Efficacy of kaolin on the control of leafhoppers in Syrah and Tempranillo varieties in Alentejo Vineyards

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MSc

Dedicated to my father that always supported my choices and education

Sworn Statement

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Sara Henriques da Silva e Costa

30 October 2022

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Resumo

O presente relatório surge de uma parceria com a Valbom do Rouxinol, Lda., que aceitou receber-me para um estágio na área da viticultura e enologia, e para a concretização, em simultâneo, de um estudo científico com vista à obtenção do grau mestre em Engenharia Agronómica na Faculdade de Ciências da Universidade do Porto (FCUP). Em relação ao estudo científico, as cigarrinhas verdes têm sido consideradas uma praga séria na viticultura do sul de Portugal e, atualmente, são poucos, e ineficazes, os inseticidas disponíveis para o controlo desta praga em modo de produção biológico (MPB). A infestação severa desta praga pode levar à necrose foliar, desfolha prematura, atraso na maturação, perda de rendimento e redução da qualidade da uva. Publicações recentes referiram o caulino como um candidato para o controle da cigarrinha verde em MPB, porém ainda é pouca a informação sobre o número ideal de tratamentos e concentrações que devem ser aplicadas para assegurar um controlo eficaz. Portanto, este estudo visa contribuir com mais informações sobre a eficácia do caulino no controlo da cigarrinha verde no setor vitivinícola, e estudar uma possível solução de tratamento. Dois estudos foram propostos de forma a investigar o impacto da aplicação preventiva de caulino no início da 2ª e 3ª geração de cigarrinhas (mais problemáticas) e, em segundo lugar, o impacto da proteção contínua de caulino desde o início da migração dos adultos para a vinha até à vindima versus uma e duas aplicações. Este último estudo visa avaliar o número ideal de tratamentos. Em ambos os ensaios, o caulino provocou uma diminuição das ninfas da cigarrinha para níveis baixos, bem como uma redução dos seus sintomas. Especificamente, não houve diferença estatística entre a realização de duas a quatro aplicações de caulino Surround WP a 2,5 g/L. Conclui-se, por razões económicas, que o tratamento com apenas duas aplicações de caulino (2KT) é considerado o mais adequado e eficiente para controlar a população de cigarrinha-verde durante o ciclo da videira.

Palavras-chave: videira, viticultura, controlo biológico, *cicadelidae*, ninfas, sintomas, necrose foliar, filme mineral, syrah, aragonês.

Abstract

This report emerges from a partnership with Valbom do Rouxinol, Lda., who agreed to receive me for an internship in the area of viticulture and winemaking and to carry out, at the same time, a scientific study in order to complete my master in Agronomic engineering at the faculty of sciences of the university of Porto (FCUP). Regarding the scientific study, leafhoppers have been considered a serious pest in viticulture in southern Portugal and, currently, the available insecticides are ineffective to control this pest in organic production. Severe infestation of this pest can lead to leaf necrosis, premature defoliation, delayed maturation, loss of yield and grape quality. Recent publications have mentioned kaolin as a candidate for the control of leafhoppers in organic vineyards, but there is still little information about the ideal number of treatments and concentrations that should be applied to ensure its control. Therefore, this study aims to contribute with more information about the effectiveness of kaolin on leafhoppers in the viticulture sector, and to study an effective treatment solution. Two studies were proposed to, firstly, understand the impact of preventive kaolin application in reducing nymph infestation in early 2nd and 3rd generation leafhopper's development (more damaging) and, secondly, the impact of continuous kaolin protection from the migration of adults to the vineyard to harvest versus one and two applications. This last study aims to evaluate the ideal number of treatments. Kaolin caused a significant decrease in leafhopper nymphs to low levels as well as a reduction in leafhopper's symptoms in both trials. In particularly, two and four pulverizations were the most and equally efficient, treatments and, for economic reasons, the treatment with only two kaolin sprays (2KT) is considered the most adequate and efficient to control the grapevine leafhopper population during the vine cycle.

Keywords: grapevine; viticulture; biologic control, *cicadellidae*, nymphs, symptoms, foliar necroses, mineral film, syrah, tempranillo.

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List of Abbreviations

0KT	NO KAOLIN TREATMENT
1KT	ONE KAOLIN PULVERIZATION TREATMENT
2KT	TWO KAOLIN PULVERIZATIONS TREATMENT
ANOVA	ANALYSIS OF VARIANCE
CKT	CONTINUOS KAOLIN PULVERIZATION TREATMENT
ED	ELECTRODIALYSIS
EIL	ECONOMIC INJURY LEVEL
HACCP	HAZARD ANALYSIS AND CRITICAL CONTROL POINT
IPM	INTEGRATED PROTECTION MANAGEMENT
KHT	POTASSIUM HYDROGEN TARTRATE
KPF	KAOLIN PARTICLE FILM
MPB	MODO DE PRODUÇÃO BIOLÓGICO
OMRI	ORGANIC MATERIALS REVIEW INSTITUTE
PK 0	PREVENTIVE KAOLIN CONTROL
PK 1	PREVENTIVE KAOLIN TREATMENT
TSS	TOTAL SOLUBLE SOLIDS

Introductory note

This report emerges from a partnership with Valbom do Rouxinol, Lda., who agreed to receive me for an internship in the area of viticulture and winemaking and to carry out, at the same time, a scientific study in order to complete my master in Agronomic engineering at FUCP.

Valbom do Rouxinol homestead is located in Évora, Portugal, and comprises 60 certified hectares in organic farming. The homestead encompasses grapevines, olive groves, cork oak forests, and a cellar for the winemaking process. Regarding the vineyard, it comprises 9.1 ha of four grapevines varieties, namely Syrah, Viognier, Tempranillo and Arinto, with which produces Dona Dorinda's white, rose, and red wine.

For the development of the scientific study, described in Chapter 1, I tried to understand, during the first month, the major viticulture struggles that could benefit from scientific research. Valbom do Rouxinol faces high populations of leafhoppers in the Syrah and Tempranillo varieties, due to the semi-arid Mediterranean climate, that lead to severe symptoms such as leaf necrosis and defoliation before ripening stage. This compromises the photosynthetic activity and consequently, the production of photoassimilates, having a negative impact on grape´s quality and yield. The current available options to control this pest in organic production proved to be insufficient and, therefore, new strategies are needed. In this study, kaolin was tested as a possible solution since it has been reported as a possible alternative to synthetic insecticides in controlling grapevine leafhoppers (Tacoli *et al.*, 2017; Tirello *et al.*, 2021). Two non-related experimental studies were performed in the two problematic and susceptible varieties in order to pursuit successful solutions to control this pest. In vineyard A, which comprises the Tempranillo variety, a preventive kaolin pulverization study was performed at the first nymph appearance of the 2nd and 3rd generation (the most damaging). On the other hand, in vineyard B, that comprises the Syrah variety, a continuous protection of kaolin (4 kaolin applications) from the beginning of adult's migration to the vineyard until harvest was compared with one and two applications. Both studies evaluated the impact on nymph infestation reduction and, particularly in the second study, the ideal number of treatments was assessed.

Concerning the internship, the Chapter 2 describes the activities developed at Valbom do Rouxinol Lda., during the 6-month internship. The main tasks performed in the vineyard were traditional organic viticulture operations, such as grapevine conduction, copper, and sulphur pulverizations for powdery and downy mildew protection, green pruning, maturation control and lastly, grape harvesting. The cellar operations for rose, white and red wine vinification comprised the cleaning and sanitation of general equipment (vats and barrels), acidity corrections, yeast inoculations, fermentation and Hazard Analysis and Critical Control Point (HACCP) control, and wine stabilization by electrodialysis.

CHAPTER 1

1. State of art

1.1. Leafhoppers as a viticulture pest

Vineyards are attacked by several arthropod pests and diseases. Among the arthropod pests (e.g., cutworms, thrips, phylloxera, mites, mealybugs, beetles, moths), leafhoppers (Hemiptera: *Cicadellidae*) are occasional pests of vineyards in temperate areas which cause direct damage to grape leaves by its feeding activity (Bournier, 1977; Bostanian *et al.*, 2003; Vincent *et al.*, 2012). Moreover, leafhoppers are also important vectors of plant pathogens, including viruses, bacteria (e.g., *Xylella fastidiosa*, the agent of Pierce's disease), and phytoplasmas that induce diseases, such as grapevine yellows through *Scaphoideus titanus* vector (Musetti, 2008; Weintraub & Beanland, 2006).

Leafhoppers can be found throughout Europe, Scandinavia, North Africa, Asia, and North America, displaying their wide adaptability. The species tend to vary from region to region, however most leafhoppers have fairly similar life cycles and are controlled by similar techniques. In Europe, *Empoasca* leafhoppers tend to be the most damaging group, especially *Empoasca vitis*, and considered a major insect pest (Pavan *et al.*, 2000; Baillod *et al.*, 1993). Regarding Portugal, leafhoppers have been considered an increasing problem in southern Portuguese viticulture. The hot summers of the recent years were one of the favourable factors for the intense increment of this pest. Their first references as a pest of the vine date back to 1980 in the Alentejo region (Quartau & Rebelo, 1992). Later, during the 1990s, the species complex extended and affected the Douro and Dão regions. Nowadays, the dominant leafhopper species in Portuguese vineyards are *Jacobiasca lybica* in Alentejo region (Quartau & Rebelo, 1992) and *Empoasca vitis* in Douro and Dão regions. In the northeast region (Verde wine), this insect is only considered an occasional enemy and not recognized as a major concern (Quartau & Rebelo, 1992; Rebelo, 1993)

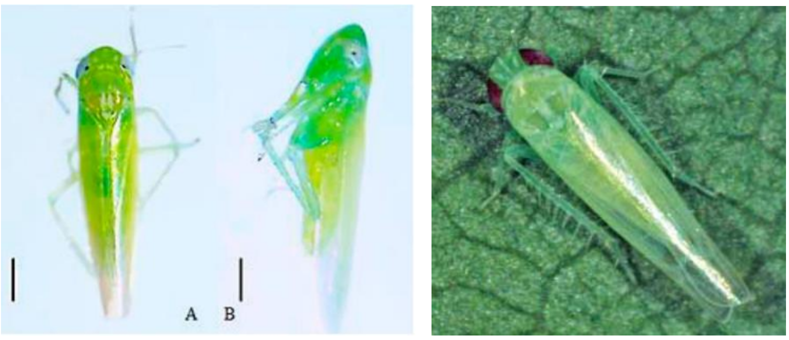

Figure 1 - Adult *J. lybica* leafhopper in Morocco: On the right side: (A) male in dorsal view and (B) lateral view, Scale bars: 0.5 mm (Khfif *et al.*, 2022). On the left side: J. lybica in Western Sicily (Bono G., 2005).

1.2. Leafhopper's cycle and morphology

J. lybica and *E. vitis* are polyphagous species that overwinters as adults on evergreen plants species (i.e., arboreal, shrubby, and herbaceous plants). Their cycle goes through three stages of development, namely egg, nymph, and adult (Figure 2), and can complete from one to four generations between, April and October, in different European grape-growing areas including Portugal (Vidano, 1963; Schvester *et al.*, 1962; Mathys *et al.*, 1968; Cerutti *et al.*, 1991).

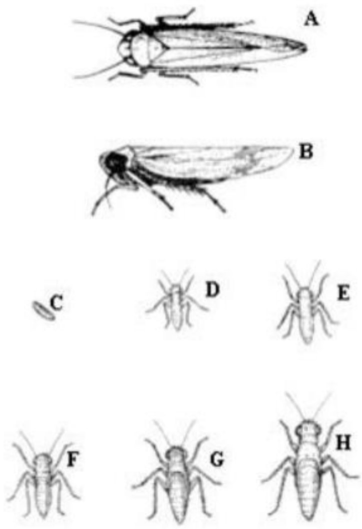

Figure 2 - Leafhopper life cycle. Adult stage (A); the five nymphal instars (D-H) and egg (C) (Lima, 2012).

