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Use of standard and setup of non conventional techniques for the elimination of viruses associated with Fig Mosaic Disease (FMD) in fig germplasm (*Ficus carica* L.).

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List of abbreviations

%	Percentage
°C	Celsius degree
µl	Microliter
µM	Micromolar
2iP	2-iso-pentenyl adenine
Ads	Adenine sulphate
BAP	Benzyleaminopurine
bp	Base pair
BYV	Beet yellows virus
Ca	Circa
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
CP	Coat protein
CPm	minor coat protein
Cvs	Cultivars
dm^{-3}	Cubic decimeter
DMBs	Double membraned-bodies
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidetriphosphate
EDTA	Ethylene diaminotetraacetic acid
EtOH	Ethanol
FAO	Food and Agriculture Organization
FBV-1	Fig Badnavirus 1
FCV	Fig cryptic virus
FFkaV	Fig fleck-associated virus
FLMaV-1	Fig leaf mottle-associated virus 1
FLMaV-2	Fig leaf mottle-associated virus 2
FLV-1	Fig latent virus 1
FMMaV	Fig mild mottling-associated virus
FMV	Fig mosaic virus
GA_3	Gibberellic acid
h	Hour
Hel	Helicase
HP	Homing protein
HSP	Heat-shock protein
IBA	Indole-3-Butyric Acid
ICTV	International Committee for Taxonomy of Viruses
kb	Kilobase
kDa	kilodalton
LB	Lysogeny broth
M	Molar
m	Meter
M	Molar
mg L^{-1}	Milligram/liter
Mg mL^{-1}	Milligram/milliliter

$MgCl_2$	Magnesium chloride
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
M-MLV	Moloney-murine leukemia virus
MP	Movement protein
MS	Murashige and Skoog
MT	meta-Topolin
MTR	Methyltransferase
MTC-SS	Meristem tip Culture Protected using the synthetic seeds technique
N°	Number
NAA	1-naphtalene acetic acid
NaI	Sodium iodide
NBP	Nucleotide-binding protein
ng	Nanogram
NLS	Sodium laurilsarcosine
nm	Nanometer
nt	Nucleotide
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PG	Phloroglucinol
PGRs	Plant growth regulators
PPFD	Photosynthetic photon flux density
Pro	Protease
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RNase	Ribonuclease
RP	Replication-associated protein
rpm	Round per minute
RT-PCR	Reverse transcription-polymerase chain reaction
sec	Second
STC	Shoot tip culture
TAE	Tris acetate-EDTA
TDZ	Thidiazuron
TNA	Total nucleic acid
TR	Translated region
U	Unit
UTR	Untranslated region
UV	Ultra-violet
V	Volt
Vol	Volume
VPg	Genome-linked protein
WPM	Woody Plant Medium
ZEA	Zeatine
µl	Micro liter
µmol m^{-2} s^{-1}	Micromole /per square meter /per second
2,4-D	2,4-Dichlorophenoxyacetic acid

Dedication

This doctoral thesis is dedicated to
the memory of my late beloved father..

I would like to thank you, daddy, for your grace, benevolence and for giving me the determination to overcome many trying moments to pursue my dreams..

Without you, I wouldn't be the woman I'm today..

Hope that you are proud of your "little girl" as you used to call me...May Allah grant you an eternal rest, Amen..

Years may come and go but your memory will never be erased.

Forever in my heart..

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Emna YAHYAOUI

Abstract

Ficus carica L. is considered one of the oldest fruit trees in the Mediterranean basin and is widely grown and harvested for the consumption of its fruits dry and fresh. This species is affected by different virus diseases, especially by Fig mosaic disease (FMD), for which Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), Fig mild mottling-associated virus (FMMaV), *Fig mosaic virus* (FMV), Fig latent virus 1 (FLV-1), Fig badnavirus 1 (FBV-1) and Fig fleck-associated virus (FFkaV) are associated. FMD is the most widespread disorder of this species, which represents a threat and a constraint for healthy fig production and germplasm exchange.

Thus, the objective of the present doctoral research was the establishment of an efficient and rapid *in vitro F. carica* propagation, sanitation and conservation of free-FMD plant material for future large-scale commercialization.

Initially, FMD-related viruses distribution was screened within the different fig plant organs (buds, leaves, syconia and seeds) of 14 Mediterranean genotypes (Palazzo, Severoni precoce, Bianca, Pilusedda, Dottato bianco, Bifera, Zidi, Baiyadi, Biancu, Brogiotto nero, Catalanisca, Houmairi, Triboiti and Turca 'Serilop') which were utilized afterward as *in vitro* plant source material. RT-PCR assays revealed that all the aforementioned viruses were present without any exception in seeds, whereas only 4 viruses (FBV, FFkaV, FLMaV-1 and FMV) were detected in buds, leaves and syconia with highly variable infection rates.

Moreover, encapsulation technology proved to be a powerful multiplication technique to sustain standard fig tissue culture protocol for three cultivars (Catalanisca, Palazzo and Bifera) and it gave high, almost similar, viability, regrowth and conversion rates. Microcutting rooting in one-step was achieved and conversion rate was comparable for the three cultivars.

Furthermore, in order to eliminate FMD associated viruses, with the exception of FBV-1 which resisted to all the sanitation attempts, Caulogenesis and Meristem Tip Culture Protected by the Synthetic Seeds technique (MTC-SS) gave the best sanitation rates.

Finally, *F. carica* (cv. Houmairi) artificial seeds conservation, for final delivery, was achieved. A high viability and moderate regrowth rates were registered with a lesser conversion rate strictly related to the plant growth regulators (PGRs) used.

Keywords: Fig, mosaic, RT-PCR, virus distribution, cytokinins, encapsulation, micropropagation, synthetic seed.

Riassunto

Ficus carica L., una fra le più antiche specie di alberi da frutto del bacino del Mediterraneo, ha un ampio areale di coltivazione considerando l'opportunità di consumo del prodotto sia fresco che essiccato. Attualmente, "Fig mosaic disease" (FMD) ad eziologia complessa è la sindrome più diffusa e dannosa. Virus differenti,quali Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), Fig mild mottling-associated virus (FMMaV), *Fig mosaic virus* (FMV), Fig latent virus 1 (FLV-1), Fig Badnavirus 1 (FBV-1) e Fig fleck-associated virus (FFkaV) sono, infatti, associati alla sindrome che costituisce una grave minaccia per la produzione e il commercio del germoplasma di fico.

In considerazione di tale problematica, il presente lavoro di tesi è stato focalizzato sulla valutazione dell'efficacia di metodologie standard e innovative per la moltiplicazione, risanamento e successiva conservazione di germoplasma di *F. carica* esente da FMD, da poter commercializzare su larga scala.

Inizialmente, la distribuzione nei vari organi dei virus associati a FMD è stata verificata in 14 differenti genotipi di *F. carica* (Palazzo, Severoni precoce, Bianca, Pilusedda, Dottato bianco, Bifera, Zidi, Baiyadi, Biancu, Brogiotto nero, Catalanisca, Houmairi, Triboiti e Turca 'Serilop') da impiegare per il risanamento *in vitro*. Nei semi, in particolare, sono stati rinvenuti tutti i virus che concorrono alla malattia, mentre solo 4 (FBV, FFkaV, FLMaV-1 e FMV) sono stati riscontrati nelle gemme, foglie e siconi, costantemente con tassi d'infezione molto variabili.

La tecnica innovativa di produzione di semi sintetici, ha consentito la propagazione del materiale vegetale di tre genotipi oggetto d'indagine (Bifera,Catalanisca, Palazzo) con valori pressoché analoghi in termini di vitalità media, ricrescita vegetativa, e paragonabili per quanto concerne la conversione e il radicamento delle microtalee incapsulate dopo la semina e senza cambiamento di mezzo.

Relativamente all'eradicazione dei virus associati a FMD dai tessuti di *F. carica*, fatta eccezione per FBV-1 rinvenuto sempre e indipendentemente dalla metodologia, l'efficacia migliore è stata ottenuta con l'impiego della callogenesi, sebbene un buon tasso di risanamento sia stato osservato anche con la cultura dei meristemi incapsulati nell'endosperma artificiale dei "i semi sintetici".

Quest'ultima tecnica in stretta correlazione al tipo di ormone utilizzato, inoltre, ha consentito per la cv. Houmairi una percentuale soddisfacente di conversionedopoconservazione a bassa temperatura.

Parole chiave: Fico, mosaico, RT-PCR, distribuzione di virus, ormone, incapsulamento, micropropagazione, seme sintetico.

Resumen

La higuera (*Ficus carica* L.) es considerada como uno de de los árboles frutales más antiguos de la cuenca mediterránea y es ampliamente cultivado y cosechado para el consumo de sus frutos tanto secos como en fresco. Esta especie se ve afectada por diversas enfermedades virales, especialmente por la denominada "Fig mosaic disease" (FMD) asociada actualemnte a los virus: Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), Fig mild mottling-associated virus (FMMaV), *Fig mosaic virus* (FMV), Fig latent virus 1 (FLV-1), Fig badnavirus 1 (FBV-1) y Fig fleck-associated virus (FFkaV). Esta enfermedad representa una amenaza y un obstáculo para la producción de higos y el intercambio de germoplasma.

El principal objetivo del presente trabajo fue establecer un método de propagación de higuera in vitro para el saneamiento y la conservación de material vegetal libre de FMD para su posterior comercialización.

Inicialmente, se estudió la distribución de los virus implicados en la enfermedad en diversos órganos de 14 genotipos de *F. carica* (Palazzo, Severoni precoce, Bianca, Pilusedda, Dottato bianco, Bifera, Zidi, Baiyadi, Biancu, Brogiotto nero, Catalanisca, Houmairi, Triboiti y Turca 'Serilop'), los cuales fueron utilizados posteriormente como fuente material vegetal *in vitro*. Los resultados obtenidos mediante RT-PCR revelaron que todos los virus mencionados estaban presentes sin excepción en las semillas, mientras que sólo cuatro de ellos (FBV, FFkaV, FLMaV-1 y FMV) fueron en brotes, hojas y siconios con tasas de infección variables.

Además, la tecnología de encapsulación demostró ser una técnica de multiplicación eficaz para poder aplicar el protocolo estándar de cultivo de tejidos de higo para tres cultivares (Catalanisca, Palazzo y Bifera) dando altas tasas de viabilidad, rebrote y conversión. Se logró el enraizamiento de microcortes en un solo paso y el índice de conversión fue comparable para los tres cultivares.

La callogénesis y el culñtivo de meristemos con la técnica de la semilla sintética (MTC-SS) fueron las técnicas que proporcionaron mayores tasas de desinfección para los virus estudiados a excepción de con FBV-1, entidad viral que no fue eliminada con ninguna de las técnicas ensayadas.

Por último, se logró la conservación de las semillas artificiales de higuera (cv Houmairi), registrándose una alta viabilidad y tasas de rebrote moderadas con un menor grado de conversión estrictamente relacionado con hormonas utilizadas.

Palabras clave: Higuera, mosaico, RT-PCR, la distribución de los virus, hormonas, encapsulación, micropropagación, y la semilla sintética.

Resum

La figuera (*Ficus carica* L.) és considerada un dels arbres fruiters més antics de la conca mediterrània i és àmpliament conreat i collit per al seu consum fresc i sec. Les malalties virals, especialment "Fig mosaic disease" (FMD), associada amb els viruses: Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), Fig mild mottling-associated virus (FMMaV), *Fig mosaic virus* (FMV), Fig latent virus 1 (FLV-1), Fig badnavirus 1 (FBV-1) i Fig fleck-associated virus (FFkaV). Esta malaltia representa una amenaça per a la producció de figues i l'intercanvi de germoplasma.

El principal objectiu d'aquest treball va ser estableixerun mètode de propagació de figuera in vitro per al sanejament i la conservació de material lliure de FMD per a su posterior commercialització. Inicialment, es va estudiar la distribució dels virus associats a FMD en diversos òrgans en 14 genotips de *F. carica* (Palazzo, Severoni Precoce, Bianca, Pilusedda, Dottato bianco, Bifera, Zidi, Baiyadi, Biancu, Brogiotto diners, Catalanisca, Houmairi, Triboiti i Turca 'Serilop'), els quals van ser utilitzats posteriorment com a font de material vegetal *in vitro*.

Els resultats obtinguts del anàlisis realitzats per RT-PCR van revelar que tots els virus eren presents sense excepció en les llavors, mentre que només quatre virus (FBV, FFkaV, FLMaV-1 i FMV) van ser detectats en brots, fulles i siconis amb taxes d'infecció variables.

A més, la tecnologia d'encapsulació va demostrar ser una tècnica de multiplicació eficaç per poder aplicar el protocol estàndard de cultiu de teixits de figa per a tres cultivars (Catalanisca, Palazzo i Bifera) donant taxesadequades de viabilitat, rebrot i conversió. Es va aconseguir l'arrelament de microtalls en un sol pas i l'índex de conversió va ser comparable per als tres cultivars.

La calogènesi i el cultiu de meristems protegits per llavors sintètiques (MTC-SS)van ser les tècniques que proporcionarem millores tases de desinfecció per als virus estudiats amb l'excepció de FBV-1 que es va resistir a tots els mètodes de sanejament.

Finalment, es va aconseguir la conservació de la llavors artificials de figuera (cv. Houmairi), registrant-ne una alta viabilitat i taxes de rebrot moderades amb un menor grau de conversió estrictament relacionat amb hormones utilitzades.

Paraules clau: Figuera, mosaic, RT-PCR, la distribució dels virus, hormones, encapsulació, micropropagació, i la llavor sintètica.

INTRODUCTION

Recently fig tree (*Ficus carica* L.) has gained high interest for its economic importance and medicinal virtues (Bayoudh *et al.*, 2015). It is considered to be one of the oldest fruit trees in the Mediterranean basin and is widely grown and harvested for its dry and fresh consumption (Dueñas *et al.*, 2008). According to El-Rayes (1995), the fig is originated from the Eastern Mediterranean region (Turkey, Syria, Saudi Arabia), from where its cultivation expanded to the whole of the Mediterranean region (Vikas and Vijay, 2011). Fig tree grows well and produces the best quality fruit in drier warm-temperate climates. Wild forms of fig are found extensively in Mediterranean countries such as Algeria, Egypt, Syria, Tunisia, Turkey, as well as in the Arabian Gulf and in Central Asia (El Rayes, 1996; Ahmad *et al.*, 2012).

F. carica, which belongs to family Moraceae genus *Ficus*, is a monoecious, deciduous tree or large shrub. The sub-genus Eusyce,to which *F. carica* belongs, is characterized by having unisexual flowers only and gynodioecism (Storey, 1975). *Ficus* comprises one of the largest genera of angiosperms with more than 800 species of trees, shrubs, hemiepiphytes, climbers, and creepers in the tropics and subtropics worldwide (Frodin, 2004).

The common fig tree is an important member of the genus *Ficus* which is divided into six subgenera based on preliminary morphology. The monoecious subgenus Urostigma is the largest with about 280 species all inclusive, and most of them display distinctive hemiepiphytic habits. *Ficus* includes 23 species of hemiepiphytes and lithophytes which produce aerial and creeping root systems (Ronsted *et al.*, 2008). *Ficus* belongs to the Eusyce section of the Moraceae, with over 1,400 species classified into about 40 genera (Watson and Dallwitz, 1999).

Fig inflorescence is unique, consisting of a syconium, which encloses many unisexual flowers that can be accessed via the ostiole by pollinating wasps that may give the true fruits (Storey, 1975).

All *Ficus* species are either dioecious, consisting of separate male and female plants, or, in the case of *F. carica*, gynodioecious, consisting of hermaphroditic and female plants. Most of the edible fig varieties are parthenocarpic (self-fertilizing). These produce seedless fruit and are propagated by cuttings (Starr *et al.*, 2003). However, some varieties require pollination by certain wasps. For every *Ficus* species, usually a unique Agaonid wasp is associated with it. Fig wasps only lay their eggs inside the florets, pollinating the fig in the process (McLeish and Van Noort, 2012). Therefore, figs that require pollination cannot be cultivated or become naturalized without the presence of the associated pollinator wasp. There are 720 known varieties of *F. carica*. Many produce edible fruits that range from pale yellow-green to deep purple on the outside, with light to deep pink flesh (Himelrick, 1999).

Moreover, several abiotic and biotic constraints limit the development of this fruit crop and contribute to the decrease in revenue and the gradual disappearance of plantations (Saddoud *et al.*, 2011). Virus diseases, especially Fig mosaic disease (FMD), for which 8 viruses are associated, is the most widespread disorder of this species and is a constraint for fig production and germplasm exchange (Condit and Horne, 1933). It represents a threat and spread in all areas of production (Saddoud *et al.*, 2007) which inhibits the development of healthy orchards and limits the production (Ashihara *et al.*, 2004).

The earliest evidence on this disease was reported by Condit and Horne (1933), but only recently its causal agent, i.e. a multipartite single-stranded negative-sense RNA virus belonging to the genus *Emaravirus* and named *Fig mosaic virus* (FMV), was identified (Elbeaino *et al.*, 2009a). This latest occurs in symptomatic plants more often than any of the other fig-infecting RNA viruses, and was ascertained to be the etiological agent of the FMD. The causal agent is transmitted by grafting using infected cuttings and, in nature, by the eriophyid mite *Aceria ficus* (Flock and Wallace, 1955), but not by seed (Martelli *et al.*, 1993). The role in symptom induction of other viruses has not been ascertained, such as the putative closteroviruses Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV- 2), Fig mild mottle-associated virus (FMMaV) (Elbeaino *et al*., 2006; 2007; 2010) and the putative marafivirus, Fig fleck-associated virus (FFkaV) (Elbeaino *et al.*, 2011b). None of the aforementioned viruses is transmitted through seeds, contrarily to Fig latent virus 1 (FLV-1) (Gattoni *et al.*, 2009) and Fig badnavirus 1 (FBV-1), the only DNA virus identified in fig so far (Laney *et al.*, 2012; Minafra *et al.*, 2012), both of which are

vertically transmitted to seedlings, in which they do not induce symptoms. Researchers from different countries noticed the widespread of this disease, especially in recent years (Castellano *et al.*, 2007; Çağlayan *et al.*, 2009; Minafra *et al.*, 2012; El Air *et al.*, 2015).

Moreover, fig trees are propagated via cutting of mature wood or grafts because seeds are non-viable. This kind of vegetative propagation insures uniformity, relatively low multiplication rates because those materials can be obtained only from upright branches which results in poor rooting (Kumar *et al.*, 1998).

Therefore, multiplication by tissue culture techniques could be advantageous due to the production of high quality disease free, true-to-type plants independent of seasonal and other environmental conditions in a comparatively smaller space (Kozai *et al.*, 1991). Tissue culture is a basic method for the propagation of plant material with high multiplication rates (Bayoudh *et al.*, 2015). Possibility of plant micropropagation was studied in fig cultivars 'Roxo de Valinhos' (Pasqual and Ferreira, 2007), 'Sultani', 'Abodi', 'White Adcy' (Mustafa and Taha, 2012), 'Conadria', 'Black Mission'(Taha *et al.*, 2013).

For FMD control, cuttings from infected trees are not to suitable for propagation. Thus, attempts to produce FMD-free trees were initiated in the mid-1960s, using heat therapy and/or *in vitro* meristem tip culture (Stretch and Scott, 1977; Faccioli, 2001; Chalak *et al.*, 2013).

1. Fig production in the Mediterranean countries

The production of the fig tree varies in the Mediterranean countries. It is largely related to the annual rainfall, climatic conditions and soil properties (El-Rayes, 1995). During 2014-2015 world fig production was estimated at 118,731 metric tons, which increased with 3% if compared with 2013-2014, but decreased with 11% compared with ten years ago (FAO, 2016).

Among the Mediterranean countries, Turkey stands as the first country in fig production in 2013 with 298,914 tones followed by Egypt, Algeria, and Morocco with 153,089, 117,100 and 101,989 tones successively (**Figure 1**). During 2014 and 2015 Turkey produced the 56 % of the world production (69,731 Tm).

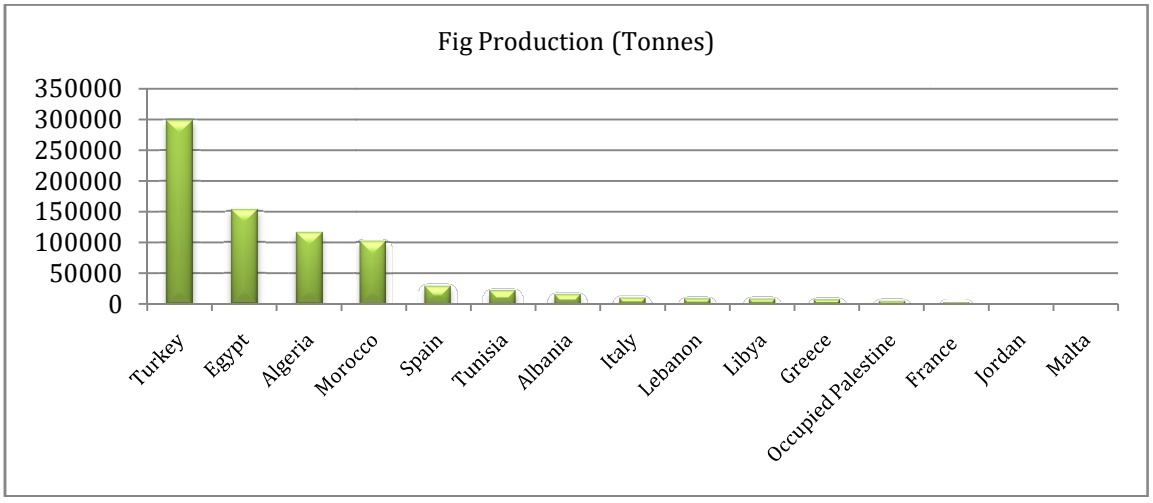

Figure 1 Main producers of fig in the Mediterranean basin (FAOSTAT, 2013)

2. Fig traditional and current uses

The genus *Ficus* is an important genetic resource due to its high economic and nutritional values and an important part of the biodiversity in the rainforest ecosystem. It is also a good source of food for fruit-eating animals in tropical areas (Ronsted *et al.*, 2008).

The syconia of *F. carica* are important worldwide for its dry and fresh consumption. Its common edible part is the syconia which is fleshy, hollow, and receptacle (Dueñas *et al.*, 2008). Syconia can be eaten raw, dried, canned, or in other preserved forms (Neal, 1965).

Dried fruits have been reported as an important source of vitamins, minerals, carbohydrates, sugars, organic acids, and phenolic compounds (Jeong *et al.*, 2001; Veberic *et al.*, 2008). The fresh and dried figs also contain high amounts of fiber and polyphenols (Vinson, 1999).

Its syconia, root, and leaves are used in traditional medicine to treat various ailments such as gastrointestinal (colic, indigestion, loss of appetite, and diarrhea), respiratory (sore throats, coughs, and bronchial problems), and cardiovascular disorders and as anti-inflammatory and anti-spasmodic remedy (Werbach, 1993; Gilani *et al.*, 2008). Furthermore, figs are used as an excellent source of minerals, vitamins, carbohydrates, and dietary fiber because it is fat and cholesterol-free and contain high number of amino acids (Slatnar *et al.*, 2011). Fruit paste is applied to swellings, tumors, and inflammation for relieving pain (Veberic *et al.*, 2008).

3. Sanitary status of fig trees

Figs are vigorous, drought tolerant trees that are relatively resistant to disease when grown in a suitable climate. However, they are grown in warm, humid climates where they became highly susceptible to various pests and diseases (Himelrick, 1999).

3.1 Fig main pests and diseases

Figs grown in warm and humid climates are highly susceptible to fungal diseases such as *Cerotelium fici* (McKenzie, 1986), a rust fungus that infects leaves, fruits and it can be problematic for growers, causing significant defoliation and yield loss (Verga and Nelson, 2014).

Armellaria mellea is the most aggressive rot pathogen of fig, known as Armillaria root disease, honey mushroom and shoestring root rot (Williams *et al.*, 1986). The pathogen causes fig rot root, stress old trees and kills young plants. Common symptoms are yellowing of the leaves, early leaf fall, premature autumn color, splitting of the bark, plant defoliation and plant death (Papachatzis *et al.*, 2008). The fungus lives as parasites on living host tissue or as saprophytes on dead plant material. *A. mellea* invades healthy trees via the roots and develops a white mat mycelium under the bark. Therefore, the infected tree wilts and dies (Singer, 1986).

Fungal infection might be observed in fig trees after fruit ripening, shriveling and falling onto the ground as well as during the drying process. Both the skin and inner cavity of

fig fruits can be contaminated by fungi (Heperkan *et al.*, 2012). Moreover, members of Aspergillus section Nigri can lead to decay in figs called fig smut (Doster *et al.*, 1996). Infection occurs on injured figs regardless of the stage of fruit development (Subbarao and Michailides, 1996).

Cylindrocladium scoparium may cause leaf spot (Crous and Wingfield, 1994) and typical symptoms caused by the pathogenic species are root rot, damping-off, wilt, leaf spotting, or necrotic lesions on fruits (Peerally, 1991).

Furthermore, nematodes of the genus *Meloidogyne* (root knot nematodes) (Goeldi, 1887) forms a very destructive pests which have a wide host range. They can severely damage growing plants, including those in the hot weather areas of Africa (Hassan *et al.*, 2010).

The black fig fly, *Lonchaea aristella* (Scotto La Massese, 1983) is the most important insect fig pest in the Mediterranean and Middle Eastern countries (Silvestri, 1917; Anagnostopoulos, 1939; Talhouk, 2002; Gonçalves *et al.*, 2005). It is considered to be highly tolerant to low temperatures when compared to other species of fruit flies (Ronald *et al.*, 1992).

In addition, other various pests are considered fig pests such as: fig wax scale *Ceroplastes rusci* (Vu *et al.*, 2006), fig fly *Silba adipata* (Raz, 1998), fig psylla *Homotoma ficus* (Jerinić-Prodanović, 2011) and the two-spotted spider mite *Tetranychus urticae* (Dupont, 1979).

3.2 Fig Mosaic Disease (FMD)

Up to 1971, enveloped round of 90-200 nm in diameter (double membraned-bodies; DMBs) (**Figure2A**), were the only anomalous intracellular structures consistently associated with FMD (Martelli *et al.*, 1993). DMBs are the likely particles of what can tentatively be referred to as *Fig mosaic virus* (FMV). DMBs have an envelope that seems to derive from the endoplasmic reticulum and consists of a lipoprotein unit membrane about 12 nm thick (**Figure 2B**), containing carbohydrates. They also contain proteinaceous material and fine fibrils and often gather around masses of convoluted, electron-dense filamentous elements (**Figure 2C**) which contain carbohydrates and are partially digested by pronase. By contrast, DMBs are insensitive to tetracycline.

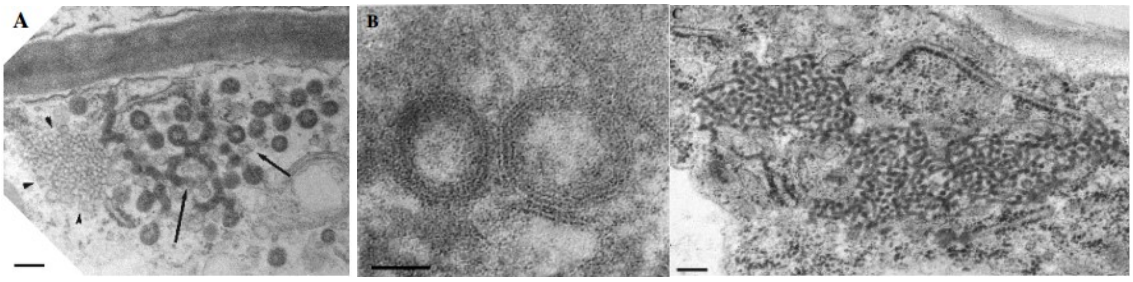

Figure 2 Aspects of DMBs in the cytoplasm of a symptomatic fig leaf. A) A group of DMBs Bar = 100 nm. B) DMBs showing the membranous envelope (Bar = 50 nm). C) Convoluted, electron dense filamentous elements accompanying DMBs Bar = 100 nm (Martelli *et al.*, 1993).

A turning point was in 2006-2007, when a putative closterovirus (Fig leaf mottle-associated virus 1; FLMaV- 1) and a putative ampelovirus (Fig leaf mottle-associated virus 2; FLMaV-2) were reported for the first time from fig in nature (Elbeaino *et al.*, 2006;2007). Before that date, all data that report the presence of viruses infecting fig are based on virus particles shape occasionally seen under electron microscope or dsRNA profiles extracted from mosaic-diseased plants.

3.2.1 Symptoms

In general, mosaic-diseased fig trees show a wide range of symptoms, mainly on leaves, as discolorations, yellowing, various patterns of chlorotic mottling and blotching, vein banding, vein clearing, chlorotic or necrotic ring spots and line patterns, variegation and mosaic (**Figure 3**) (Condit and Horne, 1933; Flock and Wallace, 1955; Plavšic and Milicic, 1980; Appiano *et al.*, 1990; Martelli *et al.*, 1993; Elbeaino *et al.*, 2009b). On fruits, yellow spots, frequently followed by premature fruit drop, may appear, which reduce fruit yield and quality (Blodgett and Gomec, 1967).

However, the virus infections associated with FMD are varied, and several viruses have been identified from figs with different mosaic-like symptoms. Nevertheless, the roles of particular viruses as causes of FMD have not been established, except for FMV (Elbeaino *et al.,* 2009). However, it cannot be excluded that FMD is a complex disease with which several fig-infecting viruses could be involved (Martelli, 2011).

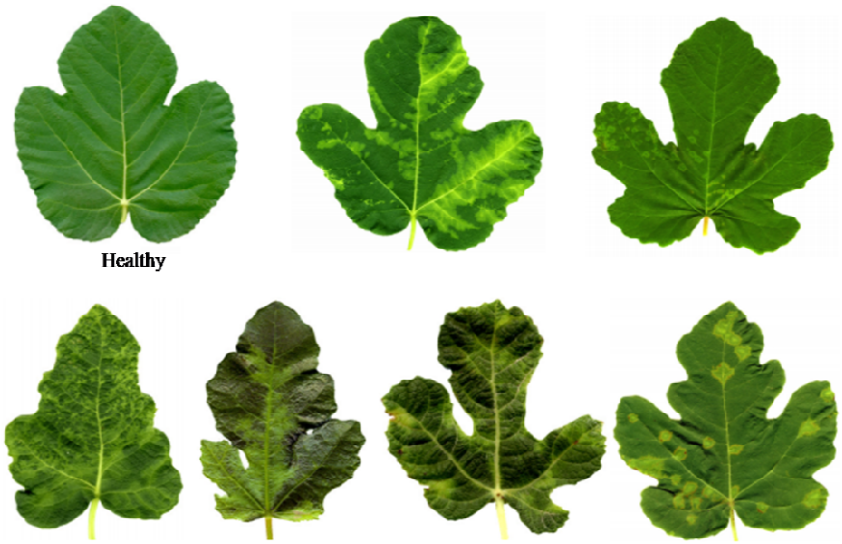

Figure 3 Fig mosaic disease range symptoms on *F. carica* leaves (Martelli, 2011)

3.2.2 Transmission

FMV, and accordingly its disease (FMD) is transmitted by *Aceria ficus* (Cotte) (**Figure 4**) in nature (Flock and Wallace, 1955), However, none of the following viruses can be transmitted through seeds, such as the three closterovirids; FLMaV-1, FLMaV-2. FMMaV (Elbeaino *et al.,* 2006; 2007; 2010), the putative marafivirus; FFkaV (Elbeaino *et al.*, 2011a), contrarily to the trichovirus; FLV-1 (Gattoni *et al.*, 2009) and the badnavirus; FBV-1, the only DNA virus identified in fig so far (Laney *et al.*, 2012; Minafra *et al.*, 2012), both of which are vertically transmitted to seedlings, in which they do not induce symptoms.

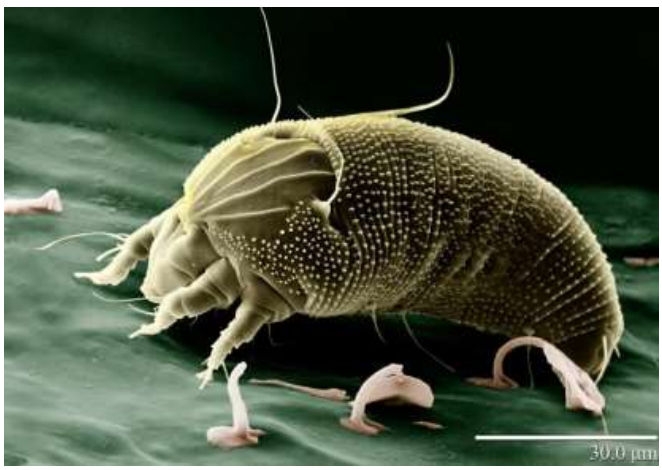

Figure 4 Environmental scanning electron microscope of a member of the species *Aceri* (Eric Erbe, USDA Agricultural Research Service, Bugwood.org.)

