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Evaluation of the effect of basic substances in the control of grapevine downy mildew

presentado por

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ABSTRACT

In this final degree project, several basic substances and seaweeds, with the capacity of stimulating the grapevine immune defence system, have been selected and tested to evaluate their direct toxicity against *Plasmopara viticola*, by measuring the sporulation and growth, in the susceptible grapevine variety *Vitis Vinifera* Tempranillo.

Three independent in vitro assays under controlled conditions have been carried out. The efficacy of the products was determined in base of the evaluation of the IC50 of the products over zoospores of the pathogen, a curative assay at different times after the inoculation and by MDA (malondialdehyde) quantification. All the treatments resulted in an evident level of protection with a significant reduction of *P. viticola* sporulation and disease index, suggesting a toxic effect.

The obtained satisfactory results of BABA, Chitosan hydrochloride, Soybean lecithin mixed with *E.arvense*, Salix, BTH and copper demonstrated that the use of basic substances and seaweeds is a good measure to implement in crop protection.

Key words: *Plasmopara vitícola,* basic substances, seaweeds, toxicity, inhition and defence mechanisms.

RESUMEN

En este trabajo de fin de grado, varias sustancias básicas y algas, con capacidad de estimular el sistema inmunológico de defensa de la vid, han sido seleccionadas y estudiadas para evaluar la toxicidad directa que presentan contra el oomiceto *Plasmopara viticola*. Este estudio se ha centrado en la variedad de vid susceptible al patógeno *Vitis vinífera* Tempranillo.

Se han llevado a cabo tres ensayos independientes en condiciones controladas. La eficacia de los productos se ha determinado a partir de la evaluación del IC50 de cada producto sobre las zoosporas del patógeno, a partir de un ensayo curativo con aplicación a diferentes tiempos tras la inoculación y por último a partir de la cuantificación de MDA (malondialdehido). Todos los tratamientos resultaron tener un nivel evidente de protección, con una reducción significativa en la esporulación de *P. viticola*. Confirmando así su efecto tóxico.

Los resultados satisfactorios obtenidos con BABA, Hidrocloruro de quitosano, Lecitina de soja mezclada con *E. arvense*, Salix, BTH y cobre demuestran que el uso tanto de sustancias básicas como de algas es una buena medida a implementar en la protección de cultivos.

Palabras clave: *Plasmopara vitícola,* sustancias básicas, algas, toxicidad, inhibición y mecanismos de defensa.

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INTRODUCTION

Grapevine downy mildew, caused by *Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & De Toni, is one of the most severe and extended grapevine diseases (Zang et al., 2019). This obligate biotrophic oomycete, in certain climatic conditions, generates considerable damage and yield losses in worldwide grapevine crops. The lack of coevolutionary genetic resistance development of *Vitis vinifera* species to downy mildew, have made grapevine plants highly susceptible to this damaging disease (Harm et al., 2011). Consequently, in order to control the pathogen, numerous fungicide applications are required. This continuous fungicide applications results in the appearance of certain strains of *P. viticola* resistant to the most commonly employed fungicides, which significantly limits the efficacy of the chemical control of the pathogen (Sudiro et al., 2022).

Among conventionally used chemical substances (Fig.1), copper is an effective component and of considerable importance in both ecological and conventional crop management. But, its abundant use over the last decades has caused serious environmental problems, due to its tendency to accumulate in the soil and nearby waters, generating in this way long-term damaging consequences for the ecosystem (Kraus et al., 2021).

The incompatibility of these substances with organic farming's booming objectives and a growing public concern around this topic, among other reasons, led the European Commission to adopt regulations to restrict the use of plant protection products containing copper compounds in agricultural soils to a maximum application rate of 4 kg ha^{-1} $year^{-1}$ (European Commission).

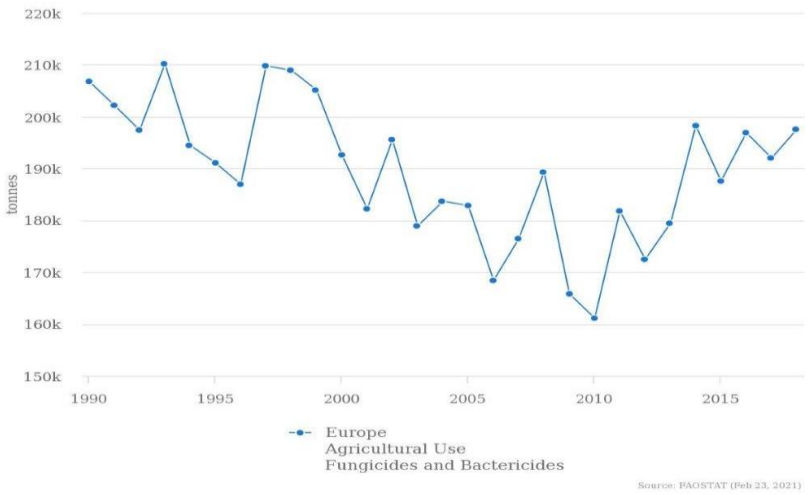


Figure 1. Agricultural use of fungicides and bactericides in Europe from 1990 to 2015.

Seeking to solve this issue, different studies for alternative methods to reduce conventional chemical treatments have been carried out. Biological control of plant pathogens has emerged as a promising alternative, since it allows reducing copper dosages maintaining an adequate efficacy against the disease, as well as being safe for people and animals health (Zang et al., 2019). Another alternative that will be developed throughout the following work, supposes the plant induction by substances, in order to improve the protection against downy mildew (Harm

et al., 2011). Among those substances, special focus will be made on basic substances (Regulation No 1107/2009).

1.2. *Plasmopara viticola*

Plasmopara viticola, the causal agent of downy mildew disease of grapevines causes serious damage worldwide, causing huge damages that have an impact in grapes and wine production.

Downy mildew was firstly noticed in North America in 1834, showing the American grape varieties genetic resistance to the disease. While the presence of the pathogen was already identified and known in the USA, Europe went through different periods in relation to the history of grape-growing. Before 1845, no important phytosanitary problems were faced. However, the following half century became problematic due to the arrival of two pathogens and an insect pest; *Erysiphe necator* causing powdery mildew, *P. viticola* causing downy mildew and the aphid *Daktulosphaira vitifoliae* causing phylloxera. With the emergence of these agents and their disastrous consequences in European vineyards the following years were mainly focused on searching solutions to these problems, hand in hand with an intensive chemical control protection (Gessler et al., 2011).

The question to ask now is how did *P. viticola* crossed borders and entered Europe. According to the latest research, downy mildew was first observed in Europe in 1878, when phylloxera-resistant wild American rootstocks were imported to solve the phylloxera epidemics. These American grape cuttings brought downy mildew with them and caused a destructive epidemic among Southern French vineyards. It was later on identified throughout Northern Italy and nearby Austrian regions and in 1881 westward, toward Spain and Portugal (Gessler et al., 2011). One of the main causes of the rapid dissemination of the pathogen over Europe was the international commerce, as France, which was taken as reference by other countries, exported the phylloxera- resistant rootstocks to the diverse new modern vineyards all around (Fontaine et et al., 2021).

The epidemics caused by the oomycete were irregular and sporadic, as they relied on favourable weather conditions as well as, in insufficient chemical control measures (Gessler et al., 2011). From the beginning of the 20th century onwards, the disease became a major problem for the European viticulture (Gäumann, 1927), which led to domesticated *Vitis vinifera* grapevines, being at the time one of the most cultivated varieties in Europe, served as source for the worldwide distribution of the disease (Fontaine et al., 2021).

1.2.1 Cycle and biology of downy mildew

P. viticola is an obligate biotrophe of grapevine, responsible of causing important yield losses to *V. vinifera*, one of the most cultivated species worldwide. This pathogen has become a real threat for the wine industry due to its capacity to affect all green tissues of grapevine (Choi et al., 2017). The taxonomic tree of the oomycete is detailed in Table 1.

Table 1. Taxonomic tree of downy mildew

Domain	Eukaryota
Kingdom	Chromista
Phylum	Oomycota
Class	Oomycetes
Order	Peronosporales

The pathogen is polycyclic, meaning that it is able to go through more than one infection cycle during a unique grapevine growing season (Massi et al., 2021). As a consequence, under adequate conditions, for two to three months, individual lesions can produce new asexual spores capable of initiating new infections.

P. viticola is characterised by going through both a sexual overwintering phase and an asexual phase during the growing season.

Along the winter sexual phase, the biotrophic oomycete survives as oospores in previously infected dead leaves (Karthick et al., 2019). The sexual reproduction takes place when an antheridium fertilizes an oogonium to form a sexual oospore (Kortekamp et al., 1998), being this oospore the primary source of inoculum for initiating the infection (Karthick et al., 2019).

Once the oospore has been formed, in spring when temperatures rise to about 10 ºC and the soil presents proper moisture, it germinates, starting in this way the contamination phase. Morphologically, oospores are spherical, containing from 14 to 16 chromosomes and covered by two inner membranes and an outer wall. They form a germ tube that leads to the emergence of a macrosporangium which contains several laterally biflagellated zoospores inside. These zoospores, disseminated by wind and rain plash, can reach the host tissues and survive in it for more than 24 hours under optimal climatic conditions. Once they are in the host tissue, zoospores move along the free water until, near the stomata and in groups of two to five, they encyst and germinate. Each spore will form a single germ tube that will penetrate the stomata and attach itself to the lower leaf surface (Vecchione et al., 2007). The germ tube generates a substomatal vesicle from which the mycelium, composed of intercellular colourless hypha, arises. During the following four hours, the hypha grows intercellularly and gives rise to the first small rounded, vesicular haustorium, contracting in this way the host cell (Vecchione et al., 2007). The hastorium keeps developing as the pathogen moves inside the tissue and parasite the mesophyll cells (Kortekamp et al,. 1998; Langcake et al., 1980), and is at this point when the sporulation phase begins. From the mycelium, sporangiophores emerge through the stoma and later on produce sporangia that mature as days go on. This sporulation requires certain conditions: 95-98% relative humidity, temperatures between 10 and 30 ºC and at least 4 hours a day of darkness (Blaeser et al., 1978; Lalancette et al., 1988; Hill, 1989)

After the asexual phase is completed a secondary infection can occur, where sporangia detach from sporangiophores and germinate, releasing zoospores that will be spread by wind-blown rain until they take over new host tissue (Blaeser et al., 1978), so that the cycle is repeated.

The period between the very first infection and the appearance of symptoms depends on climatic conditions; with an adequate temperature it normally takes an average of seven to 10 days to notice the emergence of the disease with the naked eye. In young leaves, at the upper side of the leaf, yellow translucent ‘oil spots’ with a brown halo emerge. As time goes on, the spots become dry and necrotic (Vecchione et al., 2007). Sporulation however, occurs on the lower leaf surface, where sporangia and sporangiophores appear with a white, downy, cotton appearance (Figures 2 and 3).

Figure2. Upper side of a mildew infected leave

Figure3. Underside of a mildew infected leave

1.3. Tempranillo grape variety

- Origin

It is native to the upper basin of the Ebro River, in an area between La Rioja and Navarre. It derives from two different grape varieties, Benedicto and Albillo mayor, whose spontaneous hybridization gave rise to this variety (Hidalgo, 2022).

- Cultivation area

It is one of the most important varieties in Spain and cultivated in many wine-growing regions, being the most planted red grape variety in the country in recent years, with more than 220,000 cultivated hectares (Barber, 2018). In fact, it is authorised in 38 Spanish certificates of origin, being considered as a preferred variety in 14 of them (Romero, 2021). The high quality of the grapes produced allows the elaboration of excellent quality wines, giving the variety a high prestige both in Spain and the world.

- Ampelographic characteristics

- Upper side: Dark green, curved profile with pigmentation on the main nerves
- Underside: Medium to high density long lying hairs
- Young leaves: Large, with five or seven marked lobes and pentagonal shape. They are hairy yellowish green and with reddish edges.
- Adult leaves: Large and of bluish green colour.
- Bunch: Large, compact, uniform in size and in colour of the berries.
- Berry: Medium, spherical blue-black, rather thick skin and a not pigmented soft juicy flesh.

- Agronomic characteristics

It is a short-cycle variety with a middle season sprouting and early ripening. It enjoys a high average fertility and a regular high production. Furthermore, the Tempranillo grapevines adapt easily to different types of soils and climates, offering its maximum potential in cold climates, like the continental one, characterised by warm days and cold nights.

On the one hand, the variety is highly susceptible to wood diseases as well as to downy mildew, powdery mildew and black rot. It presents a high susceptibility to cluster moths,

mites and to breakage by strong winds. On the other hand, it is little sensitive to spring colds, as its sprouting can be delayed, tolerates drought well, unless it is very extreme and it responds in a correct way to water inputs.