At the beginning of spring, with the increase in temperature and with the budbreak of the vine, the hibernating leafhopper adults abandon the winter host and settle in the vine. This period occurs, from the end of April to the beginning of May, when average temperatures exceed 10ºC (Helden, 2000). This migration to the vineyard is carried out almost exclusively by females, because males gradually disappear in early spring (Vidano, 1963). Fertilization of hibernating females occurs during winter or autumn, usually before migration to hibernating hosts (Galet, 1982). These remain in diapause throughout the hibernation period and only with the increase in the number of hours of light per day, the reproductive organs mature. According to Raposo and Amaro (2003), after a few weeks of activity in the vineyard, the females lay their eggs along the veins on the underside of the leaves. Each wintering female of *J. lybica* lays, on average, 15-

20 eggs in the first generation, which give rise to the respective first-generation nymphs, and 65-75 eggs in the second and third generations. The egg matures after 8-10 days. The egg is whitish, cylindrical and has reduced dimensions (0.6 mm in length and 0.3 mm in width), being difficult to observe (Klerks & Van Lenteren, 1991).

Before becoming an adult, it goes through five nymphal instars. The nymphs are colourless and gradually acquire yellowish-green tones in the following instars (Figure 3) (Klerks & Van Lenteren, 1991). Remaining on the underside of the leaf, the nymphs can be easily identified by their characteristic lateral movement when disturbed (Rebelo, 1993). The juvenile leafhoppers are similar to the adult, gradually increasing in size and developing the wing outlines. The individuals of this stage have an elongated and cylindrical-conical abdomen, an extended body between 0.7-0.9 mm with long antennae, but smaller than the body size (Rebelo, 1993). The five nymphal instars period last, approximately, 15 to 28 days (Klerks & Van Lenteren, 1991).

Figure 3 - *Empoasca vitis* nymphs in grapevine leaf (Bono G., 2005; Global, 2022).

In the adult stage, individuals have a light green colour tending to yellow and measure between 2.8 mm and 3.2 mm (Figure 1). As in the previous stages, maintain the biting-sucking mouth armour and are located mainly on the underside of the leaf. The body has a triangular appearance due to membranous and translucent wings, which, when at rest, are arranged as a roof like shape. The legs are long and robust, and the posterior tibias are equipped with a row of mobile spines that allow them to move in jumps (Rebelo, 1993).The antennae are located between the eyes, which have a setiform shape and are usually white, however under saturated humidity conditions can acquire brownish tones. The head is angular in shape, the pronotum is short and normally developed. Sometimes it may have white spots on the head and/or pronotum (Bono *et al.*, 2005). Females tend to be slightly larger than males but are morphologically identical. After death, they acquire a yellowish colour (Rebelo, 1993).

All leafhoppers' species are very similar in their external morphology: narrow and elongated body, greenish colour, and with similar dimensions. Specific identification is only possible through microscopic analysis of male genitalia (Quartau & Rebelo, 1992). The male genitalia are located in the abdomen and consists of the styles, aedeagus, plates, connectives and pygophore. The aedeagus (Figure 4) is the male copulatory organ through which sperm is secreted during copulation. The morphology of the aedeagus is unique to each species hence it is a diagnostic feature used for specific identification. Unlike males, female genitalia are more conserved across species (Marucci, 1998).

A **B**

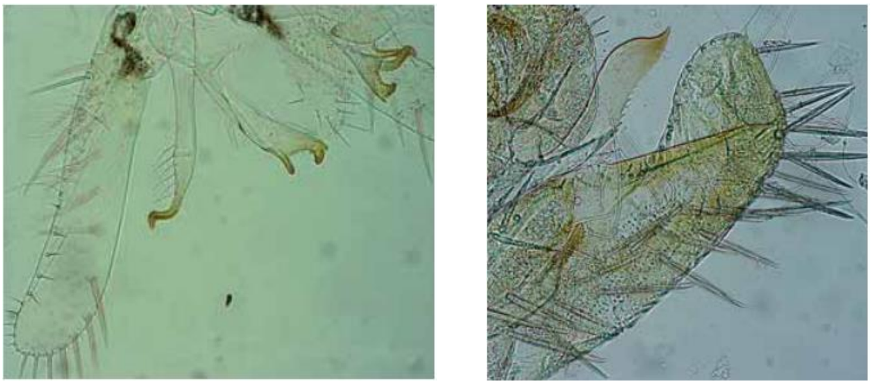

Figure 4 – Aedeagous of *J. lybica* (A) and *E. vitis* (B) (Bono G., 2005).

1.3. Leafhopper's favourable conditions of development

The favourable conditions for leafhopper's development are high temperatures and high humidity, although there are differences depending on the species, namely *E. vitis* prefers milder temperatures than *J. lybica*, which is why the latter predominates in the Alentejo (Klerks & Van Lenteren, 1991; Bono *et al.*, 2005). The leaves placed in more protected positions inside the canopy are preferred by adults for oviposition and support a higher number of nymphs. It also prefers the underside of the leaves where it spends most of its time and where it is quite active. It is known that grapevine vigour and canopy density can favour oviposition and nymph abundance in vineyards. In fact, the most vigorous shoots show higher number of leafhopper nymphs per leaf (Pavan & Picotti, 1993). The migratory movement to winter host vegetation is essential done by females and, preferably, to shrubby vegetation located next to water lines or sources (Vidano, 1963; Alvarez, 2020).

1.4. Leafhoppers associated symptoms and economic damages

In Portugal, *Empoasca vitis* and *Jacobiasca lybica* are the only species considered pests in vineyards (Pavan *et al.*, 2000; Klerks & Van Lenteren, 1991; Bono *et al.*, 2005). These leafhoppers are a phloem feeders of grapevine leaf veins, and their biting-sucking activity as well as their toxic saliva produces deformations in the leaves surface, pigmentation alterations and leaf necrosis. Symptomatology is redness or yellowing of the edges of the leaves depending on whether the affected cultivars are red or white, respectively, as demonstrated in figure 5 (B & C). In severe attacks, edge necrosis and leaf curling and coiling are observed (Figure 5: A & D). Significant damage can cause premature defoliation, making grape ripening difficult (Vidano, 1963; Carle & Moutous, 1965). Leaf symptoms are associated with physiological damage such as reductions in photosynthesis, mesophyll conductance, and transpiration rate (Candolfi *et al.*, 1993), which can lead to economic damages when the infestation exceeds one or two nymphs per leaf. These damages are related to the reduction of the foliar area, which lead to yield losses, sugar, photo assimilates and malic acid reduction content of berries (Pavan *et al.*, 2000; Baillod *et al.*, 1993; Moutous & Fos, 1971; Lehmann *et al.*, 2001).

Figure 5 – Characteristic colour change effect of vineyards attached with *J. lybica* (white and red cultivars). Leaf symptoms observed in white grapevines varieties (B), in red grapevines varieties (C), and in a more advance and severe attack phase (D) (Bono G., 2005).

The appearance of symptoms on leaves caused by leafhopper activity is not immediate, in fact, these tend to manifest 20 to 30 days after the attack. Insects may no longer be present on the damaged leaves, which sometimes leads to a misattribution of these changes to other causes such as nutritional deficiencies, viral or phytoplasma activity. In fact, the symptoms of this pest can be easily confused with other foliage alterations, caused by diseases (esca disease, curl virus and golden flavescence); mineral deficiencies (potassium, boron and magnesium), mite attacks or even phytotoxicities (Botelho, 2001). However, the activity of leafhoppers can be observed by the presence of numerous exuvia that remain on the underside of the leaf (Alvarez, 2020).

The first generation of the pest does not seem to be very harmful, as it appears at a time when the vineyard is in a period of intense photosynthetic activity and, consequently, of rapid vegetative expansion (Rebelo, 1993; Meireles, 2000). The 2nd and 3rd generations are the most disturbing, since the harmful effects are higher, generally occurring in climatic conditions favourable to its development, that is, high temperatures and low relative humidity recorded between July and August, which increases the expression of symptoms, namely the “burnt” aspect of the leaf. Moreover, according to Galet (1982), leaves exposed to the West and South are the most affected, because these not ensure sufficient nutrition to replace the water lost by prolonged exposure to the sun. This effect is even more intense the closer the plants are to water stress (Freitas, 1999). Additionally, data suggest that climatic conditions (i.e., thermal and water stresses and their seasonal variation) and agronomic factors (i.e., clone, rootstock, training system, plant density, and yield per vine) can influence the symptom expression (Pavan *et al.*, 2000; Candolfi *et al.*, 1993).

1.5. Current available treatments for leafhopper control

The control of leafhoppers involves regular monitoring of the crop, correct vineyard management and the practice of cultural control measures. The monitoring of the leafhopper can be performed with the aid of yellow chromotropic traps, where the time of appearance of the adults is detected, and by counting the number of nymphs observed in 100 leaves scattered in the vineyard. This control is essential for interventions to be opportune and to avoid crop damage (Almeida, 2021). The fight against this pest must be carried out whenever 100 nymphs are observed in 100 leaves, this value may be lower in cases of young vines or very susceptible varieties. In late attacks (July - August) due to the rapidly development, the greater intensity of the symptoms, and their consequences in terms of maturation, treatments should be carried out as soon as there is an abrupt evolution in the number of nymphs observed.

Agronomic practices that reduce leaf density, such as green pruning can decrease leafhopper infestation if it is done during egg laying (Pavan & Picotti, 2009). Limiting nitrogenous fertilization and irrigation, to avoid excessive vigour of vines, could have the same side effect as observed for other grape leafhoppers in North America (Daane *et al.*, 1995). The use of less susceptible varieties, shading, mulching, cover crops, and the diversification of cultivars and crops can also have a positive effect.

The synthetic insecticides are usually applied to control leafhoppers, specifically composed mostly of organophosphate, pyrethroids and chitin-synthesis inhibitors (Lavezzaro *et al.*, 2006; Pozzebon *et al.*, 2011). In the 1990s, organophosphates' effectiveness in controlling leafhoppers declined, probably because of the selection of strains resistant to pesticides (Girolami *et al.*, 2001). Organic-certified pesticides have been more frequently considered for the development of environmentally sound and integrated pest management (IPM) approaches. However, the origin of these natural compounds does not necessarily entail safety from the ecotoxicological perspective (Biondi et al., 2012; Biondi, Zappalà, Stark, & Desnux, 2013). For organic vineyards the only options against leafhoppers are pyrethrum (Mori & Pavan, 2014), whose efficacy is prejudiced by its low persistence, or copper products, whose amount applied per hectare is limited by legal measures due to environmental issues (Mazzini, 2010). Still in the context of IPM, other control approaches have been proposed, such as conservation biological control to promote the activity of the egg parasitoid *Anagrus atomus L.* (*Hymenoptera Mymaridae*) and other natural enemies (Cerutti *et al.*, 1991; Helden, 2000; Van Helden & Decante, 2001; Ponti *et al.*, 2005; Zanolli & Pavan, 2011), as well as the planting of grapevine cultivars of low susceptibility (Zanolli & Pavan, 2011). In the case of *S. titanus* control, citrus extracts and kaolin are also advice (Winetwork, 2017).

