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**CHEMICAL CONTROL OF THOUSAND CANKERS  
DISEASE (TCD): AN ENDOTHERAPIC APPROACH**

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*This work is dedicated to M. B.,*

*who as woman and friend, has walked with me.*



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## ABSTRACT

Borders are crossed every day by thousands of goods and phytosanitary treatments should prevent that unwished guests use these pathways as corridors to invade countries. Preventing measures may present some gaps and alien species may become a huge threat for ecosystems and human activities. In 2013, in Vicenza province, has been detected the first European record of the host-killing disease named Thousand Cankers Disease (TCD). It is caused by the conjunct action of the fungus *Geosmithia morbida* (Kolarík), and its vector *Pityophthorus juglandis* (Blackman). Hosts involved belong to *gen. Juglans* and minorly to *gen. Pterocarya*. The most threatened species is *J. nigra L.* External symptoms include premature yellowing and wilting of affected plants; under-bark necroses develop where the fungus infects the host. Plants usually die within 2-3 years since first symptoms were observed. At European level, the disease has been included in the EPPO's A2 list, but quarantine regime was not established yet. Locally, Veneto Region activated a 'Decree of mandatory control' to prevent further voluntary movements of the pathogen. The aim of this project is to assess a possible application of the endotheraphic approach against TCD. In this project, data were collected from two different black walnut plantations, and both field surveys and laboratory's experiments were conducted. Either commercial products or botanical pesticides were tested. On one hand statistical analysis led to no significant results among comparisons of natural active principles, and on the other one, commercial products showed a significant difference compared to water. A second conclusion was related with spatial distribution of injected solutions, as results suggested that number and positioning of ports may influence the effectiveness of endotheraphic treatments.





# 1. INTRODUCTION

## Foreword

The introductory chapter of this document is based on a former research performed by myself followed by the presentation of my Bachelor's dissertation: 'Il cancro rameale del noce: una nuova emergenza fitosanitaria' ([Esposito 2015](#)).

### 1.1 Biological globalization: the context

Widespread of species outside their natural range occurs since human began to trade, but the beginning of explorations since XV century resulted in a dramatic increase of this process ([Mooney and Cleland 2001](#)). Nowadays it is used to refer to these species as: alien species. A new terminology could well define the phenomena which is taking place worldwide, defined as biological globalization ([van der Weijden et al. 2007](#)). According to EU legislation n. 1143/2014 an alien species is either a living organism or a part of it able to reproduce and bring to a successful second generation. There is no distinction among plants, animals, fungi or bacteria, all of them may become an alien species if introduced in a new area. Human activities strongly influence species spreading. We may distinguish between two kinds of introduction according to voluntariness of the action. On one hand voluntary introduction is mainly related with commercial or productive purpose, on the other one, accidental introduction is due to failures of controlling systems ([van der Weijden et al. 2007](#)). Developing more efficient practises, diffused adoption of risk analysis and sharing information have key relevance to reduce potentially dangerous introductions ([Lodge et al. 2006](#)). It is important to highlight that not all the alien species have negative effects on the new environment. Those assuming an 'aggressive' behaviour are better defined as Invasive Alien Species. An alteration of evolutionary pathway both of invader and native has been proved to take place through different mechanisms (e.g. predation, competition; [Mooney and Cleland 2001](#)). These species spread uncontrollably and may threat survival capacity of local ones. IAS are the second cause of losing biodiversity ([Kettunen et al. 2008](#)), after loss of habitat. All over the world around 480 000 species are considered IAS and among all categories parasite fungi are particularly relevant ([Pimentel et all 2001](#)). A parasite is an organism which exploit a second organism, the host, to shelter or/and to nourish. Occasionally this symbiosis may lead to host's death, but generally parasites are an integrate and necessary component of an ecosystem. Damages occur when a parasite breaks the balance of an already stable system, that is the case of invasive alien

species. Seldom it is possible that native species benefit of the new partner (Reichard et al 2012), but long term dynamics are not easily predictable. Even if not many alien species turn into a serious pest, where ecological factors are favourable, some of them are causing significant economic damages and public health problems (Pimentel et al 2001). The issue is becoming every day more sensed, travels and commercial trades are heavily influencing voluntary or accidentally movement of species beyond geographical barriers (Kettunen et al. 2008). Not only the ecological aspect is threatened by these invasions, human well-being sustained by all ecosystem services (provisioning, regulating, cultural) is facing the issue as well (Pejchar and Mooney 2009).

This work is based on a recent invasion occurred in 2013, in which a fungus *Geosmithia morbida*, vectored by an alien insect *Pityophthorus juglandis* was detected for the first time in Europe in a private plantation. The fungus is the pathogenic agent of the Thousand Cankers Disease (TCD), a severe disease affecting Walnut, *gen. Juglans* (Montecchio and Faccoli 2014).

### **1.2 *Juglans* spp. and *Pterocarya* spp., the hosts**

Different level of susceptibility were found among all the species belonging to *gen. Juglans*, and the most susceptible one is *Juglans nigra*, (Black walnut) (Utley et al. 2013). Currently in Europe the threatened species are *J. nigra* and *J. regia* L. (English walnut) (Montecchio et al. 2014). Both the species are not indigenous in Italy, but if the English walnut was already cultivated since Roman age for nuts, its high-value wood and for aesthetic purpose (Ducci et al 2010), the black walnut has been only recently introduced. First European record of black walnut used as forestry species is dated in 19th century (Kremer et al. 2008). Nowadays in east Europe black walnut is becoming more important for high value timber in low lands (Nicolescu 1988, Kremer et al. 2008) or riparian forests (Salek and Hejcmanova 2011). Both the species benefited of the CEE regulation N. 2080/92, which financed new plantations of noble hardwood species. 170 000 ha have been planted since 1985 (Coletti et al. 2001) and 50% of this amount is represented by walnuts (Ducci et al 2010). In particular, mixed stands show high productivity in terms of quantity and quality of wood (Mohni et al. 2009). To assess possible development of the disease is important to deepen the knowledge of host's ecology.

Talking about Black walnut inside its natural range, located in the eastern part of USA, it is commonly found as individuals or small groups (Williams 1990). Good season lasts around

170 days, average temperature is around 13 °C and rain close to 900 mm/yr (Williams 1990). Black walnut grows well on deep, well-drained, almost neutral (pH 5-8), and moist soil (Brinkman 1965). Deep loams are probably the best soil-type, able to hold enough water also during dry season and water shortage (Williams 1990). Walnut flowers and leaves are displaying out in April-June and eventually it might face late-spring frosts (Funk 1979). Self-pollination may occur if neighbours' pollen lacks (Beineke 1974). The nuts fall after leaves, usually in October, and they are disseminated thanks mainly to gravity and animals; high production starts from 30 years up to 100 years, and mast year usually occurs every 2-3 years (Williams 1990). Seedlings come out in April-May, 1-2 years after planting (USDA 1974). Vegetative period is shorter in comparison with other species, in fact it averagely lasts 115-135 days (Brinkman 1965). Mature trees may grow 30-37 m in height and 76-102 cm in diameter at breast height (d.b.h.) if site condition are suitable (Landt and Phares 1973). The root system is a mixture between deep taproot and lateral, soil moisture and forest composition affect the final shape of roots' net (Williams 1990). Black walnut is a light demanding species (Baker 1948), especially during its first years, so to survive it must reach a dominant or codominant position (Williams 1990).

Most of the ecological requirements of *J. regia* are similar, but some important points have to be highlighted. English walnut needs at least 6 months above 10°C and 700-800 mm of rain well distributed (Becquey 1997). The natural growth presents a weaker apical dominance (Giannini and Mercurio 1997) and rooting is more developed laterally than in depth (Becquey 1997). Heights around 30 m and 2 m d.b.h are achievable according to site conditions (Gellini and Grossini 1997). Ducci et al. 2010 demonstrated that in Italy, as in Europe, there is a quite low genetic variability inside the species, likely due to the intense selection occurred during centuries to obtain high crops of nuts. This fact and the large presence of English walnut all over the territory might increase spread rate of the disease.

Even though gen. *Juglans* represents the main suitable host for developing of the disease, it is not the only susceptible genus, *Pterocarya* spp. (wingnut) in USA were found to be attackable as well (Hishinuma et al. 2016). Three species in particular were studied: *P. fraxinifolia* Lam., *P. stenoptera* C. DC. and *P. rhoifolia* (Siebold & Zucc). Very close to gen. *Juglans*, wingnut is used in USA mainly as shade tree (Hishinuma et al. 2016), but its native area is Asia, covering a corridor from Turkey to Japan. *P. fraxinifolia* or Caucasian wingnut is a relict species which grows naturally in the region between the Black Sea and the Caspian Sea; *P. stenoptera* or Chinese wingnut is spread all over the area whereas *P.*

*rhoifolia* or Japanese wingnut is mostly present in eastern China, Korea and Japan. These three species share similar ecological requirements: riparian forests, riverbanks and fresh mountain slopes with low gradient are their typical habitat. More generically, the wingnut is a light demanding genus and grows well in semi-deep and moist soil (Sheykhoulisami and Ahamadi 2009). Incidence of the disease affecting this genus is not recorded in Europe yet, but the fact that TCD might have other hosts than walnut trees should be taken in consideration when preventive measures have to be taken.

### 1.3 *Geosmithia morbida*, the pathogen

The primary causing agent of TCD is *Geosmithia morbida*, (Ascomycota: Hypocreales). Thought first official classification occurred in 2011 by Kolařík et al. 2011 through DNA sequencing and morphological comparison among several *Geosmithia* spp., Tisserat et al. 2009 proved that TCD is caused by the combined action of a fungus belonging to *Geosmithia* genus and its vector *Pityophthorus juglandis*. *Geosmithia* spp. are associated mainly with bark beetles (Coleoptera, Curculionidae, Scolytinae), but some species were identified as nourishing for species-specific ambrosia beetles. (Kolarik and Kirkendall 2010). Genetic analysis in USA proved that *Geosmithia morbida* evolved separately from other species by a long time, and it specifically established a relationship with at least one *Juglans* spp. and walnut twig beetle (Zerillo et al. 2014). Even if the growth substrate may slightly affect morphology, some common characters identify the species. Mycelium is hyaline and monilioid with yeast-like globose cells at the base; colony grows quite plane and low with lobate margins, and the surface is shared between sterile substrate and floccose-sporulating areas (Kolařík et al. 2011). No exudates were found, conidiogenesis is low-moderate and starts from the central and tougher part. Conidiophores are verrucose, penicillate; stipe 20–200 X 2.5–3 mm, penicillus 30–60 mm, terverticillate or quaterverticillate, symmetric or asymmetric, rami 15–35 X 2–3 mm, metulae (last branch) 9–11 X 2–2.5 mm, phialides 8–15 X 2–2.5 mm, 3–6 per cluster; conidia narrowly cylindrical to ellipsoid 4.5–6 X 2 mm forming chains long up to 200 µm (Kolařík et al. 2011). Although the optimum range is between 28-30 °C, the thermotolerance of the species is quite distinctive, as it is able to growth at higher temperature (Kolařík et al. 2011). Evidences of low resistance to high differences in temperatures (day/night changes) were highlighted by Freeland et al. 2012 at least on *Juglans nigra*.

Before 2013, *Geosmithia* genus was already diffused in Europe and Mediterranean countries, but different host range and geographical distance excluded almost all contacts

among vectors (Kolařík et al. 2007). It was the first plant pathogen inside the genus (Kolařík et al. 2011). Recently *Geosmithia langdonii* (Pitt) and others *Geosmithia spp.*, were likely involved in Elm dieback, similar to Dutch Elm Disease and recently recorded in Switzerland affecting a young tree of *Ulmus minor* L. (Hänzi et al. 2016). In the case of *G. morbida*, although the walnut twig beetle is proved to be the main vector, other insects were found positive to *G. morbida*. The weevil *Stenomimus pallidus* (Boheman) (Juzwik et al. 2015) or ambrosia beetles *Xylosandrus crassiusculus* (Motschulsky) and *Xyleborinus saxeseni* (Ratzeburg) could carry spores from an infected tree to a healthy one (Juzwik et al. 2016). Despite it is well documented that *G. morbida* is the causal agent of TCD, strains differ in virulence degree (Sitz et al. 2016). The differences are also demonstrated by the fact that some cankers are not able to merge because of strain's incompatibility due to the fact that anastomosis does not occur (Montecchio et al. 2016). The cause may be related with a viral infection which reduces strain's virulence and modifies mycelia's morphology (Montecchio et al. 2016), rather than genetical reasons or interaction with other pathogens as supposed by Sitz et al. 2016. The spotted presence of different haplotypes inside the same population is an evidence of past and multiple spreading of the fungus from several sources, reasonably through infected wood movements (Zerillo et al. 2014). Among phytosanitary measures tested to prevent a further widespread of the disease, kiln-drying is significant more effective than steam-heating and methyl bromide-fumigating against post-treatments colonization (Audley et al. 2016). Because of the great economic impact of the disease, *G. morbida* is one of the few forest pathogens which was sequenced to study possible ways to reduce its pathogenicity (Schuelke et al. 2016), and further studies will explore this possibility.

