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Economia circolare e approcci ecosostenibili al controllo dei fitopatogeni: analisi delle potenzialità dell'acido pirolegnoso

Circular economy and sustainable plant diseases control: analysis on the potential role of wood vinegar

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1. Introduction

1.1 Biological and traditional agriculture

In this century, a strong concern about environment and its protection is rising worldwide, so in agriculture many are the investments for the research and development of new strategies and molecules to control plant bacterial and fungal diseases of plants. As a result, many Companies and the markets have started to focalize on new and ecofriendly approaches to effectively control these plant pathogens, while maintaining high quality standards for productions. In this frame, the best options are those based on "prevention", for example with the use of resistance cultivars and a wise adoption of specific agricultural practices. Nevertheless, in most cases the use of chemicals is still unavoidable, both in biological and traditional agriculture, despite of their substantial differences (Michaels, 2014).

As far as biological agriculture is concerned, its management sinergically combines traditional farming systems and cutting-edge sustainable approaches, to promote those natural processes leading to an improvement of agroecosystems without the use of synthetic chemicals, thus to guarantee a better health status for the whole agroecosystem. Conversely, traditional agriculture allows the use of synthetic fertilizers and plant protection products, as well as of monoculture for many important crops, with heavy consequences on any living organism and microorganism in the agroenvironment. Concerning plant disease control, a fundamental element in common between biological and traditional agriculture is given by copper (hereafter Cu) (Michaels, 2014).

1.2 Copper and its evolution

The first use in plant protection of copper compounds was recorded in 1885 and they were accidently discovered by the French scientist Pierre-Marie-Alexis Millardet (Lamichhane *et al.,* 2018). He found a cure toward downy mildew disease of grape, *Plasmopara viticola,* announcing that a mixture of copper sulphate pentahydrate, lime and water was capable to defeat this fungus. This solution took the name of "Bordeaux mixture" because it was discovered in the French district of Bordeaux (Lamichhane *et al.,* 2018).

Nevertheless, the antifungal properties were detected before 1885 by the use of copper sulfate to treat wheat seeds against smut spores, but only with Bordeaux mixture as fungicide the development of Cu-based compounds took place (Lamichhane *et al.,* 2018).

Copper is an important micronutrient for most living organisms because its role as constituent of many metalloenzymes and of proteins essential in electron transport and many other reactions. On the other hand, copper can be toxic to cells, when at higher concentrations and in the ionic form Cu^{2+}. Its toxicity depends to its interaction with nucleic acids as well as with proteins having enzymatic activity (Scheiber *et al.*, 2013).

The intensive use of inorganic copper in agriculture had and still have a strong impact of human health and biodiversity. Indeed, a high level of Cu can induce stress on the plant and reduce soil fertility, causing phytotoxicity due to highly soluble Cu formulations or excessive amount. Application of these compounds in specific and sensitive plant phenological stages can also increase the risk to cause an actual abiotic stress (Lamichhane *et al.,* 2018).

Copper in high concentrations interferes with the development of plant roots and their capacity to adsorb nutrients. In this case the problem can be especially in sensitive crops during rotation. (Lamichhane *et al.,* 2018).

Another problem is the reduction of seeds germination in several crops, like sunflower, wheat, bean and maize (Lamichhane *et al.,* 2018).

At last, copper-based compounds used as bactericides and fungicides may exert a selective pressure on those microorganisms that are present in agroecosystems, including those pathogenic for plants, with the consequence to cause resistance phenomena (Cervantes and Gutierrez-Corona, 1993) as well as a drastical change of microbial communities (Chen *et al.,* 2019).

Among the most significant examples, it is worth to mention some Cu-resistance induction cases.

Xanthomonas perforans strains isolated from tomato were found resistant in different areas in Florida in 2006, making difficult the management of the disease control. The acquirement of Cu resistance lead to increase the frequency of this one due to the continuous selection pressure. It's impossible that the resistance is induced by spontaneous mutations, indeed, the way of transmission is related to chromosomal transfer of the Cu plasmids (Lamichhane *et al.,* 2019).

Resistance to copper has been studied also on *Pseudomonas syringae* pv. *tomato* (Pst)*,* causal agent of bacterial speck on tomatoes (Griffin *et al.,* 2019).

Test were done by PCR assays to detect cop (copper metabolizing) genes, cause of copper-tolerance induction in different *Pseudomonas* genus like *chicorii*, *putida* and in the same *syringae* species with pv. *actinidae*. In particular, six Cop-protein in Pst related to insurgence of this resistance: CopA, copB, CopC, CopD, CopR and CusS, located on either plasmid or chromosomal DNA. These genes were recognized as mediators of copper-tolerance in *P. syringae* pv. *tomato* (Griffin *et al.,* 2019).

Furthemore, accumulation of copper can create problem also to other soil biota in addition to bacteria and fungi, such as protozoa, mites, springtails, spiders, earthworms, insects and nematodes, going to impact on their natural processes (Lamichhane *et al.,* 2018). An example of this interference was made by comparing several soils differently polluted by copper: a decrease of functional diversity of soil communities was found by increasing copper pollution (Lamichhane *et al.,* 2018).

Copper seems to be the most useful and effective means to control both fungal and bacterial diseases of plants. However, starting from the 1st of February 2019 the EU Commission Implementing Regulation 2018/1981 of the 13rd of December 2018 is in force. It renews the approval of the active substances copper compounds for plant protection, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. At the same time the Regulation 2018/1981 affirms that copper compounds are “candidates for substitution”, and thus the threshold was reduced from 6 kg/ha/years to 4 kg/ha/years, or 28 kg in 7 years, both in traditional and biological agriculture (European Commission, 2018).

1.3 Antibiotics in agriculture and resistance

The development of antibiotic resistance genes (ARGs) is related to Cu resistance as well, and it is a resistance defined conceptually as “pollutant” by WHO (World Health Organization) (Chen *et al.,* 2019). Moreover, the acquisition of genes resistance is transmissible with horizontal transmission also to non-target microbiota (Sundin *et al.,* 2016).

Increase of these resistance genes is related to massive use of antibiotics not only in agriculture but also in therapeutics (Chen *et al.* 2019), so concerns on health and environmental increased taking the situation in Europe to a complete prohibitions and restricted use (Rezzonico *et al.*, 2009).

In the United States, antibiotic use in agriculture in less than 0.5% of the total (Rezzonico *et al.* 2009), and from 1950s antibiotics, like streptomycin, the first ever registered in the USA, were widely used to control different bacterial disease of plants (Stockwell and Duffy, 2012).

Streptomycin is an aminoglycoside antibiotic produced by soil actinomycetes, and for the 90% is used against *E. amylovora*, and with minor use to control diseases in floriculture and on potato tubers. It has the capacity to binding in an irreversibly way to the bacterial ribosome and to block the synthesis of proteins. found in orchards in the USA, New Zealand and Israel, is the spontaneous mutation of the chromosomal gene rpsL and the prevention of streptomycin binding to the ribosome.

In 1971 and in California, for the first time a resistant strain of *E. amylovora* was detected following the use of the antibiotic streptomycin to control the causal agent of fire blight (Rezzonico *et al.,* 2009). As a consequence, streptomycin resistance resulted in a limitation in the control of *E. amylovora*, as occurring in orchards in Michigan and California (Stockwell and Duffy, 2012).

Accordingly, the use of additional antibiotics was needed to control *E. amylovora* in the orchards, and streptomycin was in some Countries substituted with other antibiotics like oxytetracycline (also called terramycin), oxolinic acid and gentamicin (Stockwell and Duffy, 2012; Liu *et al.,* 2019). For all these antibiotics, three are the major strategies for resistance to develop, and that are mutations occurring on those genes related to the functionality of efflux pumps, ribosome binding, and production of specific enzymes.

Oxytetracycline is produced by *Streptomyces rimous.* It is the only antibiotic that can be used injected into the trunks to control lethal yellowing diseases, a phytoplasma disease, in palm and elm trees (Stockwell and Duffy, 2012). Biological activity of oxytetracycline consists in the inhibition of the multiplication of bacterial cells, following its reversible binding to the bacterial ribosome and thus of the block of proteins synthesis.

Gentamicin antibiotic is used to control species belonging to the genera *Pectobacterium*, *Pseudomonas*, *Ralstonia*, *Xanthomonas* and *Erwinia.* Its activity is similar to that of streptomycin, with the inhibition of protein synthesis by its binding to the bacterial ribosome. However, differently from streptomycin, gentamicin has the capacity to bind the ribosome in more than one site, and thus several mutations are needed to generate a properly resistant mutant strain. Indeed, bacterial cells became resistant only if they acquire genes encoding enzymes that modify the antibiotic.

At last, the quinolone oxolinic acid is used in Israel, in areas where *E. amylovora* already has a resistance towards streptomycin. It inhibits DNA replication, due to the binding of the quinolone to

the DNA gyrase and topoisomerase IV, with a complete block of the bacterial growth. The mechanism of resistance to oxolinic acid results from spontaneous mutations on the bacterial chromosome, leading to an alteration of the antibiotic binding sites on DNA gyrase.

Consequences of the massive use of antibiotics are not only about plants pathogen but, as expected, in clinical medicine as well, with an increase of this kind of resistance among human pathogens. The amount of antibiotics dispensed to humans in USA, estimated by the Food and Drug Administration, is around 3.300.000 tonnes for year. The 0.12% of antibiotics, 16.465 kg of the total 13.100.100 tonnes used in animal agriculture, was applied to tree fruits in 2009 in USA.
The United States Environmental Protection Agency sets a regulation about chemicals applied to crops. Antibiotics are present in these chemicals like non-toxik compounds and they are regulated about pre-harvest interval and level of permissible residues. The first for streptomycin and oxytetracycline is between 21 and 60 days, the second for oxytetracycline is 0.35 ppm (Stockwell and Duffy, 2012).

Another example of resistance mechanism is that induced by streptomycin on *Clavibacter michiganensis,* a Gram-positive bacterium causal agent of bacterial canker of tomato (Lyu *et al.,* 2019).
The test has been performed by inoculation of the bacterium on a tryptone broth with yeast agar (TBY), then the *C. michiga*nensis inoculum has been sprayed on tomato (*Solanum lycopersicum* cv. *Moneymaker*), and data have been collected.
Three strains of the bacterium showed streptomycin resistance by a point mutation in the *rpsL* gene but with a consequence on their phenotype; the strain BT-0505-R-2 has grown less than the wild-type (Lyu *et al.,* 2019).
The same test has been done on the 25 strains of *C. michiganensis* in Chile from different locations, with results that show that 21 of 25 strains (84%) were streptomycin resistant (Valenzuale *et al.,* 2019).