Nutritionally, the Tempranillo grape variety is demanding in potassium, with moderate nitrogen and phosphorus requirements and low magnesium demands.

- Oenological characteristics

The qualitative potential of this cultivar is high and its destination is very versatile. Destined for the production of both, young red wines and aged wines.

Some of the properties of Tempranillo wine are, low acidity, persistent colour and good alcohol content. It contains few tannins, which make it light but with a carbonic maceration it achieves a fruity flavour and bouquet. It complements very well with varieties such as Cabernet, Sauvignon or Merlot (*Qué es la uva tempranillo y sus características,* 2020).

1.4 Downy mildew management

The conventional agriculture is based on the overuse of fertilizers, herbicides, pesticide, heavy agricultural machinery, monocultures and deforestation (Laurin et al., 2006), which have resulted in a major environmental degradation. The massive use of phytosanitary products over the years have caused both ecological and agronomical alterations on cultivated soils, challenging in this way environmental and economic sustainability. Therefore, the agricultural sector must undergo a mayor evolutionary process. Some problems of the modern agriculture phytosanitary practices include (Riechmann, 2003):

- Loss of efficacy of plant protection products and appearance of resistance to biocidal substances
- Destruction of microfauna
- Loss of nutrients from the soil
- Contamination of soil and water with long persistence toxic compounds
- Food contamination with residues of plant protection products
- Loss of biodiversity
- Long-term damage to animal and human health
- Imbalance of the global nitrogen and phosphorus cycle
- Acid rain
- Eutrophication of aquatic systems

All those problems have led to the establishment of normative that regulate the use of phytosanitaries in agriculture, such the:

- **European Directive on the sustainable use of pesticides 2009/128/EC**

  It establishes a common legal framework for Community action to achieve the sustainable use of pesticides, taking into account precautionary and preventive approaches.

  Its main aim is to reduce the risks and effects of pesticides on the environment and human health and to promote integrated pest management methods together with non-chemical alternative techniques.

This Directive is transposed to each Member State, being in Spain the **Real Decreto 1311/2012**, **(Ministerio de Agricultura, Pesca y Alimentación, Spain),** which establishes a framework for action to achieve a sustainable use of plant protection products.

Regarding grapevine, the pesticides used have similar negative consequences for the environment and health. Aside from those, the overuse of chemicals can affect the quality of grapes and, therefore, of the wine as well. Moreover, the cost of the active substances and the labour involved in applying them can be economically challenging for farmers.

In the case of downy mildew, chemical control of the disease dates back many years. The first attempt to control the causative agent of downy mildew was in 1882 when the Bordeaux mixture was developed. This mixture was composed of a mix of copper sulphate and lime and was able to inhibit multiple metabolic processes (Massi et al., 2021; Šebela et al., 2013). Copper became the most traditionally used chemical, due to its high effectiveness against pathogens affecting the grape production and the Bordeaux mixture soon became popular along Europe (Massi et al., 2021).

Between 1970 and 1980, target site fungicides, such as benzimidazoles, morpholines, pyrimidines and azolone (Morton et al., 2008) were introduced into the market (Massi et al., 2021). Even though they had a lower toxicological profile than previously used compounds, the frequent applications of fungicides in vineyards every season lead to the development of fungicide resistance, defined as the acquired and heritable reduction in the sensitivity of a fungus to a specific anti-fungal agent (FRAC, 2020).

One of the main problems of conventional fungicides, apart from their toxicity, is the generation of fungicide-resistant strains of the pathogen. In fact, *P. viticola* has developed resistance to most of the fungicides used for its control, due to its capacity of evolution and high reproductive frequency (Calo et al., 2013). The polycyclic behaviour of the oomycete and its sexual and asexual reproduction leads to genetic changes which can cause a reduction in the sensitivity of the pathogen to a specific fungicide (FRAC, 2022). When the resistant mutants turn dominant, the pathogen can no longer be controlled by the fungicide (Hewitt, 1998).

1.5. Other methods for sanitary control: Basic substances

Active substance refers to substances including chemicals or micro-organisms that have a general or specific action against harmful organisms (*What is an active substance?*, 2022). Depending on their characteristics, some of them may fulfil the whole range of approval criteria and be approved as low-risk substances or as basic substances.

According to the article 23 of EC phytopharmaceutical Regulation No 1107/2009 the term basic substance refers to an active substance which is not a substance of concern and does not have an inherent capacity to cause endocrine disrupting, neurotoxic or immunotoxic effects. It is not predominantly used for plant protection purposes but nevertheless is useful in plant protection either directly or in a product consisting of the substance and a simple diluent.

The opportunity to use these safe products, mostly of natural origin for crop management, is in line with the ‘European Green Deal’, a roadmap that aims to protect the health of European citizens from environment-related risks and impacts (European Commission 2019).

They are generally less effective than synthetic plant protection products because they consist mainly of natural substances (Bahlai et al., 2010; Meemken et al., 2018) but the interest is already very strong and is demonstrated by the fact that in a few years the number of approved basic substances has increased very rapidly (Marchand, 2016) due to their effectiveness in plant protection.

Basic substances are categorized according to their function, either as fungicides or as elicitors. Elicitors are molecules capable of stimulating the plant's natural defence responses.

In the last years, the use of elicitors has become popular. Elicitors are compounds that can mimic an attack by virulent pathogens. Their recognition unleashes signalling cascades that activate gene expression and, subsequently, induce the natural plant defences (Steimetz et al., 2012), such as stomatal closure movements (Lee et al., 1999) that are considered part of the plant immune system (Melotto et al., 2006).

For the control of downy mildew several elicitors have been studied. Some of them are; Chitosan, Lecithins, Tagatose and *Equisetum arvense*. The list of products are periodically published in the member states, with reference to their use and authorisation (EU Pesticides database).

1.6. Mechanisms of activation of the natural defences of grapevines against the pathogen

Plants possess the ability to defend themselves against pathogens, but the success of it depends on the speed and intensity of their response (Dufour et al., 2013). In the presence of a pathogen, plants undergo deep genetic and metabolic changes to overcome the infection. These changes are regulated by two main phytohormones: salicylic acid (SA) and jasmonic acid (JA). The synergistic and antagonistic interaction between them allows the plant to respond to specific pathogens (Figueiro et al., 2015).

It is believed that resistance to biotrophic pathogens can be mediated though salicylic acid signalling, leading to hypersensitive responses followed by the establishment of systemic acquired resistance (Guerreiro et al., 2016). In the case of jasmonic acid, it is involved in the establishment of incompatible interactions with *P. viticola* establishing systemic acquired resistance.

According to published data, the levels of SA and JA increase along the first hours after the inoculation of *P. viticola*. This leads to the coordinated accumulation of pathogenesis-related proteins and phytoalexins and the reinforcement of plant cell walls.

Phytoalexins can be used as markers for resistance due to their antimicrobial properties. They are defined as low molecular weight secondary metabolites of antimicrobial nature (Ali et al., 2012). Phytoalexins are accumulated within the tissues at high concentrations to restrict in this way fungal growth.

Pathogenesis-related proteins (PR) are defined as low-molecular proteins encoded by the host plant but induced only in pathological or related situations. Besides being induced by a wide array of environmental and external cues, PRs synthesis can be triggered by internal plant developmental stimuli. One of the common features among them is their antifungal effect, whose toxicity can be accounted for, by their hydrolytic, proteinase-inhibitory and membrane-permeabilizing ability (Pierpoint et al., 1981).

Cell wall splitting enzymes induce PRs accumulation (Pierpoint et al., 1981), as well as the release of elicitors capable of inducing defence responses in plants (Mc Neil et al., 1984). The accumulation of these proteins into the plant cell wall, reinforce it and inhibit the pathogen ingress, (Benhamou, 1991;Jeun, 2000; Jeun and Buchenauer, 2001).

The success of the plant defences depends on the final output resulting from the interaction of various factors, including the genetic and physiological characteristics of both partners in the host–pathogen interaction, as well as on environmental conditions.

In this study the toxicity on *P. viticola* sporangia will be analysed.

OBJECTIVES

The aim of this study was to evaluate the toxicity of different commercial products, mainly based on basic substances and seaweed extracts, on *P. viticola*. Together with the analysis of the activation of grapevine natural defence mechanisms against *P. viticola* and their reaction to diverse stimulus under controlled conditions. For this, three specific objectives were pursued:

- Evaluate the activity of the products against the zoospores of the pathogen.
- Calculate the inhibition in the motility and viability of zoospores by different products.
- Evaluation of the oxidative stress status in grapevine plants after the application of the products.

MATERIALS AND METHODS

1. Plant material

Throughout this study, the evaluation of the effect of basic substances in the control of downy mildew has been tested on Tempranillo grape leaves, belonging to the *V. vinifera* species.

V. vinifera plants, Tempranillo variety VN40 clon, over scion 110R were provided by Vitis Navarra commercial nursery. They came up with their corresponding Plant Passport (Fig. 4). Plants were potted into containers in October 2021 and grown under greenhouse controlled conditions in NEIKER (Arcaute, Vitoria-Gasteiz), in a P2 security greenhouse. They were maintained under temperatures between 15 and 19 °C and were irrigated as needed. Two to three months after being planted, the plants were used for the assays.

The third and fourth expanded leaves from the apex of the grapevine shoots were selected and pulled up. From the greenhouse to the laboratory the leaves were moved inside a polystyrene box, so that the change in temperature did not affect the leaf tissue. With the help of a corkborer, disks of 15 mm were disposed on 90 mm diameter Petri dishes, over the wet filter paper to maintain a humid chamber. Now the vegetal material was prepared for *P. viticola* inoculation.

Fig.4 Plant Passport

Certificate of the Government of Navarre

Variety: Clone/Scion: Tempranillo (VN40)
Species: Grapevine (*V.vinifera* L.)
Type of material: Graft plant
Rootstock: 110R (237)
Lost number: 175-101-20
Year of production: 2020
Amount of plants: 300
Code of the productor: ES15310175
Product Origen: ES15310175

2. Inoculum

The *P. viticola* inoculum was obtained from previously infected leaves, collected in June 2021 in a vineyard in Etxano (Bizkaia). It was maintained in controlled conditions by weekly inoculations over healthy *V. vinifera* L. leaves.

3. Inoculation of *P.viticola* sporangia on plant leaf discs

The inoculations were done according to a published method (Díez-Navajas et al., 2007). Briefly, a portion of previously infected tissue with visible sporangiophores on it was suspended in commercial mineral water (FontVella, Grupo Danone). With the help of an agitator, the suspension was gently stirred to promote the release of sporangia. Once the dilution was prepared, 10 µl of it were applied over the Thoma cell counting chamber, to count the number of sporangia under the microscope in visible light and determine their concentration.

The frame of the Thoma cell counting chamber consists of a large central square that in turn, is divided into 16 smaller squares. 10 µL of the sample under a coverslip were scattered over the central square and then, with the 10X objective of the microscope the sporangia inside the squares were counted. The total number of sporangia in the suspension is equivalent to $2x10^4$ sporangia (sp)/ml. To determine the sporangia concentration it was also used a Flux cytometer (Beckman Coulter, CytoFLEX).

The inoculation can be done using two methods. One method implies the inoculation of the leaf disks by spraying the open dishes with the sporangia suspension. For the second method, three drops of 10 µl of the spore suspension are deposited over each leaf disk.

Once the dishes were inoculated, they were sealed with transparent film and kept overnight in total darkness on a chamber at 21 °C. 24 hours-post inoculation the drops were carefully removed with absorbent paper and incubated in the chamber with a photoperiod of 16 hours light, 8 hours darkness.

4. Products

Along this study, six different commercial products (Table 2), based on basic substances, were selected from a previous triage with the aim to test their effect in the control of downy mildew and in plants natural defence mechanisms. Apart from those products, seaweed extracts from a

national project were also tested (Towards a sustainable and circular vitiviniculture: the use of seaweed extracts for reducing chemicals and improving grapevine disease resistance. Side effects on wine quality (SEAWINES), Ref. PID2020-112644RR-C22, Convocatoria 2020 Proyectos de I+D+i - RTI Tipo RTA Coord (Table 3).

Table 2 - Products used in this study. Composition, use, approbation and regulation of them are detailed. Many of them are codified, as real names are under patent.