#### **1.4 *Pityophthorus juglandis*, the vector**

*Pityophthorus juglandis* (Coleoptera, Curculionidae, Scolytinae), also known as Walnut Twig Beetle (WTB), is a small phloem feeding bark beetle associated with *G. morbida* because with its compulsive way to feed it inoculates the pathogens through the galleries it creates (Kolařík et al. 2011). In 2013 Utley et al. described the species' morphological characters to identify it in trapping activity. WTB is long 1.5-2 mm, the ratio between length and width is more or less 3, therefore the body results quite narrow. The skin colour oscillates among brown and red shades. Females have on their frons golden hairs shaped as a round brush, the length is usually smaller the half the distance between eyes. Male also has a hairy frons, but it is reduced to small line above mandibles and they are almost sparse.

Observing pronotum frontally we can see that it is sloped upward it and reaches the top before the midpoint; it is also characterized by four to six concentric ridges, they may be discontinuous and overlap among themselves. Teeth are visible at the end of pronotum. Forewings, called elytra, are covered by punctures and short setae, the apex is rounded and the end is featured by a shallow and shiny declivity. Male can be discriminated by female because its declivity presents small granules on the first and third interstitial spaces.

*Pityophthorus juglandis* is particular because it is the only one inside the genus who feeds on walnuts. First detection of the species occurred in 1928 in North America, in New Mexico. Its range sprawled across Arizona, New Mexico and Chihuahua desert. The overlapping with *J. major*'s range led to suppose that it is the first and natural host for the species (Tisserat et al. 2009).

Even if it prefers warm climate and its native area was the Southwestern side of USA, it is currently diffuse in many countries of the East coast. Recently it found for the first time a new good substrate for living, and it invaded the natural range of Black walnut (Grant et al. 2011). The gap in temperature seems not to be an issue for the insect, which is able to survive at low temperatures (Luna et al. 2013). Winter season is spent inside galleries formerly bored in the stem or under the bark, most of the flights occur in April, but some adults forward their galleries (Newton et al. 2009). WTB is polygamous, males reach the host and release a pheromone to attract females, which land later; after breeding females dig horizontal galleries and lay eggs; larvae will move perpendicularly to maternal gallery for feeding (Seybold et al. 2010). Individuals get adults in 7 weeks, thus a second generation may occur. Adults were observed until October. Preferred site for colonizing are the bottom part of young branches with South exposition (Newton and Flower 2009). Small dimension of the insect limits the size of attackable branches, the minimum diameter where holes are recorded is 2 cm, but presence on bigger branches and on the stem is common (Tisserat et al. 2009).

### **1.5 Symptoms and epidemiology**

Tisserat et al. in 2009 described accurately the symptoms related with TCD. First of all, it should be taken in account that visible cues of the disease appear at advanced stages, after some years from the infection, though it is possible that an outbreak of WTBs speeds up the process and symptoms become evident earlier. Mortality is strictly correlated with two variables: host resistance and beetle's number (Raffa and Berryman 1983). The large number of infected holes would rapidly bring the plant to dieback status and it can be

quickly identified (Tisserat et al. 2009). Host resistance is also connected with *Juglans spp.* (Utley et al. 2013). During first phases wilting and yellowing occur, and after brown leaves start to appear on the crown. Digging activities for breeding introduce the fungus, which starts developing on phloem tissues. Even the inner part of the bark could be attacked. Affected tissues turn towards dark brown, cambium is attacked later and discolouring occurs also there. Only debarking allows to observe the necrotic area by outside, and this under bark stains usually are circular or elliptical with the major axis parallel to the sap flow. Cracks may be seen close to entering holes of the insect as well as a smooth hunch on the surface. Cankers still develop after *P. juglandis* flight away, as they often coalesce. Tree dieback and death are direct consequences. Basically, cankers girdle twigs and branches, stroking water flow and killing the above part. Flag-like leaves are clear evidences, missed production of abscisic acid due to the early season does not allow leaves falling (Annex 7.1.A). Cankers move downward, living tissues of larger branches are infected and since the first symptoms death occur within 3-4 years (Tisserat et al. 2009).

As previously described, the original range of the vector was the South West area of USA, where natural hosts were very likely *J. californica* S. Wats, *J. major* Torr. and *J. hindsii* Jeps.. Wide spreading seems to be strictly associated with movement of untreated wood, as firewood, sold outside country boundaries (Jacobi et al. 2012). Urban forestry and forests close to camping site has been demonstrated to be more susceptible to colonization pressure, because of human activities and their consequent link with entering pathway (Wiggins et al. 2014). From the first detection in Colorado in 2016 many other States recorded TCD's presence inside their territories: California, Oregon, Washington, Idaho, Utah, Nevada, Arizona, New Mexico, Tennessee, Pennsylvania, Virginia, North Carolina, Maryland, Ohio, Indiana (USDA and APHIS 2016). Moreover, in Oregon there was the first case of *J. cinerea* L., Butternut, affected by the disease. This species is already threatened by the Butternut canker, *Ophiognomonia clavignenti-juglandacearum* (Broders & Boland), and the impact on local economy might be quite strong (Serdani et al. 2013). For what regards Italy, very likely the first inoculum was introduced through untreated logs imported from USA (Montecchio et al. 2014). The dry nature of *G. morbida* spores induces also to suppose a natural spreading of the infection, but ongoing researches are assessing if these spores are really able to infect a healthy tree without vector's aid.

## 1.6 Legal framework and control measures

The broad issue of IAS has an international resonance, thus to reduce the impact the legal framework has to be based on international agreements. International Plant Protection Convention, IPPC, hosted by FAO, covers this role. IPPC Commission on Phytosanitary Measure has the assignment to set a pull of Standards able to reduce risks for every kind of plant materials. Countries may voluntarily sign the agreement, but then they are legally bound to comply its requirements. In August 2016, 37 International Standard on Phytosanitary Measures (IPSM) were approved. The heavy impact that ISPMs could have on international trades require the endorsement of WTO to be implement. Topics covered by ISPMs have a wide spectrum: guidelines for Pest Risk Analysis, regulation on wood-made packaging, guidelines for eradication programs, Integrated Risk Management fundamentals, guidelines for surveying and monitoring activities, phytosanitary certificates, etc. (IPPC 2016). Actually these measures are enhanced and monitored by national agencies, which are bundled in regional district, and for Europe this regional organization is EPPO, European and Mediterranean Plant Protection Organization. Currently 51 countries are members of this regional organization (EPPO 2016). Basing on IPPC Standards, EPPO developed its own standards to protect countries against harmful species. To simplify controlling operations, it published three lists containing organisms potentially dangerous, List A1, List A2 and an Alert list. List A1 and A2 concern about pests which should be regulated as quarantine species, the first one considers species not present inside EPPO region, and the second includes species already found inside one or more member countries (EPPO 2016). The alert list is a warning list where some pests not yet present but potentially dangerous can be found. The list is a useful tool, affected areas are defined and inspections on risky products may be intensified. Before being added to either A1 or A2, pests have to be assessed by an experts working group, which evaluate the risks of a potential spreading of the species inside the region (EPPO 2016).

The technical procedure is called Pest Risk Assessment, PRA. After this process, a pest may become an A1 or A2 organism, but the final and legal decision about the establishment of quarantine regime on it remains a European Commission right. PRA takes in account several factors: the organism itself and its monitoring and surveying system, pest's biology and current diffusion status, host's ecology and susceptibility, likelihood of establishment of the pest's inside the region, control methods, entering pathway and economic impact. In September 2015, after a Pest Risk Analysis either *Geosmithia morbida* or *Pityophthorus*



*juglandis* were included in EPPO's A2 list. In Italy the authority which physically act on territory are the Regional Phytosanitary Services. The general statement about invasive species is to prevent, if it fails eradication in early stages may be a solution, and just in case of successful settlement containing measures are applied (Veneto Region 2016). As a member of EU, Italy subscribed the 2000/29/CE Directive, which defines duties that every signer has to respect concerning plants protection against harmful organisms. Not only plants but even plants' part or products are included. Constant monitoring and regular inspecting are the targeted to reduce this risk. A phytosanitary certificate ensures plants' safety and a passport allow moving among EU's countries.

About practical control measures in the field, works are progressing. In the USA a federal regulation still not exist, but some States have implemented an exterior quarantine to prevent sales or movement of infected wood from hit areas, whole State or at county level. States are: Indiana, Kansas, Michigan, Missouri, North Carolina, Nebraska, Oklahoma, Tennessee, and Wisconsin (USDA-APHIS 2016). Moreover, logs to be trade have to be treated. ISPM N. 15 states that the product has to be heated for at least 30 min. at 56°C, and temperature measured in the core. Mayfield et al. 2014 proved that 40 min at 56°C (1 cm beneath the cambium) are enough to kill all insects. Yet, they suggested to apply the same procedure used for another insect, the Emerald Ash Borer (*Agrilus planipennis* (Fairmaire)), 60°C core temperature for 60 min. In any case, the treatment does not ensure that future colonization will not occur (Audley et al. 2016). In Italy, in 2014 Veneto Region established a local 'Decree of mandatory control' (Annex 7.1.B). Movements of walnut plants which exceed in 10 mm of diameter are forbidden outside the infested area. Debarked wood and squared up to first xylem rings can be sell as well as wood kiln-dried at least 45 min at 60°C 1 cm below cambium. Phytosanitary services have also to survey nurseries, which have to keep an updated register of purchasing (Veneto Region 2016).

### **1.7 Endotherapy**

Among the most diffuse and effective approaches for controlling pests in the field, pesticides are on top ranking. Yet, even if they are a useful tool for fighting this threat, their abuse, especially from 70's of last century, has led to several collateral effects. Insect's resistance mechanisms are considerably increasing (Whalon et al. 2008). Further, among the lethal effects on target species, sublethal consequences were also recorded on many beneficial arthropods (Desneux et al 2007). For instance, behavioural changes in honeybees were found by Thompson 2002. The negative effects chain does not stop to herbivores,

as detrimental effects of pesticides may reach higher levels of food pyramid through accumulation of active principles during long time lapses. Poisoning of raptors, vulture for example, demonstrates that even the top individuals of the food pyramid are facing this issue (Hernandez and Margalida 2008).

Nevertheless, polluting of ground water and human health are not negligible. In this scenario, in 2009 European Parliament approved an important directive about the sustainable use of pesticides, the DIRECTIVE 2009/128/EC. Quoting the first article: " *This Directive establishes a framework to achieve a sustainable use of pesticides by reducing the risks and impacts of pesticide use on human health and the environment and promoting the use of integrated pest management and of alternative approaches or techniques such as non-chemical alternatives to pesticides.*" The Article 9 is quite relevant to introduce the experiments carried out in this work. Aerial spraying of pesticides is forbidden due to drift effect which would damage the environment and human health. Few exceptions are allowed under strict controls, but in forest environment this means that almost all treatments are prohibited, and alternative approaches such as Integrated Pest Management have to be considered. When using chemicals is not avoidable, a less impacting method to perform the treatment, rather than spraying, is endotherapy: injecting of active principles directly inside the system. This technique applied to trees has a long story, first descriptions of tree injection were written by Leonardo da Vinci (Perry et al 1991), but most of the later studies were mainly addressed to understand sap flow and fluids dynamics inside the stem. Rumbold in 1920 found evidences of injecting trees for curative purpose in some articles of a Russian scientist, Ivan Shevyrev, who carried out several experiments at the end of XIX century. Nowadays this technique is fairly common, in particular in urban context where risks for health are higher, e.g: Dutch Elm Disease (*Ophiostoma novo-ulmii*) (Webber 1981), *Cameraria ohridella* on the horse chestnut tree (*Aesculus hippocastanum* L.) (Ferracini and Alma 2008), hawthorn lace bug (*Corythucha cydoniae*) on hawthorn trees (*Crataegus viridis*) (Gill et al. 1999) and others. Not less important is its implementation against diseases of high value crops: on one hand biotic as Avocado tree (Darvas et al 1984), Chestnut (Gentile 2009) both affected by a *Phytophthora* spp. and on the other hand abiotic as Olive, Peach tree (Fernandez-Escobar 1993), Apple tree (Barney et al. 1984) to overcome chlorosis due to iron deficiency. Although trunk injection reduces, quite reasonably, environmental and health risks and shows in many cases good results, it interacts physically with plant system creating a wound. Plants are

unable to heal, actually they answer to external and internal damages through a compartmentalization process (Shigo 1972). Basically, healthy tissues create a barrier to isolate the damaged part and to preclude any access to harmful organisms causing wood decay and discolouring (Shigo et al. 1977). A well done and carefully planned injection may decrease this impact: drilling a high number of holes or enlarging the same one reduce plant's mechanical resistance (Ferry and Gomez 2014). Thus, having the right tool and a skilled operator is essential for this practise. For this research I used a new tool for tree injecting filed by University of Padova: Blade for Infusion in TrEes (BITE) designed by Montecchio et al. (2012). Differently from previous tools, drilling is not necessary, a blade is inserted into the stem using a simple beating mechanism. The blade stretches sapwood fibres and the products may directly circulate throughout the transport's system without any physical loss of wood that could be caused by the instrument. Moreover, the blade's design generates a Venturi effect which improves the efficiency of transferring (Montecchio 2013). Plants recover occurs quite rapidly and compartmentalization becomes quicker due to the smaller amount of tissues damaged during the injection. In conclusion, BITE results to be either environmentally safer and "tree-friendly".

