Evaluation of chemicals against esca-related pathogens, *in vitro* and as pruning wound protectants

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Abstract

During the past decades, Grapevine (*Vitis vinifera*) Trunk Diseases (GTDs) have been under study due to their increase in incidence through all winegrowing regions worldwide. Among the most common GTDs, the esca disease complex is one of the most concerning, leading to important losses in longevity and yield. Pruning wounds are considered the main entry port for fungal spores which, if not protected, may allow the penetration of several wood pathogens, such as *Phaeomoniella chlamydospora* (Pch) and *Phaeoacremonium minimum*. In the present study, the first goal was to evaluate nine chemicals – cyprodinil + fludioxonil, copper oxychloride, copper oxychloride + summer oil, blad, fosetyl-Al, elemental silver, hydrogen peroxide, glutaraldehyde and a bituminous coating - *in vitro* against the growth of these fungi. The second goal was to evaluate four selected chemicals - cyprodinil + fludioxonil, copper oxychloride + summer oil, blad and a bituminous coating - as pruning wound protectants under field conditions against Pch in cv Cabernet Sauvignon. The pruning wounds were inoculated with Pch 1, 31 and 90 days after pruning. Three months after each inoculation the canes were collected and reisolations of the isolate were made. Our study found that most chemicals tested were effective *in vitro*, in reducing mycelial growth of both fungi. However, the results obtained under field conditions showed that one of the treatments significantly reduced the presence of Pch on the first inoculation, providing a partial protection to fresh wound. The remaining treatments were not effective as wound protectants. In summary, novel chemicals were tested that were capable to greatly inhibit *in vitro* the growth of esca-related pathogens and a chemical treatment was identified which was capable of reducing the incidence of Pch as wound protectant. This study improved the current knowledge in chemicals used against some esca-related fungi and in pruning wound protection.

Key words:
Esca disease complex, Blad, Pruning wound protection, Fungicide, Tracheomycosis

Resumo

As doenças do lenho da videira (*Vitis vinifera*) têm sido seriamente estudadas nas últimas décadas face à sua incidência crescente em todas as regiões vitícolas mundiais. Entre elas, o complexo de doenças da esca é das mais preocupante devido ao seu impacto na vinha. As feridas de poda são a principal porta de entrada para os esporos dos fungos, às quais, caso não sejam protegidas, podem permitir a penetração de muitos agentes patogénicos, como *Phaeomoniella chlamydospora* (Pch) e *Phaeoacremonium minimum.* No presente estudo, o primeiro objetivo foi avaliar o efeito de nove produtos químicos - ciprodinil + fludioxonil, oxicloreto de cobre, oxicloreto de cobre + óleo de verão, blad, fosetil-Al, prata elementar, peróxido de hidrogénio, glutaraldeído e um revestimento betuminoso - sobre o crescimento *in vitro* desses fungos. O segundo objetivo foi avaliar quatro produtos químicos - ciprodinil + fludioxonil, oxicloreto de cobre + óleo de verão, blad e um revestimento betuminoso - como protetores das feridas de poda em condições de campo, na casta Cabernet Sauvignon. Inoculações com esporos de Pch foram feitas 1, 31 e 90 dias após a poda. Três meses após cada inoculação, as varas foram colhidas e foram feitos reisolamentos do isolado. Verificou-se, no nosso estudo, que a maioria dos produtos químicos testados *in vitro*, foram eficazes na redução do crescimento micelial de fungos relacionados com o complexo de doenças da esca. Os resultados obtidos na vinha mostraram que um dos tratamentos reduziu significativamente a presença do fungo Pch na primeira inoculação fornecendo proteção parcial às feridas imediatamente depois da poda. Com este estudo, foi possível melhorar o conhecimento atual em produtos químicos usados contra alguns fungos relacionados com o complexo de doenças da esca e a proteção das feridas de poda em condições de campo.

Palavras-chave:
Esca, Blad, Doenças do lenho da videira, Fungicida, Traqueomicoses

Resumo extenso

As doenças do lenho da videira (*GTDs*; *Vitis vinifera*) têm sido seriamente estudadas durante as últimas três décadas face à sua incidência crescente em todas as regiões vitícolas mundiais. As doenças do lenho mais importantes são: a esca, a escoriose, a eutipiose e botriosferiose, que atacam os órgãos perenes da videira, levando ao seu declínio prematuro e até mesmo à morte, em alguns casos. Entre as doenças do lenho mais comuns, a esca é causada por um complexo de doenças e é considerada a doença mais preocupante devido ao seu impacto na vinha, causando perdas importantes na longevidade e produtividade das plantas, reduzindo também a qualidade do vinho. Este complexo de doenças está associado a cinco síndromes: "Brown wood streaking" doença de Petri, GLSD ("grapevine leaf stripe disease"), podridão branca e esca ("proper"). Acredita-se que este complexo de doenças é causado por diferentes fungos, sendo geralmente os mais associados, dois agentes traqueomicóticos, *Phaeomoniella chlamydospora* (Pch) e *Phaeoacremonium minimum* (Pmi), e várias espécies de basidiomicetas como a *Fomitiporia mediterranea* (mais comum na Europa) (Fmed). A evolução da doença pode ser manifestada de duas formas: a forma lenta/crónica ou a forma aguda/apoplexia. A evolução da forma crónica está associada a sintomas internos e externos. Os sintomas internos mais comuns, que afetam o tronco e braços, são caracterizados pela podridão branca (necrose branca e esponjosa) causada por Fmed e estrias negras no lenho causados por Pch e Pmi. Os sintomas externos mais comuns são visíveis nas folhas e nos bagos. Nas folhas, são visíveis manchas marginais cloróticas e necrosadas entre as nervuras, assumindo um padrão 'tiger-stripes' típico. Contudo, os sintomas foliares não ocorrem todos os anos na mesma planta. Nos bagos os sintomas são caracterizados por manchas características castanhas escuras. A forma aguda da doença é caracterizada pelo emurchecimento rápido de toda a planta, levando na maioria das vezes, à morte da mesma. Estudos têm sido realizados para encontrar um método eficaz de controlar a doença: nos viveiros, melhorando as práticas culturais (controlo preventivo), tratamentos usando produtos químicos e agentes de controlo biológico. Arsenito de sódio foi considerado o único fungicida efetivo, contudo foi banido o seu uso a nível mundial em 2003 devido aos seus efeitos carcinogénicos nos pulmões e pele humana e à sua elevada toxicidade para o ambiente. Apesar das diversas tentativas, até ao momento nenhuma estratégia de controlo definitivo foi identificada. Todos os anos, as feridas de poda são a principal porta de entrada para os esporos dos fungos, as quais, caso não sejam protegidas, podem permitir a penetração de muitos agentes patogénicos do lenho como por exemplo, Pch e Pmi. Uma estratégia possível para controlar a doença é proteger as feridas de poda com aplicações de fungicidas e de outros produtos químicos.

Devido ao sucesso limitado dos químicos já testados, existe a necessidade de testar novas substâncias ativas para encontrar uma possível alternativa ao arsenito de sódio e ser eficiente contra os fungos relacionados com o complexo da esca. No presente estudo, o nosso primeiro objetivo foi o de avaliar o efeito de nove produtos químicos sobre o crescimento *in vitro* dos fungos Pch e Pmi. Foram testadas as seguintes substâncias ativas: ciprodinil + fludioxonil, oxicloreto de cobre, oxicloreto de cobre + óleo de verão, blad, fosetil-Al, prata elementar, peróxido de hidrogénio, glutaraldeído e um revestimento betuminoso com base em emulsões aquosas de asfalto (Betkote). As presentes substâncias ativas foram avaliadas por espectrofotometria, usando microplacas de 96 poços com diluições seriadas do químico (diluições de 1:3), com uma suspensão de esporos (5×10^5) e meio de cultura liquido. O segundo objetivo foi de avaliar quatro produtos químicos selecionados como protetores das feridas de poda em condições de campo na casta Cabernet Sauvignon. Foram selecionados e testados os seguintes produtos químicos: Switch® (ciprodinil + fludioxonil), Cuprocol® + Pomorol® (oxicloreto de cobre + óleo de verão), Fracture® (blad) e Betkote tipo 3 (revestimento betuminoso). A aplicação dos tratamentos nas feridas de poda foi realizada no mesmo dia em que foi realizada a poda (dia 0), utilizando diferentes concentrações para cada substância ativa. As inoculações com os esporos de Pch foram feitas 1, 31 e 90 dias após a poda, depositando 25 µl da suspensão de esporos, com a concentração final de 1×10^5 esporos/mL. Três meses após cada infeção, as varas foram destacadas e colhidas. Foram feitos re-isolamentos do agente patogénico para identificar a presença de infeção e colonização da vara. O terceiro objetivo foi monitorizar o crescimento das plantas tratadas com os diferentes produtos químicos nas feridas de poda, através da avaliação de parâmetros específicos. Para tal, foram registados e comparados os estados fenológicos, o comprimento do lançamento do ano do gomo apical, a área foliar total, assim como também foi calculado o rácio peso fresco/peso seco.

Verificou-se no nosso estudo, que a maioria dos produtos químicos testados *in vitro*, em diferentes concentrações, foi eficaz na redução do crescimento micelial de ambos os fungos, à exceção do tratamento Betkote. Os resultados obtidos na vinha mostraram que o tratamento Betkote reduziu significativamente a presença do fungo Pch na primeira inoculação (dia 1). Este resultado mostrou que tal tratamento fornece uma proteção parcial às feridas imediatamente depois da poda. Os tratamentos Switch®, Cuprocol® + Pomorol® e Fracture® não foram eficazes como protetores de feridas, destacando a dificuldade dos produtos em controlar a entrada de Pch, em condições de campo. Estas substâncias ativas podem ter interagido com compostos da madeira ou podem ter sido inativados pelas condições climáticas ou podem ter sido “lavados” da superfície da ferida de poda, entre outras possíveis especulações. Os resultados obtidos na monitorização do crescimento das plantas mostraram diferenças significativas no tratamento Blad. Na primeira recolha de

dados dos estados fenológicos, as diferenças de crescimento vegetativo foram consideráveis. Foi também observado uma grande diferença nos primeiros registos do crescimento reprodutivo, entre o tratamento Blad e os restantes tratamentos e controlo, tendo sido observada uma diferença de 14 dias em crescimento. Através destas observações, o tratamento Blad funcionou como bioestimulante, promovendo um abrolhamento uniforme, com estados mais avançados em fenologia no inicio da fase ativa do ciclo da videira, tanto em crescimento vegetativo como reprodutivo. O estímulo de abrolhamento pode ser favorável ou desfavorável, dependendo da região/país em que é aplicado. Em regiões vitícolas com clima continental, como por exemplo a região de Champagne, pode ser desfavorável visto que as geadas tardias causam grandes prejuízos económicos. O avanço em fenologia poderia levar a prejuízos ainda maiores. Por outro lado, em regiões tropicais, como o caso do Vale de São Francisco no Brasil, esta substância ativa poderia ser usada/testada para quebrar a dormência, garantindo assim um abrolhamento uniforme. Este aumento de crescimento significativo foi também observado no comprimento do lançamento do ano, ao qual foi estatisticamente maior desde o inicio da recolha de dados (17/03/2017), até ao dia 31/03/2017. Contudo, mais estudos necessitam de ter realizados para confirmar estas especulações.

Através da realização deste estudo, foi possível identificar novos produtos químicos capazes de inibir *in vitro* o crescimento de fungos relacionados com o complexo de esca, tendo também sido identificado um tratamento químico para proteção das feridas de poda com a capacidade de reduzir a incidência de Pch. Com este estudo, foi possível melhorar o conhecimento atual em produtos químicos usados contra alguns fungos relacionados com o complexo da esca e a proteção das feridas de poda em condições de campo.

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Abbreviatures

°C	Celsius degrees
µL	Microliter
μm^2	Square micrometre
a.i.	Active ingredient
ANOVA	Analyses of variance
B	Boron
CLSI	Clinical and Laboratory Standards Institute
cm	Centimetre
CU	Copper oxychloride
CU+P	Copper oxychloride plus summer oil
cv	Cultivar
DW	Dry weight
e. g.	*exampli gratia*
EC_{50}	Concentration of fungicide that reduced growth by 50%
Fmed	*Fomitiporia mediterranea*
FW	Fresh weight
g	Gram
GLSD	Grapevine Leaf Stripe Disease
GTD	Grapevine Trunk Diseases
h	Hours
ha	Hectare
hL	Hectolitre
HWT	Hot water treatment
IPMA	Instituto Português do Mar e da Atmosfera
K	Potassium
Kg	Kilogram
L	Litre
LEAF	Linking Landscape, Environment, Agriculture and Food
m	Meter
m^2	Square metre
ml	Millilitre
mm^2	Square millimetre
NC	Negative control
nm	Nanometre
NPK	Nitrogen; Phosphorus; Potassium
ox.	Oxychloride
PC	Positive control
Pch	*Phaeomoniella chlamydospora*
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
Pmi	*Phaeoacremonium minimum*
s	Second
sin.	Synonymous

sp.	Specie
SPD	Sociedade Portuense de Drogas, S. A.
spp.	Species
USD	United States Dollar
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

1. Introduction

Grapevine (*Vitis vinifera* L.) is considered one of the most important fruit species worldwide, both due to its cultivation throughout the world and its high commercial value (Vivier *et al*., 2002). Currently, the total area of vines worldwide is approximately 7.5 million of ha (OIV, 2016) and comprises more than 5,000 cultivars that are used for the production of wine, grape juice and other foods (Ali *et al*., 2010).

Among the numerous grapevine diseases, Grapevine Trunk Diseases (GTDs) are considered the most destructive of the past three decades and, therefore, one of the most relevant challenges for the viticulture and a major concern in all wine producing countries (Bertsch *et al*., 2009; 2013).

The most important GTDs are esca, phomopsis dieback, eutypa dieback and botryosphaeria dieback, that impact the perennial organs of the plant leading to premature decline and dieback in some cases (Bertsch *et al*., 2013). Other diseases like Petri disease or Black-foot disease (*Campylocarpon* spp.*, Cylindrocladiella* spp.*, Dactylonectria* spp.*, Ilyonectria* spp. and *Neonectria* spp.) are the main diseases affecting young vineyards, reducing their productivity and lifespan, thereby causing big economical losses to the industry (Gramaje *et al.*, 2011).

In vineyards affected by GTDs the yield and the grape quality decrease (Surico *et al.*, 2000; Almeida *et al*., 2007; Lorrain *et al*., 2012). When vineyards are no longer economically sustainable to maintain, growers have no alternative but to remove and replant them, decreasing the longevity of a vineyard (Rolshausen *et al*., 2010). Hofstetter (2012) estimated that the economic cost worldwide for the replacement of GTD-linked dead grapevines was greater than 1.5 billion dollars per year (Hofstetter *et al*., 2012) and Vasquez (2007) calculated that losses due to esca disease in California in table grape production were between USD 532 and 1,970 per ha (Vasquez *et al*., 2007).

The frequency of the GTD's around the world over the past few decades has been increasing as described by several authors: in the Spanish vineyards, since 2003 (the year of sodium arsenate ban) the degree of infections grew from 1.8% to 10.5% in 2007 (Rubio *et al.*, 2011); in France, GTDs lowered potential wine production by 13% in 2014 (OIV, 2016); in Austria, a 2,7% annual increase of appearance of foliar symptoms in vineyards in a six-year study was shown (Reisenzein *et al*., 2000); in Italy, in some vineyards, the incidence of esca disease was estimated above 50% (Surico *et al*., 2006); in California, young vine decline is responsible for the replacement of 1 to

5% plants in the new established vineyards (Eskalen *et al*., 2007); in Portugal, the major GTDs are botryosphaeria dieback and esca complex disease, being spread through all over the grape growing regions, especially in Vinho Verde, Dão and Alentejo appellations (OIV, 2016). China, despite being a new wine producing country, has been highly affected specially by botryosphaeria dieback (Yan *et al*., 2013).

The increase in losses is associated to several factors:

- Since the banning of the extremely toxic sodium arsenite (the only treatment which had proven efficient in reducing the appearance of esca foliar symptoms), the incidence of GTDs has grown drastically (Rubio *et al.*, 2011).
- Broad establishment of new vineyards globally has been accompanied by a dramatic increase of young vine decline, a disease expressing similar foliar symptoms as esca but occurring in grapevine plants 1 to 9 years old (Hofstetter *et al*., 2012).
- Infections are brought to vineyards from the nurseries planting material (Rubio *et al.*, 2011).
- Cultural practices in vineyards were typically focused on grape yield, with little attention paid to pruning and poor protection of pruning wounds (Rubio *et al.*, 2011).
- Perhaps the most important factors of all are the externally asymptomatic nature characteristic of most GTDs stages and the absence of non-destructive diagnostic procedures.

All species of *Vitis* spp. and varieties of *V. vinifera* are susceptible to esca (Mugnai *et al*., 1999), but vary in degree of susceptibility. Varieties such as Cabernet Sauvignon, Sangiovese, Trebbiano Toscano, Thompson Seedless, Sauvignon blanc, Mourvèdre, Ugni blanc, Cinsault, Trousseau or Temperanillo (sin. Tinta Roriz or Aragonez) are more prone to express both foliar and wood esca internal symptoms in comparison to Merlot, Pinot Noir, Carignan, Roussane, Montepulciano (Almeida, 2007; Quaglia *et al*., 2009 Lorrain *et al*., 2012).

Cabernet Sauvignon is the main variety cultivated worldwide, with over 263,045 ha (Puckette, 2014) and therefore an important variety to be studied. Moreover, it has been demonstrated to be a susceptible cultivar to esca symptoms (Christen *et al*., 2007; Lorrain *et al*., 2012).

1.1 Grapevine Trunk Diseases

As abovementioned the most concerning trunk diseases that affect established vineyards are eutypa dieback, botryosphaeria dieback, phomopsis dieback and the esca disease complex.

Eutypa dieback, also referred as eutypiosis, is caused by the fungus *Eutypa lata* (Ascomycota, Diatrypaceae) and other species, which is a potential pathogen for more than 80 woody plant species (Bertsch *et al*., 2013). *E. lata* infects grapevines through pruning wounds during the dormant season, by means of ascospores released from perithecia after rainfall events (Webber *et al*., 2007).

Symptoms are characterized by the shrivelling of canes with short internodes which present chlorotic, wrinkled and ripped leaves with marginal necrosis and dead interveinal tissue that are easier to detect during spring (Fig. 1-AB) (Moller *et al*., 1974). Depending on the severity, eutypiosis can dry out inflorescences before opening or clusters *millerandage* (OIV, 2016). The death of the cane can occur (dead arm) and a typical wedge-shaped necrosis (Fig. 1-C) is observed in the trunk or cordons (Webber *et al*., 2007).

