58 ml. The dry mass values revealed, as well, significant differences between the two experiments, in wherein, CMF demonstrated lower values (0.14 ± 0.03 g) than the control (0.28 ± 0.01 g), as seen in figure 17B.

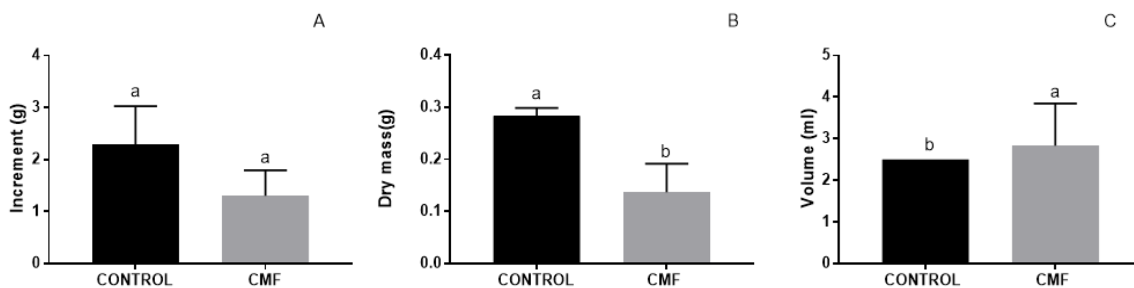


Figure 17. Influence of CMF films in non-embryogenic calluses culture. (A) Calluses increment assessment between Control and CMF, (B) Calluses dry mass evaluation between Control and CMF, (C) Total volume acquired from the calluses grown in normal medium versus calluses grown in media containing CMF. A, B and C were evaluated after 3 months of experiment. Results are presented as mean ± SD (n = 3). Different letters are statistically different by Tukey test ($p<0.05$).

Visual differences between CMF and control cells were observable, as may be seen in figure 18. Therefore, cytological analyses were required to infer if there were differences at a structural level.

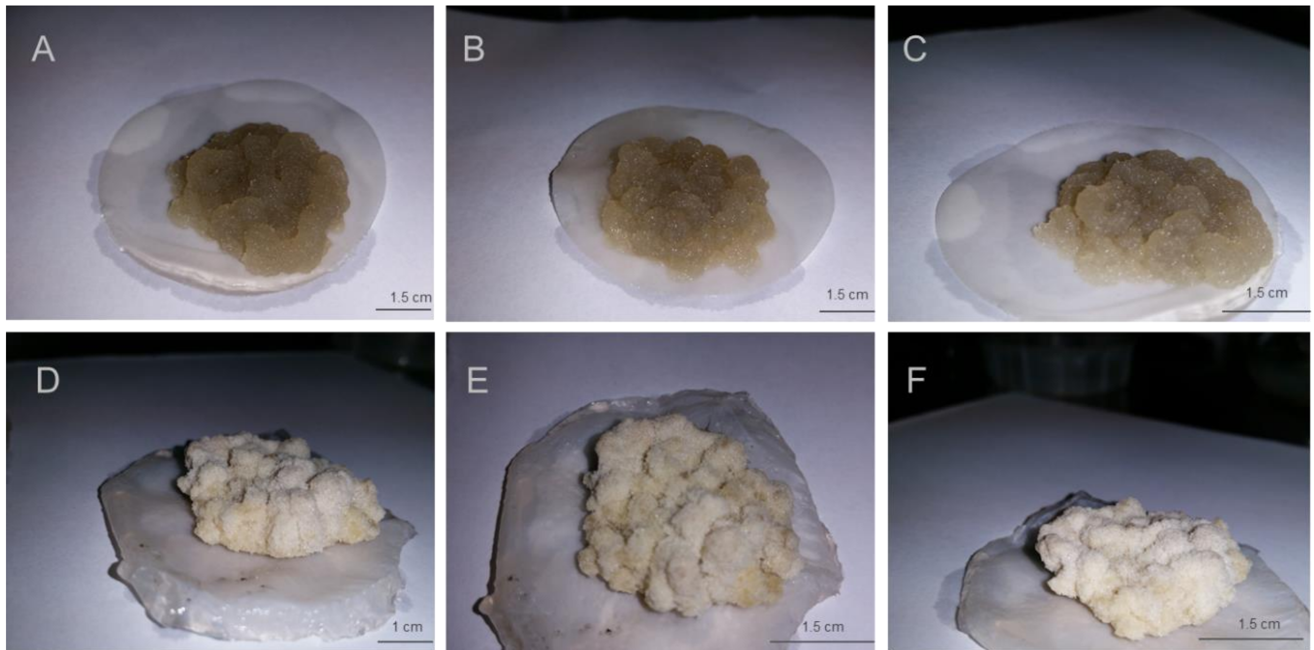


Figure 18. Calluses evolution in different culture conditions, after seven weeks. Upper images: (A, B and C) Calluses grown in a MS medium supplemented with 9% (w/v) sucrose supported with standard filters. Bottom images: (D, E and F) Calluses grown in a MS medium supplemented with 9% (w/v) sucrose supported with CMF films

As may be observed in figure 19 (next page), some differences are visible. Non-embryogenic cells grown under influence of CMF films seem to be more aggregated and its nucleus stand out more, appearing to be bigger. Beyond this, also appears to be differences in the storage cells, once, they are more visible and are present and higher quantity, when compared with the NE cells grown under control's influence.