### **1.8 Plant-based active principles**

About the products that may be injected, now I will focus on pesticides, specifically those extracted from plants. Botanical pesticides might be an alternative option to synthetic pesticides to move towards a more safety pest management, both environmentally and for humans' health. Regulatory barriers and new products more competitive are reducing market chances, few cases of botanicals are already well affirmed, like pyrethrum and neem extract (El-Wakeil 2013).

This is absolutely not related with their efficacy. Among plant chemical compounds, essential oils and specifically, monoterpenes are an interesting class that is obtaining many positive results. Some of them have a strong impact on fungi's enzymes. Essential oils have moreover an important antibacterial activity for pathogens affecting food (Burt 2004). The inhibitory action against pectin methyl esterase (PME), cellulase and polyphenol oxidase (PPO) of Thymol and (S-) Limonene was proved by Marei et al. 2012. An interesting result for endotherapeutic approach was obtained by Dal Maso et al. (2014) using also allicin, an active principle extracted from garlic. She demonstrated that it is as effective as the Thiabendazole (synthetic product) against Ash Dieback Disease (*Hymenoscyphus fraxineus*). Both didn't arrest completely the growth but slowed down cankers

development' significantly. Another product, which actually is not a botanical, that showed good results against Chestnut Ink Diseases is phosphonates (potassium phosphite, PP) (Dal Maso and Montecchio 2015). Phosphonates increase plant's defending mechanism, but formulation and concentration are as important as the active principle to avoid phytotoxic effects. (Dal Maso and Montecchio 2015). Micronutrients used as synergizing enhance plant's absorption. In general terms, essential oils demonstrated to have an actual impact on diseases, but their formulation and use have to be deepened and enlarged.

### **1.9 Objective**

The aim of this work is to assess either formulates' distribution along the translocation system or their action against Thousand Cankers Disease. Both natural active principles and classic phytosanitary products were tested. Preventive treatments were performed through endotherapeutic technique to evaluate if canker's growth may be stopped or contained. Either field experiments or laboratory tests were considered to test their efficacy.

## 2. MATERIALS AND METHODS

### Foreword

In the following experiments, selected formulations were injected inside the tree's system and lately we intervened inoculating the pathogen both *in planta* and *in vitro*. I will keep distinct site A and site B except for the paragraph about statistical analysis. For both the sites I will first describe the endotherapeutic phase and secondly the sampling and inoculating procedures that were adopted.

### 2.1 Sites description

Field work was carried out in two different sites both located in Vicenza province during the period May 2015-October 2015, apart injections in site A, which occurred in September 2014.

**Site A:** 19-years-old plantation (*J. nigra* mixed with *Paulonia tomentosa* (Thunb.)) for high quality timber located N 45°38'57.2" and E 11°39'02.5", 55-58 m a.s.l., in a private tenure in the municipality of Bressanvido. This site is where the first European record of TCD was detected in 2013 ([Montecchio and Faccoli 2014](#)).

**Site B:** 9-years-old walnut plantation (*J. nigra*, *J. regia* and the hybrid *J. x intermedia*) located N 45°39'37.6" and E 11°32'14.3", 84 m a.s.l. in the municipality of Montecchio Precalcino (loc. Levà) 8 km far from Bressanvido. This site is a property of Veneto Agricoltura.

From the climatic point of view, data released by ARPAV and collected with the local meteorological station of Montecchio Precalcino for the period 1994-2016 are taken as reference. The average annual amount of precipitation is 1285.8 mm, rains are distributed according to the typical equinoctial regime, which is characterized by two picks, the first in April-May and the second delayed in November. Average annual temperature oscillates around 13.4 °C, the minimum usually occurs in January (ave -0.8° C) and the maximum in July (ave 29.8 °C). Air moisture is quite relevant all over the year, in average 75%.

## 2.2 Trial in Site A

### 2.2.1 Endotherapy

This part of the project had exploited a running experiment, in which I was partially involved. 16 black walnuts (17-years-old) had been selected, with diameter's range from 19 to 49 cm (average 29 cm) at breast height (d.b.h.). Due to local restrictions, the 16 trees had been split in two comparable groups (Peterson et al. 2009), 10 to be treated with a formulate named Mix7 (Annex 7.2.A) and 6 to be injected with water as control. As dosage to inject 1 ml/cm of circ. had been chosen, in average 100 ml per tree. Researchers drilled 4-5 cm into the xylem using a cordless 1300 rpm drill (Makita 6271DWPE, IT). In this way they created a 0.5 cm-diameter port. With a hammer the port was sealed using a pipette tip (200 µL, Ref. 18170, Socorex Isba S.A., CH). Each port was oriented towards East direction. The pipe of a drip bag containing the solution was immediately inserted into the tip. Air inside the bag had been previously removed.

### 2.2.2 Inoculation *in planta*

In order to evaluate the distribution of the injected solution, each plant was inoculated in several points differentiated by height and orientation. The standard approach was to select 4 points: 0 m and 1 m of height; 0° and 180° from the injection port. For practical reasons, the 0 m point was actually located 15-20 cm above the injection point. Because plant's treated with water were not enough, four of them were inoculated also 90° left and 90° right from the port.

The strain used was GM4 monoconidial *Geosmithia morbida* Schio, available in TeSAF herbarium. The fungus was grown for one week at  $28 \pm 1^\circ\text{C}$  in the dark in a 94 mm-diam. Petri dish where a layer of 10 mL PDA (Potato Dextrose Agar; Difco Laboratories; Detroit, MI, USA) was laid as growing media (Annex 7.2.B; Kolarik et al. 2011). For the inoculation, the following protocol was adopted:

- i. Wear protective gloves
- ii. Flame sterilize 1 cm-diameter cork borer and use it to prepare pathogen's plugs. Preserve Petri dish from contaminations closing them immediately.
- iii. Sterilize again the tool and bore the stem until the external rings of sapwood and extract the core.

- iv. Sterilize a lancet and carefully place a plug from the Petri dish in the hole with the mycelium outwards.
- v. Using the core as stopper, close the hole.
- vi. Fix a nail 10 cm from the inoculum to easily detect the hole after a long time.

One extra tree was treated as the previous ones, but only pure PDA was used; specifically, 4 cores for each height were performed.

### **2.2.3 Detection *in planta***

Data collection occurred 100 days after the last inoculation. A sharp knife was flame sterilized before any record and the area around the core was debarked removing thin layers of bark. Width and height of the necroses (Gordon et al. 1998), that were developed, were measured using a ruler. Furthermore, to work out detailer measures later (Krokene and Solheim 1999), a picture of the necrosis was taken, using the ruler as referring dimension. Pictures were processed with an image manipulation software, GIMP 2.8 (The GIMP team, GIMP 2.8.14, [www.gimp.org](http://www.gimp.org), 1995-2014) and the borders of each necrosis were carefully highlighted by means of a graphic tablet (BambooFun CHT-661, Wacom Europe GmbH, Krefeld, DE). Using another software, ImageJ (Rasband, W.S., ImageJ 1.5b, <https://imagej.nih.gov/ij/>, 1997-2016), the area of the necrosis was extrapolated.

### **2.2.4 Branches sampling and *in vitro* experiment**

A second collaboration aimed at assessing the active principle's spatial distribution in the canopy (Aćimović et al. 2014). Wood samples were collected from two trees for treatment at the height of about 12 m using an articulated lift; in particular, three branches were cut for each cardinal direction and kept isolated in plastic bags. In laboratory four necroses were debarked and identified following a path from the crotch to the tip of every branch. Branches' samples were maintained in a humid chamber at  $24 \pm 1^\circ\text{C}$  in the dark for two weeks and mycelium development was checked every two days. Detection was based on features described by Kolarik et al. (2011) about *G. morbida* morphology.

## **2.3 Trial in Site B**

### **2.3.1 Endotherapy**

In site B, although it was an even age plantation, there were differences among trees in terms of size. Therefore, circumference at breast height of 70 asymptomatic black walnuts was measured and a label with an identifying number was pinned on the bark. Replicates were ranked according to circumference and they were subdivided into 7 homogeneous groups of 10 elements. Apart of 10 plants, which were used as control and hence were not treated, the other 6 groups were treated with different solutions: Water, Potassium phosphite, Carvon, Citral, Thymol and Thiabendazole, in accordance with the concentrations reported in annex 7.2.A. Drip bags were used for the injections. The volume of solution injected was calculated as follow: 1 ml/cm circ. + 15-20 ml. The extra amount was taken into account due to potential loss during the injections. To inject the formulates, a hand-held drill-free tool called BITE, namely, Blade for Infusion in Trees (PAN/ De Rebus Plantarum, Padova, IT), was used. The employed version was made up of three main elements: head, body and sliding hammer. The head has a perforated blade which has to be plugged into the sap system by means of the sliding hammer and a latex gasket to ensure adhesion to the bark; the body includes the tool's arm which terminates with a conical opening where syringe or pipe has to be inserted (Montecchio 2012). For each tree, injections occurred in two opposite points above the collar, 10-20 cm from the ground. A detailer protocol and picture are reported in annexes 7.2.C 7.2.D.

Each plant took between 1-2 days to absorb all the solution. Only few individuals treated with water needed more time, likely because weather conditions in that days were not so favorable. The overall process lasted 1 month (From May 25th, 2015, to June 26th, 2015).

### **2.3.2 Inoculation *in planta***

The strain used was GM4 monoconidial *Geosmithia morbida* Schio, as for the inoculation in site A. The pathogen was grown for one week at  $28 \pm 1^\circ\text{C}$  in the dark onto two different media, Potato Dextrose Agar (PDA) and Water Agar (WA) as a weaker media (Annex 7.2.B). This differentiation was done supposing that colonies which developed in limiting condition could show a more aggressive behavior (Ibrahim et al. 2002).

Inoculations started progressively after one month from each injection. They occurred along the z axis, placed in the middle of the two injection points. In particular, *G. morbida* grown on WA and on PDA was inoculated 1 m and 1.4 m above the injection height,



respectively. Further, the two plugs were displaced 2-3 centimeters towards opposite directions from z axis. The protocol applied to inoculate followed the same steps of site A. The stem was wrapped with transparent film and borders were closed with the tape to create a sort of humid chamber.

### **2.3.3 Detection *in planta***

The survey of necroses' development on the trunk followed the same protocol applied in site A.