3.2.3 Viruses associated with Fig Mosaic Disease (FMD)

FMD is a complex disorder (reviewed by Martelli, 2011) with which eight viruses of different taxonomic position are associated (**Table 1**). The following viruses have been detected in mosaic-affected fig trees, and have been partially or completely characterized. Of all the recorded diseases associated with fig crops, FMD is the most serious virus pathogen, and remains a critical pathological constraint facing fig production and germplasm exchange. The etiology of FMD has been hindered for decades (Swingle, 1928; Condit and Horne, 1933), until the discovery of FMV that was verified as the cause of the disease (Elbeaino *et al.,* 2009a; 2009b).

The wide range of symptoms, resembling those typical of FMD, observed on different varieties, incited a screening for three viruses, i.e. FLMaV-1, FLMaV-2 and FMV that were all found to be present in figs throughout the country (Elbeaino *et al.*, 2012).

Table 1 Molecularly characterized viruses found in mosaic affected fig trees.

Virus species	Genus	Reference
Fig mosaic virus (FMV)	*Emaravirus*	Elbeaino*et al*., 2009a
Fig leaf mottle associated virus 1 (FLMaV-1)a	*Closterovirus*	Elbeaino *et al.,* 2006
Fig leaf mottle associated virus 2 (FLMaV-2)	*Ampelovirus*	Elbeaino *et al.,* 2007
Fig mild mottle-associated virus (FMMaV)	*Closterovirus*	Elbeaino *et al.,* 2010
Fig fleck-associated virus (FFkaV)	*Maculavirus*	Elbeaino *et al.,*2011b
Fig latent virus 1 (FLV-1)	*Trichovirus*	Gattoni *et al.,*2009
Fig cryptic virus (FCrV)	*Alphacryptovirus*	Elbeaino *et al.,* 2011c
Fig badnavirus 1 (FBV-1)	*Badnavirus*	Laney *et al*., 2012

Viruses with italicized names are classified into genus, according to the International Committee for Taxonomy of Viruses (ICTV)

3.2.3.1 Fig mosaic virus (FMV): the causal agent of FMD

Although the eriophyid mite *A. ficus* was implicated in its transmission a long time ago, the identity of the causal agent of the disease has long been elusive (Flock and Wallace, 1955). Only recently the causal agent of FMD, a multipartite single-stranded negative-sense RNA virus belonging to the genus *Emaravirus*, was identified. Although Koch's postulates remain to be fulfilled due to the difficulty of sap inoculation (Elbeaino *et al.,* 2009a), the

strong association of FMV with FMD (Walia *et al.*, 2009) and mite transmissibility of FMV (Çaglayan *et al.*, 2010) suggest that FMV may be the causal agent of FMD.

Furthermore, FMV occurs in symptomatic plants more often than any of the other fig-infecting RNA viruses and is the major incitant of mosaic. FMV is considered as a cosmopolitan virus, since it was detected in various countries, including Italy, USA, Turkey, Lebanon, Algeria, Tunisia, Egypt, Syria, Albania, Saudi Arabia, Iran, France, Australia, Spain, Canary Islands, Costa Rica, Chile, China and Japan (Elbeaino *et al.,*2012, 2011; 2009; Walia *et al.,* 2009; Çağlayan *et al.,* 2010; Ishikawa *et al.,* 2012, Elbeshey and Elbeaino, 2011; Caglar *et al.,* 2011; El Air *et al.,* 2015; Aldhebiani *et al.,* 2015; Shahmirzaie *et al.,* 2010).

This virus is transmitted by grafting using infected cuttings and, in nature, by the eriophyid mite *A. ficus* (Flock and Wallace, 1955)but not by seed (Martelli *et al.,* 1993). It was efficiently transmitted from diseased to healthy fig seedlings by *A. ficus* (Çaglayan *et al.*, 2010) and, therefore, it can be regarded as the only causal agent of fig mosaic disease. The role in symptom induction of other viruses associated with FMD has not been ascertained (Elbeaino *et al.,* 2009a).

a. Genome structure

The complete sequence of four viral RNA segments of FMV was determined (**Figure 5**). Each of the four RNAs comprises a single open reading frame (ORF) 7,093, 2,252, 1,490 and 1,472 nucleotides in size, respectively. These ORFs encode the following proteins in the order: RNA-dependent RNA polymerase (p1 264 kDa), a putative glycoprotein (p2 73 kDa), a putative nucleocapsid protein (p3 35 kDa) and a protein with unknown function (p4 40.5 kDa). All RNA segments possess untranslated regions containing at the 50 and 30 termini a 13-nt complementary sequence. A conserved motif denoted premotif A was found to be present in addition to the five RdRp motifs A–F in RNA-1 (Elbeaino *et al*., 2009b).

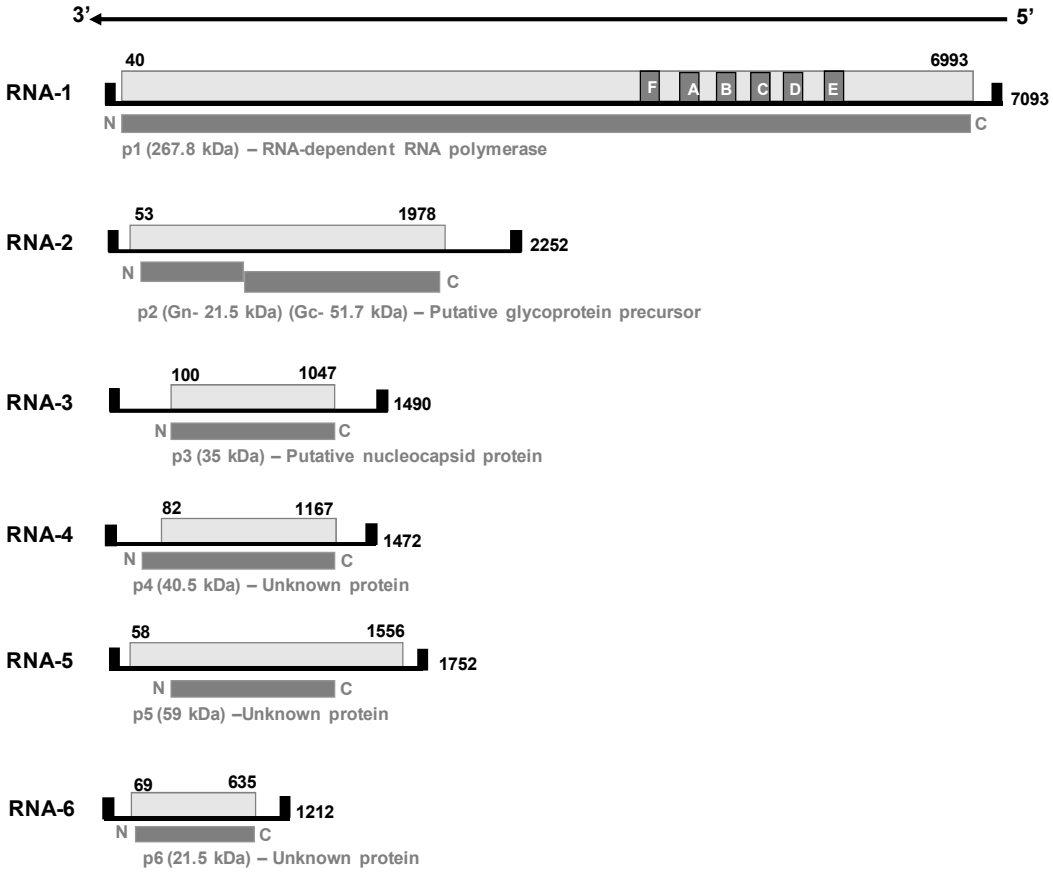

Figure 5 Schematic representation of the organization of the four FMV genomic RNA segments. The terminal 13 nucleotides conserved at the 50 and 30 termini are indicated as black boxes on each segment. Letters (A–F) represents the conserved motifs on the RdRp (RNA1) gene. Expression products of each RNA (p1 to p6) are represented as dark gray boxes. The protein function and estimated molecular weight of each segment are reported. Figure not drawn to scale (Elbeaino *et al.,* 2012, 2009b).

Two other additional FMV genomic segments were determined by RT-PCR using primers corresponding to the conserved 13 nt stretches found at the termini of FMV genomic segments (Elbeaino *et al.,* 2009b). These two novel FMV genomic RNA segments were denoted as RNA-5 and RNA-6 (Elbeaino *et al.*, 2012).

3.2.3.2 Fig leaf mottle associated virus 1 and 2 (FLMaV-1/FLMaV-2)

Fig leaf mottle-associated virus 1 (FLMaV-1) and Fig leaf mottle-associated virus 2 (FLMaV-2) were found in symptomatic figs of Italian and Algerian origin, respectively (Elbeaino *et al.*, 2006;2007). Both viruses showed to be widespread in Apulia (Southern Italy) and Tunisia. In particular, FLMaV-1 was detected in 64.9% of tested samples from Apulia (southern Italy) and in 28.8% of samples from Tunisia (Elbeaino *et al.*, 2006; Nahdi *et al.*, 2006), whereas FLMaV-2 had an incidence of 13.5% in Tunisia (unpublished information).

The positive detection of FLMaV-1 and FLMaV-2 in symptomatic fig trees from Lebanon confirms the association of both viruses with FMD, already reported from Italy (Elbeaino *et al.*, 2006;2007) and other countries (Falk *et al.*, 2006; Nahdi *et al.*, 2006). FLMaV-1 and FLMaV-2 occur in field-grown fig trees in six Mediterranean countries (Albania, Algeria, Lebanon, Syria, Tunisia and Italy) with different extent of infections (Elbeaino *et al.*, 2010a).

i. Fig leaf mottle-associated virus 1

FLMaV-1 is apparently member of the family Closteriviridae with filamentous particles c. 1800 nm long. The genome sequencing is not yet completed but most likely it has a genome organization similar to that of type-species *Beet yellows virus* (**Figure 6**).

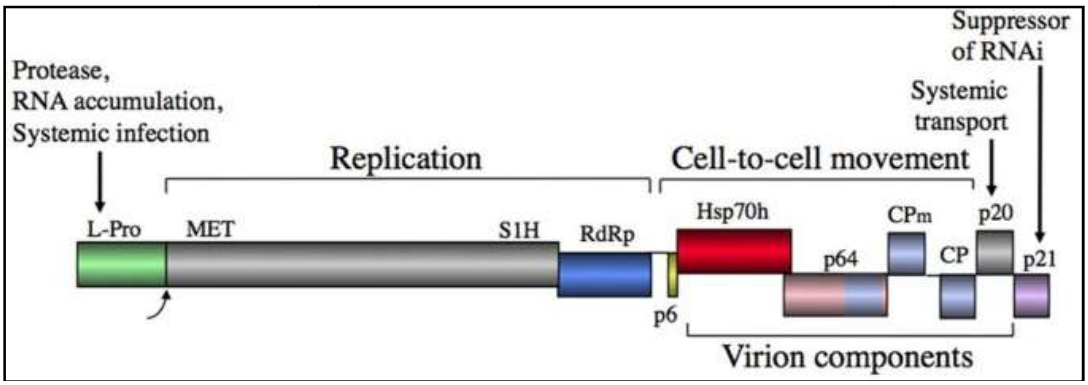

Figure 6 Diagram of *Beet yellows virus* (BYV) genome with gene functions shown the type species of the genus *Closterovirus*. L-Pro, papain-like leader protease; MET, methyl transferase; S1H, superfamily I helicase; RdRp; p6, 6-kDa protein; Hsp70h, Hsp70 homolog; p64, 64-kDa protein; CPm, minor capsid protein; CP, capsid protein; p20. 20-kDa protein; p21, 21-kDa protein (Dolja *et al.*, 2006).

FLMaV-1 is not mechanically transmissible to herbaceous hosts (Elbeaino *et al.*, 2006). Symptoms associated to FLMaV-1 are mottling, vein clearing, blotching and various patterns of chlorotic mottling and discoloration of the second and third veins of leaves (Elbeaino *et al.*, 2006;2007; Elci *et al.*, 2012).

ii. Fig leaf mottle-associated virus 2

FLMaV-2 is a member of the family Closteroviridae and a putative species of the genus *Ampelovirus*. It was recovered from symptomatic Algerian fig trees showing chlorotic mottling and clearing of the second and third veins of leaves. FLMaV-2 is not transmissible

mechanically to herbaceous hosts, similarly to FLMaV-1. Its genome consists of a double-stranded RNA of about 18 kb, having a particle exceeding 2000 nm in length, which induces a cytopathology conforming to that of the family Closteroviridae (Elbeaino *et al.*, 2007).

The genome organization of FLMaV-2 is most likely similar to the type-species of the genus *Ampelovirus*, i.e. *Grapevine leafroll associated virus 3* (GLRaV-3) (**Figure 7**). The different sizes of dsRNA and virus particle lengths of this virus and FLMaV-1, the low sequence homology, and the different phylogenetic affiliation constitute evidence this isolate belongs to a virus species different from FLMaV-1, for which the name of FLMaV-2 was proposed. Calls for further studies aimed at establishing the role, if any, of these viruses in the aetiology of FMD (Elbeaino *et al.*, 2007).

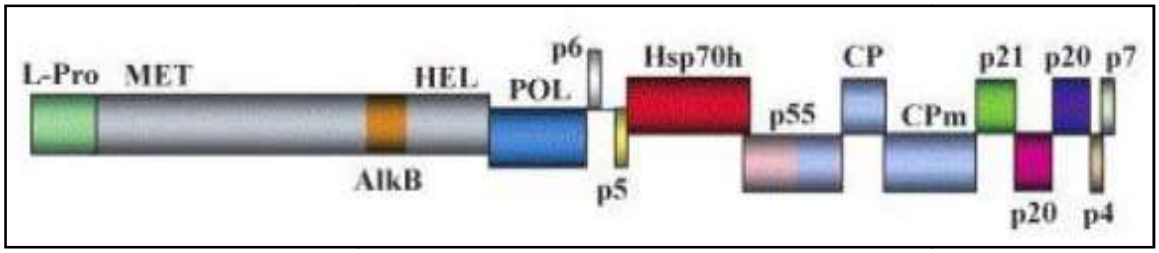

Figure 7 Diagram of *Grapevine leafroll-associated virus 3*(GLRaV-3) genome, the type species of the genus *Ampelovirus*. L-Pro: Leader Papain-like protease, MET, Hel, POL: RdRp, p6: 6 KDa protein, p5: 5 KDa protein, HSP70h: Homologue of the heat shock-proteins from the HSP70 family, p55: 55 KDa protein, CP, CPm, p21: 21 KDa protein, p20: 20 KDa protein, p4: 4 KDa protein, p7: 7 KDa protein (Dolja *et al.*, 2006).

3.2.3.3 ***Fig mild mottle-associated virus (FMMaV)***

FMMaV is a putative new closterovirus with particles ca. 2000 nm long for which the partial genome sequence was determined. The 6,290 nt long, yet sequenced from its genome, encompasses seven open reading frames (ORFs), i.e. an incomplete ORF1b encoding the putative RNA-dependent RNA polymerase (RdRp), a 25 kDa protein with unknown functions, a 6 kDa protein with putative nucleotide-binding properties, the 63 kDa homologue of the heat-shock protein 70 (HSP70h), a 64 kDa protein, the minor coat protein (CPm) of 26 kDa in size, and the incomplete coat protein (CP) (**Figure 8**). The genome organization of FMMaV is the same as that of members of the genus *Closterovirus.* In a preliminary survey carried out in southern Italian fig orchards, FMMaV was detected in three different cultivars (Elbeaino *et al.*, 2010a).

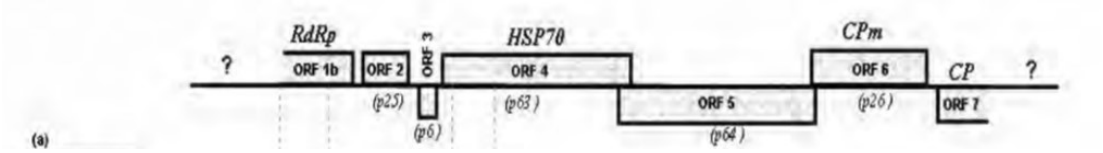

Figure 8 Schematic representation of the partial genome of FMMaV. Boxed regions correspond to ORFs and boxes with open ends correspond to incomplete ORFs. The putative function of proteins and their estimated molecular weights are reported on and above the boxes. Only 6290 nucleotides, out of 18000 predicted, are the molecular data available on its genome in the Genbank (Elbeaino *et al.*, 2010a).

This closterovirus-like virus was recovered from a fig tree (cv. Dottato bianco) from Calabria (southern Italy) showing light mottling and little or no malformation of the leaves, i.e. symptoms milder than those generally displayed by mosaic-affected fig plants in southern Italy. Electron microscope observations of leaf dips disclosed the presence of long filamentous particles with distinct cross banding that resembled very much closterovirus virions. However, no amplification was obtained when leaf extracts were assayed by RT-PCR using FLMaV-1 and FLMaV-2 specific primers (Elbeaino et al., 2006;2007). It was different from the known fig-infecting closteroviruses, therefore its provisionally nomination Fig mild mottle-associated virus (FMMaV).

3.2.3.4 ***Fig fleck associated virus (FFkaV)***

A novel virus, tentatively named Fig fleck-associated virus (FFkaV), was found in field-grown fig from six Mediterranean countries, exhibiting a discrete clearing (flecking) of some veinlets, with an incidence ranging from15% to 25%. The complete nucleotide sequence and the genome organization of this virus were determined. The viral genome is a positive-sense, single-stranded RNA of 7046 nucleotides in size excluding the 3'-terminal poly(A) tract, and comprising two open reading frames. ORF1 encodes a polypeptide of 2161 amino acids (p240), which contains the signatures of replication-associated proteins and the coat protein cistron (p24) at its 3' end. ORF2 codes for a 461 amino acid protein (p50) identified as a putative movement proteins (MP) (**Figure 9**). In phylogenetic trees constructed with sequences of the putative polymerase and CP proteins FFkaV consistently groups with members of the genus *Maculavirus*, family Tymoviridae (Elbeaino *et al.*, 2011b).

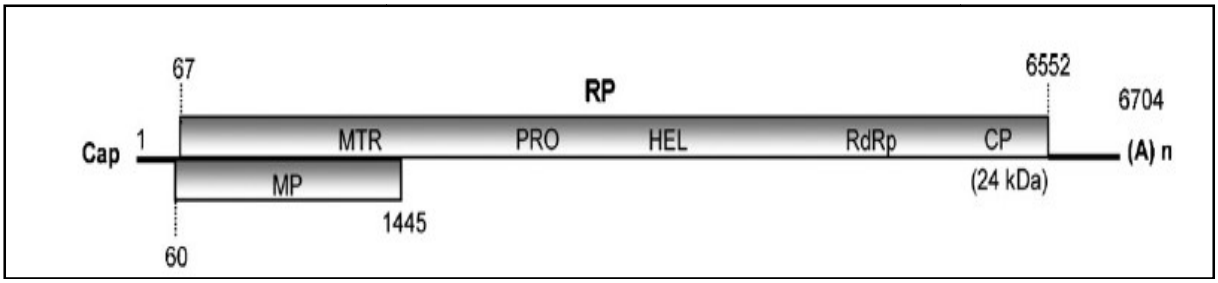

Figure 9 Schematic representation of FFkaV genome.ORF1 (upper box) codes for the replication-associated polyprotein RP containing the signatures of MTR, PRO; Hel, RdRp and the CP cistron. ORF2 (lower box) encodes the MP gene (Elbeaino *et al.*, 2011b).

3.2.3.5 Fig latent virus 1 (FLV-1)

A virus with filamentous particles of about 700 nm in length, denoted Fig latent virus 1 (FLV-1) was also identified in fig and found to be widespread in Apulian (Southern Italy) fig orchards, in trees showing or not mosaic symptoms and in symptomless seedlings. This virus resembles the members of the genus *Trichovirus*, family Flexiviridae. It is transmitted by sap inoculation to a very restricted range of herbaceous hosts without inducing apparent symptoms (Gattoni *et al.*, 2009). It was successfully purified from root tissues of infected figs.

FLV-1 viral genome is a single-stranded positive-sense RNA with an estimated size of ca. 8,000 nt, 6,620 of which have been sequenced, starting from the polyadenylated 3' terminus. Genomic RNA consists of four open reading frames encoding, in the 5' to 3' direction, the replication-associated proteins (ORF 1), a 43 kDa putative movement protein (ORF 2), the 46 kDa coat protein (ORF 3), and a 12 kDa protein with nucleic acid binding properties (**Figure 10**). FLV-1 clusters with trichoviruses in phylogenetic trees constructed with coat protein sequences. However, a distinct difference with all members of the genus rests with the size of the coat protein subunits (46 versus 22-27 kDa) and the presence of ORF 4, which is present only in three tentative species of this genus (Gattoni *et al.*, 2009).

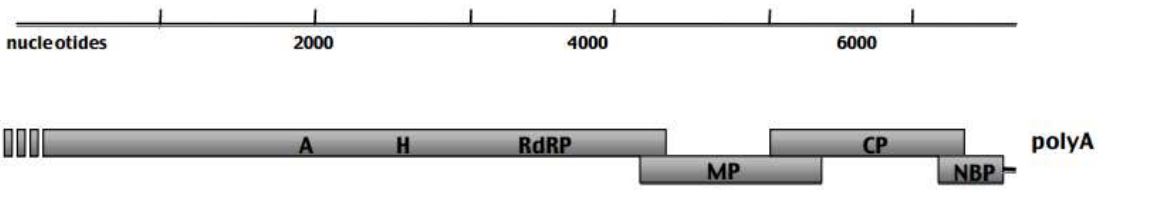

Figure 10 Diagramatic representation of the FLV-1 genome. A, AlkB domain; H, helicase domain; RpRd, RNA dependent-RNA polymerase domain; MP, movement protein; CP, coat protein; NBP, nucleotide-binding protein (Gattoni *et al.*, 2009).

FLV-1 was detected in seedlings of different age (1-year old and 4-weeks old) and different geographical origin (Italy, Greece and Turkey) where the great majority of which were symptomless. Infection rate ranged from 73 to 100% (average 92%)(Castellano *et al.*, 2009). The high transmission rate differentiates FLV-1 from most of the other seed-borne plant viruses which, in general, have a lower transmission frequency (Mink, 1993; Johansen *et al.*, 1994), and from the extant trichoviruses, none of which is apparently transmitted though seeds (Maury *et al.,* 2008). But since fig is not propagated through seeds, the epidemiological significance of seed infection would be negligible, unless FLV-1 has an yet unidentified vector-like eriophyide mite as with other *trichovirus* species (Adams *et al.*, 2005). If this were the case, seed transmission, in conjunction with secondary spread by a vector, could favor the introduction and subsequent dissemination of the virus in new environments (Castellano *et al.*, 2009).

3.2.3.6 Fig cryptic virus (FCV)

FCV is the first virus of the family Partitiviridae to be detected in fig trees and one of the few infecting woody crops in nature (Elbeaino *et al.*, 2011c). Cryptic viruses are members of the family Partitiviridae*,* which comprises isometric viruses infecting monocotyledonous and dicotyledonous plant species, currently classified in the genera *Alphacryptovirus* and *Betacryptovirus* (Ghabrial *et al.*, 2005). This family also embraces the genera *Partitivirus* and *Cryspovirus* which include fungal and protozoan dsRNA viruses, respectively (Nibert *et al.*, 2009). Plant-infecting members of the family Partitiviridae are transmitted through seeds and pollen but not by grafting or mechanical inoculation, and occur in very low concentration in the host plant (Ghabrial *et al.*, 2005), and apparently have no economic impact (Elbeaino *et al.*, 2011c).

Cryptovirus genomes known to date are generally composed of two monocistronic double-stranded RNA (dsRNA) segments 1.7–2.3 kb in size (Ghabrial *et al.*, 2005), the larger of which encodes the RNA-dependent RNA polymerase (RdRp), while the smaller codes for the coat protein (CP). However, some cryptic viruses have been reported to possess genomes consisting of three monocistronic dsRNA segments (Chen *et al.,* 2006; Salem *et al.,* 2008).

FCV is one of the few infecting woody crops in nature, therefore the limited molecular information on the species of this family currently available, makes it difficult to allocate separate genera for each of its members. Thus, the molecular characterization of FCV and its

comparison with other related viruses was pursued in this study, the results of which are hereafter reported. No visible symptoms developed in any of the herbaceous hosts manually inoculated with (FCV) extracts. All inoculated plants were also negative to dsRNA extraction and RT-PCR using FCV-specific primers (Elbeaino *et al.*, 2011c).

The complete nucleotide sequences of both RNAs were determined and found to be 1696 nts (RNA-1, Genbank accession no. FR687854) and 1415 nts (RNA-2, Genbank accession no. FR687855) in size. Each RNA contained a single open reading frame (ORF) in its plus-strand whereas no ORFs were apparently present in the minus-strand. The 5'and 3' untranslated regions (UTR) consisted of 142 and 135 nts in RNA-1 and 180 and 221 nts in RNA-2 (**Figure 11**) (Elbeaino *et al.*, 2011c).

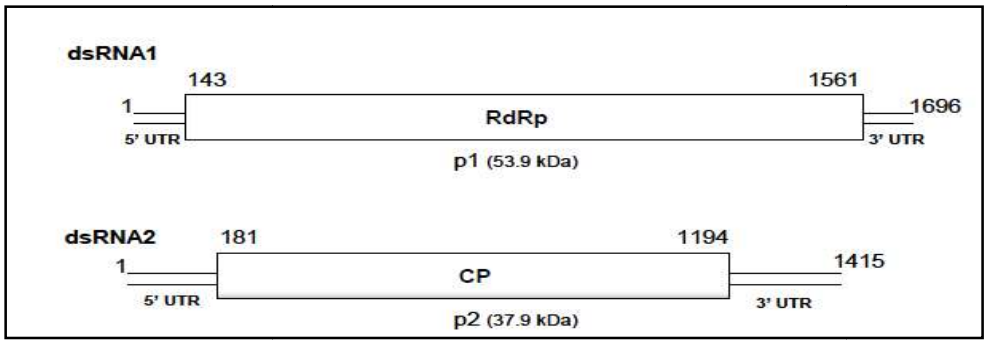

Figure 11 Schematic representation of the genome of FCV-1. DsRNA-1 contains the RdRp ORF (nt position 143-1561) and dsRNA-2 codes for a putative capsid protein (nt position 181-1194). The RdRp and the putative CP genes are represented by rectangular boxes and their estimated molecular weights are indicated between brackets. Ends of both segments contain untranslated regions (5'and 3'UTR)(Elbeaino *et al.*, 2011c).

3.2.3.7 Fig Badnavirus 1 (FBV-1)

Badnaviruses belong to the family of Caulimoviridae, the genus *Badnavirus* which are non-enveloped bacilliform DNA viruses with a monopartite genome containing about 7.2 to 9.2 kb of dsDNA with three to seven open reading frames. They are transmitted by mealybugs and a few species by aphids in a semi-persistent manner. They are one of the most important plant virus groups and have emerged as serious pathogens. Some badnaviruses are also known as endogenous viruses integrated into their host genomes and a few such endogenous viruses can be awakened, e.g., through abiotic stress, giving rise to infective episomal forms. The presence of endogenous badnaviruses pose a new challenge for the fool-proof diagnosis, taxonomy, and management of the diseases (Bhat *et al.*, 2016).The integration is assumed to

have taken place by illegitimate recombination into host genomes, and their presence is not necessarily associated with infection. However, in some cases, these copies can give rise to systemic virus infection by recombination events, for instance induced by abiotic stress like *in vitro* tissue culture process (Dallot *et al.*, 2001; Cote *et al.*, 2010) and interspecific crosses (Lheureux *et al.*, 2003).

FBV-1 causing mosaic disease in fig trees, has been detected by PCR in 19 countries in Europe, Australia, Africa, and South and North America (Laney *et al.*, 2012; Minafra *et al.*, 2012). The virus can be mechanically transmitted to several herbaceous hosts. Based on the complete genome sequence, FBV-1 was identified as a distinct species of badnavirus. The whole genome showed that it contains 7140 bp with four ORFs encoding proteins of 15.3, 16.5, 212.5, and 17.0 kD (**Figure 12**). The key badnavirus motifs, with the exception of the protease motif, were found in the 212.5kD long ORF-derived polyprotein. The virus showed a close relationship to CSSV and CYMV (Laney *et al.*, 2012).

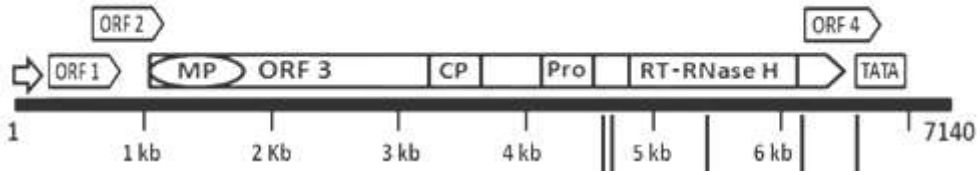

Figure 12 Linear representation of Fig badnavirus 1 genome (A) showing tRNA MET primer-binding site (denoted by an arrow); TATA-box (denoted by a box); ORF 1; ORF2; ORF 3 with movement protein (MP), capsid protein zinc-finger domain (CP), pepsin like aspartate protease (Pro), reverse transcriptase (RT) and RNase H (RNase H) motifs; and ORF 4 (Laney *et al.*, 2012).

4. In vitro fig micropropagation

Plant micropropagation is an integrated process in which cells, tissues or organs of selected plants are isolated, surface sterilized, and incubated in a growth-promoting aseptic environment to produce many clone plantlets (Altman and Loberant, 2000). Nowadays, industry has applied immensely *in vitro* propagation approach for large-scale plant multiplication of elite superior varieties (Kumar and Reddy, 2011). Moreover, multiplication by tissue culture techniques could be advantageous due to the production of high quality

disease-free, true-to-type plants independent from seasonal and other environmental conditions in a comparatively smaller space (Kozai *et al.*, 1991).

The micropropagation process involves growing mother plants under hygienic conditions and the production of stock plants in greenhouse. This step is followed by the selection of explants, disinfestations and the cultivation under aseptic conditions. A rapid regeneration and multiplication of numerous propagules is needed and called the multiplication phase, where a mass of tissues are repeatedly subcultured onto new culturing media that encourage propagule proliferation. The culture can supply shoots for the subsequent propagation phases as well as material that is required to maintain the stock. The last period of *in vitro* before transferring the plantlets to *in vivo* conditions (acclimatization) is the elongation and rooting induction phase in order to establish fully developed plantlets (Kumar and Reddy, 2011). Acclimatization is defined as the climatic or environmental adaptation of an organism, especially a plant that has been moved to a new environment (Zobayed *et al.*, 2000).

i. Culture Medium

The most adequate and effective culture medium for *in vitro* cultivation of fig tree depends on the explant type, cultivar and cultivation stage of establishment, multiplication and rooting. For *in vitro* cultivation of fig, the MS (Murashige and Skoog, 1962) and the WPM (Woody Plant Medium) media are being used.

ii. Culture establishment by shoot tips and apical buds

For the production of virus-free fig plants, the collection of shoot tips in different seasons of the year does not affect the viability and proliferation of sprouts. However, the addition of activated charcoal in the culture medium improved shoot development (Demiralay *et al.*, 1997). The age of the mother plant does not affect the establishment of shoot tip culture either.

The collection of shoot tips from 10-year-old trees and its culture in MS medium supplemented with 1 mg L^{-1} 6-Benzylaminopurine (BA) and 1mg L^{-1} 1-naphtalene acetic acid (NAA) was recommended by Günver and Ertan (1997). The *in vitro* culture establishment from apical buds is similar to that of shoot tips. However, for the selection of mother tree and

shoots as well as for the explant preparation careful attention is required (Pasqual and Ferreira, 2007).