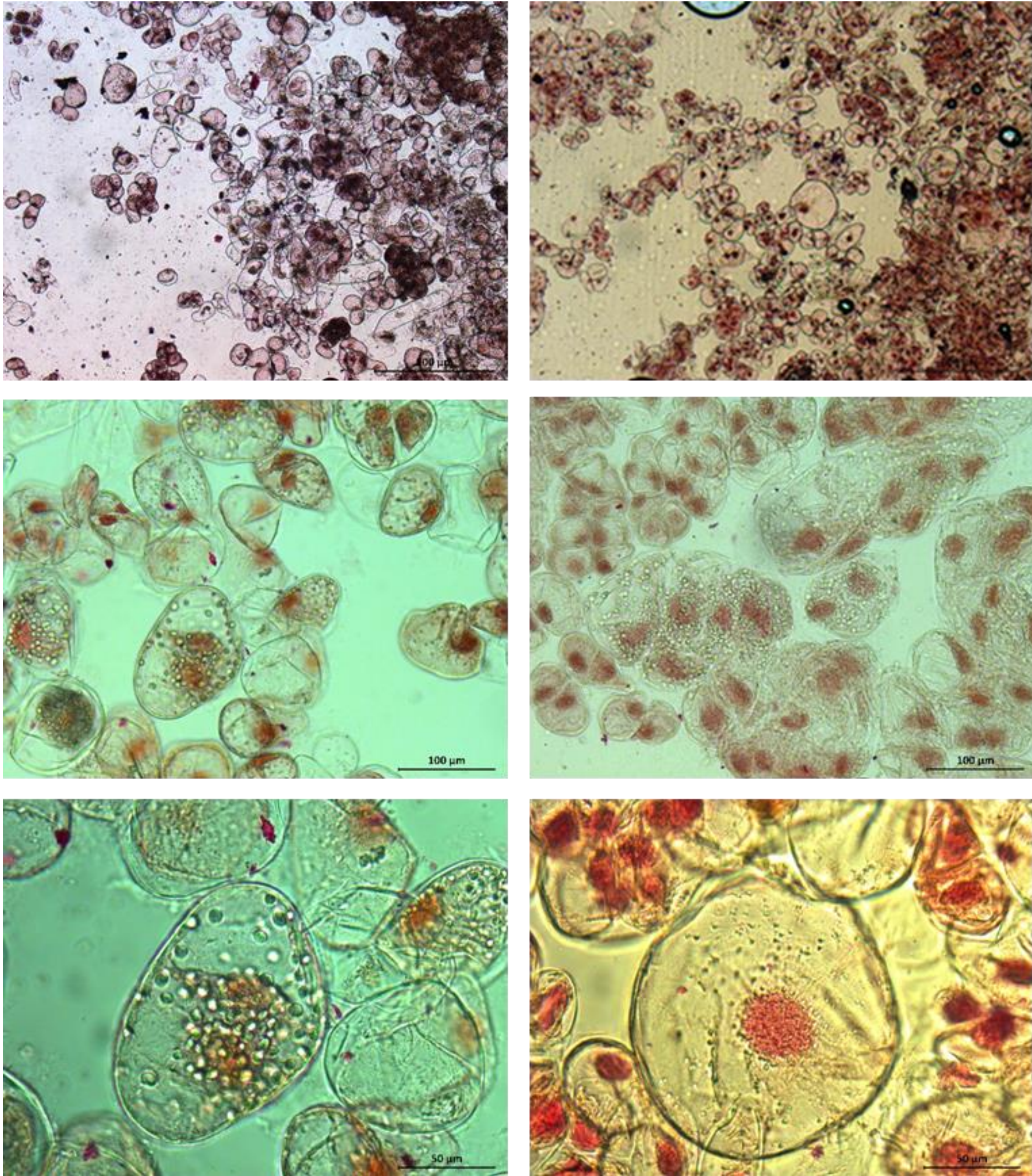


Figure 19. Comparison between non-embryogenic calluses derived from leaves of micropropagated shoots subcultured for 7 weeks on TP medium with standard filters (left side images) and grown on CMF films (right side images). All images are referred to cytological observations of non-embryogenic cells squashed and stained with acetocarmine.

4. Discussion

4.1 Physical and morphological analysis of tamarillo fruits

The evaluated fruits were originated from trees of different areas, including a sample from Colombia. This makes difficult to verify the influence of certain factors on the values of several parameters analyzed. Furthermore, abiotic factors like water intake, temperature, radiation and soil may also affect fruit composition.