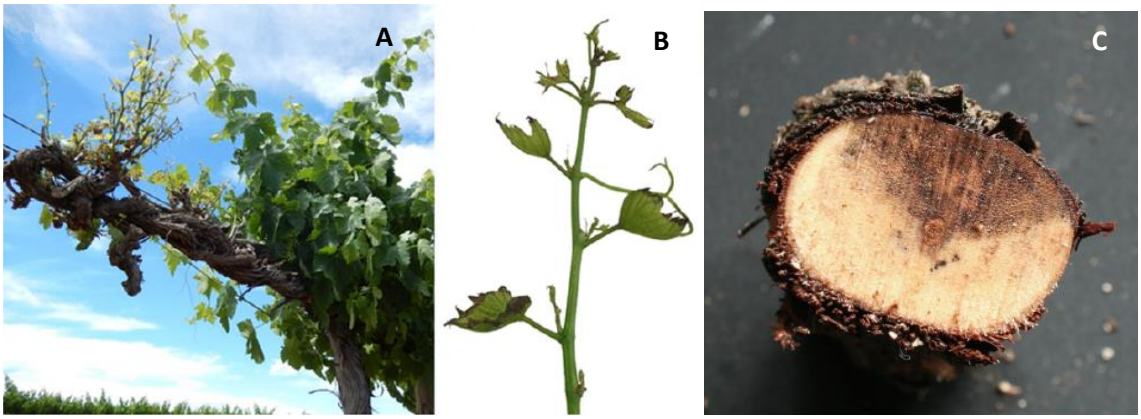

Fig. 1 – A and B - Typical leaf symptoms of *Eutypa lata*. C - Wedge-shaped necrosis, a wood symptom of eutypa dieback (adapted from Almeida *et al*., 2007; Gramaje *et al*., 2018).

Botryosphaeria dieback, also called *Botryosphaeria* canker or black dead arm, infects grapevines and several varieties of fruit trees, inducing a large number of decays with big economic impact (Bertsch *et al*., 2013; Olmo *et al*., 2017). To date, at least 21 Botryosphaeriaceae species were identified as wood pathogens to *V. vinifera* (Úrbez-Torres, 2011), with the most common species isolated worldwide: *Diplodia seriata*, *Neofusicoccum parvum, Diplodia mutila, Lasiodiplodia theobromae, Botryosphaeria dothidea, Botryosphaeria lutea, Neofusicoccum australe and Neofusicoccum luteum* (Bertsch *et al*., 2013).

Symptom expression caused by Botryosphaeriaceae species has been shown to vary from region to region, particularly due to differences in climate conditions and grapevine varieties (Úrbez-Torres, 2011). These fungi are xylem–inhabiting organisms that cause perennial cankers in the wood, expressing a typical sectorial necrosis with vascular discoloration in the trunk (OIV, 2016); leaf chlorosis, with a characteristic yellowish-orange (white cultivars) or wine-red (red cultivars) spots on leaf margins and blades (Fig. 2) (Larignon *et al*., 2001; Reis *et al*., 2016); mortality of buds and the shrivelling/drying of inflorescences or fruit cluster. These symptoms may lead to the death of the plant (Gramaje *et al*., 2011; Reis *et al*., 2016).

Fig. 2 – Two symptoms of botryosphaeria dieback. A - Drying of the inflorescence; B - the characteristic yellowish-orange spots on the margins of the leaves on a white cultivar (Sauvignon Blanc). (adapted from Bertsch *et al*., 2013).

Phomopsis dieback, is caused by *Phomopsis viticola* (OIV, 2016). Phomopsis spreads its spores during wet springs by rain splashing and is more severe in regions characterized by a humid weather through the growing season (Rawnsley, 2012; Úrbez-Torres *et al*., 2013). *P. viticola* produces cankers in the woody tissues of grapevine.

The esca disease complex is associated to five syndromes: brown wood streaking of rooted cuttings, Petri disease, grapevine leaf stripe disease (GLSD, also known as 'young esca'), white rot and esca proper (Surico *et al*., 2008). The most commonly associated fungi are *Phaeomoniella chlamydospora* (Pch), *Phaeoacremonium minimum* (Pmi) and *Fomitiporia mediterranea* (Fmed) (Bertsch *et al*., 2013). Esca occurs in both hemispheres, with Pch and Pmi widely distributed in all grape-growing regions, while Fmed is more common in Europe; in other parts of the world, wood rotting is caused by other *Fomitiporia* species (Surico *et al*., 2008; Bertsch *et al*., 2013). The syndromes produced by these fungi and their severity depends on various factors, such as the age in which the plant becomes infected, the susceptibility of grapevine cultivar and rootstock (Feliciano *et al*., 2004; Eskalen *et al*., 2007), and the concurrence of abiotic stresses (Surico *et al*., 2008). Wounds generated from vine

pruning are the main point of entry for fungal spores (Mugnai *et al*., 1999; Bertsch *et al*., 2013; OIV, 2016).

The development and evolution of esca disease may be grouped in two types: chronic and acute (Mugnai *et al.*, 1999). The evolution of chronic esca is associated to internal symptoms in the trunk and main branches and external symptoms in leaves and bunches. Internally, the most common symptoms are characterized by white rot caused by Fmed and brown wood streaking mainly by Pch and Pmi (Mugnai *et al*., 1999). Externally, symptoms on leaves are characterized by light green or chlorotic, rounded or irregular spots between veins. These spots expand progressively and coalesce, frequently becoming necrotic, so that in the end only a small strip of unaffected green tissue remains along the main veins, assuming a “tiger-stripes” pattern. However, foliar symptoms may not occur in the same plant every year. On berries, symptoms are characterized by spotting of berries called black measles (Mugnai *et al*., 1999). The esca disease complex is dealt with in more detail in the next section.

1.2 Esca disease complex

1.2.1 Fungi involved in esca disease complex

Two tracheomycotic agents, Pch and Pmi, and several basidiomycetes species, most commonly Fmed, are associated with the esca complex (Fig. 3) (Fischer, 2006; Bertsch *et al*., 2013).

Phaeomoniella chlamydospora (W. Gams, Crous, M.J. Wingf. & L. Mugnai) (Crous *et al*., 2000), is an anamorphic member of the *Herpotrichiellaceae* family (Crous *et al*., 2000). Pch occurs in grapevines worldwide and its optimal growth temperature is 25 °C (15 °C minimum; 35 °C maximum). When subjected to an optimal temperature for growth the fungus can reach a radius of 5 to 6 mm after 8 days in the dark (Crous *et al*., 2000).

Twenty species of *Phaeoacremonium* have been described to date (Gramaje *et al*., 2009), 15 of which have been isolated from grapevines worldwide (Mostert *et al*., 2006). The most common and widely distributed in vines is *Phaeoacremonium minimum* (Gramaje *et al.,* 2015), previously known as *Phaeoacremonium aleophilum* (Crous *et al*., 1996; Mostert *et al*., 2006; Gramaje *et al*., 2009). *Togninia minima* (Diaporthales) is the sexual stage of Pmi (Gramaje *et al*., 2009). Its optimal temperature for growth is at 35 °C (10°C minimum; above 35°C maximum) (Crous *et al*., 1996). When subjected to an optimal temperature for growth, the fungus can reach a radius of 4 mm after 8 days in dark. Cultures show a honey colour (Crous *et al*., 1996). *Phaeoacremonium* shares different hosts, but are mostly larvae of bark beetles, humans and wood. Host plants near a vineyard can be a potential source of inoculum for vines (Mostert *et al*., 2006).

Fomitiporia mediterranea (M. Fischer) (hymenochaetales) (Fischer, 2002) is the main causal agent of white rot that occurs inside the trunk and main branches in grapevines. It is especially common in Europe and has been isolated from a large number of woody hosts, such as *Acer negundo, Actinidia chinensis, Olea europaea and Corylus avellana* (Fischer, 2002; 2006; Bertsch *et al*., 2013; OIV, 2016). Its optimal temperature for growth lies between 15 °C and 35 °C (Fischer, 2002). Fmed spreads by means of airborne basidiospores and regularly outcrosses in nature (Bertsch *et al*., 2013). A laccase was purified from Fmed that is able to oxidize a large range of different natural phenolic and polyphenolic compounds, indicating that some oxidative processes could be related to esca symptoms (Abou-Mansour *et al*., 2009; Lorrain *et al*., 2012; Bertsch *et al*., 2013).

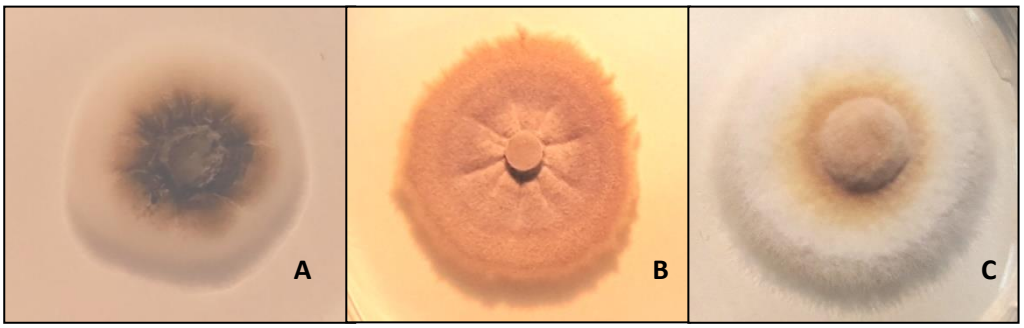

Fig. 3 - Colonies of the three main fungi involved in esca complex disease. A - *Phaeomoniella chlamydospora*; B – *Phaeoacremonium minimum*; C – *Fomitiporia mediterranea*.

1.2.2 Source of inoculum and dissemination

In the vineyard

Grapevine exhibits the highest risk of infection by airborne spores during the pruning season due of the high number of wounds inflicted on each plant, combined with the frequency of rainfall events that may occur in the same period (Eskalen *et al*., 2007). The wounds remain susceptible to infection by Pch and Pmi for up to 16 weeks after pruning, varying with the region's environment and other factors (Eskalen *et al*., 2007; Serra *et al*., 2008; Martin *et al*., 2009). Every year, a poor wound protection will favour the entrance of these fungi, including Fmed and others that can be pathogenic to vines (Surico *et al*., 2008).

The spread of Pch spores is associated with rainfall events and is made by airborne inoculum, entering through fresh wounds, causing new infections (Surico *et al*., 2008; Serra *et al*., 2008; Bertsch *et al*., 2013) However, spores of Pch can be found in the air throughout the whole year (Larignon *et al*., 2000). Pmi also penetrate the plant through pruning wounds (Larignon *et al*., 2000; Serra *et al*., 2008). However, the spores were not found during winter but during the vegetative growing season, indicating that Pmi possibly can penetrate the plant also via some other route other than pruning wounds. Spores liberation is not always linked to rainfall events (Larignon *et al*., 2000; Eskalen *et al*., 2001; Mostert *et al*., 2006).

In nurseries

Studies showed that many nursery practices are unsafe and may facilitate the infection and wood colonization by Pch and Pmi in rooted cuttings. Fungi that are involved in trunk diseases were found in rehydration baths, grafts tools, substrates in pots (e.g. sawdust) and in the water of some nurseries (OIV, 2016). Consequently, vines ready to be planted in the field can present dark streaks near the graft junction that could extend through the entire length of the cutting (Surico *et al*., 2008). The

presence of Pch and Pmi was reported to occur in canes of mother vines (Halleen *et al*., 2002; Gramaje *et al*., 2011). The ratios of the infections could increase from 40% before cutting to 70% after nursery processing (Gramaje *et al.*, 2011). Therefore, would be very important to detect infected material before plantation, to ensure longevity of newly established vineyards (Urbez-Torres *et al*., 2015).

1.1.1 Impact on the physiology of the plant and on wine

The characterization of grapevine physiology is an important key step to obtain accurate knowledge of physiological mechanisms that lead to disease development and the appearance of symptoms (Bertsch *et al*., 2013).

The wood and plant reserves

In the vineyard, esca symptoms consist in a reduced growth of canes, as well as the root system (Wagschal *et al*., 2008), leading to a decrease in the plant carbohydrate reserves during the winter rest, as a consequence of the decrease of total leaf photosynthetic rate during the previous season, thus contributing to a significant reduction in plant vigour and development during the following year or, in worse cases, to plant death (Petit *et al*., 2006; Bertsch *et al*., 2013; Fontaine *et al*., 2016). Internally, the sap flow of an asymptomatic vine decreases, in particular a week before the first leaf symptoms become visible (Bertsch *et al*., 2013). During hot days and especially when the grape load is high, when water demand is intense, the sap flow is about two times lower in symptomatic vines (when compared to asymptomatic ones) and a decrease in water transport through xylem vessels is observed, as well as in leaf transpiration rate and in the stomatal conductance (Christen *et al*., 2007; Ouadi *et al*., 2017).

The leaves

In symptomatic leaves, photosynthesis changes in cases of GLSD. According to Petit et al. (2006), external foliar symptoms and internal wood symptoms are associated with: (i) a decrease in CO_2 assimilation; (ii) a significant increase in intercellular CO_2 concentration; (iii) a significant drop in both maximum fluorescence yield and the effective photosystem II quantum yield; and (iv) a reduction of total chlorophyll (Petit *et al*., 2006). In another study, the alteration of the photosynthetic apparatus was detected two months before the first appearance of foliar symptoms (Christen *et al*., 2007).

Recent studies showed that the levels of *trans*-resveratrol and other phenolic compounds increases in asymptomatic GLSD-affected vine leaves after the first foliar symptoms start to show (Fontaine *et al*., 2016). Calzarano et al. (2016) showed that the most common phytoalexin found in symptomatic leaves was *trans*-resveratrol, being the amount of phytoalexins higher in symptomatic leaves than in asymptomatic ones. This study provided evidence that the seasonal pattern of development of GLSD symptoms might be related to the level of phytoalexins in the leaves and the grapevine growth stage (Calzarano *et al*., 2016).

Phytotoxic metabolites produced by Pch and Pmi in *in vitro* cultures were identified as two naphthalenone pentaketides (scytalone and isosclerone) and pullulans (α-glucans), polysaccharide polymers of maltotriose (Mostert *et al*., 2006; Bertsch *et al*., 2013). Scytalone caused light green to chlorotic, round to irregular, interveinal or marginal spots when applied on detached leaves of cv. Italia (Mostert *et al*., 2006). Isosclerone caused large, coalescent chlorotic and necrotic spots followed by distortion of the lamina and shrivelling (Mostert *et al*., 2006). These phytotoxic compounds were tested on different grape varieties, inducing similar symptoms to those shown by the leaves of esca-affected vines (Bruno *et al*., 2007).

The berries

Lorrain et al. (2012) analysed symptomatic bunches in Cabernet Sauvignon from the Bordeaux region for two years, demonstrating that the chemical composition was affected. In the symptomatic bunches, big differences were observed in tannin characteristics and in grape skin phenolic composition, which were significantly lower in total anthocyanins as well as in catechins and epicatechins (proanthocyanidins), compared to healthy skins (Lorrain *et al*., 2012). Infections with esca pathogens generally results in a significant increase in total phenolic compounds inside the wood tissues, especially resveratrol, proanthocyandins and anthocyanins (among others), boosting the plant defence mechanisms (Lima *et al*., 2010; Lorrain *et al*., 2012). The increase in accumulation of antifungal phenolic compounds in the wood that are known to inhibit fungal growth might be an alternative explanation for the lower phenolic compound concentrations found in esca-affected grapes, which has a big impact on wine quality (Lorrain *et al*., 2012).

Symptoms such as ‘black measles’ - characteristic dark brown spots - has a large impact in table grape varieties because the affected clusters are not marketable, leading to big economical losses (Rolshausen *et al*., 2007).

The wine

Wine made from grapes (cv Cabernet Sauvignon) containing over 25% symptomatic berries was characterized by a reduction in the sugar content, a proportional increase of total acidity and an increment of assimilable nitrogen (Lorrain *et al*., 2012). These results showed that esca-affected grapes, when compared to asymptomatic ones, are not at the same maturity stage when harvested, causing a delay in ripening due to the reduction of photosynthesis (Calzarano *et al*., 2009). Due to this delay in ripening, the wine shows a loss in quality, a decrease in the fruity aroma, and an increase in the earthy and vegetal characters ('green' aroma) in sensory analysis (Lorrain *et al*., 2012). Judges were able to distinguish and discriminate differences in wines made with healthy grapes from wines prepared with grapes including as little as 5% of esca affected fruits (Lorrain *et al*., 2012).

1.2.3 Symptomatology

Brown wood streaking of rooted cuttings

This disease is caused by the tracheomycotic fungi (Pch and Pmi) (Bertsch *et al*., 2013) and is characterized by the absence of external symptoms (Surico *et al*., 2008). Internally, different types of wood deterioration are observed, such as dark streaking, isolated or gathered into a blackish-brown bundle that starts frequently in the graft union and goes upwards (more often) or downwards infecting all plant, and black spots, surrounding the pith or sparse over the cross section surface. These black spots correspond to a kind of black gum-like exudate that oozes from those black spots. (Surico *et al*., 2008).

Petri disease

This disease was first reported in 1912 by the pathologist Lionello Petri (Eskalen *et al*., 2007; Surico *et al*., 2008) and is caused by Pch and/or by some species of *Phaeoacremonium* sp., but is more often associated to Pmi (Mostert *et al*., 2006; Bertsch *et al*., 2013). It may also be related to *Cadophora luteo-olivacea* and *Pleurostoma richardsiae* (Agustí-Brisach *et al*., 2013). Petri disease affects very young vines, from 1 to 7 years old, causing significant losses in newly planted vineyards (Eskalen *et al*., 2007).

Internally symptoms can be seen in the trunk and cordons (Mostert *et al*., 2006). Petri disease's affected plants show a darkened central pith, black dots circumscribing

the pith or sparse over the cross section surface, seen as brown to black streaking in longitudinal sections. The black dots correspond to blockage of the xylem vessels, showing a dark, gummy substance called ‘black goo’, containing polymerized phenolic compounds inside these vessels as a response by the host against the fungus growing in and around the xylem vessels (Mugnai *et al*., 1999; Mostert *et al*., 2006; Surico *et al*., 2008). Supposedly, they aim at preventing fungal dissemination within the plant.

Externally, the plant shows an interruption of growth, chlorotic leaves, yield losses and a decline in vigour leading, in some cases, to the death of the vine (Surico *et al*., 2008; Bertsch *et al*., 2013).

Grapevine leaf stripe diseases

In the past known as “young esca” (Surico *et al*., 2009), affecting vines as young as 2 to 3 years old (Surico *et al*., 2008), GLSD is caused by Pch and/or Pmi. Internally, different symptoms may appear, such as dark streaking, black spots and wood necrosis (brown or light brown) (Fig. 4-A) (Surico *et al*., 2008; Calzarano *et al.*, 2016).