Recently, new compounds replaced organophosphates in most viticultural areas and some of them (e.g., neonicotinoids) were very effective against leafhoppers (Pozzebon *et al.*, 2011; M. Delaiti, 2005; Posenato *et al.*, 2006). However, in the last years, several active ingredients have been banned in Europe and some of the remaining insecticides showed lower effectiveness and persistence against leafhoppers occurring in vineyards, which often requires a large number of treatments. Outbreaks were detected in conventional vineyards despite the use of broad-spectrum insecticides as well as in organic vineyards treated with pyrethrins. The selection of active ingredients has become more important than in the past and chemical control must be integrated with agronomic and cultural measures to obtain adequate control of grapevine leafhoppers (Chuche & Thiéry, 2014; Mori *et al.*, 2020; Pavan *et al.*, 2012). Therefore, the identification of effective control tools is required. Recent studies evaluated the most effective insecticides to be applied in conventional and organic vineyards. The most effective synthetic insecticides were acetamiprid, flupyradifurone and lambda-cyhalothrin, while the most effective natural product was kaolin (Tirello *et al.*, 2021).

1.6. Kaolin particle film as pest control

Particle films are effective against many key orders of arthropod pests affecting crops, including homopterans, coleopterans, lepidopterans, dipterans, and rust mites, as well as the family *Eriophyidae* (Glenn & Puterka, 2010).

Arthropods use the senses of touch, taste, sight, and smell in the processes of locating and accepting plants as a host for feeding and reproduction (Miller & Strickler, 1984). During this process, the four senses interact in such manner that insect's sense positive and negative cues, the sum of which provokes a positive or negative behaviour in insects. For example, when the accumulation of positive cues outweighs negative cues, an acceptance behaviour (i.e., feeding, oviposition) will occur. Plant tissues coated with particle films are obviously altered visually and tactilely to insects, and could possibly alter the taste or smell of the host plant (Glenn *et al.*, 1999). Choice and no-choice laboratory bioassays with various insects revealed that the primary mechanism of action was repellence of adults from treated foliage with particle films that results in reduced feeding and oviposition (Glenn & Puterka, 2010).

To be effective on plant tissues, the particle film needs to have certain characteristics: (1) chemically inert mineral particle, (2) particle diameter < 2 μm, (3) formulated to spread and create a uniform film, (4) porous film that does not interfere with gas exchange from the leaf, (5) transmits photosynthetically active radiation (PAR) but excludes ultraviolet (UV) and infrared (IR) radiation to some degree, (6) alters insect/pathogen behaviour on the plant, and (7) can be removed from harvested commodities. Many of these characteristics are similar to natural plant defences, consisting of increasing cuticle thickness and pubescence to reduce water and heat stress (Levitt, 1980) and to interfere with disease and insect damage (Neinhuis & Barthlott, 1997; Barthlott & Neinhuis, 1997). Additionally, these particle films can be combined with pesticides, as a pesticide delivery system, providing the efficacy of a full rate of that pesticide and allowing a pesticide concentrations reduction by 50% (Puterka, Glenn, *et al.*, 2000).

As an alternative to synthetic insecticides, kaolin particle film (KPF) technology is proposed for arthropod pest control, being listed for use in organic food production by the Organic Materials Review Institute (OMRI) (Glenn & Puterka, 2010; Glenn *et al.*, 1999). Kaolin Surround® (Engelhard Corp., Iselin, NJ, USA) is a potential alternative pest management product with improved safety to pesticide handlers and reduced environmental impact (Glenn et al., 1999). Studies have shown that formulations of KPF can effectively protect host plants from insect pests including leafhoppers (Tacoli *et al.*, 2017) lace bugs (Marcotegui *et al.*, 2015), thrips (Tyler-Julian *et al.*, 2014), aphids (Pissinati & Ventura, 2015), psyllids (Puterka *et al.*, 2005; Butler *et al.*, 2011), scales (Butler *et al.*, 2011), and against chewing pests such as tephritid fruit flies (Butler *et al.*, 2011), tortricid moths (Pease *et al.*, 2016), and blossom weevils (Marko *et al.*, 2008). As a water suspension, hydrophilic kaolin Surround® WP can be sprayed on the surface of crops to form a protective film (Glenn *et al.*, 1999; Puterka, Sekutowski, *et al.*, 2000), as shown in figure 6, changing the tactile and visual features (high brightness) of the leaf, and consequently repel leafhoppers, deter oviposition, disrupt feeding, and even compromise grasping (Glenn & Puterka, 2010; Glenn *et al.*, 1999). According to Tacoli *et al.* (2017), feeding inhibition is the main route of action by which kaolin affects nymph populations, being more effective on the nymphal instars. Moreover, Glenn *et al.* (1999) reported that kaolin contributed to control fungal and bacterial plant pathogens by preventing the formation of a liquid film on the leave's surface. Given the product's high cost and moderate effectiveness, the optimization of its application doses should be promoted.

Figure 6 – Kaolin particle film in Syrah grapevines with a 2,5 g/L Surround WP pulverization

Kaolin application significantly influences the grapefruit metabolome in a way that provides grapes with higher phenolic compounds, tartaric and malic acids, total acidity, and lower sugar content (Dinis *et al.*, 2020). Besides, it is essential to reinforce that a good influence was observed on wine, such as higher acidity and lower alcohol levels (current food tendency and an aim for Alentejo's wines), and seems to improve the aroma. In sum, foliar kaolin application in grapevine leaves demonstrates great potential as an insect repellent strategy, resulting in increased fruit yield and quality (Dinis *et al.*, 2020).

Kaolin is a white, non-porous, non-swelling, low-abrasive, fine-grained, plate-shaped, aluminosilicate mineral [$Al_4Si_4O_{10}(OH)_8$] that easily disperses in water and is chemically inert over a wide pH range (Glenn & Puterka, 2010). Kaolin clay has been widely used in a variety of industrial applications including paints, cosmetics, and pharmaceuticals (International, 2022). Concerning the agriculture sector, at the present time, a commercial particle film material, Surround® crop protectant (95% kaolin), is being used in about 90% of the Pacific Northwest pear market for the early season control of pear psylla, and, approximately, 20% of the Washington State apple market to reduce sunburn damage (Werblow, 1999; Heacox, 2001).

Concerning the viticulture sector, only two studies are reported in the literature evaluating kaolin application on the control of leafhoppers infestation. One evaluated the effectiveness of insecticides to be applied in conventional and organic vineyard during the 2nd generation of the species *E. vulnerate*. It reported that the most effective natural product was kaolin (2 applications at a 4 Kg/l concentration) and that it could be an alternative to pyrethrins in organic vineyards or used as a complementary tool in conventional vineyards (Tirello *et al.*, 2021). The second study evaluated the preventive (at the beginning of the 2nd generation) and curative (at the peak of the third generation) application of kaolin (rate of 2% w/v on two occasions separated by 5–6 days) to control *E. vitis* leafhoppers infestation. According to Tacoli *et al.* (2017), both the preventive and curative kaolin applications caused a significant decrease in the populations. Based on these outcomes, this study stated kaolin as valuable alternative to synthetic insecticides in controlling grapevine leafhoppers (Tacoli *et al.*, 2017).

2. Objectives

Leafhoppers have been considered a serious pest in southern viticulture of Portugal and, currently, there are few and low efficient insecticides available for organic production to control this pest. Recently publications referred kaolin as a candidate for controlling leafhoppers in organic vineyards, however little information still exists regarding the number of treatments and concentrations that should be applied in viticulture to control efficiently this pest. Hence, this study aims to contribute with more information about kaolin effectiveness toward leafhoppers in the viticulture sector and propose an effective treatment solution. Therefore, two studies were performed in order to firstly, understand the impact of preventive kaolin application on nymph infestation reduction at the beginning of the 2nd and 3rd leafhopper generation, and, secondly, the impact of continuous kaolin protection from the migration of adults to the vineyard to harvest versus one and two applications, to assess the ideal number of treatments.

3. Material and methods

3.1. Site description

The vineyard A is a six-year-old organic vineyard with grapevines growing using the double arched Guyot training system with distances between and along rows of 2.5 m and 0.8 m. With a 500 grapevines/ ha density, it is composed of two types of cultivars, namely Tempranillo (70 %) and Syrah (30%), but the study was only performed on Tempranillo grapevines (Figure 7). This vineyard comprises 1.5 ha and is located in Évora, Portugal (N 38º 38' 10.666' W 7º 54´35.593'').

The vineyard B is a sixteen-year-old organic vineyard with grapevines growing using the double arched Guyot training system with distances between and along rows of 2.5 m and 0.8 m, respectively. It is composed of two types of cultivars, namely Syrah (80 %) and Viognier (20%), however the study was only performed on the susceptible grapevine variety Syrah (Figure 7). This vineyard comprises 2.0 ha and is located in Évora, Portugal, (N 38º 38' 10.666' W 7º 54´35.593'').

The same green pruning, fertilization, and irrigation practices, as well as fungicide treatments, were applied to all treatments and controls.

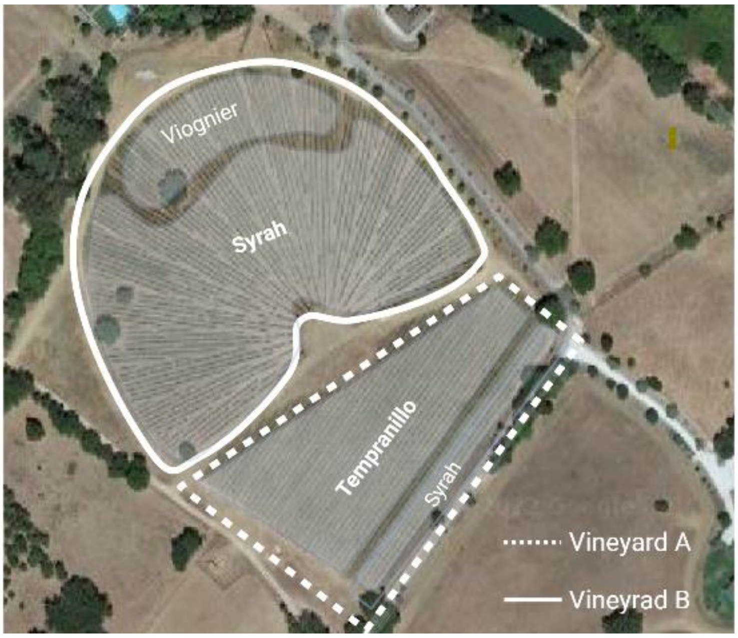

Figure 7 - Satellite image of vineyard A (——) and B (·····), and their respective cultivars.

3.2. Experimental design

For vineyard A, a preventive treatment was addressed by kaolin spraying at the beginning of the 2nd and 3rd leafhopper generations and compared with a control (no kaolin application). To achieve this, the pulverizations were performed when the nymphs of the 2nd generation and 3rd generation were firstly observed. These two generations are considered the most dangerous and detrimental and, therefore, considered the priority to control. Each kaolin application consisted of two kaolin sprayings with a seven-day interval, as recommended by the manufacture (Thomassen, 2010), for improved leaf coverage and kaolin efficiency. Therefore, in total, four applications of kaolin were performed at a 2,5 g/L concentration in the preventive kaolin treatment (PK1), minimum advised for vineyards by the manufacture of Surround WP (Thomassen, 2010), and no application in the preventive kaolin control (PK0). In order to protect the controls lines of kaolin pulverizations, plastic covers were put during the pulverizations (Figure 8).

Figure 8 – Plastic covers used to protect the control line from kaolin pulverization.

For each treatment, three replicates were established, thus the experimental design accounted for six experimental units. Four rows comprising six grapevines each were set up as an experimental unit, and from the four central grapevines, as despite in figure 9, all data was collected.