### **2.3.4 Branch sampling and *in vitro* experiment**

At the same time of trunk inoculations, samples of branches were collected for *in vitro* tests. Using a telescopic pruner, four branches (A, B, C, D) were cut for each tree. A cross scheme was followed to choose the first four branches, A-B above the injection's points whereas C-D perpendicular to them. Branches were resized to pieces 20 cm long and with a diameter of 2.5 cm. Sticks were stored in plastic bags and immediately brought in the laboratory for the inoculations. After a previous cleaning under running water, the next operations occurred in a biohazard vertical laminar flow hood to prevent contamination by and to external environment. Branches were sterilized dipping them into oxygenated water 35 % v/v for 10 minutes. They were washed with demineralized water to stop the drying process and posed vertically to remove external moisture. An adapted version of the elm inoculation protocol of Webber and Hedger (1986) was employed. A 0.5 cm-diameter borer was flame sterilized and plugs of the same pathogen's strain, GM4 *G. morbida* Schio grown for one week at  $28 \pm 1^\circ\text{C}$  in the dark, were prepared. The tool was flame sterilized a second time and used to incise bark and phloem. The external layer was removed by means of a lancet. A plug was carefully positioned in the hole with the mycelium outwards and protected with the wood material previously removed. The area was sealed with parafilm (Parafilm M; Pechiney Plastic Packaging, Chicago, IL, USA) as well as the stick's edges. The whole sample was wrapped with transparent film. The samples were maintained 90 days at  $28 \pm 1^\circ\text{C}$  in the dark (Kolarik et al. 2011). Elapsed this time, the inoculum's area was debarked and samples were resized to be able to put them into a 94 mm-diam. Petri dish on moist tissue paper. After 10 days, pathogen's presence above the debarked area was assessed. Detection is based on features described by Kolarik et al. (2011) about *G. morbida* morphology. Mycelium and conidia developed from 50% of positive samples were plated on PDA with 0.25 % w/v of streptomycin for further verifications.

## **2.4 Statistical analysis**

For statistical analysis three software were used: Minitab (Minitab Inc, State College, PA, USA), Microsoft Excel (Microsoft Corporation, Redmond, WA, USA), R cran (R Core Team, 2016).

### **2.4.1 *In planta***

Analysis performed either in site A or site B considered data corrected subtracting the core dimension. Further the square root of the corrected area was calculated to get normally distributed data. Moreover, an index was designed as  $\ln \frac{Height(cm)}{Width(cm)}$  to assess possible differences of growth's dynamic. Before testing possible differences among categories, analysis of residuals, normality test and Levene's test for variances ( $p < 0.01$ ) were run. If these assumptions were satisfied, T test or ANOVA test ( $p < 0.05$ ) were performed, otherwise Kruskal-Wallis test ( $p < 0.05$ ) was chosen for analysis.

In site A data was compared according to: treatments, inoculation's height and orientation.

In site B data were compared according to: treatments, growing media and diameter's class. Trees were subdivided in three equipotent categories of circumference: 0-49 cm; 50-59 cm; >60cm.

### **2.4.2 *In vitro***

In both cases a preliminary table with absolute frequency of presence and absence of *G. morbida* was prepared and Fisher's test used to evaluate differences among all the categories. If p-value showed a significant evidence ( $p < 0.05$ ) to accept the alternative hypothesis, we tested singularly each couple applying the chi square post hoc test.

### 3. RESULTS

#### 3.1 Results Site A

##### 3.1.1 *In planta* experiment

The average size of necroses' area comparing water and mix7 was respectively 2.29 cm<sup>2</sup> (SD = 0.65) and 2.08 cm<sup>2</sup> (SD= 0.61). Analysis of variance showed no significant difference between trees which had been treated with these two solutions (p = 0.144; Figure 3.1.1.1). Comparing lesions' areas at 0 m and 1 m within the same treatment, the test resulted in no significant value as well. In particular, p values were 0.053 and 0.124 for water and mix7 respectively (Table 3.1.1.1).

On the other side, comparing this measure among different orientations (0° - 90° - 180°), one significant statistical difference (p = 0.039; Figure 3.1.1.2) was observed among areas of mix7 at 0 m height. At 0° the average area was 1.6 cm<sup>2</sup> (SD = 0.53), whereas at 180° the average was 2.26 cm<sup>2</sup> (SD = 0.77).

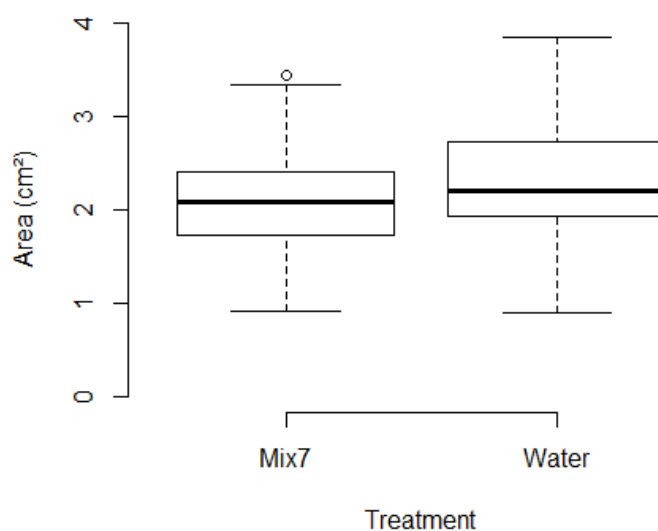


Figure 3.1.1.1 Difference in necroses' area between treatments

	Water		Mix7	
	0 m	1 m	0 m	1 m
Mean (cm <sup>2</sup> )	2.08	2.48	1.93	2.23
SD (cm <sup>2</sup> )	0.74	0.49	0.73	0.44
p-value	0.053		0.124	

Table 3.1.1.1 Comparative analysis for necroses' area according to factor "height", for water and mix7 treatments separately

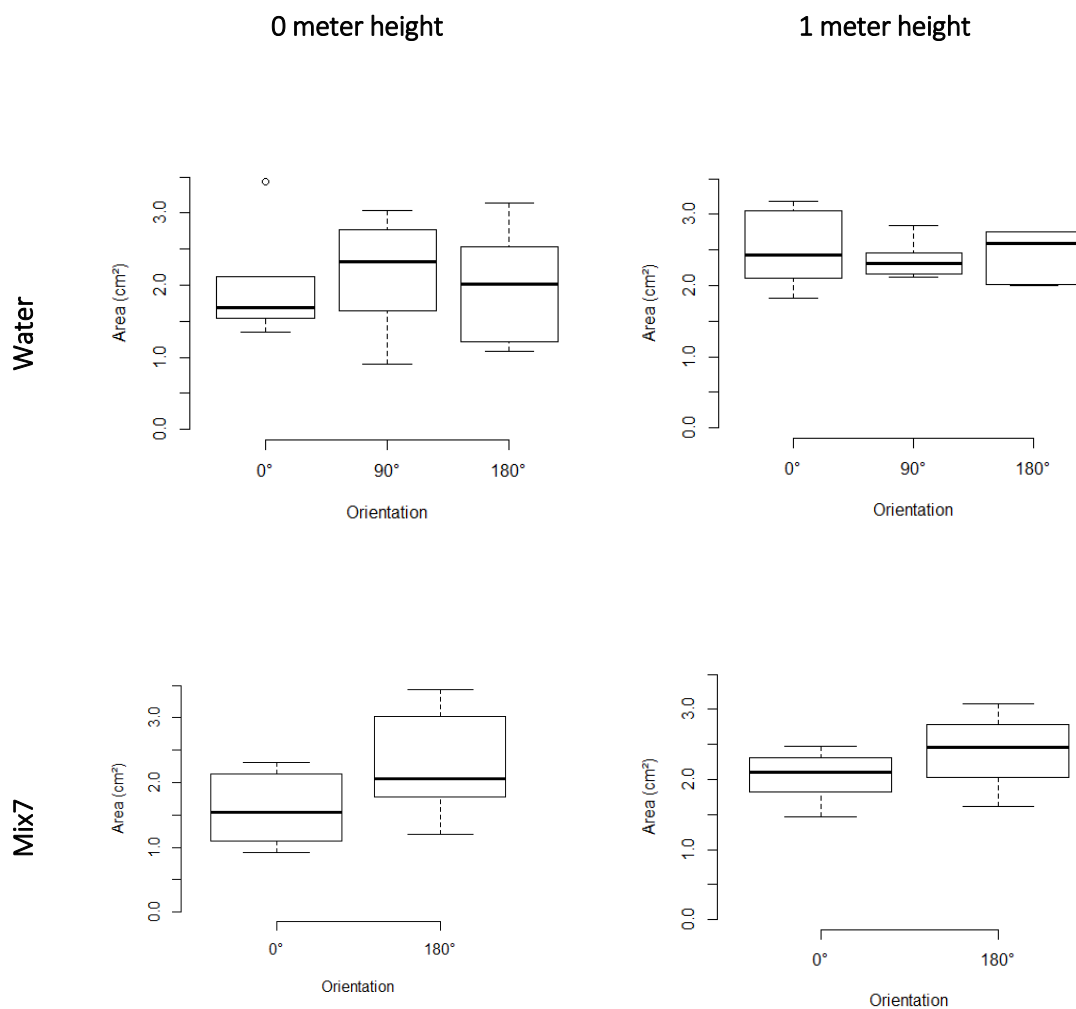


Figure 3.1.1.2 Differences in necroses's area according to orientation, for each combination of treatment and height

Considering the index  $\ln \frac{H}{W}$ , statistical analysis did not indicate any significant difference for all the comparisons. Table 3.1.1.2 resumes the values obtained from these comparisons.

	Water			Mix								
Mean	1.36			1.40								
SD	0.44			0.59								
p-value	0.733											
	0 m		1 m		0 m		1 m					
Mean	1.26		1.45		1.24		1.55					
SD	0.53		0.30		0.72		0.37					
p-value	0.167				0.099							
	0 °	90 °	180 °	0 °	90 °	180 °	0 °	90 °	180 °			
Mean	1.19	1.29	1.27	1.47	1.37	1.54	1.15	1.34	1.45	1.65		
SD	0.26	0.42	0.85	0.19	0.29	0.42	0.97	0.37	0.43	0.27		
p-value	0.959			0.571			0.576			0.246		

Table 3.1.1.2 Analysis of variance for the index  $\ln H/W$  according to treatments, injection's height and orientation from the port

### 3.1.2 *In vitro* experiment

Figures 3.1.2.1-5 show results obtained after the assessment of *G. morbida* presence/absence test. Fisher's Exact Test demonstrated that a significant difference exists between trees treated with water and trees treated with mix7 ( $p < 0.001$ ). A second significant result came from the comparison among cardinal directions within trees treated with mix7 ( $p = 0.034$ ). Yet, the following post-hoc test appeared to confute this result, adjusted p-value according false discovery rate method didn't confirm the first evidence ( $p=0.109$ ). There are no other relevant results from this test subdividing data according to orientation or position.

## Geosmithia morbida Presence/Absence test

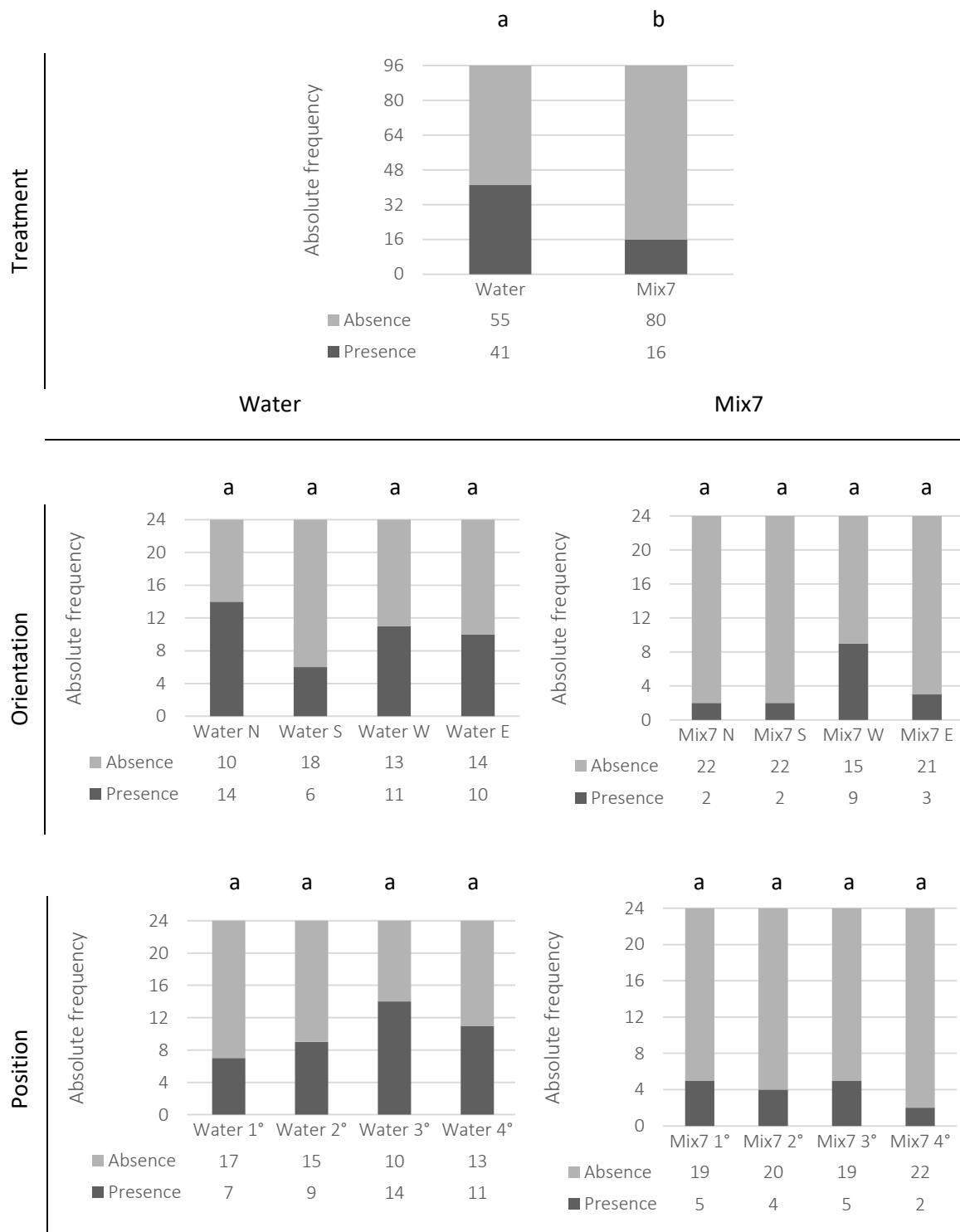


Figure 3.1.2.1-5 *G. morbida*'s viability after a period of 300 days from the injection, according to treatment and each combination of orientation and position

## 3.2 Results Site B

### 3.2.1 *In planta* experiment

The analysis of variance among the six treatments, either for lesion's area or for the index, showed no significant level of difference both where the inoculation occurred with a PDA's plug and with a WA's plug. Table 3.2.1.1 and Table 3.2.1.2 resume results obtained from these tests.