Due to the increase of resistance by bacteria, the EU Commission of Health and Consumer Protection completely ban 4 antibiotics (*i.e.* monensin, salinomycin, avilamycin and flavophospholipol) usable as feed additives for livestock starting from January 1st, 2006. EU already banned antibiotics, but this new Feed Additives Regulation complete the old one (European Commission, 2005).

Determination of which Countries permit antibiotics use is difficult: China, USA and Mexico are an example of legalized use. Also, in emergency situations antibiotics can be used in Germany, Switzerland and Austria (Stockwell and Duffy, 2012).

1.4 Plant disease epidemics caused by bacteria in Italy

Bacterial diseases of plants are difficult to control because the only antibacterials available so far are copper and antibiotics, the last just in those Countries where are allowed for agriculture.

Several epidemics of bacterial diseases of plants have occurred in Italy in recent times. One of these epidemics was caused by *R. solanacearum* in 2017 in Emilia Romagna, on potato and tomato plants. *R. solanacearum* is a Gram-negative bacterium of 0.5-1.5 µm length thermophile (35-37°C) and it has the capacity to survive in the soil and in water, as well as in crop residues (until 2-3 years) and in numerous spontaneous plants like *Solanum nigrum* or *Portulaca oleracea*. It causes diseases in 200 host plants of *Solanaceae*, such as potato, tomato, pepper, eggplant and tobacco (Testi *et al.,* 2017). The infection starts with seed tubers (*i.e.* potato) or plants (*i.e.* tomato and others), irrigation waters or infected tools with the consequence of bacterial penetration inside wounded root. Symptoms are multiple and occurring on different anatomical parts: the main damage is in the vascular system and the first symptoms are leaves wilting and plant dwarfism, then the entire plant darkens and dries (Testi *et al.,* 2017).

In Emilia Romagna, many locations were hit by *R. solanacearum*. The identification of the bacterium was done by laboratory molecular techniques as SMSA, immunofluorescence and selective PCR, with analyzes on different hit cities of the country. In Ferrara, two companies had fields of tomato infected for a total of 10.3 ha, while in Parma also four companies had problems caused by the bacteria, always on fields of tomato and for a total of 31.7 ha. Also, in Budrio (BO) a potato field from tuber seeds of 8.8 ha had the infection.

The problem is that the direct control of the disease after the inoculation is practically impossible, so the only way in this case is the prevention. Indeed, in this case was express in 4 points:

- Certified seeds free from the bacterium with disinfestation by hydrochloric acid and well conservation;
- Healthy controlled plants;

- Elimination of the infected plants and control of spontaneous ones;
- Avoid contaminations in field and wounds on the plants to prevent bacterium entrance;

The contaminated unit of soil were treated with pyroherbicide and other treatments like burial with deep plowing while machineries, stores and various structures were decontaminated by steam and sodium hypochlorite (Testi *et al.,* 2017).

Pseudomonas syringae pv. *actinidiae* (*Psa*) is another bacterium which is associated to different epidemics occurring in Italy. The pathogen was first isolated in 1992 from *Actinidia deliciosa* and considered agent of sporadic damage but starting from 2008 *Psa* became more present than the past, in particular in central Italy, more precisely in Lazio (Ferrante and Scortichini, 2010).
Psa attacks preferably *A. chinensis* and *A. deliciosa* (Ferrante and Scortichini, 2010) and can penetrate inside plants thanks to stomata and generally openings, mostly in temperature between 12°C and 18°C and high humidity; spring, first summer and autumn-winter too can be adapted to spread the disease with these conditions, with the consequence dispersion of the inoculum (Scortichini *et al.,* 2012).
Symptoms are viewable first on the leaf with necrotic spots surrounded by brown-black spots, then on blossoms showing browning and necrosis and on trunk by typical cancer with red and orange exudates (Scortichini *et al.,* 2012).
The best way to control this phytopathogen is an integrated approach, by precise spray treatments with bactericides and equilibrated plant nutrition plus preventive measures to reduce the bacterial inoculum. Indeed, use of resistant cultivars and pollinators, biocontrol agents and compounds with the capacity to induce systemic defense on the plant can be fundamental for it survive (Scortichini *et al.,* 2012).

Another recent example is the bacterium *Xylella fastidiosa*, a pathogen agent attacking in the summer 2013 many olive trees in the Italian region Puglia. Many plants near Lecce, in the southern Italy, showed identical symptom like drying of the crowd in isolated parts or the entire plant, internal browning and leaves partially dried out. Different pathogen agents were recognized as well as *X. fastidiosa*: lepidoptera *Zeuzera pyrina* and some vascular woody fungi of genus *Phaeoacremonium* and *Phaemoniella spp.* The commune action of these pathogens is called “Olive Quick Decline Syndrome” or simply reported as OQDS (Ciervo, 2015).

X. fastidiosa is a Gram-negative bacterium that multiplies in the xylematic vessels and has many host plants like oleander tree, different citrus species and, of course, olive tree too. Its capacity to move from the high part of the plant to the low is the reason why it's possible to find it in the root.
However, the best propagation way is by insects that use xylematic lymph as feed because of its rapidity; indeed, latency period is not present in this case (Ciervo, 2015).
As always, prevention is the best one solution: resistant cultivars, appropriate cultivation practices and chemical/biological control of vector insects; an example of biological control of *Xylella* is on *Homalodisca vitripennis,* one of the most dangerous vector, by *Gonatocerus ashmeadi* while chemicals that are normally used are systemic insecticides like neonicotinoids (Irvin *et al.,* 2014).

1.5 Plant disease epidemics caused by fungi in Italy

Not only bacteria had caused epidemics in Italy and other Countries, but also fungi have played an important role. One of these is surely *Plasmopara viticola,* an oomycete agent of grapevine downy mildew originally from North America and then in Europe starting from the end of XIX century.
First symptoms are visible in the lower page of the younger leaf with decolorated spots that become yellow chlorotic and translucid, than they can brown and necrotize. In conditions of high humidity, it's also possible the observation of whit efflorescence by the fungi near these zones that, in particular favorable conditions, appear without the decolorated ones (Ash, 2000).
The infection is strongly related to climate parameters like air's temperature, rain's intensity and duration, leaf wetting and relative humidity, so prevention with the use of models is one of the best strategies to reveal the disease. The most used and easy model is based on the "three ten" rule: 10 cm of branches length, 10 mm of fallen rain in 24/48h and minimal temperature of 10°C, although 12°C is more preferable as reference. The strong difference between fungi control and bacteria control is the possibility to use chemicals products as prevention but also as a direct cure (Ash, 2000).
Normally, after the detection of the best period by models, coating products like dithiocarbamates or Bordeaux mixtures in prevention can be used, while in post-infection are normally used fungicides like phosphonate phenylamides and carboxylic acid amide (Ash, 2000).

Powdery mildew is another fungus disease that affects many host plants, including grapevine. Pathogen agents belong to the order of fungi ascomycete *Erysiphales,* in particular the most reported is *Erysiphe necator* (or *Uncinula necator)* (Angeli and Pertot, 2007).

It is normally present in Italy, especially in the southern part, with recognizable symptoms and growth conditions very different than grapevine downy mildew; it's more independent by the climatic conditions and with the best ones it can spread much more than *P. viticola* (Angeli and Pertot, 2007).

Powdery mildew is viewable on both leaf edges starting with clear spots and subsequently a white film, but in case of a strong attack it's possible the appearance of browning and necrosis. Young branches can be attacked by the fungus with brown reticulated spots viewable until the complete lignification, however the biggest damage is on the florescence, susceptible already before flowering, and then on the grapes. Indeed, after the post-flowering infection, grapes necrotize and, without the capacity to support the pulp growth, split leaving possibility to other infections to take advantage of the openings. If the attack is weaker, grapes show little spots and browning with the typical white efflorescence of the *E. necator* (Angeli and Pertot, 2007).

Control of the powdery mildew is generally based on copper sulfate, a solution totally efficiency towards the fungus, but in the last years a product, called AQ10, based on *Ampelomyces quisqualis* start to be used as alternative. It is an hyperparasite of *E. necator* belonged to the *Dothideomycetes* class (Angeli and Pertot, 2007).

Another example of epidemic problem on grapevine and many other plants is botrytis or gray mold, a disease caused by the pathogen *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana),* an ascomycotan. It attacks branches, grapes, leaves, shoots and other plant parts on vegetables (lettuce, broccoli, beans) and fruit crops (strawberry, raspberry). On apical branches it's possible the appearance of necrosis while on the older brown notches that can be cover by mold, beside flowers suffer on pre-flowering, taking the forming grapes to dry and fall. The formed grapes in veraison are not hit but the fungus can wait into flowers residuals until the favorable period, then with the penetration starts the typical symptoms of gray mold areas. Signs of the botrytis are findable also on the peduncle with rot and consequences fall of the entire brunch (Williamson *et al.,* 2007).

For the disease management there are different possibilities. Practices like provide to an adequate air movement and an increase of light interception can prevent it, especially with moderate temperature and high humidity. Chemical use started 35 years ago by the use of methyl benzimidazole carbamate (MBC) that generated a resistance towards the fungicide, so use of mixed

fungicides reduce this risk and permit the maximum effect of each one used as ingredient (Williamson *et al.,* 2007).

Another solution is the biological control by the use biocontrol agents. Use of formulations with fungi as *Trichoderma harzianum, Ulocladium oudemansii* and *Chlonostachys rosea* are some examples, but also yeast like *Candida oleophla* or the bacteria *Bacillus subtitils, P. syringae* and *Streptomyces griseoviridis* are normally used with this purpose (Williamson *et al.,* 2007).

1.6 Alternatives to copper in plant protection

The new thresholds permitted in EU for copper use, and the problems related to bacterial Cu-resistance, together an increasing sensibility about climate change and environmental sustainability, have taken producers and consumers to questioning about alternative to Cu-based products, and not only.

Among the possible solution to alternatives to copper are biocontrol agents (BCAs) and botanicals or bio-stimulants.

1.6.1 Biocontrol agents

BCAs are based on microorganisms (*i.e.* bacteria, fungi and viruses) that are pathogens or predators/parasitoids for the plant pathogen or pest to be controlled, and that offer the possibility to control plant pathogens and pests without their complete eradication. The biological activities of BCAs have a large spectrum based on the combination of different modes without risk for biodiversity and of development of pathogen population resistance (Lamichhane *et al.,* 2018).