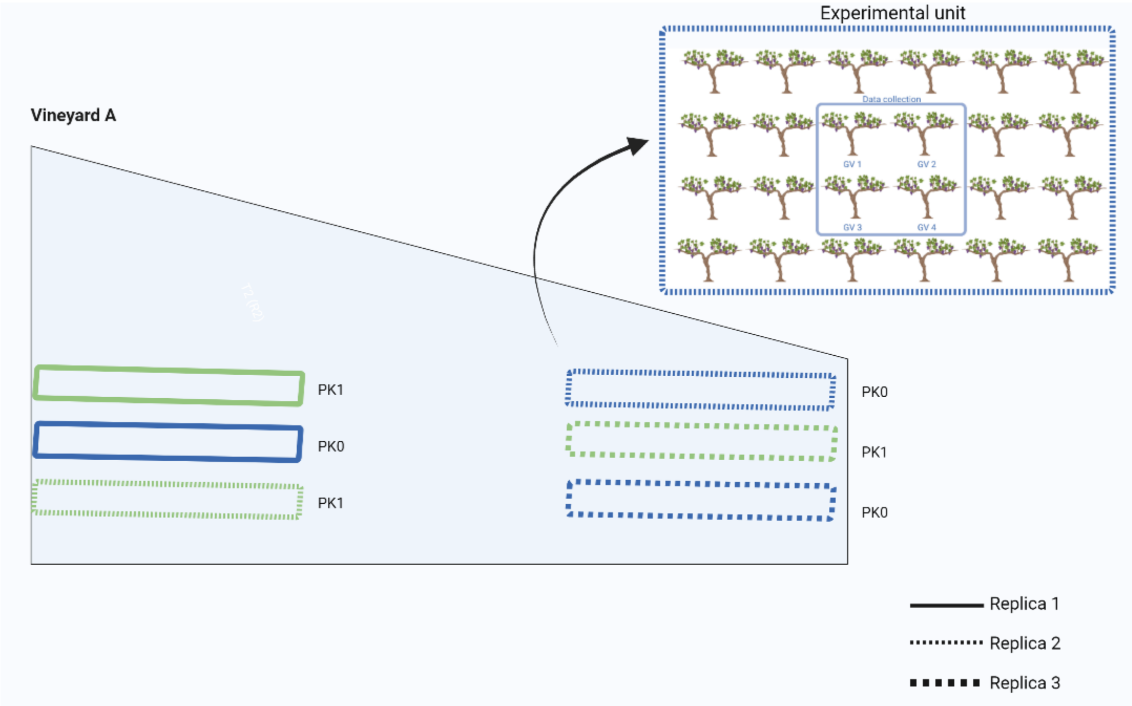

Figure 9- Description of the experimental design in vineyard A with the cultivar Tempranillo, namely the representation of an experimental unit and the distribution of the two treatments: control (PK0) and preventive kaolin (PK1), as well as the three replicas.

For vineyard B, the continuous kaolin application from the migration of adults to the vineyard to harvest day, versus no kaolin application (control) was addressed. The kaolin spraying was performed once in twenty-one days, period defined by the manufactured as kaolin's maximum protection period (Thomassen, 2010). Additionally, the ideal number of treatments was studied along the continuous application. Hence, two more treatments were defined in this vineyard, specifically (1) one kaolin application at the beginning of adult migration and (2) two kaolin applications: one at the beginning of adult migration and another twenty-one days after. Therefore, in total, four treatments regarding kaolin application were performed, namely no kaolin application (0KT), one kaolin treatment (1KT), two kaolin treatments (2KT), and continuous kaolin treatment (CKT). Each application was performed by one pulverization of a water kaolin solution at 2,5 g/ L concentration, minimum advised for vineyards by the manufacture of Kaolin Surround WP (Thomassen, 2010). In order to protect the controls lines of kaolin pulverizations, plastic covers were put during the pulverizations (Figure 8).

For each treatment three replicates were established, thus the experimental design accounted for twelve experimental units. Four rows comprising six grapevines each were set up as an experimental unit, and from the four central grapevines, as despite in figure 10, all data was collected.

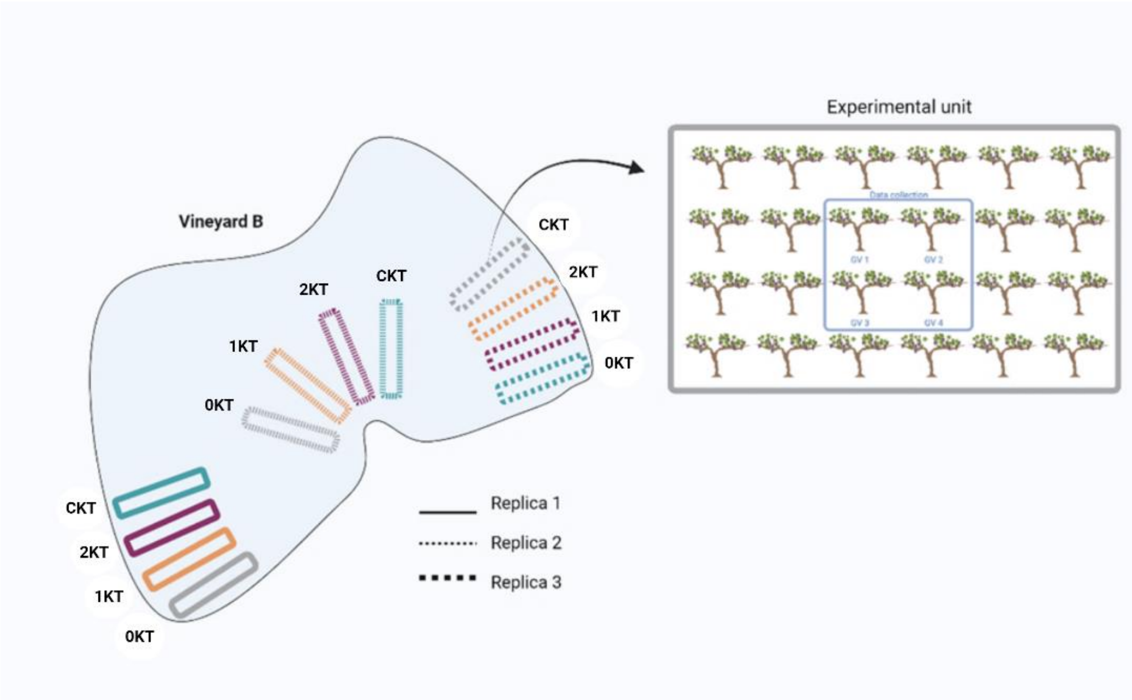

Figure 10- Description of the experimental design in vineyard B with the cultivar Syrah, namely the representation of an experimental unit and the distribution of the four treatments: no kaolin treatment (0KT), one kaolin treatment (1KT), two kaolin treatment (2KT), and continuous kaolin treatment (CKT), as well as the three replicas.

3.3. Leafhopper's life cycle

Adult population curves were assessed through yellow chromotropic sticky traps (40 mm x 25 mm). Every week from the end of April (at the beginning of the vegetative cycle, when average temperatures exceed 10ºC) until harvest day, new traps were placed in the central rows of each treatment (about 0.5 m high in the canopy level), as represented in figure 11. The traps were replaced weekly, and the number of adult leafhoppers recorded to construct a flight path curve. Binocular loupe confirmation of the counted leafhoppers was performed.

Figure 11 – Representation of the chromotropic yellow traps setting in an experimental unit.

3.4. Nymph infestation level

Leafhopper densities were estimated weekly by monitoring and recording the number of nymphs on twelve aleatory leaves per grapevine at the same hour. In vineyard A, the weekly recording started from Mid-March until August (when harvest started), and in vineyard B, the weekly recording started from end of April until early September (when harvest started).

3.5. Symptom expression – leaf necrosis analysis

The symptoms cause of leafhopper is reflected in leaf necrosis, reddish of leaves, in the case of red grapes varieties, or even complete dryness. Therefore, in order to evaluate the effect of kaolin on damage intensity by leafhoppers, the visual spectral region of the grapevine central canopy was evaluated at harvest time for all treatments by ImageJ software, as represented in Figure 12. Firstly, the background was removed and then, by adjustment of hue, saturation and brightness values, the total canopy area, and the total damaged canopy area (number of pixels) was measured to calculate the percentage of damaged canopy area. This data has great error associated, due to the overlapping leaves (frontal image of the whole canopy) however, is used as supplementary data in order to understand damage intensity tendency. For more reliable results, aleatory leaves should have been sampled and analysed separately.

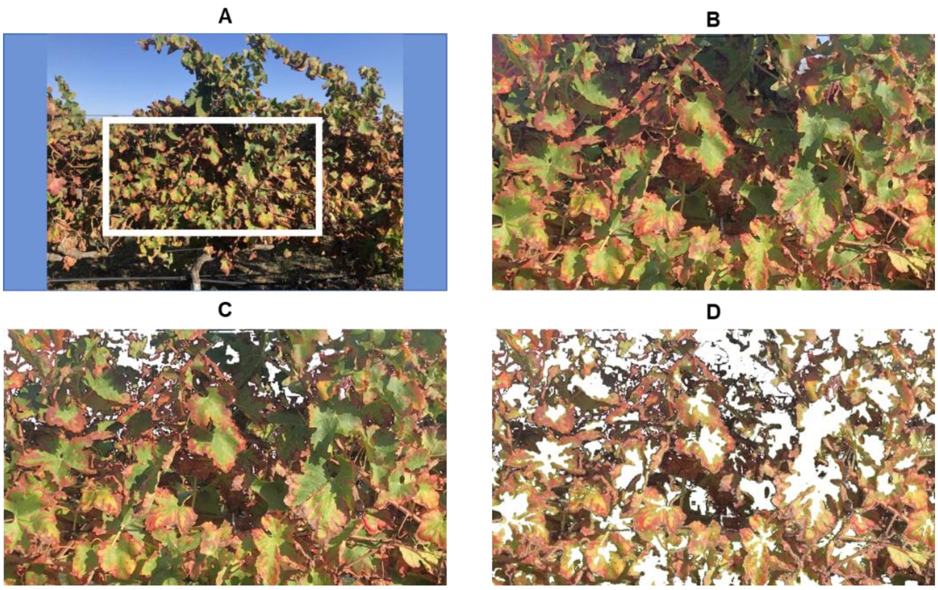

Figure 12 – Schematic illustration of grapevine central canopy image processing to calculate the percentage of damaged canopy: A – Grapevine and central canopy selection for evaluation; B - Central canopy selection for evaluation; C - Central canopy background removal; D – Damage central canopy.

3.6. Statistical Analysis

The level of nymph infestation and symptom’s expression at harvest time, for both trials, was evaluated by One-way analysis of variance (ANOVA) using IBM® SPSS ºStatistics 26.0 software, after normality and homogeneity validation. Moreover, multiple comparisons were performed through post hoc Tuckey’s tests. All tests were applied at a 95% confidence interval.

4. Results and discussions

4.1. Leafhopper flight path

The largest population peaks of the grapevine leafhoppers occurred in midsummer, demonstrating the insect's good ability to develop under conditions of higher temperatures, as presented in figure 13. During the sampling period it was possible to observe, at least, two adult generations, namely the 1st that migrated to the vineyard and the second that resulted in the development of the nymphs of the 3rd generation. Specifically, the migration flight of the 1st generation started around mid-May reaching its peak in the beginning of June. After a month and a half, it was observed the peak of flight of the adults of the 2nd generation (Figure 13). The knowledge of these timings is useful to effectively decided when to apply the treatments. In the case of the trial on vineyard B, it was essential to know when the migration flight took place, so the kaolin pulverization could have an impact not only on feeding disruption but also on oviposition.

Figure 13 – Leafhopper population dynamics of vineyard B from the begging of May until mid of August.

4.2. Preventive treatment trial (vineyard A)

4.2.1. Nymph infestation level

The number of nymphs was evaluated on the four central grapevines of each treatment and registered after 1 (T_1), 2 (T_2), 3 (T_3), 4 (T_4), 5 (T_5), 6 (T_6), 7(T_7), 8 (T_8), 9

(T_9), 10 (T_{10}), 11 (T_{11}), 12 (T_{12}), and 13 (T_{13}) weeks (Figure 14). The 1st pulverization was set to 25 of May, when the firsts nymphs of the 2nd generation appeared, the 2nd pulverization on 2 June, the 3rd pulverization was set on 16 of June, when the first nymphs of the 3rd generation emerged, and the 4th pulverization at 28 of June (Table 1).