Fruit quality can be evaluated by a series of parameters, *e.g.* weight, organoleptic characteristics, internal and external color, firmness, caliber, among others. Being also defined as the range of characteristics that determine its value for the consumers. Fruit weight and its caliber are significant factors in quality and, generally, the consumer prefer fruits with superior size. Hereupon, tamarillo fruits from the JBUC demonstrated to have a lower weight ~ 33 g when compared with the purchased variety (TCOL ~ 108 g). Furthermore, according to the literature, our samples, have lower weight when compared with reference values of commercialization, since yellow-gold and purple-red varieties from Ecuador possess values ranging from 107 ± 6.0 g until 188 ± 21.0 g. Thus, the higher the caliber, the highest would be the price to pay for the fruit and the Portuguese market is exigent in terms of acceptance, not accepting smaller sizer fruits, or too big, as well. Standard values sustain that tamarillo diameter should range from 4.6 until 7 cm (red variety) and from 3.9 to 5 cm for the yellow variety. In terms of length the standard values lie between 4.6 and 8 cm. According to our results, all samples satisfy the standards, expect for the Colombian variety that exceed them. In terms of diameter, the values ranged from 3 (C3) to 5.5 cm (TCOL), against standard values ranging between 3.9 until 7 cm. It is important to clearly underline that standards were established among country's that marketed tamarillo fruits for a while (New Zealand, Ecuador and Colombia) and in our case, there is not regulation and therefore no commercialization capacity (Duarte,1996; Vasco *et al.*, 2009; Schotsmans, 2011)

According to Kader & Saltviet (2003), in sensorial terms, the most important properties of fruits is the texture, which together with appearance, flavor and firmness are the key elements to influence the consumers. There have been made few studies about tamarillo firmness. Nevertheless, the methods used are not suitable for the evaluation made in this research and therefore cannot be considered as standard values for comparison. In literature, other fruits from the Solanaceae family (*Solanum lycopersicum*) presented firmness values ranging from 45–80% that could be used as standard, since the same procedures were used. Firmness values, in this research, ranged from a minimum of 58.5% (TCQ) and a maximum 84% (C5). The minimum value presented by TCQ can be explained by having exceeded the commercial maturity. Establishing a comparison between the two *Solanum* species referred earlier, the tamarillo firmness fitted the standard values.

Regarding non-climacteric fruits, such as tamarillo, its potential quality cannot be improved during processing, but is possible to maintain until it reaches the final consumer. In fact, this was possible to observe, once samples from Colombia were sent without peduncle. Peduncle length and thickness gives us the following information: thin and longer stems are more flexible making easier to collect and transport the fruits. In contrast, thick and shorter peduncles are more likely to damage fruit quality and should be cut above the sepal's insertion. Base peduncle thickness can be related to the fruit commercial maturation stage, since peduncle abscission occurs causing an accelerate water loss and chlorophyll degradation and, ultimately, detaches. No previous reports are known about this parameter, for this reason there are no standard values. Resuming the three analyses made, fruits of the TR variety displayed the longer peduncle, and the lowest values for medium and base peduncle thickness, making it an interesting material for

future breeding programs. Concerning base peduncle thickness, PC and TCQ were distinguished, for having the higher and the lower value, respectively.

Moisture content analysis is a critical parameter for evaluation of fruit quality and essentially a function of quality control. Currently, many moisture analysis methods are available and the AOAC official methods (Horwits, 2000) have been the most used according to the literature, despite not being the elect to conduct this analysis. Our results showed that, even though moisture content from C9 variety has revealed the highest value, over 50%, fruits and vegetables have an average moisture content of 70 to 95% (Brown, 2007). For this reason it seems reasonable to assume that our values cannot be validated and new performances are necessary.

Differences have been observed among cultivars for some relevant compositions traits. For example, the red variety had highest SSC and lowest TA, suggesting that this variety had a sweeter taste than the remaining ones. In agreement to Boyes & Strübi (1997), the orange group also revealed the highest TA value (1.81 ± 0.01%), which indicates that this cultivar will have a more acidic taste. Generally, SSC and TA values are in accordance with the literature (Vasco *et al.*, 2009). Further, according to (Paes *et al.*, 2015) a SSC value over 12% can qualify tamarillo either for raw consumption or industrial processing. The main organic acid detected in tamarillo samples was citric acid, which occurs together with much smaller amounts of malic acid. These results are in agreement with previous studies (Schotsmans, 2011).

Additionally, when the fruits were sliced, to perform TA, SSC and moisture procedures, some abnormal structures were found in all varieties, except in TR. In the literature there are no existing information concerning this issue, but efforts should be made to characterize it, once, appear to be potentially hazardous structures to consumers.