<b>PDA</b>	<b>Water</b>	<b>Carvone</b>	<b>Citral</b>	<b>Potassium phosphite</b>	<b>Tecto 20 S</b>	<b>Thymol</b>
Mean (cm <sup>2</sup> )	2.00	2.77	2.57	2.88	2.20	2.35
SD (cm <sup>2</sup> )	0.87	1.18	1.01	0.94	0.98	0.73
p-value	0.302					

<b>WA</b>	<b>Water</b>	<b>Carvone</b>	<b>Citral</b>	<b>Potassium phosphite</b>	<b>Tecto 20 S</b>	<b>Thymol</b>
Mean (cm <sup>2</sup> )	3.00	2.93	2.54	3.15	2.80	3.24
SD (cm <sup>2</sup> )	0.73	1.00	1.07	0.63	0.580	1.21
p-value	0.569					

Table 3.2.1.1 Comparison of the lesion's area among treatments

<b>PDA</b>	<b>Water</b>	<b>Carvone</b>	<b>Citral</b>	<b>Potassium phosphite</b>	<b>Tecto 20 S</b>	<b>Thymol</b>
Mean	2.39	2.71	2.39	2.82	2.80	3.10
SD	0.48	0.83	0.67	0.64	1.16	0.56
p-value	0.323					

<b>WA</b>	<b>Water</b>	<b>Carvone</b>	<b>Citral</b>	<b>Potassium phosphite</b>	<b>Tecto 20 S</b>	<b>Thymol</b>
Mean	2.51	2.66	2.70	2.82	2.80	2.78
SD	0.42	0.66	0.85	0.89	0.87	0.80
p-value	0.949					

Table 3.2.1.2 Comparison of the index  $\ln H/W$  among treatments

Considering results of T-test between the two growing media on necroses' areas, within each treatment there was only one significant difference in case of water (PDA: Ave = 2.00, SD = 0.87; WA: 3.00, SD = 0.73; Table 3.2.1.3; Figure 3.2.1.1). On the other side, statistical analysis did not indicate any significant statistical difference for the index  $\ln H/W$  (Table 3.2.1.4).

	Water		Carvone		Citral		Potassium phosphite		Tecto 20 S		Thymol	
	PDA	WA	PDA	WA	PDA	WA	PDA	WA	PDA	WA	PDA	WA
Mean (cm <sup>2</sup> )	2.00	3.00	2.77	2.93	2.57	2.54	2.88	3.15	2.20	2.80	2.35	3.24
SD (cm <sup>2</sup> )	0.87	0.73	1.18	1.00	1.01	1.07	0.94	0.63	0.98	0.58	0.73	1.21
p-value	0.012		0.746		0.956		0.471		0.116		0.062	

Table 3.2.1.3 Comparison of the necroses' area between growing media for each treatment

	Water		Carvone		Citral		Potassium phosphite		Tecto 20 S		Thymol	
	PDA	WA	PDA	WA	PDA	WA	PDA	WA	PDA	WA	PDA	WA
Mean	2.39	2.51	2.71	2.66	2.39	2.70	2.82	2.82	2.80	2.80	3.10	2.78
SD	0.48	0.42	0.83	0.66	0.67	0.85	0.64	0.89	1.16	0.87	0.56	0.80
p-value	0.576		0.892		0.379		0.991		0.992		0.329	

Table 3.2.1.4 Comparison of the index  $\ln H/W$  between growing media for each treatment



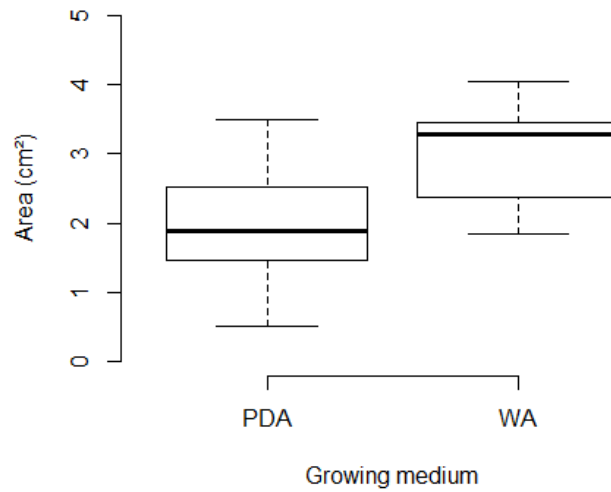


Figure 3.2.1.1 Difference in area's size according to growing media, among trees treated with water

Comparing diameter's classes effect for each growing medium, Kruskal - Wallis test was adopted because not all the categories were normally distributed. The test did not highlight any difference among categories, either for necroses' area or for index  $\ln H/W$  (Table 3.2.1.5-6).

<b>PDA</b>	<b>Class 1</b>	<b>Class 2</b>	<b>Class 3</b>
Mean (cm <sup>2</sup> )	2.51	2.60	2.26
SD (cm <sup>2</sup> )	0.91	1.08	0.95
p-value	0.576		
<b>WA</b>	<b>Class 1</b>	<b>Class 2</b>	<b>Class 3</b>
Mean (cm <sup>2</sup> )	2.81	2.79	3.16
SD (cm <sup>2</sup> )	0.82	0.86	1.02
p-value	0.663		

Table 3.2.1.6 Comparisons of necroses' area among diameter's classes

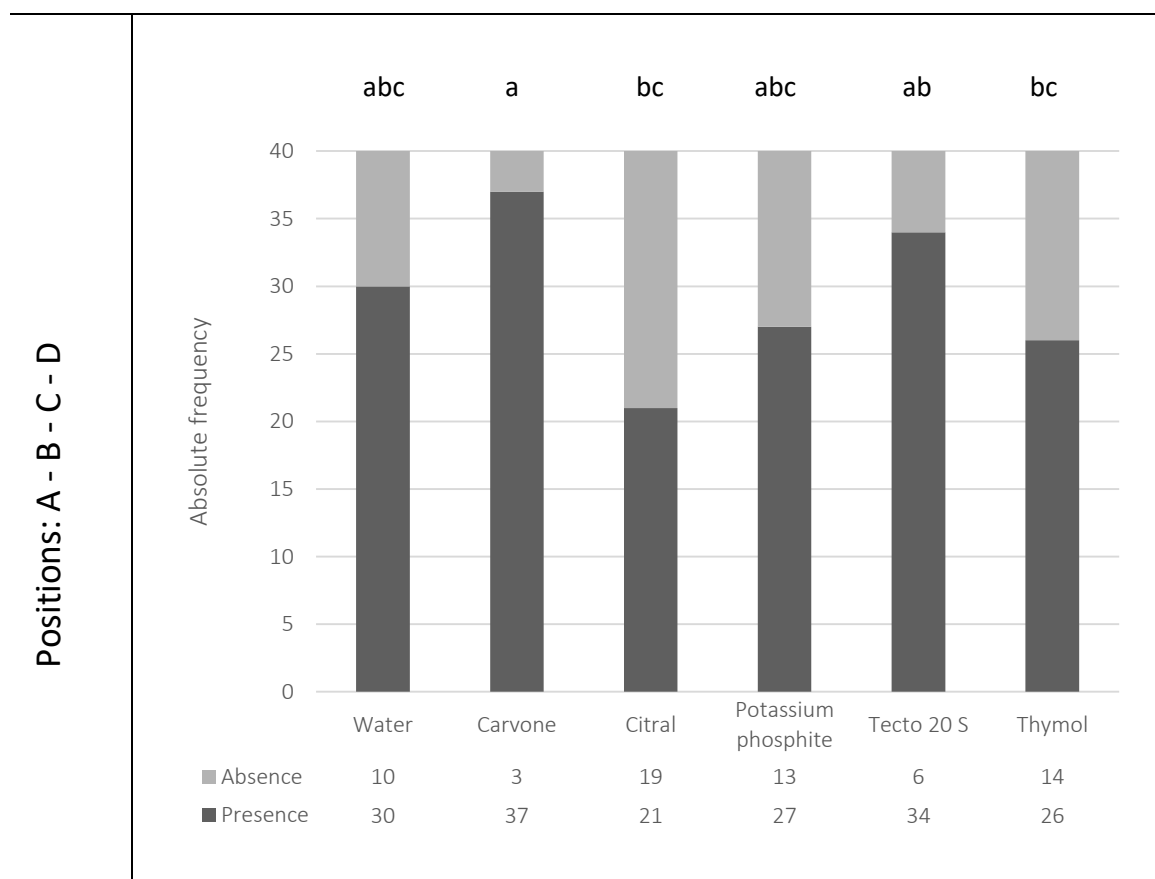
<b>PDA</b>	<b>Class 1</b>	<b>Class 2</b>	<b>Class 3</b>
Mean	2.67	2.90	2.63
SD	0.79	0.68	0.84
p-value	0.516		
<b>WA</b>	<b>Class 1</b>	<b>Class 2</b>	<b>Class 3</b>
Mean	2.81	2.85	2.44
SD	0.66	0.92	0.58
p-value	0.189		

Table 3.2.1.5 Comparisons of the index  $\ln H/W$  among diameter's classes

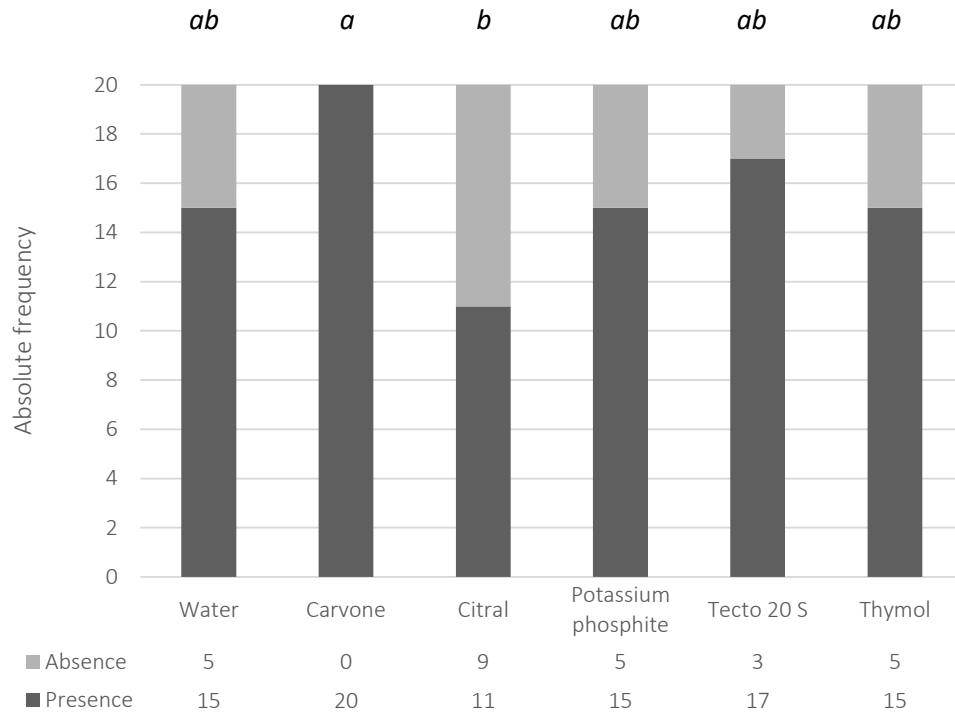
### 3.2.2 *In vitro* experiment

Figures from 3.2.1.1 to 3.2.1.3 show results obtained after assessment of *G. morbida* presence/absence test. Fisher's Exact Test on treatments, applied considering all the positions together (A – B – C – D), demonstrated that there were significant differences among trees treated with tested solutions ( $p < 0.001$ ). Despite of this result, post hoc tests did not confirm that any treatment was more effective in comparison with water. Running the Fisher's Exact Test to compare the final percentages of *G. morbida*'s presence among treatments considering only A and B positions, a significant value ( $p = 0.015$ ) was obtained, yet, the post hoc test didn't show any significant value comparing treatments and water. A p-value not significant ( $p = 0.052$ ) was obtained applying the same test considering only C and D positions.