Externally, the main symptom is the typical leaf symptom of esca called ‘tiger stripes’, with leaves showing interveinal chlorotic areas and reddening of leaves resulting in necrotic stripes with only a narrow green stripe along the midrib (Fig. 4-B) (Surico *et al*., 2008). It was demonstrated by Sparapano et al.s (2001) that the first leaf symptoms occurs years after infection by wood pathogens. Leaf symptoms normally appear in late spring/early summer (Surico *et al*., 2008).

On the berries, particularly on white varieties, ‘black measles’ - characteristic dark brown spots (Fig. 4-C) - may occur on the skin (Mugnai *et al*., 1999; Surico *et al*., 2008). Berries with heavy spotting often show skin cracks and become prey to soft rotting fungi or bacteria (Mugnai *et al*., 1999).

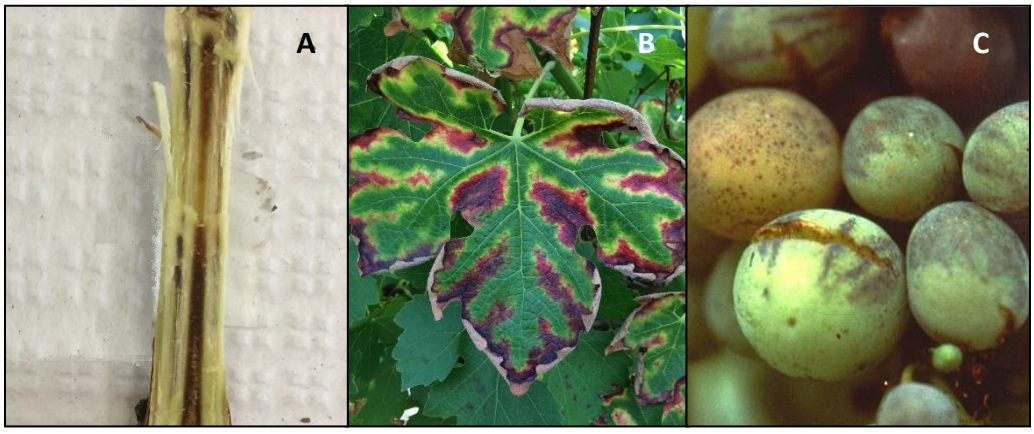

Fig. 4 – A – Internal symptom characterized by brown wood streaking observed in a 1-year-old cane in cv Cabernet Sauvignon, Lisbon region (Photo: João Costa); B – Foliar symptom with the typical “tiger stripe” pattern in cv Cabernet Sauvignon in early June, Lisbon region (Photo: João Costa); C – Spotting of grape berries with some visible cracks (Adapt from: Mugnai *et al*., 1999).

White rot

White rot occurs in vine trunks and branches of mature vines and is caused by Fmed and/or other basidiomycetes (Surico *et al*., 2008). The rot caused by this fungus is a typical white decay, transforming the wood into a spongy, soft whitish-yellow mass that is delimited by a dark necrotic area surrounding the rotten wood separating the apparently healthy tissues from the decayed ones (Fig. 5) (Surico *et al*., 2008). The rotten wood is completely non-functional. However, if only the last 2 or 3 wood rings maintain their capacity to transport water and solutes, the vine will continue to vegetate normally and will be productive. In contrast, if the last 2 or 3 wood rings also become affected by tracheomycosis or apoplexy, the vine will die (Surico *et al*., 2008).

Fig. 5 - Cross section of a grapevine trunk showing white rot, caused by *Fomitiporia* spp., highlighting the yellowish, soft and spongy rotted wood surrounded by a marginal band of brownish red wood (Adapted from: Bertsch *et al*., 2013).

Esca proper

This syndrome identifies plants that exhibit the effects of the three major fungi associated with esca complex: Pch, Pmi and Fmed. Therefore, symptoms are associated to those of the tracheomycosis and with those of white rot (Fig. 6-A) (Surico *et al*., 2008).

As mentioned before, the development and evolution of esca disease may follow two routes: chronic and acute. Chronic esca includes internal and external symptoms, characterized by the typical symptoms of Pch, Pmi and Fmed (Mugnai *et al*., 1999), already described. Foliar symptoms may not appear in the same plant every year (Surico *et al*., 2008).

Apoplexy, also known as acute or severe form of esca, is characterized by the sudden wilting, followed by the shrivelling and drying of the berries and leaf drop. The healthy-looking leaves dry up in a few days (Fig. 6-B) (Mugnai *et al*., 1999). Normally, this event happens in late spring/early summer, when a rainfall event is followed by dry and hot weather, which may lead to an imbalance between foliar transpiration and root absorption (Mugnai *et al*., 1999; Surico *et al*., 2006; Surico *et al*., 2008). After this

event, the plants can recover growth in the current season or the following one, but, more often, the vine dies (Bertsch *et al*., 2013).

The most accepted theory is that apoplexy is caused mainly by a dysfunction of the conducting xylem caused by white rot and tracheomycosis (Surico *et al*., 2008).

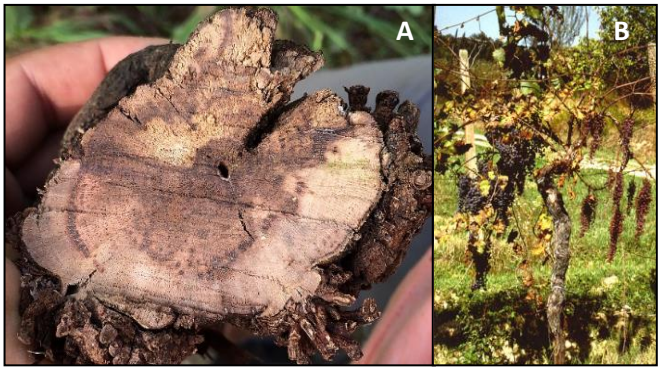

Fig. 6 – A - Cross section through the trunk of an old vine (over 30 years old) showing black spotting, brown-red wood characterized by the presence of tracheomycotisis fungi (Pch and/or Pmi) and symptoms of white rot (Fmed), in cv Touriga Nacional; B – Leaf dissection and fall are characteristic symptoms of apoplexy. Leaves with healthy appearance quickly wither, drying completely in a few days (Adapted from: Mugnai et al., 1999).

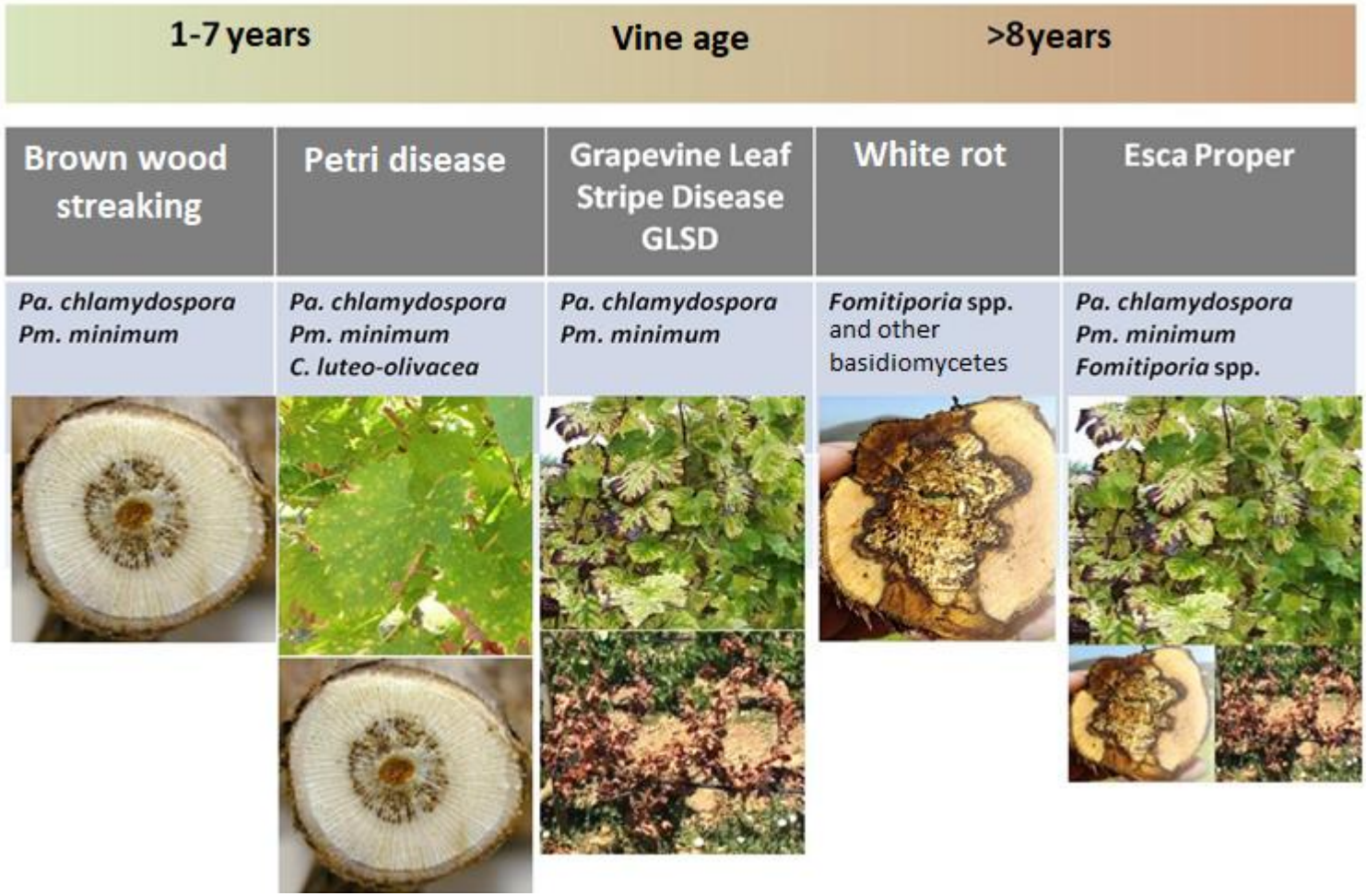

Fig. 7 – The different syndromes of esca complex disease, according to the age of the vine, to the type of wood and foliar symptoms and/or pathogens infecting and acting into the vine. Adapted from: Mondello *et al.,* 2017).

1.3 Control strategies and mitigation

1.3.1 In nurseries

The implementation of appropriate sanitary conditions is crucial to ensure that perfect healthy plants are being produced to be delivered to grape producers for the successful beginning and sustainability of all vineyards (Surico *et al*., 2008; Gramaje *et al*, 2011).

Hot Water Treatment (HWT) has been used for disinfection of propagating material to obtain commercial plants in good sanitary conditions (Moretti *et al*., 2005). HWT is generally performed at 50 °C for 30 min. However, it is not immune of collateral effects concerning the viability of the plant material treated (Moretti *et al*., 2005; Bertsch *et al*., 2013). If the treatment is not correctly applied, it may result in loss of plant material. *V. vinifera* cultivars display different degrees of sensitivity to HWT and the range of temperatures used depends on the pathogens that need to be neutralized (Bertsch *et al*., 2013). A study showed that HWT at 50 °C for 30 min reduced the presence of Pch by 78% (Larignon *et al.,* 2009). At the same temperature and time, on the rootstock (110R and 101-14 Mgt) immediately before grafting, a significant decrease in the incidence of Pch and Pmi was observed at the base of the rootstock of uprooted grafted grapevines (Fourie *et al*., 2004).

Formulations of biological agents such as *Trichoderma* spp. to control Pch and Pmi in the nursery produced good results, improving the root mass and plant development, leading to lower rates of pathogen infection (Fourie *et al*., 2001; Di Marco *et al*., 2004).

The use of electrolyzed acid water in cutting hydration after the cold-stored period showed that it was effective in reducing conidial germination of Pch and Pmi without affecting plant growth and development in nursery fields (Di Marco *et al*., 2009).

1.3.2 Cultural practices

Preventive control under vineyard conditions is very important to reduce the risk of infection. A study by Surico et al. (2008) recommends the following points:

- Eliminate the infected vines that display severe symptoms of esca;

- Remove and burn the canes and pruning residues as well as dead/dying vines to reduce the inoculum load in the field since the propagules are in all lignified parts of the vine;
- Whenever possible, use propagation material in good sanitary conditions;
- Application of fungicides and wound protectant sealants against esca and other GTD pathogens to achieve good disease control and to reduce the spread of diseases to other vines;
- Identify and record the infected vines and then prune them separately;
- Adapt the pruning period to the region and their climate;
- Avoid mechanical harvest.

Other authors also gave some additional recommendations:

- Before plantation, chose the variety and rootstock considering the degree of sensitiveness to trunk diseases. Rootstock crosses of *V. riparia* × *V. berlandieri* can be less susceptible to Petri disease pathogens (Gramaje *et al.,* 2010);
- Chose a vine training and pruning system that causes smaller wounds to cicatrize faster, giving less opportunity for esca fungi to penetrate the vines (Lecomte *et al*., 2017);
- Prune grapevines in dry weather since airborne fungi are almost absent under these conditions (Rolshausen *et al*., 2010).

Regarding the pruning of grapevine, two factors should be taken in consideration: (i) the period of the year when esca-related fungal spores are more easily spread, and (ii) the speed with which pruning wounds will cicatrize. Taking these factors in account, it is recommended to adapt the pruning period to the climate of the region (Surico *et al*., 2008). In regions with more severe winters, pruning should be delayed ensuring that when the vines are pruned, the wounds that were made will heal more rapidly, and in regions with milder winters, pruning should take place as early as possible, to heal the wounds before they become infected by the newly disseminated spores (Surico *et al*., 2008).

Vine training and pruning decisions have a big impact in the amount of esca-affected plants and the simplification of trunk structure observed in recent decades might have favoured the increasing incidence of this disease. In fact, it was demonstrated that, on cv Merlot, vines trained in lyra had 14% esca incidence (both leaves and wood symptoms), compared to 36% for those trained as Guyot. Also, the pruning decision is very important. For example, in cv Chardonnay with Guyot training system, vines pruned normally had 16% esca incidence compared to 38% for those subjected to a severe pruning regime (Lecomte *et al*., 2017). Another pruning system

that showed the potential to reduce trunk diseases is Guyot Poussard (Osti *et al*., 2017).

An ancient custom and interesting fact regarding esca, particularly in Mediterranean countries, was to open the trunk in the middle with an axe and to insert a stone as a wedge to leave the rotted tissues exposed to the air. This practice delayed the recurrence of symptoms for some years (Marin, 2000; Surico *et al*., 2008).

1.3.3 Chemical treatments

As introduced before, fresh pruning wounds are considered the main infection route for fungal disease pathogens (Mugnai *et al*., 1999; Martin *et al*., 2009; Díaz *et al*., 2013). It is therefore important to protect pruning wounds against esca-related fungi and other wood pathogens. Chemical control is based on the protection of exposed wounds using fungicides in order to prevent grapevine infection. To date, the number of available commercial products that can protect pruning wounds is limited (Bertsch *et al*., 2013).

Sodium arsenite was considered the only effective fungicide available to reduce the impact of esca complex disease (Bertsch *et al.,* 2013; OIV, 2016; Mondello *et al*., 2017). This fungicide was banned worldwide in 2003 because of its potent toxicity to all live forms, its carcinogenic effects on human lung and skin, high toxicity to environment and the accumulation in the food chain (Di Marco *et al.*, 2000; Bertsch *et al.,* 2013; Mondello *et al*., 2017). Nowadays, this chemical has been in focus of attention, for research purpose, to better understand its efficacy and mode of action in order to find an effective substitute product (Vallet *et al*., 2017).

Several studies were conducted with different active compounds to control trunk diseases on pruning wounds, but only a few gave positive results. For example, as paintbrush treatment was tested, thiophanate-methyl (Topsin M®), cyproconazole + iodocarb treatment (Garrison® – comercial tree wound past), boric acid in a wound-sealant paste (Biopaste®) and a pyraclostrobin formulation (Cabrio®) showed positive results as wound protectants, but with variations in the efficacy among species (Eskalen *et al*., 2007b). Also, myclobutanyl applied as a spray treatment in the pruning wounds gave positive results against Pch and Pmi (Herche, 2009). In another study, spraying a wound with pyraclostrobin + boscalid (Tessior®) lead to a decrease of infection of grape wood and showed long-lasting activity during the susceptible phase of wounds (Kuhn *et al*., 2017).

Other substances like tebuconazole, flusilazole, benomyl, prochloraz (Rolshausen *et al*., 2010), prothioconazole + tebuconazol, fluazinam (Gramaje *et al*,

2010), mancozeb, fenarimol and procymidone (Amponsah *et al.,* 2012) showed positive effects against GTDs *in vitro*, but some of them cannot be used because of health and safety concerns (Bertsch *et al.,* 2013).

Chitosan, due to its fungicide property, was studied both *in vitro* and *in vivo*. Results showed that chitosan was effective in reducing mycelial growth of Pch and of other grapevine trunk diseases' related fungi. The application of chitosan improved the vine growth (plant height and number of roots) and reduced fungal wood colonization compared to untreated plants (Nascimento *et al*., 2007).

Despite the tentative use of alternative chemically active compounds, no long-term and effective control was attained to date (Bertsch *et al.,* 2013).

1.3.4 Biological control agents (BCA)

Inoculation with different species of *Trichoderma* spp. at various stages of rooted cuttings resulted in more vigorous plants and reduced, indirectly, their susceptibility to esca pathogens, inducing resistance mechanisms (Fourie *et al*., 2001; Di Marco *et al*., 2004). *Trichoderma* spp. have been tested as wound protectants against pathogens related to esca complex disease, botryosphaeria and eutypa dieback (Bertsch *et al*., 2013). Healthy vines can be inoculated with *Trichoderma harzianum,* which colonizes the woody tissues (trunk and cordon) and lives in association with the pith parenchyma cells of healthy vine tissues, working as a vaccine against pathogens (John *et al*., 2001; Hunt, 2004). The effectiveness can be compromised because it needs a period for complete colonization, during which the pruning wounds are vulnerable to infection by pathogens (John *et al*., 2008). Another experiment tested a bio-fungicide comprising *Trichoderma asperellum* and *Trichoderma gamsii* (Remedier®), sprayed on the fresh pruning wounds (1 to 2 days after pruning) (Aloi *et al*., 2017). Results showed a 45 to 65% reduction of leaf symptoms in treated vines when compared to the untreated vines, and in older vineyards a reduction on vines showing leaf symptoms and also a reduction in the mortality of plants were observed.

Some species of *Epicoccum* have been studied, both *in vitro* and *in planta*, against Pch. The strains tested *in vitro* showed inhibition of growth of Pch. The most efficient strain was after evaluated *in planta.* The results showed a significant reduction in re-isolations of the pathogen, suggesting it worked as an antagonist against Pch's spread (Del Frari *et al*., 2017).