Indeed, many are the examples of control disease with these agents. A valid one is *Coniothyrium minitans,* a fungi mycoparasites normally used to control the genus *Sclerotinia* and *Sclerotium*. A 5-year trial was tested to prove the efficiency of this BCA. A rotation crop with potato (*Solanum tuberosum*), bean (*Phaseolus vulgaris*), carrot (*Daucus carota*) and chicory (*Cichorium intybus*) was sprayed with *C. minitans* (C10A, C15, IVT1 and IVT5 isolates combined) but also another biocontrol agent: *Trichoderma spp*. Results show a failure of *Trichoderma spp*. treatments but in certain occasions they stimulated production or survival of *S. sclerotiorum,* 2,4 times higher than non-treated control. In other hand, *C. minitans* demonstrated its use as BCA due to the reduction of soil contamination by *S. sclerotiorum,* in particular spraying on crops and turning residues into the soil

considerably reduced survival of sclerotia. So, this treatment wouldn't prevent damage and decreased yield when the infection is in progress, but it can reduce the inoculum for the subsequent years (Gerlagh *et al.,* 1998).

Biological control can be also by bacteria. Indeed, use of the them to control *X. fragariae* (*Xf*, IVIA XF349-9A and CECT549 strains), *X. arboricola* pv. *pruni* (*Xap*, CFBP3894 and CFBP5563 strains*)* and *P. syringae* pv. *actinidiae* (NCPPB3739 and IVIA 3700-1 strains) was tested by the use of 55 lactic acid bacteria (LAB) from *Lactobacillus plantarum* and *Leuconostoc mesenteroides.* First, an *in vitro* test was done on Petri dishes. Results showed that Cluster 1 (17 LABs) was moderate/high active on *Xf*, moderate on *Xap* and inefficiency on *Psa*, Cluster 2 (33 LABs) was low/inefficiency on *Psa* and moderate/high on *Xap* and *Xf*, but the most important result was Cluster 3 (Daranas *et al.,* 2019).
This one, composed by 5 LABs (*L. plantarum* CC100, PM411, TC92 and *L. mesenteroides* CM160, CM209) resulted with the highest activity towards the three pathogens. The second part of the test was directly on kiwi *A. chinensis* cv. *Hayward*, *Prunus amygdalus × Prunus persica* and strawberry *Fragaria × ananassa.* Valuations in quarantine greenhouse EPPO A2, semi-field and orchards. In quarantine greenhouse the 5 LABs of Cluster 3 were tested comparing them to water and streptomycin treatments as control while in semi-field the comparison was different and enlarged with more compounds:

- Kiwi with PM411, *Bacillus amyloliquefaciens* D747 and copper oxide;
- *Prunus* and strawberry with PM411, TC92, *B. subtilis* QST713, chitosan, ASM (acibenzolar-S-methyl), copper hydroxide and antibiotic kasugamycin;

The third and last test in orchard was done on *A. chinensis* in an ambient where *P. syringae* symptoms were just present. The thesis tested were PM411, *B. amyloliquefaciens* D747 and copper oxide (Daranas *et al.,* 2019).
Results were very different. The first (quarantine greenhouse EPPO A2) showed a positive response toward the incidence of *Psa*, *Xap* and *Xf* by *L. plantarum* PM411 and TC92. On kiwi the reduction of the symptoms by TC92 was 84.5-96.3% and by PM411 was 70-75.4%, on Prunus 59.1-69.3% by TC92 and 45.5-65.5% by PM411, on strawberry 35.4-69.2% by TC92 and 45.8-92.3% for PM411 (Daranas *et al.,* 2019).

The situation in semi-field conditions was a little bit different. Efficiency on reduction *Xap* on *Prunus* was around 41,5-55% by TC92, better than non-treated but not then Bacillus QST713, chitosan, ASM, kasugamycin and also copper. Similar situation on strawberry by PM411 toward *Xap* symptoms, with an incidence of 63,6-75% it was better than non-treated ones without big difference than QST713, ASM, copper and kasugamycin. PM411 on *Psa* (kiwi) had a good incidence, 54,2%, then non-treated but with similarity to *B. amyloliquefaciens* D747 and copper oxide.

In the orchards, PM411 had an incidence around 20% similar to D747 and copper but lower than non-treated (Daranas *et al.,* 2019).

So, BCAs are valid alternatives to Cu-based products. Formulations of antagonistic strains of bacteria and fungi to control bacteria and fungi are used in biological and traditional agriculture. Indeed, more than 209 strains are used for this purpose and registered for commercial use; 94 of these are directly registered for disease control (Daranas *et al.,* 2019).

Different products registered in different countries are used as BCAs, for example *Aureobasidium pullulans* DSM 14940 and 14941 in EU, Canada and USA, *Pantoea agglomerans* C9-1 In Canada and USA, *P. agglomerans* p10c in New Zealand and *P. agglomerans* E325 in Canada. Also, *Pseudomonas fluorescens* A506 in the USA, used to control fire blight in pome fruits, *Pseudomonas rhodesiae* HAI-0804 used in Japan to control *P. syringae* and *P. fluorescens* CL145A, always in Japan, is used on bacterial rots in cabbage and lettuce. More examples are *Bacillus pumilis* QST 2808, *B. subtilis* QST713 used to control *Venturia spp.* in USA and bacteriophages, in the USA, registered for the control of *C. michinganensis* pv. *michiganensis* and *Xanthomonas campestris* pv. *vesicatoria* (Lamichhane *et al.,* 2018).

1.6.2 Botanicals

The other category of alternatives to Cu-products called botanicals (or bio-stimulants) have a different origin and they works strengthening the plant toward abiotic and biotic stresses. Some species of plants can contain compounds toxic to pathogens and when extracted from them, they assume the name botanical pesticides or simpler, as write before, botanicals. Extraction is based on the use of specific solvents that solubilize compounds of similar polarity and extraction technology for separate active fractions and inert components (Lamichhane *et al.,* 2018).

The choice of the solvent is essential, starting from the best properties like low toxicity, rapid absorption, preservative action and low heat but clearly depending by the nature of the extract.

Solvents normally use are water, ethanol, methanol, chloroform, dichloro-methanol, ether and acetone, often combined to obtain the best one for the extraction, and they are specific to the compounds. Botanicals can be extracted from different plant parts and are divided in three groups: antimicrobial secondary metabolites, volatile oils and essential oils. Antimicrobial metabolites are naturally synthesized in response to microbial infection and have different mechanism of action. The Table 1 represents these molecules (Lamichhane *et al.,* 2018).

Table 1. Molecules extracted from the plant and their mechanisms (da Lamichhane *et al.*, 2018)

Compounds	**Mechanisms**
Flavonoids	Inactivate enzymes, complex with cell wall, bind to adhesins
Tannins	Substrate deprivation, enzyme inhibition, bind to proteins
Alkaloids	Intercalate into cell wall
Phenolics	Membrane disruption, substrate deprivation
Phenolics acids	Inactivate enzymes, complex with cell wall, bind to adhesins
Coumarins	Interaction with eukaryotic DNA
Lectins and polypeptides	Form disulfide bridges
Terpenoids, essential oils	Membrane disruption

Plants normally used for extracted are *Azadirachta indica* (Neem), *Eucalyptus globolus, Zingiber officinale, Nicotiana tabacum* (tobacco) and *Curcuma longa* (Lamichhane *et al.,* 2018).

Volatile oils are another category of botanicals. These compounds have a small molecular weight and play an important role in defense system with the inhibition of pathogen growth. They are isolated by hydro distillation or steam and are represented by terpinoides like monoterpenes, quiterpenes and diterpenis extracted from plant like *Piper nigrum*, *Syzygium aromaticum, Myristica fragrans, Origanum vulgare* and *Thymus vulgaris* (Lamichhane *et al.,* 2018).

The last category is essential oils, fungistatic used against post-harvest pathogens. They are terpenoides and aromatic compounds extracted from *Urtica spp., Thymus vulgaris, Eucalyptus globulus, Melaleuca alternifolia, Ruta graveolens* and *Cymbopogon fexuosus* (Lamichhane *et al.,* 2018).

Botanicals have a large spectrum of use, and in Table 2 it is possible to have some examples.

Table 2. Example of botanicals extracted from different plants parts

Plant	Part used	Preparations	Diseases
Origanum hercleoticum	Leaf	Essential oil	*Fusarium oxysporum, Phoma tracheiphila*
Azaridachta indica, Annona squamosa, Oscimum sanctum	Leaf, stem, bark, root	Crude extract	Anthracnose of pepper
Fragaria spp.	Fruit	Volatile compound	Anthracnose of strawberry
Nerium oleander	Leaf	Crude extract	*Bipolaris oryzae*
Syziugium aromaticum, Curcuma longa, Allium sativum, Ocimum sanctum	Leaf, seed, fruit	Crude extract	*Aspergillus flavus*
Brassica napus, Lycopersicon esculentum	Leaf, stem	Water extract	Bacterial disease on onions
Ocimum gratissimum, Aframomum melegueta	Leaf	Crude	Post-harvest yam (*Diascorea* spp.)

Botanicals can be considered as alternatives due to their capacity to reduce crop losses, to be easily bio-degradable and cheaper but a standard extraction method doesn't exist, so the management of these compounds is not so easy. More, they are quickly degraded and it's necessary the development of formulations (Lamichhane *et al.,* 2018).

However, also these alternatives have a legislative problem. On June 25, 2019, was published the new Regulation 2019/1009 of European Parliament and of the Council of 5 June 2019 just about these products. The full application is provided for July 16, 2022, when the entry of CE marked fertilizers and bio-stimulants, but botanicals and bio-stimulants too are excluded from been products destinated to phytosanitary protection (European Commission, 2019).

Among botanicals can also be recorded those substance tested in the present work, mainly belonging to the polyphenols family or to defense inducers from algae and yeasts (Lamichane *et al.*, 2018).
It may be worthwhile to spend some more words on another botanical having its origin in wood, having very ancient origins and called "Wood vinegar", which is the liquid associated to the wood pyrolysis. The other oily dark material produced by pyrolysis, called Tar, has been used back to Middle Palaeolithic. Products like birch bark pitch, a fraction of tar, are findable in Italy and Germany from 80.000 years ago but with different purpose then agricultural use (Tiilikkala *et al.,* 2010).

Indeed, evidences lead to think that the first use was medical like clean and refresh teeth and also as universal adhesive and mastic. Use in agriculture of plant derivates belong to the last 2000 years starting from China, Egypt, Greece and India but, more precisely, wood vinegar use as fertilizers and growth-promoting agent it's even more recent. First surely attempt as agriculture use is in 1930's because it's difficult to find scientific evidence of its use before these years in modern information retrieval (Tiilikkala *et al.,* 2010).

Liquid products by pyrolysis contain many organic compounds but the exact composition is still complicated to detect. However, fundamental compounds are methanol and acetic acids, also that acetone, furan, furfural, formic, propionic and butyric acids, methyl acetone, acetaldehyde and allyl alcohol (Tiilikkala *et al.,* 2010).