*Geosmithia morbida* Presence/Absence test



Positions: A - B



Positions: C - D

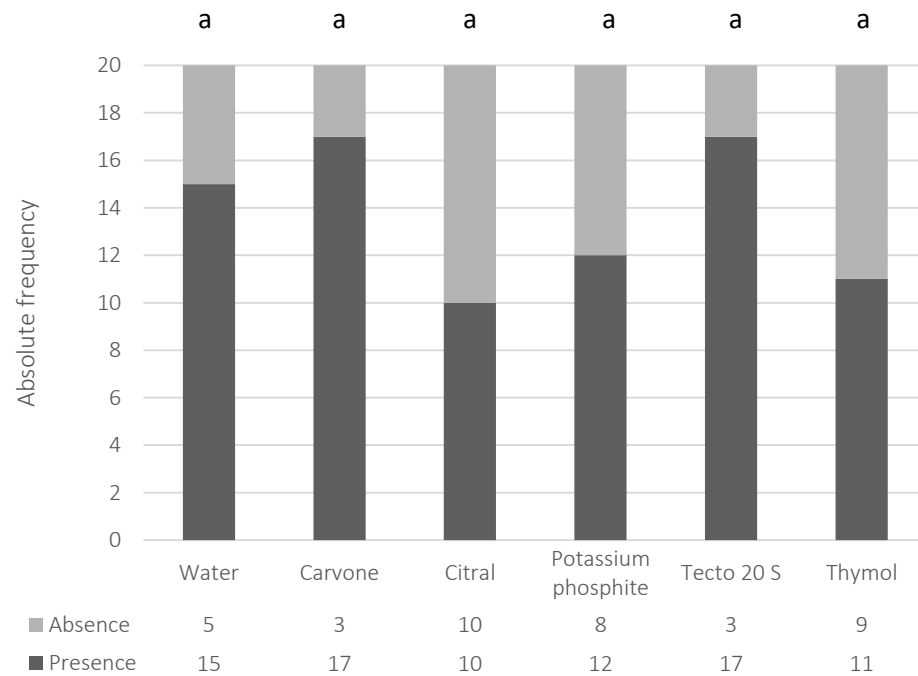


Figure 3.2.2.1-3 *G. morbida*'s viability after a period of 100 days from the injection

#### 4. DISCUSSION

The use of endotherapy to heal trees, specifically in urban context, and its efficacy has been documented since 1920 (Rumbold). Yet, an extensive application of this technique was reported during the 80's, when Dutch Elm Disease threatened many countries (Webber 1981). Tools to inject chemicals evolved during last decades to prevent losses and collateral damages to trees. With this aim, BITE tool was designed (Montecchio 2013). Recently, the interest for botanical pesticides was rediscovered. These substances and their interaction with plant's system are currently studied to reduce as much as possible negative impacts of classic synthetic products. For instance, positive results in forestry sector were obtained against Ash Dieback using allicin (Dal Maso et al. 2014).

The experiments' scope was focused on the assessment of the potential implementation of the endotherapeutic approach as preventive method against TCD, in order to reduce the widespread of the disease where it is already present and to avoid further infection in susceptible areas.

Field test carried out in site A showed no particular difference between trees treated with the mix of selected commercial products (mix7) and controls treated with water. Even the comparison between necroses developed at different heights from the injecting ports proved that, at least in this close range, there were no significant results. On the other hand, a relevant result was detected between necroses caused at 0° and 180 ° at 0 m of trees treated with the mix7. Considering that, also laboratory tests on pathogens viability showed a partially positive outcome for the variable "orientation", so we suppose that spatial distribution of the injected solution was not uniform in the crown. Further tests may consider if the number of ports influence or not this phenomenon in *J. nigra* as previously reported by Aćimović et al. (2014) for apple tree. Presence/absence test demonstrated, oppositely to field's test, that at crown level mix7 was significantly effective compared to controls. The distance from the main branch did not affect the efficacy of the product against the fungus. This results should be interpreted within the time scale of the experiment. Indeed, considering that injection occurred 300 days before inoculations, it may likely be that formulates have been undertaken to photodegradation when it reached the canopy (Escalada et al. 2008). Further and specific experiments are required to test this hypothesis.

In site B the main objective was to assess the efficacy of natural active principles for preventing TCD. Tested formulations did not bring about either a significant reduction in lesion's development in comparison with controls, or a difference on necrosis's shape according to the index  $\ln \frac{H}{W}$ . On the other side, a positive result among comparisons between media, suggests that growing *G. morbida* on PDA or WA may lead to different necroses' development rate. Trees size did not affect both lesion's area and the index, probably because dosage in trunk injections was balanced according to the size or because tree's size did not affect pathogen's development (Kauffman and Jules 2006). *In vitro* test corroborated the same conclusion of *in planta* test. In particular, there was neither a significant action of any formulation in comparison with water nor an unequal spatial distribution in the crown. A final comment has to be kept for the approach used to inject the solutions. BITE did not cause visible and extensive collateral damages to trees, the system drip bag-BITE was demonstrated to be efficient and rather autonomous. Even if the application of this technique in the forestry sector may be quite limited, urban or ornamental trees could benefit of this low-impact device.

In conclusion, although former researches demonstrated that natural active principles may substitute commercial products, results obtained from this specific work does not corroborate this thesis. Specifically, tested formulations were demonstrated to be not more effective than controls treated with water, but on the other side, no phytotoxic symptoms were detected. Speculating about the results, even if not demonstrated by statistical test, some ideas for further experiments may be formulated. In site B, bar graphs of *in vitro* experiment showed where citral, thymol, potassium phosphite as well were injected, the presence of *G. morbida* had the lowest values. For that, the next step could be to test the same products injecting different dosages and several concentrations, or to develop new formulations with other botanical pesticides.

## **5. ACKNOWLEDGMENTS**

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## 6. REFERENCES

1. Abràmoff, M. D., Magalhães, P. J., & Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics international*, 11(7), 36-42.
2. Aćimović, S. G., VanWoerkom, A. H., Reeb, P. D., Vandervoort, C., Garavaglia, T., Cregg, B. M., & Wise, J. C. (2014). Spatial and temporal distribution of trunk-injected imidacloprid in apple tree canopies. *Pest management science*, 70(11), 1751-1760.
3. ARPAV (2017) Principali variabili meteorologiche Anni 1994-2016. <http://www.arpa.veneto.it/dati-ambientali/open-data/clima>
4. Audley, J., Mayfield, A. E., Myers, S. W., Taylor, A., & Klingeman, W. E. (2016). Phytosanitation methods influence posttreatment colonization of *Juglans nigra* logs by *Pityophthorus juglandis* (Coleoptera: Curculionidae: Scolytinae). *Journal of economic entomology*, 109(1), 213-221.
5. Barney, D., Walser, R. H., Nelson, S. D., Williams, C. F., & Jolley, V. D. (1984). Control of iron chlorosis in apple trees with injections of ferrous sulfate and ferric citrate and with soil-applied iron-sul. *Journal of Plant Nutrition*, 7(1-5), 313-317.
6. Becquey, J. (1997): Les noyers à bois. Institut pour le Développement Forestier (IDF), Paris
7. Beineke, Walter F. 1974. Recent changes in the population structure of black walnut p. 43-46. In Proceedings, Eighth Central States Forest Tree Improvement Conference, University of Missouri, Columbia, MO.
8. Brinkman, Kenneth A. 1965. Black walnut (*Juglans nigra* L.). In *Silvics of forest trees of the United States*. H. A. Fowells, comp. U.S. Department of Agriculture, Agriculture Handbook 271. 762 p.
9. Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International journal of food microbiology*, 94(3), 223-253.
10. By Trunk Injection *Proceedings of the 7th Congress on Plant Protection*. Plant Protection Society of Serbia, IOBC-EPRS, IOBC-WPRS, Belgrade, 2015, pp. 225 - 229
11. Colletti L. 2001 - Risultati dell'applicazione del Regolamento CEE 2880/92 in Italia. *Sherwood – Foreste ed Alberi Oggi* 70: 23-31.
12. Dal Maso & Montecchio, L. (2013) Comparative trials of four potassium phopsite
13. Dal Maso, E., Cocking, J., & Montecchio, L. (2014). Efficacy tests on commercial fungicides against ash dieback in vitro and by trunk injection. *Urban Forestry & Urban Greening*, 13(4), 697-703.
14. Darvas, J. M., Toerien, J. C., & Milne, D. L. (1984). Control of avocado root rot by trunk injection with phosethyl-A1. *Plant disease*.
15. Desneux, N., Decourtye, A., & Delpuech, J. M. (2007). The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.*, 52, 81-106.
16. Ducci F, De Rogatis A, Proietti R 2010 2010 *Juglans regia* L., phenotypic selection and assessment of genetic variation within a simulated seed orchard *Ann. CRA - Centro Ric. Selv. - Vol. 36, 2009 - 2010: 139 - 150*
17. El-Wakeil, N. E. (2013). Botanical pesticides and their mode of action. *Gesunde Pflanzen*, 65(4), 125-149.

18. Escalada, J. P., Gianotti, J., Pajares, A., Massad, W. A., Amat-Guerri, F., & García, N. A. (2008). Photodegradation of the acaricide abamectin: a kinetic study. *Journal of agricultural and food chemistry*, 56(16), 7355-7359.
19. Esposito, D. (2015) Il cancro rameale del noce: una nuova emergenza fitosanitaria, Università degli studi di Padova, Italy
20. Fernández-Escobar, R., Barranco, D., & Benlloch, M. A. N. U. E. L. (1993). Overcoming iron chlorosis in olive and peach trees using a low-pressure trunk-injection method. *HortScience*, 28(3), 192-194.
21. Ferracini, C., & Alma, A. (2008). How to preserve horse chestnut trees from *Cameraria ohridella* in the urban environment. *Crop Protection*, 27(9), 1251-1255.
22. Freeland, E., Cranshaw, W., & Tisserat, N. (2012). Effect of *Geosmithia morbida* isolate and temperature on canker development in black walnut. *Plant Health Progress. Online publication. doi, 10.*
23. Funk, David T. 1979. Black walnuts for nuts and timber. In *Nut tree culture in North America*. p. 51-73. Northern Nut Growers Association, Hamden, CT.
24. Gellini, R. and P. Grossoni (1997): *Botanica forestale*. CEDAM, Padova.
25. Gentile, S., Valentino, D., & Tamietti, G. (2009). Control of ink disease by trunk injection of potassium phosphite. *Journal of Plant Pathology*, 565-571.
26. Giannini, R. and R. Mercurio (1997): *Il Noce comune per la produzione legnosa*. Avenue media, Bologna.
27. Gill, S., Jefferson, D. K., Reeser, R. M., & Raupp, M. J. (1999). Use of soil and trunk injection of systemic insecticides to control lace bug on hawthorn. *Journal of Arboriculture*, 25, 38-42.
28. Gordon, T. R., Wikler, K. R., Clark, S. L., Okamoto, D., Storer, A. J., & Bonello, P. (1998). Resistance to pitch canker disease, caused by *Fusarium subglutinans* f. sp. pini, in Monterey pine (*Pinus radiata*). *Plant pathology*, 47(6), 706-711.
29. Grant J. F.\*, Windham M. T., Haun W. G., Wiggins G. J. and Lambdin P. L. 2011 Initial Assessment of Thousand
30. Hishinuma, S. M., Dallara, P. L., Yaghmour, M. A., Zerillo, M. M., Parker, C. M., Roubtsova, T. V., ... & Seybold, S. J. (2016). Wingnut (Juglandaceae) as a new generic host for *Pityophthorus juglandis* (Coleoptera: Curculionidae) and the thousand cankers disease pathogen, *Geosmithia morbida* (Ascomycota: Hypocreales). *The Canadian Entomologist*, 148(01), 83-91.
31. Ibrahim, L., Butt, T. M., & Jenkinson, P. (2002). Effect of artificial culture media on germination, growth, virulence and surface properties of the entomopathogenic hyphomycete *Metarhizium anisopliae*. *Mycological Research*, 106(6), 705-715.
32. Jacobi, W. R., Hardin, J. G., Goodrich, B. A., & Cleaver, C. M. (2012). Retail firewood can transport live tree pests. *Journal of economic entomology*, 105(5), 1645-1658.
33. Juzwik, J., Banik, M. T., Reed, S. E., English, J. T., & Ginzler, M. D. (2015). *Geosmithia morbida* found on weevil species *Stenomimus pallidus* in Indiana.