An *in vitro* study showed antagonistic activity produced by *Bacillus subtilis* strain (AG1) as it inhibited mycelial growth of Pch and Pmi (Alfonzo *et al*., 2009).

Other possible BCA like *Fusarium lateritium* (John *et al*., 2005), *Erwinia herbicola* (Rolshausen *et al*., 2001), *Cladosporium herbarum* (Rolshausen *et al*., 2005), *Aureobasidium pullulans* and *Rhodotorula rubra* (Munkvold *et al*., 1993) were also reported in different studies as effective against GTDs pathogens, alone or combined with fungicides. Despite this efficacy, some of these studies were just conducted *in vitro* or in nurseries, and further research is required (Bertsch *et al*., 2013).

Collectively, these studies have shown the potential of antagonistic organisms to partially protect grapevine pruning wounds but, to date, their use is still very limited.

2. Aim of the study

Due to the limited success of the currently available chemicals, there is a need of testing other different active ingredients in order to find an alternative to sodium arsenite and to be efficient against esca related fungi. The evaluation of either existing or novel chemicals that can be used to reduce the incidence of esca complex disease and other GTDs has been a major priority for industry and researchers during the past two decades (Mondello *et al*., 2017). This study was conducted to improve the current knowledge in chemicals against fungi and pruning wound protection, using different types of chemicals in order to find a possible alternative treatment that could provide wound protection, thus avoiding or reducing new infections.

The present study had the following aims:

I – Testing different chemicals, *in vitro*, on the mycelial growth of *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*;

II – Testing selected chemicals as pruning wound protectants against Pch on cv Cabernet Sauvignon, under field conditions;

III – Monitoring the treated plant's growth by evaluation of specific parameters.

By doing this study, (i) we aim to categorize novel chemicals, *in vitro*, as capable of greatly inhibiting the growth of Pch and Pmi, and that can be used as pruning wound protectants, for trunk injections or in propagation processes in nurseries; (ii) and to identify any of the chemicals tested as pruning wound protectants as an efficient protection that may last until pruning wounds cicatrize, therefore preventing infections by Pch.

3. Material and Methods

3.1 Testing different concentrations of fungicides on mycelial growth of Pch and Pal *in vitro*

3.1.1 Fungi tested

The fungi used for the *in vitro* experiments were *Phaeomoniella chlamydospora* (CBS 161.90) and *Phaeoacremonium minimum* (CBS 110713), both isolated in South Africa.

The cultures were maintained in 90 mm vented Petri dishes containing 15 ml of 3.9 % w/v Potato Dextrose Agar (PDA) (Difco, Becton, Dickinson and C, Franklin Lakes, NJ, USA), and stored in an incubator at 25 °C, in the dark, for 2 weeks.

3.1.2 Fungicides

Several fungicides were chosen to test their efficacy against the selected fungi. The concentrations of fungicides were selected by preliminary trials.

- Cyprodinil (37.5% w/w) + Fludioxonil (25% w/w) (Switch®, Syngenta) used in a wide range of vegetables and fruit crops. In grapevine is used as a systemic and contact fungicide against *Botrytis* spp. (More info: https://www.syngenta.co.uk/product/crop-protection/fungicide/switch);
- Copper Oxychloride (36.5% w/w) (Cuprocol®, Syngenta) is a fungicide with preventive action, used in several crops. In grapevine is used against *Plasmopara viticola* (the causal agent of downy mildew) and it was also reported to control *Phomopsis* spp.. (More info: https://www.syngenta.pt/product/crop-protection/fungicida/cuprocol).
- Blad (20% w/v of Blad) (Fracture®, CEV/CONVERDE), discovered in 1991 (Instituto Superior de Agronomia, Lisbon University, Portugal), is a naturally-occurring 20 kDa polypeptide which is extracted from lupine (*Lupinus albus*) plantlets, having a powerful and broad-spectrum antifungal activity and being non-toxic for humans, animals and the environment. This fungicide is used as a contact fungicide with penetration properties and it is a multi-target fungicide; one of its modes of action involves degradation of fungal chitin (Monteiro *et al*.,

2015). In grapevine it was successfully tested against *Botrytis spp.* and *Erisiphe necator* (the causal agent of powdery mildew). It is also bacteriostatic and exhibits a powerful plant biostimulant activity. (More info: http://www.cev.com.pt/);

- Fosetyl-Al (74.6% w/w of fosetyl-Al) (Aliette Flash®, Bayer) is a systemic fungicide used on several crops, mainly against *Pythium* spp. and *Phytophthora* spp.. Indirectly, this product is also a plant-defence elicitor (More info: http://www.bayercropscience.pt/internet/produtos/produto.asp?id_produto=97#propriedades).
- Elemental silver (1,000 ppm of elemental silver) (AgroArgentum, BioBac®, M.H.I Compania de Ingenerie S.R.L.), is mostly used as a plant defense elicitor for a wide range of crops. This product has a systemic effect, enhancing the production of phytoalexins, which in turn boost the plants vigor. No reports were found about fungicidal activity of the product. (More info: https://www.bh-solutions.eu/en-product-information).
- Hydrogen peroxide (30% v/v) (Sigma-aldrich®) is characterized by a broad-spectrum of efficacy against yeasts, bacteria and viruses (Block *et al*., 2000). (More info: https://www.sigmaaldrich.com/catalog/product/sigma/h1009?lang=pt&region=PT).
- Glutaraldehyde (25% w/v) (VWR® chemicals) is often used in industrial, laboratory, agricultural and medical settings (Ballantyne *et al*., 2001), and can be used as a fungicide, bactericide and anti-viral (PPDB, 2016). (More info: https://pt.vwr.com/store/product/2343937/glutaraldeido-25-solucao-aquosa-technical).
- Betkote type 3 (SPD, S.A.) is a dark coloured bituminous coating based on aqueous asphalt emulsions. The manufacturers suggest possible applications in agriculture as graft union protectant and pruning wound protectant in trees, although to the best of our knowledge no scientific research supports their claim. No reports were found about fungicidal activity. (More info: http://www.amccunha.pt/admin/ficheiros/artigosFicheiros/11180_05312013041638_BETKOTE%20TIPO%203%20FT.pdf).

A table summarizing the fungicides' details and their highest concentrations tested is found in Annex 1. The chosen concentrations for the *in vitro* tests were selected based on either the manufacturer indications, previous works with such chemicals within this research unit or personal communications from collaborators.

3.1.3 Experimental setup

Spore counting

In order to prepare a spore suspension of the concentration of 5 x 10^5 spores/mL:

1. A two-week-old culture of fungal pathogen (grown in PDA medium, at 25 °C ± 1 °C, in the dark) was flooded, the surface of the colony was scraped with a plastic scalpel and the spores and mycelia suspension were filtered through a sintered glass spore filter (PYREX Gooch Type Filtering Crucibles) into a beaker.
2. Spore counting was performed using a hemocytometer (Marienfeld®, 2.5 μm^2), in an optical microscope (Leitz® HM LUX 3: 40x optic). To determine the number of spores using the hemocytometer, the spores were counted in each small grid, in diagonal (Fig. 8).
3. Determine the number of spores in a specific volume, by using the formula:

$$\mathbf{spores/mL} = \frac{1000 \ * \text{counting average} \ * \ 4000}{16}$$

4. Calculate the final volume to make a dilution with the desired concentration (5 x 10^5 spores/mL).

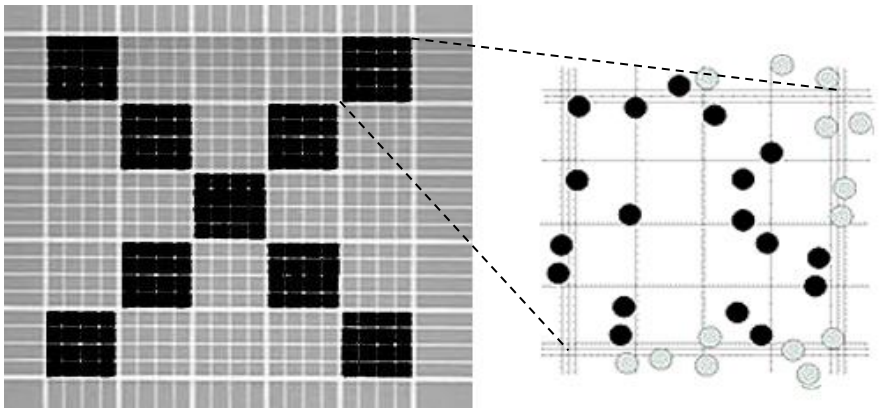

Fig. 8 - Schematic representation of part of an hemotocytometer (Marienfeld®, 2.5 μm^2). Black dots represent the spores that are counted, and the grey dots represent the ones that are not included in the spore counting. (Extracted from http://www.lo-laboroptik.de/deutsch/info/info.html).

Preparation of the 96 wells microplate to test fungicide activity of different chemicals

After preparing a spore suspension of the desired concentration, stock solutions and the respective dilutions were prepared. From the stock solution, 1:3 serial dilutions were made, having in a total 11 different concentrations of each chemical.

Under sterile conditions, in each well of the microplate (with flat bottom), it was loaded:

1. 80 µL of the liquid medium potato dextrose broth (PDB, Difco, Becton, Dickinson and C, Franklin Lakes, NJ, USA) amended with 250 mg/L of chloramphenicol (AppliChem GmbH, ITW company, Germany);
2. 80 µL of spore suspension, under continuous stirring with a magnetic stirrer;
3. 80 µL of the chemical, in a total of 4 replicates per treatment and per concentration tested (Annex 2).

Negative controls (NC) (wells with medium, chemical at the highest concentration and water) were added. Positive controls (PC) (wells with medium, spore suspension and water) were also added.

To avoid losses in volume due to evaporation, parafilm was placed around the lid during the incubation period. The microplates stayed inside an incubator in the dark, for 72 h at 25 °C ± 1 °C.

Samples were analysed in a microplate reader (BIO-TEK Synergy HT) with GEN5 software. All the samples were analysed at 630 nm, at 25 °C. This wavelength measures turbidity and it was selected based on previous works (Jorge, 2014).

In order to confirm the results, the whole set of experiments was repeated once more, at a distance of 1 month.

3.2 Testing of selected fungicides to protect pruning wounds under field conditions

3.2.1 Vineyard

The field trial took place in the Almotivo vineyard, located in the Instituto Superior de Agronomia's premises, in Lisbon (coordinates: 38°42'29.5" N; 9°11'14.5" W). Detailed meteorological data from the field trial period are found in Annex 3.

The chosen grapevine cultivar was Cabernet Sauvignon grafted with 140 RU rootstock, 19 years old (planted in 1998). The vineyard has not irrigation system. By the classification "World Reference Base for Soil Resources" (WRB, 2006), the soil is classified as a vertisoil. The vineyard has a density of 3,333 plants/ha and there are

four main training systems – Lys, Sylvoz, Lira and Cordon Royat Bilateral. Vines from all these training systems were used in our experiment. The chemical treatments applied during the year of the experiment can be consulted in Annex 4.

3.2.2 Experimental setup

For this experiment, only Pch (CBS 161.90) was tested.

Three fungicides and the grafting mastic were selected from the experiments *in vitro* (Table 1). In this study copper combined with summer oil was used (Pomorol®, Nufarm) for a better adhesion of copper oxychloride to the pruning wounds.

Table 2 - Characteristics of chemicals and concentrations of the different active ingredients used against Pch, *in vivo*.

Active ingredient (a. i.)	Trade name	Manufacturer	Formulation	Concentration tested a. i.
Cyprodinil + Fludioxonil	Switch®	Syngenta	37.5% Cyprodinil + 25% Fludioxonil	0.9375 g/L
Copper oxychloride + Summer oil	Cuprocol® + Pomorol®	Syngenta + Nufarm	36.5% Copper oxychloride + 80% (w/w) summer oil	36.5 g/L copper ox. + 5.28 g/L summer oil
Blad	Fracture®	CEV/CONVERDE	20% (w/v) Blad	60 g/L
Bituminous coating	Betkote type 3	SPD, S.A.	Industrial secret	100 %
Water (Control)	-	-	-	-

One-year-old canes were selected randomly among different plants that had not shown GLSD foliar symptoms over the previous two years. A total of 150 canes were divided as such: 3 groups of 50 canes. Each group of 50 contains 10 biological replicates for each treatment under study and the control (Annex 5).

The *in vivo* experiments started in 19 December 2016 and ended in 19 June 2017 (Fig. 9), following this timeline:

- Day 0 – Pruning of all canes at a length of 25 cm (±2 cm), leaving on average 5 buds. After pruning the canes, 50 µL of the chemical treatments were applied onto all wounds, and the location of the treated plant was recorded on a map.

The Bituminous coating was applied with a paintbrush and it was placed to cover the entire surface of the wound.

- Day 1 – Preparation of a spore suspension of Pch with the final concentration of 1 x 10^5 spores/mL. In the first group of 50 biological replicates, a droplet of 25 µL of the spores suspension was deposited onto the fresh pruning wound. Each wound was wrapped with parafilm (Parafilm® M) and left for 15 days after infection and then removed.
- Day 31 - In the second group of 50 biological replicates, a droplet of 25 µL of the spores suspension of Pch was deposited onto the pruning wounds. Each wound was wrapped with parafilm (Parafilm® M) and left for 15 days after infection and then removed.
- Day 90 - Collection of the first batch of 50 canes artificially infected on day 1. All the canes were harvested at 25 cm and separated by treatment.
- Day 91 - In the last group of 50 biological replicates a droplet of 25 µL of the spores suspension of Pch was deposited onto the pruning wounds. Each wound was wrapped with parafilm (Parafilm® M) and left for 15 days after infection and then removed.
- Day 120 - Collection of the second batch of 50 canes artificially infected on day 31. All the canes were harvested at 25 cm and separated by treatment.
- Day 150 - Collection of the last batch of 50 canes artificially infected on day 91. All the canes were harvested at 25 cm and separated by treatment.

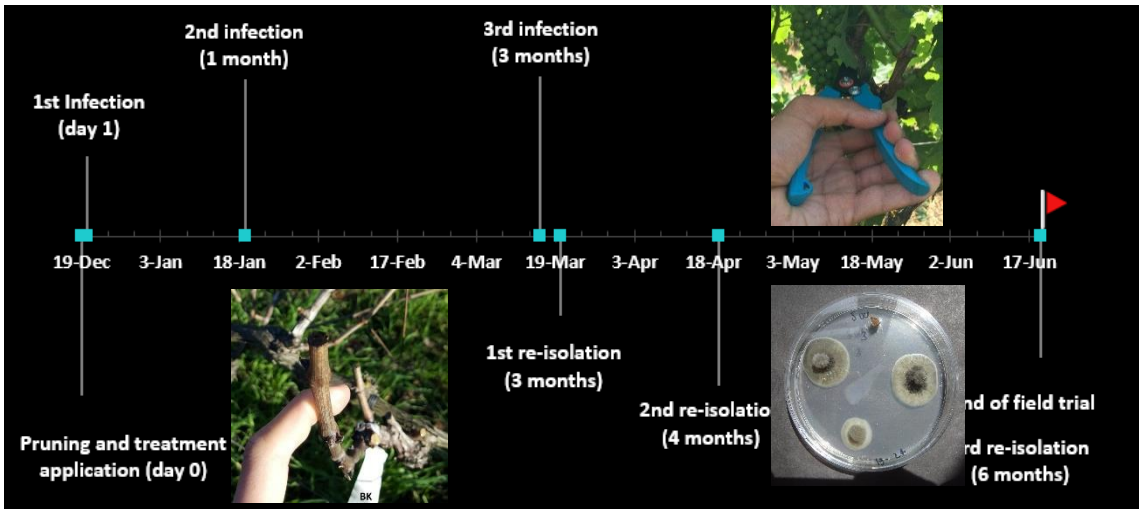

Fig. 9 - Timeline with the field experiment setup, from the pruning and treatment (day 0) to the last reisolation procedure (6 months after applying the treatments) (Photos: João Costa and Giovanni del Frari).

3.2.3 Determination of internal wood symptoms and pathogen re-isolation

After harvesting, the canes were handled as explained below.

For each cane, the bark was removed and the cane was sliced in 4 portions (lengthwise), to examine and record the presence of streaking, from the infection point until the end of the streak.

For the pathogen re-isolation, in each cane, 1 cm of wood (lengthwise, from the infection point) of was discarded, and then 4 uniform pieces were sliced (3 mm). Each piece of wood was processed, following the steps:

1. Dipped in ethanol (98% v/v);
2. Passed on the flame for 3 s;
3. Immersed in sodium hypochlorite (7% v/v), for approximately 30 s;
4. Dipped in sterile water, for 30 s;
5. Dipped again in sterile water, in another beaker, for 30 s;
6. Dried in a sterile filter paper.

The 4 pieces of wood were then placed in a Petri dish with potato dextrose agar amended with chloramphenicol (bacteriostatic antibiotic). The Petri dishes with the wood pieces were stored in an incubator at 25 °C, in the dark. Fungal growth from plated wood pieces was monitored daily and sub-cultured if necessary.

The incidence of Pch and other fungi in each of the treated pruning wounds were recorded.

3.3 Other parameters evaluated

3.3.1 Record of phenological stages, shoot length and estimation of leaf area

Phenological stages and shoot length:

The phenological stages were followed by the BBCH-scale in which the development of the grapevine is divided by growth phases (principal growth stages 0 to 9) and each phase is subdivided in growth steps (secondary growth stages 0 to 9) (Lorenz *et al*., 1995) (Annex. 6).

Since bud burst, all green shoots that bursted from the top bud of each vine in study were recorded (twice a week) for both the phenological stages and the green shoot length.

Total leaf area:

The total leaf area was recorded once following the methodology described by Lopes et al. (2005). Using these models, the percentage of secondary shoots was also calculated. These parameters were evaluated to compare and detect differences of growth by treatment and infection, in order to observe if there is any correlation between growth or inhibition and the treatment applied onto the pruning wounds.

3.3.2 Fresh/dry weight

For all canes harvests, the shoots which were grown from the top bud of the canes that we treated were collected and measured. A total of 5 shoots per treatment were randomly selected and the fresh (FW) and the dry weights (DW) were recorded.

After separated and numbered, the shoots were weighed, and after 72 h in the oven at 70 °C were again weighed to obtain the dry weight. The ratio FW/DW was then calculated.

3.4 Data and statistical analyses

EC_{50} values consist in the concentration of chemical which reduce mycelial growth by 50% (Jasper, 2001). Mean absorbances were used to calculate growth rate inhibition for each chemical treatment, following the steps below:

1. The mean absorbances were all converted in percentages, with 100% corresponding to no growth inhibition.
2. Percentages were plotted, showing a dose/response curve.
3. Regression functions (Y = a X + b) were fitted for the different chemical treatments (“Y” is the dependent variable; “X” is the independent variable; “a” is the slope of the line; “b” is the y-axis intercept).
4. By using the regression function, the respective EC_{50} value was calculated for each chemical under study.