Efficiency of products with these origins is fundable in a weak series of scientific publications. Liquid produced from bamboo and broad-leaved trees were effective to control sapstaining fungi in 0.10-10% of concentrations without been toxic to the environment (Tiilikkala *et al.,* 2010).

Also, a good efficacy as insect repellant can be found in scientific literatures (Tiilikkala *et al.* 2010). Wood tars from alder, larch and birch were effective to control insects like *Clethrionomys rufocanus bedfordiae* in Japan and tars from birch is effectively efficient on *Arion lusitanicus* and *Aranta arbustorum*.

Another use is like herbicides but there is a lack of scientific evidence to prove it; vinegar at high concentrations can kill plant cells and at low concentrations can stimulate their growth (Tiilikkala *et al.,* 2010). This possibility was tested in China in a field experiment with a mixture of biochar, the solid part of pyrolysis process, and pyroligneous acid, showing a positive result on plant growth stimulation (Tiilikkala *et al.,* 2010).

Treatments based on bamboo vinegar have also a potential use for this purpose (Tiilikkala *et al.,* 2010).

2. Aim of the WORK

As a consequence of its effectiveness in controlling plant pathogenic bacteria and fungi, since more than 150 years ago copper has created a sort of addiction both in traditional and biological agriculture.

However, the increasing occurrence in agroecosystem of copper-resistant strains, both in phytopathogenic bacteria and fungi, has significantly reduced the effectiveness of copper-based plant protection products. Additionally, due to the copper toxicity and to the environmental concerns derived from its wide use, on the 1st of February 2019 a new Regulation of the European Commission established a reduction for thresholds of copper use in plant protection. However, several unclear points still remain unsolved such as how to control and verify the correct copper distribution. For instance, to use more concentrated copper solutions than what allowed in a restricted area, by exploiting the total copper amount as calculated on the total owned area.

The paramount importance to protect and maintain the environment is out of question. However, other questions are important too, such as are the potential alternatives both effective and economically sustainable? Is it realistic nowadays to affirm that they can really substitute copper-based products?

In order to find a shortcut that has now to be followed in EU for the registration of a plant protection product, many small and even big companies have focused to produce products having bio-stimulant activity, and thus an indirect increase in the plant ability to defend itself. Together with several different biocontrol agents (BCAs) they are among the candidates to substitute “traditional” copper-based plant protection products. The scientific community has a pivotal role in this context, that is to analyze any feature of these putative alternatives using the same critical spirit applied so far for copper antimicrobials, including questions concerning the future occurrence of resistance phenomena, their phytotoxicity as well as their impact on human health and on environment.

This work will focus on several botanicals, that are Wood vinegar, “Distillato di Legno”, “Frontiers 2.0”, Silvateam Agritan BQ and Agritan C-plus.

The so called “Distillato di Legno” as well as Wood vinegar have been kindly provided by BioDea. The “Distillato di Legno” is a liquid botanical obtained by the extraction from biomass of autochthone

chestnut. The extraction is based on gasification. It is produced in Val di Chiana (Arezzo, Tuscany) by BioDea and rich in acetic acid, polyphenol, tannins and over 300 organic substances, as reported on its label. It is suggested to be used by spraying on leaves or by fertirrigation. The so-called Wood vinegar is produced by pyrolysis, a physical decomposition obtained by high temperatures in inert atmosphere and contains pyroligneous acid. Both these mixtures are reported to induce plant defense and a more powerful root system.

The third botanical here used is “Frontiere 2.0” (Kalòs), an extract of yeast with fermented brown algae. These algae are reported to have been collected in Icelandic coasts, washed, selected, grinded and extracted in acid solution. According to the label, Frontiere 2.0 contain 1% of organic nitrogen and it works by inducing plant defense, thanks to the simulation of a biotic attack by putative fungi and bacteria pathogens by the carbon polymeric chains that are presents in their cellular walls.

The last two products here tested are Agritan BQ and Agritan C-plus (Silvateam SpA), both based on tannins.

Their phytotoxic and phytostimulant activities have been tested, firstly *in vitro* by several tests carried out on model plants such as *Arabidopsis thaliana* and *Nicotiana tabacum.* Then analogous field experiments on *N. tabacum* (cv. *Kentucky* and cv. *Virginia*) have been carried out.

Concerning their potential effectiveness in plant protection, *in vitro* tests have been carried out to ascertain their antimicrobial activity against phytopathogenic fungi and bacteria, including some quarantine microorganisms. Then, their anti-infectivity on Gram negative plant pathogenic bacteria has been also verified, as well as.

Afterwards, the hypersensitive response (HR) of leaves of *N. tabacum* has been tested and then tests of the inhibition capacity on different bacteria and fungi pathogens has been tested.

3. Materials and methods

3.1 Root elongation assay

Root elongation assay is a standard test adapted to verify the similar-auxin effect by any substance including the botanicals here tested. In this case, test has been carried out on *A. thaliana* and *N. tabacum* seedlings with "Distillato di Legno", "Frontiere 2.0", Silvateam Agritan BQ, Silvateam Agritan C-plus, and by using IAA and distilled sterile water as positive and negative controls, respectively.

A. thaliana seeds have been sterilized in 1ml of ethanol, keep for 10 minutes and shaked, then in a sterilization solution (5 drops of Tween-20 in 1ml of sodium hypochlorite), kept for 5 minutes and shaked every 2 minutes. After this, seeds have been washed with 1 ml of distillated sterile water for 5 times and maintained in 500 µl of sterile distilled water.
Then, preparation of Murashige and Skoog 0 (Murashige and Skoog, 1962) plates has been made; the "zero" indicates the lack of sucrose. 1L of MS for medium is prepared with the formula reported in Table 3.

Table 3. MS0 composition

Components	MS0
MS powder	2.22 g
Phytoagar (7%)	7.5 g
Distillate water	Added to reach 1 L of final volume

After autoclaving at 121°C for 20 minutes, the medium has been poured in Petri dishes. After their solidification, *A. thaliana* seeds have been put on and incubate for 48h at 4°C for vernalization. After that time, seeds have been put in growth chamber at 25°C with a photoperiod of 16h of light and 8h of dark.

After 5 days of germination, 7 seedlings have been put on new MS (same composition of MS0 with addition of sucrose at 0,5% concentration) plates, put at a 65°C angle to allow the best root propagation in the medium. Seedlings have been positioned 4 cm distant a line where the substances

to be tested have been then spotted. Test have been set up with a 15 µl spot for each decimal dilution (0.1, 0.01 and 0.001), and for the negative and positive controls given by distilled sterile water and by IAA, respectively. As far as IAA is concerned, the concentrations used have been 10, 50, 100 and 200 µM. The lenght of root apparatus and its architecture have been evaluated after 15 days of growth.

The radication test carried out on *N. tabacum* has been very similar to that on *A. thaliana*. The main differences are about the seeds sterilization, where the solution of sodium hypochlorite plus Tween-20 has been substituted by sodium hypochlorite at 5% concentration. Seeds have been put directly in MS plate in growth chamber at 25°C with a photoperiod of 16h of light and 8h of dark without the vernalization, because *N. tabacum* seeds have been preserved in fridge. Data have been collected at 5, 10 and 15 days of growth.

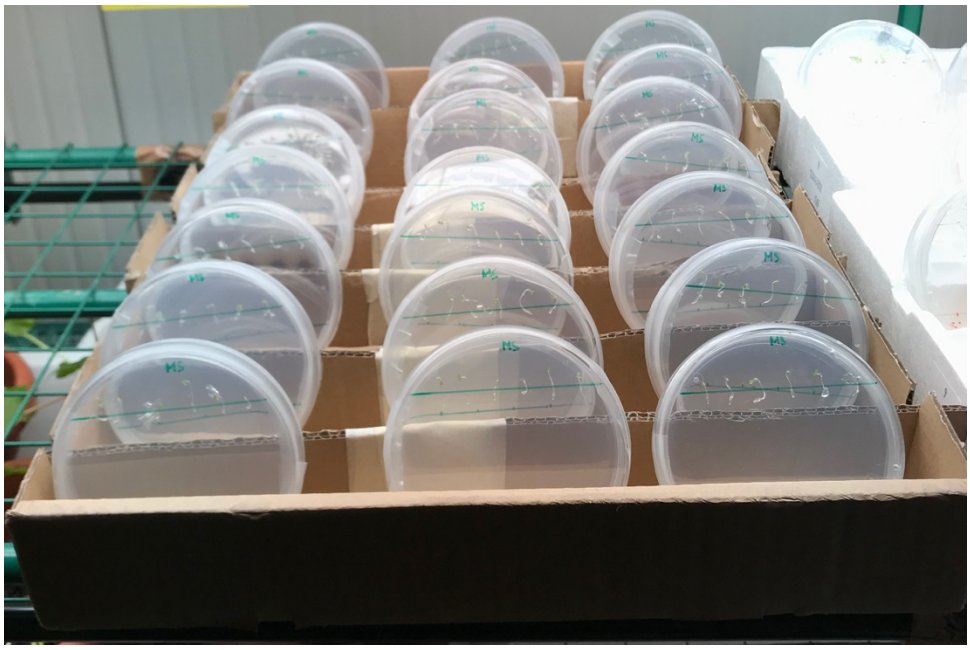

Figure 5. Root elongation assay.

3.2 Ion Leakage

Briefly, plants have the capacity to autoinduce localize cell death, thus to stop an infection just where the pathogen tried penetration. This process is called “Hypersensitive Response” (HR), and allows the suppression of disease progress, totally and not.

HR is induced by different organisms like fungi, bacteria and viruses but also insects and nematode thanks to microbial molecules known as microbe and pathogen-associated molecular patterns (MAMPs and PAMPs) (Balint-Kurti, 2019).

The recognition is related to a specific class of proteins acting as cognate receptors, called Patterns Recognition Receptors (PRRs). They have the capacity to activate a defense response in the plant called MAMP/PAMP-triggered immunity (MTI/PTI). These two are qualitatively similar to another specific response induced by NBS-LRR proteins (NLRs), proteins that possess a nucleotide-binding site (NBS) and leucine-rich repeat domains (LRR) (Balint-Kurti, 2019).

These proteins are encoded by R-gene, a gene the mediated resistance strongly associated to HR response (Balint-Kurti, 2019).

Hypersensitive response lead to a rapid programmed cell death (PCD) on the area of the attack, and with the early production of reactive oxygen species (ROS) and with the release from the plant cells of K^{+}/H^{+} ions. Indeed, ions release can be determinate by placing pathogen-inoculated plant in water and measuring the increase of conductivity, due to the electrolytes release by the dying plant cells (Johansson *et al.,* 2015).