A protocol for *in vitro* multiple shoots induction and plantlets regeneration, through apical buds with a maximum of 4.8 shoots per shoot tip explant after 8 weeks of culture, was developed by Kumar *et al.* (1998). However the development of large scale production system is still needed. A limited number of 5.1 shoots per nodal segment shoots was obtained by Fráguas *et al.* (2004) for *F. carica* micropropagation and 9.2 shoots per shoot tip after 2 months of culture were obtained (Hepaksoy and Aksoy, 2006). Yakushiji *et al.* (2003) and Kim *et al.* (2007) reported protocols for adventitious shoot regeneration through leaf explants of fig. Qrunfleh *et al*. (2013) obtained 5.8 shoots per explant after 6 weeks of culture while studying the effect of different concentrations of carbon sources, salt concentration and gelling agents on *in vitro* shoot growth using shoot tip explants. Recently Sharma *et al.* (2015) improved a protocol for direct shoot multiplication inducing a maximum of 26.8 shoots per nodal segment and 15 shoots per shoot tip with mean shoot length of 8 and 4.3 cm, respectively, after 45 days of culture. MS medium added with 7.5 µM 6-benzyladenine (BA) was the best culture medium, but the leaves exhibited a rudimentary structure.

Recently, a reliable protocol for *in vitro* regeneration, via shoot-tips, of virus-free fig plants from three important local female varieties and one selected caprifig (pollinator) was developed in Tunisia (Bayoudh *et al.*, 2015). Three major Tunisian local *F. carica* varieties, Zidi (ZDI), Soltani (SNI), Bither Abiadh (BA) and one rare and recalcitrant caprifig Assafri (ASF), were multiplied. The best initiation of shoot-tips with sizes 0.5, 1 and 1.5 mm was obtained on medium containing 0.2 mg L^{-1} Benzyleaminopurine (BAP), 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} gibberellic acid (GA_3). The variety SNI showed the highest shoot-tip initiation potentialities for the establishment step with 100% of explant development rate. The shoot multiplication and plantlet development were provided by medium supplemented with 0.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA. The best rooting rate (83.34%) was favored by medium supplemented with half-strength MS and 1 mg L^{-1} Indole-3-Butyric Acid (IBA) (Bayoudh *et al.*, 2015).

iii. Micropropagation via meristem culture

In vitro propagation through meristem culture is the best possible means of virus elimination and produces a large numbers of plants in a short span of time(Kumar and Reddy, 2011). This technique consists on removing a small (0.2–0.5 mm) piece of tissue from the meristematic area and culturing on a nutrient medium; it can result in a pathogen-free plantlet for regeneration. The explants sometimes are used from the apical dome, although in most cases one to several primordia leaves of the sub apical region are also included. Generally, apical meristem without primordia leaves have the highest probability to produce virus-free plantlets. However, those lacking the primordia leaves have the lowest probability to survive in the culture medium (Sastry and Zitter, 2014). Moreover, viruses elimination depends on the virus concentration in plant tissue and the physiological condition of the mother plant from which a meristem has been isolated (Verma *et al.*, 2004).

iv. Culture Establishment by Leaf Segments

Yakushiji *et al.* (2003) reported the first protocol of organogenesis and plant regeneration from vegetative organs of *F. carica* cv. Masui Dauphine. Leaf fragments of fig tree obtained from *in vitro* shoot cultures were grown on MS medium supplemented with different combinations of 2, 4-D, TDZ, and 0.5 mM phloroglucinol. The addition of 2,4-D induced root formation and the presence of phloroglucinol increased root formation significantly. When the combination of 2,4-D and TDZ was added to MS medium containing phloroglucinol, the explants produced adventitious buds on the edges. The addition of phloroglucinol was also effective in inducing adventitious bud formation. Regenerated plantlets were successfully established in soil after a short period of acclimatization.

v. Micropropagation via somatic embryogenesis

Somatic embryos, which are bipolar structures, arise from individual cells and have no vascular connection with the maternal tissue of the explants (Haccius, 1978). Embryos may develop directly from somatic cells (direct embryogenesis) or development of recognizable embryogenic structures is preceded by numerous, organized, non-embryogenic mitotic cycles (indirect embryogenesis). There are advantages and disadvantages of somatic embryogenesis in large-scale plant multiplication (Jain, 2001).

The major advantages are the large-scale somatic embryo production for bioreactors, encapsulation, cryopreservation, genetic transformation and clonal propagation. The major limitations are genotypic dependence of somatic embryo production and poor germination rate (Kumar and Reddy, 2011). The process of cell differentiation from callus causes a high frequency of somaclonal variation, while direct somatic embryo formation appears to give rise to a relatively uniform clone material (Denchev *et al.*, 1990).

vi. Shoot Multiplication

For 'Roxo de Valinhos' fig cultivar, WPM culture medium supplemented with 0.5 mg L^{-1} BA or 0.5 mg L^{-1} kinetin is efficient for shoot multiplication. Long shoots of *F. carica* cv. 'Kalamon' were obtained after 8 weeks on the medium containing 0.5 mg L^{-1} BA (Pontikis and Melas, 1986). Kumar *et al.* (1998) developed a reliable procedure for multiple-shoot induction and plantlet regeneration with apical buds collected from 7- to 8-year-old trees of *F. carica* using MS medium supplemented with 2.0 mg L^{-1} 6-benzylaminopurine and 0.2 mg L^{-1} 1-naphthaleneacetic acid. The *in vitro*-regenerated shoots were further multiplied on MS medium supplemented with 2.0 mg L^{-1} 6-benzylaminopurine and 0.2 mg L^{-1} 1-naphthaleneacetic acid and an average multiplication rate of four per subculture was established with 90% success. Excised shoots were rooted in liquid half strength MS medium supplemented with 2.0 mg L^{-1} indole-3-butyric acid and 0.2% activated charcoal. Regenerated plantlets were successfully established in soil, with a success rate of 68%.

For the *in vitro* proliferation of cvs. 'Brown Turkey' and 'Smyrna' and their subsequent use in genetic transformation, Yancheva *et al.* (2005) used MS medium supplemented with 0.25 mg L^{-1} BA, 0.05 mg L^{-1} IBA and 0.05 mg L^{-1} GA_3. The shoots were placed horizontally in shoot proliferation medium. The cultures were maintained in the culture room, illuminated by white fluorescent light (32 µM m^{-1} s^{-1}) for 16-h photoperiod at 25 °C for 4 to 5 weeks before removing the explant leaves. The highest shoot regeneration rate achieved was on the basic MS medium amended with 1 mg L^{-1}thiamine-HCl, 4% sucrose, 2.0 mg L^{-1} thidiazuron (TDZ), and 2 mg L^{-1} IBA. The regeneration depended directly on the explant orientation. As a multiplication medium, the MS medium containing 1 mg dm^{-3} Indole-butyric acid (IBA), 1 mg dm^{-3} gibberellic acid and 5 mg dm^{-3} 6-benzyladenine was the best for *F. carica* cv. Sarilop (Hepaksoy and Aksoy, 2006).

vii. Encapsulation technology via synthetic seeds technique

Encapsulation technology has drawn tremendous attention in recent years because of its wide use in conservation and delivery of tissue cultured plants of commercial and economic importance. Production of synthetic seeds by encapsulating somatic embryos, shoot buds or any other meristematic tissue helps in minimizing the cost of micropropagated plantlets for commercialization and final delivery (Rai *et al.*, 2009). Sharma *et al.* (2015) reported for the first time the encapsulation of nodal segments of *F. carica,* storage and post-hardening survival of plantlets. However, encapsulated propagules failed rooting with a single step (on growth medium) for plantlets recovery.

Maximum shoot growth (95.6 %) was recorded on MS medium supplemented with 7.5 µM BA (6-benzyladenine), 0.5 µM NAA and 50 µM Ads (Adenine sulphate). Encapsulated nodal segments stored at 4 °C for 1 to 6 weeks sprouted at variable frequencies in successive weeks of transfer. Plantlets were acclimatized and established in field where they grew without any detectable variations after 6 months of transfer (Sharma *et al.*, 2015).

viii. Rooting phase

For root induction and growth of fig cv. 'Roxo de Valinhos' , the addition of auxins to the culture medium is not necessary (Brum, 2001; Fráguas *et al.*, 2004). However, the rooting of cv. 'Sarilop' is successful on MS medium amended with 1.2 and 2.5 µM IBA or NAA (Hepaksoy and Aksoy, 2006).

ix. Acclimatization phase

Substantial amounts of *in vitro* micropropagated plants do not survive during the direct transfer to the greenhouse or field conditions. The greenhouse and field have substantially lower relative humidity, high light intensity, and hygienic conditions that are stressful to the micropropagated plants compared to *in vitro* conditions. Most species grown *in vitro*, including the fig tree, require an acclimatization process before transferring them to the greenhouse as well as to the field. This practice ensures the high survival rate and vigorous growth of *in vitro* plantlets when transferred to soil. Researchers are successful in improving the survival index of *in vitro* fig plantlets during acclimatization. A 90-95% survival rate was obtained for fig plantlets according to the following steps (Pasqual and Ferreira, 2007).

5. Fig sanitation attempts by in vitro tissue culture

Tissue culture techniques are likely to provide methods for production of virus-free fig planting material. Shoot-tip culture, combined or not with thermotherapy, has been widely used for the elimination of viruses from plants (Stretch and Scott, 1977; Faccioli, 2001; Chalak *et al.*, 2013). Recently, these techniques have also been assessed on figs to remove some viruses associated with FMD (Sahraroo *et al.*, 2009; Chiumenti *et al.*, 2013) with the exception of the only DNA virus FBV-1 associated with FMD which resisted to all attempts of elimination, a behavior that confirms indirectly its hypothesized integration in the fig genome as proposed by Laney *et al.* (1992) (Chiumenti *et al.*, 2013).

Plant tissue culture is a technique based in the isolation of small parts of plants (tips, meristem and somatic embryos) and growing them on artificial media in adequate conditions so the parts of plants can grow and develop into complete plants (Hollings, 1965). Moreover, this technique can be used to produce virus-free plants. The size of tissue like shoot- tip (5-10 mm) or meristem portion (0.2-0.7 mm) is the critical point for the achievement of virus eradication, considering that smaller portion of tissue can be characterized by a lower virus concentration (Faccioli, 2001).

F. carica regenerated plantlets, from infected adult mother plant by FMV, were subjected to *in vitro* meristem tip culture technique. Plantlets showed to be still infected with 93.8% of elimination rate (Chiumenti *et al.*, 2013). The use of shoot-tip culture without thermotherapy has always been adopted (Stretch and Scott, 1977; Faccioli, 2001; Chalak *et al.*, 2013), although with a limited extent, depending on the virus (and phytoplasma) type to be eliminated and on the availability of plant material. Shoot-tip culture technique was applied on the two Lebanese fig cvs. ‘Biadi’ and ‘Aswad’ which were infected by FLMaV-1, FLMaV-2 and FMV. This technique was reliable for the elimination of 60 to 100%. Regeneration ability was twice as great for stem cutting explants (39-42%) than for shoot-tip explants (16-17%) with poor survival mostly resulting from the oxidation problem (Chalak *et al.*, 2015).

5.1 Somatic embryogenesis

Somatic embryogenesis has been used to eliminate viruses from various plants using explants such as anthers, ovaries or leaves cultivated on a callus induction medium. The kind

of infected tissue used interfered with elimination rates (Sutan *et al.*, 2010). The calli were transferred to an embryo differentiation medium, producing embryo-derived plantlets able to be micropropagated by cultural apical cuttings. The presence of virus particles in callus and the regeneration of healthy embryos or plantlets is related to the virus distribution and mechanisms of virus movement in the tissues, and most probably to the characteristics of the callus and its evolution after several months of culture (Panattoni *et al.*, 2013).

Organogenesis induction from leaf fragments of *F. carica* using phloroglucinol (PG) was experimented by Yakushiji *et al.* (2003)but adventitious bud differentiation frequency was relatively low and no adventitious buds without the addition of PG were observed. Soliman *et al.* (2010) reported that the best medium for callus formation from leaf segment of the Egyptian cv. of *F. carica* 'Sultani' (fresh consumption) was MS supplemented with 2 mg L^{-1} 2,4-D and 0.2 mg L^{-1} kinetin. The best plantlet differentiation was obtained at concentrations of 30 mg L^{-1} 2iP (2-iso-pentenyl adenine) and 7 mg L^{-1} TDZ with 0.25 mg L^{-1} NAA (with a regeneration efficiency of 83% and 79%, respectively). Moreover, the obtained callus failed to induce organogenesis on media containing a combination of BA and kinetin. The highest shoot formation percentage (89%) was obtained when using 2 mg L^{-1} TDZ and mg L^{-1} 2iP. The highest percentage of shoots forming roots (95%) was obtained when using MS medium supplemented with 1 mg L^{-1} IBA. Regenerated plantlets were not molecularly assessed for FMD associated viruses.

In vitro plant regeneration of four fig cultivars, namely 'Brown Turkey', 'Conadria', 'Deanna' and 'Poona', using leaf explants obtained from *in vitro* established shoots showed that the best callusing medium was MS supplemented with 2 mg L^{-1} TDZ and 4mg L^{-1}2iP. Among all tested genotypes, the cv. 'Brown Turkey' showed maximum response to callusing (85.8%) with shooting on same medium. Earliest callus induction was observed on MS medium supplemented with 2 mg L^{-1} TDZ and 2 mg L^{-1} IBA. Shooting was induced from callus by transfer to MS medium supplemented with 0.5 mg L^{-1} NAA and 7 mg L^{-1} TDZ. Shoots developed roots following transfer to half strength MS medium supplemented with 1 mg L^{-1} IBA and 2 g L^{-1} activated charcoal (Dhage *et al.*, 2012).

5.2 Thermotherapy combined or not with tissue culture

Thermotherapy is the technique most frequently applied in sanitation protocols during the time frame 1991-2010 (Panattoni *et al.*, 2013). This treatment consists of keeping plants,

or more frequently a part of them, at temperatures between 35°C and 54°C, within the physiological tolerance limits of each plant, for an appropriate period. In practice, the selected temperature represents the best compromise between virus degradation and plant survival, taking into account that the threshold of thermal sensitivity of some viruses is lower than that of plant cells and that damage caused to plant tissues by the thermal stress can more easily be reversed than viral damage (Spiegel *et al.*, 1993).

The combination of thermotherapy with meristematic-tip culture was tried throughout the world and has eliminated the virus pathogen and produced virus-free plant material of many species (Verma *et al.*, 2004; Kumar *et al.*, 2009).

Trials to produce FMD-free trees were initiated in the middle of 1960s, using heat therapy or *in vitro* meristem tip culture (Martelli, 2011) giving encouraging results by regenerating symptomless sanitized plantlets. Moreover, growing fig in tissue culture with alternating high-temperature regime resulted in fig plants with no external symptoms of fig mosaic disease (Lopez-Delgado *et al.*, 1998). Thermotherapy did not affect the stem cuttings responses of both fig Lebanese varieties ‘Biadi’ and ‘Aswad’. Furthermore, ‘Biadi’ cultivar infected with FLMaV-1 reached the 36% elimination rate via stem cutting culture submitted to thermotherapy and 66% via shoot-tip culture. When infected with FLMaV-2, virus elimination rates in ‘Biadi’ was 81% via stem cutting culture submitted to thermotherapy and reached 100% after shoot-tip culture. Whereas, elimination rates in ‘Aswad’ cultivar infected with FLMaV-1 did not exceed 60%, even after shoot-tip culture, and were 70 to 73% for this variety infected with FLMaV-2 (Chalak *et al.*, 2015). For FMV, elimination rates from shoot-tip cultures were 80% in ‘Aswad and 86% in‘Biadi’. Only 60% of ‘Aswad’ shoots deriving from stem cutting culture with thermotherapy, and 72% of ‘Biadi ‘shoots, were free of FMV.

FLV-1 could not be totally removed when explants were subjected to meristem-tip culture or to a single cycle (35 days at 38°C) of *in vitro* heat therapy (81.3 and 87.5% successful elimination, respectively) but was eradicated (100% sanitation) by protocols encompassing shoot-tip culture combined with heat therapy, or two heat therapy cycles. High sanitation rates from FLV-1 (81 to 100%) were registered using *in vitro* heat therapy alone (two cycles) or combined with tissue culture.

Very high sanitation rates (90-100%) from FMV were also obtained with all sanitation procedures adopted, with special reference to *in vitro* heat therapy alone or combined with tissue culture. FMV was eradicated (100% sanitation) by shoot-tip culture combined with heat therapy or *in vitro* heat therapy (Chiumenti *et al.*, 2013).

FLMaV-2 was more susceptible to thermotherapy and easier to eradicate than FLMaV-1 and FMV. Stem cutting culture coupled with thermotherapy was the most effective for shoot regeneration (40% of reactive explants), while elimination of the three viruses was possible even though with lower rates of removal (from zero to 81%) were achieved (Chalak *et al.*, 2015). *In vitro* heat therapy alone or combined with tissue culture succeeded to eliminate FLMaV-1, FLMaV-2 and FMV after 70 days at 38°C (Sahraroo *et al.*, 2009; Chiumenti *et al.*, 2013; Chalak *et al.*, 2015). Shoot-tip culture combined to thermotherapy can oxidize and/or compromise the regeneration of explants (Chalak *et al.*, 2015).

OBJECTIVES

The main objective of this doctoral research is to establish a complete protocol for fig *in vitro* multiplication, sanitation from Fig mosaic disease (FMD) associated viruses and conservation for the large-scale commercialization.

Thus, the interest to initially focus on screening for FMD-related viruses distribution within the different fig plant organs (buds, leaves, syconia and seeds) of 14 Mediterranean genotypes which will be afterward utilized as an *in vitro* source plant.

Moreover and in order to provide an efficient and rapid *in vitro F. carica* propagation using both standard technique based on the classical phases of shoot induction, multiplication and rooting besides to the non-conventional one using the synthetic seeds technology which summarize all the aforementioned phases in just one step.

Since for FMD control, cuttings from infected trees are not to be used for propagation, old and improved sanitation approaches were applied and their efficiency was evaluated during our research in order to produce FMD-free plantlets serving as source plant material.

Finally, in order to ensure the delivery of sanitized and healthy *F. carica* L. plantlets serving for future large-scale commercialization, we tested the practical applicability of fig conservation as encapsulated nodal segments for 45 days at 4°C

EXPERIMENTS

Chapter 1.

1. Viruses infecting different Mediterranean genotypes of *Ficus carica* and their distribution in different plant organs

1.1 ABSTRACT

Seven Mediterranean F. *carica* genotypes, i.e. *cv.* Palazzo, Severoni precoce, Bianca, Pilusedda, Dottato bianco, Bifara and Zidi, were screened for the presence of seven fig-infecting viruses associated with fig mosaic disease (FMD) in order to explore their distribution in different plant organs (leaf, bud and syconium) that will be utilized as a plant source material in different sanitation techniques. RT-PCR assays conducted on reverse-transcribed TNA extracted from leaves, apical buds and syconia (1.5-2 cm) of each genotype for the presence of Fig leaf mottle-associated virus 1 (FLMaV-1), Fig mild mottling-associated virus (FMMaV), *Fig mosaic virus* (FMV), Fig latent virus 1 (FLV-1), Fig Badnavirus 1 (FBV-1) and Fig fleck-associated virus (FFkaV) showed that the infection rates in tested cultivars were 72.2% for FBV-1 followed by FFkaV (27.4%), FLMaV-1 (18,7%) and FMV (11.1%); whereas FMMaV and FLV-1 were absent. Virus distribution within the different explants showed that FBV-1 was the most prevalent in all explants with infection rates ranging between 50% and 83%, whereas FFkaV was mostly concentrated in syconia (39%) and to a lesser extent in buds (32%) and leaves (10%). The highest infection with FLMaV-1 was found in syconia (50%), whereas buds and leaves showed a mild level of infection (25%). FMV was present in 25% and 8% of tested buds and leaves, respectively, whereas it was absent in syconia.

Keywords: Fig, mosaic, RT-PCR, virus distribution

1.2 MATERIAL AND METHODS

1.2.1 Field survey and collection of plant material

Seven Mediterranean *F. carica* genotypes, i.e. cv. Palazzo, Severoni precoce, Bianca, Pilusedda, Dottato bianco, Bifara nera and Zidi, originated from a germplasm collection at CIHEAM-IAMB in Apulia and from three fig orchards located at Pollina, Petralia, Soprana and Santa Maria di Gesù in Sicily (Southern Italy) were collected. Fig leaves, buds and syconia were screened for the presence of FLMaV-1, FLMaV-2, FMMaV, FMV, FLV-1, FFkaV and FBV-1 using RT-PCR technique (**Figures 13**, **14** and **15**).

Figure 13 *F. carica* leaves showing mosaic, vein banding, leaf deformation and chlorotic blotching symptoms collected from Santa Maria di Gesù in Sicily and the CIHEAM-IAMB germplasm collection.

Figure 14 Fig buds collected from Santa Maria di Gesù and the CIHEAM-IAMB germplasm collection.

Figure 15 Fig syconia of *cv*. Dottato biancocollected from Santa Maria di Gesù.

1.2.2 Extraction of total nucleic acids and RT-PCR

RT-PCR tests were made on total nucleic acids (TNAs) extracted from 0.2 g of leaves, buds and syconia using "silica capture" method as described by Foissac *et al.* (2001) (**Figure 16**).

Using the liquid nitrogen, the frozen explant was grinded by three metal balls throw the vortex machine, then obtained powder was macerated with 1 mL of grinding buffer (**Annex 1**). The homogenized solution was transferred into labeled tubes to which 100 µl NLS 10% was added. The mixture was incubated at 70°C, with an intermediate shaking, and then placed inside the ice for 5 min. Following a centrifugation at 13,000 rpm for 10 min, 300 µl of supernatant were transferred into new eppendorf tubes mixed with 300 µl NAI 6M, 50 µl Silica and 150 µl of absolute ethanol. The mixture was shacked gently at room temperature for 30 min and centrifuged at 6,000 rpm for 1 min. The pellet was washed three times with 500 µl of washing buffer and centrifuged for 1 min at 6,000 rpm (**Annex 1**).

The pellet was dried at room temperature for 10 min, re-suspended in 150 µl of sterile distilled water and incubated at 70°C for 4 min. Following a centrifugation at 13,000 rpm for 3 min, 150 µl of the supernatant was transferred into new eppendorf tube and stored at -20°C.

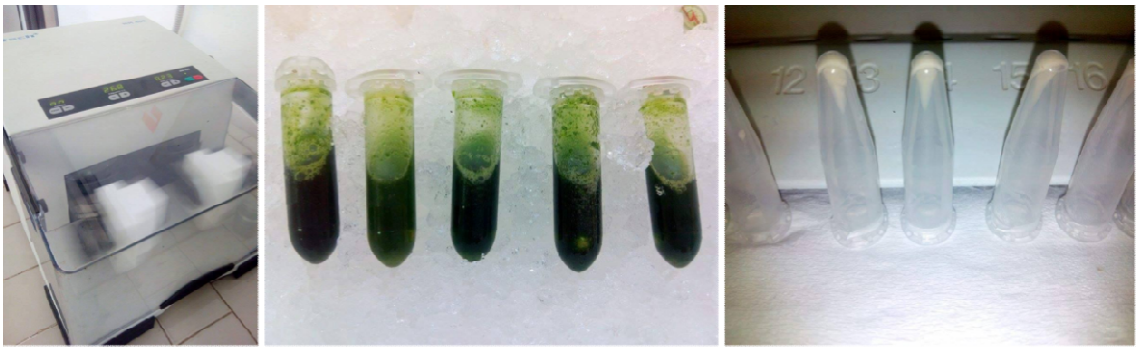

Figure 16 Total nucleic acids (TNAs) extraction according to Foissac *et al*. (2001).

For the molecular test, TNAs were denatured by boiling at 94°C for 5 min, then reverse-transcribed (with the exception of FBV-1) with random primers and M-MLV reverse transcriptase (In vitrogen Laboratories, USA) for 1 h at 39 °C. 2.5 µl cDNA reaction mix was used for PCR amplification using GoTaq (Promega Company, USA).

The mixture contained 0.5 µl of primers (**Table 2**), 0.5 µl of dNTPs, 5 µl GoTaq DNA polymerase buffer (5x), 1.5 µl $MgCl_2$ (25 mM) and 0.25 µl Go Taq DNA polymerase (5U/µl) in a final volume of 25 µl adjusted with sterile distilled water.

PCR amplifications were conducted in a Bio-Rad C1000 thermal cycler. PCR cycles were as the following: denaturation at 94°C for 4 min followed by 35 cycles at 94°C for 30 sec, primers annealing at 58°C for 35 sec, elongation at 72°C for 35 sec. Then the amplified products were elongated at 72°C for 5 min and cooled to 4°C (**Figure 17).** Ten µl of the PCR reactions were electrophoresed in 1.2% agarose gel in 1× TAE buffer (**Annex 2**) and stained with Red gel nucleic acid stain (Biotium, Milan, Italy). The amplified DNA fragments were visualized and photographed under UV light.

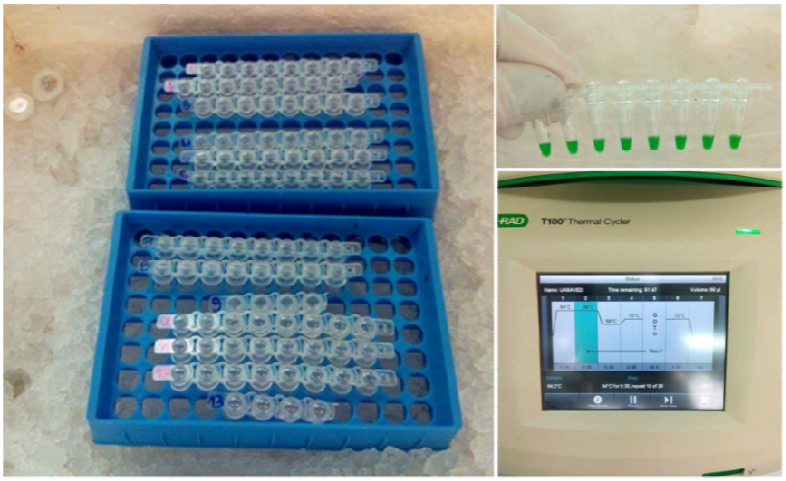

Figure 17 Polymerase Chain Reaction (PCR) conducted on fig samples.

Table 2 List of sense and antisense virus specific primers used in RT-PCR for the genome amplification of viruses associated with FMD.

Viruses	Primers	Primers sequences (5'-3')	Amplicon (bp)	Gene target	References
FLMaV-1	N 17/4-s	CGTGGCTGATGCAAAGTTTA	352	HSP70	Elbeaino *et al*., 2006
	N17/4-a	GTTAACGCATGCTTCCATGA			
FLMaV-2	F1- s	GAACAGTGCCTATCAGTTTGATTTG	360	HSP70	Elbeaino *et al*., 2007
	F1- a	TCCCACCTCCTGCGAAGCTAGAGAA			
FMMaV	LM3- s	AAGGGGAATCTACAAGGGTCG	311	HSP70	Elbeaino *et al*., 2010
	LM3- a	TATTACGCGCTTGAGGATTGC			
FMV	E5-s	CGGTAGCAAATGGAATGAAA	302	RdRp	Elbeaino *et al*., 2009
	E5-a	AACACTGTTTTTGCGATTGG			
FLV-1	FF up	CGCTTTGCCCCAATGTGCAGAT	200	CP	Gattoni *et al*., 2009
	FF down	TCGAAGGCCAGAGTTGATGCA			
FFKaV	D8-s	TCAATCCCAAGGAGGTGAAG	280	RdRp	T. Elbeaino, unpublished
	D8-a	ACACGGTCAATGAGGGAGTC			
FBV-1	Badna 3500s	GACGGGAAGGTTATCCAACA	220	RNA poly	Minafra *et al*., 2012
	Badna 3500 a	ATCACTCGCCACTTCTTCGT			

1.3 RESULTS AND DISCUSSION

Based on RT-PCR results, all fig plants from different origins showed to be infected with at least one virus but most of them harbored mixed infections (**Table 3**). The infection rates found in tested cultivars, and based on multiple explants, were 72.2% for FBV-1, followed by FFkaV (27.4%), FLMaV-1 (18.7%) and FMV (11.1%); whereas FMMaV and FLV-1 were absent (**Table 4**). Virus distribution within the different explants showed that FBV-1 was the most prevalent virus in all explants with infection rates ranging between 50% and 83% (**Figure 18**), whereas, FFkaV was mostly concentrated in syconia (39%) with a minor presence in buds (32%) and leaves (10%).

The highest infection with FLMaV-1 was found in syconia (50%) and with a lesser extent in buds and leaves (25%). FMV was present in 25% and 8% in tested buds and leaves, respectively, whereas was not detected in syconia (**Table 5**). In general, the comparison between the infection rates found in different plant organs showed that fig viruses are more persistent in syconia and buds rather than in leaves (**Figure 19**).

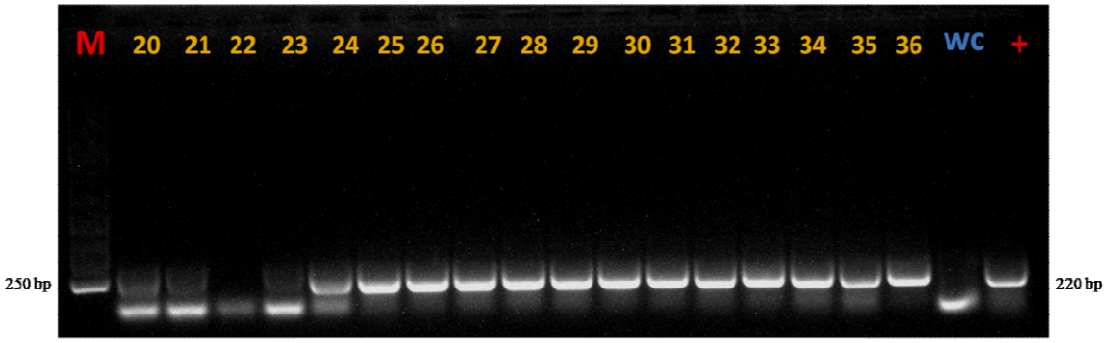

Figure 18 Electropherogram showing PCR amplicons (220 bp) from FBV-1 infected fig trees. M: Ladder marker; WC: Water control; +: FBV-1-infected sample used a positive control for PCR reaction.

Table 3 Sanitary status of field-grown adult fig accessions as determined by PCR.

Countries	Origin	Genotypes	FLMaV-1	FMMaV	FLV-1	FMV	FFkaV	FBV-1
Italy	CIHEAM-IAMB	Palazzo P2(CA1)	+	-	-	-	-	-
		SeveroniPrecoceP1 (Ca10)	-	-	-	-	+	-
	Pollina (Sicily)	Bianca S1	+	-	-	+	+	+
	Petralia Soprana (Sicily)	Pilusedda S6	+	-	-	+	+	+
	Santa Maria di Gesù (Sicily)	Dottato bianco 1	-	-	-	-	+	+
		Dottato bianco 3	-	-	-	-	+	+
		Bifara nera 2	-	-	-	+	+	-
Tunisia	CIHEAM-IAMB	Zidi T132	-	-	-	-	+	+

Table 4 Total virus infection in the tested plant organs.

Viruses	FLMaV-1	FMV	FFKaV	FBV-1
Infected/tested	6/32	8/72	33/120	78/108
Infection rate (%)	**18.7**	**11.1**	**27.4**	**72.2**

Table 5 Total virus distribution in the tested plant organs.

Viruses	FLMaV-1	FMV	FFKaV	FBV-1
Syconia infected/tested	4/8	0/24	16/41	30/36
Infection rate (%)	**50**	**0**	**39**	**83**
Buds infected/tested	3/12	6/24	13/40	30/36
Infection rate (%)	**25**	**25**	**32.5**	**83**
Leaves infected/tested	3/12	2/24	4/40	18/36
Infection rate (%)	**25**	**8.3**	**10**	**50**

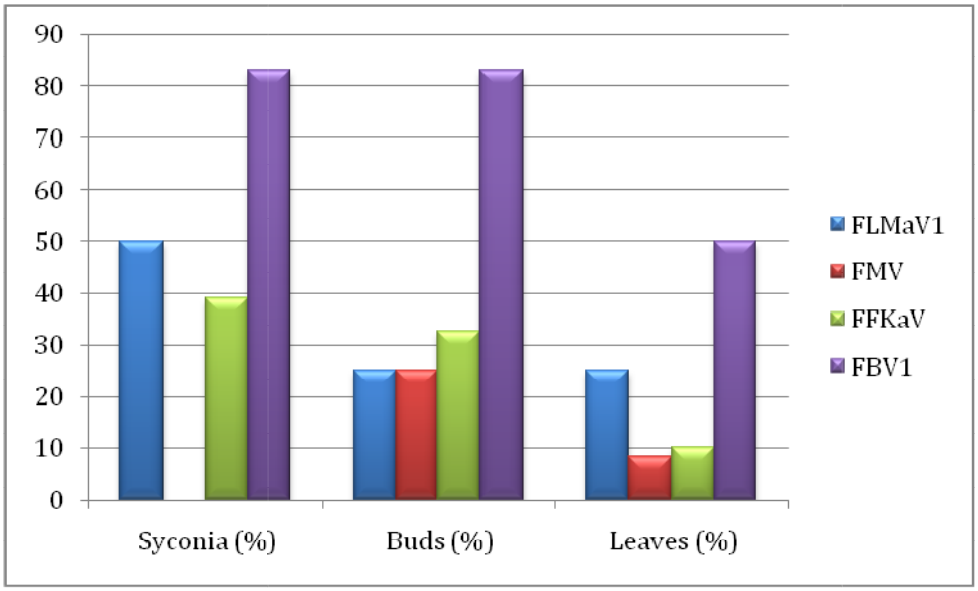

Figure 19 Comparison between the infection rates found in different plant organs.