34. Juzwik, J., McDermott-Kubeczko, M., Stewart, T. J., & Ginzel, M. D. (2016). First Report of *Geosmithia morbida* on Ambrosia Beetles Emerged From Thousand Cankers-diseased *Juglans nigra* in Ohio. *Plant Disease*.
35. Kauffman, M. J., & Jules, E. S. (2006). Heterogeneity shapes invasion: host size and environment influence susceptibility to a nonnative pathogen. *Ecological Applications*, 16(1), 166-175.
36. Kettunen, M., Genovesi, P., Gollasch, S., Pagad, S., Starfinger, U. ten Brink, P. & Shine, C. 2008. Technical support to EU strategy on invasive species (IAS) - Assessment of the impacts of IAS in Europe and the EU (final module report for the European Commission). Institute for European Environmental Policy (IEEP), Brussels, Belgium. 44 pp. + Annexes.
37. Kolarik, M, Kirkendall LR. 2010. Evidence for a new lineage of primary ambrosia fungi in *Geosmithia* Pitt (Ascomycota: Hypocreales). *Fungal Biol* 114:676–689
38. Kolarik, M, Kostovcik M, Pazoutova S. 2007. Host range and diversity of the genus *Geosmithia* (Ascomycota: Hypocreales) living in association with bark beetles in the Mediterranean area. *Mycol Res* 11:1298–1310
39. Kolarik, M.; Freeland, E.; Utley, C.; Tisserat, N. *Geosmithia morbida* sp. nov., a new phytopathogenic species living in symbiosis with the walnut twig beetle (*Pityophthorus juglandis*) on *Juglans* in USA. *Mycologia* 2011, 103, 325-332.
40. Kolarik, M.; Freeland, E.; Utley, C.; Tisserat, N. *Geosmithia morbida* sp. nov., a new phytopathogenic species living in symbiosis with the walnut twig beetle (*Pityophthorus juglandis*) on *Juglans* in USA. *Mycologia* 2011, 103, 325-332.
41. Kremer D Cavlovic J Bozic M Dubravac T 2008 Distribution and Management of Black Walnut (*Juglans nigra* L.) in Croatia *Periodicum Biologorum* Vol.110, No 4, 317–321, 2008
42. Krokene, P., & Solheim, H. (1999). What do low-density inoculations with fungus tell us about fungal virulence and tree resistance?. *Colloques de l'INRA (France)*.
43. Landt, Eugene F., and Robert E. Phares. 1973. Black walnut ... an American wood. USDA Forest Service, FS-270. Washington, DC. 7 p.
44. Lodge, D. M., Williams, S., MacIsaac, H. J., Hayes, K. R., Leung, B., Reichard, S., ... & Carlton, J. T. (2006). Biological invasions: recommendations for US policy and management. *Ecological Applications*, 16(6), 2035-2054.
45. Luna E. K., Sitz R. A., Cranshaw W. S. and Tisserat N. A. 2013 The Effect of Temperature on Survival of *Pityophthorus juglandis* (Coleoptera: Curculionidae) *Environmental Entomology* 42(5):1085-1091.
46. Marei, G. I. K., Rasoul, M. A. A., & Abdelgaleil, S. A. (2012). Comparative antifungal activities and biochemical effects of monoterpenes on plant pathogenic fungi. *Pesticide biochemistry and physiology*, 103(1), 56-61
47. Mayfield A. E., Fraedrich S. W., Taylor A., Merten P. and Myers S. W. Efficacy of Heat Treatment for the Thousand Cankers Disease Vector and Pathogen in Small Black Walnut Logs *J. Econ. Entomol.* 107(1): 174-184

48. Mohni, C., Pelleri, F., & Hemery, G. E. (2009). The modern silviculture of *Juglans regia* L.: a literature review. *Die Bodenkulture*, 60(3), 21-34.
49. Montecchio, L. (2012). BITE: A LOW IMPACT TOOL FOR TRUNK INJECTIONS. *Journal of Plant Pathology*, 94(4).
50. Montecchio, L. (2013). A Venturi effect can help cure our trees. *Journal of visualized experiments: JoVE*, (80)
51. Montecchio, L., & Faccoli, M. (2014). First record of thousand cankers disease *Geosmithia morbida* and walnut twig beetle *Pityophthorus juglandis* on *Juglans nigra* in Europe. *Environmental Entomology*, 44, 1455-1464.
52. Montecchio, L., & Faccoli, M. (2014). First record of thousand cankers disease *Geosmithia morbida* and walnut twig beetle *Pityophthorus juglandis* on *Juglans nigra* in Europe. *Environmental Entomology*, 44, 1455-1464.
53. Montecchio, L., Fanchin, G., Berton, V., & Scattolin, L. (2015). Vegetative incompatibility and potential involvement of a mycovirus in the Italian population of *Geosmithia morbida*. *Phytopathologia Mediterranea*, 54(3), 465.
54. Montecchio, L., Fanchin, G., Simonato, M., & Faccoli, M. (2014). First record of thousand cankers disease fungal pathogen *Geosmithia morbida* and walnut twig beetle *Pityophthorus juglandis* on *Juglans regia* in Europe. *Plant Disease*, 99(8), 1183-1183.
55. Montecchio, L., Risato, D., & Presti, N. (2012). *U.S. Patent Application No. 14/233,039*.
56. Mooney, H. A., & Cleland, E. E. (2001). The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences*, 98(10), 5446-5451.
57. Newton L., Fowler G., 2009 Pathway Assessment: *Geosmithia* sp. and *Pityophthorus juglandis* Blackman movement from the western into the eastern United States USDA-APHIS
58. Newton, L., Fowler, G., Neeley, A. D., Schall, R. A., & Takeuchi, Y. (2009). Pathway Assessment: *Geosmithia* sp. and *Pityophthorus juglandis* Blackman movement from the western into the eastern United States. *US Department of Agriculture Animal and Plant Health Inspection Service: Raleigh, NC, USA*, 50.
59. Nicolescu, N. V. (1998). Considerations regarding black walnut (*Juglans nigra*) culture in the north-west of Romania. *Forestry*, 71(4), 349-354.
60. Pejchar, L., & Mooney, H. A. (2009). Invasive species, ecosystem services and human well-being. *Trends in ecology & evolution*, 24(9), 497-504.
61. Perry, T. O., Santamour, F. S., Stipes, R. J., Shear, T., & Shigo, A. L. (1991). Exploring alternatives to tree injection. *Journal of Arboriculture*, 17(8), 217-226.
62. Petersen, J. E., Kennedy, V. S., Dennison, W. C., & Kemp, W. M. (2009). *Enclosed experimental ecosystems and scale* (p. 221). Springer-Verlag, New York.
63. Pimentel, D., McNair, S., Janecka, J., Wightman, J., Simmonds, C., O'Connell, C., Wong, E., Russel, L., Zern, J., Aquino, T. and Tsomondo, T. 2001. Economic and environmental threats of alien plant, animal, and microbe invasions. *Agriculture, Ecosystems and Environment*, 84: 1–20
64. R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.

65. Raffa, K. F., & Berryman, A. A. (1983). The role of host plant resistance in the colonization behavior and ecology of bark beetles (Coleoptera: Scolytidae). *Ecological monographs*, 53(1), 27-49.
66. Reichard, M., Vrtílek, M., Douda, K., & Smith, C. (2012). An invasive species reverses the roles in a host–parasite relationship between bitterling fish and unionid mussels. *Biology letters*, 8, 601-604 pp
67. Rumbold, C. (1920). The injection of chemicals into chestnut trees. *American Journal of Botany*, 1-20.
68. Šálek, L., & Hejmanová, P. (2011). Comparison of the growth pattern of black walnut (*Juglans nigra* L.) in two riparian forests in the region of South Moravia, Czech Republic. *Journal of Forest Science*, 57(3), 107-113.
69. Schuelke, T. A., Westbrook, A., Broders, K., Woeste, K., & MacManes, M. D. (2016). De novo genome assembly of *Geosmithia morbida*, the causal agent of thousand cankers disease. *PeerJ*, 4, e1952.
70. Serdani, M., Vlach, J. J., Wallis, K. L., Zerillo, M., McCleary, T., and Tisserat, N. A. 2013. First report of *Geosmithia morbida* and *Pityophthorus juglandis* causing thousand cankers disease in butternut. Online. Plant Health Progress
71. Seybold, S. J., D. Haugen, J. O'Brien, and A. Graves. 2010. Pest Alert: Thousand Cankers Disease. USDA Forest Service, Northeastern Area, State and Private Forestry. Rev. August 2010
72. Sheykhoulislami, A., & Ahmadi, T. (2009). The Study of Caucasian Walnut (*Pterocarya fraxinifolia* (Lam.) Spach.) in Forests of Mashelak (Noshahr, Iran). *Botany Research Journal*, 2(2), 28-33.
73. Shigo, A. L. (1972). Successions of microorganisms and patterns of discoloration and decay after wounding in red oak and white oak. *Phytopathology*.
74. Shigo, A. L., Marx, H. G., & Carroll, D. M. (1977). Compartmentalization of decay in trees.
75. Sitz, R. A., Luna, E. K., Caballero, J. I., Tisserat, N. A., Cranshaw, W. S., & Stewart, J. E. (2016). Virulence of Genetically Distinct *Geosmithia morbida* Isolates to Black Walnut and Their Response to Coinoculation with *Fusarium solani*. *Plant Disease*, PDIS-04.
76. Thompson, H. M. (2003). Behavioural effects of pesticides in bees—their potential for use in risk assessment. *Ecotoxicology*, 12(1-4), 317-330.
77. Tisserat, N., Cranshaw, W., Putnam, M. L., Pscheidt, J., Leslie, C. A., Murray, M., Hoffman, J., Barkley, Y., Alexander, K., and Seybold, S. J. 2011. Thousand cankers disease is widespread in black walnut in the western United States. Online. Plant Health Progress
78. Tisserat, N., Cranshaw, W., Leatherman, D., Utley, C., and Alexander, K. 2009. Black walnut mortality in Colorado caused by the walnut twig beetle and thousand cankers disease. Online. Plant Health Progress E. K.
79. U.S. Department of Agriculture, Forest Service. 1974. Seeds of woody plants in the United States. p. 454-459. C. S. Schopmeyer, tech. coord. U.S. Department of Agriculture, Agriculture Handbook 450. Washington, DC Baker,
80. Utley, C., Nguyen, T., Roubtsova, T., Coggeshall, M., Ford, T. M., Grauke, L. J., Graves, A. D.,

- Leslie, C. A., McKenna, J., Woeste, K., Yaghmour, M. A., Cranshaw, W., Seybold, S. J., Bostock, R. M., and Tisserat, N. 2013. Susceptibility of walnut and hickory species to *Geosmithia morbida*. *Plant Dis.* 97:601-607.
81. Utley, C., Nguyen, T., Roubtsova, T., Coggeshall, M., Ford, T. M., Grauke, L. J., Graves, A. D., Leslie, C. A., McKenna, J., Woeste, K., Yaghmour, M. A., Cranshaw, W., Seybold, S. J., Bostock, R. M., and Tisserat, N. 2013. Susceptibility of walnut and hickory species to *Geosmithia morbida*. *Plant Dis.* 97:601-607.
82. Webber J.F., & Hedger J. (1986). Comparison of interactions between *Ceratocystis ulmi* and elm bark saprobes in vitro and in vivo. *Transactions of the British Mycological Society* 86, 93–101.
83. Webber, J. (1981). A natural biological control of Dutch elm disease. *Nature* **292**, 449 – 451
84. Whalon, M. E., Mota-Sanchez, D., & Hollingworth, R. M. (Eds.). (2008). *Global pesticide resistance in arthropods*. Cabi.
85. Wijden van der, W., Leewis, R. & Bol, P. 2007. Biological globalisation – Bioinvasions and their impacts on nature, the economy and public health. KNNV Publishing, Uthrecht, the Netherlands. 223 pp
86. Williams, R. D. 1990. *Juglans nigra* L. Black walnut, pp. 391–399. In R. M. Burns and B. H. Honkala (eds.), *Silvics of North America*, vol. 2, *Hardwoods*. Agriculture Handbook 654, U.S. Department of Agriculture and Forest Service, Washington, DC.
87. Zerillo, M. M., Caballero, J. I., Woeste, K., Graves, A. D., Hartel, C., Pscheidt, J. W., Tonos J, Broders K., Cranshaw W., Seybold S., Tisserat, N. (2014). Population structure of *Geosmithia morbida*, the causal agent of thousand cankers disease of walnut trees in the United States. *PloS one*, 9(11), e112847.