The test is based on the opportunity to measure the ion leakage induced also in absence of on infection by simulating a pathogenic attack. Here "Distillato di Legno" and "Frontiere 2.0" have been used. As positive control, *Pseudomonas syringae* pv. *tomato* (DC3000) has been selected. Pre-culture of the bacterium has been done in liquid KB (Table 8) medium is reported in 3.5.2.

The protocol of ion leakage starts with the preparation of leaf disks, having a 0.7 cm diameter, obtained with a sterile cork borer from leaves of *N. tabacum* cv. *Virginia* 6178. Then, 6 disks have taken and immerged in 10 ml of products with different dilutions:

- "Distillato di Legno" as such, 1:10, 1:100, 1:500, 1:1000 plus a spot with a negative control represented by demineralized water;
- "Frontiere 2.0" as such, 1:10, 1:100, 1:1000 plus two spots with a negative control (demineralized water) and a positive control*;*

A negative pressure has been applied (2.0 atm for 5 minutes) to allow the adsorption by the cell into mesophyll. After this, the disks have been dried up with filter paper and resuspended in 10 ml of

demineralized water in 6 spots plates. Measures of conductivity of the demineralized water have been taken at T_0, T_3, T_6, T_{24} and T_{48} maintaining the disks in agitation for all the times (Johansson *et al.,* 2015).

3.3 HR test on Tobacco

HR test on tobacco plants, that is *N. tabacum* cv. *Kentucky* in this case, is a standard protocol based on the infiltration of bacterial Gram-negative cells into intact leaves (Umesha *et al*., 2008). It is generally used to test any feature of Gram-negative plant pathogenic bacteria related to their TTSS, as well as to test induction of plant defense responses by soluble substances. In this work, it has been used to test the ability of "Distillato di Legno" and "Frontiere 2.0" to activate HR and, thus the programmed cell death. The dilutions used have been for "Frontiers 2.0" 1:500 and 1:1000 and for "Distillato di Legno" 1:500 and 1:1000. The choice of the products to be teste das well as of their dilutions was related to their density that, when very high, make impossible infiltration. As positive control, *P. syringae* pv. *tomato* DC3000 was used. Data has been collected after 24h from the products and bacterium inoculation.

3.4 Conservation of fungi and bacteria

3.4.1 Fungi and bacteria tested

The ability of the botanicals here tested to inhibit fungal and bacterial growth has been assayed, using different fungi and bacteria selected from the collection of the Laboratory of Molecular Plant Pathology of the Department of Agri-food and Environmental Sciences (DAGRI), Università degli Studi di Firenze (UNIFI).

The phytopathogenic fungi here tested are *Sclerotinia rolfsii, S. sclerotiorum, Fusarium graminearum, F. culmorum, F. avenaceum, F. poae, Alternaria alternata, Cryphonectria parasitica, Phytophthora cinnamomi, P. infestans, Gnomoniopsis castaneae, Monilinia fructigena* and *Verticillum longisporum* (Table 4).

S. rolfsii is a fungus typical of tropical and sub-tropical region, in Italy presents in the central-southern regions. It's a polyphagous pathogen that attack the root part with symptoms viewable as leaf yellowing due to a general weakening of the plant (Tarabbia, 2002).

S. sclerotiorum, pathogen agent of white mold, can be observed with immediate wilting of leaves and then plant death. It can attack a large number of plant and it produce typical black survival structures known as Sclerotia (Watson and Smith, 2013).

The genus *Fusarium* is typical on rice, bean, soybean and other cereals, causing disease generally reported under the name of fusariosis. The species contained in *Fusarium* genus are over 20 and generally cause root, stem and fruit rot, vascular wilt and post-harvest decomposition. They are considered weak pathogen agents and symptoms can be viewable on stressed or wounded plant (Askun, 2018).

A. alternata is a pathogen agent in this case isolated from apple but active on different plants like kiwi, tomato and so on. Typical in high humidity areas, it attacks leaves and stem generating spots of variable diameter (Esmailzadeh *et al*, 2008).

Cortical chestnut cancer is a disease caused by *C. parasitica,* a fungus active both on younger and older plant with specific symptoms as cortical reddening and consequence desiccation of the apical part. Hit chestnuts react by emitting new branches that, however, can show symptoms in the following years (Rigling *et al.,* 2016).

The genus *Phytophthora* tested, *infestans* and *cinnamomi,* belong to the *Oomycota* class, *Chromista* reign (Nowicki *et al.,* 2011).

Despite this difference, *P. infestans* attack tomato and potato plants causing rot on stems, root, bulbs, tuber, leaves and fruit and it is recognizable by the watery spots and white mold on the leaves (Nowicki *et al.,* 2011).

While *P. cinnamomi* is more typical on ornamental plant and symptoms are not too much different than *infestans,* causing wilting and yellowing on leaves and darkening on roots (Cahill *et al.,* 2008).

Carrying on, *G. castaneae* is the pathogen agent of brown (or chalky) rot of chestnut (Lione *et al.,* 2018).

Symptoms start from inside the fruit and are not viewable on the outside. First, the endosperm becomes brown and then harder, assuming a whitish and chaklis consistence, beside the pulp that became soft and inconsistent, making the fruit not marketable (Lione *et al.,* 2018).

M. fructigena is a pome-associated fungus that hits fruits and young shoots; on the firsts it's provokes soft rot, on the seconds localized cancer and dieback. In particular, the fruits are hit starting from early stages of development with the subsequently dark mold formation (Xu and Robinson, 2003).

The last tested fungus is *V. longisporum,* the cause of *Verticillum* wilts of oilseed rape and generally *Brassicaceae* plant hosts. The disease symptoms are dark stripes on the stems of healthy-looking plants beside the formation of microsclerotia beneath the epidermis (Depotter *et al.,* 2016).

The plant pathogenic bacteria here tested are *P, savastanoi* pv. *nerii* strain Psn23, *P. savastanoi* pv. *nerii* strain Psn23 pT3-GFP and pv. *savastanoi*, *C. michiganensis* subsp. *michiganensis* (NCPB 382), four pathovars of *P. syringae* (*actinidiae, tomato, phaseolicola* and *tabaci*), eight pathovars of *X. axonopodis* (*euvesicatoria, vesicatoria, juglandi, perforans, gardneri, alpha-alpha, pelargoni* and *phaseoli*) and five pathovars of *C. flaccumfaciens* (*ilicis, ortii, flaccumfaciens, betae* and *poinsettiae*) (Table 4).

P. savastanoi survive as endophyte and epiphyte on different parts of the plant like leaves and fruits, preferring summer and autumn because of better climatic conditions, and it enters moving on rainwater or irrigation water using plant wounds (Lutri, 2015). Pathovar *savastanoi* has identical symptom of pv. *nerii* but differently its plant host is olive tree (Hosni *et al.,* 2011).

Psn23 wild-type is identical to Psn23 pt3-GFP strain except for the integration by this last of the plasmid pLPCM_T3_GFP (Chicaybam *et al.,* 2017).

The plasmid contains a reporter gene called Green Fluorescent Protein (gfp), a protein extracted from the Pacific Northwest jellyfish *Aequorea victoria* (Ormö *et al.*, 1996) that absorbs ultraviolet light and emits a lower-energy light (Goodsell, 2003).

pLPCM_T3_GFP plasmid has been set up in the Laboratory of Molecular Plant Pathology of the Department of Agri-food and Environmental Sciences (DAGRI), Università degli Studi di Firenze (UNIFI). *Gfp* gene transcription is activated by a promoter called T3 related to *hrpA*, a gene of the type III secretion system (T3SS), with a consequence measurable fluorescent emission.

P. syringae is a Gram-negative bacterium. The species includes over 60 pathovars and in this work have been tested 4 of them, each one specific for a host plant: *actinidiae* for kiwi plant, *tomato* for tomato, *phaseolicola* for bean and *tabaci* for tobacco. The access inside the plant is through stomata and biological/provoked wounds (Xin *et al.,* 2018), generating symptoms recognizable as cancer of the plant as reported in paragraph 1.4.

X. axonopodis species is a Gram-negative pathogen that attack different plant hosts (Gottwald *et al.,* 2002).

The bacterium has the capacity to produce similar symptoms on different parts of the plant like pods, leaves, seed and rarely stems (Gottwald *et al.,* 2002). These symptoms are water soaked small spots

on the leaves that enlarge and became necrotic and brown, often surrounded by a yellowing on the tissues around the spots. On stems and pods the spots are similar than leaves but, in these cases, they became brown without chlorosis (Gottwald *et al.,* 2002).

C. flaccumfaciens is a Gram-positive bacteria species tested in this work and attack several plant hosts with peculiar symptoms that are on the leaf with wilt and chlorosis (Francis *et al.,* 2011; Sammer and Reiher, 2012).

Another Gram-positive bacterium it's also been tested: *C. michiganensis* pv. *michiganensis* strain NCPB 382. The bacterium is causal agent of bacterial canker of tomato, one of the most destructive bacterial disease on this plant (Lyu *et al.,* 2019).

The symptoms are stem cankers, spots on the fruit, chlorosis on leaves with wilting and a general inhibition of plant growth (Lyu *et al.,* 2019).

Table 4. Fungi and Bacteria tested in this work

Fungi	Bacteria
S. rolfsii	*P. savastanoi* pv. *nerii* strain Psn23
S. sclerotiorum	*P. savastanoi* pv. *nerii* strain Psn23 pT3-GFP
F. graminearum	*P.savastanoi* pv. *savastanoi*
F. culmorum	*C. michiganensis* subsp. *michiganensis* NCPB 382
F. avenaceum	*P. syringae* pv. *actinidiae*
F. poae	*P. syringae* pv. *tomato* DC3000
A. alternata	*P. syringae* pv. *phaseolicola*
C. parasitica	*P. syringae* pv. *tabaci*
P. cinnamomi	*X. axonopodis* pv. *euvesicatoria*
P. infestans	*X. axonopodis* pv. *vesicatoria*
G. castaneae	*X. axonopodis* pv. *juglandi*
M. fructigena	*X. axonopodis* pv. *perforans*
V. longisporum	*X. axonopodis* pv. *gardneri*
	X. axonopodis pv. *alpha-alpha*
	X. axonopodis pv. *pelargori*
	X. axonopodis pv. *phaseoli*
	C. flaccumfaciens pv. *ilicis*
	C.flaccumfaciens pv. *ortii*
	C. flaccumfaciens pv. *flaccumfaciens*
	C. flaccumfaciens pv. *betae*
	C. flaccumfaciens pv. *poinsettiae*

3.4.2 Growth media and culture maintenance

Fungi and bacteria have been grown on specific culture media accordingly to their needs.