1.4 CONCLUSIONS

The present study expands the knowledge on the sanitary status of seven Mediterranean fig genotypes and provides further information about the virus incidence and distribution within the trees (Yahyaoui *et al*., 2016a).

All fig-infecting viruses were present in the surveyed cultivars with the exception of FMMaV and FLV-1. These results are not surprising considering the mode of propagation of this species (by rooted cuttings and grafting) and the presence of very efficient virus vectors (eriophyid mites, mealybugs and aphids), both factors that promote the transmission of viral agents in nature. FBV-1, the only DNA virus, showed to be the most widespread virus in all cultivars. This outcome is strongly explained by the fact that FBV is vectored primarily by mealybugs and aphids, thus it may share vectors with the closteroviruses that infect the crop.

Furthermore FBV-1 was found to infect all different *F. carica* organs (syconium , leaf and bud) which confirms its vertical transmission to seedlings and its hypothesized integration in the host genome (Laney *et al*., 2012). FMV, the causal agent of FMD, was found to have a low infection rate directly related to its eriophyid mite (*A. ficus*) transmissibility, also to be more concentrated in buds then leaves and absent in syconia, confirming no transmissibility through seeds (Martelli *et al.,* 1993). Contrary to FFkaV and FLMaV-1 which were present and mostly concentrated in syconia.

The outcome of this study, although is still preliminary, from one hand gives an insight on the best explants to be used in a sanitation program for the regeneration of 'healthy' plants and, from another hand, increases the possibilities to compare the different sanitation techniques based on the explants available.

Chapter 2.

2. Viruses infecting seeds from different Mediterranean genotypes of *Ficus carica*. L.

2.1 ABSTRACT

Five Mediterranean *F. carica* genotypes, i.e. *cv.* Pilusedda, Dottato bianco, Bifara nera, Turkia and Zidi, were seeds extracted and screened for the presence of seven fig-infecting viruses associated with fig mosaic disease (FMD). RT-PCR assays conducted on reverse-transcribed TNA extracted from seeds of each genotype for the presence of Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), Fig mild mottling-associated virus (FMMaV), *Fig mosaic virus* (FMV), Fig latent virus 1 (FLV-1), Fig Badnavirus 1 (FBV-1) and Fig fleck-associated virus (FFkaV) showed that FBV-1 was the most prevalent in all tested seeds with 100% of infection rate, followed by FMV (52.7%), FFKaV (50.9%), FLV-1 (40%). A mild level of infection was given by FLMaV-1 (29.1%), FLMaV-2 (16.4%) and FMMaV (14.5%). Infection rates within the different genotypes revealed that FMV and FFKaV were highly and equally present in Pilusedda seeds (70%) as well as FLV-1 in both Bifera nera and Zidi seeds (62.5%). FLMaV-1 was mostly concentrated in Dottato 1 (33.3%) whereas, FLMaV-2 and FMMaV showed to be present only in Bifera nera (56.2% and 50%, respectively).

Keywords: *Fig,* mosaic, RT-PCR, virus, seeds infection

2.2 MATERIAL AND METHODS

2.2.1 Seeds collection and isolation

A total of 55 fig syconia used in this experiment, were seeds extracted from five Mediterranean *F. carica* genotypes, i.e. cv. Pilusedda, Dottato bianco, Bifara nera, Turkia and Zidi and collected from a germplasm collection at CIHEAM-IAMB and two fig orchards located at Petralia Soprana and Santa Maria di Gesù.

Initially freshly collected mature syconia were cut, one by one, using a sharp lade, then, they were manually de-pulped using a high pressure running tap water with a continuous mashing between fingers. Seeds were separated from the fleshy part using two plastic sieves with different whole sizes. The sieve with a bigger size was placed on the top of the small one in order to first separate seeds from the pulped part then collect the seeds. This step was repeated three times in order to obtain a well separated and clean seeds (**Figure 20**).

A beaker was placed under the two sieves in order to avoid seeds lost. Seeds were then placed on a filter paper, in a single layer, and were air-dried at room temperature for 24 hours.

Figure 20 Steps of seeds extraction from fig different Mediterranean syconia cultivars.

2.2.2 Extraction of total nucleic acids and RT-PCR

For molecular tests, TNAs were extracted from 0.2 g of each syconium seeds according to the aforementioned "silica capture" method described by Foissac *et al.* (2001), then were reverse transcribed and amplified by RT-PCR in order to screen for the presence of FLMaV-1, FLMaV-2, FMMaV, FMV, FLV-1, FFkaV and FBV-1 using specific primers pairs (**Table 2**). PCR product was visualized by electrophoresis in 1.2% agarose gel.

2.3 RESULTS AND DISCUSSION

Results of RT\PCR showed that all viruses associated with FMD were present in the tested seeds without any exception (**Figure 21**). In particular, FBV-1 was found to be present in all seeds (100% of infection) while FMMaV showed the lower seeds infection rate (14.5%) (**Figure 22**).

FMV and FFkaV showed good levels of infection rate in seeds with 52.7% and 50.9%, respectively. FLMaV-1 was present in 29.1% of seeds, whereas FLMaV-2 showed a low infection rate of 16.4% (**Table 6**).

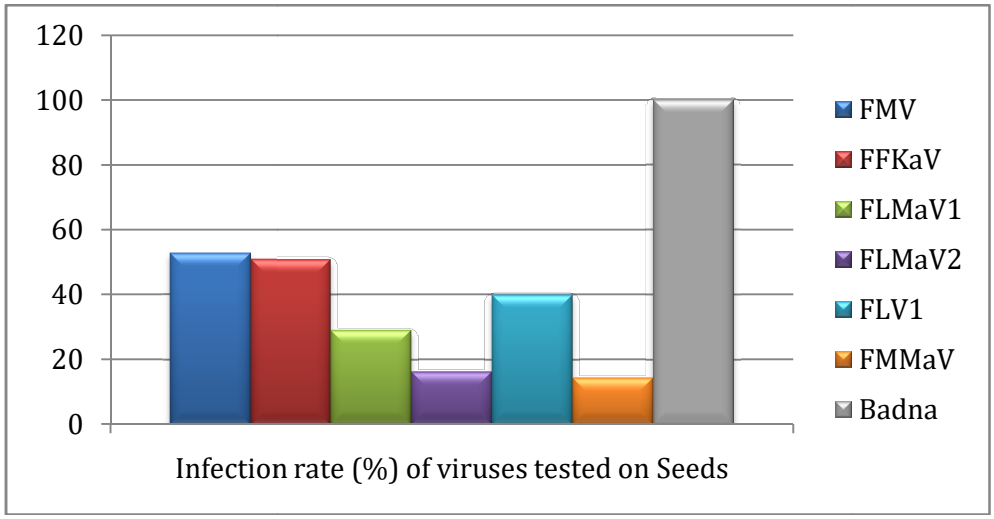

Figure 21 Infection rates of all tested viruses associated with FMD in fig seeds.

Table 6 RT-PCR results of the extracted seeds from various *F. carica* L. cultivars.

Varieties	Symbols	FMV	FFKaV	FLMaV-1	FLMaV-2	FLV-1	FMMaV	FBV-1
Pilusedda	P1	+	-	-	-	-	-	+
	P2	+	+	-	-	-	-	+
	P3	+	-	-	-	-	-	+
	P4	-	+	-	-	-	-	+
	P5	+	+	-	-	-	-	+
	P6	+	+	-	-	-	-	+
	P7	-	+	-	-	-	-	+
	P8	-	+	-	-	-	-	+
	P9	+	+	-	-	-	-	+
	P10	+	-	-	-	-	-	+
Dottato (Tree 1)	D11	+	-	-	-	-	-	+
	D12	+	+	-	-	+	-	+
	D13	-	+	-	-	+	-	+
	D14	-	-	-	-	-	-	+
	D15	+	+	-	-	-	-	+
	D16	+	+	-	-	-	-	+
	D17	-	-	-	-	-	-	+
	D18	-	-	-	-	-	-	+
	D19	+	-	-	-	-	-	+
	D20	+	+	-	-	-	-	+
	D40	+	+	+	-	-	-	+
	D41	-	-	+	-	+	-	+
	D42	-	-	+	-	-	-	+
	D43	-	-	+	-	+	-	+
	D44	+	+	+	-	+	-	+
Bifera nera (Tree 2)	B21	+	+	-	+	-	+	+
	B22	-	+	-	+	-	+	+
	B23	+	-	-	+	-	+	+
	B24	+	+	-	+	+	+	+
	B25	+	+	-	+	+	+	+
	B26	+	+	-	+	+	-	+
	B27	-	-	-	+	+	+	+
	B28	-	-	-	+	-	-	+
	B29	+	+	-	+	-	+	+
	B30	+	+	-	-	+	+	+
	B50	-	-	-	-	-	-	+
	B51	-	-	+	-	+	-	+
	B52	-	-	-	-	+	-	+
	B53	-	-	+	-	+	-	+
	B54	+	-	+	-	+	-	+
	B55	-	-	+	-	+	-	+
Zidi P6	Z31	+	+	-	-	+	-	+
	Z32	-	-	-	-	+	-	+
	Z33	+	-	+	-	+	-	+
Zidi P4	Z45	-	-	-	-	-	-	+
	Z46	-	-	-	-	+	-	+
	Z47	-	-	-	-	-	-	+
	Z48	-	-	-	-	+	-	+
	Z49	-	-	-	-	-	-	+
Turkia	T34	+	+	+	-	+	-	+
Dottato (Tree 3)	D35	-	+	+	-	-	-	+
	D36	+	+	+	-	+	-	+
	D37	-	+	+	-	-	-	+
	D38	+	+	+	-	-	-	+
	D39	+	+	+	-	-	-	+
Total positives		29/55	28/55	16/55	9/55	22/55	8/55	55/55
Total infection%		**52.7**	**50.9**	**29.1**	**16.4**	**40**	**14.5**	**100**

RT-PCR revealed that FLV-1 was detected with an adequate infection rate (about 40%) anyway lesser than the seeds transmission reported by Castellano *et al*. (2009), ranging from 73 to 100% with an average of 92%.

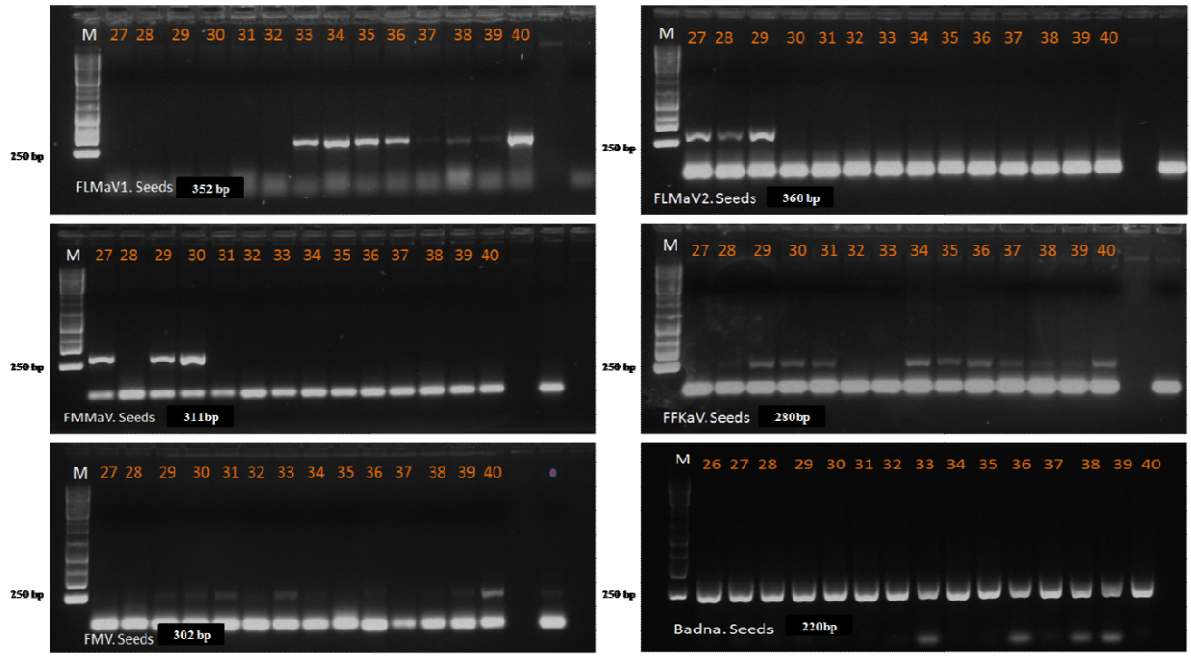

Figure 22 Electropherogram of RT-PCR reactions conducted on seeds extracted from field of different cultivars presenting infections with FLMaV-1, FLMaV-2, FMMaV, FFKaV, FMV and FBV-1 for the same infected explants.

Moreover, the four viruses with the highest infection rate FMV, FFKaV, FLV-1 and FBV-1 gave 18.18% as a mixed infection but more data are needed to determine the effect of mixed infections to symptomatic and epidemiological aspects of FMD (Elci *et al.*, 2012).

Varieties having an equal or a superior number to eight fruits were summarized in **Table 7** in order to screen for seeds infection rates within each variety.

FBV-1 was present in all cultivars seeds with 100% of infection rate. FMV revealed a high infection rate in Pilusedda seeds with 70% followed by Dottato1 and Bifera nera with 53.3% and 50% respectively whereas the lowest one was observed in Tunisian seeds (25%).

Table 7 FMD associated viruses within *F. carica* L. different cultivars seeds.

Varieties (Seeds)	Origin	FMV	FFKaV	FLMaV-1	FLMaV-2	FLV-1	FMMaV	FBV-1
Pilusedda	Petralia Soprana	7/10	7/10	0/10	0/10	0/10	0/10	10/10
		70%	**70%**	**0%**	**0%**	**0%**	**0%**	**100%**
Dottato (Tree 1)	Santa Maria di Gesù	8/15	7/15	5/15	0/15	5/15	0/15	15/15
		53.3%	**46.7%**	**33.3%**	**0%**	**33.3%**	**0%**	**100%**
Bifera nera		8/16	7/16	4/16	9/16	10/16	8/16	16/16
		50%	**43.7%**	**25%**	**56.2%**	**62.5%**	**50%**	**100%**
Zidi (P6+P4)	Tunisia	2/8	1/8	1/8	0/8	5/8	0/8	8/8
		25%	**12.5%**	**12.5%**	**0%**	**62.5%**	**0%**	**100%**

FFKaV was more present in Pilusedda seeds (70%) with a less infection rate in Dottato1 (46.7%), Bifera nera (43.7%) and Zidi (12.5%). Moreover, Dottato1 seeds were the most infected with FLMaV-1 with 33.3% followed by Bifera nera (25%) and Zidi (12.5%). Pilusedda seeds were free from this virus. In addition, FLMaV-2 and FMMaV were present only in Bifera nera seeds with 56.2% and 50%, respectively.

Bifera nera and Zidi seeds showed to be equally infected with FLV-1 with 62.5% followed by Dottato1 (33.3%) while Pilusedda was free from this virus.

2.4 CONCLUSIONS

Molecular analysis confirmed the detection of the pre-tested FMD associated viruses in *F. carica* L. seeds (Yahyaoui *et al*., Unpublished). In particular, the high transmission rate of FLV-1 differentiates from most of the other seed-transmitted plant viruses which, in general, have a lower transmission frequency, and from the extant trichoviruses, none of which is apparently transmitted through seeds.

The low infection percentage observed for FMMaV don't take on important epidemiological significance both because previous reports on its presence inside seeds or their cuticles there are not and because the aforementioned viruses are all hypothetically not transmissible through seeds, with the exception of FLV-1 and FBV-1. This last in fact gave the maximum value of seed infection rate followed by FMV and FFKaV. This outcome is strongly explained by the fact that FBV-1 is vectored primarily by mealybugs and a few species by aphids in a semi-persistent manner (Bhat *et al.*, 2016), thus it may share vectors with the closteroviruses that infect the crop. Besides to its high vertical seeds transmissibility to seedlings (Laney *et al.*, 2012; Minafra *et al.*, 2012).

Furthermore, this genus is known as endogenous badnaviruses (Hohn *et al.*, 2008; Staginnus *et al.*, 2009) since, depending on the recombination events, they are present as integrated sequences in some host plant genomes. In some cases, however these copies can give rise to systemic virus infection induced by abiotic stress like *in vitro* tissue culture process and inter-specific crosses (Dallot *et al.*, 2001; Cote *et al.*, 2010). The integration in the host genome explains the high infection rate within all parts of fig plant. Thus, seed transmission of FBV-1, in conjunction with secondary spread by a vector, could favor the introduction and subsequent dissemination of the virus in new environments.

Since there are no previous reports on the presence of FMD-associated viruses inside seeds or their cuticles, such epidemiological significance remains negligible because the aforementioned viruses with the exception of FLV-1 and FBV-1 are all hypothetically not transmissible through seeds. In general, fig is not propagated through seeds and such presence of viruses significance of seed infection would be negligible.

Chapter 3.

3. *In vitro Ficus carica.* L multiplication via the standard technique.

3.1 ABSTRACT

Infected *Ficus carica.* L mother trees by seven viruses associated with FMD showing symptoms and/or confirmed by RT-PCR conducted on reverse-transcribed TNAs, extracted from leaves for the presence of Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), Fig mild mottling-associated virus (FMMaV), *Fig mosaic virus* (FMV), Fig latent virus 1 (FLV-1), Fig Badnavirus 1 (FBV-1) and Fig fleck-associated virus (FFkaV) were chosen for in vitro multiplication serving as source plant material for future sanitation techniques.

Apical and lateral buds of 14 *Ficus carica* L. Mediterranean genotypes, i.e. cv. Palazzo, Severoni precoce, Brogiotto nero, Triboiti, Catalanisca, Bianca, Biancu, Dottato, Pilusedda, Turca 'Serilop', Baiyadi, Houmairi, Zidi and Bifera nera were cultured on (MS-1) medium supplemented with 4 mg L^{-1} BAP and 0.2 mg L^{-1} NAA, shoot multiplied on (MS-2) supplemented with half strength BAP and equal NAA and rooted in MS-hormone free medium. Regeneration rates were recorded and best genotypes were selected for future sanitation attempts. Obtained results showed that the total percentage of bud breaking was acceptable (50.7%) but variable according to the varieties used. Apical buds showed to give the best total regeneration rate (63.6%) if compared to the auxiliary ones (34.8%). Bianca and Biancu varieties reached the 100% of survival rate, followed by Turca 'Serilop' (74.2%), Palazzo (68.6%), Bifera nera (68.1%), Triboiti (62.5%), Dottato S4 (62.5%), Dottato 1 (58.1%) and Houmairi (54.5%) successively. The Turkish cultivar Turca 'Serilop' registered a very high regeneration rate but regenerated plantlets were very small showing a very rudimentary leaves.

From vigor point of view, four fig genotypes showed to be the well adapted for *in vitro* multiplication and the best candidates for future sanitation program. Palazzo occupied the first position with 68.6% of regeneration rate followed by Bifera nera (68.1%), Houmairi (54.5%) and Catalanisca (41.7%). Those four genotypes were easily rooted on MS-hormone free medium. Severoni precoce revealed the lowest bud breaking percentage with 18% of regeneration rate after Brogiotto nero (30.8%), Zidi (35.6%), Pilusedda (40%) and Baiyadi (43.7%). Those cultivars showed to have the weakest in vitro multiplied plant material.

Key words Fig, in vitro multiplication, propagation

3.2 MATERIALS AND METHODS

3.2.1 Explant sterilization

Cuttings of 15 to 20 cm length without leaves (containing 4 to 8 buds) were collected from infected, with some viruses associated with FMD, fig trees belonging to 14 Mediterranean genotypes, i.e. *cv.* Palazzo, Severoni precoce, Brogiotto nero, Triboiti, Catalanisca, Bianca, Biancu, Dottato, Pilusedda, Turca 'Serilop', Baiyadi, Houmairi, Zidi and Bifera nera (**Table 8**) in order to be multiplied and conserved as *in vitro* source plant material for further sanitation techniques.

Cuttings were cut into small portions (microcuttings of 1 to 2 cm of length), each portion containing one bud (apical or lateral), and were put into a Beaker (**Figure 23**).

Figure 23 Sterilization of cuttings collected from Petralia Soprana and Pollina.

Small portions were washed under running tap water for 45 min, in order to remove dust particles and the phenolic substances. Then they were surface sterilized by immersion in a 1% solution of a detergent (10 mL L^{-1} of water) for 10 min followed by 2% solution of "Folicur Fungicide" (2g L^{-1}) for 10 min, ethanol (70% in sterile distilled water) for 10 min using the vacuum under the laminar flow hood and 30% solution of sodium hypochlorite solution containing a few drops of Tween-20 for 20 min. Explants were then transferred into new sterilized beakers and were rinsed three times with sterile distilled water, each for 3 min.

At the 3rd wash, 250 µl of the "Ticarcillin disodium and clavulanate potassium (15:1)" antibiotic was added for each 50 mL and let for 30 min. Finally, sterilized microcuttings were dried under sterile laminar flow hood for 10 min.

Table 8 Sanitary status of initial fig mother trees belonging to various Mediterranean genotypes.

Countries	Origin	Samples	Genotypes	FLMaV-1	FLMaV-2	FMMaV	FLV-1	FMV	FFkaV	FBV-1
Italy	IAMB	Palazzo P2 (CA1)	Palazzo	+++	-	-	-	-	-	+
		Severoni Precoce P1 (Ca10)	Severoni Precoce	-	-	-	-	-	+	-
		Brogiotto Nero P10 (Ca12)	Brogiotto Nero P10	-	-	+	-	-	-	-
(Italy) Sicilan Var	Pollina	S3	Triboiti	nt	nt	nt	nt	nt	nt	nt
		S2	Catalanisca	+	-	+	+	+	+	+
		S1	Bianca	+	-	+	+	+	+	+
	Petralia Soprana	S5	Biancu	nt	nt	nt	nt	nt	nt	nt
		S4	Dottato	-	-	-	+	+	+	+
		S6	Pilusedda	+	-	+	+	+	+	+
	Santa Maria di Gesu	Tree 1	Dottato Bianco	-	-	-	-	-	+	+
		Tree 3		-	-	+	-	-	+	+
		Tree 2	Bifara nera	-	-	-	+	+	+	+
Turkey		Turca 'Serilop' P1 (Ca2)	Serilop	++	-	-	++	-	++	-
Lebanon		NB35	Baiyadi	+	+	-	-	+	-	+
		Nb8	Houmairi	-	+	-	+	+	-	+
Tunisia		T132 (P4 campo)	Zidi	-	-	-	+	-	+	+

+: Infected; +++: Highly infected; -: Negatif; nt: Not tested

3.2.2 Fig multiplication via buds

Scales were removed and both apical and auxiliary buds were excised from the sterilized microcuttings and plated on a solid shooting medium (MS-1). This latest is based on MS (Murashige and Skoog, 1962) mineral salts and vitamin mixture (**Table 9**), supplemented with 30 g L^{-1} sucrose, 4 mg L^{-1} BAP, and 0.2 mg L^{-1} NAA (**Table 10**) (Kumar *et al.*, 1998), and solidified with 6.5 g L^{-1} plant agar (Micropoli, Cesano Boscone, Italy) (**Figure 24**). After the pH adjustment to 5.8, the medium was autoclaved at 120 °C for 20 min. The cultures were then sealed with Parafilm, labeled and maintained under light conditions (16/8-h light/dark photoperiod), with light supplied by cool-white fluorescent lamps (TMN 30 W/84; Philips, Suresnes, France) at a photosynthetic photon flux density (PPFD) of 35 µmol m^{-2} s^{-1}.

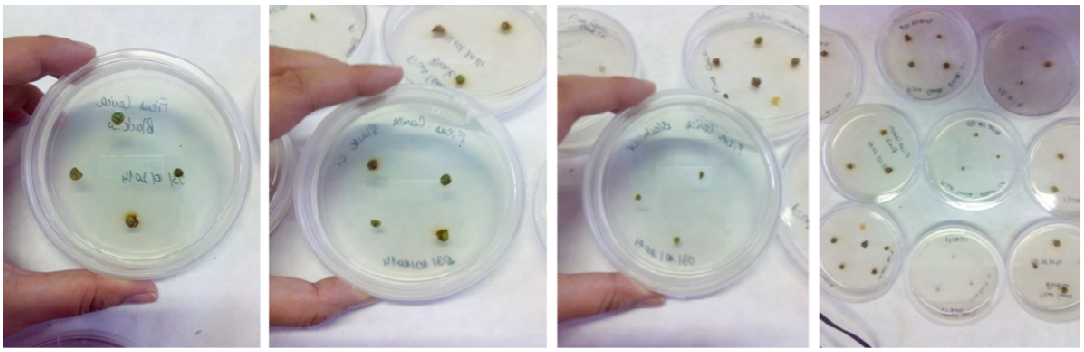

Figure 24 Fig multiplication via buds.

The cultures were sub-cultured at least twice at 3-weeks intervals in fresh medium, in order to remove the black or brown accumulations at the bottom tip of the explants. Contamination was checked within three days interval (**Figure 25**). Percentage of shoots initiated was calculated.

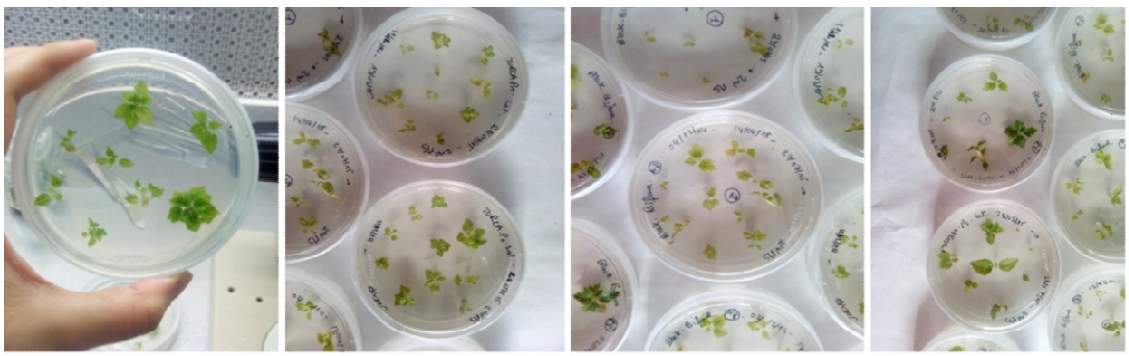

Figure 25 Subculture of fig plantlets into on a solid shooting medium (MS-1).

After 35 days of development on the culture shoot formation medium, young and vigorous microshoots were transferred into the induction of multiple shoots medium (MS-2) supplemented with 2 mg L^{-1} of BAP (**Figure 26**) (**Table 10**).

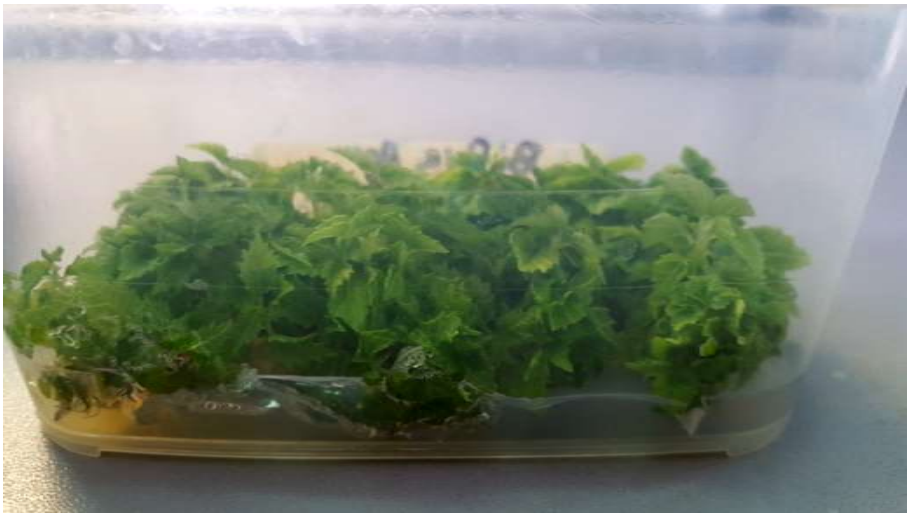

Figure 26 Multiple shoots induction of fig cv. Bifera nera on MS-2 medium.

The cultures were properly sealed, labeled and randomly placed in culture room. Proliferating shoots obtained from *F. carica* buds were monthly subcultured in 500-mL glass jars containing 100 mL of the same medium for three months. The *in vitro* multiplied plantlets were transferred into a bigger plastic jars where they were rooted on hormone-free MS medium (**Figure 27**).

Figure 27 Rooted cv. Bifera nera (a), Palazzo (b) and Catalanisca (c) on hormone -free MS medium.

Table 9 Murashige and Skoog, 1962 (MS) basal culture medium.

Constituents	***Mg L^{-1}***	***Constituents***	***Mg L^{-1}***
Macronutrients		*Iron Source (Fe, EDTA)*	
NH4NO3	1650		
KNO3	1900	FeNA EDTA	36.7
CaCL2	332,02		
MgSO4	180.54	*Vitamins*	
KH2PO4	170		
		Nicotinic acid	0.5
		Pyridoxine hydrochloride	0.5
Micronutrients		Thiamine hydrochloride	0.5
KI	0.83	Glycine	2.0
H3BO3	6.2	Myo-inositol	100.0
MnSO4 4H2O	16.9	Sucrose	30 x 103
ZnSO4 7H2O	8.6		
NaMoO4 2H2O	0.25	pH	5.8
CuSO4 5H2O	0.025	Agar	8500.0
CoC12 6H2O	0.025		

Table 10 Growth regulators concentration in MS Fig media.

Medium name	**Salts and vitamins**	**Growth regulators (mg L^{-1})**
MS-1	Murashige and Skoog	4 BAP and 0.2 NAA.
MS-2	Murashige and Skoog	2 BAP and 0.2 NAA.

BAP*= 6-Benzylaminopurine; NAA*= 1-Naphthaleneacetic acid

3.3 RESULTS AND DISCUSSION

Apical and axillary buds initiation was observed after two to three weeks from culture on MS-1 medium supplemented with 4 mg L^{-1} BAP and 0.2 mg L^{-1} NAA. The percentage of bud breaking from nodal was variable according to the varieties used (**Figure 28**). Registered results in **Table 11** showed that all varieties succeeded to break the buds with different frequency where Bianca and Biancu varieties reached the 100% of survival rate followed by Turca 'Serilop', Palazzo, Bifera, Triboiti, Dottato, Dottato1 and Houmairi successively. The Turkish cultivar Turca 'Serilop' registered a very high regeneration rate (74.2%) but regenerated plantlets showed to be small with a very rudimentary leaves.