The average EC_{50} values and respective standard deviations were calculated for the two experiments done.

For the reisolations of the pathogen, statistical analysis was performed as described above, considering 4 wood pieces per cane. The canes were rated as either infected or not infected. The percent re-isolation was calculated. To perform the statistical analysis, the data were treated following the steps below:

1. The total number of wood pieces (from 0 to 4) by cane that were artificially infected were recorded and transformed in percentage. Each piece of wood from which the pathogen was re-isolated counted as 25%.
2. Percentage infection was arcsine square root transformed (arcsine $\sqrt{(x/100)}$) prior to analysis, as described by Díaz et al. (2013).
3. A single factor analysis of variance (ANOVA) and a Tukey's post-test for comparison of the treatments with the control ($P < 0.05$) was performed for all re-isolation times.

For the determination of internal wood symptoms, cane length, total leaf area and fresh/dry weight ratio, average and standard deviations were calculated, followed by a single factor analysis of variance (ANOVA) and a Tukey's post-test for comparison of the treatments with the control ($P < 0.05$).

All data were analysed using Past version 3 statistical software (Hammer & Harper, Oslo, Norway) (Hammer *et al*., 2001).

4. Results and Discussion

4.1 Testing different fungicides on mycelial growth of Pch and Pmi

Different methods were applied as preliminary tests for the effect of different fungicides on mycelial growth of Pch and Pmi:

1. Chemicals dissolved in the medium and one mycelial plug in the middle, measuring the fungal colony diameters, as explained by Nascimento et al. (2007) and Martín et al. (2013).
2. Imbibed discs of filter paper with the chemicals and then placed in the surface of the medium on the Petri dish with a suspension of spores, as explained by Espinel-Ingroff et al. (2007), following the general guidelines of CLSI M44-A.
3. By spectrophotometry using a 96 wells microplate with serial dilutions of the chemicals amended with the spores suspension and medium.

The method that gave the best results was the third because:

1. All fungicides could be tested;
2. It needs only 72 h of incubation period;
3. It is possible to evaluate a wide range of concentrations in the same experiment;
4. It needs limited amounts of material and time to prepare the experiment.
5. It is a reliable method and gave consistent results.

4.1.1 Fungicide activity towards *Phaeomoniella chlamydospora* (CBS 161.90)

Pch was inhibited by all chemicals at different concentrations, with the exception of Betkote (Table 2). An example of interaction is shown in Fig. 10.

- Cyprodinil + fludioxonil: the mean EC_{50} value obtained in this study was 0.012 mg/L. Our result was very similar to the one described by Jasper (2001) (EC_{50} 0.019 mg/L), for the same pathogen. These results confirm our method's reliability.

- Fosetyl-Al: the mean EC_{50} value obtained in this study was 147.055 mg/L. This active ingredient was the one that needed a much higher concentration of fungicide to observe the same effectiveness as the other chemicals under study. Bibliographic searches were not successful in finding previous works

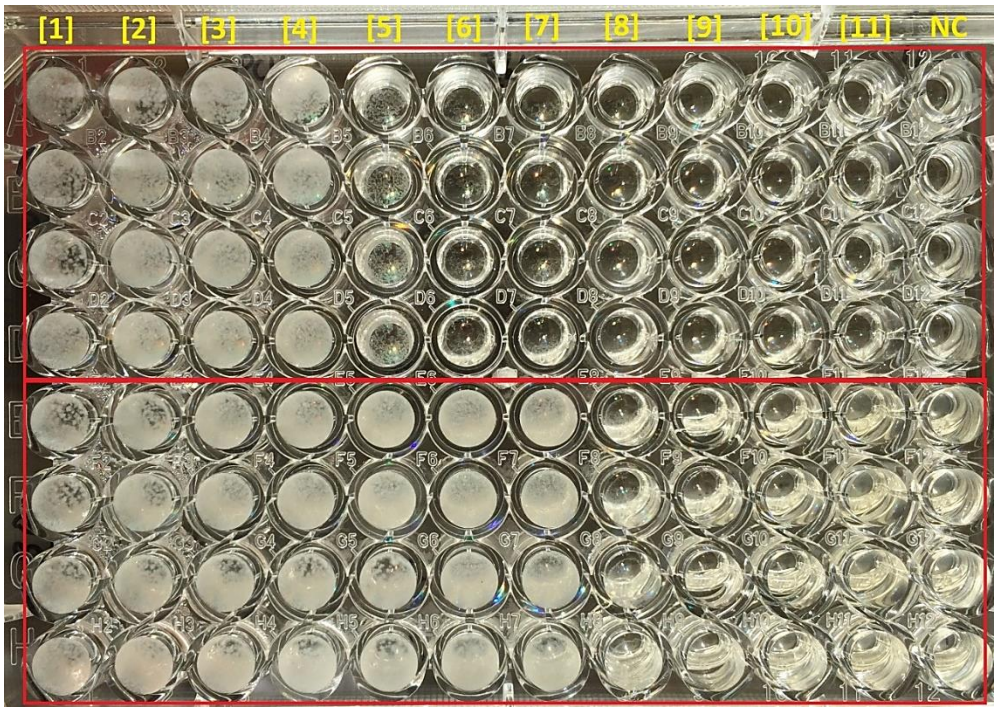

Fig. 10 - Result of the interaction of chemical concentration/fungus after 72 h of incubation period against Pch. 11 serial dilutions were tested to determine the EC_{50}. This microplate was composed by four replicates of Switch (A to D) and four replicates of Blad (E to H). NC is the negative control, chemical without spores. Microplates were analysed in the microplate reader.

where this chemical was tested *in vitro* against Pch.

- Elemental silver: to the best of our knowledge, we have been the first ones to test this a. i. against Pch. The mean EC_{50} value determined in this study was 0.462 mg/L.
- Blad: as far as we are aware, we have been the first ones to test this a. i. against Pch. The EC_{50} value determined in this study was 16.154 mg/L.
- Copper oxychloride: the mean EC_{50} value determined in this study was 15.954 mg/L. Our result was 11 times higher than the one described by Di Marco et al. (2011) (EC_{50} 1.449 mg/L), but with lower values when compared to the results obtained by Gramaje et al. (2009) (EC_{50} 100 mg/L). These differences can be due to several factors, such as the different strains used and/or the manufacturer formulation of the chemical.
- Hydrogen peroxide: as far as we are aware, we have been the first ones to test this a. i. against Pch. The mean EC_{50} value determined in this study was 17.648 mg/L.

- Glutaraldehyde: to the best of our knowledge, we have been the first ones to test this a. i. against Pch. The mean EC_{50} value determined in this study was 71.203 mg/L.
- Bituminous coating: to the best of our knowledge, we have been the first ones to test this a. i. against Pch. This chemical did not show any inhibition on mycelial growth at the tested concentrations.

Table 2 - Results of the activity of different active ingredients against Pch assessed by regression analysis with the respective standard deviation.

Active ingredient (a.i.)	**Trade name**	**EC_{50} Pch (CBS 161.90) Average (mg/L)**
Cyprodinil + fludioxonil	Switch	0.012 ± 0.001
Fosetyl-Al	Aliette	147.055 ± 0.762
Elemental silver	BioBac	0.462 ± 0.084
Blad	Fracture	16.154 ± 0.749
Copper oxychloride + Summer oil	Cuprocol + Pomorol	14.453 ± 0.885
Copper oxychloride	Cuprocol	15.954 ± 1.193
Hydrogen peroxide	H_2O_2	17.648 ± 11.305
Glutaraldehyde	Glutaraldehyde	71.203 ± 34.730
Bituminous coating	Betkote (%)	-

The Fig. 10 shows the graphics with the dose/response curves by chemical treatment (from A to I) used in the present study. The graphics express one experiment by chemical treatment, with the respective mean EC_{50} values.

4.1.2 Fungicide activity towards *Phaeoacremonium minimum* (CBS 110.713)

Pmi's growth was inhibited by all chemicals tested, at different concentrations, except for Betkote:

- Cyprodinil + fludioxonil: as far as we are aware, we have been the first ones to test this a. i. against Pmi. The mean EC_{50} value obtained in this study was 0.307 mg/L.

- Fosetyl-Al: the EC_{50} value obtained in this study was 334.892 mg/L. This active ingredient was again the one that required a much higher concentration of fungicide to induce the same effectiveness as the other chemicals under study. Bibliographic searches were not successful in finding previous works where this chemical was tested *in vitro* against Pmi.
- Elemental silver: to the best of our knowledge, we have been the first ones to test this a. i. against Pmi. The mean EC_{50} value determined in this study was 9.313 mg/L.
- Blad: to the best of our knowledge, we have been the first ones to test this a. i. against Pmi. The EC_{50} value determined in this study was 73.438 mg/L.
- Copper oxychloride: the mean EC_{50} value determined in this study was 49.94 mg/L. Our result was higher than the one described by Di Marco et al. (2011) (EC_{50} 11.249 mg/L), but lower than the one described by Gramaje et al. (2009) (EC_{50} 100 mg/L). The variations of the different results may have some explanations, that were mentioned above.
- Hydrogen peroxide: to the best of our knowledge, we have been the first ones to test this a. i. against Pmi. The EC_{50} value determined in this study was 60.493 mg/L.
- Glutaraldehyde: As far as we are aware, we have been the first ones to test this a. i. against Pmi. The EC_{50} value determined in this study was 27.294 mg/L.
- Bituminous coating: to the best of our knowledge, we have been the first ones to test this a. i. against Pmi. This chemical did not show any inhibition on mycelial growth at the tested concentrations.

Table 3- Results of the activity of different active ingredients against Pmi assessed by regression analysis with the respective standard deviation.

Active ingredient (a.i.)	Trade name	EC_{50} Pmi (CBS 110713) Average (mg/L)
Cyprodinil + Fludioxonil	Switch	0.307 ± 0.021
Fosetyl-Al	Aliette	334.892 ± 2.664
Elemental silver	BioBac	9.313 ± 0.047
Blad	Fracture	73.438 ± 9.583
Copper oxychloride + Summer oil	Cuprocol + Pomorol	50.947 ± 0.197
Copper oxychloride	Cuprocol	49.943 ± 1.753
Hydrogen peroxide	H_2O_2	60.493 ± 29.899
Glutaraldehyde	Glutaraldehyde	27.294 ± 15.451
Bituminous coating	Betkote (%)	-

The Fig. 11 represents the graphics with the dose/response curves by chemical treatment (from A to I) used in the present study. The graphics express one experiment by chemical treatment with the respective mean EC_{50} values.

4.1.3 Comparison of the results obtained

Both fungi were inhibited but with different required concentration of chemicals necessary to reach the EC_{50} values. However, that of Pch was much lower than that of Pmi for all the chemicals where inhibition was observed, except for glutaraldehyde. To inhibit the growth of both fungi simultaneously, the highest concentration of the respective fungicide found in Table 2 and 3 should be applied.

Similar values for EC50 were observed for both copper formulations, demonstrating that the addition of summer oil (Pomorol®) has no interference on the bioactivity of copper towards the pathogens tested.

As far as we are aware, most of the chemicals used in our study have never been tested before against these fungi. These results are therefore a starting point for the application of these fungicides against other wood pathogens in vitro, as well as for their evaluation in vivo in future works, such as in nurseries, in trunk injections or in wound protection.

Three fungicides – Switch, Copper oxychloride and Blad, as well as the grafting mastic – Betkote – were further evaluated under field conditions as pruning wound protectants.

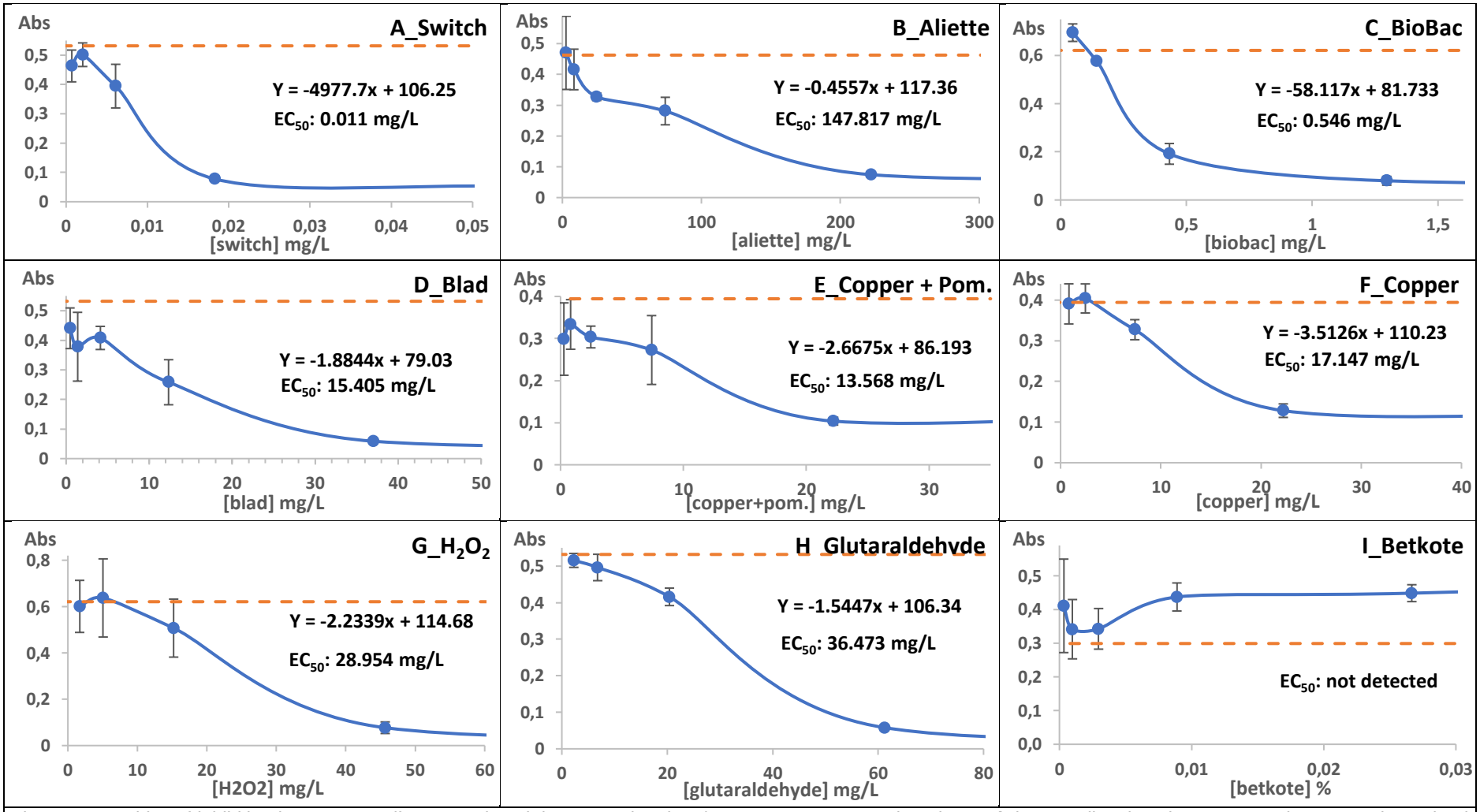

Fig. 11 – Graphics of inhibition by treatment (from A to I) and the respective dose/response curve, tested against **Pch** in 96 wells microplates. EC_{50} values were determined for each treatment. The blue line represents the average of 4 replicates by chemical concentration with the respective standard deviation (vertical bars). The orange dashed line represents the positive control (PC), for which there is no fungal inhibition. Each graphic does not show all the points representing the tested concentrations. Rather, only the points where the slope of the dose/response curve showing the inhibition of the fungal growth is visible are represented.

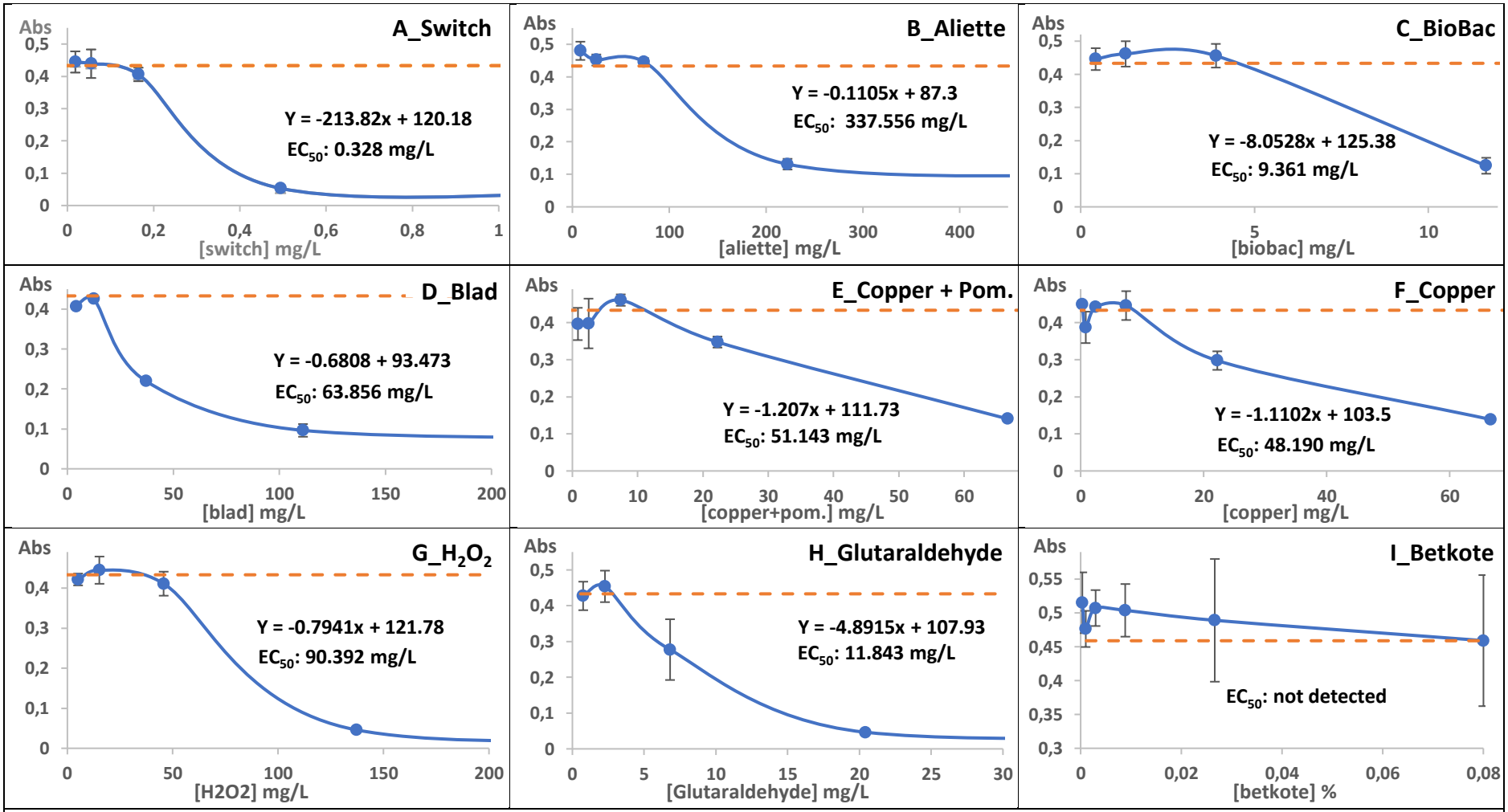

Fig. 12 – Graphics of inhibition by treatment (from A to I) and the respective dose/response curve, tested against **Pmi** in 96 wells microplates. EC_{50} values were determined for each treatment. The blue line represents the average of 4 replicates by chemical concentration with the respective standard deviation (vertical bars). The orange dashed line represents the positive control (PC), for which there is no fungal inhibition. Each graphic does not show all the points representing the tested concentrations. Rather, only the points where the slope of the dose/response curve showing the inhibition of the fungal growth is visible are represented.