Products	Composition	Use	Approval	Legislation
Product 1*	3-Aminobutanoic acid (BABA)			
Product 2	Chitosan hydrochloride	Elicitor, having a fungicide and bactericide effect via the stimulation of natural defence mechanisms	Yes	Directive 91/414/EEC SANCO/12388/2013 -rev.3
Product 3	Soybean lecithin and *Equisetum arvense* L.	Lecithin: Fungicide Equisetum: Fungicide with an eliciting action on the crop's self-defence mechanism	Yes	Directive 91/414/EEC SANCO/12798/2014 -rev.4 Directive 91/414/EEC SANCO/12386/2013 -rev.5
Product 4	Soybean lecithin	Stimulates plant's natural defences	Yes	Directive 91/414/EEC SANCO/12798/2014 -rev.4
Product 5	Salix spp. cortex	Fungicide with an eliciting action on the crop's self-defence mechanism	Yes	Directive 91/414/EEC SANCO/12173/2014 -rev.4

* no basic substance: Non-protein amino acid that primes the defence system to protect against a wide range of stresses, including various microbial pathogens, drought and salt stress.

Table 3. Seaweeds used in this study. The active matter and concentration of them are detailed.

Seaweeds	Active matter	Concentration
T1	H_2O	
T2	Benzothiadiazole (BTH)BION®	50 WG 2g/l
T3	Ulva ohnoi crude extract	6 g/l
T4	Ulva ohnoi purified extract	6 g/l
T5	Rugulopterix okamurae crude extract	6 g/l
T6	Rugulopterix okamurae purified extract	6 g/l
T7	Copper (Nordox)	75 WG 0,15%

All seaweed extracts included 1 ml/l of Retenol as coadyuvant.

5. Microscopic observation of vine leaf tissue infected with *P. viticola*

To observe the intercellular leaf tissues infected by *P. viticola*, samples were treated according to the KOH-aniline blue fluorescence method (Díez-Navajas et al., 2007). To assess the KOH efficiency in discolouring plant pigments, the leaf disks were introduced into a 2 ml Eppendorf tube with 2 ml of 1M KOH. Subsequently, the tubes containing the samples were simultaneously incubated at 100°C in a thermo-block during 20 minutes. The incubation was followed by three 20 minutes washes in distilled water of the leaf disks. Once the samples were washed they were introduced into 0, 05% aniline blue for 20 minutes. Aniline blue is used to visualize infection structures laying on the leaf surface. Afterwards, another 20 minutes wash in distilled water of the disks was carried out. The last step before examining the samples by epifluorescence under the microscope (NIKON, Intensilight C-HGFI) was to mount them on glass slides in a water drop. Images were taken with a Canon EOS 300D, incorporated to the microscope, by using the 10X, 20X and 40 X objectives.

6. Evaluation of the IC50 of the products against the pathogens zoospores

The objective of this assay was to calculate the half-maximal inhibitory concentration (IC50) of the basic substances that were used along the assays. This value represents the concentration at which a substance exerts half of its maximal inhibitory effect. It is typically used to characterize an antagonist of a biological process and to measure its efficacy (IC50 Calculator, n.d.). The IC50 has been calculated based on the number of spores produced by the pathogen per unit area.

To calculate the IC50 as described at (Krzyzaniak et al., 2018), five products at different concentrations (Table 4) and seven seaweeds at a unique concentration (Table 7) were prepared, which were then mixed with a solution of *P. viticola* sporangia.

The concentrations used for measuring the sporulation are detailed in Table 4 and 7.

Table 4- Products at the different used concentrations in the indicated units.

Product	C1	C2	C3	C4	C5	C6	C7
BABA(mM)	0	0,2	1	2	4	8	16
Product 2 (ml/L)	0	0,2	1	2	4	8	16
Product 3 (ml/L)	0	0,2	1	2	4	8	16
Product 4 (ml/L)	0	0,2	1	2	4	8	16
Product 5 (ml/L)	0	0,2	1	2	4	8	16
Sporangia concentration				2.10^4 sp/ml			

Once the product concentrations were prepared the following dilutions were carried out:

Table 5. Prepared dilution for Product 1

0,0164g of BABA in	C2= 320 ml of water
	C3= 160 ml of water
	C4= 80 ml of water
	C5= 40 ml of water
	C6= 20 ml of water
	C7= 10 ml of water

Table 6. Prepared dilutions for Products; 2, 3, 4 and 5

In 10 ml of water	C2= 2 µl of product
	C3= 10 µl of product
	C4= 20 µl of product
	C5= 40 µl of product
	C6= 80 µl of product
	C7= 160 µl of product

Out of that, 1 ml of each of the concentrations was mixed with 1 ml of the inoculum solution, whose concentration was of $2x10^4$ sp (sporangia)/ml.

Table 7. Seaweeds at the used concentrations. Fixed concentration for all of them.

Seaweeds	Concentration
T1	Unique
T2	Unique
T3	Unique
T4	Unique
T5	Unique
T6	Unique
T7	Unique

Subsequently, 1ml of each seaweed extract was mixed with 1 ml of the sporangia dilution with a concentration of $2x10^4$ sp/ml.

Application of the final solution

Three drops of 10 µl of the prepared final solutions were inoculated into each leaf disk. We had five products times seven concentrations, that is to say 35 different conditions, adding the seven seaweeds at unique concentrations and one control, the final number of conditions were of 43. For each condition six disks were established, so it can be concluded that the total number of disks was of 258.

Once the petri dishes were prepared, they were introduced into a chamber under controlled conditions (18-22 °C with a photoperiod of 16/8 hours light/obscurity, respectively) for seven days.

After seven days, if sporulation was observed, it was captured by a stereomicroscope that included the LEICA software. Subsequently, the percentage of inhibition for each product and condition was calculated.

Measurement of sporangia germination and zoospore mobility

Once the pathogen-product mixture was in contact, two hours later the sporangia germination and the zoospore mobility were evaluated.

- **Germination of sporangia**: Three measurements of the number of empty sporangia were carried out. For this re-counting the Thoma cell counting chamber was used.

  10 µL of the sample under a coverslip were deposited over the central square and then, with the 10X objective of the microscope the empty sporangia inside the squares were counted. Subsequently, the percentage of inhibition for each product and condition was calculated.

- **Zoospore mobility**: Three measurements of the observed mobile zoospores per minute were carried out. The counting was also done with the Thoma cell counting chamber. After the results were obtained, the percentage of inhibition for each product and condition was calculated.

The trial was repeated three independent times.

7. Curative assay

For this assay, disks of fully expanded 3rd, 4th or 5th leaves of the Tempranillo grape variety were unattached. The leaf disks were randomly mixed and placed on petri dishes, differentiated from each other by condition and time. Afterwards, the samples were inoculated with *P. viticola* sporangia at 2.10^4 sp/ml, spraying the disks with the inoculum twice. 24 hours after the inoculation, the remaining drops on the leaves were carefully removed with absorbent paper. They were then sprayed with the commercial products (Table 2) and seaweeds (Table 3) at the required concentrations (Table 8), at 24, 48, 72 and 96 hours after the infection. We had 13 products times four times, that is to say 52 different conditions. For each condition six leaf disks were established, being 312 the total number of disks required for this assay.

Table 8- Products at the different used concentrations in the indicated units.

Product	Concentration
Product 1	2 mM
Product 2	2,5 ml/l
Product 3	3,33 ml/l
Product 4	3,75 ml/l
Product 5	4,16 ml/l
Seaweeds	Fixed

The disks were kept at 18-22 ºC inside a chamber, together with a photopheriod of 16 hours of light and 8 hours of darkness. After seven days, images of the sporulated disks were taken on a stereomicroscope that included the LEICA software and the reduction of the infection was evaluated. The trial was repeated three independent times and as prerequisite it always needed to begin on Mondays, so that the 24, 48, 72 and 96 spraying hours were fulfilled along one week.

8. Disease incident evaluation

Seven days after the IC50 and curative assays, inoculated disks were checked. In case of observing sporulation over the leaf disks, the disease severity was evaluated. For this purpose, disks were observed under stereomicroscope (LEICA) and images taken (software LEICA). Images were processed with Image J, following the methodology described by Peresotti *et al.*, (2011).

The analysis of all the images was done in the same way. First of all, the image was selected. Afterwards, by drawing a circle around, the leaf disk was defined and subsequently, its colour balance was adjusted by ("image/adjust/colour balance/all/auto/apply").Then, the outside was cleared out ("edit/clear outside"). By transforming the image into an 8-bit format ("image/type/8-bit"), it turned black and white. Thereafter, the background was subtracted ("process/subtract background"). To better observe the sporulated relative area over the dark background, the threshold was adjusted by ("image/adjust/threshold"). Finally, the selected area was calculated ("analyse/measure"). The area was given in pixels, so it has been determined that 1 cm^2 is equivalent to 1.134.000 pixels, as all the images were taken with the 2592 x 1944 resolution.

9. MDA quantification

The method proposed by Heath and Packer 1968 was used for the quantification of malondialdehyde (MDA).

For this assay, the leaves were homogenized in liquid nitrogen to obtain a frozen powder. A quantity, around 50 mg, was then weighed and recorded. Subsequently, it was dissolved in 1ml of 1% w/v of trichloroacetic acid, after which it was centrifuged at maximum speed for 10 minutes. Then 800 µl of the supernatant were mixed with 800 µl of 20% w/v TCA and 0, 5% w/v thiobarbituric acid. The mixture was heated to 95 °C for 30 minutes, cooled in ice and centrifuged for five minutes at 10000g. Finally, the absorbance of each sample was measured with a UV-VIS recording Spectrophotometer (UV-2401 PC) at 532 nm and subtracting at 600 nm the non-specific absorption. With these data and the molar extinction coefficient for malondialdehyde, the amount of malondialdehyde present was calculated and relativized by expressing it in nanomoles per gram of tissue.

10. Statistical analysis

All data were analysed and graphed using the JASP 0.16.2.0 software. Values are presented mean ± standard deviation and standard error of the mean, of three independent assays; Evaluation of the IC50 of the products against the zoospores of the pathogen, curative assay and MDA quantification assay, of which for the first two, three and for the last one, one biological repetitions respectively were carried out. The sporulated relative area and the MDA quantification were calculated by an ANOVA. Tuckey's test was carried out among the different treatments for each time point and concentration, and among different time points and concentrations for each treatment, where differences were considered significant at $P \leq 0, 05$. Conditions with different letters were significantly different.

RESULTS

1. Microscopic observation of vine leaf tissue infected with *P.viticola*

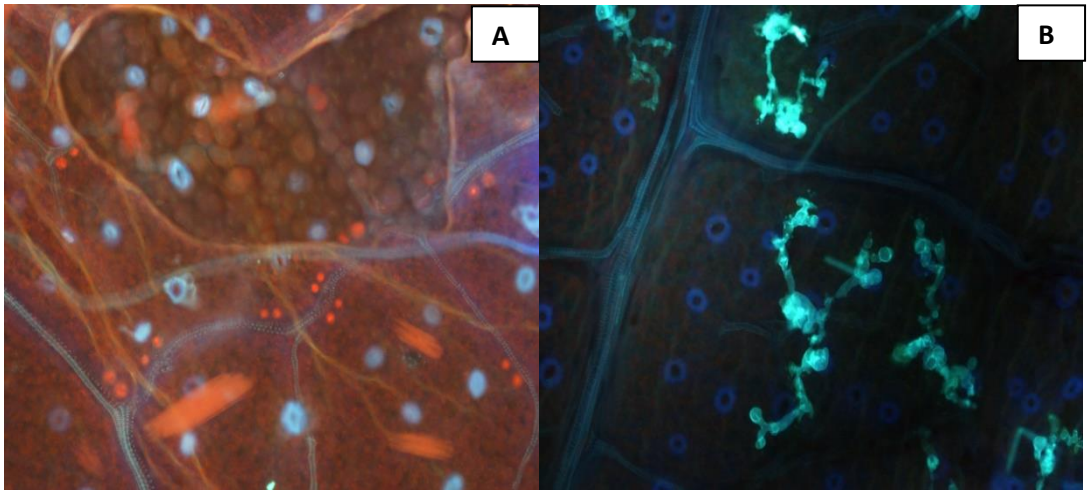
After *P. viticola* inoculation over the leaf disks, samples were processed at different times according to the KOH-aniline blue fluorescence method in order to observe the different stages of *P. viticola* infection cycle. In Figure 5 diverse phases of the pathogens life cycle are displayed. The samples have been examined by epifluorescence under the microscope (NIKON, Intensilight C-HGFI) and captured with the camera included in the microscope. Using the information given by the images, it was possible to assume at what point of the infection cycle the product was having an effect on the pathogen.

In Figure **5A** the leaf tissue before the inoculation can be observed, at this point the stomata were not still invaded by zoospores. Nevertheless, in Figure **5B** it can be observed the mycelium of germinated zoospores that emerged a germ tube that grows thought the stomata, generating in this way an intercellular mycelium that after arises from the stomata.