4.2 Genetic assessment studies in *S. betaceum* through the use of molecular markers.

RAPD assays has been used to investigate genetic studies in several species of the genus *Solanum* (Arias *et al.*, 2010; Onamu *et al.*, 2015). For this reason, no standard values are available for comparison. Our work represents the first study of genetic diversity among 16 tamarillo genotypes using RAPD (4 genotypes from adult trees and 12 hybrids obtained from artificial hybridyzation).

From the 20 primers tested only 16 generated amplification PCR products and from these only 4 (OPC 6, OPC 11, OPC 13 and OPC 15), scored a total number of 48 polymorphic bands. The selected primers gave clear and reproducible patterns. The patterns distinguished between the plants and their analysis established an approach to distinguish them based on RAPD markers. The dendrogram available clearly differentiated all the 16 genotypes and combining its analysis with the similarity index some differences were found: the genotypes that share a wider genetic information between themselves are in the second sub-cluster (C1– H2). From these study we deduce that the 4 adult tree genotypes that share more genetic information between all samples were C1 (58.63%) and the C9 (61.58%), revealing the possibility of the first to be the potential male parent and the second one the female parent. The results of our study clearly demonstrate that tamarillo cultivars could be identified based on their RAPD fingerprints, but more studies are required to complement this information.

4.3 Culture conditions improvement through the use of CMF

These studies were carried out to determine the characteristics of absorption, IAA release and diffusion assays of CMF in order to use this material in plant tissue culture assays. In these exploratory experiments IAA was used, not only because of its importance in the control of physiological and stress phenomena, but also because it is a small molecule and possess a weak acidic nature that accounts for some diffusion-driven transport at near IAA isoelectric point. Therefore, due to these physical characteristics its physiological importance as well as the availability of an optimized colorimetric procedure for quantification, we chose to use this hormone for these assays.

The absorption assay revealed that CMF did not apparently retain IAA when in contact with an aqueous buffered solution of this hormone. The transport mechanism in this particular case, may have been inhibited because of pH difference established between the aqueous media and the membrane, as the pH of the CMF is difficult to measure accurately. In addition, in the two media with similar pH, separated by CMF membranes, there seems to have occurred a diffusion phenomenon, this effect could be explained assuming membranes impermeability properties. Nevertheless, further research is required to consolidate this studies.

Overall, these assays seem to indicate that CMF are not potential auxin controlled delivery systems, as IAA release mostly took place in the initial 3 hours. However, this membranes appear to have porous features allowing mass transfer phenomena (concentration driven transport phenomena, such as Fick's diffusion) with a constant rate of IAA diffusion. This interesting property could potentially be applied for an interface in solid/liquid culture system with continuous refreshing of the liquid phase.

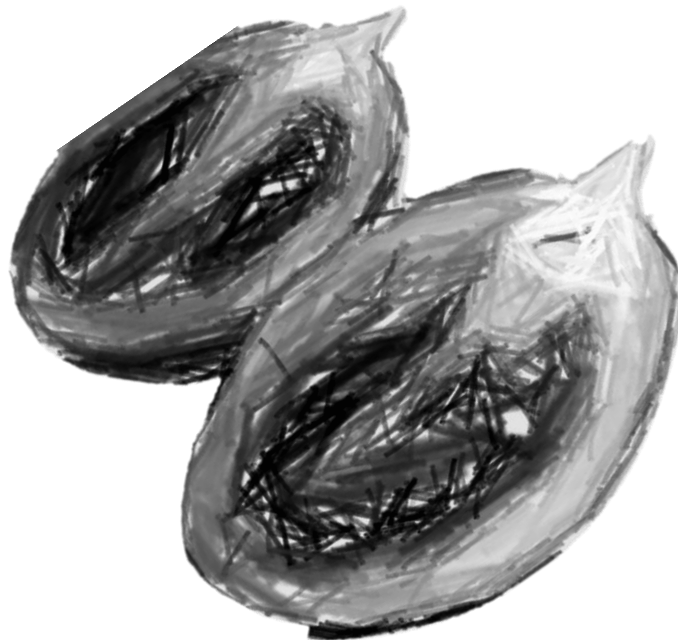
4.4 Use of CMF as an alternative support to *in vitro* culture

The inclusion of CMF as a component to media for *in vitro* culture of plants was evaluated through the culture of nodal segments of tamarillo. The results showed that shoots developed in CMF supplemented medium reached a higher height than those obtained on CMF-free medium, although the results were not significantly different. Further, a significant difference was observed in terms of nodal segments and adventitious roots, having been superior in CMF shoots in the first case and in control shoots, in the second one. This could be related to the development responses behind the influence of the hormone used, once BAP is usually very effective for nodal explant regeneration, boosts cell division and elicit plant growth (Adrian *et al.,* 2003). Hereupon, CMF derived shoots, apparently, had a better capacity to retain this hormone than the shoots from the CMF-free medium. On the other hand, the superior development of secondary roots by CMF-free medium shoots can be related to two possible factors: the influence by the high levels of endogenous auxins in tamarillo or either by the highest synthesis of BAP in this target location. Additionally, the shoots' leaves from the CMF medium presented a greenish-yellow color, potentially indicating a nutritional deficit, such as lack of macronutrients. Hence, more studies to test the chlorophyll content are required to understand if there are effective differences.