4.2 Testing the selected fungicides to protect pruning wounds under field conditions

4.2.1 Pathogen reisolation

The four products tested in our field trial, except Betkote, were originally selected because they all contain different active ingredients and all showed efficacy *in vitro* against Pch.

Previous work by members of the physiology group at LEAF have never reisolated Pch from Almotivo vineyard while examining one-year-old canes (n > 100). This result (data not published) allowed us to exclude the addition of negative controls from the experiment.

On the first re-isolation, Betkote significantly reduced the presence of Pch three months after the inoculation took place. This means that the chemical acted as a physical barrier and prevented the infection of the pathogen. The remaining treatments had close values to the positive control, indicating that they were not effective as fresh wounds protectants.

On the second re-isolation, no chemical treatment was statistically significantly different from the positive control. The treatment Betkote (20%), despite not being statistically significant with the positive control (50%), had the lowest incidence of Pch in wood compared with the remaining chemical treatments. The incidence of Pch in the Switch (80%) and Copper ox. + summer oil (70%) treatments were higher (albeit not statistically different) than the control, while in Blad remained similar, but no statistical differences were observed.

On the third re-isolation, again no chemical treatment was statistically significantly different from the positive control. The treatment Betkote (30%), despite not being statistically significant when compared to the positive control (60%), had once again the lowest incidence of Pch in wood compared with the remaining treatments. Blad (40%), had close results to Betkote, and had lower incidence of the pathogen compared to the positive control, but not significant ($P < 0.05$). Previous work indicated that the susceptibility of pruning wounds to infection decreases over time (Munkvold *et al*., 1995; Eskalen *et al*., 2007), but remain susceptible to Pch infection for up to 4 months (Eskalen *et al*., 2007; Serra *et al*., 2008). In our study, the positive control was statistically different in the 2nd and 3rd re-isolations, when compared to fresh wounds (1st re-isolation) ($P < 0.05$), and pruning wounds remained susceptible to infection even 3 months after pruning and treatment application (Fig. 13). Reduced infection in spring has been linked to faster growth of non-pathogenic microorganisms on wound surfaces (Munkvold *et al*., 1995), but in addition we suggest an important role played by an active plant defence system.

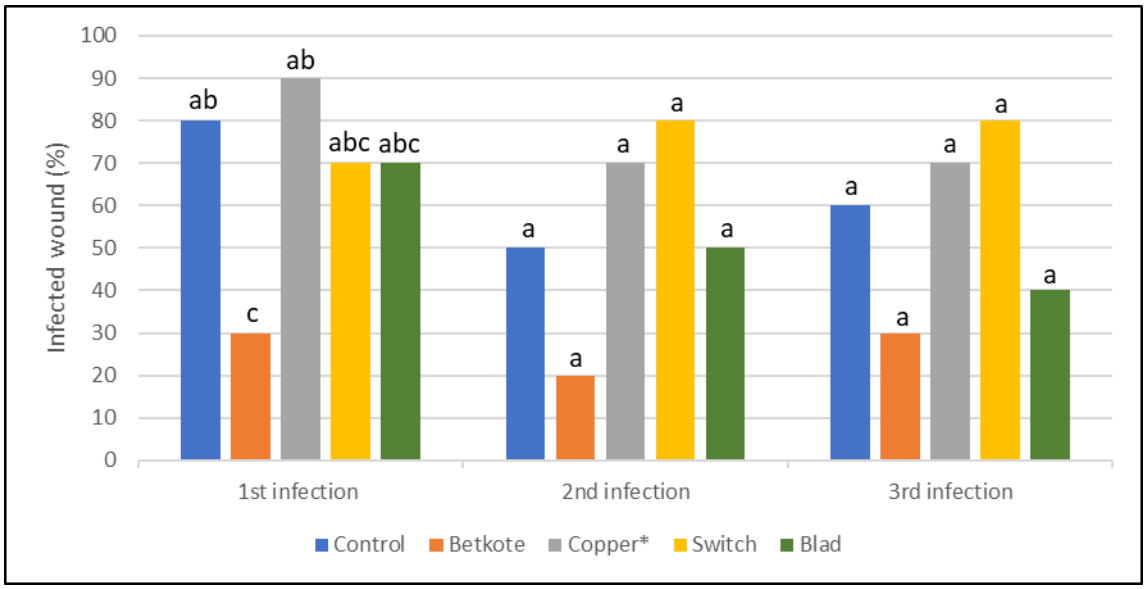

Fig. 13 - Percentages of infected wounds based on fungal reisolation of Pch on cv. Cabernet Sauvignon. The canes were harvested 3 months after each inoculation. The percentage of re-isolations were converted in arcsine $\sqrt{(x/100)}$ values before the statistical analysis, but the non-transformed data are presented. One-way ANOVA was performed. Letters indicate the significance statistical differences among control and chemical treatments in study, according to Tukey's pairwise multiple comparison test ($P < 0.05$).

Discussion of the efficacy of selected fungicides as pruning wound protectants:

- Betkote: this treatment was the best in all re-isolations in this study (Fig. 12-B), despite no statistical difference in the second and third re-isolations. This result showed that a physical barrier, even without any fungicide activity, may give a partial protection to fresh wound during, at least, 1 day after pruning. This wound protection, created by a protective layer, was enough to reduce infections during the critical period, preventing therefore the spread of this pathogen through vines by winter pruning wounds. As far as we are aware, this was the first study that used a physical barrier without fungicide activity or in combination with other fungicide.
- Copper oxychloride + summer oil: the combination of these chemicals has never before been tested as pruning wound protectant against Pch. As shown by the results, this formulation did not work well as wound protectant. Copper may have interacted with compounds in the wood, may have gone through some sort of inactivation by climatic conditions or, most likely, may have been washed away from the surface of the wound.
- Switch (cyprodinil + fludioxonil): as far as we are aware, this chemical was never used before as pruning wound protectant against Pch. As observed in the results, this chemical did not work well as wound protectant for the pathogen under study. The reasons may be the same of those given for the treatment copper oxychloride + summer oil.

- Blad: this chemical was never before used as pruning wound protectant against Pch as well. The reasons may be the same of those suggested for the treatment copper oxychloride + summer oil.

Overall, this study demonstrates that infections caused by Pch can be significantly reduced using a single grafting mastic application (Betkote) after pruning.

4.2.2 Vascular streaking length

Brown to black vascular streaking is known to be the most common internal symptom attributed to Pch (Surico *et al*., 2008; Bertsch *et al*., 2013). Vascular streaking length is considered as another parameter to evaluate the efficacy of fungicides, as reported by Díaz and Latorre (2013).

Vascular discoloration was observed and recorded in all the sampled spurs artificially infected with Pch (Fig. 14). The measurements of the length of wood discoloration can be observed in Fig. 15.

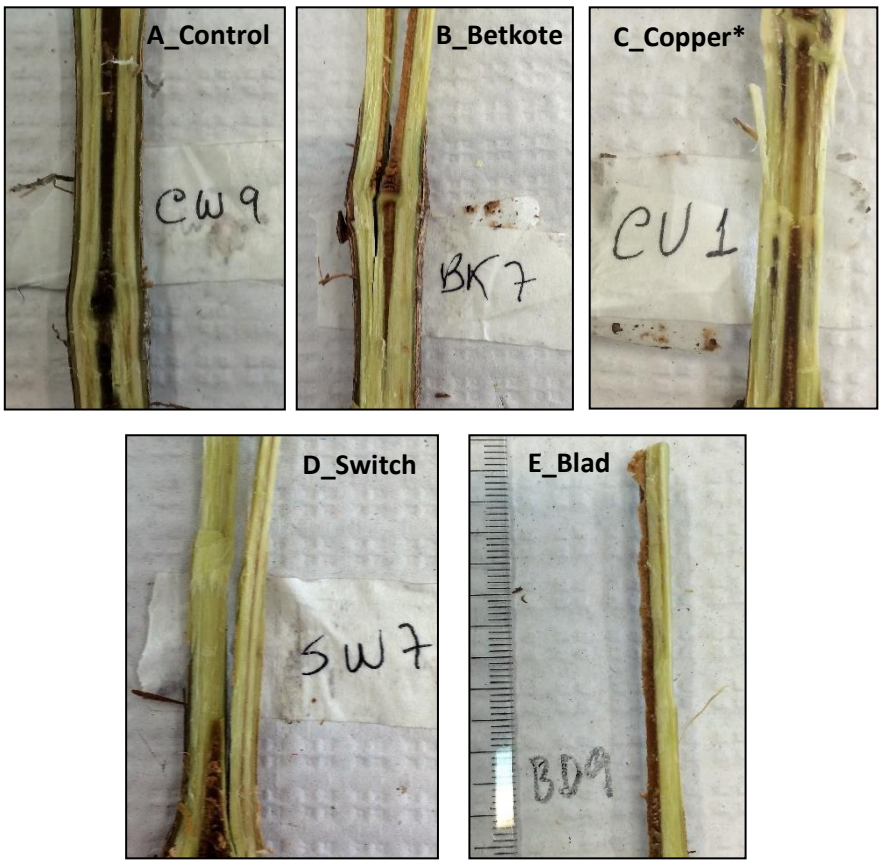

Fig. 14 – Presence of vascular streaking, with different intensities in colour, in control (PC) and in the chemical treatments under study (from A to E), observed at the time of the third re-isolation. Asterisk is referred to the treatment copper oxychloride with the summer oil.

On the first shoot harvest record (3 months after pruning), Betkote was statistically different from the positive control ($P < 0.05$), showing a shorter length of vascular discoloration. This result and the result obtained in the 1st re-isolation (Fig. 13), showed that this physical barrier was effective to protect the fresh wounds against the pathogen evaluated. The remaining chemical treatments were not statistically significant against the control ($P < 0.05$).

On the second shoot harvest record (4 months after pruning), Betkote was also statistically different ($P < 0.05$), both against control and the remaining treatments, except for Switch. The remaining chemical treatments produced results which were not statistically different from the control ($P < 0.05$).

On the third shoot harvest record (6 months after pruning), no statistically significance was observed among samples.

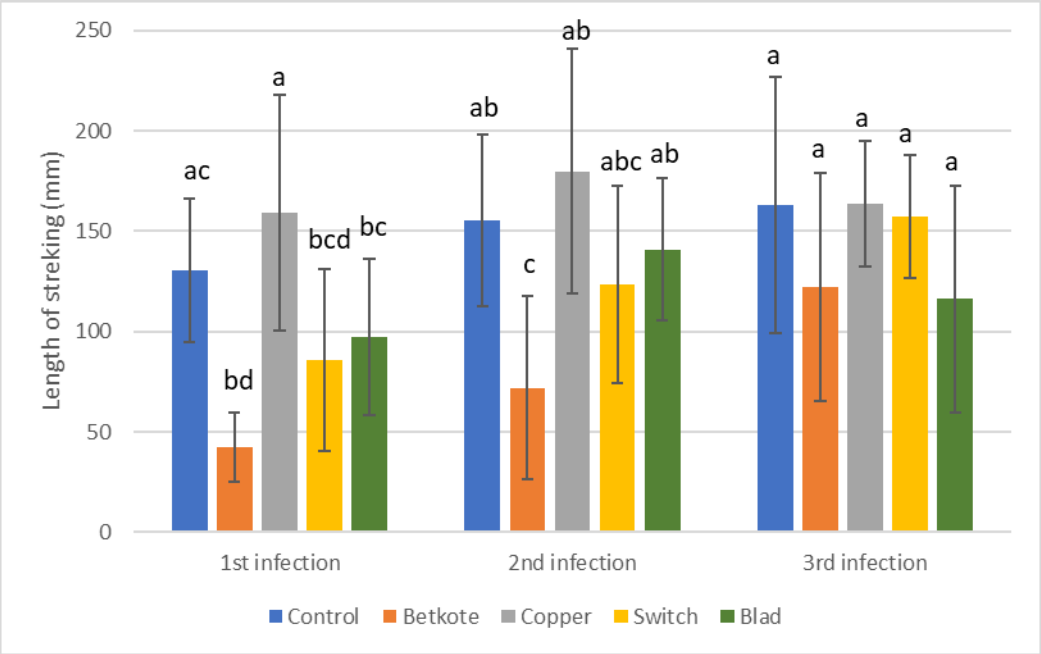

Fig. 15 - Length of vascular streaking (mm) in canes of cv Cabernet Sauvignon, separated by inoculation time with the respective standard deviation. One-way ANOVA was performed. Letters indicate the significance statistical differences among control and chemical treatments under study, according to Tukey's pairwise multiple comparison test ($P < 0.05$). Bars represent the standard errors.

Similar works, on the same cultivar, of artificial inoculation of pruning wounds with Pch under field conditions revealed different results of infectivity/streaking compared to ours. For example, Eskalen et al. (2007) found that in all the records of vascular streaking lengths were lower than 60 mm. In our study in all the records, positive controls had values higher than 130 mm of vascular streaking length. These differences may be due to the long inoculation periods (4 months) utilized in the present study, to the inoculation time of the year, to the climatic conditions (which will certainly influence the behaviour of fungicides and fungi on pruning wounds surface), to the susceptibility of pruning wounds to fungal

pathogens, which will vary depending on the specific isolate used in the different studies (Rolshausen *et al*., 2010), among other factors.

Based on our results, some explanations are provided below in an attempt to understand the big variability (error bars) observed in the data depicted in Fig. 15:

1. The streaking was present but Pch was not re-isolated, meaning that another wood pathogen caused such symptoms in the wood or the symptoms were already present at the moment of pruning.
2. Infection by Pch was present, as the pathogen was re-isolated *in vitro*, but no streaking was observed. Brown streaking may sometimes involve a single or a few xylem vessels, being hard to identify by visual observation.
3. Infection and vascular streaking are present, but the pathogen spread (and therefore the brown streaking) was limited. This may be due to competitive interactions among pathogen-plant-microbiome.

4.3 Other parameters evaluated

In the beginning of the season, large differences in bud burst were observed among the different treatments. Since then, records were taken in order to evaluate differences in growth. The phenological stages, the shoot length as well as the leaf area were recorded.

4.3.1 Phenological stages record

Since 17/03/2017, 16 phenological stages were recorded, twice a week, until 16/05/2017. Results can be seen on Fig. 16. It is important to mention that the first record was made when some of the buds had already broken dormancy, mainly in the Blad-treated canes.

As can be seen in the first record in every treatment, differences were considerable. On the first record, on average, while Blad was already in the leaf development phase (phenological stage 11 – “first leaf unfolded and spread away from the shoot”), the other treatments were still in the sprouting growth stages (from 5 to 8 – between the “wool stage” and almost in the bud burst).

Another difference observed was the first records of the reproductive growth. On Blad treated canes, the first record was made in 21/03/2017. For the control and the other treatments, the first record of the reproductive growth was observed in 04/04/2017. A considerable difference in time (14 days) was observed between the Blad-treated canes and the other samples to reach the same growth stage. Then, the control and the other treatments recovered over time, with all samples reaching full flowering at the same time: for

treatments Blad and Switch full flowering was recorded on 02/05/2017 and for the control and the remaining treatments 3 days later.

With the observations above mentioned, the treatment Blad worked as a biostimulant enhancing a uniform bud break with advanced stages in the beginning of the season, both on vegetative and reproductive growth.

Depending on the wine growing region/country, bud break stimulation has pros and cons. In Europe and specially in regions with continental climate, such as Champagne region (Roiter *et al*., 2013), the advance in phenological stages might be a problem since the late frosts affect the green shoots, leading to even higher economic losses. In contrast, in tropical wine regions may be beneficial, such as in the São Francisco river Valley in Brazil, where it is possible to have more than one cycle and harvests per year (Tonietto *et al*., 2012). In such regions chemicals are applied, such as hydrogen cyanamide (H_2CN_2), to break dormancy efficiently and to ensure a uniform bud break (Albuquerque *et al*., 1987; Potjanapimon *et al*., 2007). A uniform bud break was also observed for the Blad treatment in our study and hence it could also act as a bud break elicitor in tropical regions.

Further studies must be carried out to confirm the obtained data and for further applications of this product, not only in grapevine but also in other crops.