Figure **5C** displays the formation of a dense net of mycelia. They penetrate the cell walls of the mesophyll and this give rise to the primary hypha. Once the hypha has arised, the mycelium keeps developing and increasing, that is exactly what it is shown in Figure **5D**.

Figure **5E** and **F** represent the emergence of sporangiophores with sporanges attached.

In Figure **5G** and **H** the final phase of the infection cycle can be observed. From the sporangiophores sporangia emerge, that is actually what it can be observed in Figure **5G**. Over the leaf surface the sporangia are seen in a with a white, downy, cotton appearance.



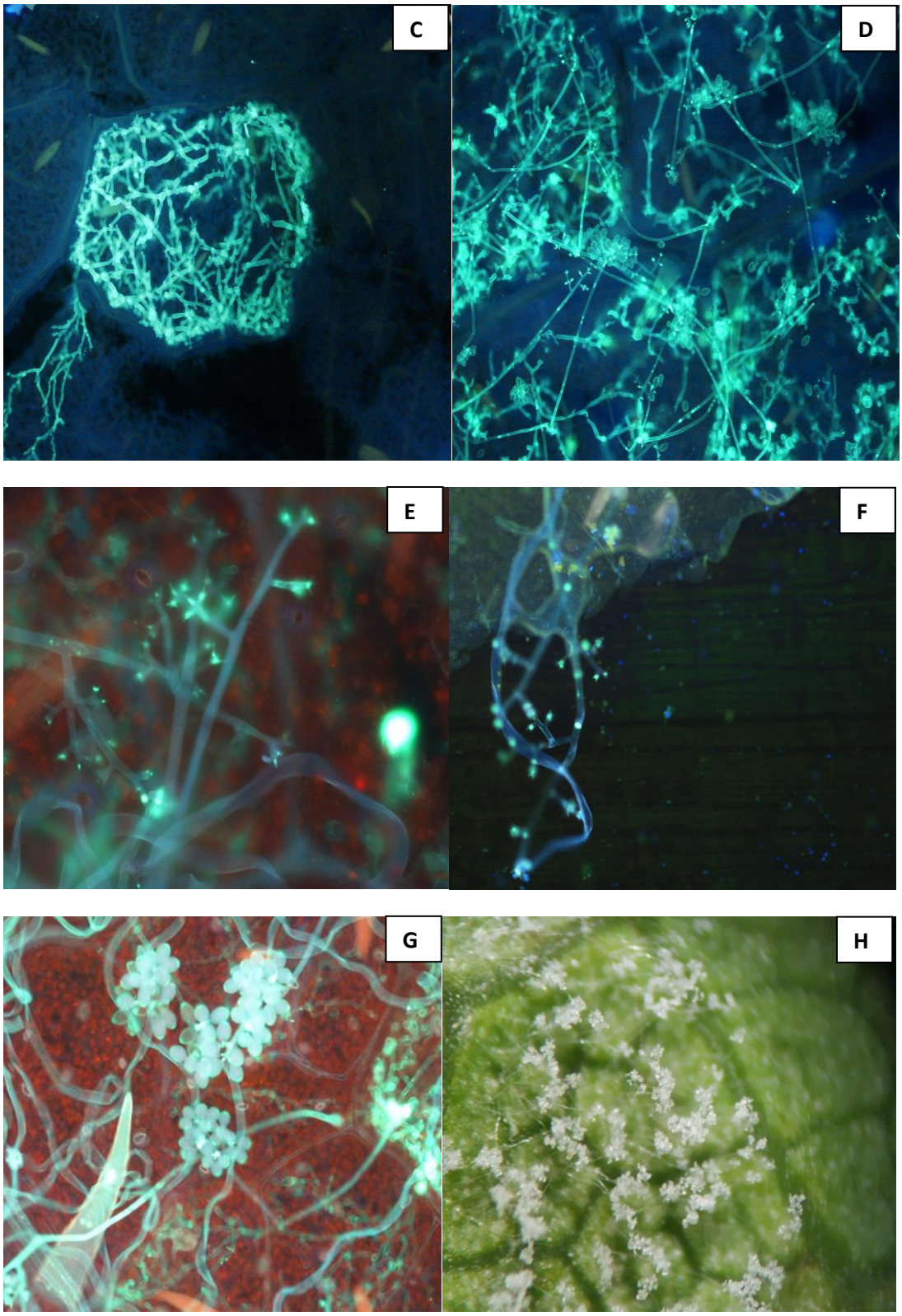


Figure 5. Samples examined by epifluorescence under the microscope at different stages of the infection cycle

Evaluation of the IC50 of the products against the pathogens zoospores.

For the evaluation of the IC50 the different products (Table 2) at different concentrations (Table 4) and seaweed extracts (Table 3) at unique concentrations (Table 7), were mixed with a solution of *P. viticola*, with a concentration of $2x10^4$ sporangia (sp)/ml. Two hours after, the data including the number of empty sporangia and the number of mobile zoospores per minute was recorded. This data is represented in **Figures 8 and 9**.

Seven days after the inoculation of the products and the inoculum over the leaf disks, the number of spores produced by the pathogen per unit of area was measured, in all the figures the relative area represents, the sporulated area in pixels ($1cm^2$ equal to 1.134.000 pixels). The data of the three independent repetitions of the assay is shown in two ways, but it contains the same information:

- **Figure 6**: Application of the same product (Table 2) at different concentrations(Table 4).

- **Figure 7**: Same concentration (Table 4 and 7) of the different products (Table 2) and seaweed extracts (Table 3).

2. Evaluation of the IC50: Same product, different concentrations

Figure 6 shows the application of same product (Table 2) at different concentrations (Table 4) seven days after the inoculation.

All the graphs, except **Fig.6A**, manifest a downward trend of the infected area as the dose of the products gradually increase. This indicates that the products can present toxicity against *P. viticola*. In the case of 3-aminobutanoic acid (BABA, **Fig.6 A**) , it can be seen that the relative area at concentrations C2 and C3 increased in relation to the control (C1), concentration at which there was no product, just inoculum. At higher concentrations it can be seen the product effectiveness against the pathogen and a maximal protection level, as the *P. viticola* sporulation symptoms were completely inhibited. There were significant differences between C3 and C4, as the infected area was over the half from one to the other. However, no significant differences were observed between C4 and C5, but do between C5, C6 and C7 and lower concentrations.

In the case of **Fig.6B**, it can be seen that until the product concentration equivalent to 1ml/l (C3) was reached, high levels of sporulation symptoms appeared, not existing any significant differences among C1 and C2, but yes between these two concentrations and the rest, as well as between C3 and the rest of the concentrations.

In **Fig.6C** and **E**, the same pattern was observed. The concentration that only includes the inoculum (C1) as it was expected, presented high levels of infection, expressing significant differences in comparison with the rest of the concentrations. Both products presented toxicity against the pathogen, as very little or no infection was observed above C5.

The different concentrations at which product 4 was applied, showed significant differences between C1 and C3,C4,C5,C6 and C7. Moreover, differences exist between C2 and C5, C6 and C7. It was the only product that presented extensive sporulation at every concentration, being even the infected area with *P. viticola* at the highest product concentration, higher than at C6.

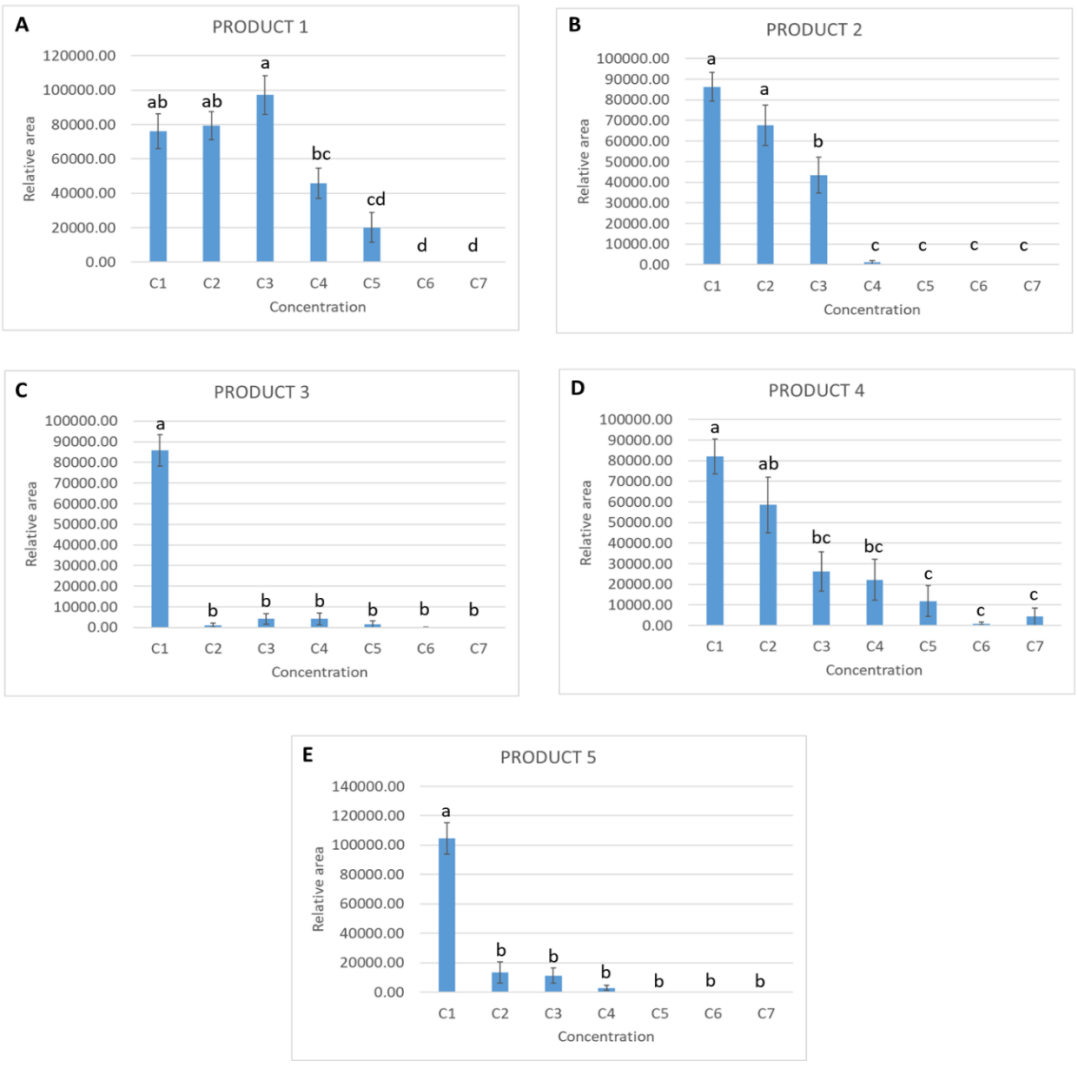


Figure 6. Each graphic shows the relative sporulated area (pixels) seven days after the application of the same product (Table 2) at different concentrations (Table 4), together with *P. viticola.*

3. Evaluation of the IC50: Same concentrations, different products

Figure 7 represents the same concentrations of the diverse products seven days after the infection. It can be clearly seen how as the product concentration increased the sporulated relative area decreased, unless in the case of the control, which clearly makes sense.

Fig.7B reflects the effectiveness of product 3 against *P. viticola* at very low concentrations, as the sporulated area was drastically reduced. The same pattern was followed along higher concentrations of the product, to the point of not perceiving any infection symptoms at C7. Same happened with product 5 (**Fig.7 E**), being it totally effective against the pathogen from C5 on. No significance differences have existed between these two products at any concentration.

The only product that was not able to completely control the pathogen at C7 concentration was product 4 (Table 2), even though the infected area was significantly reduced, its trend has not decreased at all times.

In **fig. 7C**, it can be observed that product 1 was significantly different to the other ones, as well as to the control. The control was significantly different from product 3 and 5, what makes sense with what it has been discussed in the second paragraph.

In **Fig.7D**, the toxicity of product 2 against the pathogen at C4 is clearly observed, comparing it with the caused infection when the third concentration was applied. Significant differences exist between the control and products; 2, 3, 4 and 5 and between the product 1 and product 2, 3 and 5. In the case of C5 (**Fig.7E**), no sporulation emerged with the application of product 2 and 5 and the only present significant difference, was between the control and the rest of the products. The same pattern can be observed in **Fig.7F** and **G**.

Figure 7H, represents the seaweeds (Table 3) at unique concentration. No significant differences can be observed among the seaweeds but there are present between the control and the seaweeds. Copper has been totally effective against the oomycete. In the case of Ulva EP the infected area has been the highest.