The calluses development in media with standard filters was more effective, since it occurred a higher mass increment as well as dry mass, with the dry mass revealing statistic differences. This can be related to a more effective delivery of nutrients into the cells, since CMF films are more impermeable thus preventing the normal absorption of nutrients. Despite this, the calluses grown in CMF films presented a peculiar appearance and had a higher volume. This may be related with an increase of the mitotic index or to an increase in cell volume. Cytological observations made in non-embryogenic calluses,

subcultured for 7 weeks on TP media, showed that cells on CMF possessed more plastids *per* individualized cell and the nucleus was more prominent. This may indicate a change on metabolism due to the modification on the nutrients that are accessible for the cells when culture was carried out under CMF membranes. How CFMs interfere with nutrient absorption and influences cell metabolism needs further analysis.

5. Conclusions and Future Perspectives



Our results indicate that wide variation in the characteristics of the fruits occurs among tamarillo accessions. All samples assessed fulfill the firmness standards, meaning that the fruits from all origins presented good quality. However, C5 variety should be the chosen for breeding programmes since it presented the highest firmness values. The caliber (length and diameter) and firmness values were highly satisfactory, but the same does not happen in terms of weight, once our samples possessed much lower values when compared with the standard for marketing, being a quite unsatisfactory factor, concerning an overall evaluation. To overcome this issue, some production strategies are required. Apart from watering, fertilizing, weeding and clearing, the fruit removal from the tree can be done in order to get sizes and weights suitable for commercialization. Peduncle and calyx are also structures that may affect fruit quality. When these structures are removed from the fruit, accelerated water loss and chlorophyll degradation occurs. In our research, TR variety revealed to be the best variety, once it expressed longer and thinner peduncles, being a good line to select to cross with others with potential interesting traits. Concerning tamarillo endogenous organic acids, red varieties revealed less acidity, since citric and malic acid values were lower, as well as TA. Concerning this, PC reveled to be the sweetest variety, since it revealed the lowest values for TA and the highest for SSC, and that should be taken into consideration for improvement assays. Regarding moisture assays, the values were below what should be expected, not reaching the average moisture content (70 to 95%). In order to validate the standard, new performances are required and the methodology needs improvement. Nonetheless, there are good prospects for the selection of tamarillo with improved organoleptic, nutritional and functional quality. Hereupon, breeding programmes and production strategies will help in developing tamarillo as a commercially important crop, especially in Portugal.

The reproducibility of RAPD amplification is recognized to be greatly influenced by experimental conditions, but proves to be particularly suited for studies in species in which the genome is poorly known. Our data indicated that these markers are proper for an initial approach and 16 tamarillo accessions were analyzed. The results obtained clearly displayed genomic variations between the DNA of 4 adult trees (C1, C5, C7 and C9) and the 12 hybrids (H1 – H12). Through the cluster analysis and similarity indices it can be concluded that the hybrids share more traits with the C9, C1 and C7 genotypes. Specifically, H7 and H8 genotypes reveal highly conserved specific genes from the C9 genotype, once they share ~ 80% of similarity, for this reason its phenotype will be very similar to this yellow variety. The same happens with H3 and H12 genotypes, which share more than 70% with C1 genotype. Hereupon, its phenotype will resemble with this red crop. Although, the accuracy of these results should be confirmed with studies using a different markers such as microsatellites and/or AFLP and the hybrids phenotype diagnosis will be confirmed as soon as the trees exhibit its fruits.

In this work it was tried to improve *in vitro* culture conditions through the use of a bio-based material, *i.e.*, CMF. Regarding preliminary assays of CMF-IAA release and diffusion, IAA proved to be a suitable factor to characterize the CMF's behavior. CMF seems not to be able to retain IAA, but some diffusion phenomenon happened, indicating that CMFs are not well suited biomaterials for auxin release. Nonetheless, CMF membranes apparently had porous features since mass transfer phenome occurred. Even so, CMF films were applied to observe if there were disparities in the development in non-embryogenic calluses cultures. It was possible to observe some differences, mostly in mass increment (media with standard film), and in terms of volume (media with CMF film) concluding that, probably, the films due to intrinsic properties *e.g.* impermeability not allowed the transfer of nutrients. Finally, the use of CMF films in order to substitute

the standard filters showed to be ineffective and, although some interesting results were achieved, more test are required to obtain more strong answers. In contrast, CMF as complement to *in vitro* propagation showed positive outcomes, since it was achieved an increase in shoot height and a greater number of nodes when CMF was added to the media. Although this could be related to the PGRs influence, it seems reasonable to admit that the results were promising.

Globally, it is expected that our results have helped to increase the knowledge and contributed to the development of tamarillo as an important crop.