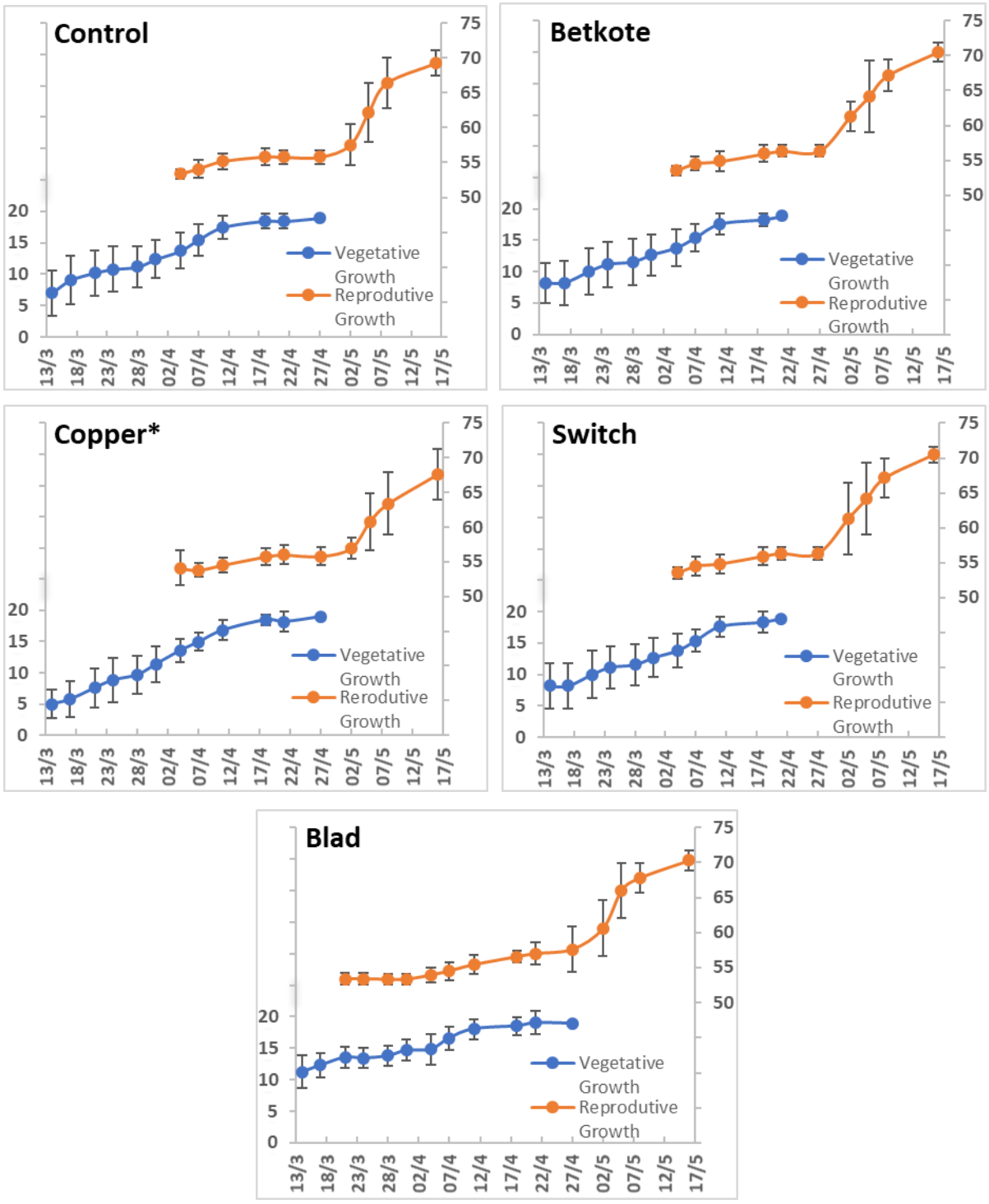

Fig. 16 - Phenological stages from the different treatments applied in Almotivo vineyard, cv Cabernet Sauvignon. Data were recorded by the BBCH scale (Lorenz *et al.*, 1995). Error bars indicate the standard deviation. The scale on the left comprises two principal growth stages – Sprouting (from 0 to 9) and leaf development (from 11 to 19). The scale on the right comprises three principal growth stages – Inflorescence emergence (from 53 to 57), flowering (from 60 to 69) and development of fruits (from 71 to 79). Asterisk refers to the treatment copper oxychloride with the summer oil.

4.3.2 Shoots length

In adition to the phenological stages record, the shoot length was also recorded. Data was recorded from 17/03/2017 until 25/05/2017.

During the experiment the number of biological replicates decreased over time. In the beginning, 30 biological replicates by chemical treatment were followed until the first shoot harvest (19/03), then decreasing to 20 until the sencond shoot harvest (19/04) and until the end of the experiment, 10 biological replicates were recorded – This procedure may lead to an increasing standard deviation. For future studies, more biological replicates should be used to decrease those values.

On the first and second graphics (Fig. 17 - A and B), the growth of the shoot over time may be observed. The graphic with the whole data is presented in Annex 7. The second graphic (Fig. 16-B) shows the shoot length in greater detail by chemical treatment, with the respective differences ($P < 0.05$).

Blad was statistically different from the control ($P < 0.05$), from the beginning of the recordings until 31/03. Visually, the differences of growth can be easily detected from the control and the other treatments (Fig. 17-C). The remaining treatments were not statistically significant when compared with the control ($P > 0.05$) (Annex 8).

Harman (2000) described that by enhancing vine and root development, the tolerance to stress would increase. Some authors also described that stress predisposes the plants to attack by several pathogens, including Pch (Ferreira *et al*., 1999; Fourie *et al*., 2001). By the re-isolation results (Fig. 13-B), Blad was not statistically significant from the control ($P > 0.05$), but less isolations of the pathogen were found in the first and third re-isolations, giving the idea that Blad might enhanced the plant vigour and therefore reduce slightly the pathogen incidence. However, more studies need to be done in order to confirm these speculations.

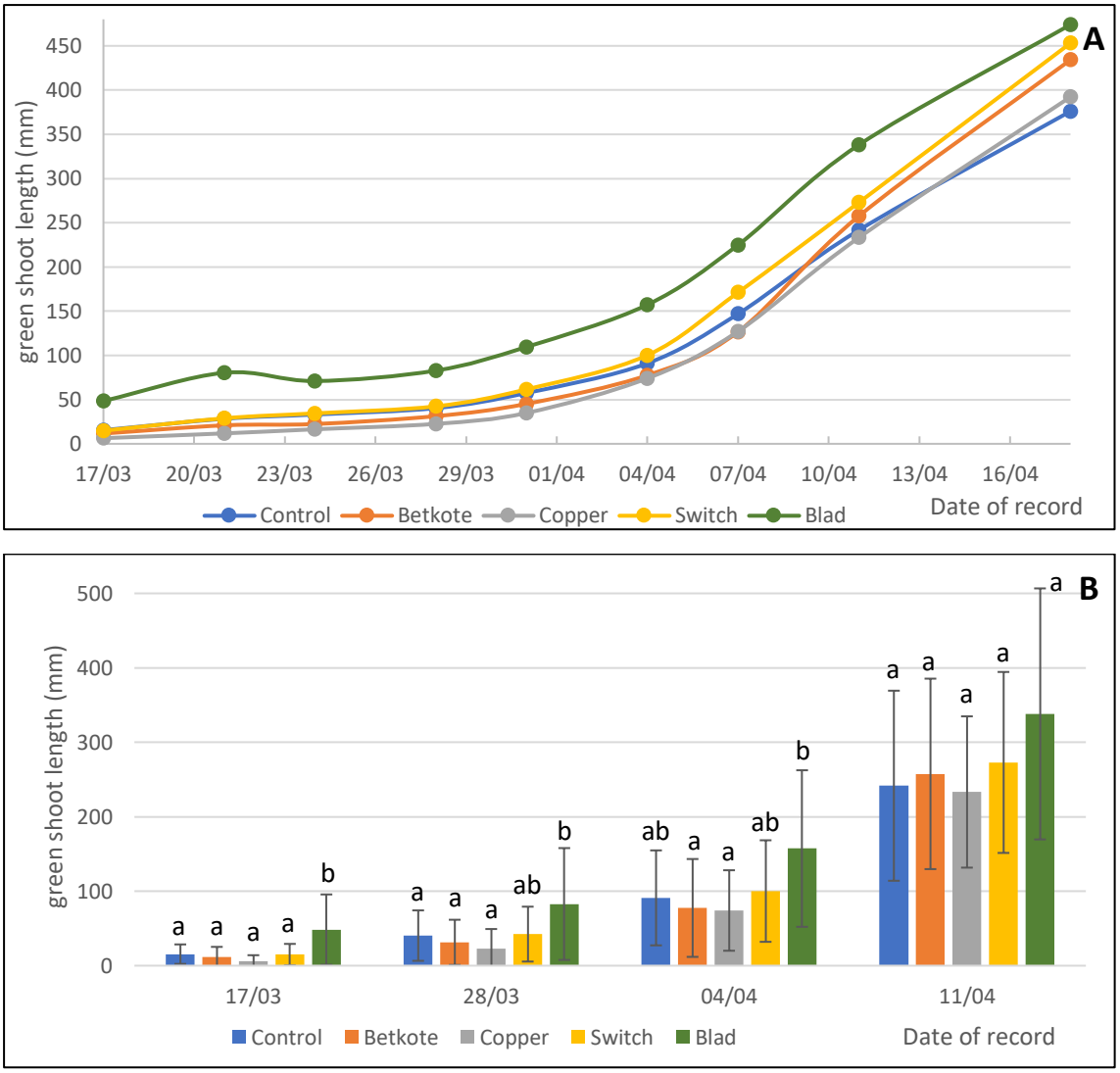

Fig. 17 – Evolution of the shoot length records during vegetative growth in cv Cabernet Sauvignon, Lisbon region, 2017; A – Graphic with the shoot length (mm) measured under the different treatments; B – Detailed graphic showing the differences of shoot length. One-way ANOVA was performed. Letters indicate the significance statistical differences among control and treatments, according to the Tukey's pairwise multiple comparison test ($P < 0.05$); C – Pictures taken on 19/03/2017, showing the differences in growth, between control (C1) and Blad treatment (C2).

4.3.3 Shoot biomass

The biomass was recorded to reinforce the findings mentioned above. The results are shown in Table 4.

The results obtained from the first shoot harvest are the most important to compare because it was when higher differences in growth were observed between Blad, the control and the remain treatments ($P < 0.05$), with Blad showing a higher biomass, which is in accordance with the findings from the green shoot length (Fig. 16-B).

As observed in Fig. 16-B, from the record of 11/04/2017 on, no statistical significance was observed. The biomass of the shoots followed the same trend, showing no statistical significance between chemical treatments and the control ($P < 0.05$).

Table 4 – Biomass of the shoots from the top bud by each shoot harvest time. Results are presented as the average and the respective standard deviation. Units are represented in grams (g). One-way ANOVA was performed, and treatments were compared by Tukey's pairwise multiple comparison test ($P < 0.05$). Values followed by a different letter in each row were significantly different, according to Tukey's pairwise multiple comparison test.

	Control	**Betkote**	**Copper***	**Switch**	**Blad**
1st re-isolation (19/03/2017)(g)	0.888 ± 0.441 ab	0.598 ± 0.371 ab	0.148 ± 0.103 a	1.208 ± 0.722 b	4.994 ± 0.591 c
2nd re-isolation (19/04/2017)(g)	37,81 ± 9,812 abcd	13.71 ± 5.523 c	29.7 ± 12.062 bc	36.384 ± 11.522 bd	53.858 ± 10.054 d
3rd re-isolation (19/06/2017)(g)	57,31 ± 18,467 a	65.054 ± 11.233 a	51.534 ± 8.576 a	64.422 ± 27.426 a	73.754 ± 17.092 a

Asterisk refers to the treatment copper oxychloride with the summer oil.

4.3.4 Leaf area and percentage of secondary shoots

In addition to the other parameters evaluated, the total leaf area and the percentage of secondary shoots were also recorded once (16/05/2017), but no statistically significant differences ($P < 0.05$) were observed among the control and the treatments. The results can be seen on Table 5.

Table 5 - Total leaf area and percentage of secondary shoots by the different treatments. Record was made on 16/05/2017. One-way ANOVA was performed. Values followed by a different letter in each row were significantly different, according to Tukey's pairwise multiple comparison test ($P < 0.05$).

	Control	**Betkote**	**Copper***	**Switch**	**Blad**
Total leaf area (m^2)	0.667 a	0.558 a	0.601 a	0.615 a	0.649 a
Secondary shoots (%)	40.377 a	36.900 a	39.172 a	39.250 a	33.420 a

Asterisk refers to the treatment copper oxychloride with the summer oil.

By the measurements effectuated in that date, all vines from the different treatments were in the same stage of growth ($P < 0.05$). In the absence of statistical difference, we did not continue these measurements.

4.3.4 Fresh/dry weight

Fresh and dry weight from the shoot of the top bud was recorded and their ratio calculated, in order to compare the weight by the different chemical treatments applied to the pruning wounds.

The results obtained can be seen on Table 6 with the statistical significance.

For all shoot harvest times the ratio was not statistically different ($P < 0.05$), explaining that the differences of the fresh/dry weight were essentially in water content.

Table 6 - Ratio fresh/dry weight results obtained from the three shoot harvests by the different treatments. One-way ANOVA was performed, and treatments were compared by Tukey's pairwise multiple comparison test ($P < 0.05$). Values followed by a different letter in each row were significantly different, according to Tukey's pairwise multiple comparison test.

	Control	**Betkote**	**Copper***	**Switch**	**Blad**
1st reisolation (g)	4.484 ± 0.879 a	4.096 ± 0.596 a	4.933 ± 1.420 a	4.137 ± 0.748 a	5.034 ± 0.120 a
2nd reisolation (g)	4.881 ± 0.521 a	4.960 ± 0.121 a	4.541 ± 0.281 a	4.642 ± 0.111 a	4.076 ± 0.615 a
3rd reisolation (g)	3.426 ± 0.454 a	2.930 ± 0.236 a	3.133 ± 0.247 a	3.155 ± 0.329 a	3.271 ± 0.219 a

Asterisk refers to the treatment copper oxychloride with the summer oil.

5. Conclusion

The issue of GTDs is ever more concerning and immediate action is needed to prevent the spread of GTD-related pathogens. This study collaborated in furthering the research in the field of pruning wounds protection to reduce the risk of infection by Pch, and *in vitro* by testing novel active ingredients.

In our study *in vitro,* we found novel chemicals with different active ingredients that were effective in reducing mycelial growth of Pch and Pmi. These findings were made by using a different method from those commonly used in the literature, capable of analysing all the different chemicals studied.

The results obtained under field conditions showed that the treatment Betkote reduced significantly the presence of Pch on the first re-isolation (day 1). This result showed that such treatment provides partial protection to fresh wounds immediately after pruning. However, the remaining treatments were not effective as wound protectants, highlighting the difficulty of these products to control the pathogen under field conditions.

The results obtained by monitoring the treated plants' growth, we concluded that the treatment Blad, in all the parameters evaluated, worked as a biostimulant in the beginning of the season. This treatment had promoted:

- an earlier and uniform bud break with advanced stages in the beginning of the season, both on vegetative and reproductive growth.
- the growth of the shoots, with statistical significance from the control since the beginning of the recordings until the date 31/03.

Overall, this study was important to increase the current knowledge in chemicals used against some esca-related fungi and in pruning wound protection under field conditions because we tested different active ingredients that have never been tested against Pch and Pmi, *in vitro.* We also tested novel chemicals as pruning wounds protectants that were never used against Pch, under field conditions. Those chemicals that we tested can be a starting point for new trials against other wood pathogens *in vitro*, as well as the evaluation *in vivo* in future works such as in nurseries or trunk injections or as wound protection.

In this study all treatments application was handmade being economically expensive in large scale. It is necessary to implement and improve mechanical spray applications to protect pruning wounds, reducing the costs, minimizing the chemical inputs and being less time consuming. For future studies, these improvements should be taken in account.

The next goals in this research are:

- Test the same chemicals but with different pruning periods in order to find the best timing for application of treatments to ensure protection of wounds for the duration of wound susceptibility.
- Evaluate more active ingredients already tested *in vitro*, against other wood fungi, for example Pmi, under field conditions.
- Possibly, combine Betkote with some fungicide in order to improve the wounds protection, reducing therefore the risk of infection.

This study opened new possibilities for research in GTDs and gave new insights for future works in this topic.

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7. Annexes

Annex 1

Table 7 – Detailed characteristics of fungicides and highest concentration rate used against Pch and Pmi *in vitro* experiment.

Active ingredient	Trade name	Manufacturer	Formulation	Concentrations tested (g/L)
Cyprodinil + fludioxonil	Switch®	Syngenta	37.5 % Cyprodinil + 25 % Fludioxonil	0.01333
Copper oxychloride	Cuprocol®	Syngenta	36.5 % Copper oxychloride	0.06667
Copper oxychloride + summer oil	Cuprocol® + Pomorol®	Syngenta + Nufarm	36.5 % Copper oxychloride + 80 % summer oil	0.06667 of copper ox. + 0.04695 of summer oil
Blad	Fracture®	CEV/CONVERDE	20 % Blad	1.0
Fosetyl-Al	Aliette Flash®	Bayer	80 % Fosetyl-Al	0.66667
Elementar silver	BioBac®	M.H.I Compania de Ingenerie S.R.L.	1000 ppm Elementar silver	0.01167
Glutaraldehyde	-	VWR chemicals	25 % Glutaraldehyde	14.88097
Hydrogen peroxide	-	Sigma-aldrich	30 % Hydrogen peroxide	11.100
Bituminous coating	Betkote tipo 3	SPD, S.A.*	Industrial secret	0.08 %

*Sociedade Portuense de Drogas

Annex 2

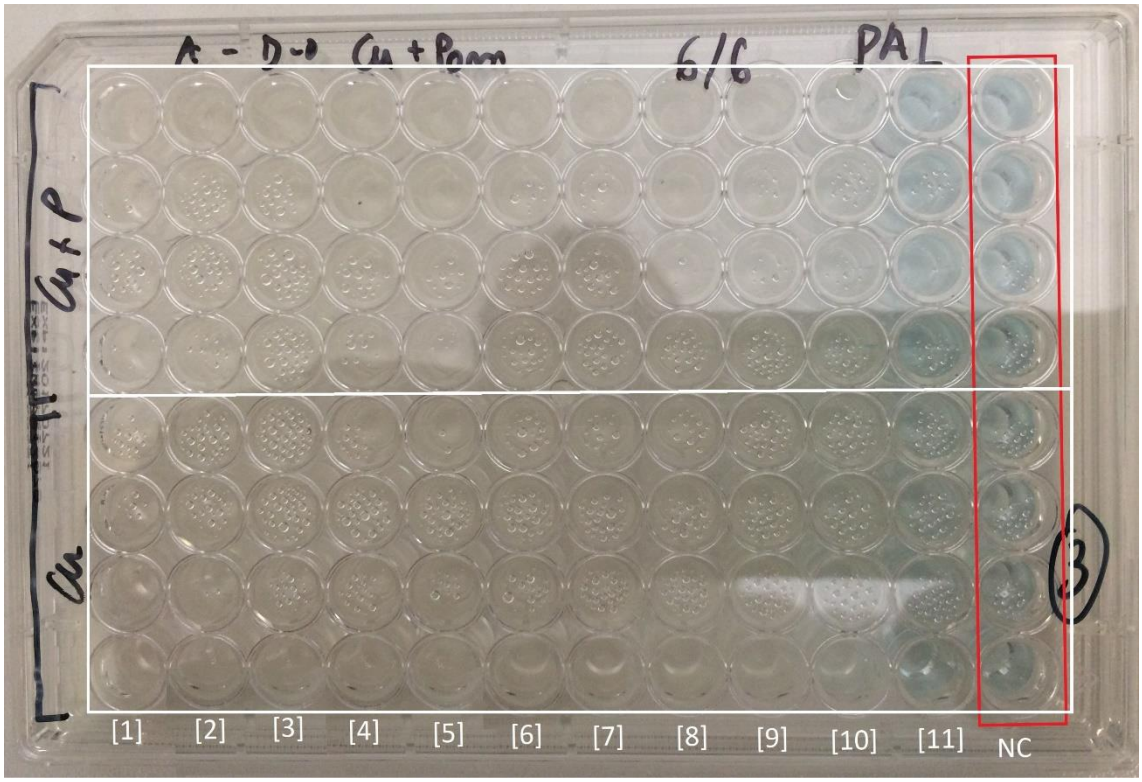

Fig. 17 - Microplate with 96 wells flat bottom with two different treatments tested - Copper oxychloride + summer oil (CU+P) and Copper oxychloride (CU). Each treatment had 4 replicates. 11 different serial dilutions, from the left (less concentrated) to right (highest concentration). In the 12th column is representing the NC, with the same concentration as the highest.