Table 1– Kaolin pulverization (2,5 g/L) dates on: no kaolin treatment (PK0) and preventive kaolin treatment (PK1), in vineyard A.

Treatment	1º Pulverization	2º Pulverization	3º Pulverization	4º Pulverization
PK0				
PK1	25-05-2022	02-06-2022	16-06-2022	28-06-2022

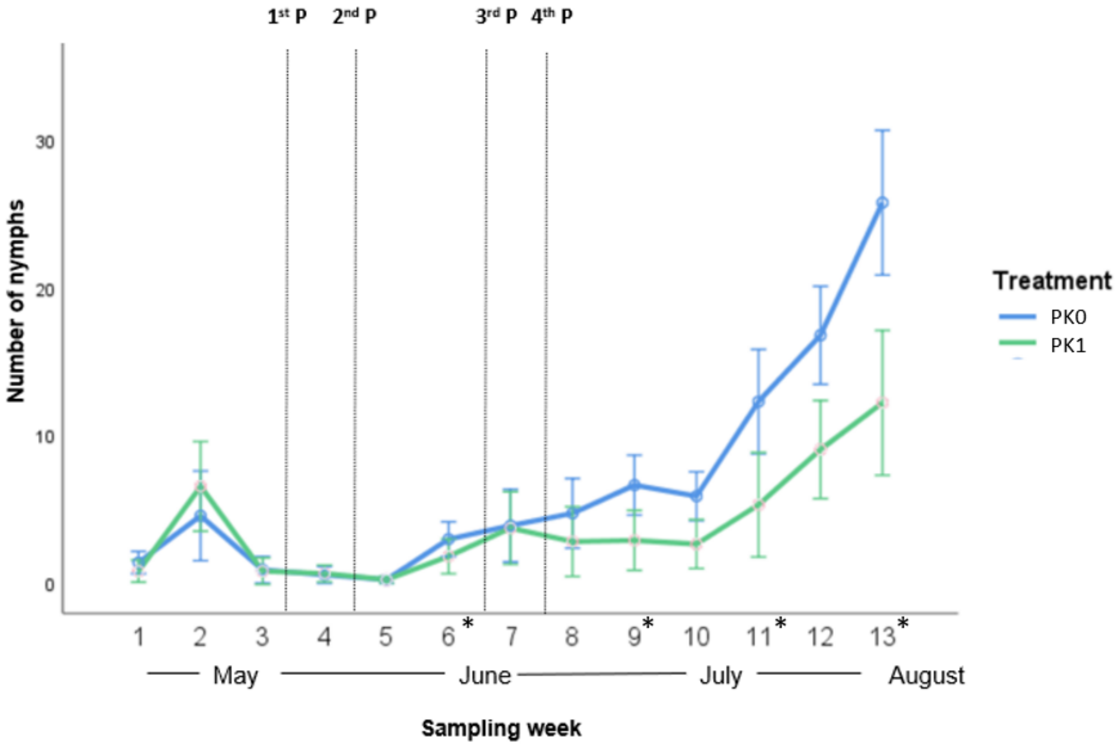

Figure 14 – Preventive application trials (Kaolin, Surround WP, 2,5 g/L). Nymphs recorded during the sampling period in vineyard A, under two treatments: no kaolin treatment (PK0) and Preventive kaolin treatment (PK 1). Kaolin in the PK1 treatment was applied on 25 of May, 2 of June, 16 of June and 28 of June. Week analysed for statistical significance variance (*).

Statistical significance of the number of nymphs was analysed at 4 periods, namely 2 weeks after the 1st pulverization (T_6), 2 weeks after the 2nd pulverization (T_9), 4 weeks after the 2nd pulverization (T_{11}), and 6 weeks after the 2nd pulverization (T_{13}), as described in Table 2.

Table 2 - Preventive kaolin trial (Kaolin, Surround WP, 2,5 g/L). Number of nymphs recorded during the sampling period in vineyard A, in the two treatments: no kaolin treatment (PK0) and preventive kaolin treatment in the beginning of the 2nd and 3rd generation (PK1), with a 95% confidence level. Kaolin was applied on 25 of May and 2 of June (beginning of 2nd generation), and in 16 and 28 of June (beginning of 3rd generation). Different letters among treatments at the same date indicate significant (α=0.05).

		Number of nymphs observed		
	Observation period	**PK0**	**PK1**	**One-Way ANOVA**
	1st week	$1,3^a \pm 1,4$	$0,8^a \pm 1,1$	
	2nd week	$4,5^a \pm 3,7$	$6,2^a \pm 6,2$	
	3rd week	$0.8^a \pm 1,8$	$0,8^a \pm 1,2$	
2nd generation preventive pulverization	*4th week*	$0,5^a \pm 1,0$	$0,6^a \pm 0,9$	
	5th week	$0,2^a \pm 0,4$	$0,2^a \pm 0,4$	
	6th week	$2,9^a \pm 2,5$	$1,8^a \pm 1,2$	$F(1,22)=2.110; p=0.160$
3rd generation preventive pulverization	*7th week*	$3,8^a \pm 4,7$	$3,7^a \pm 3,5$	
	8th week	$4,7^a \pm 4,4$	$2,8^a \pm 3,4$	
	9th week	$6,6^a \pm 4,4$	$2,8^b \pm 1,8$	$F(1,22)=7.349; p=0.013$
	10th week	$5,8^a \pm 3,5$	$2,6^b \pm 1,8$	$F(1,22)=8.270; p=0.009$
	11th week	$12,3^a \pm 7,9$	$5,3^b \pm 2,8$	$F(1,22)=8.395; p=0.008$
	12th week	$16,8^a \pm 6,4$	$9,0^b \pm 4,6$	$F(1,22)=11.655; p=0.002$
	13th week	$25,8^a \pm 10,3$	$12,2^b \pm 5,3$	$F(1,22)=16.435; p=0.001$

Treatments were compared in One-Way ANOVA, revealing the existence of significant differences in infestation level at T_9 and at the following weeks until harvest (up to 5 weeks). Thus, despite no significant difference was observed during the development of the 2nd generation, the preventive treatment was persistent and had a positive impact in the reduction of nymphs of the 3rd generation (the most damaging). During this last 5 weeks it was possible to observe, on average, a 5-fold reduction on the number of nymphs, at the treated grapevines. Higher concentrations of kaolin could be tested to see if it could reduce the infestation degree to lower levels, or even to their total absence.

4.2.2. Symptom expression

Symptom expression by leafhopper's feeding activity was evident at vintage time in vineyard A for the variety Tempranillo, more extensively on the non-treated grapevines, as can be observed in figure 19 in the attachment section. The percentage of symptomatic leaf surface (change in colour or dryness) was estimated by ImageJ and statistical difference was observed ($F_{(1,4)}=35.481$; $p<0.004$) between the two treatments through One-way ANOVA. The percentage of leaf surface with symptoms was lower in the preventive kaolin (PK1) treatment (42,67%) than the control (79,31%), as can be observed in figure 15, suggesting the same results obtained on nymph infestation evaluation.

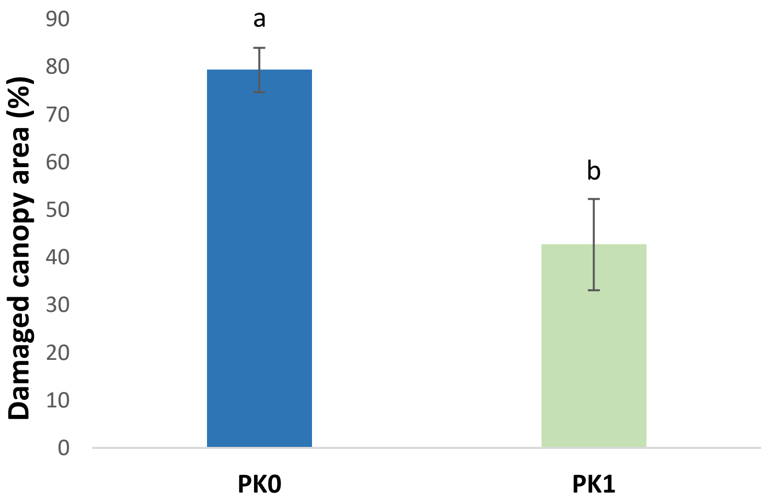

Figure 15 – Results of One-way ANOVA performed on the percentage of damaged central exposed canopy area (mean ± standard deviation) for each treatment: no kaolin pulverization treatment (PK0) and preventive kaolin pulverization treatment (PK1), in vineyard A. Different letters represent significant differences between treatments (p<0.05).

4.3. Number of treatments screening trial (vineyard B)

4.3.1. Nymph infestation level

The pulverizations dates of this trial were based on the weekly observations of caught adults on the chromotropic traps. The first pulverization was set to 28 of May, when an increased growth of caught adults was first register (beginning of the oviposition), and the following treatments were set with 21 days intervals (Table 4).

Table 3 – Kaolin pulverization (2,5 g/L) dates on: no kaolin treatment (0KT); one kaolin treatment (1KT), two kaolin treatments (2KT) and continuous kaolin treatment CKT), in vineyard B.

Treatment	**1º Pulverization**	**2º Pulverization**	**3º Pulverization**	**4º Pulverization**
0KT				
1KT	28-05-2022			
2KT	28-05-2022	18-06-2022		
CKT	28-05-2022	18-06-2022	08-07-2022	29-07-2022

The number of nymphs was evaluated on the four central grapevines of each treatment and registered after 1 (T_1), 2 (T_2), 3 (T_3), 4 (T_4), 5 (T_5), 6 (T_6), 7(T_7), 8 (T_8), 9 (T_9), 10 ($T1_0$), 11 (T_{11}), 12 (T_{12}), 13 (T_{13}), and 14 (T_{14}) weeks. In figure 16 it is possible to see a tendency of nymph density reduction with the increase of the number of kaolin pulverizations. Moreover, it is also clear, for all treatments, that the largest population peak of the leafhopper nymphs occurred in midsummer, between July and August, corresponding to, on average, 34,1 $\pm$ 12,4 nymphs in on the 0KT, related to the 3^{rd} generation.

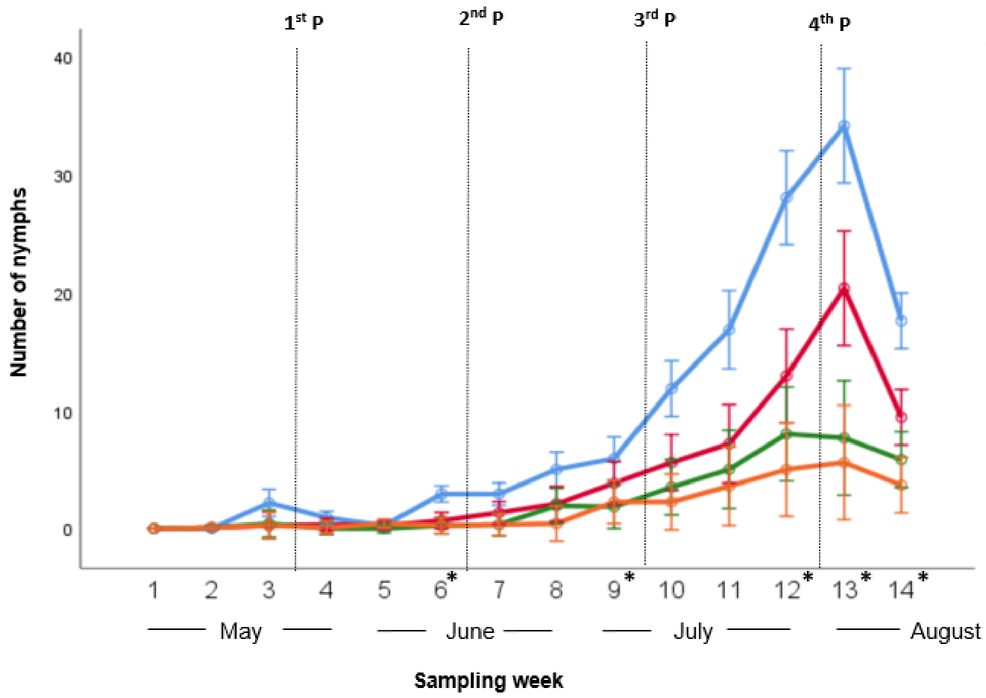

Figure 16 - Nymphs recorded during the sampling period in vineyard B, in the four treatments: no kaolin treatment (0KT); one kaolin treatment (1KT), two kaolin treatments (2KT) and continuous kaolin treatment CKT) with a 95% confidence level. Kaolin applied on 28 of May (1st P) on 1KT, 2KT and CKT treatments, 18 of June (2nd P) on 2KT and CKT treatments, 8 of July (3rd P) and 29 of July (4th P) on CKT. Week analysed for statistical significance variance (*****).