6. References

Abdul Khalil HPS, Davoudpour Y, Islam MN, Mustapha A, Sudesh K, Dungani R, Jawaid M. (2014) Production and modification of nanofibrillated cellulose using various mechanical processes: a review. Carbohydrate Polymers 99: 649-665.

Acosta-Quezada PG, Martínez-Laborde JB, Prohens J. (2011) Variation among tree tomato (*Solanum betaceum* Cav.) accessions from different cultivar groups: implications for conservation of genetic resources and breeding. Genetic Resources and Crop Evolution 58: 943-960.

Acosta-Quezada PG, Vilanova S, Martínez-Laborde JB, Prohens J. (2012) Genetic diversity and relationships in accessions from different cultivar groups and origins in the tree tomato (*Solanum betaceum* Cav.). Euphytica 187: 87-97.

Acosta-Quezada PG, Raigón MD, Riofrio-Cuenca T, García-Martínez MD, Plazas M, Burneo JI, Figueroa JG, Vilanova S, Prohens J. (2014). Diversity for chemical composition in a collection of different varietal types of tree tomato (*Solanum betaceum* Cav .), an Andean exotic fruit. Food chemistry 169: 327-335.

Adrian, S, Nigel, S. and Mark, F. (2003). *Plant* Biotechnology: The genetic manipulation of plants. Oxford University Press, UK.

Angulo-Bejarano PI & Paredes-López O (2011) Development of a regeneration protocol through indirect organogenesis in prickly pear cactus (*Opuntia ficusindica* (L.) Mill). Scientia Horticulturae, 128: 283-288.

Arias Y, Peteira B, González I, Martínez Y, Miranda I. (2010) Variabilidad genética entre genotipos promisorios de tomate (*Solanum lycopersicum*) obtenidos en programas de mejoramiento frente al TYLCV 25: 190-193.

Barghchi M (1986) *In vitro* rejuvenation of *Cyphomandra betacea* (tamarillo). Plant Physiology Division Biennial Report, Department of Scientific and Industrial Research (DSIR), New Zealand, pp. 52.

Barghchi M (1998) *In vitro* regeneration, plant improvement and virus elimination of tamarillo [*Cyphomandra betacea* (Cav.) Sendt)]. In: Davey M, Alderson P, Lowe K, Power J. (Eds.) Tree Biotechnology – Towards the Millennium. Nothingham, Nothingham University Press, pp. 173-185.

Bohs L. (1989) Ethnobotany of the genus *Cyphomandra* (Solanaceae). Economic Botany, pp. 143-163.

Boyes S, Strubi P. (1997) Organic acid and sugar composition of three New Zealand grown tamarillo varieties (*Solanum betaceum* (Cav.)). New Zealand Journal of Crop and Horticultural Science, Wellington, v. 25: 79-83.

Brown AC. (2007) Understanding Food: Principles and Preparation (3rd edition) Cengage Learning, pp. 25.

Caeiro AF. (2015) Influência das auxinas endógenas e de proteases extracelulares no processo de embriogénese somática em tamarilho (*Solanum betaceum*). Tese de Mestrado, Universidade de Coimbra, Portugal, pp. 13-21.

Carnevali A. (1974) Il tamarillo: una nuova possibilità per la frutticoltura meridionale. Frutticoltura 36: 31-37.

Canhoto JM. (2010) Biotecnologia Vegetal, da clonagem de plantas à transformação genética, Imprensa da Universidade de Coimbra, Portugal.

Canhoto JM, Lopes ML, Cruz GS. (2005) Protocol for somatic embryogenesis: tamarillo (*Cyphomandra betacea* (Cav.) Sendt.). In: SM Jain, PK Gupta (Eds.) Protocol for Somatic Embryogenesis in Woody plants, Dordrecht, Springer, pp. 379-389.

Cohen D, Elliot D (1979) Micropropagation methods for blueberries and tamarillos. Combined Proceedings. International Plant Propagators Society 29: 177-179.

Correia SI. (2011) Somatic embryogenesis in *Cyphomandra betacea* (Cav.) Sendt (tamarillo): optimization and molecular analysis. Tese de Doutoramento, Universidade de Coimbra, Portugal.

Correia SI, Canhoto JM. (2012) Biotechnology of tamarillo (*Cyphomandra betacea*): From *in vitro* cloning to genetic transformation. Scientia Horticulturae 148: 61-168.

Correia SI, Lopes ML, Canhoto JM. (2011) Somatic embryogenesis induction system for cloning an adult *Cyphomandra betacea* (Cav.) Sendt. (tamarillo). Trees.

Currais L, Loureiro J, Santos C, Canhoto JM. (2013) Ploidy stability in embryogenic cultures and regenerated plantlets of tamarillo. Plant Cell, Tissue and Organ Culture 114: 149-159.

Duarte O, Alvarado E. (1997) Tratamientos para mejorar la propagación del tomate de árbol (*Cyphomandra betacea* Sendt.) por semillas y estacas. Proc. Interamer. Soc. Trop. Hort. 41: 248–251.