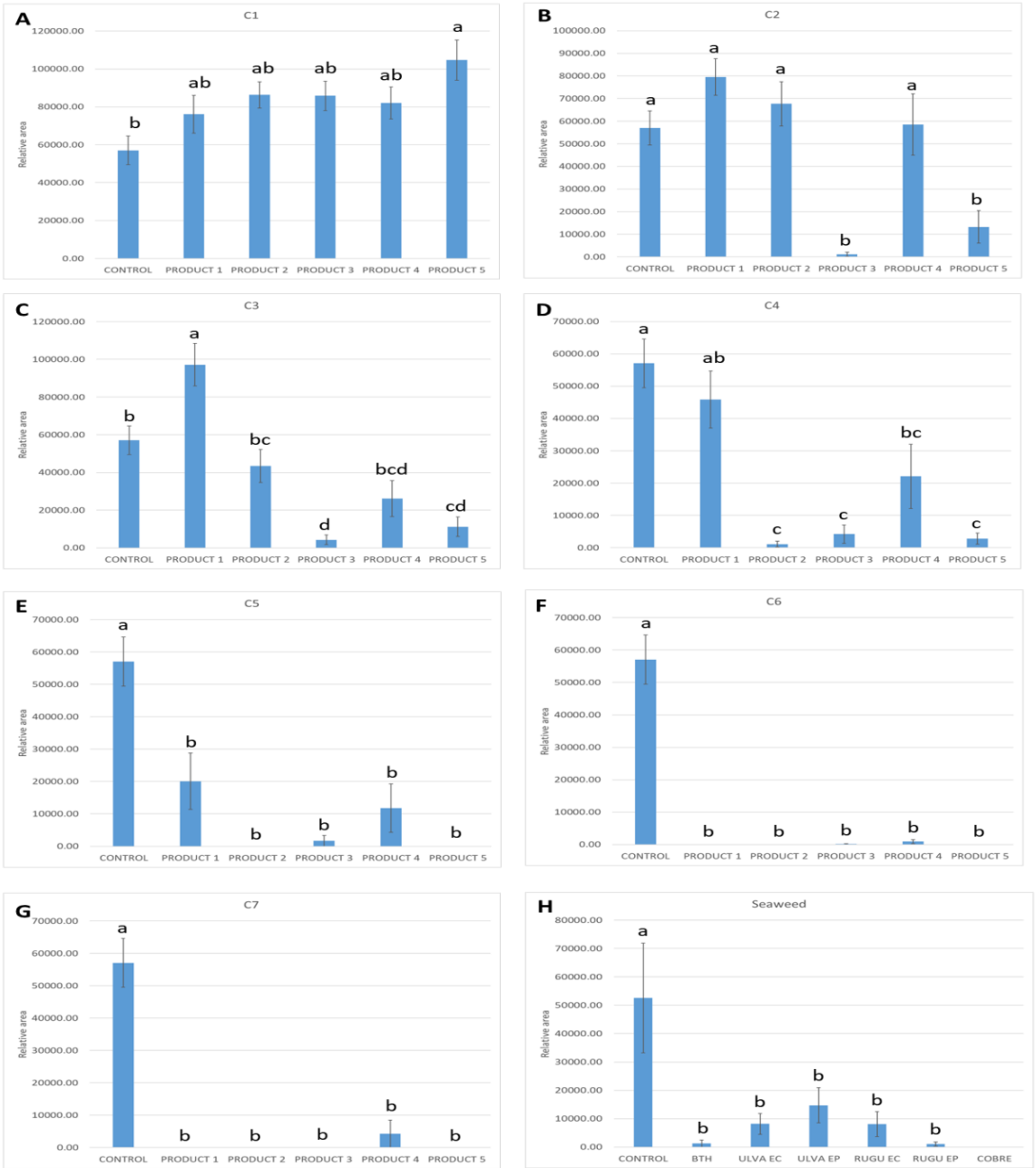


Figure 7. Each graphic shows the relative sporulated area (pixels) of all the products (Table 2 and 3) at the same concentration (Table 4 and 7), seven days after the inoculation of *P. viticola.*

4. Measurement of sporangia germination and zoospore mobility

Figure 8 represents the number of empty sporangia two hours after the products and seaweeds extracts (Table 2 and 3) at different concentrations (Table 4 and 7) were mixed with the *P. viticola* suspension 2 x 10^4 sp/ml.

In all cases, the number of empty sporangia was highest in the control, when the pathogen was not in contact with any of the products (C1). As the products at different concentrations were mixed with the inoculum, a smaller number of empty sporangia appeared. The products did not follow a constant downward trend in any of the cases. In the case of products 1 and 4, empty sporangia were observed at the highest concentration although none were observed at concentration C7 with the remaining products. Additionally, no empty sporangia were observed with the concentration evaluated of seaweeds T5 and T6, but with the application of copper the higher rate of empty sporangia was observed.

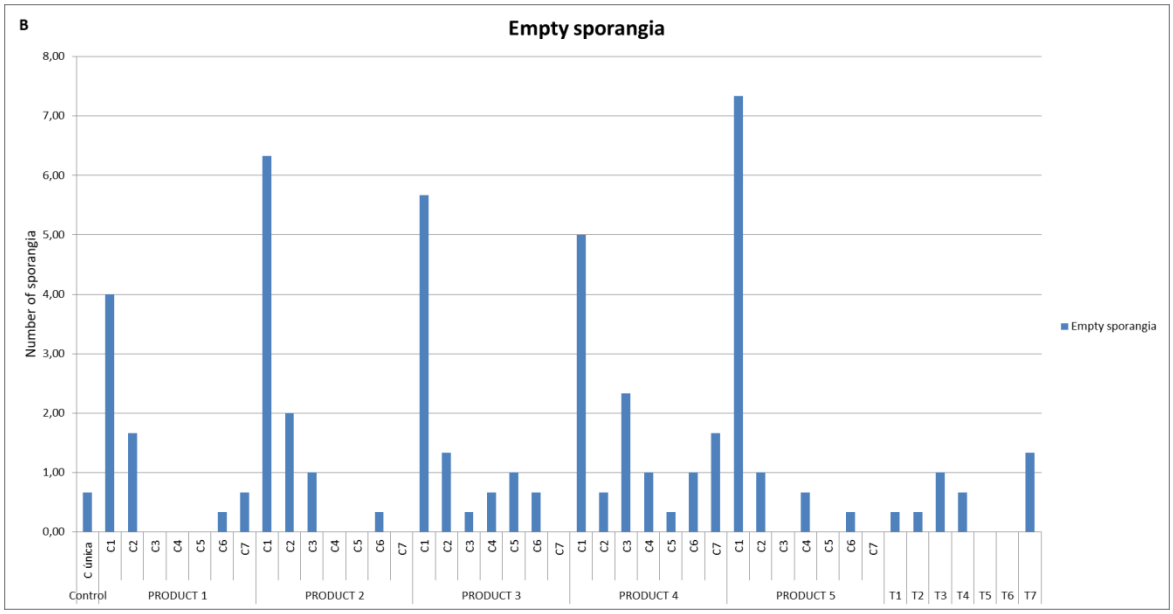


Figure 8. Number of empty sporangia two hours after the application of the solution of different products (Table 2 and 3) at different concentrations (Table 4) with *P. viticola*.

Figure 9 represents the number of mobile zoospores per minute two hours after the products and the inoculum were put together. A connection can be observed between the number of empty sporangia (Fig. 8) and the zoospore motility per minute. As empty sporangia increased the quantity of zoospores and their motility increased. The same pattern was observed throughout product 1, 2 and 3, lower motility of zoospores as the concentration decreased. However, the contrary was evidenced in the case of products 4 and 5, what did not coincide with what it was demonstrated on Figure 8.

When testing seaweeds, practically no motility of zoospores could be observed.

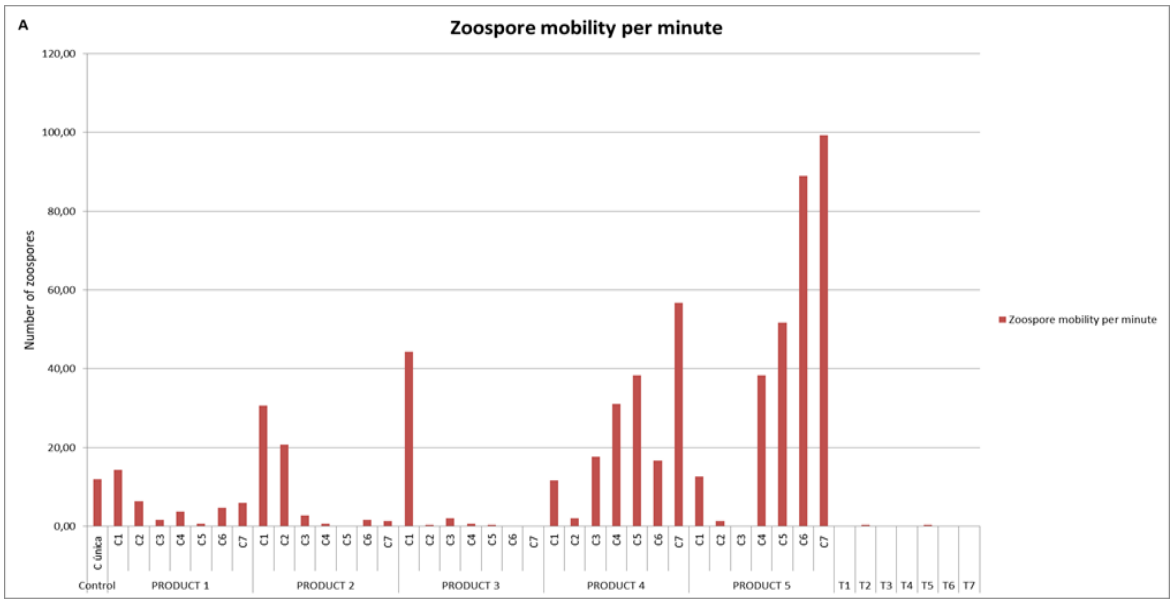


Figure 9. Number of zoospores moving two hours after the application of the solution of different products (Table 2 and 3) at different concentrations (Table 4) with *P. viticola.*

CURATIVE ASSAY

For the curative assay, leaf disks were inoculated with a *P. viticola* suspension of $2x10^4$. Then at the different time points after the inoculation (24, 48, 72 and 96 hours), the products (Table 2) and seaweed extracts (Table 3), at different concentrations (Table 8) were applied. Seven days after each application time-point the number of spores produced by the pathogen per unit of area was measured, in all the figures the relative area represents the sporulated area in pixels (1cm 2 equal to 1.134.000 pixels). The data of the three independent repetitions of the assay is shown in different ways, but it contains the same information:

- Different products at the same application time-point/ Different seaweeds at the same application time point

- Same product at different application time- points/ Same seaweed at different application time-point.

5. Curative assay: Different products at the same time

In Figure 10 the different products (Table 2) sprayed at the same time post inoculation have been evaluated. Product 5 completely inhibited the growth of the pathogen 24 hours after the inoculation. Product 1 has been effective as well, as at 24 hours the infected area was around 5000 pixels and at 48, 72 and 96 hours post inoculation nearly inexistent.

Proucts 2, 3 and 4 follow similar pattern at the different times of application post inoculation. No significant differences were observed between the control and products 2, 3 and 4, indicating, that these products lacked a defence stimulation function against *P. viticola* in the conditions used.