Annex 3

Table 8 – Meteorological data from the automatic station Lisboa/Tapada da Ajuda (IPMA): Latitude, 38º 42' N; Longitude, 9º 11' W; Altitude, 60 m. Each week of the experiment include the average of 7 days, starting from 19th December 2016 and finishing on 30th June 2017. Mean, max. and min. temperatures were recorded at 1,5 m high. Relative humidity of the air was recorded at 1,5 m high. The wind speed was recorded at 10 m high. Total precipitation consists in the sum of precipitation of respective week.

Week of experiment	Mean temperature (°C)	Max. temperature (°C)	Min. temperature (°C)	Relative humidity (%)	Mean wind speed (m/s)	Total precipitation (mm)
1	10,8	14,6	6,3	80,6	2,9	0,0
2	10,8	14,3	7,6	77,1	2,8	10,3
3	11,4	16,7	7,6	82,9	2,0	0,5
4	9,9	15,4	5,2	69,6	2,0	0,2
5	8,7	14,3	3,5	71,9	1,7	29,6
6	12,9	15,7	10,5	87,5	2,7	19,6
7	11,0	15,2	7,3	84,6	2,6	25,6
8	12,8	17,9	9,0	78,6	2,4	24,9
9	13,7	18,2	10,3	68,4	2,5	2,7
10	12,9	17,2	9,1	84,3	1,9	37,4
11	15,6	21,3	11,2	63,8	2,9	0,3
12	13,3	19,1	8,6	66,1	2,1	8,7
13	12,6	17,3	8,2	80,9	2,1	35,6
14	17,1	24,7	10,1	56,6	2,2	0,0
15	17,4	25,1	11,4	66,4	1,8	0,0
16	18,6	25,3	13,0	62,3	2,2	0,0
17	15,1	21,3	9,1	61,8	2,4	4,3
18	17,6	22,9	12,2	72,4	2,3	27,7
19	18,5	23,7	13,5	73,1	2,7	25,5
20	20,9	28,2	13,9	61,6	2,1	0,0
21	18,8	24,5	14,2	74,6	2,3	5,0
22	20,8	27,6	14,6	59,8	2,3	0,0
23	25,1	34,4	17,1	56,8	1,8	0,1
24	21,4	27,6	16,2	68,8	2,1	0,2
25	18,9	23,8	14,3	66,7	2,9	1,2

Annex 4

Table 9 - Treatments application during the year of the experiment (2017) in cv Cabernet Sauvignon with the respective active ingredient and dose applied.

Date	Trade name/manufacturer	Active ingredient	Reason of application	Dose applied
22/02	Cuprocol (Syngenta); Garbol (Bayer).	Copper oxychloride; summer oil	Fungi; Hibernating forms of insects and mites	200 ml/hL 1,75 L/hL
24/02	Touchdown (Syngenta); Chikara (Belchim).	Glifosate; Flazasulfuron	Control of weeds in lines	2 L/ha 150 g/ha
10/03	Quadris (Syngenta).	Azoxystrobin	*Phomopsis* spp.	75 ml/hL
24/03	Quadris (Syngenta); Score (Syngenta).	Azoxystrobin; Difenoconazol	*Phomopsis* spp.	100 ml/hL 50 ml/hL
30/03	Nergetic Zimactiv (ADP Fertilizantes)	NPK (30-0-0)	Plant nutrition	50 g/plant
27/04	Ridomil Gold MZ Pepite (Syngenta); Topaze (Syngenta); Zetaminol (Syngenta); Stimufol K (Syngenta).	Metalaxyl-M + Folpet; Penconazole; Fertilizer NPK (18-11-8) with amino acids; Fertilizer NPK with high content of K and B.	*Plasmopara viticola; Erisiphe necator;* Foliar fertilization; Foliar fertilization	2,5 Kg/ha 0,3 L/ha 100 g/hL 400 g/hL
16/05	Pergado F (Syngenta); Dynali (Syngenta); Zetaminol (Syngenta); Stimufol K (Syngenta).	Mandipropamide + Folpet; Ciflufenamide + Difenoconazole; Fertilizer NPK (18-11-8) with amino acids; Fertilizer NPK with high content of K and B.	*Plasmopara viticola; Erisiphe necator;* Foliar fertilization; Foliar fertilization.	2,5 Kg/ha 650 ml/ha 100 g/hL 400 g/hL
01/06	Dynali (Ciflufenamida + Difenoconazol); Zetaminol (Syngenta); Stimufol K (Syngenta).	Ciflufenamide + Difenoconazole; Fertilizer NPK (18-11-8) with amino acids; Fertilizer NPK with high content of K and B.	*Erisiphe necator;* Foliar fertilization; Foliar fertilization	650 ml/ha 100 g/hL 400 g/hL
29/06	Quadris (Syngenta); Zetaminol (Syngenta); Stimufol K (Syngenta).	Azoxystrobin; Fertilizer NPK (18-11-8) with amino acids; Fertilizer NPK with high content of K and B.	*Erisiphe necator;* Foliar fertilization; Foliar fertilization	1 L/ha 100 g/hL 400 g/hL

Annex 5

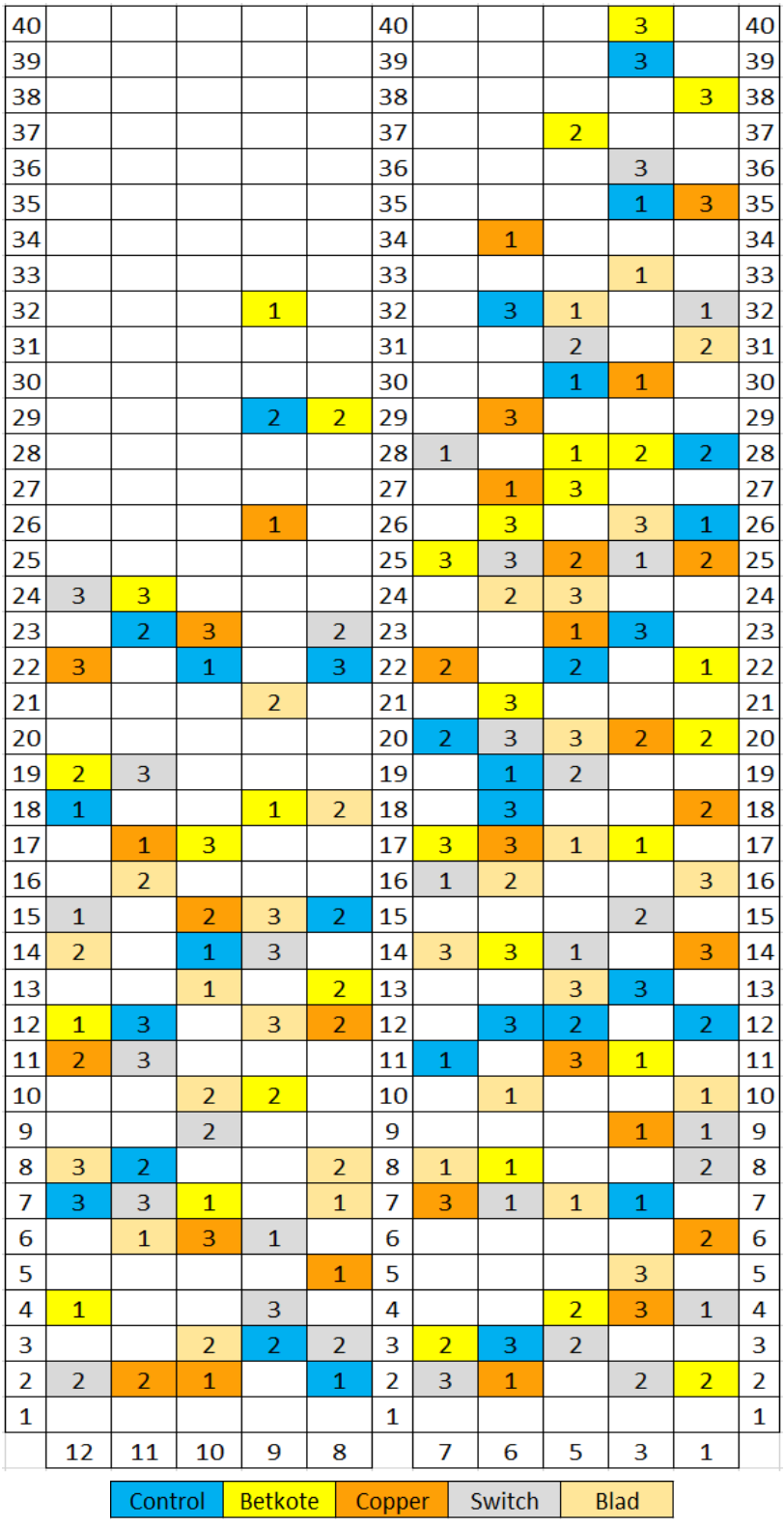

Fig. 18 - Field map of the cv Cabernet Sauvignon in Almotivo vineyard. The numbers on the vertical (both on the right and left) represents the vines. The numbers on the horizontal (on the bottom) represents the number of the lines. The different colours show the treatments in study and inside, the numbers represent the infection time of those canes.

Annex 6

Table 10 – Description of the phenological stages of the grapevine according to the extended BBCH scheme. The records on the vineyard followed this scale. (Adapted from Lorenz *et al*., 1995).

BBCH-Code	Description
Principal growth stage 0	**Sprouting**
00	Dormancy: winter buds pointed to rounded, bright or dark brown according to cultivar, bud scales more or less closed according to cultivar
01	Beginning of bud swelling: buds begin to expand inside the bud scales
03	End of bud swelling: buds swollen, but not green
05	"Wool stage": brown wool clearly visible
07	Beginning of bud burst: green shoot tips just visible
09	Bud burst: green shoot tips clearly visible
Principal growth stage 1	**Leaf development**
11	First leaf unfolded and spread away from shoot
12	Two leaves unfolded
13	Three leaves unfolded
14	Four leaves unfolded
15	Five leaves unfolded
16	Six leaves unfolded
19	Nine or more leaves unfolded
Principal growth stage 5	**Inflorescence emergence**
53	Inflorescence clearly visible
55	Inflorescence swelling, flowers closely pressed together
57	Inflorescences fully developed, flowers separating
Principal growth stage 6	**Flowering**
60	First flowerhoods detached from the receptacle
61	Beginning of flowering: 10% of flowerhoods fallen
63	Early flowering: 30% of flowerhoods fallen
65	Full flowering: 50% of flowerhoods fallen
68	80% of flowerhoods fallen
69	End of flowering
Principal growth stage 7	**Development of fruits**
71	Fruit set: fruits begin to swell, remains of flowers lost
73	Berries groat-sized, bunches begin to hang
75	Berries pea-sized, bunches hang
77	Begin of berry touch
79	Berry touch complete
Principal growth stage 8	**Ripening of berries**
81	Beginning of ripening: berries begin to brighten in colour
83	Berries brighting in colour
85	Softening of berries
89	Berries ripe for harvest
Principal growth stage 9	**Senescence**
91	After harvest: end of wood maturation
92	Beginning of leaf discoloration
93	Beginning of leaf fall
95	50% of leaves fallen
97	End of leaf fall
99	Post-harvest treatments

Annex 7

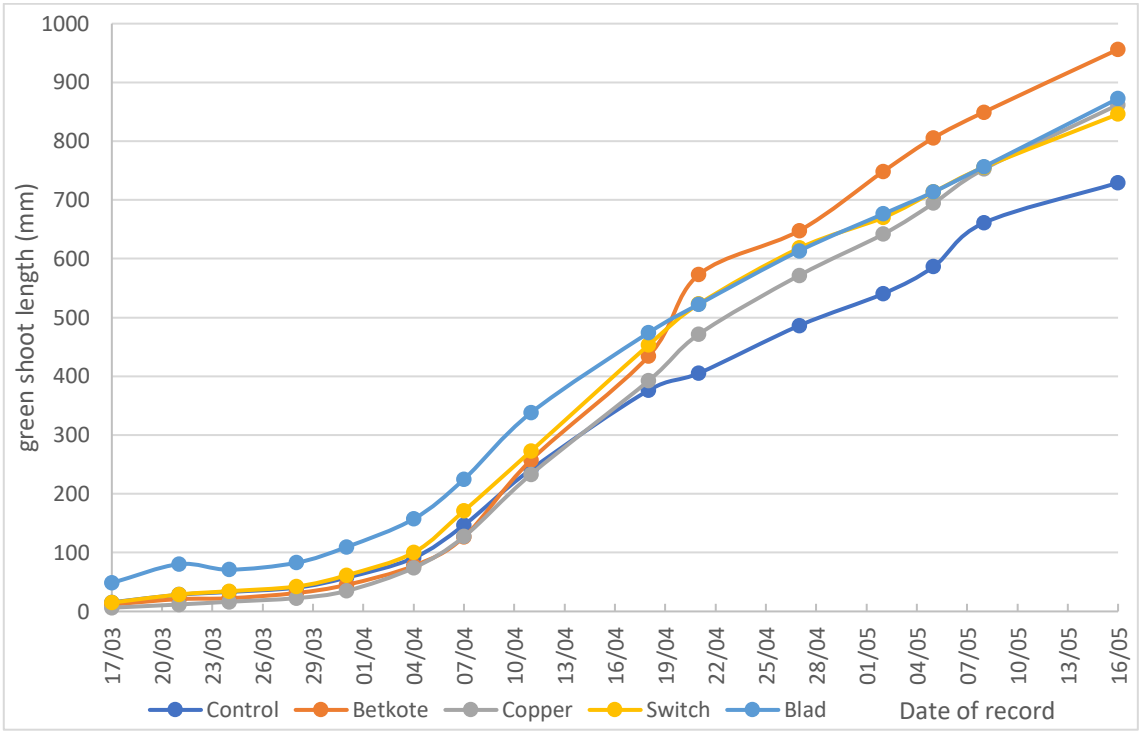

Fig. 19 - Shoot length records (in mm) of the control and the different treatments, during the vegetative growth in cv Cabernet Sauvignon, Lisbon region, 2017. Asterisk refers to the treatment copper oxychloride with the summer oil.

Annex 8

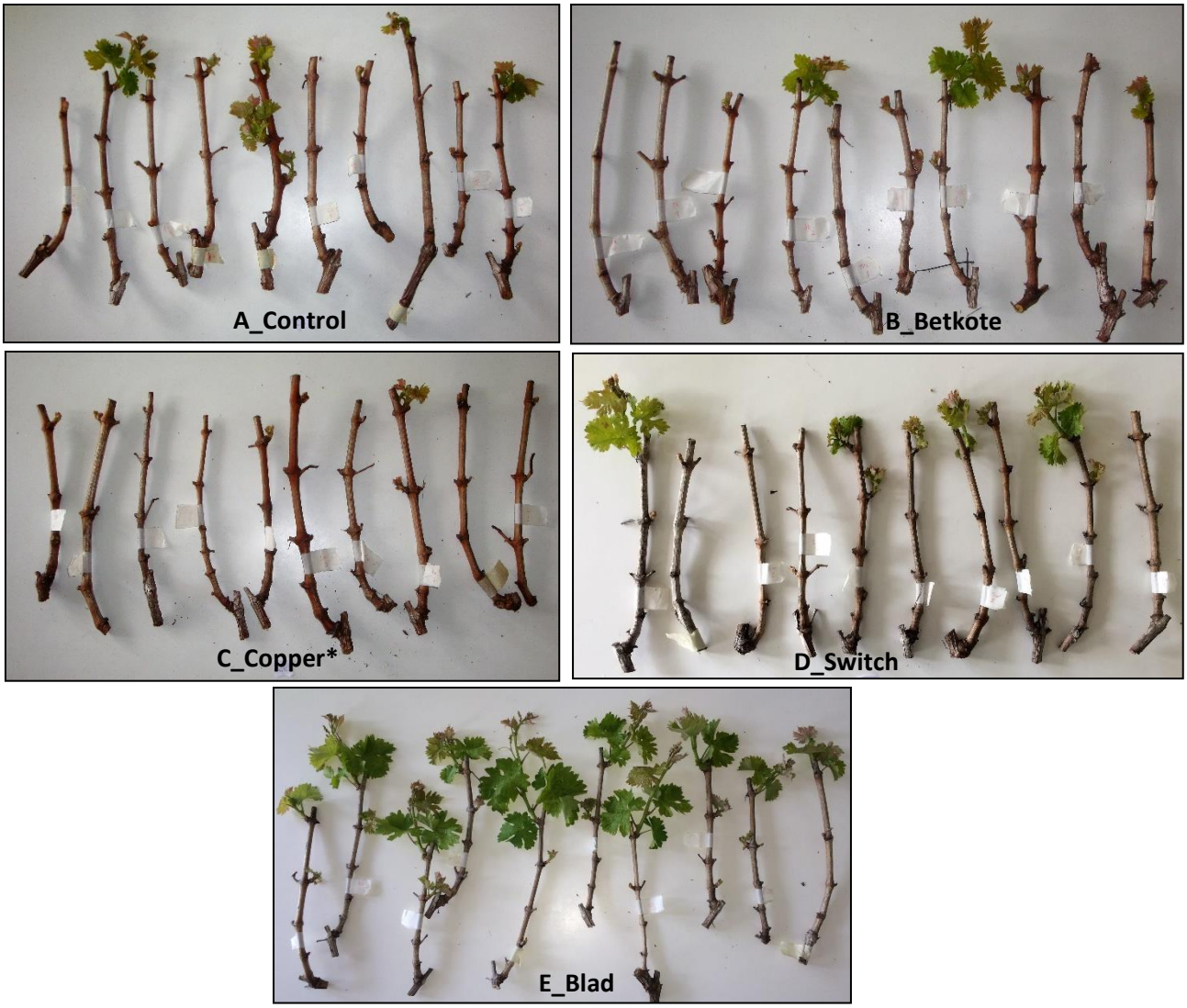

Fig. 20 – Visual differences in length of the shoots from the top bud of the different treatments in study (from A to E) (19/03/2017). Blad was statistically different ($P < 0.05$) from the control and remain treatments. Asterisk refers to the treatment copper oxychloride with the summer oil. (Photos: João Costa).