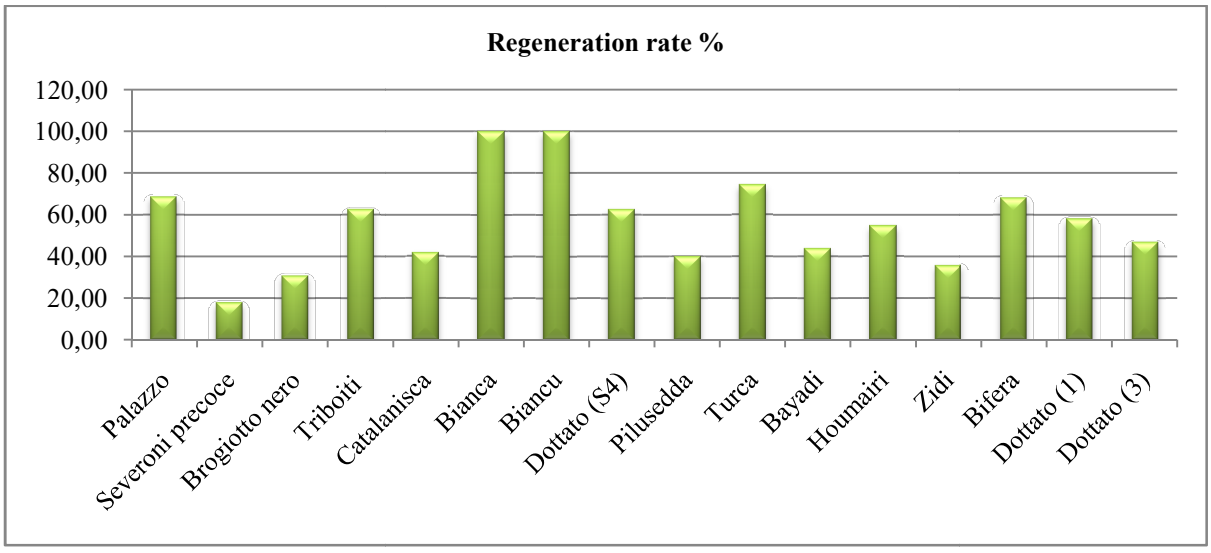

Figure 28 Total regeneration rate (%) of the initial cultured *in vitro* cultivars via apical and lateral buds.

Severoni precoce cultivar showed to have the lowest bud breaking percentage with 18% of regeneration rate followed by Brogiotto nero (30.8%), Zidi (35.6%), Pilusedda (40.0%) and Baiyadi (43.7%). Those cultivars showed to have the weakest *in vitro* multiplied plant material if compared to the other obtained varieties.

Table 11 General *in vitro* multiplication table of *F. carica*.

Cultivars	Ap	Ap reg	% Ap	Lat	Lat reg	% Lat	TOT Buds	TOT reg	TOT%
Palazzo	15	12	80.0	20	12	60	35	24	68.6
Severoni precoce	16	9	56.2	34	0	0.0	50	9	18.0
Brogiotto nero	12	8	66.7	14	0	0.0	26	8	30.8
Triboiti	3	3	100	5	2	40.0	8	5	62.5
Catalanisca	8	4	50.0	16	6	37.5	24	10	41.7
Bianca	11	11	100	0	0	0.0	11	11	100
Biancu	6	6	100	0	0	0.0	6	6	100
Dottato (S4)	3	3	100	5	2	40.0	8	5	62.5
Pilusedda	20	8	40.0	0	0	0.0	20	8	40.0
Turca 'Serilop'	12	7	58.3	19	16	84.2	31	23	74.2
Baiyadi	10	4	40.0	6	3	50.0	16	7	43.7
Houmairi	12	7	58.3	10	5	50.0	22	12	54.5
Zidi	27	11	40.7	18	5	27.8	45	16	35.6
Bifera nera	28	25	89.3	19	7	36.8	47	32	68.1
Dottato (Tree 1)	24	16	66.7	7	2	28.6	31	18	58.1
Dottato (Tree 3)	21	11	52.4	11	4	36.4	32	15	46.9
Total	228	145	**63.6**	184	64	**34.8**	412	209	**50.7**

Ap: Apical; **Ap reg:** Apical regenerated; **Lat:** Lateral; **Lat reg:** Lateral regenerated; **Tot:** Total; **Tot reg:** Total regenerated.

Moreover and according to our results, *F. carica* cultivars showed to be well proliferated via apical buds more than the lateral ones, where the total regeneration rate reached the 63.6% and 34.8% respectively (**Figure 29**).

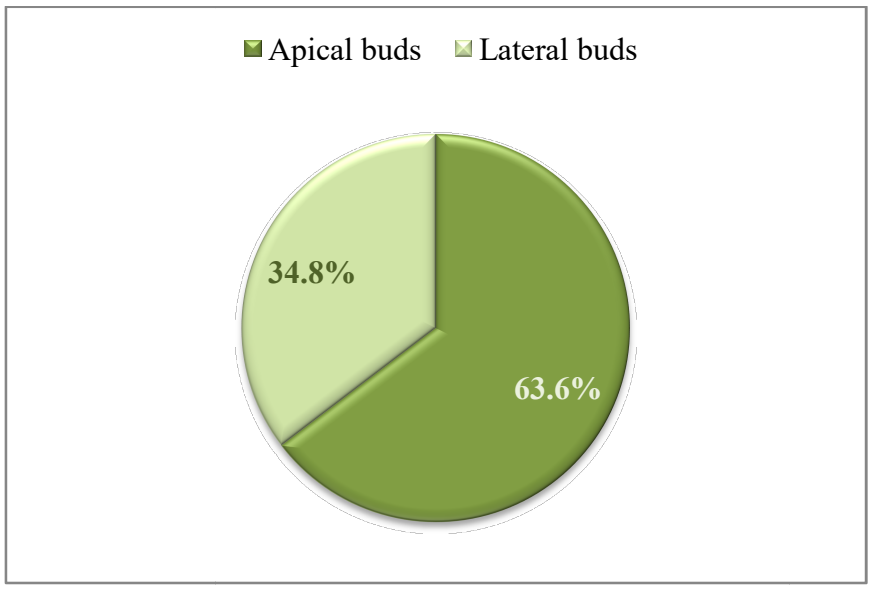

Figure 29 Total regeneration rate of fig via apical and lateral buds.

The newly developed young shoots, obtained during the initiation phase using MS-1, were harvested and subcultured on fresh MS-2 medium supplemented with 2 mg L^{-1} BAP and 0.2 mg L^{-1} NAA for shoot multiplication, in order to increase the stock of *in vitro* plant material which will serve for the sanitation trial and the total multiplied plantlets were summarized in the **Table 12.** Actually, it was recently confirmed by Bayoudh *et al.* (2015) that the proliferation of fig shoots was mainly provided by medium containing small amounts of BAP and NAA.

Table 12 Total stock of *in vitro* multiplied plant material of *F. carica*

Origin	Genotypes	Plant n° via buds multiplication
Pollina-Sicily	Catalanisca	233
Petralia Soprana-Sicily	Dottato	64
	Biancu	8
Santa Maria di Gesù-Sicily	Bifera	449
Lebanon	Houmairi Nb8	4
	Baiyadi	6
IAMB	Palazzo P2	135
Turkey	Turca 'Serilop' P1	27
Total *in vitro* stock varieties		**926**

Multiple shoots were emerged directly from the cultured explants after four weeks. We registered a variation of the explant response in the multiplication medium, where Bifera nera gave the highest multiplied plantlets number, followed by Catalanisca and Palazzo. Those three fig cultivars showed to be easily propagated and well adapted for *in vitro* multiplication.

Differently, Houmairi cultivar showed the ability to form callus instead of regenerating an entire plantlet (**Table 12**). Biancu, Turca 'Serilop' and Baiyadi were found to be the worst *in vitro* propagated cultivars, where they revealed the lowest regenerated plantlets number having rudimentary leaves.

Moreover, the contamination during the subculture participated in reducing the raised plantlets number subjected to the multiplication phase besides to the fact that shoot proliferation is significantly dependent on varieties and culture media used (Bayoudh *et al.*, 2015).

3.4 CONCLUSIONS

Regarding the *in vitro* standard *F. carica* multiplication, the adopted sterilization procedure gave good results. Several pathogens such as bacteria and fungi were eliminated, as well as, the prevention of browning of explants during fig tissue cultures by daily transfer to fresh media.

Moreover, our results confirmed the positive combination effect of BAP and NAA on adventitious *in vitro* shoot multiplication and regeneration of *F. carica* already adopted by Kumar *et al.* (1998) (Yahyaoui *et al*., Unpublished). Moreover we found that the regeneration rate via apical buds was superior to that obtained via lateral ones. Thus, for a mass *in vitro* multiplication ad propagation of *F. carica*, the use of apical buds is very advantageous and recommended.

In addition, Palazzo showed to be the best well adopted cultivar to the *in vitro* multiplication where it revealed a satisfying regeneration rate followed by Bifera nera and Catalanisca. Those three genotypes form the best candidates for the future sanitation program. On the contrary, the following cultivars, Severoni precoce, Brogiotto nero, Zidi, Pilusedda, and Baiyadi, showed to be the less adapted to the *in vitro* multiplication. This heterogeneous behavior among the regeneration rates of fig cultivars may due to various factors as genotype, combination of growth regulators (Meena *et al.*, 2010; Sharma *et al.*, 2011) or an early oxidation problem as reported by Chalak *et al.* (2015).

Chapter 4.

4. *In vitro Ficus carica.* L multiplication via synthetic seeds

4.1 ABSTRACT

In this study, the encapsulation technology based on the calcium alginate coating was applied to some *F. carica* L. genotypes. Uninodal microcuttings (3-4 mm long), excised from *in vitro* proliferating shoots of three Italian cultivars, (Bifera, Palazzo and Catalanisca) were employed. The influence of three different plant growth regulators (PGRs): 6-Benzylaminopurine (BAP), Meta-Topolin (MT) and Zeatine (ZEA), added to the artificial endosperm, were evaluated. Particularly, the viability, regrowth and conversion parameters of the synthetic seeds were recorded after 60 days from the sowing on a hormone-free medium. Results showed that the cultivars Catalanisca and Palazzo showed the highest viability average rate (90%), followed by Bifera nera (80%). Encapsulated microcutting regrowth percentage was highest for Palazzo (86.7%), followed by Catalanisca (81.7%) and Bifera nera (66.7%), while the conversion rate was comparable for the three cultivars (43.4, 40.0 and 48.3%, respectively). A strong interaction between cultivars and PGRs was observed. Moreover, regarding the effect of the PGRs, BAP and MT provided the best results about regrowth and conversion, even if the cultivar Catalanisca presented 100% of regrowth with ZEA.

To our knowledge, this is the first time that encapsulation technology has been applied to Italian cultivars of *F. carica*.

Keywords: cytokinins, encapsulation, fig, micropropagation, synthetic seed.

4.2 MATERIAL AND METHODS

4.2.1 Plant material, encapsulation procedure, and culture conditions

Shoots were randomly chosen from the *in vitro* proliferated plantlets and used to provide propagules for encapsulation. Microcuttings (3-4 mm long) without leaves and with one auxiliary bud were excised from the *in vitro*-proliferated shoots and subjected to the encapsulation procedure. This one consisted of immersing microcuttings in a sodium alginate (medium viscosity; Sigma code A-2033; Sigma-Aldrich, St. Louis, MO) solution (2.5%, w/v) enriched with artificial endosperm (half-strength proliferation medium supplemented with 54 g L^{-1} sucrose). The alginate-coated propagules were then complexed for 35 min in a mixture of $CaCl_2$ (1.1%, w/v), containing the artificial endosperm components (**Figure 30a**; **30b**). The hardened alginate capsules were washed for 15 min in the sterile artificial endosperm solution (Micheli and Standardi 2005). Three different Murashige and Skoog (MS) media were used for the artificial endosperm, each one contained 0.7 mg L^{-1} GA_3 and 0.4 mg L^{-1} NAA and differently supplemented with 0.5 mg L^{-1} of BAP, or meta-Topolin (MT) or zeatine (Zea).

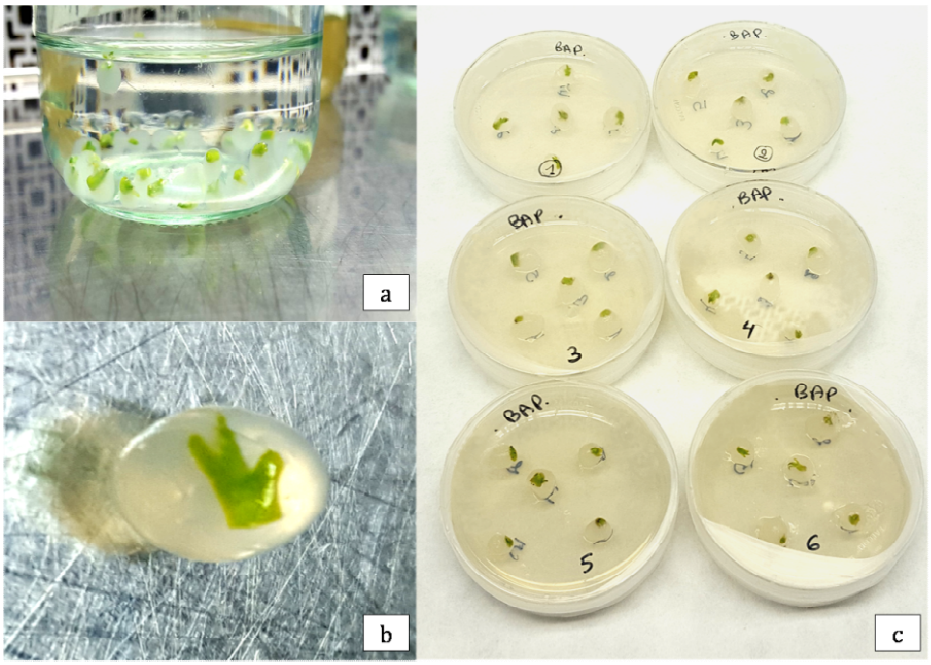

Figure 30 (a) Alginate-coated propagules complexed with $CaCl_2$ mixture containing the artificial endosperm components (b) A single alginate-encapsulated propagule containing one nodal segment of 3-4 mm length (c) Cv. Bifera nera encapsulated microcuttings using BAP.

Synthetic seeds were then sown in Petri dishes (60 x 15 mm) containing 10 mL of the above-mentioned proliferation MS medium (**Table 9**). Five synthetic seeds were placed in each Petri dish, and five repetitions were prepared for each treatment (**Figure 30c**). The cultures were incubated under the same conditions of the *in vitro* plant multiplication. Data were collected at 60 days post-sowing for all experiments and they regarded the following parameters: viability (explants with a green appearance, without necrosis or yellowing), regrowth (encapsulated microcuttings that produced shoots (≥4 mm), and conversion (simultaneous extrusion of shoots and roots at least 4 mm long from encapsulated microcuttings).

4.2.2 Statistical analysis

Recorded data were statistically analyzed by two-way ANOVA using SPSS version 17 (SPSS Inc., Chicago, IL, USA). Factorial experiments were designed to quantify interactions and to identify the optimal combination of factors with the aim of improving the response of explants. With respect to the effects of genotypes and plant growth regulators (PGRs) added, two factors were considered: (C) Cultivars and (P) PGRs. Seven parameters were registered: viability, regrowth, conversion of encapsulated microcuttings, number and length of shoots, number and length of roots. Each parameter was analyzed by two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. The significance of differences among means was analyzed using Tukey's test at $P \leq 5$ %.

4.3 RESULTS AND DISCUSSION

Encapsulated *F. carica* microcuttings placed in growth chamber showed a satisfactory **viability** rate associated with green and well proliferated leaves (**Figures 31** and **32**). Thus, it can be concluded that the gelling matrix of sodium alginate showed no toxic effect on the viability of produced artificial seeds and 2.5% sodium alginate can be adopted as a concentration instead of 4% applied by Sharma *et al.* (2015).

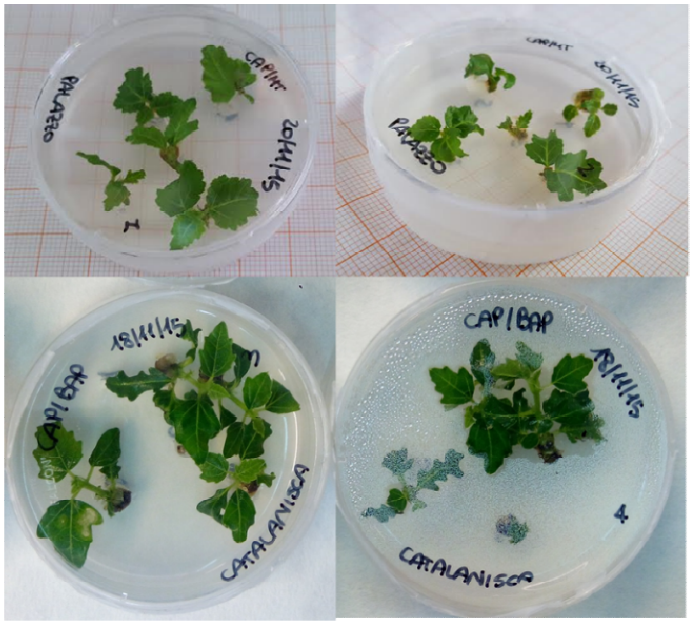

Figure 31 Encapsulated microcuttings of cv. Palazzo fig using MT (upper part) and Catalanisca using BAP (down part).

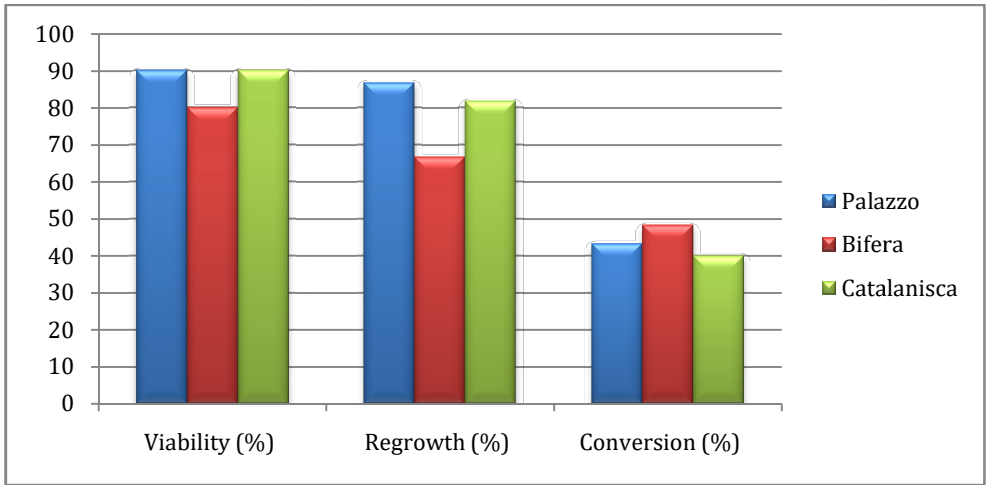

Figure 32 Comparison among the viability, regrowth and conversion rates obtained from synthetic seeds of Palazzo, Bifera nera and Catalanisca Italian cultivars.

Statistical analysis did not show significant difference between viability average rate of plantlets obtained after 60 days of culture (P=0.285) at 5% level. This percentage was quite

high and comparable for the three Italian cultivars tested: both Palazzo and Catalanisca cultivars showed 90% of viability rate while Bifera nera 80% (**Table 13**).

Table 13 Effects of cultivars and type of PGRs added to the medium on viability, regrowth and conversion rate in *F. carica* synthetic seeds, after 60 days from sowing.

Factors/treatment	Viability (%)	Regrowth (%)	Conversion (%)
Cultivars (C)			
Palazzo	90.0	86.7	43.3
Bifera	80.0	66.7	48.3
Catalanisca	90.0	81.7	40.0
PGRs (P)			
MT	88.3	80.0	50.0
BAP	88.3	83.3	53.3
ZEA	83.3	71.7	28.3
Statistical analysis of factors [a]			
C	0.285	0.015	0.730
P	0.722	0.211	0.050
C x P	0.084	<0.001	0.092

[a]Two-way ANOVA (C, P), followed by Tukey's test $p \leq 0.05$

PGRs did not affected significantly *F. carica* encapsulated microcuttings viability (*P*=0.722). Furthermore, no significant difference was obtained for PGRs and cultivars interaction at 5% level (*P*=0.084). Moreover, Bifera nera cultivar showed a higher viability response with both PGRs: BAP and MT (90%), when compared with ZEA (60%).

However, cultivar Catalanisca responded differently this last PGR, providing 100% of viability rate and 85% with the other PGRs. The third cultivar Palazzo presented an equal viability rate with all the PGRs (90%). However, the best average viability rate was provided by BAP and MT (88.3%), followed by ZEA (83.3%) for all tested cultivars (**Table 13**).

Moreover, data presented in **Table 13** showed that, after 60 days from sowing, encapsulated microcuttings revealed different **regrowth** percentages, depending on the type of PGR used. This variability was confirmed since a highly statistical significant difference was obtained by cultivars at 5% level (*P*= 0.015). The cultivar Palazzo showed the best

regrowth rate (86.7%), followed by Catalanisca (81.7%) and Bifera nera cultivar which showed the lowest rate (66.7%).

PGRs added at 0.5 mg L^{-1} concentration, expressed no significant effect on the regrowth rate of artificial seeds (*P*=0.211), but a very statistical significant interaction was observed between cultivars and PGRs (*P*=<0.001). Bifera nera cultivar exceeded the 4 mm of shoot length with a high regrowth rate using the MT (90%) and BAP (85%) PGRs, while the ZEA gave the lowest rate (25%). However this latest assessed at 100% of regrowth rate for Catalanisca cultivar followed by BAP (80%) then MT (65%) (**Table 14**).

Table 14 Effect of PGRs added to the medium on viability, regrowth and conversion rate for the three *F. carica* synthetic seeds cultivars, after 60 days from sowing

	Viability (%)				Regrowth (%)				Conversion (%)			
PGRs (P)	**BAP**	**MT**	**ZEA**	**TOT**	**BAP**	**MT**	**ZEA**	**TOT**	**BAP**	**MT**	**ZEA**	**TOT**
Cultivars (C)												
Bifera	85	90	40	71.7	85	90	25	66.7	75	60	8	44.6
Catalanisca	70	70	100	80	80	65	100	81.7	45	35	40	40
Palazzo	90	90	85	88.3	85	85	90	86.7	40	55	35	43.3

Significance			
C	0.048	0.015	0.753
P	0.398	0.211	0.034
C x P	< 0.001	< 0.001	0.055

[a]Two-way ANOVA (C, P), followed by Tukey's test $p \leq 0.05$

Moreover, the three PGRs gave a comparable regrowth rate for Palazzo cultivar (90% ZEA and 85% BAP and MT). However, the best PGR effect on *F. carica* regrowth was provided by BAP (83.3%), followed by MT (80%) and then by ZEA (71.7%). Cultivars and their interaction with PGRs indicated a highly statistical significant effect on shoot length (*P*=<0.001) (**Table 15**).

Mean shoot length per explant was recorded for all cultivars and Palazzo provided the best length (1.75 cm), followed by Catalanisca (1.43 cm) then Bifera nera (1.09 cm). No statistical significant effect was observed for all PGRs on shoot length at 5% level (*P*= 0.166) (**Table 15**)

.

Table 15 Effects of cultivars and different PGRs added to the medium on re-growth and shoot production in synthetic seeds of *F. carica* after 60 days from sowing.

Factors/PGRs	**Shoots**	
	Number/s. seed (*n*)	**Length (cm)**
Cultivars (C)		
Palazzo	1.00	1.75
Bifera	1.00	1.09
Catalanisca	1.00	1.43
PGRs (P)		
MT	1.00	1.31
BAP	1.00	1.55
ZEA	1.00	1.45
Statistical analysis of factors [a]		
C	_	<0.001
P	_	0.166
C x P	_	<0.001

[a]Two-way ANOVA (C, P), followed by Tukey's test $p \leq 0.05$.

In addition, microcuttings from encapsulated nodal segments showed the ability to convert and to produce shoots and roots on the sowing media (**Figure 33**), differently to what was reported by Sharma *et al.* (2015). In that report, Authors changed medium, using another additional rooting MS medium supplemented with salicylic acid at 2.5 µM, because the microshoots failed to root in the previous growth medium.

PGRs showed a statistical significant effect on the **conversion** rate at 5% level (*P*= 0.05), while no effect was recorded for cultivars (*P*= 0.730) and PGRs/cultivars interaction (*P*= 0.092). The optimal conversion rate was provided by the Bifera nera cultivar (48.3%), followed by Palazzo (43.3%) then Catalanisca (40%). Medium added with ZEA showed the lowest rate for all tested cultivars (28.3%), if compared with MT (53.3%) and BAP (50.0 %) (**Table 16**). A statistical significant effect of PGRs was observed on roots number (*P*= 0.033), as well as length ($P \leq 0.001$) (**Table 16**).

Mean root number per shoot was higher for Palazzo cultivar (2.28), followed by Catalanisca (1.81) and Bifera nera (1.40), with a mean length of 1.33, 1.27 and 1.04 cm, respectively. BAP and MT resulted to be the best PGRs for enhancing roots number (mean number of 2.27 and 2.0 respectively) and roots length (mean length of 1.17 and 1.58 cm, respectively). On the other side, synthetic seeds in medium ZEA showed the lowest mean root number (1.20) and length (0.89 cm), within all PGRs tested (**Table 16**).

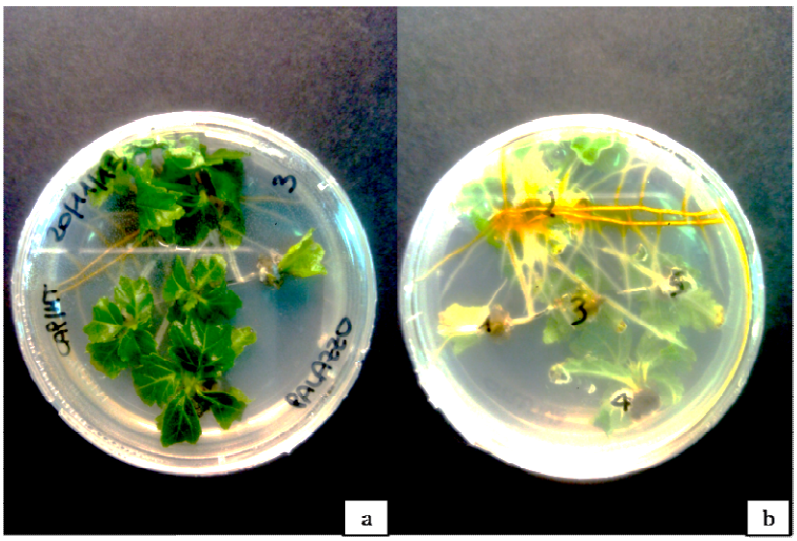

Figure 33 Conversion of Palazzo encapsulated microcuttings cultivar using MT PGR (a) and plantlets rooting in a single step after 60 days from sowing (b).

For all genotypes of *F. carica* tested, the use of MT and BAP at 0.5 mg L^{-1} concentration provided a very good results in terms of viability, regrowth and conversion rates, on the contrary of ZEA, which showed to be the worst. The three Italian fig cultivars tested showed a high viability rate (86.7%), where BAP and MT gave the best rates (88.3%), followed by ZEA (83.3%).

Furthermore, the regrowth rate reached 78.3 % where the best PGR for regrowth was BAP (83.3%), followed by MT (80.0 %) and by ZEA (71.7%). A good and a promising preliminary result in term of conversion rate was registered by all cultivars (43.9%). ZEA showed to have the lowest conversion rates for all tested cultivars (28.3%), when compared with MT (53.3%) and BAP (50.0%).

Table 16 Effects of cultivars and different PGR treatments on conversion and root production in synthetic seeds of *F. carica* after 60 days from sowing.

Factors/PGRs	**Roots**	
	Number/ s. seed (*n*)	**Length (cm)**
Cultivars (C)		
Palazzo	2.28	1.33
Bifera	1.40	1.04
Catalanisca	1.81	1.27
PGRs (P)		
MT	2.00	1.58
BAP	2.27	1.17
ZEA	1.20	0.89
Statistical analysis of factors [a]		
C	0.122	0.218
P	0.033	<0.001
C x P	0.001	0.088

[a]Two-way ANOVA (C, P), followed by Tukey's test $p \leq 0.05$.

4.4 CONCLUSIONS

The results of our study on calcium alginate encapsulation of *in vitro*-derived microcuttings of *F. carica* confirmed the practical applicability of this technology for propagation of this important fruit crop (Yahyaoui *et al*., 2016b). Fig showed to be highly suitable for encapsulation, with the production of good quality artificial seeds and, moreover, encapsulation proved to be a powerful multiplication technique to sustain standard and traditional fig tissue culture protocols.

Encapsulation technology application demonstrated that Catalanisca and Palazzo cultivars showed the highest viability average rate, followed by Bifera nera. The regrowth percentage was highest for the cv. Palazzo, followed by Catalanisca and Bifera nera, while the conversion rate was comparable for the three cultivars. A strong interaction among cultivars and PGRs was observed. BAP and MT provided the best results about regrowth and conversion, even if the cultivar Catalanisca reached its maximum regrowth rate with ZEA.

According to our knowledge, the success of microcuttings rooting in a single step after sowing as well as the application of the encapsulation technology to the Italian fig cvs are reported for the first time in the present study.

Fig encapsulated microcuttings showed to have a specific aptitude to root, thus they can be considered suitable for artificial seeds production confirming the suitability of the adopted procedure. A high performance of rooting for all tested fig genotypes was recorded, also when compared with what was previously obtained from *in vitro*-derived propagules of Carrizo citrange (Germana *et al.*, 2011) and from *F. carica* encapsulated seeds (Sharma *et al.*, 2015) on the same sowing medium. Further investigations on the conservation of fig synthetic seeds are in progress.

Chapter 5.

5. Fig sanitation via standard and improved approaches

5.1 ABSTRACT

Four Mediterranean *F. carica* genotypes, i.e. *cv.*Palazzo, Houmairi, Bifera nera and Catalanisca cultivars initially infected by Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), Fig mild mottling-associated virus (FMMaV), *Fig mosaic virus* (FMV), Fig latent virus 1 (FLV-1), Fig Badnavirus 1 (FBV-1) and Fig fleck-associated virus (FFkaV) were subjected to three sanitation techniques: Shoot Tip Culture (STC), Meristem Tip Culture Protected using The Synthetic Seeds technique (MTC-SS) and Caulogenesis via meristem and leaves, in order to produce virus-free plant material. The sanitary status of all genotypes was assayed by RT-PCR using viruses-specific primers. Obtained results revealed that all sanitation trials proved to eliminate FMD associated viruses with different sanitation rates. FLMaV-1 was completely eradicated by MTC-SS and Caulogenesis while with a lesser sanitation rate by STC. Moreover, FLMaV-2 was easily eradicated via STC and Caulogenesis. Plantlets regenerated via MTC-SS and Caulogenesis were well and likewise healed from FMMaV, but lesser sanitized by STC.

FLV-1 was eliminated with a low sanitation rate via STC while MTC-SS and Caulogenesis techniques gave a higher rate. Caulogenesis showed to be the best technique in eliminating FMV followed by MTC-SS and STC. Caulogenesis and MTC-SS permitted a high sanitation rate from FFKaV if compared to STC. The only DNA virus FBV-1 resisted to all sanitation attempts confirming, indirectly, its presence as integrated sequence in fig genome.

Key words: fig mosaic disease, Caulogenesis, tissue culture, synthetic seeds, sanitation, RT-PCR.

5.2 MATERIAL AND METHODS

5.2.1 Shoot Tip culture (STC)

Well proliferated *in vitro* plantlets were subjected to the shoot tip culture (STC) sanitation technique. Small explants (0.7 to 0.9 mm) were removed from *in vitro* micro-cuttings of eight various Mediterranean genotypes, i.e. *cv.* Palazzo, Catalanisca, Biancu, Dottato1, Bifera nera, Houmairi, Baiyadi and Serilop and plated on MS-1 medium (**Table 9**) supplemented with 4 mg L^{-1} BAP combined with 0.2 mg L^{-1} NAA. Petri dishes were then incubated under the same aforementioned culture conditions (**Chapter 3, Section 3.2.2**). The buds were sub-cultured at least twice per 3-weeks intervals into a fresh medium.

The *in vitro* regenerated plantlets were then transferred into a MS-2 medium (**Table 10**) supplemented with 2 mg L^{-1} of BAP combined with 0.2 mg L^{-1} NAA for multiple shoots induction. Explants were sub-cultured, each 30 days of interval, onto a fresh medium for three months. After 30 additional days, individual elongated shoots were used for the induction of roots on hormone-free MS medium.

Regenerated plantlets were screened for the same viruses of their mother plants by RT-PCR. Preliminary sanitized plantlets were immersed in 0.3% Fungicide for 20 min and transferred to plastic pots containing an autoclaved artificial soil mix. Potted plantlets were covered with polythene covers to ensure high humidity around the plants. For the first 10 days, the plantlets were irrigated with 1/6-strength MS basal medium (devoid of sucrose and inositol) and subsequently with tap water. After 15 days, polythene covers were removed and plantlets were acclimatized for one week. The plantlets were then transplanted to pots containing natural soil and kept under shade for 2 weeks before transferring to the *in vivo.*

5.2.2 Caulogenesis via leaves and buds

A total of 40 fig leaves, of 30 days old, and buds of five *in vitro* multiplied cultivars (Houmairi, Bifera, Catalanisca, Bianca and Dottato1) were selected to induce morphogenesis. For callus formation, the entire leaves were cultured on solidified and MS Plus (MS+) supplemented with 4 mg L^{-1} of BAP combined with 0.4 mg L^{-1} NAA medium. Each Petri dish contain 6 to 12 leaves (according to the leaf size) and placed in different positions (top surface, upside down and angled) (**Figure 34**).