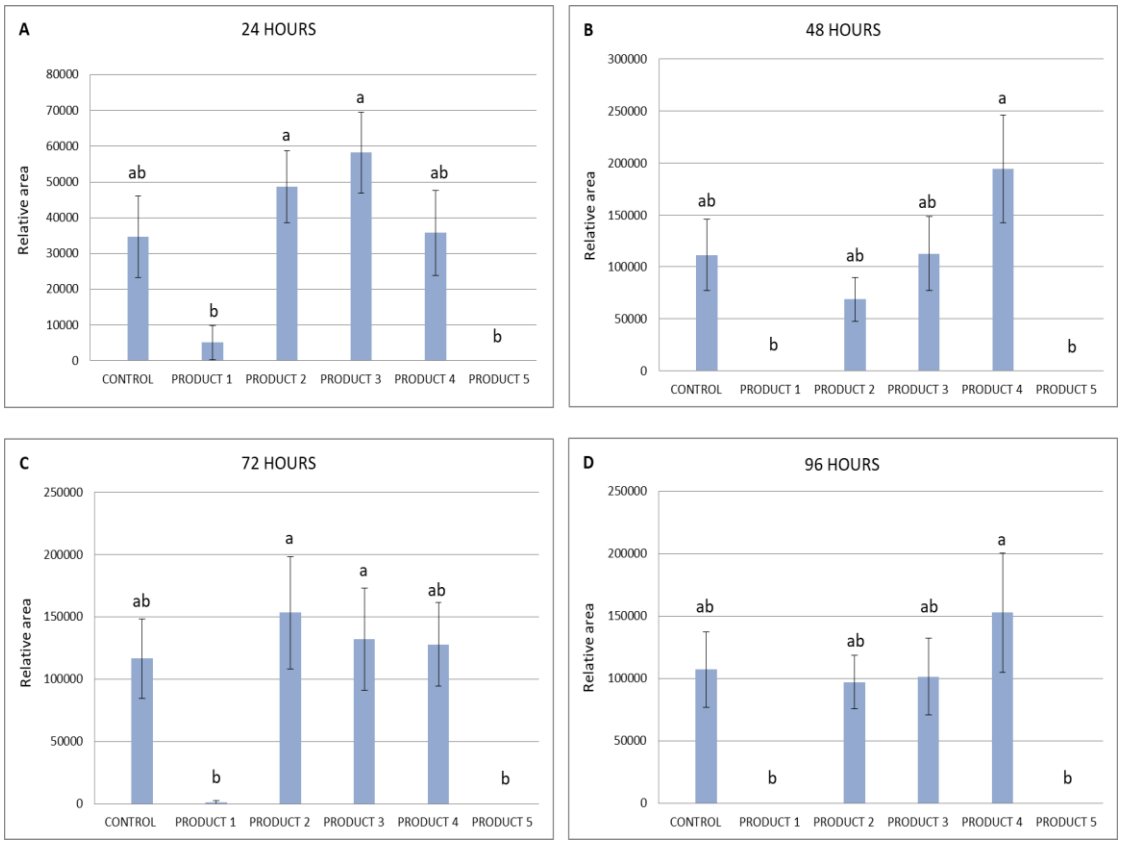


Figure 10. Relative sporulated area (pixels) seven days after the different applicacion time-points. Same time-point after inoculation with *P.viticola* , different products (Table 2) at different concentrations (Table 8).

6. Curative assay: Different seaweeds at the same time

Figure 11 evaluates the different seaweeds (Table 3) sprayed at the same time post inoculation, each of them after seven days. No significant differences were observed between the control and the algae in any of the cases.

At 24 and 48 hours post inoculation (**Fig. 11A and B**) BTH and copper were the ones that have lower sporulation rates, in comparison with the other substances. There was only one significant difference present in the whole Figure 11, and it was between Rugu Ep and copper.

The application of the algae extracts 24 hours post inoculation (**Fig.11A**), concluded in lower sporulation rates than the applications at 48, 72 and 96 hours post inoculation. The latest did not present important differences among them.

There were no significant differences between the crude and purified extracts in both Ulva and Rugu extracts. However, a bigger sporulated area emerged with the spraying of the Ulva crude extract in comparison with the application of the purified one (**Fig.11 A, B, C)**. The contrary was seen in **Fig. 11A, C** and **D**, as the application of the Rugu crude extract carried out less sporulation than the one of the purified one. It can be observed that at 72 and 96 hours Rugu EC is quite effective against the pathogen.

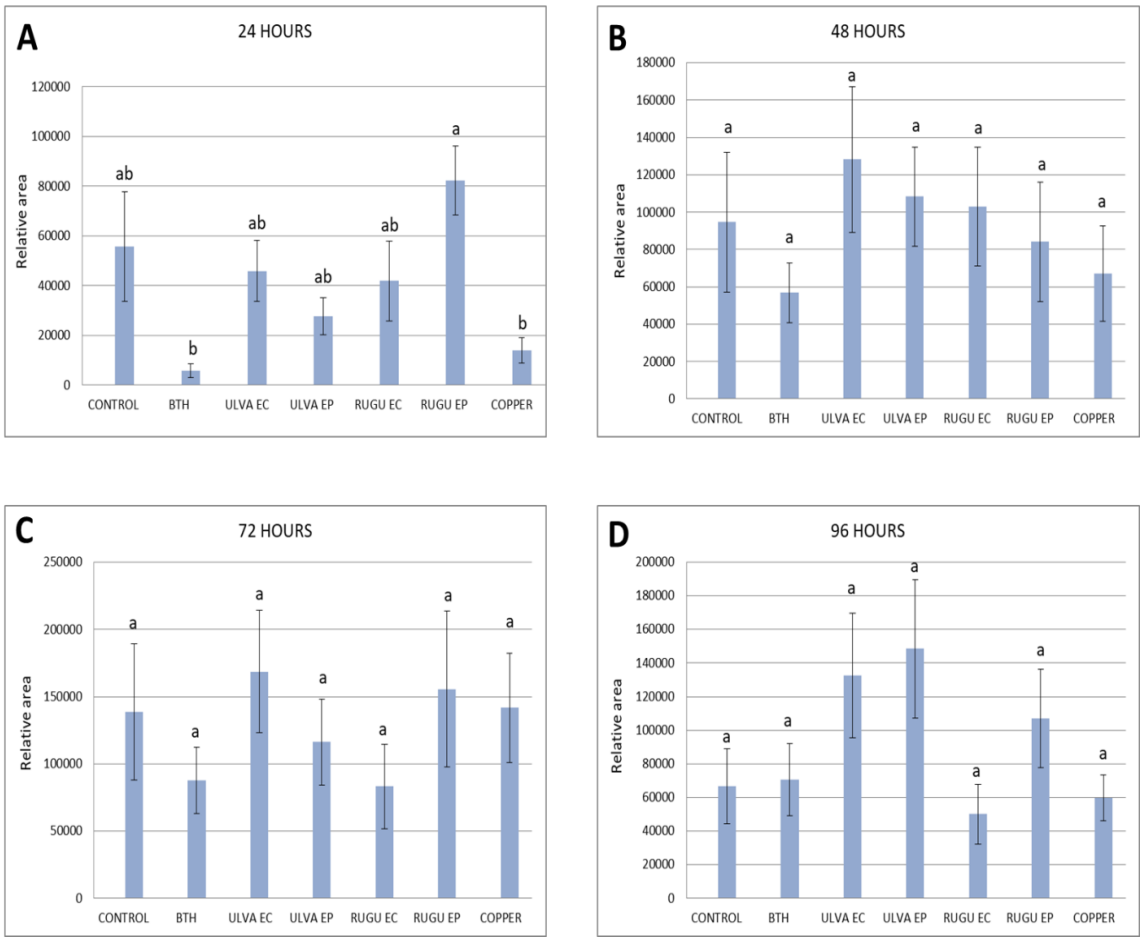


Figure 11. Relative sporulated area (pixels) seven days after the different applicacion time-points. Same time-point after inoculation with *P.viticola*, different seaweeds (Table 3) at unique concentrations (Table 8).

7. Curative assay: Same product at different times

Figure 12 represents effect of the same product (Table 2) at the four times of the application and after seven days.

In **Fig.12A**, there were not significant differences between the times at which the product was applied after the inoculation, but it was observed that at 48 and 96 hours the growth of the pathogen was totally inhibited.

Fig.12 B and **C** followed the same pattern, the sporulated area follows an upward trend and at the 96 hours application, it was seen that the infection was lower than the one of previous days. In the case of **Fig.12B** there were significant differences between 24 hours and 72. No significant differences were between the different times when product 3 is applied (**Fig.12 C**). In the case of product 4 (**Fig.12D**) the infection was the highest when the product was applied 48 hours post inoculation. Significant differences exist between 24 and 48 hours.

As it was observed in **Fig.12E**, the application of product 5 at the different times was totally effective as it absolutely inhibited the development of *P. viticola*.

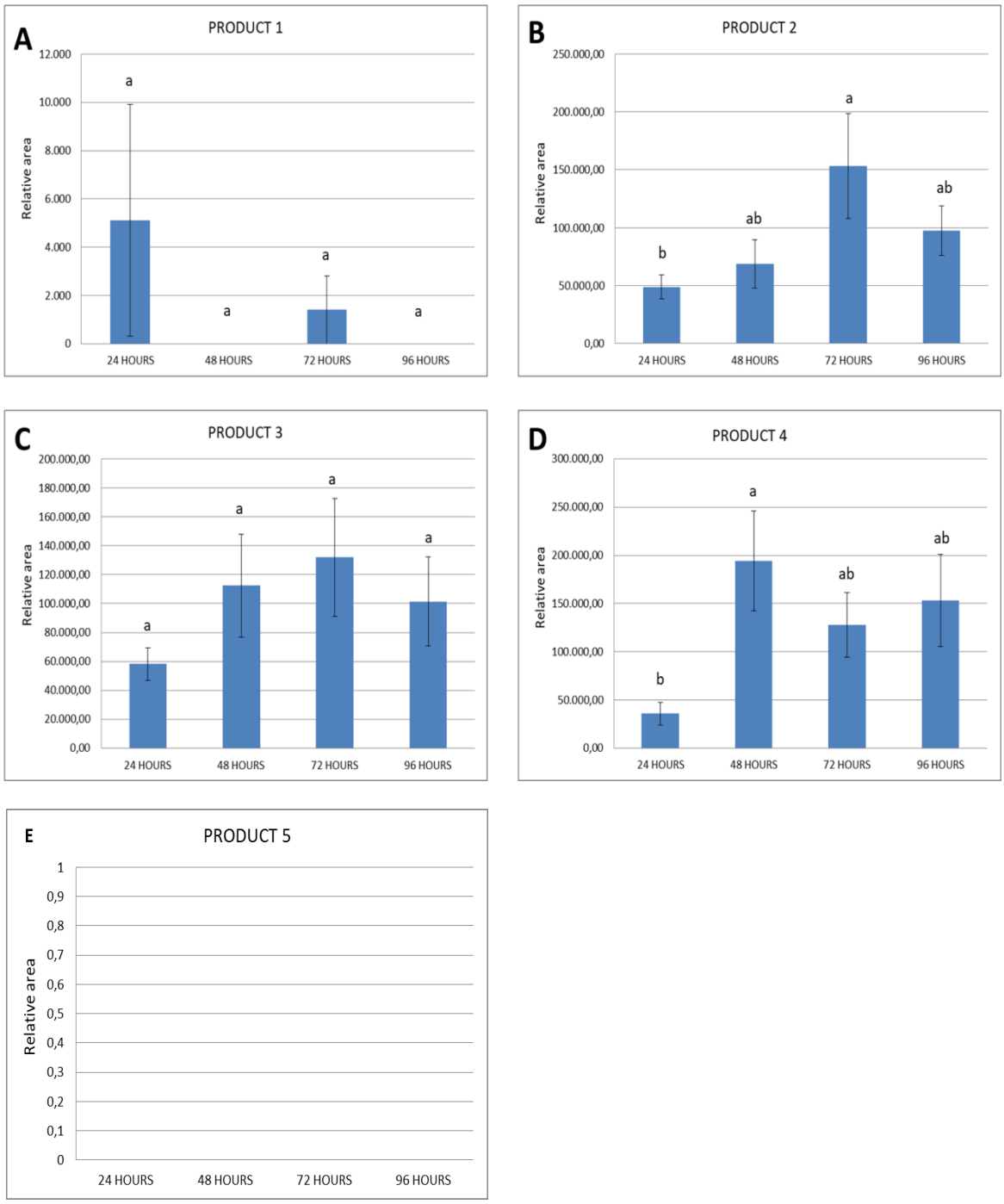


Figure 12. Relative sporulated area (pixels) seven days after the different applicacion time-points. Same product (Table 2) at different concentrations (Table 8) after inoculation with *P.viticola at different time-points.*

8. Curative assay: Same seaweed at different times

Figure 13 represents the effect of the same seaweed (Table 3) at the four time-points after the application and after seven days post application.

It was observed in **Fig.13 A, B, E** and **F** that they followed the same pattern, lower sporulated areas after seven days of being sprayed 24 hours post inoculation and an upward trend of infected area over the leaves at 48 and 72 hours. A decrease of the signs of *P. viticola* infection was visible when the application was done 96 hours after the inoculum was pulverized over the leaves. Significance differences were observed along the different times in the application of

BTH and copper, 24 hours and 72 hours post inoculation. No other significant differences pop up among these graphics.

In the case of Ulva EP, it followed and upward trend as time goes on and significance differences were distinguished at the first and last times of application. No significant differences were observed in the case of the pure and crude Rugu algae extracts.