Statistical significance variance of the number of nymphs was analysed at 5 periods (Figure 17 & Table 5), namely 3 weeks after the 1st pulverization (T_6), 3 weeks after the 2nd pulverization (T_9), 3 weeks after the 3rd pulverization (T_{12}), and after the following weeks until harvest (T_{13} & T_{14}), in order to understand the influence of the pulverizations on the 2nd and 3rd generations of leafhoppers, as well as their persistence effect until harvest (T_{14}).

Table 4 –Nymphs recorded during the sampling period in vineyard B, in the four treatments: no kaolin treatment (0KT); one kaolin treatment (1KT), two kaolin treatments (2KT) and continuous kaolin treatment CKT), with a 95% confidence level. Kaolin applied on 28 of May (1st pulverization) on 1KT, 2KT and CKT treatments, 18th of June (2nd pulverization) on 2KT and CKT treatments, 8 of June (3rd pulverization) and 29 of June (4th pulverization) on CKT. Different letters among treatments at the same date indicate significant differences according to post hoc Tukey tests (α=0.05).

		Number of nymphs observed				
	Observation period	**0KT**	**1KT**	**2KT**	**CKT**	**One-Way ANOVA**
	1st week	0,0^{a} ± 0,0	0,0^{a} ± 0,0	0,0^{a} ± 0,0	0,0^{a} ± 0,0	
	2nd week	0,0^{a} ± 0,0	0,1^{a} ± 0,3	0,1^{a} ± 0,3	0,1^{a} ± 0,3	
	3rd week	2,2^{a} ± 3,7	0,3^{a} ± 0,6	0,4^{a} ± 0,9	0,3^{a} ± 0,5	
1st Pulverization >						
	4th week	0,9^{a} ± 1,6	0,3^{a} ± 0,9	0,0^{a} ± 0,0	0,1^{a} ± 0,3	
	5th week	0,3^{a} ± 0,6	0,3^{a} ± 0,9	0,0^{a} ± 0,0	0,4^{a} ± 0,7	
	6th week	2,9^{a} ± 2,0	0,7^{b} ± 1,0	0,3^{b} ± 0,5	0,3^{b} ± 0,5	F(3,44)=14.377; p=0.000
2nd Pulverization >						
	7th week	2,9^{a} ± 2,7	1,3^{a,b} ± 1,6	0,3^{b} ± 0,7	0,3^{b} ± 0,7	
	8th week	5,0^{a} ± 4,0	2,1^{b} ± 2,2	1,9^{b} ± 2,1	0,4^{b} ± 0,7	
	9th week	5,3^{a} ± 47	3,1^{a,b} ± 2,7	1,8^{b} ± 2,0	2,2^{b} ± 2,5	F(3,44)=4,166; p=0.011
3rd Pulverization >						
	10th week	11,8^{a} ± 6,7	5,6^{b} ± 3,1	3,5^{b} ± 2,2	2,3^{b} ± 2,5	
	11th week	16,8^{a} ± 8,0	7,2^{b} ± 5,8	5,0^{b} ± 4,0	3,6^{b} ± 4,0	
	12th week	28,0^{a} ± 12,0	13,0^{b} ± 4,3	8,0^{b,c} ± 2,5	5,0^{c} ± 4,4	F(3,44)=19.368; p=0,000
4th Pulverization >						
	13th week	34,1^{a} ± 12,4	20,3^{b} ± 9,1	7,7^{c} ± 3,8	5,6^{c} ± 5,1	F(3,44)=30.036; p=0.000
	14th week	17,6^{a} ± 5,2	9,4^{b} ± 3,3	5,8^{b,c} ± 4,2	3,7^{c} ± 3,1	F(3,44)=27.434; p=0,000

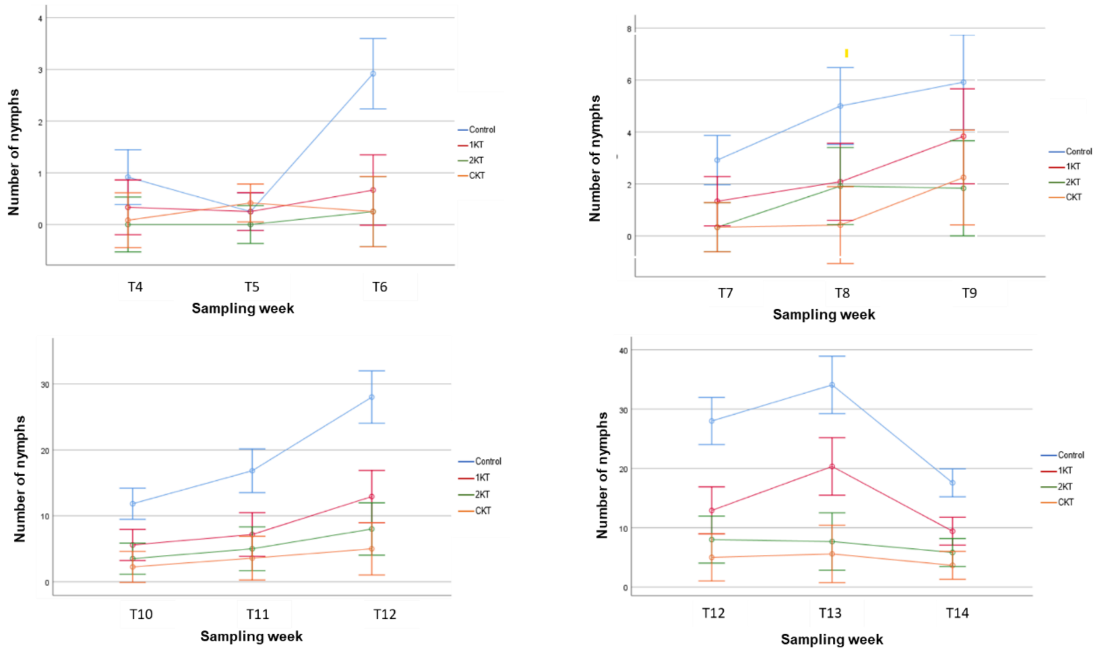

Figure 17 – Number of treatments screening trial (Kaolin, Surround WP, 2,5 g/L). Nymphs recorded during the 4 periods chosen to analyse statistically significance for all treatments: no kaolin treatment (0KT); one kaolin treatment (1KT), two kaolin treatments (2KT) and continuous kaolin treatment CKT) with a 95% confidence level. Kaolin applied before T4 (1st pulverization) on 1KT, 2KT and CKT treatments, before T7 (2nd pulverization) on 2KT and CKT treatments, T10 (3rd pulverization) and T13 (4th pulverization) on CKT.

Regarding the 1st pulverization, on week 6 (T_6), 1KT, 2KT and CKT are the same because they only had one pulverization so far. Through one-way ANOVA we can see a significant reduction of nymphs with only one pulverization, compared with 0KT, as described in table 4. In fact, on average, 1 to 0 nymphs were observed in the treated grapevines.

On the 11th week (T_{11}), 2KT and CKT are the same because both had two pulverizations so far. The grapevines treated twice showed again, on T_{11}, significant reduction on the nymph infestation level in comparison to the control. 1KT, that did not take the 2nd pulverization does not show statistical difference from the control and 2KT and CKT treatments.

On the 12th week (T_{12}), only CKT was treated again with another kaolin pulverization. It presented no statical significance difference from 2KT, however statistical differences from 1KT and 0KT. Both 1KT and 2KT statically differ from the control, showing the persistence of the kaolin protection.

On the 13th and 14th week (T_{13} & T_{14}), CKT, despite having another pulverization with kaolin, did not show, again, statistical difference from 2KT, however showed when compared with 1KT and the control. Moreover, 1KT and 2KT continued to present statical difference from 0KT, demonstrating once again kaolin's effect persistence.

Overall, CKT grapevines in all periods investigated never displayed statistical difference in infestation level between 2KT, and only one pulverization (1KT) showed persistent effect until harvest (up to for 9 weeks).

4.3.2. Symptom expression

Symptom expression by leafhopper's feeding activity was very evident at vintage time in vineyard B for the variety Syrah, more extensively on the non-treated grapevines, as can be observed in figure 20 in the attachment section. The percentage of symptomatic leaf surface (change in colour or dryness) was estimated by ImageJ and statistical difference was observed ($F(1,3)=27.983$; $p=0.0000$) between the four treatments through One-way ANOVA. In the figure 18 we can see a tendency of leave damage reduction with the increment of the number of kaolin pulverizations, however, there is no statistical difference between CKT and 2KT, suggesting the same results obtained on nymph infestation evaluation. One kaolin pulverization (1KT) does not differ from the remaining treatments.

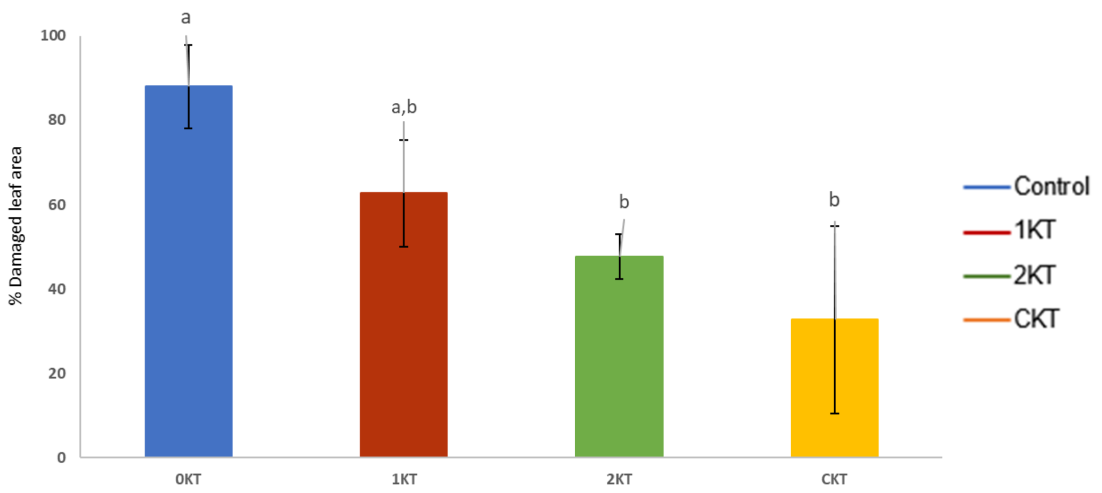

Figure 18 - Results of One-way ANOVA performed on the percentage of damaged central exposed canopy area (mean ± standard deviation) for each treatment: no kaolin pulverization treatment (0KT), one kaolin pulverization treatment (1KT), two kaolin pulverizations treatment (2KT) and continuous kaolin pulverization treatment (CKT)) after harvest, in vineyard B, for the optimum number of treatments trial for the Syrah variety. Different letters represent significant differences between treatments, as per Tukey HSD ($p<0.05$).