Dufresne A. (2012) Nanocellulose, From nature to high performance tailored materials, Walter de Gruyter GmbH, Berlin/Boston.

Gamelas JAF, Pedrosa J, Lourenço AF, Ferreira PJ. (2015) Surface properties of distinct nanofibrillated celluloses assessed by inverse gas chromatography. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 469: 36-41.

Gamelas JAF, Pedrosa J, Lourenco AF, Mutjé P, González I, Chinga-Carrasco G, Singh G, Ferreira PJ. (2015) On the morphology of cellulose nanofibrils obtained by TEMPO-mediated oxidation and mechanical treatment. Micron 72: 28-33.

Guimarães ML, Tomé MC, Cruz GS, (1996) *Cyphomandra betacea* (Cav.) Sendt. (tamarillo) In: Bajaj, Y.P.S. (Ed.), Biotechnology in Agriculture and Forestry vol. 35, Trees IV. Springer Verlag, Berlin, pp. 120–137.

Guimarães ML, Cruz GS, Montezuma-de-Carvalho JM (1988) Somatic embryogenesis and plant regeneration in *Cyphomandra betacea* (Cav.) Sendt. Plant Cell, Tissue and Organ Culture 15: 161-167.

Henriksson M & Berglund LA (2007) Structure and Properties of Cellulose Nanocomposite Films Containing Melamine Formaldehyde. Journal of Applied Polymer Science. 43: 2817-2824.

Henriksson M, Henriksson G, Berglund LA, Lindström T. (2007) An environmentally friendly method for enzyme-assisted preparation of microfibrillated cellulose (MFC) nanofibers. European Polymer Journal 43: 3434-3441.

Herrick FW, Casebier RL, Hamilton JK, Sandberg KR. (1983) Microfibrillated cellulose: morphology and accessibility. J Appl Polym Sci: Appl Polym Symp 37: 797–813.

Hicks GS (1994) Shoot induction and organogenesis *in vitro*: a developmental perspective. *In Vitro* Cellular & Developmental Biology-Plant 30: 10-15.

Hubbe MA, Rojas JO, Lucia LA, Sain M. (2008) Cellulosic Nanocomposites: a review. BioResources, 3: 929-980.

Kader AA & Saltviet ME. (2003) Atmosphere Modification. In: Posthar-vest Physiology and Pathology of Vegetables, Bartz, J. A. and Brecht, J. K. (eds.), Marcel Dekker, New York pp. 229–246.

Kiziltas EE, Kiziltas A, Bollin SC, Gardner DJ (2015) Preparation and characterization of transparent PMMA – cellulose-based nanocomposites. Carbohydrate Polymers, 127: 381–389.

Klemm D, Heublein B, Fink HP, Bohn A. (2005) Cellulose: Fascinating Biopolymer and Sustainable Raw Material. Angewandte Chemie - International Edition, 44: 3358-3393

Kou M, Yen J, Hong J, Wang C. (2008) *Cyphomandra betacea* Sendt. phenolics protect LDL from oxidation and PC12 cells from oxidative stress. LWT - Food Science and Technology, 42: 458–463.

Kumar P, Gupta VK, Misra AK, Modi DR, Pandey BK. (2009). Southern Cross Journals. 2009 Potential of Molecular Markers in Plant Biotechnology, 2: 141–162.

Lacerda DR, Acedo MDP, Lovato, MB. (2002) A técnica de RAPD : uma ferramenta molecular em estudos de conservação de plantas. 3: 87-92.

Lavoine N, Desloges I, Bras J (2014) Microfibrillated cellulose coatings as new release systems for active packaging. Carbohydrate Polymers, 103: 528–537.

Lewis DH, Considine JA, 1999a. Pollination and fruit set in the tamarillo (*Cyphomandra betacea* (Cav.) Sendt.) 1. Floral biology. N. Z. J. Crop Hort. Sci. 27: 101–112.

Lim TK. (2013) *Solanum betaceum*. Edible Medicinal and Non-Medicinal Plants, v.6.

Lopes ML, Ferreira MR, Carloto JM, Cruz GS, Canhoto JM (2000) Somatic embryogenesis induction in tamarillo (*Cyphomandra betacea*). In: SM Jain, PK Gupta, RJ Newton (Eds.) Somatic Embryogenesis in Woody Plants, vol. 6. Dordrecht, Kluwer Academic Publishers, pp. 433-455. Morton JF (1987) Tree Tomato. In: JF Morton (Ed.) Fruits of Warm Climates. Miami, FL, pp. 437- 440.

McCane J, Widdowson DA (1992) In: Fruit and nut (Suppl. To the composition of foods.) 5th Ed. London, Holland, Unwin & Buss, pp. 74-77.

Mullis KB, Faloona FA. (1987) Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol, 155: 335-350.

Murashige T., Skoog F. (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant, 15: 473-497.