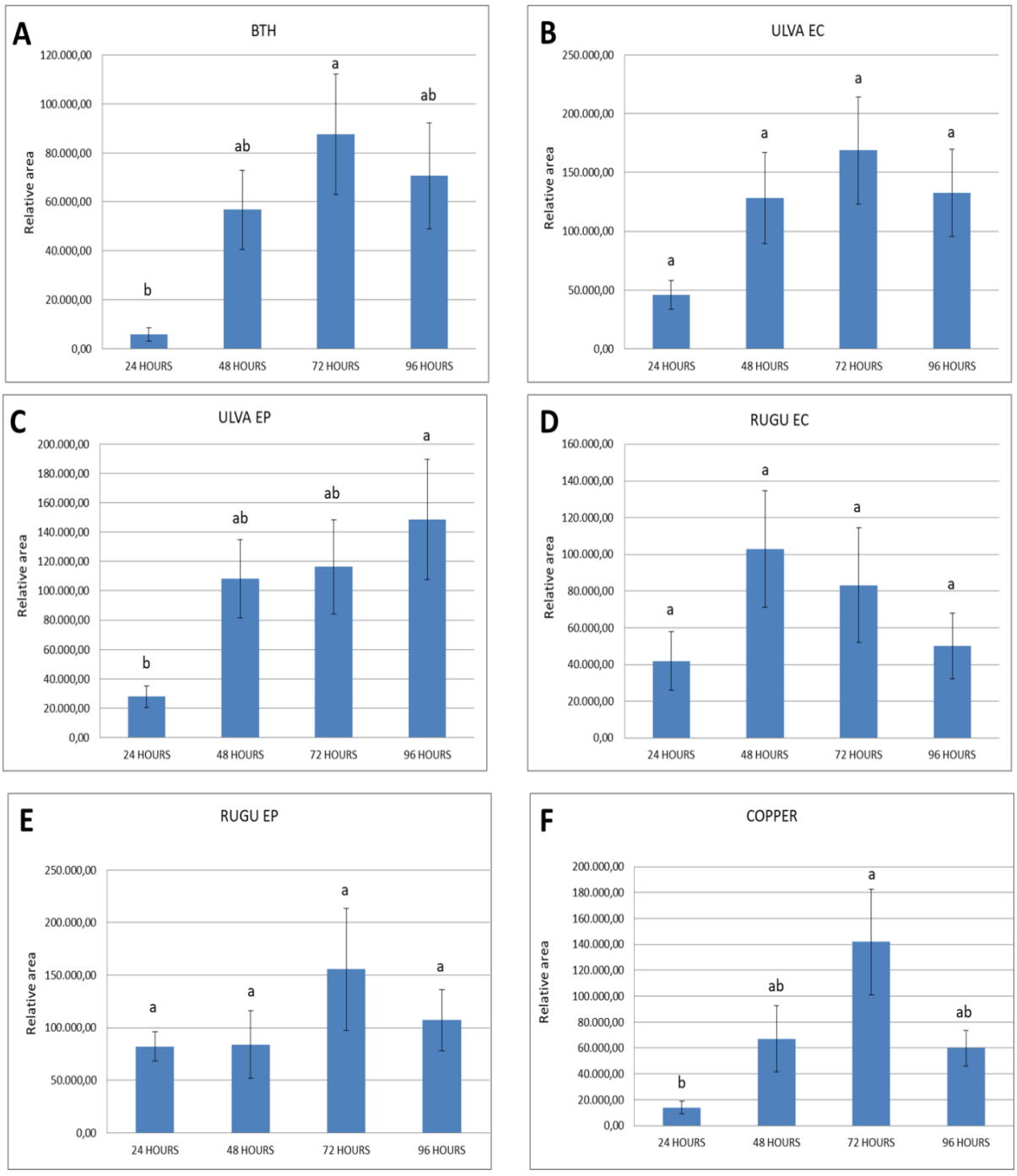


Figure 13. Relative sporulated area(pixels) seven days after the different applicacion time-points. Same seaweed (Table 3) at unique concentrations (Table 8) after inoculation with *P.viticola at different time-points.*

9. MDA quantification

Figure 14 represents the quantity of MDA produced by the different products (Table 2) at two, eight and 14 days post inoculation.

Significant differences were only observed in **Fig.14.A**, in which the control and products three and five were significantly different.

Graphics at **Fig.14A** and **C** followed the same pattern. The main difference between them was that 14 days after the inoculation the quantity of MDA was lower than two days after the inoculation.

The highest quantity of MDA in all the cases was obtained with product 5. Comparing the three graphs it was observed that the quantity of MDA from the different products 8DP was the highest.

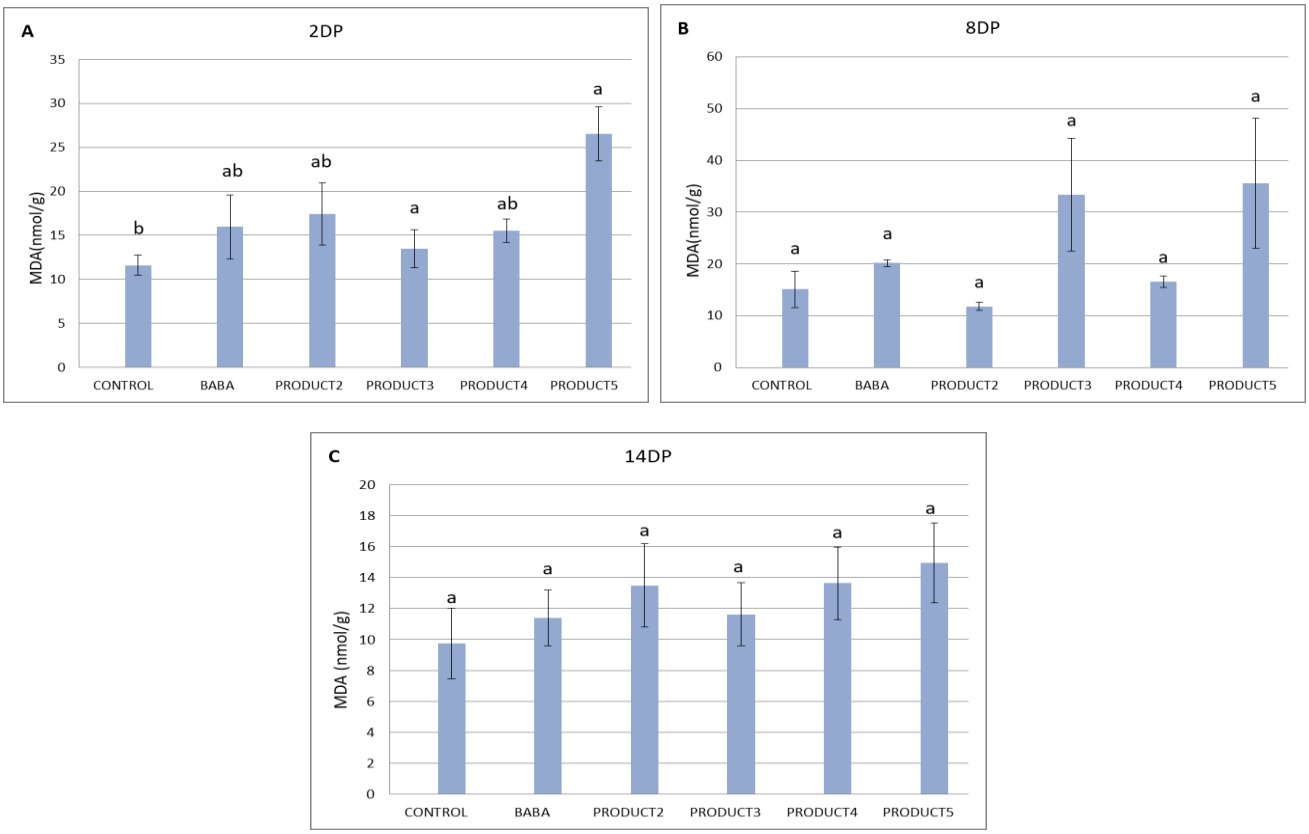


Figure 14. MDA quantity of the different products (Table 2) the same day post inoculation

Figure 15 shows the quantity of MDA of each product at the different times points.

It was observed that the highest MDA content came from product 5. However, the lowest, as it was expected, came from the control. In all the cases, except in the case of product 2 that is at 2DP, the highest quantity of malondialdehyde was achieved eight days post inoculation. The only obtained significant difference was with the application of product 1, between 8DP and 14DP.

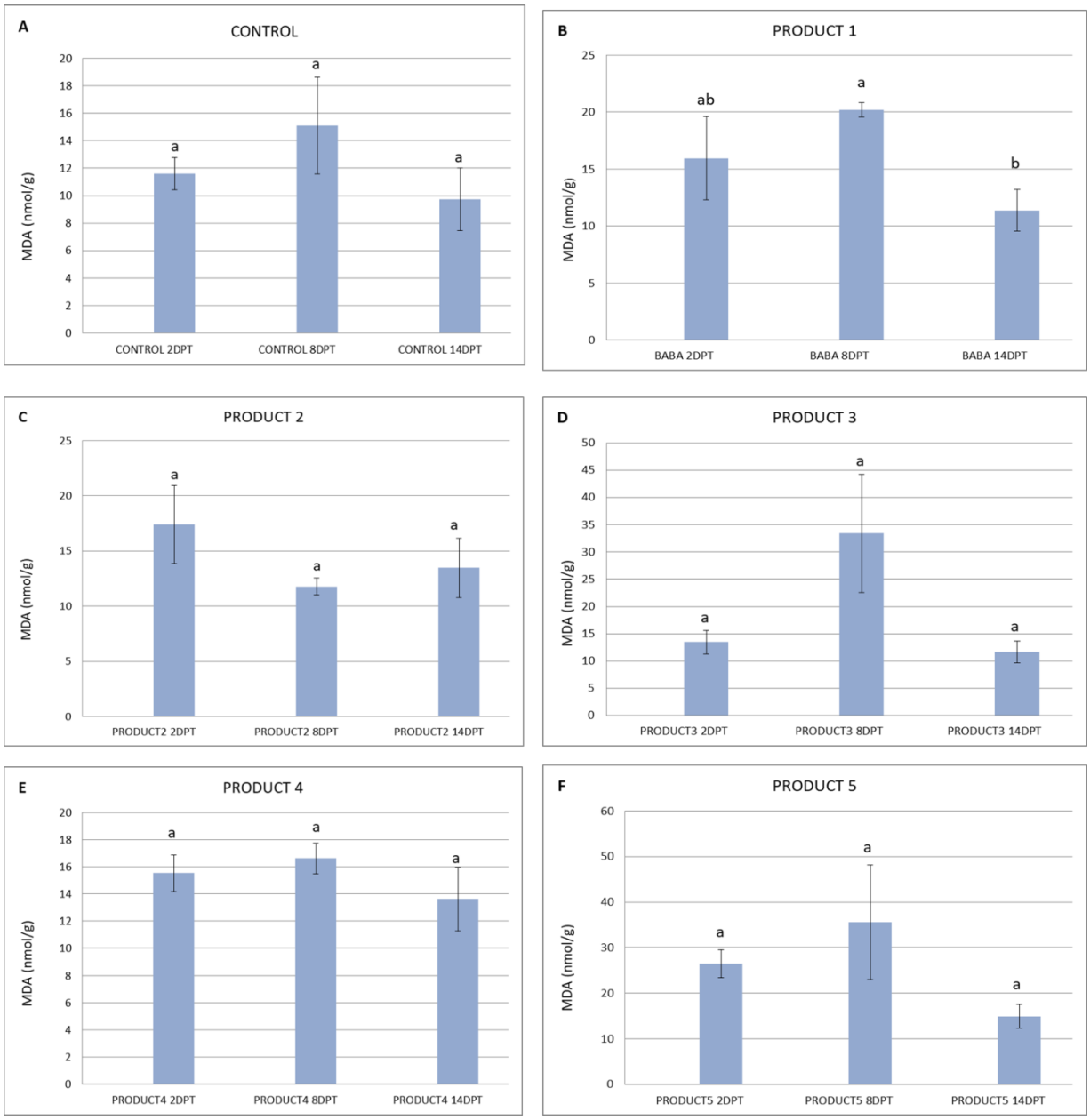


Figure 15. MDA quantity of each product (Table 2) at the different days after the inoculation with *P.viticola*

DISCUSSION

This study aimed to value the use of five different products and diverse seaweeds as means to protect grapevine from downy mildew, within the current biocontrol strategy context. Leaf disks sporulation assays, sporangia germination, and zoospore mobility measurements enabled me to reveal the toxicity and efficacy of these substances against *P. viticola*. The microscopic observation of the infected leaf tissues encouraged me to better understand the infection cycle of the pathogen and in this way be able to better discuss the effect the product can have over the pathogen.

Oomycetec *P. viticola* infection requires direct contact with the leaf surface, once the pathogen pour into the leaf tissue, zoospores need to invade the stomata, for the infection to begin (Verseci et al., 1999). After this, the mycelium arises and spreads within the intercellular tissue.

Sporangiophores emerge and they penetrate through the stomata, generating in this way a white downy mildew layer at the leaf surface. At the following step, sporangia are formed with the later release of zoospores (El Hadrami et al., 2010). The cycle can be repeated several times along the culture season.