5. Conclusion

5.1. Preventive trial vineyard A

Kaolin caused a decrease in the field populations leafhopper's nymphs at low levels in the preventive criteria. The effect of kaolin in the preventive applications was persistent as differences from the control last for up to 5 weeks after the final pulverization. The reduction in leafhopper nymph densities was associated with a 2-fold reduction on 3rd generation nymphs' infestation, and a 2-fold on damaged canopy area. The level of leafhopper control is comparable to that obtained with the most effective synthetic insecticides (Pozzebon *et al.*, 2011; Posenato *et al.*, 2006), however higher concentrations of kaolin could be tested to see if it could reduce the infestation level to lower levels, or even to their total absence

5.2. Ideal number of treatments trial vineyard B

Nymph's abundance was significantly affected by kaolin applications ($p<0.01$). Kaolin caused a decrease of leafhopper's nymphs at low levels, as well as a reduction in leafhopper's symptoms. Particularly, 2KT and CKT treatments were the most and equally efficient, that was due to economic reasons, the treatment with only two kaolin sprays (2KT) is considered the most adequate and efficient treatment to control the grapevine leafhopper population. During the peak of the 3rd generation, the treatment 2KT has shown a 4,4-fold reduction on the number of nymphs, and on the 2nd generation's peak it was observed a 2,6-fold reduction. Regarding the damaged leaf canopy, the 2KT achieved almost a 2-fold reduction.

During the second generation, it was possible to see, on average, the absence of nymphs in the treated grapevines. On the other hand, during the 3rd generation (higher infestation compared with the 2nd) the nymph reduction was not so intense. This suggests that for the preventive treatment of the 3rd generation, a higher concentration of kaolin could be needed to control the higher infestation level and maintain lower levels or, even, their total absence.

The level of leafhopper control is comparable to that obtained with the most effective synthetic insecticides (Pozzebon *et al.*, 2011; Posenato *et al.*, 2006). Moreover, similar results to control the 2nd generation of leafhoppers used by Tacoli *et al.* (2017) were obtained with 2KT treatment.

6. Future work

The next step of this study is to understand the effect of each treatment in the organoleptic properties of wine, since clearly KPF has an impact on grape and wine quality as a result of many molecular and biochemical changes in key primary/secondary metabolic pathways (Dinis *et al*., 2020). The grapes were already collected and are being analyse (20 berries per grapevine) for total soluble solids (TSS), [malic acid], [acid tartaric acid], total polyphenols, anthocyanins, tannins, and aluminium. This last parameter is to understand if aluminium is kept as a residue on the grape and, consequently in wine.

Considering the positive results of this evaluations, the next step would be to perform a screening test of the concentration of kaolin to use, to understand the most suited to control leafhoppers, principally at the 3rd generation.

CHAPTER 2

1. Introduction

This chapter describes the activities developed during the 6-month internship at Valbom do Rouxinol Lda., Évora, Alentejo. The main tasks performed in the vineyard were traditional organic viticulture operations, such as grapevine conduction, copper and sulphur pulverizations for powdery and downy mildew protection, green pruning, maturation control, and lastly, grape harvesting. The cellar operations for rose, white and red wine vinification comprised the sanitation of general equipment (vats and barrels), acidity corrections, yeast inoculations, fermentation and Hazard Analysis and Critical Control Point (HACCP) control, and wine stabilization by electrodialysis. This internship allowed to deepen my knowledge in viticulture and oenology and to gain professional experience in these areas.

2. Company

Valbom do Rouxinol homestead is located in Évora, Portugal, and comprises 60 certified ha in organic farming. The homestead encompasses grapevines, olive groves, cork oak forests, and a cellar for the winemaking process. The main objective of the company is to produce high quality organic wines.

The vineyard is located in a typical semi-arid Mediterranean climate, with warm summers and cool winters. The annual precipitation is approximately 900 mm, concentrated between October and May, and the drought period can last from May to September. Irrigation is performed by a drip system aligned with weather stations which measure temperature and relative humidity to provide the irrigation controller with near real-time weather information. The soils have a sandy loam texture and are moderately basic. The vineyard comprises 9.1 ha of four grapevines varieties, namely Syrah, Viognier, Tempranillo and Arinto, distributed in 5 fractions:

F1: Syrah & Viognier (2.0 ha)

F2: Syrah & Tempranillo (1.9 ha)

F3: Viognier (1.6 ha)

F4: Viognier & Arinto (2.2 ha)

F5: Syrah (1.4 ha)

3. Viticulture operations

3.1 Grapevine conduction

The first activity performed in the vineyard, upon my arrival to the company, was the conduction of grapevines to the bilateral Guyot system. This process prepares the vine for the fruiting process, where the vine branches are bent and tied to the wire, allowing the upright positioning of the shoots, adequate light penetration, and air movement through the canopy (Magalhães, 2008).

3.2 Green pruning

During April and May, the removal of excess stems appearing from the base was performed. These branches compete with the branches left in the pruning, creating density in the hedge with consequent negative reflections on the quality of the production. The elimination of these branches should be carried out as soon as possible, in order to avoid the vine to invests reserves in unproductive material. Moreover, these are normally located near the ground, and therefore more susceptible to mildew infections (Magalhães, 2008).

Shoot trimming was performed to maintain canopy shape, reduce vine vigour, improve microclimate in the fruiting zone, increase the efficiency of disease treatment, facilitate harvest and the access of the tractors (Magalhães, 2008). This practice was performed manually during the fruit development.

Defoliation was made between fruit setting and *veraison* in order to improve the exposure to light and, consequently, enhance the colour and maturation of the berries, as well as to improve air circulation around the clusters (Magalhães, 2008). However, due to the warm climate, the leaf removal was undertaken with precaution to prevent the risk of berry overheating and burning.

3.3 Treatment and control of pests and diseases

The most common diseases throughout this vintage were powdery mildew (*Erysiphe necator*) and downy mildew (*Plasmopara viticola*), which required special monitoring, control, and intervention. In terms of pests, the green leafhoppers were the major concern, which were attended in the scientific study, previously described in Chapter 1.

Regarding powdery and downy mildew, several pulverizations of water solutions of sulphur and copper were initiated based on the automatic weather stations installed in the vineyard and by local monitoring to confirm possible infections. The weather stations collect information on temperature, rainfall, leaf wetness and humidity and process the data for the likelihood of an infection event. For downy mildew, the favourable conditions set were wet soils for at least 16h or high humidity, and temperatures higher than 10 ºC. For powdery mildew, the favourable conditions were defined as low to moderate light (mild cloudy weather), temperatures within 6-33°C range, and humid conditions.

In the case of powdery mildew, a typical disease progression begins on the leaves as chlorotic spots on the upper leaf surface. Signs of the pathogen appear a short time later as white mycelium on the lower leaf surface. As spores are produced, the infected areas take on a white, powdery, or dusty appearance. On fruit and rachises, the pathogen appears as white, powdery masses that may colonize the entire berry surface. Black to brown web scarring can be seen on mature fruit, which represents former colonies (Magalhães, 2008).

In the case of downy mildew, it is a fungus that attacks all green parts of the vines, particularly the leaves. On young leaves (in spring), the disease will appear on the upper surface as small yellow spots referred to as oil spots. They are about 10 mm diameter, often with a brown halo. These spots tend to grow to about 50 mm diameter, as they mature, while the halo fades. After warm humid temperatures, a dense, raised, white cottony growth develops on the underside of the yellow oil spots. As the spots age naturally, the centres dry out and become a reddish brown with a yellow outer ring (Magalhães, 2008).

3.4 Maturation control

In order to determine the optimum harvest date, berries were sampled periodically, in the early morning, after *verasion* to evaluate the levels of sugar, pH, acids and flavour compounds. This sampling collection was performed randomly in zigzag and delivered to Eurofins Laboratory for technical analysis. The determination of the harvest date was set based on these evaluations (mainly on the balance between acidity and sugars) and the weather forecast. Since rainfalls were predicted during the optimal ripening stage, harvest was anticipated (one week) in order to avoid must dilution and microbial development.

3.5 Grape Harvesting

Grape harvesting was performed manually and under cool temperatures in order to avoid hydrolysis of aromatic precursor, oxidation, and the development and action of undesirable microorganism. For small fractions, harvesting was performed early in the day, when the temperatures were low, and immediately refrigerated at 5º C until grape processing. For the larger fractions (Syrah), harvest was performed during the night and processed immediately at the cellar.

4. Cellar operations

3.6 Cellar cleaning and sanitation

To prepare the cellar for the winemaking process all cellar, crusher, press, tanks, hoses, and general equipment was sanitized using a high-pressure machine with hot water. Moreover, the same sanitation process was performed to every equipment once used during the winemaking process. Small equipment and hoses were also put in contact with a sulphur dioxide water solution (5% w/w) of in order to sanitize them.

3.7 Barrel sanitation

One of the activities in the cellar was the sanitation of barrels which were previously filled with wine from previous vintages. To achieve the barrel's sanitation, firstly, the barrels were empty and thoroughly rinsed to get rid of all residues (i.e., tartrate crystals), and left to dry out completely. A sulphur disc attached to a wire was lit and suspended into the barrel, releasing $S0_2$ to remove all oxygen, which could lead to bacteria and mould build up. After the disc has finished burning, it was removed, and the bung replaced tightly. Afterwards, the barrels were filled with the new vintage's wine for their ageing process.

3.8 Red winemaking process

Grape sorting was performed by hand at a sorting table in order to accomplish a high-quality wine. Undesired grapes were discarded, such as unripe or rotten grapes, and other materials (i.e., branches and leaves), and kept only the healthy and ripe grapes for the vinification. Subsequently, the selected grapes entered the stalk remover (de-stemmer) to separate the stalk from the berries to avoid over-extraction of tannins and unwanted flavours in the fermentation process. This process took place through rotating perforated drums in which grape berries, having a smaller calibre, are separated from the woody materials. The grape stems were not discarded but transferred to the compost pile. After de-stemming, the grapes underwent a light crushing process and introduced into cooled tanks with the objective of delaying the fermentation. During this step, additions were performed to the must, specifically sulfur dioxide to stop bacterial contamination, nutrition supplement based on organic yeast derivatives for the support of yeast metabolism, organic nitrogen, vitamins, trace elements, and fermentation additives (FermControl Bio), tartaric acid for acidity correction, and wood chips for organoleptic complexity. Punch down grapes by stomping (3 times a week) and pump over the must (twice a day) were the two techniques used during maceration to extract the optimal amount of colour, aroma compounds and tannins from the skins of the grapes. When the fermentation started, the cooling was adjusted to a higher temperature allowing it to rise to 26 ºC. To ensure optimal fermentation and guarantee high quality, continuously monitorization of the fermentation process was performed daily by measuring the density and the temperature of all vats. Regular pump-overs continued during the alcoholic fermentation until enough colour and tannins were extracted, as well as to allow some must aeration. When the alcoholic fermentation finished the wine, and the skins were transferred to the pneumatic press. All pressed wine was relocated to a new stainless-steel vat where the malolactic fermentation occurred, during which the malic acid is converted to lactic acid, leading to a reduction in acidity (smother wines) and the production of new aroma and flavour compounds (Jackson, 2020).

3.9 White winemaking process

Grape sorting was performed by hand at a sorting table in order to accomplish a high-quality wine. Undesired grapes were discarded, such as unripe or rotten grapes, and other materials (i.e., branches and leaves), and kept only the healthy and ripe grapes for the vinification. Successively, the selected grapes entered the stalk remover (de-stemmer) to separate the stalk from the berries, were then lightly crushed, and transferred to a pneumatic press to separate the juice form the skins. Afterwards, the must was pumped into a cooled stainless-steel tank (4ºC), where it settled for 24 hours to achieve must clarification before the initiation of the alcoholic fermentation. Additions of sulphur dioxide and supplementary yeast nutrition were also made (FermControl Bio).