Mwithiga G, Inzoberi M, Shitanda D, Nyota P. (2007) Evaluation of the effect of ripening on the sensory quality and properties of tamarillo (Cyphomandra betaceae) fruits. Journal of food Engineering, 79: 117-123.

Onamu R, Porfirio Legaria-Solano J, Sahagún-Castellanos J, Rodriguéz-de-la-O JL, Pérez-nieto J. (2015) Diversidad genética entre variedades de papa (*Solanum tuberosum* L.) cultivadas en méxico 38: pp. 7-15.

Osong SH, Norgren S, Engstrand P. (2016) Processing of wood-based microfibrillated cellulose and nanofibrillated cellulose , and applications relating to papermaking : a review. Cellulose, 23: 93-123.

Osorio C, Hurtado N, Dawid C, Hofmann T, Heredia-Mira FJ, Morales AL (2012). Chemical characterisation of anthocyanins in tamarillo (*Solanum betaceum* Cav.) and Andes berry (*Rubus glaucus* Benth.) fruits. Food Chemistry, 132: 1915-1921.

Paes AS, Quast LB, Quast E, Raupp DS (2015) Desenvolvimento de Geleia Light de Tamarillo Contendo Alto Teor de Polpa Development of Tamarillo Light Jelly with High Pulp Content. , 17: 293–306.

Pongjaruvat W (2008) Effect of modified atmosphere on storage life of purple passion fruit and red tamarillo, Palmerston North, New Zealand, Massey University.

Popenoe HL, King SR, León J, Kalinowski LS, Vietmeyer ND (1989). Lost Crops of the Incas: Little-known Plants of the Andes with Promise for Worldwide Cultivation, Washington, D.C., the National Academy Press.

Pratt, HK , Reid MS (1976) The Tamarillo : Fruit Growth and Maturation , Ripening , Respiration , and the Role of Ethylene, pp.399–404.

Prohens J, Nuez F. (2000) The Tamarillo (*Cyphomandra betacea*): A review of a promising small fruit crop. Small Fruits Review, 1: 43-68.

Prohens J, Ruiz JJ, Nuez F (1996) Advancing the tamarillo harvest by induced postharvest ripening. HortScience 31: 109-111.

Rafalski, J.A., 1991. RAPD markers- a new technology for genetic mapping and plant breeding. Ag. Biotech. News and Information 3: 645-648.

Rose RJ, Mantiri FR, Kurdyukov S, Chen SK, Wang XD, Nolan KE, Sheahan MB (2010). Developmental biology of somatic embryogenesis. In: Plant developmental biology-biotechnological perspectives, v.2 Springer, pp. 3-26.

Schotsmans WC, East A, Woolf A. (2011) Tamarillo (*Solanum betaceum* (Cav.)). In: Postharvest Biology and Technology of Tropical and Subtropical Fruits. Woodhead Publishing Limited, pp. 427 - 441, 442e.

Siró I, Plackett D. (2010) Microfibrillated cellulose and new nanocomposite materials: A review. Cellulose, 17: 459-494.

Syverud, K, 2014. Highlights from ten years of nanocellulose research at PFI Recent advances in cellulose nanotechnology research.

Syverud K, Stenius P (2009) Strength and barrier properties of MFC films. Cellulose 16: 75-85.

Sugiyama M (1999) Organogenesis *in vitro*. Current Opinion in Plant Biology 2: 61-64.

Vasco C, Ruales J, Kamal-Eldin A. (2008). Total phenolic compounds and antioxidant capacities of major fruits from Ecuador. Food Chemistry, 111: 816–823.

Vasco C, Avila J., Ruales J, Svanberg U, Kamal-Eldin A. (2009). Physical and chemical characteristics of golden-yellow and purple-red varieties of tamarillo fruit (*Solanum betaceum* Cav.). International Journal of Food Sciences and Nutrition, 60: 278-288.

Tonon G, Capuana M, Di Marco A. (2001) Plant regeneration of *Fraxinus angustifolia* by *in vitro* shoot organogenesis. Scientia Horticulturae 87: 291-301.

Thorpe T (1980) Organogenesis *in vitro*: structural, physiological, and biochemical aspects. International review of cytology Suppl. 11: 71-111.

Turk BA, Swartz HJ, Zimmerman RH. (1994) Adventitious shoot regeneration from *in vitro* cultured leaves of *Rubus* genotypes. Plant Cell, Tissue and Organ Culture 38: 11-17.

Turbak AF, Snyder FW, Sandberg KR (1983) J Appl Polym Sci: Appl Polym Symp 37: 815–827.

Ummartyotin S, Manuspiya H. (2015) A critical review on cellulose: From fundamental to an approach on sensor technology. Renewable and Sustainable Energy Reviews, 41: 402-412.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18: 6531-6535.

Yang X, Zhang X. (2010) Regulation of Somatic Embryogenesis in Higher Plants. Critical Reviews in Plant Sciences 29: 36 - 57.

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell, 5: 1411-1423.