## 7. ANNEXES

### 7.1.A Symptoms



Spotted cankers on the trunk

Author: Prof. Lucio Montecchio



Early yellowing of the crown

Author: Esposito Davide



Overlapping cankers along a branch

Author: Prof. Lucio Montecchio



Flag-like leaves

Author: Prof. Lucio Montecchio

## 7.1.B 'Decree of mandatory control' and map of the area currently officially affected

**Bur n. 19 del 24/02/2015**

(Codice interno: 291936)

DECRETO DEL DIRIGENTE DEL SETTORE SERVIZI FITOSANITARI n. 8 del 06 febbraio 2015

**Misure fitosanitarie di controllo di *Geosmithia morbida* in Regione Veneto. Aggiornamento della zona delimitata.**  
[Agricoltura]

Note per la trasparenza:

Con il presente provvedimento viene approvato l'allargamento della zona delimitata di cui al Decreto dirigenziale n.43 del 6 novembre 2014 (Decreto di ampliamento della zona delimitata di cui al Decreto dirigenziale n. 30 del 14 agosto 2014, ove vengono adottate le misure fitosanitarie di controllo per contrastare la diffusione dell'organismo nocivo *Geosmithia morbida*, agente del cancro rameale del noce, in Regione Veneto).

Il Dirigente

VISTA la normativa fitosanitaria vigente e in particolare la direttiva 2000/29/CE e il decreto legislativo 19 agosto 2005 n. 214 attuazione della direttiva 2002/89/CE che dispongono l'adozione di misure di protezione contro l'introduzione e la diffusione nel territorio nazionale e comunitario di organismi nocivi ai vegetali o ai prodotti vegetali;

VISTO il proprio Decreto n. 30 del 14 agosto 2014 "*Misure fitosanitarie di controllo ed eradicazione per contrastare la diffusione dell'organismo nocivo *Geosmithia morbida*, agente del cancro rameale del noc, in Regione Veneto*" con il quale sono stati definiti in Allegato i confini della zona delimitata costituita dalla zona infestata (poligoni i cui vertici corrispondono ai focolai individuati) e la zona cuscinetto (zona perimetrale di 2 Km oltre la zona infestata);

VISTO il proprio Decreto n. 43 del 6 novembre 2014 "*Misure fitosanitarie di controllo di *Geosmithia morbida* in Regione Veneto. Aggiornamento della zona delimitata*" con il quale viene approvato l'allargamento della zona delimitata di cui al Decreto dirigenziale n. 30 del 14 agosto 2014;

CONSIDERATO che nel corso di un monitoraggio invernale sono stati individuati ulteriori 2 focolai esterni alla zona precedentemente definita;

VISTO l'art.16.2 della Direttiva 2000/29/CE che prevede l'obbligo degli Stati membri di adottare misure di protezione al fine di prevenire la diffusione nel territorio anche di altri Stati membri di organismi nocivi non regolamentati;

CONSIDERATO che *Geosmithia morbida* rappresenta una minaccia per la coltura del noce nero (*Juglans nigra*), ampiamente diffuso nel territorio della pianura padana nel corso degli ultimi vent'anni, ma anche del noce europeo (*Juglans regia*) e probabilmente dei suoi ibridi, anche se di questi attualmente non è nota la suscettibilità;

RITENUTO necessario aggiornare i confini della zona delimitata ove adottare misure regionali di contrasto alla diffusione di *Geosmithia morbida*;

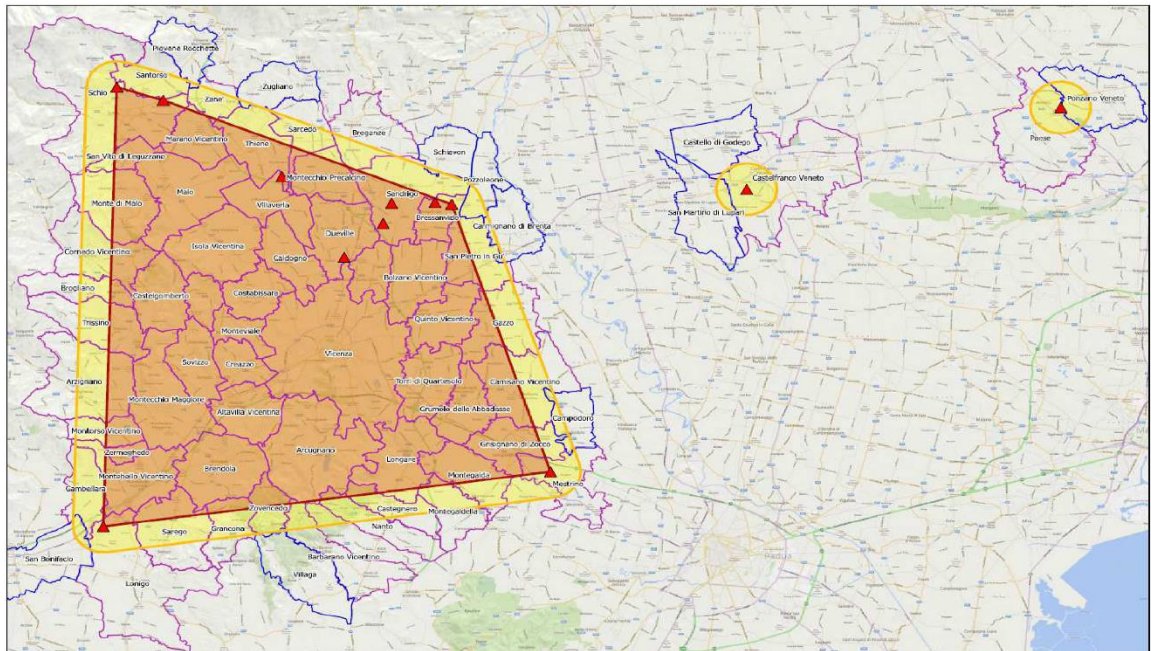
decreta

1. di aggiornare i confini della zona delimitata, comprendente la zona infestata (poligono i cui vertici corrispondono ai focolai finora individuati) e la zona cuscinetto (zona perimetrale di 2 Km oltre la zona infestata) come riportato in **Allegato A**, parte integrante del presente decreto;
2. di prevedere che nella zona delimitata si adottino le seguenti misure fitosanitarie obbligatorie:
  - a. Divieto di trasporto fuori dalla zona di vegetali destinati alla piantagione (comprese marze e portainnesti) con diametro massimo superiore ai 10 mm appartenenti ai generi *Juglans* e *Pterocarya*;
  - b. Divieto di trasporto fuori dalla zona di legname e suoi derivati ramaglie e corteccia ad esclusione dei seguenti casi:
    1. Squadratura del legname fino a completa rimozione di corteccia, strato floematico e prime cerchie xilematiche;
    2. Trattamento termico fino al raggiungimento della temperatura di 60 °C per almeno 45 minuti a livello delle prime cerchie xilematiche;

c. Le aziende vivaistiche che coltivano o commercializzano piante appartenenti ai generi *Juglans* o *Pterocarya* all'interno della zona delimitata sono oggetto di specifici controlli da parte del Settore Fitosanitario e hanno l'obbligo di tenere registrate le movimentazioni delle piante;

3. chiunque non ottemperi alle disposizioni di cui al presente decreto è punito con le sanzioni amministrative previste dall'art. 54 del decreto legislativo 19 agosto 2005 n. 214;
4. di dare atto che il presente provvedimento non comporta spesa a carico del bilancio regionale;
5. di pubblicare il presente atto integralmente nel Bollettino ufficiale della Regione.

Giovanni Zanini





## 7.2.A Formulates

### Site A

Mix7				
	Prochloraz	Tetraconazole	Carbitol <sup>TM</sup>	Abamectin
Unit	g/L	g/L	g/L	g/L
Value	19.09 <sup>b</sup>	3.4 <sup>b</sup>	838.4	9 <sup>c</sup>

b = Binal PRO (GOWAN ITALIA S.r.l., Faenza (RA) IT), commercial product

c = Vertimec EC (Syngenta AG, Basel, CH), commercial product

### Site B

Potassium Phosphite	
Fosfisan <sup>a</sup>	
Unit	v/v %
Value	35

a = (Adriatica S.p.A., Loreo (Ro), IT), commercial product, P<sub>2</sub>O<sub>5</sub> 30%, K<sub>2</sub>O 20%

Thiabendazole	
Unit	g/L
Value	22 <sup>d</sup>

d = Tecto 20S (Decco Italia, Belpasso (CT), IT), commercial product

Thymol formulation					
	Thymol	Ethanol	Glicerol	Tween 20S <sup>e</sup>	Fosfisan <sup>a</sup>
Unit	g/L	g/l	g/l	g/l	v/v %
Value	16.42	62.8	32.8	16.4	30

e = commercial products

a = (Adriatica S.p.A., Loreo (Ro), IT), commercial product, P<sub>2</sub>O<sub>5</sub> 30%, K<sub>2</sub>O 20%

Citral formulation					
	Citral	Ethanol	Glicerol	Tween 20S <sup>e</sup>	Fosfisan <sup>a</sup>
Unit	g/L	g/l	g/l	g/l	v/v %
Value	16.64	62.8	32.8	16.4	30

a = (Adriatica S.p.A., Loreo (Ro), IT), commercial product, P<sub>2</sub>O<sub>5</sub> 30%, K<sub>2</sub>O 20%

Carvone formulation					
	Carvone	Ethanol	Glicerol	Tween 20S <sup>e</sup>	Fosfisan <sup>a</sup>
Unit	g/L	g/l	g/l	g/l	v/v %
Value	16.42	62.8	32.8	16.4	30

a = (Adriatica S.p.A., Loreo (Ro), IT), commercial product, P<sub>2</sub>O<sub>5</sub> 30%, K<sub>2</sub>O 20%

Ethanol, glycerol, and Tween 20S were purchased from Sigma-Aldrich Co. St. Louis MO, US.

## **7.2.B Growing Media**

### PDA Preparing from Commercial Medium Powder

1. Add 39 g of Commercial PDA Powder to 1 L of distilled water.
2. Boil and stir to dissolve completely the medium.
3. Autoclave 15 min at 121°C to sterilize.

### WA Preparing from Commercial Medium Powder

1. Add 20 g of Commercial Agar Powder to 1 L of distilled water.
2. Boil and stir to dissolve completely the medium.
3. Autoclave 15 min at 121°C to sterilize.

## **7.2.C Injecting Protocol**

Treating each plant we pursued the following protocol:

- i. Wear protective gloves and safety glasses.
- ii. Fill a drip bag with the volume of formulate, close the pinch clamp and fix it on the trunk as high as possible to help the gravity lead process.
- iii. Open the pinch clamp and fill the pipe up to the tip.
- iv. Select two opposite points above the collar, 10-20 cm from the ground, to create two ports.
- v. Black walnut's bark is characterized by a surface quite irregular and deep cracks, thus it is need to slightly smooth the two selected area with a knife, formerly flame sterilized.
- vi. Put a small piece of mastic on the gasket and insert the BITE perpendicularly to the trunk using the hammer.
- vii. If the gasket is pressed enough against the surface no air should be able to enter the system. To check so, with a syringe half-filled of water lift back the plunger. All the air inside the body should be drawn and the plunger should tend to come to its original position.
- viii. Remove the syringe, rapidly insert the tip and open the pinch clamp

7.2.D Endotherapy site B





Injecting system working during field test in site B. (Drip bag and bites plugged into the trunk above the collar)

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