For each fungus, except for *P. infestans* and *cinnamoni,* the medium used has been the "homemade version" of Potato Dextrose Agar or simpler PDA (Table 5) (Beever and Bollard, 1970), because several fungi are unable to grow on commercial PDA. Preparation starts with boiling 200 g of sliced potatoes in 1 L of distilled water for 30 minutes, then potatoes are removed and the liquid filtered to eliminate solid residues. The filtrate is then mixed with dextrose, phytoagar and distilled water to reach the final volume of 1 L, thus to replace the amount of distilled water evaporated during the initial potatoes boiling. The final step is to autoclave the medium at 121°C for 20 minutes.

Only for the *Phytophthora* genus have been used a more specific medium called Pea Agar Medium (PAM) (Tomada *et al.,* 2016), prepared with 125g of frozen peas, phytoagar and distilled water (Table 6). The peculiarity of this medium is the necessity of two cycles of autoclaving at 121°C for 20 minutes: the first with only peas and water, the second without peas and just for medium sterilization.

Table 5. Growth media composition used for fungi

Composition	Potato Dextrose Agar (PDA)	Pea Agar Medium (PAM)
Distilled water	1 L	1 L
Phytoagar	20 g	12-13 g
Potatoes	200 g	
Peas		125 g
Dextrose	20 g	

Transfer of fungi in a new plate is made under sterile hood with a sterilized-by-fire bistoury and then cutting and removing a little piece of 5 mm that is put on the new agar.

However, as solid bacteria medium is been used solid KB for all the bacteria of genus *Pseudomonas*, nutrient sucrose agar or NSA (Vidaver, 1980) for the pathovars of *Curtobacterium* and *Clavibacter* and yeast extract dextrose or YDC (Schaad, 1985) for Xanthomonas. The composition of this media is reported in Table 6.

For *Pseudomonas*, the antibiotic nitrofurantoin is added to the solid medium starting from a stock solution of 10 mg/ml to a final of 10 µg/ml. Calculation is by the standard Ci*Vi=Cf*Vf in a final volume of 1L.

$$10\ mg/ml * X = 10\ \mu g/ml * 1000\ ml$$

The quantity of antibiotic to add is 1000 µL (1ml). The operation is done waiting 20-30 minutes after autoclaving; this avoid the risk of antibiotic deactivation.

All the bacteria are transferred under sterile hood with a sterile loop. So, single colony are taken and strips on the new medium by loop.

Table 6. Growth media composition used for bacteria

Composition	Solid KB	Nutrient Sucrose Agar (NSA)	Yeast Extract Dextrose (YDC)
Distilled water	1 L	1 L	1 L
Tryptone (DIFCO)	10 g		
Proteose peptone	10 g	5 g	
Yeast extract		3 g	10 g
K_2HPO	1.5 g		
$CaCO_3$			20 g
NaCl		5 g	
Sucrose		5 g	
D-glucose			20 g
Glycerol	10 gr or 7.93 ml		
$MgSO_4\ 7H_2O$	1.5 gr or 6.1 ml		
BactoAgar	15-20 g	12 g	15 g

Bacteria and fungi plates have been transferred into an incubator at 25°C for 3-5 days. Then, colonies can be transferred in fridge at 4°C for conservation.

3.5 *In vitro* growth inhibition test on fungi and bacteria

3.5.1 Fungal inhibition test

Fungal cultures of 5-6 days have been taken from the growth plates and transferred on the new ones using the specific media for each one: Peas Agar Medium (Tomada *et al.,* 2016) for *P. infestans* and *cinnamomi,* Potato Dextrose Agar (Beever and Bollard, 1970) for the others.

Transfer is done by sterilized scalpel cutting a piece of agar from the original colony and putting it on a new plate.

Test on *S. rolfsii* has been done with “Distillato di Legno”, “Frontiere 2.0”, Silvateam Agritan BQ and C-plus with two replicates for each one. Plates have been divided in 6 parts and a different approach on the two replicates have been applied. Indeed, the test on the first replicate has been with the direct application of a 15 µL spots on the agar, the second with sterile cellulose disks soaked by 15 µL of products. Also, a plate divided in 6 parts with sterilized water as negative control has been prepared; 3 parts with direction application of 15µL spots on agar and the other 3 with sterile cellulose disks soaked.

Dilutions of products used have been 2 spots at 0.1, 2 spots 0.01 and the last 2 spots 0.001, both for agar-directly and for cellulose disks.

The same products have been tested on the other fungi *S. sclerotiorum, F. graminearum, F. culmorum, F. avenaceum, F. poae, A. alternata, C. parasitica, P. cinnamomi, P. infestans, G. castaneae, M. fructigena* and *V. longisporum* but with a different formulation.

Fungi have been transferred by sterilized bistoury in the same way of *S. rolfsii,* but products application has been difference. Plates have been divided in 5 parts, excavated in the agar with a cork borer of 7 mm of diameter and the dowels removed with a sterilized bistoury. Subsequently, wells have been filled with 50 µL of products as such and water as negative control.

All the preparade plates have been put in incubation at 25°C for 6 days to then measure the radius of the single fungus and see the possible inhibition effect of the products.

3.5.2 Bacterial inhibition test

Bacteria used *in vitro* test have been taken from the original colony plates previously prepared and transferred in specific liquid medium for pre-culture preparation. Liquid media are specific for each bacterium tested and, in this work, have been used KB (Stacy and King, 1954) for *Pseudomonas,* LB “Luria and Bertani” (Miller, 1972) for *Curtobacterium* and *Clavibacter* and NB “Nutrient brooth” (Fay and Persley, 1983) for *Xanthomonas* (Table 7).

Table 7. Liquid media composition used for bacteria pre-cultures

Composition	Liquid KB	LB	NB
Distilled water	1 L	1 L	1 L
Yeast extract		5 g	2 g
Tryptone (DIFCO)	10 g	10 g	
Proteose peptone	10 g		5 g
Beef extract			1 g
K_2HPO_4	1.5 g		
NaCl			5 g
Glycerol	10 g or 7.93 ml		
$MgSO_4$ $7H_2O$	1.5 g or 6.1 ml		

The preparation of the pre-cultures started after autoclaving the media. 20 ml of the liquid medium have been taken and put in a 50 ml Falcon, then, in the case of KB two antibiotics have been added: gentamycin and nitrofurantoin. Calculation of the quantity has been done starting from a stock solution of 10 mg/ml to reach a final concentration of 10 µg/ml. The equation used is the standard Ci*Vi=Cf*Vf. So, for each the antibiotics the calculate is:

$$10\ mg/ml * X = 10\ \mu g/ml * 20\ ml$$

The result is 20 µl for gentamycin and 20 µl for nitrofurantoin. So, bacterium has been added to the medium by an inoculation loop and left growth in agitation overnight for 12 hours.
The day after 1ml has been measured $OD_{600,}$ optical density at 600 nm that is a value of absorbance at 600nm essential to monitor the bacterial growth, reaching 1.284. Normally, as referment it's used the value 0.5, so the equation Ci*Vi=Cf*Vf is used to calculate the volume to use. Formula is:

$$1{,}285 * X = 0{,}5 * 20\ ml$$

With the result of 7.8 ml, this quantity has been taken from the 20ml and put in a new falcon. The next part is washing the bacterium culture and suspend in saline solution called physiologic solution, which is obtain with distilled water (500 ml) and NaCl (0.85%), autoclaved for 20 minutes at 121°C. The resuspension of the pellet is obtained by centrifugation at 5000 g for 10 minutes and then, after throwing away the old liquid, washed with 7.8 ml of physiologic solution; the process has been repeated for 3 times.

The pre-cultures preparation of *Xanthomonas*, *Curtobacterium* and *Clavibacter* genus was the same expect for of quantity of liquid medium used, 15 ml instead of 20 ml, and the absence of any antibiotics.

Bacteria cells have been left in agitation overnight at 25°C and the next day OD_{600} has been measured. Application of the equation Ci*Vi=Cf*Vf is necessary to calculate the culture volume to obtain a final volume of 20ml with the same concentration OD_{600} measured.
200 µl for each bacterium have been taken and plated on new KB, YDC and NSA plates (Table 7), then the agar has been excavated with a cork borer of 7 mm of diameter and dowels removed with a sterilized bistoury.
Spots have been filled with "Distillato di Legno" and Wood Vinegar as such and diluted 1:10, 1:100 and 1:1000. For each bacterium 4 plates divided in 6 parts have been used and organized as:

- A plate with 3 spots filled with "Distillato di Legno" as such and the other 3 with "Distillato di Legno" 1:10;
- A plate with 3 spots filled with "Distillato di Legno" 1:100 and the other 3 with "Distillato di Legno" 1:1000;
- A plate with 3 spots filled with Wood Vinegar as such and the other 3 with Wood Vinegar 1:10;
- A plate with 3 spots filled with Wood Vinegar 1:100 and the other 3 with Wood Vinegar 1:1000;

The prepared plates have been put in the incubator at 25°C for 5 days and then measures of the diameter of the areas of inhibition of growth around the wells have been collected and reported as average.

3.5.3 Biomolecular method: anti-infective activity on *Pseudomonas savastanoi* pv. *nerii* strain Psn23 and Psn23 pT3-GFP

Transformed *Pseudomonas savastanoi* pv. *nerii* strain Psn23 with pLPCM_T3_GFP plasmid and *Pseudomonas savastanoi* pv. *nerii* strain Psn23 have been tested with Distillato di Legno. The high

density of the other products and their spectra did not allow their use in this test, where the fluorescent emission by Green Fluorescent Protein (*gfp*) of Psn23 pT3-GFP was measured.

Pre-culture have been prepared in liquid KB (Table 7) + nitrofurantoin for Psn23 and liquid KB + nitrofurantoin + gentamycin for Psn23 pT3-GFP, both starting from a stock solution of 10 mg/ml to a final of 10 µg/ml in a final volume of 13 ml for both the bacterium.

Bacteria have been grown overnight and used with OD_{600} = 0.5, centrifuged at 5000g for 10 minutes and washed two times with sterile physiologic solution. Then, the supernatant has been removed and the bacterium pellet has been resuspended in liquid KB and MM.

Minimal Medium (MM) (Huynh *et al.*, 1989) is a medium capable to imitate the same conditions inside plant apoplast, inducing the activity of the type III secretion system in the bacterium; its composition is reported in Table 8. At the end, it's sterilized by filtration and it must have a pH of 5.7, essential to the activation of the T3SS pH correction.