In this study, four basic substances and seaweed extracts, with the capacity of stimulating the grapevine immune defence system, have been selected and tested to evaluate their direct toxicity against the pathogen, by measuring the sporulation and growth. All the treatments resulted in an evident level of protection with a significant reduction of *P. viticola* sporulation and disease index, suggesting a toxic effect.

BABA (Product 1) was not toxic towards *P. viticola* at low concentrations in leaf disk sporulation assays. However, in concentrations above 1 ml/l the sporulated area followed a gradual reduction and when concentrations were above 8 ml/l the pathogen was completely inhibited. Although the number of empty sporangia decreased in comparison with the control, zoospore mobility was not totally inhibited. That is because BABA has not a direct effect against zoospores (Harm et al., 2011), but it induces defence responses in plants.

The curative effect showed great inhibition results, being one of the most effective products among this study. When the application was done at 48 and 96 hours after the inoculation, it was able to completely inhibit the pathogen's growth, this is due to the immune stimulation effect BABA has. It is able of inhibiting the fungal expansion by the activation of jasmonic acid signal pathway, that increase the production of phytoalexins PR proteins and lignification (Harm et al., 2011). It can be concluded that BABA is highly efficient even when it was applied hours after the inoculation.

Film-forming substances, such as chitosan, have already been studied and its efficacy against pathogenic fungi and bacteria have been demonstrated in previous researches (El Hadrami et al., 2010; El Guilli et al., 2016). To test if **chitosan hydrochloride** (Product 2) acted to prevent *P. viticola* infection, it was put in contact with the pathogen at different concentrations and times after the inoculation. Leaf disks sporulation assays, in which the product was directly in contact with the inoculum, revealed that significance differences exist between the different concentrations application. Despite those variations, at concentrations above 2 ml/l, the maximal protection level was obtained, as evidenced by significant inhibition of the pathogens sporulation symptoms. That was directly related with the number of empty sporangia, a reduction in the number of sporangia and as consequence in the quantity of liberated zoospores and in their mobility, evidenced the efficacy of the product. This makes it not surprising that, from C4 concentrations on, no empty sporangia were identified on the samples. The effectiveness of chitosan is due to its ability to stimulate plant defence mechanisms, inducing plant resistance against the pathogen (Costantini et al., 2022). It promotes the accumulation of phytoalexins, PR proteins and reinforces the plant cell wall, generating physical barriers around the sites of a pathogen attack (Muñoz-Bonilla et al., 2013). Moreover, it interferes directly with the fungal growth, binding the fungi DNA (Muñoz-Bonilla et al., 2013), which may explain the toxic effect observed in this study.

However, when the curative assay was carried out, different results were obtained. The infected area increased, except at 96 hours, and was not eliminated at any of the times. The maximum was reached when the application of the product was done 72 hours after the inoculation. An answer to that can be the relationship between the activity of chitosan as biocide and its

physicochemical state (molecular structure, chemical functionality and physical aspect). The physical state of chitosan is a crucial factor that affects to its activity (Muñoz-Bonilla et al., 2013) and in this case the molecular weight and the degree of acetylation could not be the same at the different times. Apart from that, the substance is mainly employed for the control of bacteria, so the test carried out could have been affected by these two aspects. That is why chitosan hydrochloride is used as a powerful elicitor rather than a direct antimicrobial agent (Muñoz-Bonilla et al., 2013).

Another of the substances, whose toxicity was analysed, was the one that mixed **Soybean lecithin and *Equisetum arvense* L** (Product 3). The results of the evaluation of the IC50 showed, the effectiveness of the product when it was directly in contact with *P. viticola,* at different concentrations. No significant differences were observed from C2 on, meaning that the product is able to inhibit the growth at lower concentrations than the rest of the products. However, empty sporangia have been detected along the different concentrations except the highest one, which is an unexpected observation that should be further investigated. Regarding zoospore mobility, a low liberation of zoospores and, as consequence, low motility was observed, evidencing the toxicity.

The toxicity of Product 3 may be derived from its composition of *Equisetum arvense* L and lecithins. *E. arvense* is rich in silicon in the form of silicilic acid and the presence of silicon in plants alleviates many biotic and abiotic stresses (Curri et al., 2007) and triggers a broad range of natural defences, such as the reduction of the water excess of the plant to limit the growth of the pathogen (Costantini et al., 2022). The silica on leaves acts as a physical barrier to the penetration of *P. viticola.* In the case of lecithin, they are considered rich in bioactive compounds naturally produced in plants as secondary metabolites and they have an antifungal effect that stops or inhibits the development of the mycelia, reducing in this way the sporulation of the fungal pathogen (Castillo et al., 2012).

As in the case of chitosan, in the curative assay, the highest levels of sporulation among all the products appeared at 24 hours, the sporulated area increased at 48 and 72 hours and at 96 hours it decreased. This may be because when spraying 24 hours after the inoculation, as the pathogen cycle is in its early stages, the product could not get in contact with the pathogen itself, so it's able to continue growing. However, 96 hours after inoculation, the infection symptoms over the leaf surface are starting to be visible and the spraying of the product has a direct effect over pathogen, getting in touch with the sporangia and therefore drying them.

Soybean lecithin (Product 4) was also tested by its own. Leaf disks sporulation assays showed the presence of significant differences at low concentrations between the mixture of (Soybean lecithin + *Equisetum arvense*.L) and the treatment of soybean lecithin. This basic substance at low concentrations showed low inhibitory effect against the pathogen, following a downward trend as the concentration increases. Remarkably, it is the only product that was not able to inhibit the pathogen sporulation at C7. Checking the empty sporangia and the zoospore mobility, empty sporangia are present at the maximum concentration level, implying a high rate of zoospore mobility. It could be concluded that this product is not the most toxic against *P. viticola*, even though, lecithins have demonstrated antifungal activity. The highest toxicity of product 3 might be explained by the presence of *E. arvense* extract in the composition or by a different extraction method of the lecithins, as the products are from different companies. *E. arvense* could have an antigerminative activity against the pathogen, as previously demonstrated (La Torre et al., 2019), which would cause a smaller amount of zoospores to enter the tissue

reducing the final sporulation. Moreover, it has been demonstrated that *E. arvense* extract is rich in fatty acids, which could act as antifungal agents by entering fungal plasma membrane and destabilizing cell integrity (Langa-Lomba et al., 2021). Therefore, the *E. arvense* present in Product 4 could as well contribute in this manner against downy mildew.

Regarding the curative assay, Product 4 was not effective at decreasing the sporulation at any of the times tested. Its behaviour was similar to that observed with products 2 and 3, as not a single significant difference was observed with these products. The only differences were observed with product 1 (BABA) and 5 (SALIX) which have a clear inhibitory effect when applied in a curative mode.

Salix was also tested in this assay. It follows a decreasing trend as the product concentration increases. A significant difference exists between C1 and the rest of the concentrations, mimicking the behaviour of Product 3. Above concentrations of 4 ml/l, Salix provides the maximal protection level, as evidenced by the significant inhibition of *P .viticola* sporulation symptoms, being a very effective product from very low concentrations. This can be seen in the decrease of empty sporangia. However, an error occurred when counting the number of mobile zoospores per minute and the residues of the product got mixed up with zoospores, so the data related to product five present at Figure 9 is not correct. If the data had been collected in a correct way, the inhibition of zoospore release and their mobility would have probably been observed.

Salix extract composition has been previously analysed and high levels of polyphenols and flavonoids were detected. This could be an explanation for the toxic effect against the pathogen as an antigerminative effect, previously reported by (Andreu et al., 2018) was detected when used in greenhouse (Chovelon, 2006) or field (Dagostin et al., 2006). The Salix extract was also effective in reducing the pathogen disease incidence and severity.

The effectiveness of Salix can be clearly observed in the curative assay. The product has been able to achieve the maximal protection level at the four different application time-points. There is a short phase in the life cycle of the oomycete when it is very vulnerable between the hatching from the sporangium and the encystment of stomata (Krzyzaniak et al., 2018). Salix semms to prevent stomatal encystment. As a consequence, zoospores are unable to reach the stomata and therefore to develop and cause sporulation. It has been studied that Salix is able to supress downy mildew as copper, reference product against *P. viticola* (Dagostin et al., 2008).

One of the key processes in early plant defence signalling is enhanced lipid peroxidation. Lipid peroxidation products, such as MDA act as signalling molecules and regulate several transcription factors sensible to stress (Figueiredo et al., 2017). The production of lipid peroxides is induced by the pathogen, indicating disruption of cellular membrane and causing a loss of cellular integrity (Nascimiento et al., 2019).

The MDA content was measured at 2, 8 and 14 days after the applications of the products (1 to 5). The highest MDA concentration was observed at 8DPT for most of the products, except for product 2. This observation could mean that at 8 days after the product application the plant will be more protected against the pathogen that in the other two time points, as fatty acids would activate the defence mechanisms and induce the plants systemic acquired resistance (Nascimiento et al., 2019), reducing the mycelial growth. Significant differences among the quantity of MDA produced by the application of the different products were only observed at two days post inoculation, because the observed MDA quantity was similar between all the

products. Among all, Product 4 (SALIX) generates the highest increase in MDA, which could be related to the stimulation capacity of the product.

Seaweeds have also been tested along these bioassays. Few studies have been carried out about the toxicity of different algae against *P. viticola,* but some of their antifungal activities may guide their application towards crop protection. Copper and BTH were used as reference products in order to compare the efficacy of two different seaweeds; Ulva ohnoi and Rugulopterix okamurae extracts, in both crude and purified.

Copper (T7) is one of the most employed substance against the oomycete *P. viticola*, its effectiveness at the time of inhibiting the pathogen growth, has made it a key treatment when managing the disease. The results show, how in the evaluation of the IC50 of the products against the pathogen, the maximal level of protection was achieved with the application of copper, reinforcing in this way its power in fighting downy mildew. However, when the inoculum and the product were not directly in contact it was not so effective, as it did not inhibit *P. viticola* sporulation. In the case of BTH (T2), it was observed that when directly getting in contact with the pathogen, the development of the pathogen was restricted. BTH is a salicylic acid analog that strengthens plant defence mechanism. Its activity is related to induce resistance on plants. The accumulation of salicylic acid on the plant led to the accumulation of phytoalexins, PR proteins and to the reinforcement of the plants cell wall (Dufour et al., 2013). At the curative assay, it has been the most effective substance, due to the expression of all this secondary metabolites.

The seaweed Ulva ohnoi (T3,T4), in comparison with the control produced a lower sporulated relative area and it did had effect over the mobility of zoospores, completely reducing it. Purified ulvan extracts induces defence gene expression and plant defence responses via the jasmonic acid pathway (Jaulneau et al., 2011). This gives them the ability of modulating immune responses after the infection of the pathogen (Fernández-Díaz et al., 2017). Not good results were obtained in the curative assay. That may be because the quality and content of active compounds extracted from natural sources can vary during various time periods, as they can be easily degraded.

The other tested seaweed was Rugulopterix okamurae (T5,T6). In the developed assays it was observed that this seaweed is more effective that Ulva ohnoi. It succeeded in inhibiting at good percentage the sporulated area seven days after the inoculation, at levels similar to BTH. No empty sporangia were visible after its application, neither zoospore mobility. This is due to the development an antifouling defence mechanism that produces and secretes allelochemicals and halogenated terpenoids, capable of damaging the pathogen and therefore limiting its growth (Sahoo et al., 2015).

CONCLUSIONS

In this study, the efficacy of diverse basic substances and seaweeds against grape downy mildew was demonstrated by in vitro inoculation assays. All substances upon coming into direct contact with the pathogen were able to reduce sporulation levels, probably due to the presence antifungal components in their composition. The toxicity of BABA, Chitosan hydrochloride, Soybean lean lecithin combined with *E. arvense*, Salix, BTH and copper was successfully proved, as they successfully reduced sporulation levels. Soybean lecithin, Ulva ohnoi and Rugulopterix okamurae were the least toxic products and Salix was the most toxic.

Curatively, only BABA and Salix were effective as the rest of the products were not able to inhibit the sporulation. However, a stronger inhibition is observed for all the products at 24 after infection. This highlights that the pathogen is more sensitive to these products during the first 24 hours and that, later, this sensitivity disappears.

Although in vitro studies are a very valuable tool for screening effective products against *P. viticola*, they cannot completely illustrate the interaction between the pathogen and the plant. To deepen the knowledge, complementary genetic, metabolic and proteomic studies are always desirable. Further research is needed for the incorporation of these powerful substances into actual crop management strategies. In fact, every product should have a proven efficacy against the pathogen in the field, as the successful observations in the laboratory are not always maintained there.

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