After the 24-hour period, the must was decanted to another stainless-steel vat at a higher temperature (15ºC) to allow the initiation of the alcoholic fermentation. To ensure optimal fermentation and guarantee high quality, continuously monitorization of the fermentation process was performed daily by measuring the density and the temperature. Half through the alcoholic fermentation must was transferred to used 300 L French oak barrels to enhance aromas, and to increase the wine's structure and complexity.

3.10 Wine stabilization

The tartaric stabilization of wines before bottling is an important and common step during wine production to avoid the precipitation of tartaric acid salts. Potassium hydrogen tartrate (KHT) is a natural constituent of grapes and alcoholic fermentation leads to a decrease in its solubility due to the presence of ethanol (Jackson, 2020). To overcome the problem of tartaric precipitation in the Dona Dorinda's Rose 2021 vintage wine, before bottling, wine stabilization was performed trough electrodialysis (ED). In more detail, positively charged ions (as K^+ and $Ca2^+$) can be separated from the negatively charged bitartrate ion (HT^-) by pumping the wine through an ED cell and applying a charged current. As the wine passes through the ED, the ions migrate from the wine towards a brine solution which collects them. The resulting wine has a minimized concentration of HT^- ions, which makes it less likely to form and precipitate KHT crystals (El Rayess & Mietton-Peuchot, 2016).

3.11 Winery management software implementation

Valbom do Rouxinol was implementing a new winery management software named Vintrace. This software allows to plan and schedule the fruit intake, manage crush-pad operations, keep track of additions, laboratory analysis data, contents of the tanks, barrel usage and to take conclusions about wine productivity and manufacturing costs. One of my main responsibilities was to learn how this software works and to configurate it for the Valbom do Rouxinol reality. It was necessary to import all data related to the vineyards and grape varieties, and to migrate all cellar's equipment and inventory, namely vats, tanks, barrels, and the list of additives.

Annexes

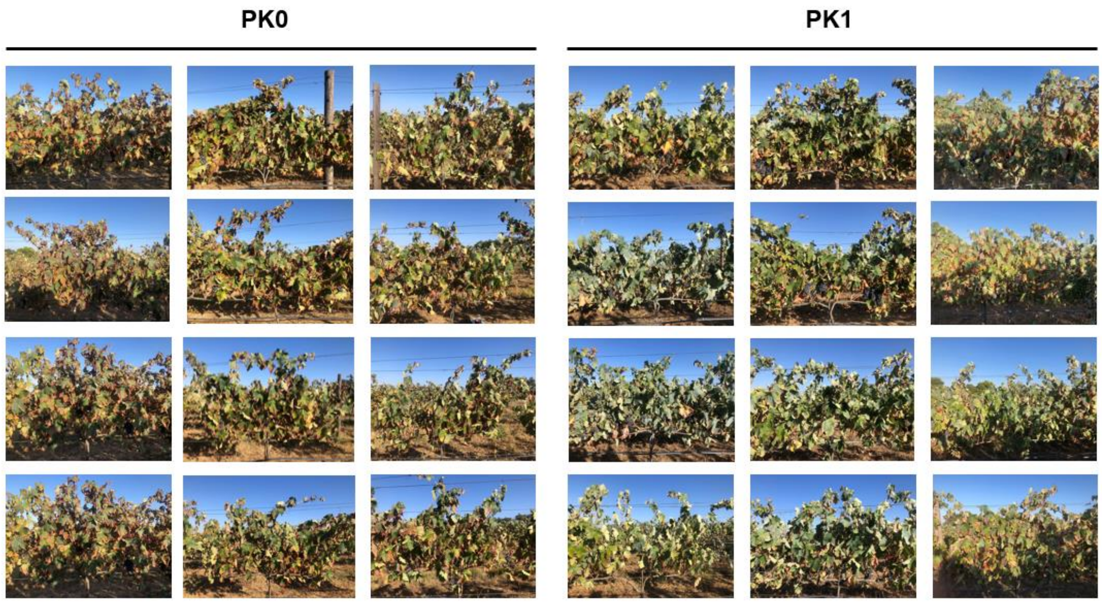

Figure A.1 – Grapevine's canopy of the control (PK0) and preventive treatment (PK1) at harvest time in vineyard A.

Figure A.2 – Grapevine's canopy of the control (0KT), one kaolin application (1KT), two kaolin application (2KT), and continuous kaolin application

Table A.1 – Data from the preventive study in vineyard A, regarding nymph infestation level.

					1ºP	2ºP		3º P	4º P						
			T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	T_{11}	T_{12}	T_{13}
Replica	**Grapevine**	**Treatment**	**10/5/22**	**17/5/22**	**24/5/22**	**31/5/22**	**7/6/22**	**14/6/22**	**21/6/22**	**28/6/22**	**5/7/22**	**12/7/22**	**19/7/22**	**26/7/22**	**2/8/22**
1	1	PK0	2	1	0	0	0	2	2	2	5	7	9	16	49
	2	PK0	3	3	0	0	1	0	2	5	15	2	23	25	35
	3	PK0	2	6	6	2	0	1	2	9	6	9	8	21	33
	4	PK0	4	10	2	0	0	2	0	9	10	2	7	13	19
2	1	PK0	0	7	0	3	1	3	6	1	2	3	22	19	15
	2	PK0	0	2	0	0	0	5	1	0	4	2	2	14	16
	3	PK0	0	1	0	0	0	7	9	0	5	8	8	11	14
	4	PK0	0	2	0	0	0	1	4	0	1	5	4	16	25
3	1	PK0	0	2	0	1	0	2	0	3	6	9	10	10	21
	2	PK0	3	1	0	0	0	0	0	5	11	6	9	7	21
	3	PK0	1	11	1	0	0	5	4	9	2	13	22	20	29
	4	PK0	1	8	1	0	0	7	16	13	12	4	23	29	32
1	1	PK1	0	11	1	0	0	0	0	4	2	3	1	6	13
	2	PK1	1	10	0	0	0	2	4	1	4	4	1	8	12
	3	PK1	0	11	0	3	1	3	4	11	1	1	5	5	7
	4	PK1	0	8	2	1	0	2	6	8	0	7	5	11	13
2	1	PK1	0	2	0	0	0	0	1	0	3	1	5	2	3
	2	PK1	0	1	0	0	1	1	1	0	4	2	9	14	14
	3	PK1	1	12	0	0	0	3	7	1	5	1	9	10	13
	4	PK1	2	19	3	0	0	1	2	3	2	3	8	18	24
3	3	PK1	3	0	3	1	0	1	0	1	1	2	4	7	8
	3	PK1	0	0	0	1	0	4	12	1	2	1	5	10	15
	3	PK1	2	2	0	1	0	2	3	1	4	2	3	13	16
	3	PK1	0	2	0	0	0	2	4	2	6	4	8	4	8

Table A.2 - Data from the number of treatments screening study in vineyard B, regarding nymph infestation level.

				1st P			2nd P			3rd P			4th P		
		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14
Replica	Treatment	10/5/22	17-mai-22	24-mai-22	31-mai-22	7-jun-22	14-jun-22	21-jun-22	28-jun-22	5-jul-22	12-jul-22	19-jul-22	26-jul-22	2-ago-22	9-ago-22
1	0KT	0	0	11	1	0	4	6	1	6	4	11	20	26	17
	0KT	0	0	4	0	1	1	3	4	10	14	18	31	31	12
	0KT	0	0	3	4	0	3	2	3	2	3	6	22	19	19
	0KT	0	0	8	4	0	1	0	1	1	11	22	42	17	10
2	0KT	0	0	0	0	0	3	8	6	6	8	14	23	44	22
	0KT	0	0	0	0	0	6	1	9	8	15	21	17	36	20
	0KT	0	0	0	2	0	1	2	6	2	7	14	47	60	12
	0KT	0	0	0	0	0	7	5	13	9	7	8	21	39	27
3	0KT	0	0	0	0	0	2	1	7	2	23	32	24	26	21
	0KT	0	0	0	0	0	2	6	9	18	17	28	14	32	22
	0KT	0	0	0	0	2	4	1	0	5	10	9	25	49	17
	0KT	0	0	0	0	0	1	0	1	2	23	19	50	30	12
1	1KT	0	0	0	0	0	0	3	3	4	11	22	19	23	12
	1KT	0	0	0	0	0	2	3	1	5	1	2	7	17	10
	1KT	0	0	1	0	0	0	0	2	1	5	4	13	11	13
	1KT	0	0	0	1	0	1	0	0	4	6	5	10	18	10
2	1KT	0	0	0	0	0	0	1	8	2	4	7	16	11	4
	1KT	0	0	0	0	0	1	5	2	8	9	5	9	20	9
	1KT	0	0	2	0	0	0	1	3	7	6	13	14	9	4
	1KT	0	1	0	0	0	3	2	2	6	10	12	8	26	8
3	1KT	0	0	0	0	0	0	0	0	1	3	5	9	29	15
	1KT	0	0	0	0	0	0	0	1	3	4	3	14	20	11
	1KT	0	0	0	0	3	1	0	0	3	6	3	20	42	10
	1KT	0	0	0	3	0	0	1	3	2	2	5	16	18	7
1	2KT	0	0	1	0	0	0	0	0	0	2	4	9	13	12
	2KT	0	0	0	0	0	1	0	0	0	4	7	10	3	2
	2KT	0	0	0	0	0	0	0	0	2	2	5	8	5	8
	2KT	0	0	0	0	0	0	0	0	0	1	2	5	9	5
2	2KT	0	0	0	0	0	0	2	4	1	7	11	8	10	14
	2KT	0	1	1	0	0	0	0	0	2	1	0	10	2	1
	2KT	0	0	0	0	0	1	0	4	5	5	10	8	5	9
	2KT	0	0	0	0	0	0	0	5	0	2	4	7	14	2
3	2KT	0	0	0	0	0	0	1	0	3	6	11	10	7	7
	2KT	0	0	3	0	0	0	0	3	6	2	2	12	9	4
	2KT	0	0	0	0	0	1	1	4	2	3	0	3	10	3
	2KT	0	0	0	0	0	0	0	3	1	7	4	6	5	3
1	CKT	0	0	1	0	0	0	0	0	0	0	2	0	1	0
	CKT	0	0	0	0	0	0	0	0	0	2	0	1	1	0
	CKT	0	0	1	0	0	0	0	1	2	1	1	1	0	1
	CKT	0	0	0	0	0	0	0	0	1	0	0	2	0	0
2	CKT	0	0	0	0	0	1	0	0	2	4	2	3	11	9
	CKT	0	0	0	0	1	0	1	0	2	8	14	4	3	5
	CKT	0	0	0	0	0	0	0	0	2	5	2	9	5	6
	CKT	0	0	0	1	0	1	0	0	1	2	8	15	12	8
3	CKT	0	0	0	0	1	0	0	2	9	1	5	6	7	5
	CKT	0	0	0	0	0	1	1	1	4	3	5	3	10	4
	CKT	0	1	1	0	1	0	0	0	0	1	2	8	3	2
	CKT	0	0	0	0	2	0	2	1	4	0	2	8	14	4

Table A.3 – Data of percentage damaged area in the grapevines of vineyard A.

Treatment	% Damaged Area
PK0	80,63
PK0	83,17
PK0	74,14
PK1	46,52
PK1	31,76
PK1	49,73

Table A.4 – Data of percentage damaged area in the grapevines of vineyard B.

Treatment	% Damaged Area
0KT	76,63
0KT	93,17
0KT	94,14
1KT	76,52
1KT	51,76
1KT	59,73
2KT	53,66
2KT	45,50
2KT	43,68
CKT	10,19
CKT	54,49
CKT	33,34

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