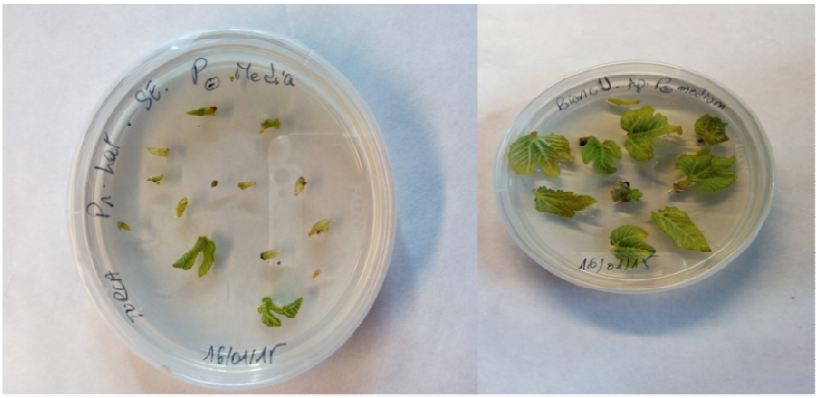

Figure 34 Leaves excised from *in vitro* proliferated plantlets of cv. Turca 'Serilop' and Biancu plated on MS plus medium.

Explants were sub-cultured every four weeks into fresh medium for three months. After 2 to 4 months from culture starting, active cells were formed on the surface of the callus initiating the micro shoots development. The sprouted micro shoots were transferred into test tubes containing (MS-1) medium and maintained under the same culture conditions for fig multiplication. The cultures were maintained for a period of almost 4 to 6 weeks in order to induce plant development with a continuous subculture every two weeks. After 2 months of incubation, regenerated plants were transferred to a new MS-2 media for shoot multiplication. Regenerated plantlets were rooted according to the aforementioned medium used for plant multiplication via buds (**Chapter 3, Section 3.2.2**).

5.2.3 Meristem Tip Culture Protected by Synthetic Seeds technique (MTC-SS)

A total of thirty meristem tips of about 0.3-0.5 mm in size, were excised from the *in vitro* proliferated plantlets of cvs. Palazzo, Bifera nera and Catalanisca and explants with one or two primordial leaf were encapsulated by applying the synthetic seeds technique. Murashige and Skoog (MS) media was used supplemented with 0.7 mg L^{-1} GA_3 and 0.4 mg L^{-1} NAA and 0.5 mg L^{-1} of meta-Topolin (MT). Five synthetic seeds were then sown in Petri dishes (60 x 15 mm) containing 10 mL of the above-mentioned proliferation MS hormone free medium (**Table 9**). The cultures were incubated in a growth chamber at 23 ± 1 °C under a 16/8-h (light/dark) and a PPFD of 40 µmol m^{-2} s^{-1}. Rooted plantlets were subcultured monthly in a fresh MS hormone free medium.

5.2.4 Thermotherapy combined with Meristem Tip protected by synthetic seeds

a) Initial plant material

In vitro rooted plantlets, cvs. Palazzo, Bifera nera and Catalanisca, grown in MS hormone free basal medium, regenerated from the previous sanitation techniques were selected and screened (0.2 g of leaves) for the presence of the original viruses infecting the mother plant by RT-PCR using universal pair of primers. Only infected and not preliminary sanitized plantlets were chosen for the application *of in vitro* thermotherapy (**Figure 35**).

Figure 35 *In vitro* rooted plantlets for the thermotherapy technique.

b) In vitro cultures

Under a sterile laminar flow hood, plantlets were transferred in glass jars, each with 25 ml of MS hormone free medium gelled with 7.5 g Agar. The pH of the medium was adjusted to 5.8 before autoclaving. No contaminations were recorded since jars and media were separately sterilized at 120 °C for 20 min each, and then versed under the sterilized flow hood.

c) Thermotherapy associated to synthetic seeds technique

The *in vitro* cultured plantlets cvs. Palazzo, Bifera nera and Catalanisca were placed in a heat chamber, where the temperature was raised gradually to 38 ± 0.5 °C (**Figure 36**).

Figure 36 Application of the thermotherapy technique on fig cvs. Palazzo, Bifera nera and Catalanisca kept under 38 ± 0.5 °C for 20 days.

After one period of heat treatment (20 days), meristem tips of about 0.3-0.5 mm in size comprising one or two primordial leaf, developed during the high temperature period, were excised and subjected to the encapsulation procedure as described before. Murashige and Skoog (MS) media was used supplemented with 0.7 mg L^{-1} GA_3; 0.4 mg L^{-1} NAA and 0.5 mgL^{-1} of MT. Individual synthetic seeds were then sown in Petri dishes (60 x 15 mm) containing 10 mL of the above-mentioned proliferation MS hormone free medium (**Table 9**; **Figure 37**).

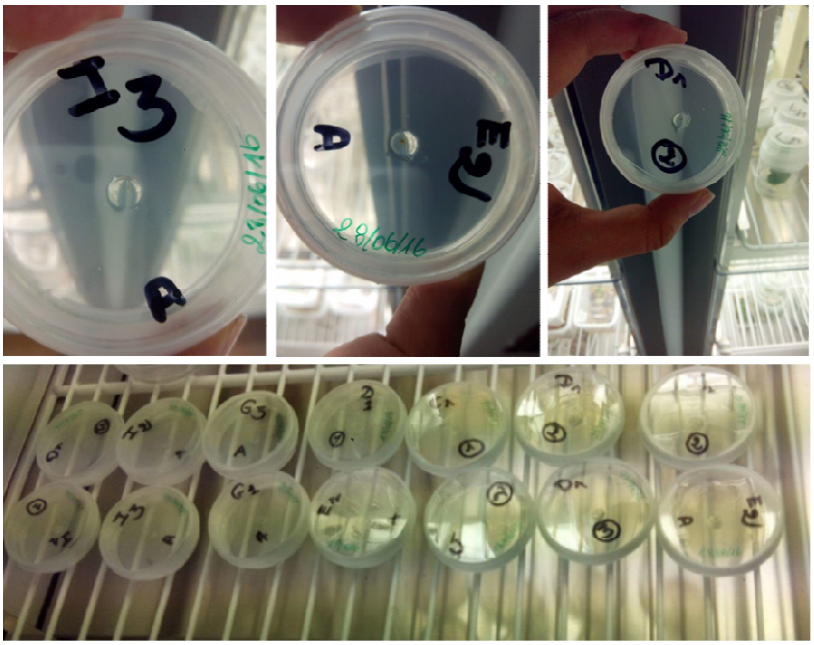

Figure 37 Encapsulated meristem tips of about 0.3-0.5 mm, of heat-treated plantlets for one cycle (20 days), using encapsulation technology.

The cultures were incubated in a growth chamber at 23± 1 °C under a 16/8-h (light/dark) and a PPFD of 40 µmol m^{-2} s^{-1}. Rooted plantlets were subcultured monthly in a fresh MS hormone free medium. Leaves from *in vitro* plantlets derived from artificial seeds, were sampled and tested by RT-PCR for the same viruses as in the initial *in vitro* plants before the thermotherapy application. Virus-free samples tested by RT-PCR were further multiplied and *in vitro* rooted. Positive results of RT-PCR testing were subjected to another period of thermotherapy (20 days).

5.2.5 Molecular analysis via Polymerase Chain Reaction (PCR)

Regenerated plantlets subjected to all aforementioned sanitation techniques were screened for the presence of the same viruses detected in their mother plants by RT-PCR. Preliminary sanitized plantlets were immersed in 0.3% fungicide for 20 min and transferred to plastic pots containing an autoclaved artificial soil mix. Potted plantlets were covered with polythene covers to ensure high humidity around the plants. For the first 10 days, the plantlets were irrigated with 1/6-strength MS basal medium (devoid of sucrose and inositol) and subsequently with tap water. After 15 days, polythene covers were removed and plantlets were acclimatized for one week. The plantlets were then transplanted to pots containing natural soil and kept under shade for 2 weeks before transferring to the *in vivo*.

5.3 RESULTS AND DISCUSSION

A total of 72 regenerated plantlets from Palazzo, Houmairi, Bifera nera and Catalanisca cultivars initially infected by FLMaV-1, FLMaV-2, FMMaV, FLV-1, FMV, FFKaV and FBV-1 (**Table 17**) were chosen from the various sanitation techniques, 8 well proliferated and representative plantlets per treatment, in order to screen for FMD associated virus elimination.

Table 17 Sanitary status of cultivars before all sanitation techniques

Origin	Varieties	FLMaV-1	FLMaV-2	FMMaV	FLV-1	FMV	FCV	FFkaV	FBV-1
Pollina	Catalanisca	+	-	+	+	+	-	+	+
Santa Maria di Gesu	Bifara (Black)	-	-	-	+	+	-	+	+
Lebanon	Houmairi Nb8	-	+	-	+	+	-	-	+
Italy	Palazzo	+++	-	-	-	-	-	-	+

+: Infected; -: Not infected; +++: Highly infected

i. Shoot tip culture (STC)

Shoot-tip size is a very important factor in the elimination of viruses from plants (Verbeek *et al.*, 1995; Panattoni *et al.*, 2013). In our attempts of fig sanitation via shoot-tip culture, we excised about 0.7 to 0.9 mm of length from fig mosaic infected *in vitro* multiplied cultivars and multiplied using MS-1 medium.

The total raised plantlets via shoot tip culture was 65.4%, where the cultivar Palazzo, showed to be the most well adapted to this technique with 93.1% of regeneration rate followed by Catalanisca (74.2%) and Bifera nera (66.1%) (**Table 18**).

Table 18 Regeneration rate of *in vitro* multiplied shoot tips cultivars

Countries	Origin	Sample	Cultivar	Initial n°	Regenerated n°	Regeneration rate %
Apulia (Italy)	**IAMB**	Palazzo P2 (CA1)	**Palazzo**	130	121	93.1
Sicily (Italy)	**Pollina**	S2	**Catalanisca**	198	147	74.2
	Petralia Soprana	S5	**Biancu**	7	3	42.9
		S4	**Dottato1**	78	28	35.9
			Bifera	322	213	66.1
Lebanon		Nb8	**Houmairi**	63	20	31.7
			Baiyadi	5	0	0.0
Turkey		Turca 'Serilop' P1 (Ca2)	**Serilop**	11	0	0.0
TOTAL multiplied samples via STC				**814**	**532**	**65.4**

Biancu, Dottato and Houmairi cultivars did not exceed the 50% of regeneration rate via shoot tip culture where they revealed the ability to produce a callus. The Lebanese Baiyadi and the Turkish cultivars did not succeed to survive (**Table 18**).

Eight well developed regenerated plantlets via shoot tip culture were chosen from Palazzo, Catalanisca, Bifera nera and Houmairi genotypes in order to screen for FMD associated viruses elimination. The results obtained by molecular analysis of the sanitary status of each cultivar subjected to STC are reported in **Table 19**.

RT-PCR revealed that FLMaV-1 was completely eradicated from Palazzo and eliminated from cv. Catalanisca with 75 % rate Furthermore, FLMaV-2 was eliminated at 100% from the Lebanese variety Houmairi, whereas only 25 % of regenerated plantlets from cv. Catalanisca were sanitized from FMMaV.

FLV-1 showed to be eliminated with 25%, 12.5% and 37.5% from Houmairi, Bifera nera and Catalanisca cultivars successively. FMV, the causal agent of FMD, was removed with 62.5%, 50% and 37.5% from Houmairi, Bifera nera and Catalanisca successively. FFKaV registered an elimination rate of 62.5% and 50% from Bifera nera and Catalanisca regenerated plantlets respectively, whereas the badnavirus FBV-1 has not been eliminated.

STC technique showed to be able to eliminate FLMaV-1 with 87.5% from the total initially infected plantlets besides to a complete eradication of FLMaV-2 (100% elimination rate). Only 25% of the experimented plantlets showed to be sanitized from FMMaV as well as FLV-1. Furthermore, 56.2% of regenerated plantlets were sanitized from FFKaV in opposite to the badnavirus FBV-1 which resisted to the STC sanitation attempt and showed to be present in all tested plantlets with 100%.

Table 19 Sanitary status of cultivars subjected to the Soot tip culture technique (STC) using RT-PCR.

Cultivars	Code	FLMaV-1	FLMaV-2	FMMaV	FLV-1	FMV	FFkaV	FBV-1
Palazzo	A1	-						+
	A2	-						+
	A3	-						+
	A4	-						+
	A5	-						+
	A6	-						+
	A7	-						+
	A8	-						+
Sanitized/tested		*8/8*						*0/8*
Sanitation rate (%)		**100%**						**0%**
Houmairi Nb8	D1		-		+	-		+
	D2		-		+	-		+
	D3		-		-	-		+
	D4		-		-	+		+
	D5		-		+	+		+
	D6		-		+	-		+
	D7		-		+	-		+
	D8		-		+	+		+
Sanitized/tested			*8/8*		*2/8*	*5/8*		*0/8*
Sanitation rate (%)			**100%**		**25%**	**62.5%**		**0%**
Bifera	E1				+	-	-	+
	E2				+	-	-	+
	E3				-	-	-	+
	E4				+	+	+	+
	E5				+	+	+	+
	E6				+	-	-	+
	E7				+	+	-	+
	E8				+	+	+	+
Sanitized/tested					*1/8*	*4/8*	*5/8*	*0/8*
Sanitation rate (%)					**12.5%**	**50%**	**62.5%**	**0%**
Catalanisca	G1	-		+	-	-	-	+
	G2	-		+	+	-	-	+
	G3	-		+	-	-	-	+
	G4	-		+	+	+	+	+
	G5	+		-	+	+	+	+
	G6	-		+	+	+	+	+
	G7	+		-	-	+	-	+
	G8	-		+	+	+	+	+
Sanitized/tested		*6/8*		*2/8*	*3/8*	*3/8*	*4/8*	*0/8*
Sanitation rate (%)		**75%**		**25%**	**37.5%**	**37.5%**	**50%**	**0%**
Total sanitized/tested		*14/16*	*8/8*	*2/8*	*6/24*	*12/24*	*9/16*	*0/32*
Total sanitation rate (%)		**87.5%**	**100%**	**25%**	**25%**	**50%**	**56.2%**	**0%**

ii. Meristem Tip Culture Protected using the synthetic seeds technique (MTC-SS)

It seems that the size of excised shoot-tip is not only important to produce virus-free plants of many crops, but also to determine the survivability of the explants in culture (Malaurie *et al.*, 1995; Manganaris *et al.*, 2003). In this context, Parmar *et al.* (2013) mentioned that *in vitro* shoot growth of *Clerodendrum inerme* was higher with larger size explants than the smaller one. This may be due to the high number of leaf primordia in large shoot-tips. Sahraroo *et al.* (2009) encountered many difficulties to regenerate *F. carica* plantlets from 0.2 to 0.4 mm meristem since they failed to grow on the media and smaller explants (ca. 0.3 mm) often necrotize (Chiumenti *et al.*, 2013).

Regarding this problem of survivability, low rates and the frequently encountered difficulties of plantlets regeneration from this size of explants, we tested, for the first time according to our knowledge, to protect the small meristem using the synthetic seeds technology where it forms an artificial seed containing all nutrients and hormones necessary for their development without necrosis.

Therefore, a total of thirty meristem tips of about 0.3 to 0.5 mm were excised from the three cultivars Palazzo, Bifera nera and Catalanisca and then were encapsulated via synthetic seeds technology using MT hormone, placing four seeds per plate (**Figure 38** and **39**).

Figure 38 Encapsulated meristem tips (0.3-0.5 mm) of Catalanisca cultivar via the synthetic seeds technology.

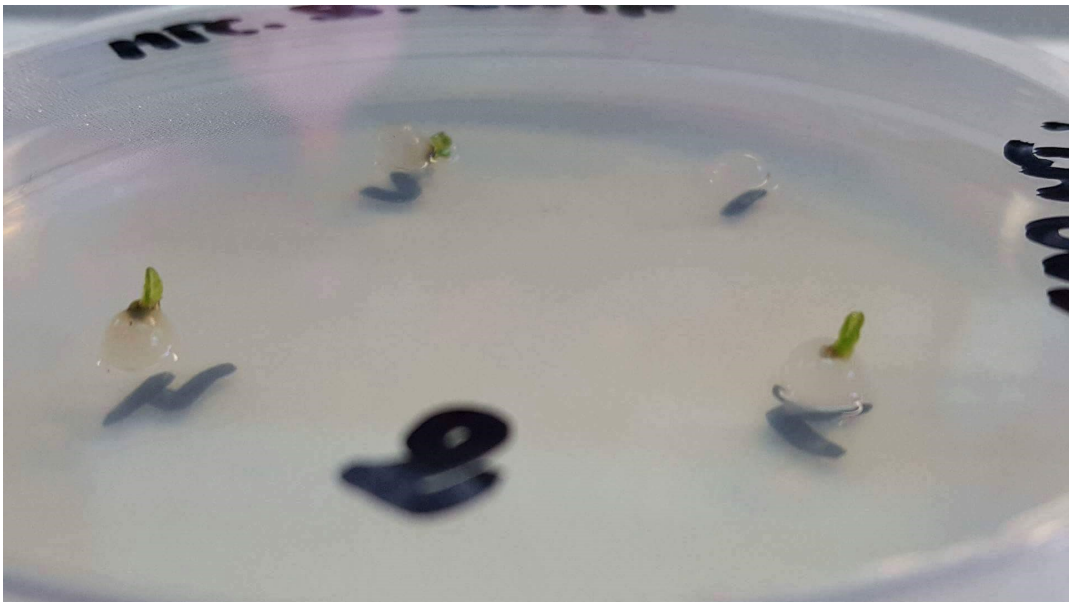

Figure 39 Bifera nera cultivar encapsulated meristem tips using the synthetic seeds technology.

The total registered regeneration rate of protected meristem tips reached 35.5%. In particular, Bifera nera showed the best regeneration rate with 40%, followed by Palazzo and Catalanisca with 36.6% and 30%, respectively (**Table 20).**

Regarding the sanitary status evaluation by MTC-SS, the results obtained are reported in **Table 21**. RT-PCR molecular analysis revealed that FLMaV-1 was totally eradicated at100 % of elimination rate from both cultivars Palazzo and Catalanisca using the MTC-SS technique. Moreover, FMMaV showed a very high elimination rate (75%) from cv. Catalanisca and FLV-1 was eliminated with 75% and 50% from Bifera nera and Catalanisca varieties respectively.

Plantlets subjected to MTC-SS were sanitized from FMV with 62.5% (cv. Bifera) and 50% (cv. Catalanisca).FFKaV was eliminated, for the first time, with 87.5% and 62.5% from Bifera nera and Catalanisca cultivars respectively. FBV-1 resisted to the MTC-SS technique.

The total elimination of some FMD associated viruses from 24 plantlets regenerated via MTC-SS and associated to three cultivars; Palazzo, Bifera nera and Catalanisca revealed that FLMaV-1 was completely eradicated from the regenerated plantlets. FMMaV and FFkaV were equally eliminated by 75% followed by FLV-1 (62.5%) and FMV (56.2%). The only DNA virus (FBV-1) resisted to this sanitation attempt.

Table 20 Regenerated rates registered for cultivars subjected to the Meristem Tip Culture Protected using the synthetic seeds technique (MTC-SS).

Cultivars origin	Cultivars name	
Apulia IAMB	**Palazzo**	
Regenerated/tested		*11/30*
Regeneration rate		***36.6%***
Sicily Santa Maria di Gesù	**Bifera**	
Regenerated/tested		*12/30*
Regeneration rate		***40%***
Sicily-Pollina	**Catalanisca**	
Regenerated/tested		*9/30*
Regeneration rate		***30%***
Total regenerated/tested		*32/90*
TOTAL regeneration rate (%)		***35.5%***

Table 21 Sanitary status of regenerated plantlets subjected to the MTC-SS technique

Cultivars	Code	FLMaV-1	FLMaV-2	FMMaV	FLV-1	FMV	FFkaV	FBV-1
Palazzo SS (MT)	B1	-						+
	B2	-						+
	B3	-						+
	B4	-						+
	B5	-						+
	B6	-						+
	B7	-						+
	B8	-						+
Sanitized/tested		*8/8*						*0/8*
Sanitation rate (%)		***100%***						***0%***
Bifera nera SS MT	F1				+	-	-	+
	F2				-	-	-	+
	F3				-	-	-	+
	F4				-	-	-	+
	F5				-	+	-	+
	F6				+	+	-	+
	F7				-	+	-	+
	F8				-	-	+	+
Sanitized/tested					*6/8*	*5/8*	*7/8*	*0/8*
Sanitation rate (%)					***75%***	***62.5%***	***87.5%***	***0%***
Catalanisca SS MT	H1	-		+	-	-	-	+
	H2	-		-	-	-	-	+
	H3	-		-	+	-	-	+
	H4	-		+	-	+	-	+
	H5	-		-	+	+	+	+
	H6	-		-	-	+	+	+
	H7	-		-	+	+	-	+
	H8	-		-	+	-	+	+
Sanitized/tested		*8/8*		*6/8*	*4/8*	*4/8*	*5/8*	*0/8*
Sanitation rate (%)		***100%***		***75%***	***50%***	***50%***	***62.5%***	***0%***
Tot sanitized/tested		*16/16*		*6/8*	*10/16*	*9/16*	*12/16*	*0/24*
Tot sanitation rate (%)		***100%***		***75%***	***62.5%***	***56.2***	***75%***	***0%***

iii. *Caulogenesis*

Caulogenesis in indirect morphogenesis is the shoot development in callus, due to low auxin and high cytokinin concentration in the culturing medium, where the callus induction was applied for 20 buds and 20 leaves of the five following cultivars: Houmairi, Bifera, Catalanisca, Bianca and Dottato1, using Murashige and Skoog (MS-plus) supplemented with 4 mg L^{-1} BAP and 0.4 mg L^{-1} NAA (**Figure 40**).

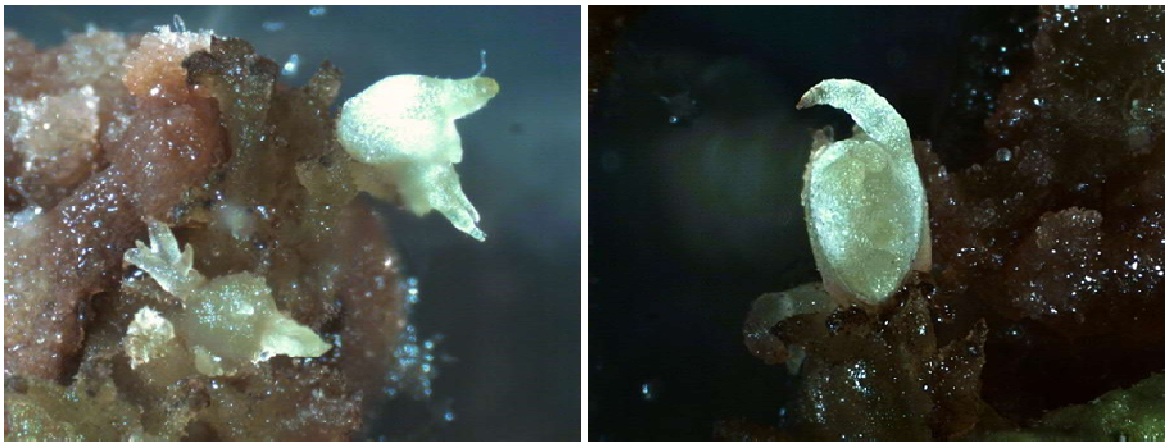

Figure 40 Caulogenesis from fig leaves cv. Catalanisca using MS-plus.

The best total Caulogenesis rate was achieved by cv. Houmairi with 62.5%, where meristem revealed the highest rate (80%) if compared to leaves (45%) (**Figure 41**). Catalanisca cultivar occupied the second position with 17.5%, as total regeneration rate, where only leaves showed the Caulogenesis ability with 35%.

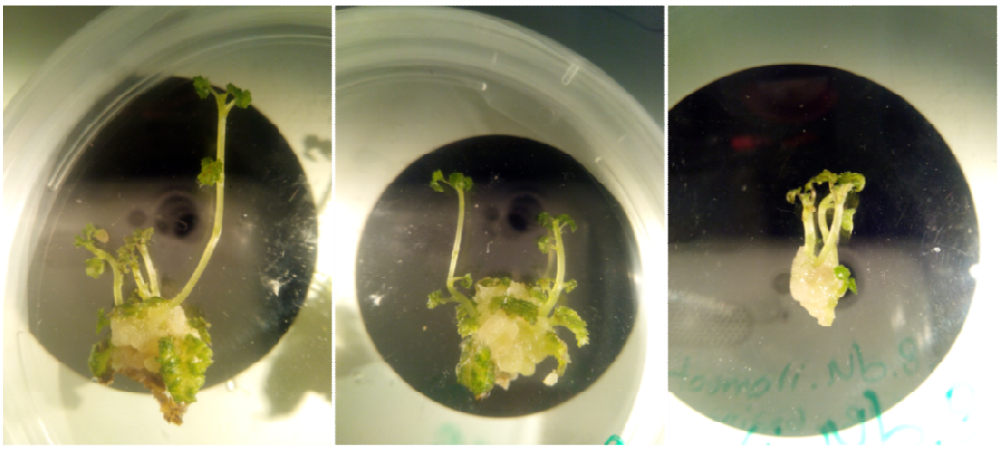

Figure 41 Plantlets regenerated by Caulogenesis from fig buds cv. Houmairi Nb8 in MS-Plus medium.

Caulogenesis from the remaining cultivars did not exceed the 10% with a very delicate plantlets. In general, the total Caulogenesis rate was low and only 10.5% of the tested explants was able to regenerate shoots from callus (**Table 22**). *In vitro* regenerated shoots via Caulogenesis were elongated and multiplied on MS-1 and MS-2 and then rooted on MS hormone free medium with the exception of cv. Houmairi which was multiplied using only MS-2 and MS-hormone free medium because of its high ability to form callus (**Figure 42** and **43**).

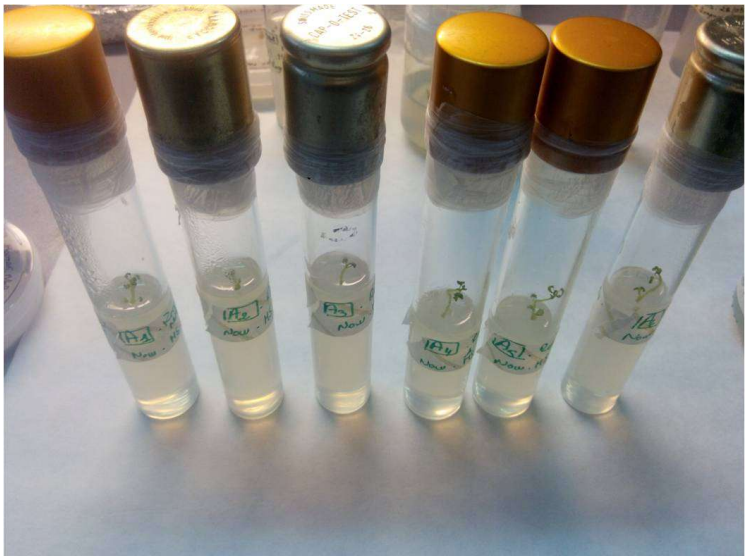

Figure 42 Individual transfer of plantlets regenerated by Caulogenesis from fig buds cv. Houmairi Nb8 in MS-2 medium.

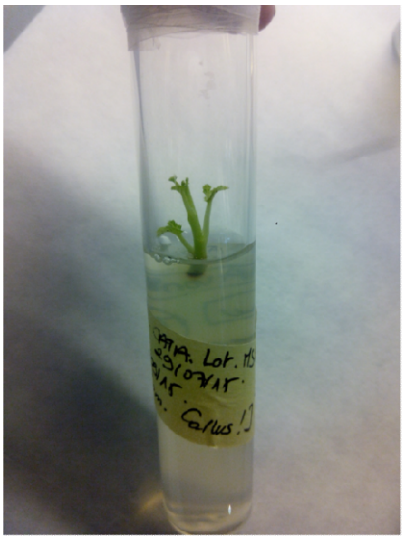

Figure 43 Shoots multiplication of the regenerated shoot by Caulogenesis from fig leaves cv. Catalanisca in MS-2 medium**.**

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Table 22 Total regeneration rate of plantlets via Caulogenesis of *F. carica*

Cultivars			**Meristem**		**Leaves**		**TOTAL**	
Origin		***Name***	***regenerated/tested***	***Regeneration rate %***	***regenerated/tested***	***Regeneration rate %***	**N°**	**Rate (%)**
Lebanon		Houmari Nb8	16/20	80	9/20	45	25	62.5
Sicily	Santa Maria di Gesù	Bifera	0/20	0	4/20	20	4	10
	Pollina	Catalanisca	0/20	0	7/20	35	7	17.5
	Pollina	Bianca	3/20	15	0/20	0	3	7.5
	Santa Maria di Gesù	Dottato3	2/20	10	1/20	5	3	7.5
TOTAL			**21**	**21%**	**21**	**21%**	**42**	**10.5 %**

A total of 240 plantlets was obtained via *in vitro* multiplication of regenerated plantlets via Caulogenesis, where only two cultivar Houmairi and Catalanisca succeeded to survive and to be *in vitro* multiplied having as a final stock 229 and 11 plantlets, successively. Plantlets regenerated via Caulogenesis from callus formed on buds and leaves revealed an important virus elimination rate where FLMaV-1 was eradicated (100% elimination rate) from cv. Catalanisca. FLMaV-2 was completely removed from the Lebanese cultivar Houmairi (100% elimination rate). FMMaV was eliminated with 75% via Caulogenesis from Catalanisca cultivar leaves. FLV-1 was partially eliminated from Houmairi cultivar with 50% of elimination rate and with 75% from Catalanisca cultivar.

FMV was eliminated with 75% from both Houmairi and Catalanisca cultivars. FFKaV was eliminated with 87.5% from cv. Catalanisca whereas FBV-1 was present in all regenerated plantlets (**Table 23**).

Table 23 Sanitary status of Houmairi and Catalanisca cultivars regenerated plantlets via Caulogenesis.

Cultivars	Code	FLMaV-1	FLMaV-2	FMMaV	FLV-1	FMV	FFkaV	FBV-1
Houmairi NB.8 (Callus buds)	C1		-		+	+		+
	C2		-		-	-		+
	C3		-		-	-		+
	C4		-		-	-		+
	C5		-		+	-		+
	C6		-		-	-		+
	C7		-		+	-		+
	C8		-		+	+		+
Sanitized/tested			*8/8*		*4/8*	*6/8*		*0/8*
Sanitation rate (%)			***100%***		***50%***	***75%***		***0%***
Catalanisca (callus Leaves)	I1	-		-	+	-	-	+
	I2	-		+	-	-	-	+
	I3	-		-	-	-	-	+
	I4	-		-	-	-	-	+
	I5	-		-	-	-	-	+
	I6	-		+	-	+	-	+
	I7	-		-	-	-	-	+
	I8	-		-	+	+	+	+
Sanitized/tested		*8/8*		*6/8*	*6/8*	*6/8*	*7/8*	*0/8*
Sanitation rate (%)		***100%***		***75%***	***75%***	***75%***	***87.5%***	***0%***
Total sanitized/tested		*8/8*	*8/8*	*6/8*	*10/16*	*12/16*	*7/8*	*0/16*
Total sanitation rate (%)		***100%***	***100%***	***75%***	***62.5%***	***75%***	***87.5%***	***0%***

In general, the three aforementioned sanitation techniques applied; Soot tip culture (STC), Meristem Tip Culture Protected using the Synthetic Seeds Technique (MTC-SS) and Caulogenesis via meristem and leaves of *in vitro* cultivars proved to eliminate FMD associated viruses with different sanitation rates (**Figure 44**).