Table 8. Minimal Medium (MM) preparation

Composition	Minimal Medium (MM)
Distilled Water	500 ml
Potassium Phosphate Buffer	250 ml
$(NH_4)SO_4$	0.5 g
NaCl	10 g
$MgCl_2$ (1M)	850 µL
Fructose	0.9 g

Table 9. Potassium phosphate buffer preparation for MM

Composition	Potassium Phosphate Buffer
Distilled water	900 ml
K_2HPO_4	8.5 ml
KH_2PO_4	91.5 ml

The multiwell plates (24 wells each) have been inoculated with bacteria, treated or untreated with "Distillato di Legno" as reported in Table 10. Absorbance at 600nm and fluorescence at 485nm/535nm have been measured at done at T_0, T_2, T_{24} and T_{48} from the inoculation. Data obtained have been then used to calculate the relative fluorescence as following:

$$\frac{Fluorescence\ (485nm/535nm)}{Adbsorbance\ (600nm)}$$

Table 10. Organization of the three plates used to test anti-infective activity of "Distillato di Legno"

KB plate	MM plate	"Whites" plate
1 ml of Psn23 + IAA 200 µM	1 ml of Psn23 + IAA 200 µM	1 ml of KB
1 ml of Psn23 pT3-GFP + IAA 200 µM	1 ml of Psn23 pT3-GFP + IAA 200 µM	1 ml of MM
1 ml of Psn23 + "Distillato di Legno" 1:10	1 ml of Psn23 + "Distillato di Legno" 1:10	1 ml of KB + IAA 200 µM
1 ml of Psn23 + "Distillato di Legno" 1:100	1 ml of Psn23 + "Distillato di Legno" 1:100	1 ml of MM + IAA 200 µM
1 ml of Psn23 + "Distillato di Legno" 1:1000	1 ml of Psn23 + "Distillato di Legno" 1:1000	1 ml of KB + "Distillato di Legno" 1:10
1 ml of Psn23 pT3-GFP + "Distillato di Legno" 1:10	1 ml of Psn23 pT3-GFP + "Distillato di Legno" 1:10	1 ml of KB + "Distillato di Legno" 1:100
1 ml of Psn23 pT3-GFP + "Distillato di Legno" 1:100	1 ml of Psn23 pT3-GFP + "Distillato di Legno" 1:100	1 ml of KB + "Distillato di Legno" 1:1000
1 ml of Psn23 pT3-GFP + "Distillato di Legno" 1:1000	1 ml of Psn23 pT3-GFP + "Distillato di Legno" 1:1000	1 ml of MM + "Distillato di Legno" 1:10
		1 ml of MM + "Distillato di Legno" 1:100
		1 ml of MM + "Distillato di Legno" 1:1000
		1 ml of KB + Psn23
		1 ml of KB + Psn23 pT3-GFP
		1 ml of MM + Psn23
		1 ml of MM + Psn23 pT3-GFP

3.6 In field tests

The two cultivars of *N. tabacum* Virginia ITB and Kentucky Foiano have been tested directly into field with "Distillato di Legno" and "Frontiere 2.0".

Seedlings have been planted with peat burying in 3 of June 2019 in Pistoia, Toscana, in clayey-silty field worked with a shallow plowing. Planting distance used have been different between the two cultivars: inter-row distance for Virginia has been 30 cm and for Kentucky 1 m, while the distance between the rows has been 1 m for each the cultivars (Figure 6). All the 184 plants (92 Virginia and 92 Kentucky) have been fertilized with urea (36% of N) and irrigated every two days to maintain a high vigor.

Figure 6. Planting of *N. tabacum* cv. Virginia

The two products have been dispensed in two different ways: the first as 1:600 dilution on the leaves, and as 1:1200 dilution as fertirrigation, while the second just as such and on leaves.
The calendar of treatments with each product is reported in Table 11.

Table 11. Calendar of the different treatments in field

Number of treatment	"Distillato di Legno" 1:600 foliar	"Distillato di Legno" 1:1200 fertirrigation	"Frontiere 2.0" as such foliar
1°	13/06/2019	13/06/2019	05/07/2019
2°	19/06/2019	19/06/2019	
3°	26/06/2019	26/06/2019	
4°	02/07/2019	02/07/2019	
5°	11/07/2019	11/07/2019	

For a better management of the treatments, plants have been divided in three parcels: parcel 1 (92 plants) has been dedicated to "Frontiere 2.0" and no-treatment, parcels 2 (46 plants) and 3 (46 plants) respectively to foliar treatments and fertirrigation with the Distillato.

In September 2019 data have been collected with measure of the height of the plants, number and weight of leaves and photosynthetic efficiency measurement to see the possible difference between treated and no treated. This last measure has been done thanks to an instrument called Handy PEA by Hansatech Instruments (Hansatech Instruments).

Handy PEA (Plant Efficiency Analyzer) measures chlorophyll fluorescens signals emitted from green plants thanks to the application of leafclips (Figure 7). These can be closed to pre-darken the leaf in an area of 4 mm and, after 20 minutes, re-opened with the application of the sensor head of the instrument. The sensor has the capacity to produce a red light of 650 nm readily absorbed by chloroplasts and collect the data directly on the internal storage of the machine.

Figure 7. Leafclips used to predarken the leaf

Data collected are:

- F_0, used to indicate the loss of energy during the transfer between the antenna and the reaction center of the photosystem II (Bussotti *et al.*, 2012);

- $F_{m,}$ the maximum fluorescence value observable when the leaf receives a light impulse after pre-darken, usually measurable after 1 second (Bussotti *et al*., 2012);
- F_v, the variable component of fluorescence obtainable by subtraction of F_0 from F_m. Low value indicate a low activity of the photosystem II (Bussotti *et al*., 2012);
- Φ_{P0} (F_v/F_m), a value that indicates the maximum quantum yield of primary photochemistry in the induced dark state. It is the maximum probability of energy absorption by reaction center of the photosystem II (Bussotti *et al*., 2012);
- Ψ_0, the probability that an electron captured by the reaction center is transfered other the plastoquinone A (Q_A^-) in the photosystem II (Bussotti *et al*., 2012);
- Performance Index (PI_{abs}), expression of the potential capacity of energy conservation (Bussotti *et al*., 2012);

The plants analyzed have been picked randomly putting the leafclips on high-positioned and low-positioned leaves, two for plants, to collect difference on the same plant of tobacco.
The number of analyzed subjects has been 20 plants for foliar treatment and 20 for fertirrigation treatment by "Distillato di Legno" (10 Kentucky and 10 Virginia), 10 plants for foliar treatment by "Frontiere 2.0" (5 Kentucky and 5 Virginia) and 20 for no treatment (10 Kentucky and 10 Virginia).

4. Results

4.1 Root elongation test in *A. thaliana* and *N. tabacum*

As reported in Materials and Methods 3.1, tests of root elongation have been done on *A. thaliana* and *N. tabacum,* both using seedlings grown on MS medium.
For the first measurements have been done after 15 days of growth, while on *N. tabacum* in 5, 10 and 15 days, using the products "Distillato di Legno", "Frontiere 2.0", Silvateam Agritan BQ and C-plus, and two control, a negative with water and a positive with IAA.
Data have been recorded as root length (cm ± standard error) (Figure 8 and Table 13, Table 14, 15 and 16).

A. thaliana

Table 13. *A. thaliana* roots measures in cm at 15th day

	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6	Plant 7
"Distillato di Legno" 1 (WV1)	3.3 ± 0.59	1.5 ± 0.59	3.8 ± 0.59	4.2 ± 0.59	1.3 ± 0.59	0.45 ± 0.59	0.65 ± 0.59
"Distillato di Legno" 2 (WV2)	3 ± 0.93	5.4 ± 0.93	1.2 ± 0.93	6.5 ± 0.93	0.4 ± 0.93	6 ± 0.93	1.95 ± 0.93
IAA 1	0.55 ± 0.33	0	0.45 ± 0.33	0.5 ± 0.33	1.85 ± 0.33	0.5 ± 0.33	2.35 ± 0.33
IAA 2	4.55 ± 0.93	5.5 ± 0.93	5.8 ± 0.93	0.45 ± 0.93	5.6 ± 0.93	0.45 ± 0.93	5.6 ± 0.93
C-plus	0	1.55 ± 0.28	2.35 ± 0.28	1.6 ± 0.28	2.05 ± 0.28	1.25 ± 0.28	1.5 ± 0.28
BQ	0.6 ± 0.80	3.5 ± 0.80	0.55 ± 0.80	4.6 ± 0.80	1.95 ± 0.80	5.6 ± 0.80	0.45 ± 0.80
"Frontiere 2.0"	0.2 ± 0.65	0.45 ± 0.65	0	0.55 ± 0.65	0	4.85 ± 0.65	0.8 ± 0.65

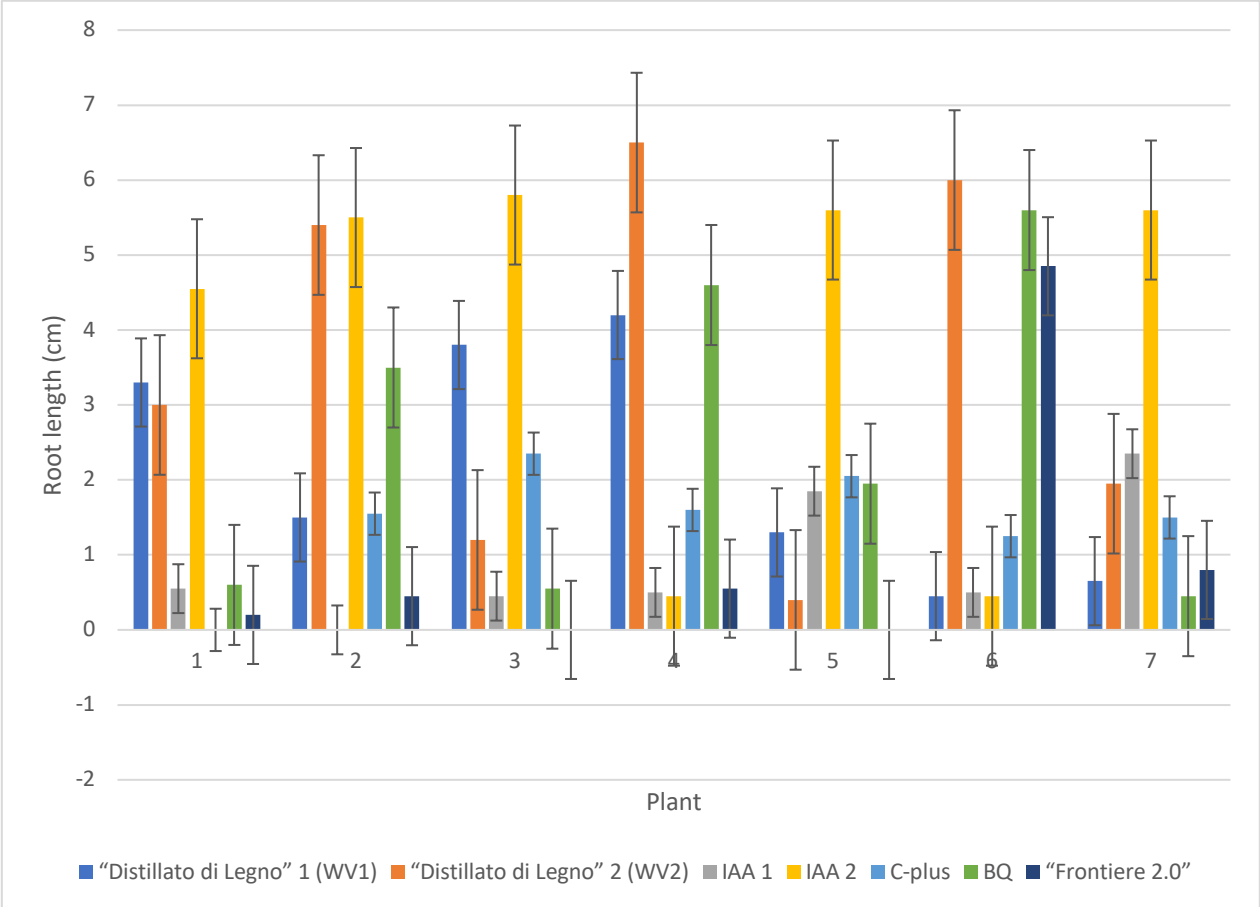

Figure 8. Comparison between root length of *A. thaliana* with different the treatments