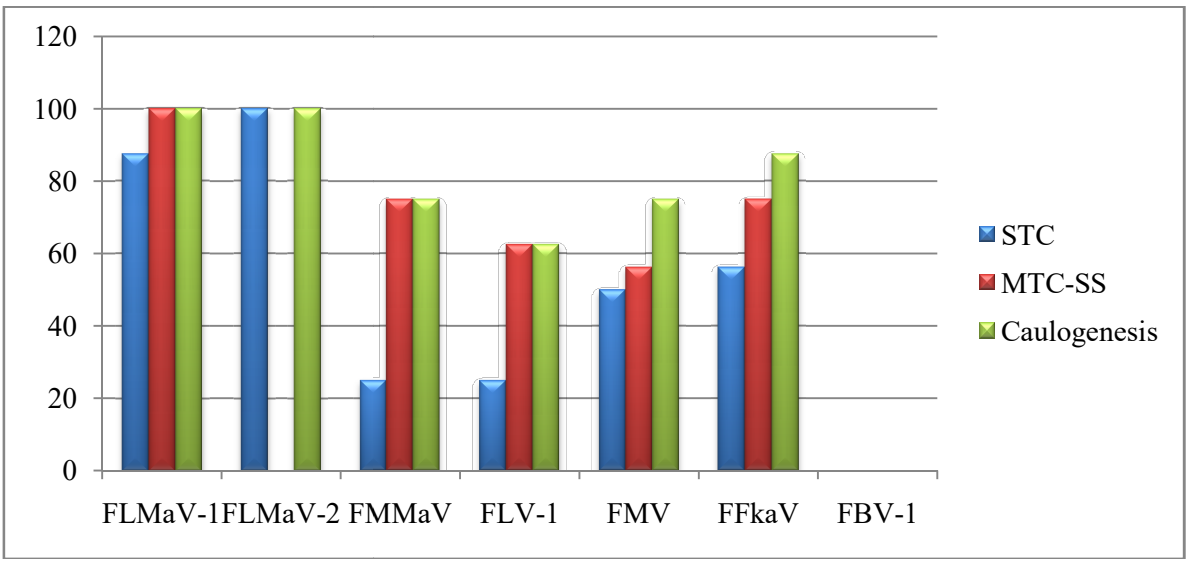

Figure 44 Total sanitation rates using the three sanitation techniques STC, MTC-SS and Caulogenesis

FLMaV-1 was completely eradicated (100% sanitation rate) by the two techniques MTC-SS and Caulogenesis while eliminated with only 87.5% by STC. Moreover, FLMaV-2 was easily eradicated via STC and Caulogenesis (**Table 24**).

Plantlets regenerated via MTC-SS and Caulogenesis were equally sanitized from FMMaV with 75% of elimination rate whereas it was eliminated only with 25% via STC. FLV-1 was eliminated with only 25% via STC while equally sanitized from regenerated plantlets via MTC-SS and Caulogenesis with 62.5%.

Caulogenesis technique showed to be the best in eliminating the causal agent of FMD, FMV, by 75% followed by MTC-SS with 56.2% while STC gave only 50% of sanitation rate. FFkaV was highly sanitized via Caulogenesis (87.5%) as well as MTC-SS (75%) from the newly regenerated plantlets while it was eliminated via STC. with only 56.2%. Furthermore, this is the first report of FFkaV elimination attempt from *F. carica*.

The only DNA virus FBV-1 resisted to the three previous sanitation attempts with 0% of elimination rate. This extremely low sanitation rate is not surprising since *Badnavirus* genus is known to be present as integrated sequences in some host plant genomes (Hohn *et al.*, 2008; Staginnus *et al.*, 2009).

Table 24 Sanitation rate comparison between the three techniques

	FLMaV-1	FLMaV-2	FMMaV	FLV-1	FMV	FFkaV	FBV-1
STC	***87.5%***	***100%***	***25%***	***25%***	***50%***	***56.2%***	***0%***
MTC-SS	***100%***	***-***	***75%***	***62.5%***	***56.2***	***75%***	***0%***
Caulogenesis	***100%***	***100%***	***75%***	***62.5%***	***75%***	***87.5%***	***0%***

iv. Thermotherapy combined with MTC-SS

After one cycle (20 days) of *in vitro* heat therapy at to 38 ± 0.5 °C, the total regeneration rate of excised and protected meristem tips (0.3-0.5 mm) via synthetic seeds technique reached 38.5%. Furthermore, a big number of capsules did not survive because of an early oxidation problem which may be caused by the high temperature exposure of the tender meristem. The total registered regeneration rate was comparable to the previous one obtained via protected meristem tips without thermotherapy (35.5%) but it seemed to be highly superior if compared with the poor survival rate (16% to 17%) obtained via normal meristem tips of about 0.6 mm in size cultured directly into the medium (Chalak *et al.*, 2015).

The actual surviving explants are not ready yet for the RT-PCR test because of their current small size and the need to develop well their leaves. Thus, virus elimination via heat treatment and subsequent removing of apical meristematic region and its encapsulation using the synthetic seeds technique is still in progress.

5.3 CONCLUSIONS

As consequences of the use of standard and improved sanitation approaches in order to sanitize the pre-tested infecting fig cultivars, Caulogenesis and the protected meristem tips using the synthetic seeds techniques (MTC-SS) proved to be more effective in eliminating FMD associated viruses than the standard technique of shoot-tip culture (STC) (Yahyaoui *et al.*, Unpublished).

Viruses elimination rates were strictly depended on the virus, the cultivar and the technique applied. FLMaV-1 was completely eradicated using the MTC-SS and Caulogenesis techniques and eliminated with high percentage 87.5% using STC. This latest rate showed to be extremely higher than what was recently reported for the Aswad and Baiyadi Lebanese cultivars (Chalak *et al.*, 2015).

Moreover, FLMaV-2 was easily eradicated via both Caulogenesis and STC sanitation techniques. This elimination rate was in agreement with the one obtained by STC applied on the Lebanese Baiyadi cultivar but extremely higher than Aswad cultivar (Chalak *et al.*, 2015). In addition, plantlets regenerated via MTC-SS and Caulogenesis were equally sanitized from FMMaV and FLV-1 with an important elimination rate contrary to STC technique which showed to be the less effective. Furthermore, Caulogenesis technique showed to be the best in eliminating the causal and unique agent involved in the symptomatology of FMD (FMV) followed by the MTC-SS and STC. FFKaV was highly sanitized via Caulogenesis as well as MTC-SS from the newly regenerated plantlets, while it was less eliminated via STC. This is the first report of FFKaV elimination attempt from *F. carica* L. The only DNA virus FBV-1 resisted to the three previous sanitation attempts confirming its presence as integrated sequences in the host plant genomes (Hohn *et al.*, 2008; Staginnus *et al.*, 2009). Those results are in agreement with what was reported by Chiumenti *et al.* (2013), where sanitized plantlets showed to be still infected with FBV-1 even after one year from sanitation. The obtained molecular results are preliminary and needs to be assessed *in vivo* in order to avoid the false negatives previously reported for FLV-1 and FMV when the test was repeated a year following the sanitation attempts (Chiumenti *et al.*, 2013). But since both viruses FBV-1 and FLV-1 were commonly found in asymptomatic plants in nature, their presence in the sanitized explants could be conceivable.

The standard shoot-tip culture technique, adopted in our research, gave a higher percentage of raised plantlets than what was reported by Chalak *et al.* (2015), using smaller explants of both shoot tip and stem cutting. Moreover, a good total regeneration rate has been reached using the protected Meristem Tips Technique (MTC-SS). It seems that the size of excised shoot-tip is not only important to produce virus-free plants of many crops, but also to determine the survivability of the explants in culture (Malaurie *et al.*, 1995; Manganaris *et al.*, 2003).

Chapter 6.

6. Fig artificial seeds conservation via synthetic seeds technique

6.1 ABSTRACT

A refined protocol for long-term storage and conservation based on the calcium alginate coating of *F. carica.* L was developed. Uninodal microcuttings (3-4 mm long), excised from *in vitro* proliferating shoots of a cv. Lebanese (Houmairi) was employed. The influence of two plant growth regulators (PGRs): 6-Benzylaminopurine (BAP) and Meta-Topolin (MT), added to the artificial endosperm, were evaluated and the viability, regrowth and conversion parameters of the synthetic seeds were registered after 45 days of conservation at 4 °C. Obtained results on encapsulated microcuttings revealed a very high viability and regrowth rates using MT or BAP hormones where no statistical significant effect of PGRs was recorded. Regenerated Houmairi plantlets exceeded the 4 mm with a total mean shoot length of 0.743 cm. Conserved encapsulated nodal segments showed a low ability to convert and to produce shoots and roots on the sowing media. PGRs showed a statistical significant effect on the conversion rate where MT gave the best rate if compared with BAP. Recorded total mean root length was 0.098 cm with no statistical significant effect of PGRs used. Nevertheless the cold conservation of artificial seeds, during this period at 4 °C, reduced slightly both viability and regrowth rates but seemed to ameliorate the conversion rate.

To our knowledge, this is the first report of rooting achievement in a single step of long-term storage of encapsulated *Ficus carica* nodal segments.

Keywords: cytokinins, encapsulation, fig, conservation, synthetic seed.

6.2 MATERIAL AND METHODS

Since *F. carica* confirmed the practical applicability of the synthetic seeds technology (Yahyaoui *et al.,* 2016b), storage at 4 °C of 60 encapsulated *in vitro* nodal segments (3-4 mm) of *F. carica* cv. Houmairi originated from Lebanon was conducted according to the aforementioned synthetic seeds technique using two different Murashige and Skoog (MS) media, each one contained 0.7 mg L^{-1} GA_3 and 0.4 mg L^{-1} NAA and differently supplemented with 0.5 mg L^{-1} of BAP or MT (**Figure 45**).

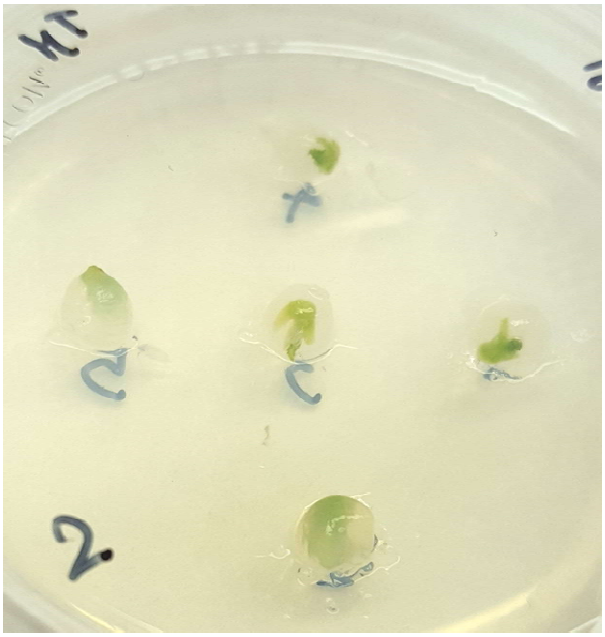

Figure 45 Encapsulated *in vitro* nodal segments, using MT hormone, of fig cv. Houmairi (originated from Lebanon) and stored at 4 °C for 45 days.

Artificial seeds were conserved for a period of 45 days and then cultured under the previous mentioned conditions. Viability, regrowth and conversion data were collected after 45 days from culture and confronted with those of not-conserved seeds. Data were statistically analyzed by two-way ANOVA using SPSS version 17 (SPSS Inc., Chicago, IL, USA). Two factors were considered: (T) Type of test (Conserved or not) and (P) PGRs. Parameters were analyzed by two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. The statistical significance of differences among means was analyzed using Tukey's test at $P \leq 5\%$.

6.3 RESULTS AND DISCUSSION

In the present study, conserved encapsulated *F. carica* (*cv*. Houmairi) microcuttings placed at 4°C for 45 days , showed the ability to recover and sprout normally with a green and well proliferated leaves in growth chamber only two weeks after sowing (**Figure 46**).

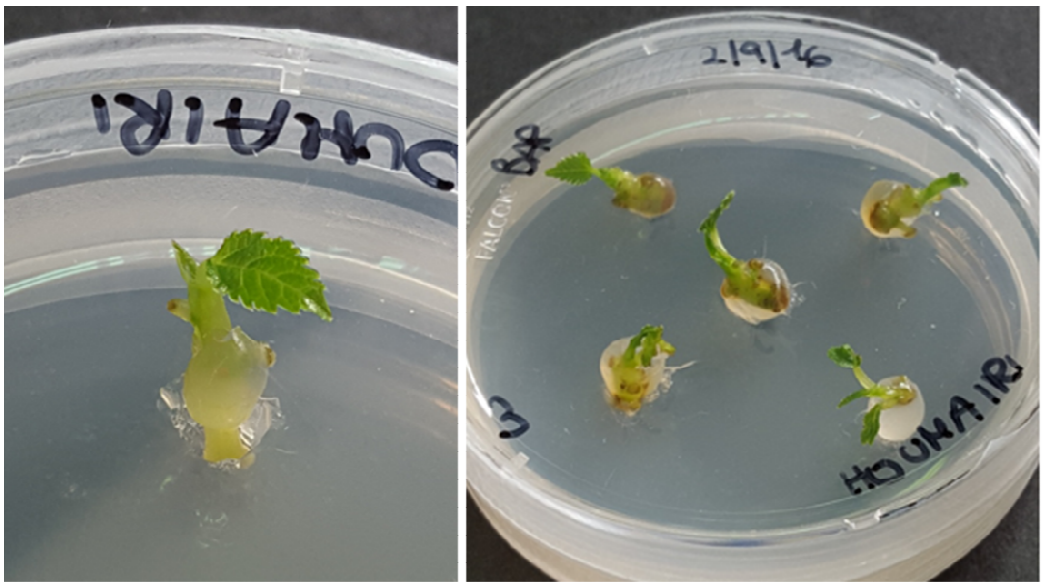

Figure 46 Houmairi cultivar recover in growth chamber after two weeks from conservation at 4 °C using BAP hormone.

PGRs did not affect significantly *F. carica* **conserved** encapsulated microcuttings **viability** obtained after 45 days of culture (*P*=0.82) at 5% level. This percentage was quite high (73%) with (83%) using BAP hormone and (63%) using MT (**Table 25**).

Moreover, obtained data showed that, after 45 days from sown of conserved encapsulated seeds a comparable **regrowth** rate (47%) was registered. PGRs added at 0.5 mgL^{-1}concentration, showed no statistical significant effect on the regrowth rate of artificial seeds (*P*= 0.612) and gave a regrowth rates of 43% and 50% using BAP and MT respectively. PGRs revealed no statistical significant difference of shoots length (*P*= 0.743). Regenerated Houmairi cultivar from conserved artificial seeds, exceeded the 4 mm with a total mean shoot length of 0.743 cm. Mean shoot length enhanced by BAP and MT were 0.527 and 0.577 cm respectively (**Table 26**).

Table 25 Effects of PGRs added to the medium on viability, regrowth and conversion rate in *Ficus carica* conserved synthetic seeds, after 45 days from sowing.

Factors/treatment	Viability (%)	Regrowth (%)	Conversion (%)
Conserved seeds (C)			
Houmairi Nb 8	73.0	47.0	13.0
PGRs (P)			
BAP	83.0	43.0	3.0
MT	63.0	50.0	23.0
Statistical analysis of factors [a]			
PGRs (P)	0.82	0.612	0.023

[a]Two-way ANOVA (T, P), followed by Tukey's test $p \leq 0.05$.

Furthermore, microcuttings from conserved encapsulated nodal segments showed a low ability to convert and to produce shoots and roots on the sowing media (13%). PGRs showed a statistical significant effect on the **conversion** rate at 5% level (*P*= 0.023) where MT gave the best rate (23%) if compared with BAP (3%) (**Table 25**). Recorded total mean root length was 0.098 cm with 0.16 and 0.037 cm given by MT and BAP respectively. Non statistical significant effect was recorded by PGRs on obtained roots length (*P*= 0.069) (**Table 26**).

Table 26 Shoots and roots length measurement after 45 days from *in vitro* culture of cv. Houmairi conserved seeds.

Factors/PGRs	Shoots and roots length (cm)	
	Shoots (cm)	Roots (cm)
Conserved seeds (C)		
Houmairi Nb.8	0.552	0.098
PGRs (P)		
BAP	0.527	0.037
MT	0.577	0.16
Statistical analysis of factors [a]		
PGRs (P)	0.743	0.069

[a]Two-way ANOVA (T, P), followed by Tukey's test $p \leq 0.05$.

Concerning the **not-conserved** seeds, a normal germination of plantlets was observed (**Figure 47**). PGRs showed no significant effect on *F. carica* encapsulated microcuttings **viability** obtained after 45 days of culture (*P*=0.321) at 5% level. The viability rate was very high (98%). BAP showed to give the best viability rate (100%) followed by MT (97%) (**Table 27**).

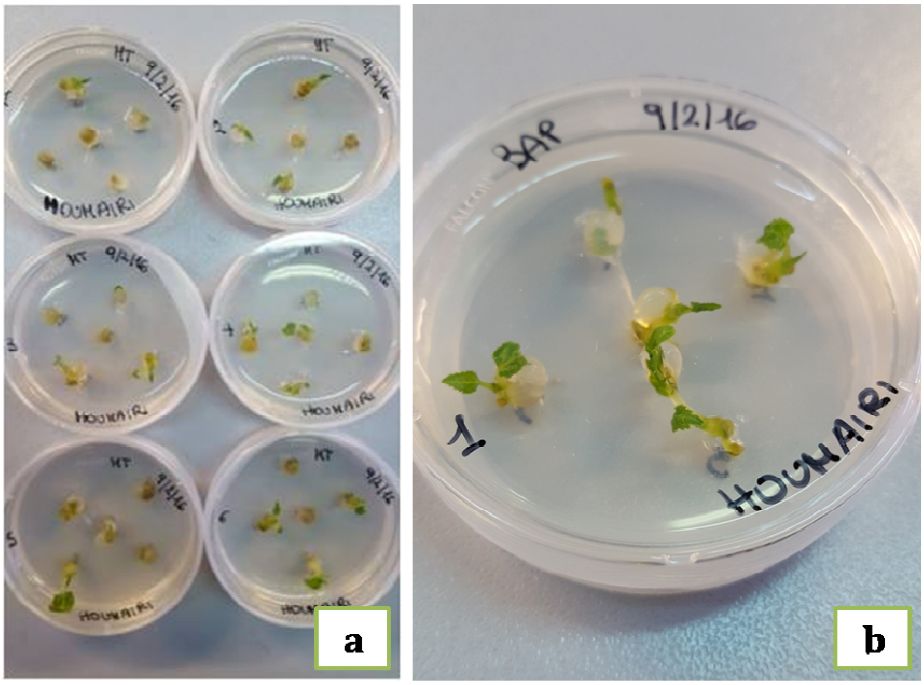

Figure 47 Not-conserved Houmairi cultivar regenerated plantlets using MT (a) and BAP (b) plant growth regulators.

Regrowth rate for **not-conserved** artificial seeds was high (73%). PGRs added at 0.5 mgL^{-1}concentration, showed a highly statistical significant effect on seeds regrowth rate (*P*=0.019). The hormone BAP gave an optimum rate (87%) if compared with MT (60%) which remains also high rate. PGRs revealed a significant difference on shoots length (*P*= 0.009) (**Table 27**). Regenerated plantlets gave a total mean shoot length of 0.88 cm. BAP was the best to increase shoots length with a mean length of 1.09 cm, followed by MT with only 0.67 cm (**Table 28**).

Table 27 Effects of PGRs added to the medium on viability, regrowth and conversion rate in fig non conserved synthetic seeds, after 45 days from sowing.

Factors/treatment	Viability (%)	Regrowth (%)	Conversion (%)
Not-conserved seeds (NC)			
Houmairi Nb 8	98.0	73.0	7.0
PGRs (P)			
BAP	100	87.0	3.0
MT	97.0	60.0	10.0
Statistical analysis of factors [a]			
PGRs (P)	0.321	0.019	0.309

[a]Two-way ANOVA (T, P), followed by Tukey's test $p \leq 0.05$.

Conversion rate of **not-conserved** encapsulated nodal segments showed a very low ability to convert and to produce shoots and roots on the sowing media (7%). PGRs showed no statistical significant effect on the conversion rate at 5% level (*P*= 0.309) where MT supported a better rate (10%) when compared with BAP (3%) (**Table 27**). The total mean root length was 0.045 cm with 0.08 and 0.001 cm given by MT and BAP respectively. No statistical significant effect was recorded by PGRs on obtained roots length (*P*= 0.173) (**Table 28**).

Table 28 Shoots and roots length after 45 days from *in vitro* culture of cv. Houmairi not-conserved synthetic seeds.

Factors/PGRs	Shoots and roots length (cm)	
	Shoots (cm)	Roots (cm)
Not-conserved seeds (N C)		
Houmairi Nb.8	0.88	0.045
PGRs (P)		
BAP	1.09	0.01
MT	0.67	0.08
Statistical analysis of factors [a]		
PGRs (P)	0.009	0.173

[a]Two-way ANOVA (T, P), followed by Tukey's test $p \leq 0.05$.

a. ***Influence of the type of test: conserved or not on all parameters***

The type of test showed a highly statistical significant effect on the viability rate after 45 days from artificial seeds sowing on the medium ($P< 0.001$) at 5% level. The optimum viability rate was obtained with the not-conserved seeds with 98% when compared with the conserved one (73%). Thus, the conservation of the artificial seeds reduces the viability rate (**Table 29** and **Figure 48**).

Table 29 Statistical analysis of viability, regrowth and conversion rates according to the type of test (conserved or not) and PGRs used.

	Viability (%)			Regrowth (%)			Conversion (%)		
PGRs (P)	**BAP**	**MT**	**TOT**	**BAP**	**MT**	**TOT**	**BAP**	**MT**	**TOT**
Type of test (T)									
Conserved	83	63	**98**	43	50	**47**	3	23	**13**
Not conserved	100	97	**73**	87	60	**73**	3	10	**7**

Significance			
P	0.05	0.245	0.014
T	< 0.001	0.002	0.216
P x T	0.16	0.054	0.216

[a]Two-way ANOVA (T, P), followed by Tukey's test $p \leq 0.05$.

Moreover, the type of test showed a highly significant effect on the regrowth rate after 45 days from artificial seeds sowing on the medium ($P= 0.002$) at 5% level. The optimum regrowth rate was obtained with the not-conserved seeds with 73% when compared with the conserved one (47%). Thus, the conservation of the artificial seeds reduces the regrowth rate (**Table 29** and **Figure 48**).

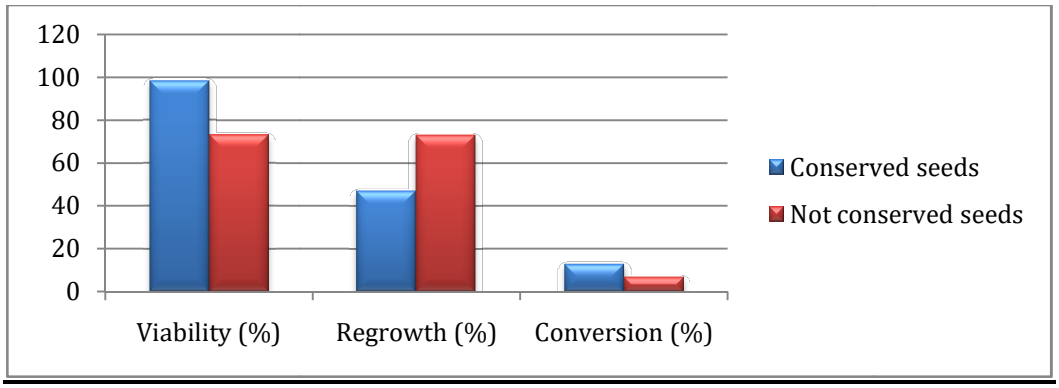

Figure 48 Viability, regrowth and conversion rates according to the type of test used conserved seeds or not.

No statistical significant effect was recorded for the type of test on the conversion rate (*P*= 0.216) at 5% level. Even though the conversion rate was low, the conservation of synthetic seeds improved the conversion rate which passes from 7% (not conserved) to 13% (conserved).

b. PGRs effect on viability, regrowth and conversion rates for both conserved and not-conserved artificial seeds

The registered viability rate was very important using the two PGRs (86%), where BAP gave the best rate with 92% followed by MT with 80%. PGRs revealed no statistical significant effect on viability rate for both conserved and not-conserved artificial seeds (*P*= 0.068) at 5% level. Thus BAP and MT could be used equally for artificial conserved and not-conserved optimum viability (**Figure 49** and **Table 30**).

The total obtained regrowth rate for all seeds was fairly high (60%), with (65%) given by BAP and (55%) by MT. Furthermore, no statistical significant effect was registered on regrowth rate for both conserved and no conserved artificial seeds (*P*= 0.267) at 5% level (**Table 30**).

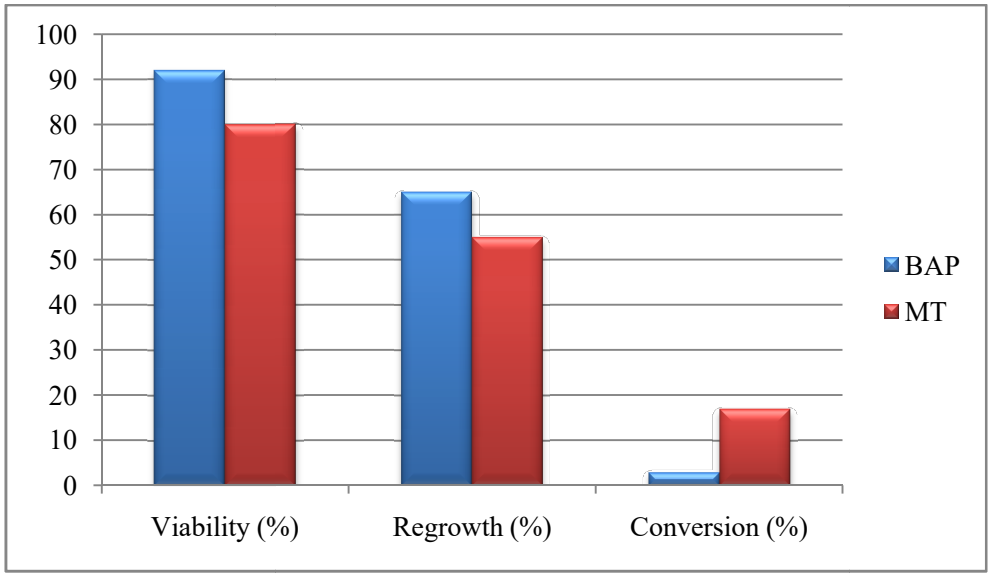

Figure 49 Viability, regrowth and conversion rates according to PGRs used for all artificial seeds.

The obtained conversion rate was too low for all experimented artificial seeds (10%) but PGRs registered a statistical significant effect at 5% level (*P*= 0.015), where MT showed to give the best rate (17%) if compared with BAP (3%) (**Table 30**).

Table 30 Statistical analysis of viability, regrowth and conversion rates according to PGRs used.

Parameters	***PGRs used***			***Significance***
Viability rate (%)	**BAP** 92%	**MT** 80%	**Tot** 86%	0.068
Regrowth rate (%)	**BAP** **65%**	**MT** **55%**	**Tot** **60%**	0.267
Conversion rate (%)	**BAP** 3%	**MT** 17%	**Tot** 10%	0.015

[a]Two-way ANOVA (T, P), followed by Tukey's test $p \leq 0.05$.

6.4 CONCLUSIONS

We report here for the first time a refined protocol for *F. carica* (cv. Houmairi) artificial seeds conservation for 45 days at 4 °C and their ability to regenerate and converse in one step (on the same medium) (Yahyaoui *et al*., Unpublished).

Moreover, we registered a good regeneration ability with vigor and well proliferated plantlets besides to a very high viability and regrowth rates using MT or BAP PGRs. However, the conversion rate was low and strictly related to the type of PGRs used, where MT gave better results if compared to BAP. Nevertheless the conservation of artificial seeds, during this period at 4 °C, reduced slightly both viability and regrowth rates but seemed to improve the conversion rate.

CONCLUDING REMARKS

The present thesis expands the knowledge on the sanitary status of various Mediterranean fig genotypes and provides further information about the incidence of virus associated to FMD and their distribution within the trees where all fig-infecting viruses were present in the surveyed cultivars. Moreover, new sanitation and conservation opportunity of healthy fig are suggested to use for the successful establishment of commercial production.

The high incidence of the syndrome is not surprising considering the mode of propagation of this species (by rooted cuttings and grafting) and the presence of very efficient virus vectors (eriophyid mites, mealybugs and aphids), both factors that favor the transmission of viral agents in nature. FBV-1, the only DNA virus, showed to be the most widespread virus infecting all different *F. carica* organs (leaf, bud and syconium). FMV, the causal agent of FMD, was found to have a low infection rate (11.1%) directly related to its eriophyid mite (*Aceria ficus*) transmissibility, also to be more concentrated in buds then leaves and absent in syconia, confirming its no transmissibility through seeds (Martelli *et al.*, 1993). In a different way, FFkaV and FLMaV-1 were present and mostly concentrated in synoconia.

In conclusion, the outcome of this study, although not yet final, it provides an insight on the FMD viruses distribution within the fig tree. RT-PCR assays conducted on reverse-transcribed TNA extracted from leaves, apical buds and syconia of each genotype showed the highest infection rate for FBV followed by FFkaV, FLMaV-1 and FMV; whereas FMMaV and FLV-1 were absent.

Virus distribution within the different explants showed that FBV-1 was the most prevalent in all explants with a high infection rates, whereas FFkaV was mostly concentrated in syconia and to a lesser extent in buds and leaves. The highest infection with FLMaV-1 was found in syconia while buds and leaves showed lower level of infection. FMV was present as well in buds and leaves, but with low rates and it was absent in syconia. Infection rates in fig seeds revealed that all the pre-tested viruses were present without any exception. FMV and FFkaV showed the highest levels of infection rate followed by FLV-1, FLMaV-1, FLMaV-2

and FMMaV. FBV-1 was found to be present in all seeds and reached the maximum percentage.

Furthermore, this doctoral research studied the use of both conventional *in vitro* multiplication and the no conventional using the synthetic seeds technology. Encapsulation technology proved to be a powerful multiplication technique to sustain standard fig tissue culture protocol. Catalanisca, Palazzo and Bifera nera cultivars gave high, almost similar, viability, regrowth and conversion. Microcutting rooting in one step was achieved revealing their specific aptitude to root where a comparable conversion rates for the three cultivars were obtained. Benzyleaminopurine (BAP) and meta-Topolin (MT) plant growth regulators (PGRs) provided the best results about regrowth and conversion.

Moreover, this study permitted anyway to compare old and new sanitation trials for obtaining virus-free *F. carica* germplasm. Shoot tip culture (STC) showed to be less effective than Meristem Tip Culture Protected by the Synthetic Seeds technique (MTC-SS) and Caulogenesis from meristem and leaves of *in vitro* cultivars. In particular FLMaV-1 was eradicated only using MTC-SS and Caulogenesis, even if it was eliminated in a good percentage by the STC.

This last technique and the Caulogenesis permitted the easy eradication of FLMaV-2. FMMaV was equally sanitized via MTC-SS and Caulogenesis as well as for FLV-1. This latest was sanitized with a lesser extent by STC. Caulogenesis technique showed to be the best in eliminating the causal agent of FMD (FMV) followed by MTC-SS and STC. FFkaV was highly eliminated, for the first time and with high percentages via Caulogenesis followed by MTC-SS and STC with a lesser extent. By contrast, the DNA virus FBV-1 resisted all attempts of elimination, a behavior that reinforces indirectly its hypothesized integration in the fig genome.

Finally, *F. carica* (cv. Houmairi) artificial seeds conservation, for final delivery, was achieved. A high viability and moderate regrowth rates were registered with a lesser conversion rate strictly related to the PGRs used where MT gave better results if compared to BAP. Those preliminary results need a field application in order to evaluate the soil survival rate, known limitation for this technology (Jung *et al.*, 2004).

Moreover, the genetic stability of regenerated plantlets has to be ascertained by molecular analysis. Following these tests, the optimized protocol for large-scale production of good quality and healthy fig plants, as well as their conservation as encapsulated propagules, could be adopted and applied for successful establishment of commercial production.

Publications and Congress

✓ ***Articles accepted and soon will be published***

Yahyaoui E., Frasheri D., Germanà M.A., Burruano S., D'Onghia A.M., Elbeaino T. (2016). Viruses infecting different Mediterranean genotypes of *Ficus carica* and their distribution in different plant organs. *Acta Hort.*

Yahyaoui E., Casamento D., Frasheri D., D'Onghia A.M., Germanà M.A. (2016). Encapsulation and evaluation of some growth regulator effects on *in vitro*-derived microcuttings of three Italian *Ficus carica* L. genotypes. *Acta Hort.*

✓ ***Articles in preparation***

- Incidence of Fig Mosaic Disease associated viruses in various Mediterranean *Ficus carica* seeds.

- *In vitro Ficus carica* L. multiplication and evaluation using both traditional and non conventional techniques.

- Fig virus-free production and survival rate improvement using meristem tip culture technique associated to the encapsulation technology.

- Elimination of viruses associated with Fig Mosaic Disease using standard and improved sanitation trials for various Mediterranean *Ficus carica* L. cultivars.

- Conservation and evaluation of encapsulated microcuttings of *Ficus carica* Lebanese cultivar.

- ✓ **Congress**

Yahyaoui E., Frasheri D., Germanà M.A., Burruano S., D'Onghia A.M., Elbeaino T. (2015). Viruses infecting different Mediterranean genotypes of *Ficus carica* and their distribution in different plant organs. *V International Symposium on Fig, Napoli (Italy).*

Yahyaoui E., Casamento D., Frasheri D., D'Onghia A.M., Germanà M.A. (2016). Encapsulation and evaluation of some growth regulator effects on *in vitro*-derived microcuttings of three Italian *Ficus carica* L. genotypes. *IX International Symposium on In Vitro Culture and Horticultural Breeding, Giza (Egypt).*

Annexes

Annex 1 Buffers and solutions used for the total nucleic acid (TNA) extraction.

Buffers	Components	Quantity	Note
Grinding Buffer	Guanidine thiocyanate	4.0M	
	NaOAc EDTA KOAc PVP-40	0.2M 25mM 1.0M 2.5% wt/vol	Ph 5.2 Stored at 4°C
NaI	Na2SO3 NaI (Sigma S8379)	0.75g 36g	dissolved in 40 mL of H_2O Stored in dark at 4°C
Silica solution	silica particles (Sigma S5631)	60g	Added to 500 mL of H_2O Ph 2.0 Stored in dark at 4°C
Washing buffer	Tris-HCl EDTA NaCl EtOH	10.0 mM 0.5 mM 50.0 mM 50%	pH 7.5 Store at 4 °C

Annex 2 Solutions used for agarose gels

Buffer	Materials	Quantity	Note
TAE 10% (stock solution)	Tris-HCl Sodium acetate EDTA	0.4M 0.4M 0.02M	pH 7.2 Sterilized by autoclaving

References

Adams M., Antoniw J., Fauquet C. (2005). Molecular criteria for genus and species discrimination within the family Potyviridae. *Archives of Virology*, 150(3): 459-479.

Ahmad K. S., Kayani W. K., Hameed M., Ahmad F., Nawaz T. (2012). Floristic diversity and ethnobotany of Senhsa, District Kotli, Azad Jammu and Kashmir (Pakistan). *Pakistan Journal of Botany*, 44(Suppl. 2): 195-201.

Aldhebiani A.Y., Elbeshehy E. K., Baeshen A. A., Elbeaino T. (2015). Four viruses infecting figs in Western Saudi Arabia. *Phytopathologia mediterranea*, 54(3): 497.

Altman A. and Loberant B. (2000). Micropropagation of plants, principles and practices. *Encyclopedia of Cell Technology*.

Anagnostopoulos P. (1939). The enemies of fruit trees. *Athens (in Greek)(cit. in Katsoyannos, 1983)*.

Appiano A., Conti M., Lovisolo O. (1990). Mosaico del fico. Stato attuale delle conoscenze e nuove osservazioni. *Agricoltura Ricerca*.

Ashihara W., Kondo A., Shibao M., Tanaka H., Hiehata K., Izumi K. (2004). Ecology and control of eriophyid mites injurious to fruit trees in Japan. JARQ 38: 31-41.

Anonymous (2013). Food and Agriculture Organization of United Nations, Statistical Data (FAOSTAT). Available at/ http://www.fao.org/statistics/fr/.

Bayoudh C., Labidi R., Majdoub A., Mars M. (2015). In vitro Propagation of Caprifig and Female Fig Varieties (*Ficus carica* L.) from Shoot-tips. *Journal of Agricultural Science and Technology*, 17(6): 1597-1608.

Bhat A. I., Hohn T., Selvarajan R. (2016). Badnaviruses: The Current Global Scenario. *Viruses*, 8(6): 177.

Blodgett E. and Gomec B. (1967). Fig mosaic. *Plant Dis. Reptr*, 51: 893-896.

Brum G. R. (2001). *Micropropagação da figueira (Ficus carica L.)'Roxo de Valinhos'*. Universidade Federal de Lavras.

Caglar B. K., Fidan H., Guldur M. E., Elbeaino T. (2011). The prevalence of three viruses infecting fig in southern Turkey. *Journal of Phytopathology*, 159(3): 181-183.

Chen L., Chen J.S., Zhang H., Chen S.N. (2006). *Arch. Virol.* 151, 2077–2083.

Çağlayan K., Medina V., Gazel M., Serçe Ç. U., Serrano L., Achon A., Soylu S., Çalişkan O., Gümüş M. (2009). Putative agents of fig mosaic disease in Turkey. *Turkish Journal of Agriculture and Forestry*, 33(5): 469-476.

Çaglayan K., Serçe Ç. U., Barutçu E., Kaya K., Medina V., Gazel M., Soylu S., Çaliskan O. (2010). Comparison by sequence-based and electron microscopic analyses of *Fig mosaic virus* isolates obtained from field and experimentally inoculated fig plants. *Plant Disease*, 94(12): 1448-1452.

Castellano M., De Stradis A., Minafra A., Boscia D., Martelli G. (2009). Seed transmission of Fig latent virus 1. *Journal of Plant Pathology*: 697-700.

Castellano M., Gattoni G., Minafra A., Conti M., Martelli G. (2007). Fig mosaic in mexico and south Africa. *Journal of Plant Pathology*: 441-444.

Chalak L., Elbeaino T., Elbitar A., Fattal T., Choueiri E. (2015). Removal of viruses from Lebanese fig varieties using tissue culture and thermotherapy. *Phytopathologia mediterranea*, 54(3): 531.

Chalak L., Elbitar A., Mourad N., Mortada C., Choueiri E. (2013). Elimination of grapevine Bois Noir Phytoplasma by tissue culture coupled or not with heat therapy or hot water treatment. *Advances in Crop Science and Technology*, 2013.

Chiumenti M., Campanale A., Bottalico G., Minafra A., De Stradis A., Savino V., Martelli G. (2013). Sanitation trials for the production of virus-free fig stocks. *Journal of Plant Pathology*: 655-658.

Condit I. J. and Horne W. (1933). A mosaic of the fig in California. *Phytopathology*, 23: 887-896.

Cote F. X., Galzi S., Folliot M., Lamagnere Y., Teycheney P. Y., Iskracaruana M. L. (2010). Micropropagation by tissue culture triggers differential expression of infectious endogenous Banana streak virus sequences (eBSV) present in the B genome of natural and synthetic interspecific banana plantains. *Molecular plant pathology*, 11(1): 137-144.

Crous P. and Wingfield M. J. (1994). A monograph of Cylindrocladium, including anamorphs of Calonectria. *Mycotaxon*, 51: 341-435.

Dallot S., Acuna P., Rivera C., Ramirez P., Cote F., Lockhart B., Caruana M. L. (2001). Evidence that the proliferation stage of micropropagation procedure is determinant in the expression of Banana streak virus integrated into the genome of the FHIA 21 hybrid (Musa AAAB). *Archives of Virology*, 146(11): 2179-2190.

Demiralay A., Yalcin-Mendi Y., Aka-Kacar Y., Cetiner S. (1997). In vitro propagation of *Ficus carica* L. var. Bursa Siyahi through meristem culture.In: (eds). *I International Symposium on Fig 480*.

Denchev P., Velcheva M., Dragijska R., Kuklin A., Atanassov A. (1990). Somatic embryogenesis in Medicago. *Biotechnology and Biotechnological Equipment*, 4(5-6): 66-70.

Dhage S., Pawar B., Chimote V., Jadhav A., Kale A. (2012). In vitro callus induction and plantlet regeneration in fig (*Ficus carica* L.). *Journal of Cell and Tissue Research*, 12(3): 3395.

Dolja V. V., Kreuze J. F., Valkonen J. P. (2006). Comparative and functional genomics of closteroviruses. *Virus Research*, 117(1): 38-51.

Doster M., Michailides T. and Morgan D. (1996). *Aspergillus* species and mycotoxins in figs from California orchards. *Plant Disease*, 80(5): 484-489.

Dueñas M., Pérez-Alonso J. J., Santos-Buelga C., Escribano-Bailón T. (2008). Anthocyanin composition in fig (*Ficus carica* L.). *Journal of Food Composition and Analysis*, 21(2): 107-115.

Dupont L. M. (1979). On gene flow between *Tetranychus urticae* Koch, 1836 and *Tetranychus cinnabarinus* (Boisduval) Boudreaux, 1956 (Acari: Tetranychidae): synonomy between the two species. *Entomologia experimentalis et applicata*, 25(3): 297-303.

El-Rayes R. (1995). The fig tree in the Mediterranean region and in Syria. *Options Mediterr*, 13: 79-83.

El Air M., Mahfoudhi N., Digiaro M., Dhouibi M., Elbeaino T. (2015). Incidence and distribution of viruses in Tunisian fig orchards. *Journal of Plant Pathology*, 97(2).

El Rayes R. (1996). Fig tree in Syria.In: (eds). *1. MESFIN International Meeting on Tropical and Sub-Tropical Fruit Germplasm Conservation. La Laguna, Tenerife (Espana). 2-4 Oct 1995.*

Elbeaino T., Digiaro M., Alabdullah A., De Stradis A., Minafra A., Mielke N., Castellano M. A., Martelli G. P. (2009a). A multipartite single-stranded negative-sense RNA virus is the putative agent of fig mosaic disease. *Journal of General Virology*, 90(5): 1281-1288.

Elbeaino T., Digiaro M., De Stradis A., Martelli G. (2006). Partial characterisation of a closterovirus associated with a chlorotic mottling of fig. *Journal of Plant Pathology*, 88(2): 187-192.

Elbeaino T., Digiaro M., De Stradis A., Martelli G. (2007). Identification of a second member of the family Closteroviridae in mosaic-diseased figs. *Journal of Plant Pathology*: 119-124.

Elbeaino T., González Rodríguez Á.M., Grajal-Martín M.J., Digiaro M. (2011a). Survey of fig viruses in the Canary islands. *Journal of plant pathology,* 93(3): 737-739.DOI:doi.org/10.4454/jpp.v93i3.3659.

Elbeaino T., Digiaro M., Heinoun K., De Stradis A., Martelli G. (2010). Fig mild mottle-associated virus, a novel *closterovirus* infecting fig. *Journal of Plant Pathology*, 92(1): 165-172.

Elbeaino T., Digiaro M., Martelli G. (2012). RNA-5 and-6, two additional negative-sense RNA segments associated with *Fig mosaic virus*. *Journal of Plant Pathology*, 94(2): 421-425.

Elbeaino T., Digiaro M., Martelli G. P. (2009b). Complete nucleotide sequence of four RNA segments of *Fig mosaic virus*. *Archives of Virology*, 154(11): 1719-1727.

Elbeaino T., Digiaro M., Martelli G. P. (2011b). Complete sequence of Fig fleck-associated virus, a novel member of the family Tymoviridae. *Virus Research*, 161(2): 198-202.

Elbeaino T., Kubaa R. A., Digiaro M., Minafra A., Martelli G. P. (2011c). The complete nucleotide sequence and genome organization of fig cryptic virus, a novel bipartite dsRNA virus infecting fig, widely distributed in the Mediterranean basin. *Virus Genes*, 42(3): 415-421.

Elbeshehy E. K. F. and Elbeaino T. (2011). Viruses infecting figs in Egypt. *Phytopathologia mediterranea*, 50(2): 327-332.

Elci E., Serçe Ç., Gazel M. (2012). Molecular detection and comparative sequence analysis of viruses infecting fig trees in Turkey. *Journal of Phytopathology*, 160(7-8): 418-423.

Faccioli G. (2001). Control of potato viruses using meristem and stem-cutting cultures, thermotherapy and chemotherapy. In: (ed). *Virus and Virus-like Diseases of Potatoes and Production of Seed-potatoes*. Springer, pp.365-390.

Falk B. W., Salem N., Davis C. (2006). Fig mosaic.

Flock R. and Wallace J. (1955). Transmission of fig mosaic by the eriophyid mite *Aceria ficus*. *Phytopathology*, 45: 52-54.

Foissac X., Savalle-Dumas L., Gentit P., Dulucq M., Candresse T. (2001). Polyvalent detection of Fruit tree Tricho. *Capillo and Res*(45): 33-35.

Fráguas C. B., Pasqual M., Dutra L. F., Cazetta J. O. (2004). Micropropagation of fig (*Ficus carica* L.)'Roxo de Valinhos' plants. *In Vitro Cellular and Developmental Biology-Plant*, 40(5): 471-474.

Frodin D. G. (2004). History and concepts of big plant genera. *Taxon*, 53(3): 753-776.

Gattoni G., Minafra A., Castellano M., De Stradis A., Boscia D., Elbeaino T., Digiaro M., Martelli G. (2009). Some properties of Fig latent virus 1, a new member of the family Flexiviridae. *Journal of Plant Pathology*, 91(3): 555-564.

Germana M. A., Micheli M., Chiancone B., Macaluso L., Standardi A. (2011). Caulogenesis and encapsulation of in vitro-derived propagules of Carrizo citrange *Citrus sinensis* (L.) Osb.× *Poncirius trifoliata* (L.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, 106(2): 299-307.

Ghabrial S., Nibert M., Maiss E., Lesker T., Baker T., Tao Y. (2005). Family partitiviridae. *Virus Taxonomy: Eighth Report of the International Committee for the Taxonomy of Viruses*: 581-590.

Gilani A. H., Mehmood M. H., Janbaz K. H., Khan A., Saeed S. A. (2008). Ethnopharmacological studies on antispasmodic and antiplatelet activities of *Ficus carica*. *Journal of ethnopharmacology*, 119(1): 1-5.

Goeldi E. A. (1887). Relatorio sobre a molestia do cafeeiro na provincia do Rio de Janeiro.

Gonçalves M., Andrade L., Almeida L., Pica M. (2005). Study of *Ceratitis capitata* and *Lonchaea aristella* on Fig Trees.In: (eds). *III International Symposium on Fig 798*.

Grassi G. and Santonastaso M. (1997). The fig growing in Italy: The present state and problems.In: (eds). *I International Symposium on Fig 480*.

Günver G. and Ertan E. (1997). A study on the propagation of figs by the tissue culture techniques.In: (eds). *I International Symposium on Fig 480*.

Haccius B. (1978). Question of unicellular origin or non-zygotic embryos in callus cultures. *Phytomorphology*.

Hassan M., Chindo P., Marley P. and Alegbejo M. (2010). Management of root knot nematodes (*Meloidogyne* spp.) on tomato (*Lycopersicon lycopersicum*) using organic wastes in Zaria, Nigeria. *Plant Protect. Sci*, 46(1): 34-38.

Hepaksoy S. and Aksoy U. (2006). Propagation of *Ficus carica* L. clones by in vitro culture. *Biologia plantarum*, 50(3): 433-436.

Heperkan D., Moretti A., Dikmen C. D. and Logrieco A. F. (2012). Toxigenic fungi and mycotoxin associated with figs in the Mediterranean area. *Phytopathologia mediterranea*, 51(1): 119-130.

Himelrick D. (1999). Fig Production Guide. *Alabama A and M Auburn Universities, Alabama Cooperative Extension System, ANR-1145*, 1(7).

Hohn T., Richert-Pöggeler K. R., Staginnus C., Harper G., Schwarzacher T., Teo C. H., Teycheney P.-Y., Iskra-Caruana M.-L. and Hull R. (2008). Evolution of integrated plant viruses. In: (ed). *Plant Virus Evolution*. Springer, pp.53-81.

Hollings M. (1965). Disease control through virus-free stock. *Annual review of phytopathology*, 3(1): 367-396.

Ishikawa K., Maejima K., Komatsu K., Kitazawa Y., Hashimoto M., Takata D., Yamaji Y., Namba S. (2012). Identification and characterization of two novel genomic RNA segments of *Fig mosaic virus*, RNA5 and RNA6. *Journal of General Virology*, 93(7): 1612-1619.

Jain S. M. (2001). Tissue culture-derived variation in crop improvement. *Euphytica*, 118(2): 153-166.

Jeong H., Mason S. P., Barabási A.-L., Oltvai Z. N. (2001). Lethality and centrality in protein networks. *Nature*, 411(6833): 41-42.

Jerinić-Prodanović D. (2011). The first finding of the fig psylla *Homotoma ficus* L.(Hemiptera, Psylloidea, Homotomidae) in Serbia. *Pesticidi i fitomedicina*, 26(3): 205-212.

Johansen E., Edwards M. C., Hampton R. O. (1994). Seed transmission of viruses: current perspectives. *Annual review of phytopathology*, 32(1): 363-386.

Jung S., Yoon E., Jeong J., Choi Y. (2004). Enhanced post-germinative growth of encapsulated somatic embryos of Siberian ginseng by carbohydrate addition to the encapsulation matrix. *Plant cell reports*, 23(6): 365-370.

Kim K. M., Kim M. Y., Yun P. Y., Chandrasekhar T., Lee H. Y., Song P. S. (2007). Production of multiple shoots and plant regeneration from leaf segments of fig tree (Ficus carica L.). *Journal of Plant Biology*, 50(4): 440-446.

Kozai T., Debergh P., Zimmerman R. (1991). Micropropagation technology and application. *Micropropagation-technology and aplication*.

Kumar N. and Reddy M. (2011). In vitro plant propagation: A review. *Journal of Forest and Environmental Science*, 27(2): 61-72.

Kumar S., Khan M., Raj S., Sharma A. (2009). Elimination of mixed infection of Cucumber mosaic and Tomato aspermy virus from Chrysanthemum morifolium Ramat. cv. Pooja by shoot meristem culture. *Scientia Horticulturae*, 119(2): 108-112.

Kumar V., Radha A., Chitta S. K. (1998). In vitro plant regeneration of fig (*Ficus carica* L. cv. Gular) using apical buds from mature trees. *Plant cell reports*, 17(9): 717-720.

Laney A. G., Hassan M., Tzanetakis I. E. (2012). An integrated badnavirus is prevalent in fig germplasm. *Phytopathology*, 102(12): 1182-1189.

Lheureux F., Carreel F., Jenny C., Lockhart B., Iskra-Caruana M. (2003). Identification of genetic markers linked to banana streak disease expression in inter-specific Musa hybrids. *Theoretical and Applied Genetics*, 106(4): 594-598.

Lopez-Delgado H., Dat J. F., Foyer C. H., Scott I. M. (1998). Induction of thermotolerance in potato microplants by acetylsalicylic acid and H2O2. *Journal of Experimental Botany*, 49(321): 713-720.

Malaurie B., Thouvenel J. C., Pungu O. (1995). Influence of meristem-tip size and location on morphological development in Dioscorea cayenensis-D. rotundata complex 'Grosse Caille' and one genotype of *D. praehensilis*. *Plant cell, tissue and organ culture*, 42(2): 215-218.

Manganaris G., Economou A., Boubourakas I., Katis N. (2003). Elimination of PPV and PNRSV through thermotherapy and meristem-tip culture in nectarine. *Plant cell reports*, 22(3): 195-200.

Martelli G. (2011). Fig mosaic disease and associated pathogens, APS Press: St. Paul, MN, USA**:** 281-287.

Martelli G., Castellano M., Lafortezza R. (1993). An ultrastructural study of fig mosaic. *Phytopathologia mediterranea*: 33-43.

Maury Y., Duby C., Khetarpal R.K. (2008). Seed certification for viruses. In: Hadidi A., Khetarpal R.K., Koganezawa H. (eds). *Plant Virus Disease Control*: 237-248. APS Press, St. Paul, MN, USA.

McLeish M. J. and Van Noort S. (2012). Codivergence and multiple host species use by fig wasp populations of the Ficus pollination mutualism. *BMC evolutionary biology*, 12(1): 1.

McKenzie E. (1986). New plant disease record in New Zealand: Fig rust (*Cerotelium fici*) on *Ficus carica*. *New Zealand journal of agricultural research*, 29(4): 707-710.

Meena M. C., Meena R., Patni V. (2010). High frequency plant regeneration from shoot tip explants of *Citrullus colocynthis* (Linn.) Schrad.–An important medicinal herb. *African Journal of Biotechnology*, 9(31): 5037-5041.

Minafra A., Chiumenti M., Elbeaino T., Digiaro M., Bottalico G., Pantaleo V., Martelli G. (2012). Occurrence of fig Badnavirus 1 in fig trees from different countries and in symptomless seedlings. *Journal of Plant Pathology*, 94(4).

Mink G. (1993). Pollen and seed-transmitted viruses and viroids. *Annual review of phytopathology*, 31(1): 375-402.

Morton H. (1987). Industry perspectives in nematology.

Murashige T. and Skoog F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, 15(3): 473-497.

Mustafa N. and Taha R. A. (2012). Influence of Plant Growth Regulators and Subculturing on In Vitro Multiplication of Some Fig (*Ficus Carica*) Cultivars. *Journal of Applied Sciences Research*, 8(8): 4038-4044.

Nahdi S., Elbeaino T., Digiaro M., Martelli G. (2006). First record of Fig leaf mottle-associated virus 1 in Tunisia. *Journal of Plant Pathology*: S70-S70.

Neal M. C. (1965). In gardens of Hawaii. Special Publication 50. Bernice P, Bishop museum press, Honolulu, HI.

Nibert M. L., Woods K. M., Upton S. J., Ghabrial S. A. (2009). *Cryspovirus*: a new genus of protozoan viruses in the family Partitiviridae. *Archives of Virology*, 154(12): 1959-1965.

Panattoni A., Luvisi A., Triolo E. (2013). Review. Elimination of viruses in plants: twenty years of progress. *Spanish Journal of Agricultural Research*, 11(1): 173-188.

Papachatzis A., Eliopoulos P., Statharas G. and Vagelas I. (2008). Ficus carica root rot disease caused by *Armillaria mellea* and *Rosellinia necatrix* in Greece. *University of Craiova*, 13: 143-148.

Parmar V. R., Patel H. A., Jasrai Y. T. (2013). Developing normal plants of Clerodendron from viral infected stock through meristem culture. *Cibtech. J. Bio-Protocols*, 2: 1-5.

Pasqual M. and Ferreira E. (2007). Micropropagation of Fig tree (*Ficus carica* sp). In: (ed). *Protocols for Micropropagation of Woody Trees and Fruits*. Springer, pp.409-416.

Peerally A. (1991). The classification and phytopathology of *Cylindrocladium* species. *Mycotaxon*, 40: 323-366.

Plavšic B. and Milicic D. (1980). Intracellular changes in trees infected with fig mosaic.In: (eds). *V International Symposium on Virus diseases of Ornamental Plants 110*.

Pontikis C. and Melas P. (1986). Micropropagation of *Ficus carica* L. *HortScience*, 21(1).

Qrunfleh I. M., Shatnawi M. M., Al-Ajlouni Z. I. (2013). Effect of different concentrations of carbon source, salinity and gelling agent on in vitro growth of fig (*Ficus carica* L.). *African Journal of Biotechnology*, *12*(9).

Rai M. K., Asthana P., Singh S. K., Jaiswal V., Jaiswal U. (2009). The encapsulation technology in fruit plants—a review. *Biotechnology advances*, 27(6): 671-679.

Raz D. (1998). The phenology of the fig fly and its control. *ISHS Acta Horticulturae*, 480: 207-208.

Ronald F., Mau L., Jayma L. and Kessing M. (1992). Department of Entomology. *Honolulu, Hawaii*.

Ronsted N., Salvo G., Savolainen V. (2007). Biogeographical and phylogenetic origins of African fig species (Ficus section Galoglychia). *Molecular phylogenetics and evolution*, 43(1): 190-201.

Ronsted N., Weiblen G., Clement W., Zerega N., Savolainen V. (2008). Reconstructing the phylogeny of figs (Ficus, Moraceae) to reveal the history of the fig pollination mutualism. *Symbiosis (Rehovot)*, 45(1): 45.

Saddoud O., Baraket G., Chatti K., Trifi M., Marrakchi M., Mars M., Salhi-Hannachi A. (2011). Using morphological characters and simple sequence repeat (SSR) Markers to characterize Tunisian fig (*Ficus carica* L.) cultivars. *Acta Biologica Cracoviensia Series Botanica*, 53(2): 7-14.

Saddoud O., Chatti K., Salhi-Hannachi A., Mars M., Rhouma A., Marrakchi M., Trifi M. (2007). Genetic diversity of Tunisian figs (*Ficus carica* L.) as revealed by nuclear microsatellites. *Hereditas*, 144(4): 149-157.

Sahraroo A., Babalar M., Ebadi A., Habibi M. K., Khadivi-Khub A. (2009). Influence of apical meristem culture and thermotherapy on production of healthy fig plants. *Horticulture environment and Biotechnology*, 50(1): 45-50.

Salem N.K., Golino D.A., Falk B.W., Rowhani A. (2008).*Arch. Virol.* 153, 455–462.

Sastry K. S. and Zitter T. A. (2014). Management of virus and viroid diseases of crops in the tropics. In: (ed). *Plant virus and viroid diseases in the tropics*. Springer, pp.149-480.

Scotto La Massese C. (1983). The main pests of fig-tree (*Lonchaea aristella*, *Ceroplastes rusci*; insecticide). *Phytoma (France)*.

Shahmirzaie M., Rakhshandehroo F., Zamanizadeh H., Elbeaino T., Martelli G. (2010). First report of *Fig mosaic virus* from fig trees in Iran. *Journal of Plant Pathology*, 92(4): S120-S120.

Sharma M. M., Singh A., Verma R. N., Ali D. Z., Batra A. (2011). Influence of PGRS for the in vitro plant regeneration and flowering in *Portulaca oleracea* L: a medicinal and ornamental plant. *Int J Bot*, 7(1): 103-107.

Sharma S., Shahzad A., Mahmood S., Saeed T. (2015). High-frequency clonal propagation, encapsulation of nodal segments for short-term storage and germplasm exchange of *Ficus carica* L. Trees, 29(2): 345-353.

Silvestri F. (1917). Sulla *Lonchaea aristella* Beck.(Lonchaeidae) dannosa alle infiorescenze e fruttescenze del caprifico e del fico. *Bollettino del Laboratorio di Zoologia Agraria in Portici*, 12: 123-146.

Singer R. (1986). *The Agaricales in modern taxonomy*.

Slatnar A., Klancar U., Stampar F., Veberic R. (2011). Effect of drying of figs (*Ficus carica* L.) on the contents of sugars, organic acids, and phenolic compounds. *Journal of agricultural and food chemistry*, 59(21): 11696-11702.

Soliman H. I., Gabr M., Abdallah N. A. (2010). Efficient transformation and regeneration of fig (*Ficus carica* L.) via somatic embryogenesis. *GM crops*, 1(1): 40-51.

Spiegel S., Frison E. A., Converse R. H. (1993). Recent developments in therapy and virus-detection procedures for international movement of clonal plant germ plasm. *Plant Disease*, 77(12): 1176-1180.

Staginnus C., Iskra-Caruana M., Lockhart B., Hohn T., Richert-Pöggeler K. (2009). Suggestions for a nomenclature of endogenous pararetroviral sequences in plants. *Archives of Virology*, 154(7): 1189-1193.

Starr F., Starr K. and Loope L. (2003). Ficus carica, United States Geological Survey–Biological Resources Division, Haleakala Field Station, Hawaii.

Storey W. (1975). Advances in fruit breeding. Figs in Janick J, Moore JN, eds, Purdue University Press: West Lafayette, Indiana, USA.

Stretch A. and Scott D. (1977). Production of highbush blueberries free of red ringspot virus disease. *Plant Disease Reporter (USA)*.

Subbarao K. V. and Michailides T. J. (1996). Development of phenological scales for figs and their relative susceptibilities to endosepsis and smut. *Plant Disease*, 80(9): 1015-1021.

Sutan A. N., Popescu A., Isac V. (2010). In vitro culture medium and explant type effect on Caulogenesis and shoot regeneration in two genotypes of ornamental strawberry. *Romanian biotechnological letters*, 15(2): 12-18.

Swingle W. (1928). New crops for Arizona and improvement of old ones. *Assoc Ariz Producer*, 7(3): 5.

Taha R. A., Mustafa N., Hassan S. (2013). Protocol for Micropropagation of Two Ficus carica Cultivars. *World Journal of Agricultural Sciences*, 9(5): 383-388.

Talhouk A. M. (2002). *Insects and mites: injurious to crops in Middle Eastern countries*. American University of Beirut Press.

Veberic R., Colaric M., Stampar F. (2008). Phenolic acids and flavonoids of fig fruit (*Ficus carica* L.) in the northern Mediterranean region. *Food Chemistry*, 106(1): 153-157.

Verbeek M., van Dijk P., van Well P. M. (1995). Efficiency of eradication of four viruses from garlic (*Allium sativum*) by meristem-tip culture. *European Journal of Plant Pathology*, 101(3): 231-239.

Verga A. and Nelson S. (2014). Fig rust in Hawai'i. *College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa*.

Verma N., Ram R., Hallan V. a., Kumar K., Zaidi A. (2004). Production of Cucumber mosaic virus-free chrysanthemums by meristem tip culture. *Crop Protection*, 23(5): 469-473.

Vikas V. and Vijay R. (2011). *Ficus carica* Linn-An overview. *Res J Med Plant*, 5: 246-253.

Vinson J. A. (1999). The functional food properties of figs. *Cereal foods world*, 44(2): 82-87.

Vu N. T., Eastwood R., Nguyen C. T., Pham L. V. (2006). The fig wax scale *Ceroplastes rusci* (Linnaeus)(Homoptera: Coccidae) in south-east Vietnam: Pest status, life history and biocontrol trials with *Eublemma amabilis* Moore (Lepidoptera: Noctuidae). *Entomological Research*, 36(4): 196-201.

Walia J. J., Salem N. M., Falk B. W. (2009). Partial sequence and survey analysis identify a multipartite, negative-sense RNA virus associated with fig mosaic. *Plant Disease*, 93(1): 4-10.

Watson L. and Dallwitz M. (1999). *The families of flowering plants: descriptions, illustrations, identification, and information retrieval*. University of New Orleans.

Werbach M. (1993). Healing with food. *New York: HarperPerennial*, 54: 287.

Williams R., Wargo P. and Sites W. (1986). Armillaria root disease.

Yahyaoui E., Frasheri D., D'Onghia A.M., Germanà M.A. *In vitro Ficus carica* L. multiplication and evaluation using both traditional and non-conventional techniques. Unpublished manuscript.

Yahyaoui E., Frasheri D., Germanà M.A., Burruano S., D'Onghia A.M., Elbeaino T. (2016a). Viruses infecting different Mediterranean genotypes of *Ficus carica* and their distribution in different plant organs. *ISHS Acta Horticulturae.* In press.

Yahyaoui E., Frasheri D., Germanà M.A., D'Onghia A.M., Elbeaino T. Incidence of Fig Mosaic Disease associated viruses in various Mediterranean *Ficus carica* seeds. Unpublished manuscript.

Yahyaoui E., Frasheri D., Germanà M.A., D'Onghia A.M., Elbeaino T. Elimination of viruses associated with Fig Mosaic Disease using standard and improved sanitation trials for various Mediterranean *Ficus carica* L. cultivars. Unpublished manuscript.

Yahyaoui E.,Casamento D., Frasheri D., D'Onghia A.M., Germanà M.A. (2016b).Encapsulation and evaluation of some growth regulator effects on *in vitro*-derived microcuttings of three Italian *Ficus carica* L. genotypes. *ISHS Acta Horticulturae.* In press.

Yahyaoui E.,Casamento D., Frasheri D., D'Onghia A.M., Germanà M.A. Conservation and evaluation of encapsulated microcuttings of *Ficus carica* Lebanese cultivar. Unpublished manuscript.

Yakushiji H., Mase N., Sato Y. (2003). Adventitious bud formation and plantlet regeneration from leaves of fig (*Ficus carica* L.). *The Journal of Horticultural Science and Biotechnology*, 78(6): 874-878.

Yancheva S. D., Golubowicz S., Yablowicz Z., Perl A., Flaishman M. A. (2005). Efficient Agrobacterium-mediated transformation and recovery of transgenic fig (*Ficus carica* L.) plants. *Plant science*, 168(6): 1433-1441.

Zobayed S., Afreen-Zobayed F., Kubota C., Kozai T. (2000). Mass propagation of *Eucalyptus camaldulensis* in a scaled-up vessel under in vitro photoautotrophic condition. *Annals of Botany*, 85(5): 587-592.