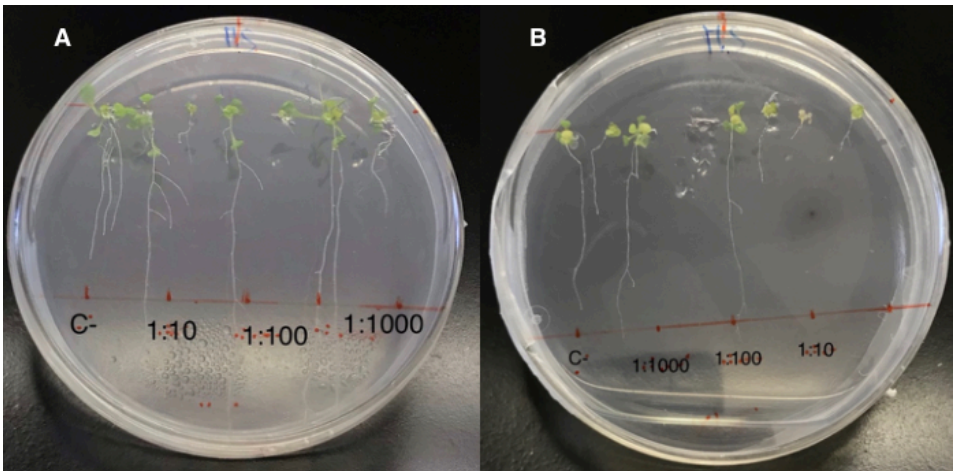

Figure 9. WV2 (A) and WV1 (B) plates. The two replicates have spots positioned in different way to analyze the effect on root growth

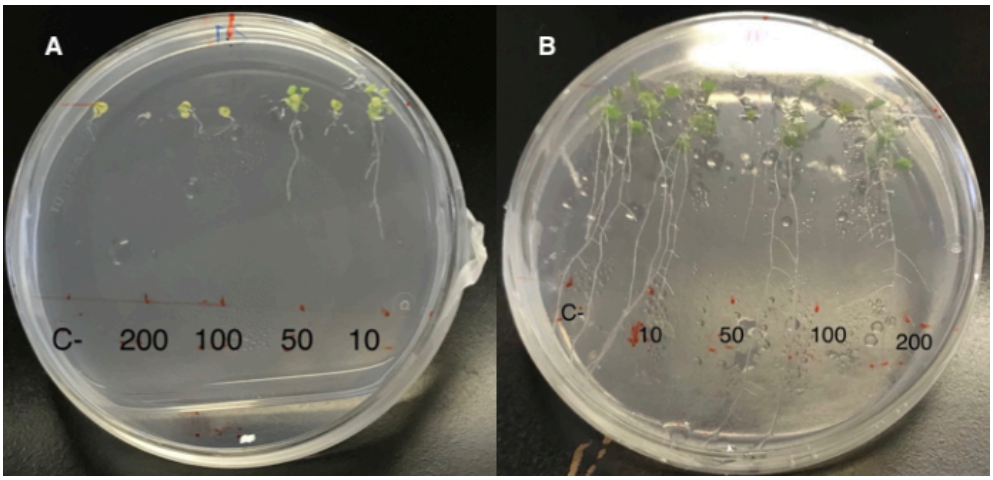

Figure 10. IAA1 (A)and IAA2 (B) plates. The two replicates have spots positioned in different way to analyze the effect on root growth

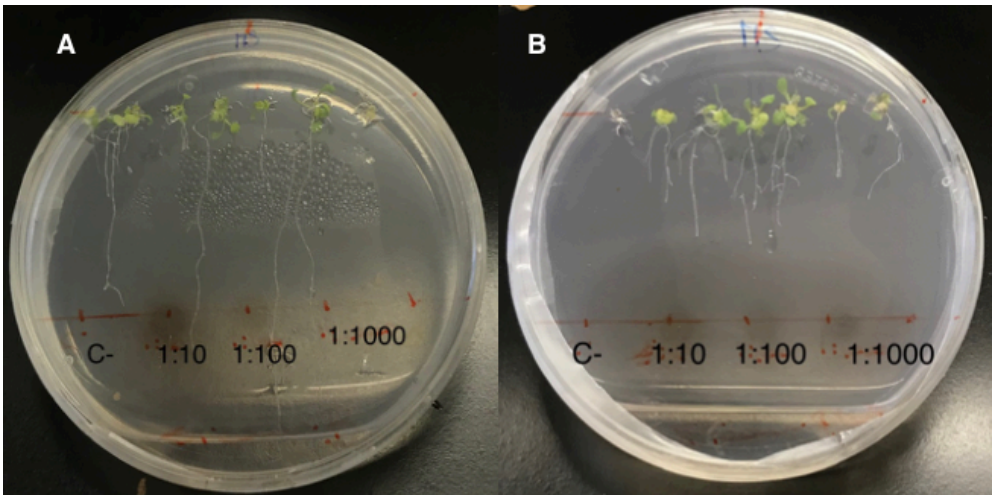

Figure 11. Silvateam Agritan BQ (A) and C-plus (B) plates

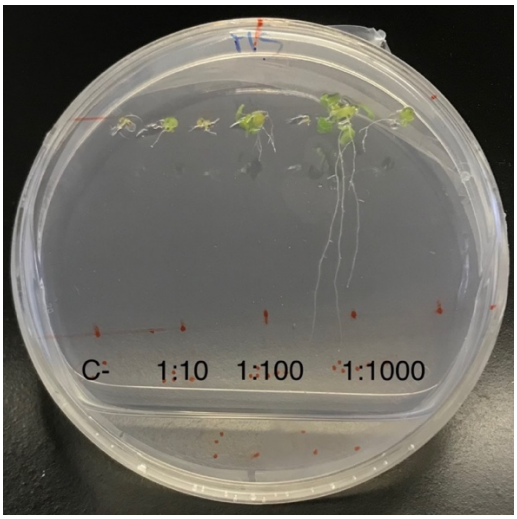

Figure 12. "Frontiere 2.0" plates

N. tabacum

Table 14. *N. tabacum* roots measures in cm ± standard error at 5th day. "Distillato di Legno" data is reported as "D" and "Frontiere 2.0" data as "FR"

	Plant 1	**Plant 2**	**Plant 3**	**Plant 4**	**Plant 5**	**Plant 6**
D as such	0	0.6 ± 0.14	0.6 ± 0.14	0.4 ± 0.14	0.5 ± 0.14	0.4 ± 0.14
D 1:10	1.3 ± 0.14	1.5 ± 0.14	1.6 ± 0.14	2 ± 0.14	2.1 ± 0.14	2 ± 0.14
D 1:100	3.1 ± 0.14	1.8 ± 0.14	1.5 ± 0.14	2.1 ± 0.14	1.5 ± 0.14	1.6 ± 0.14
D 1:1000	1 ± 0.14	1.3 ± 0.14	1 ± 0.14	1.4 ± 0.14	0.9 ± 0.14	1.1 ± 0.14
FR as such	0	0.9 ± 0.10	1.6 ± 0.10	0.8 ± 0.10	1.4 ± 0.10	1.25 ± 0.10
FR 1:10	0.5 ± 0.10	1.2 ± 0.10	0.3 ± 0.10	0	1.05 ± 0.10	0.5 ± 0.10
FR 1:100	1.6 ± 0.10	1.2 ± 0.10	1.6 ± 0.10	0.7 ± 0.10	1.1 ± 0.10	1.5 ± 0.10
FR 1:1000	0.4 ± 0.10	0.5 ± 0.10	1.1 ± 0.10	0.3 ± 0.10	1.1 ± 0.10	1.05 ± 0.10
C-plus as such	1.3 ± 0.07	0.9 ± 0.07	0.5 ± 0.07	1.35 ± 0.07	1.4 ± 0.07	0.9 ± 0.07
C-plus 1:10	0.9 ± 0.07	0.3 ± 0.07	0.7 ± 0.07	0.3 ± 0.07	0.8 ± 0.07	0.5 ± 0.07
C-plus 1:100	0.6 ± 007	1.2 ± 0.07	0.7 ± 0.07	1.2 ± 0.07	1.1 ± 0.07	1 ± 0.07
C-plus 1:1000	1.1 ± 0.07	0.85 ± 0.07	1.5 ± 0.07	1.2 ± 0.07	1.5 ± 0.07	1.2 ± 0.07
BQ as such	1.5 ± 008	1.5 ± 0.08	1.4 ± 0.08	1 ± 0.08	1.9 ± 0.08	1.1 ± 0.08
BQ 1:10	0.5 ± 0.08	1.6 ± 0.08	1.6 ± 0.08	1.2 ± 0.08	1.8 ± 0.08	1.1 ± 0.08
BQ 1:100	1.4 ± 0.08	0.5 ± 0.08	2 ± 0.08	1.1 ± 0.08	1.9 ± 0.08	1.5 ± 0.08
BQ 1:1000	1.1 ± 0.08	1.4 ± 0.08	1.5 ± 0.08	1.1 ± 0.08	1.6 ± 0.08	1.65 ± 0.08
C-	1.1 ± 0.08	1 ± 0.08	1.2 ± 0.08	1.5 ± 0.08	1.2 ± 0.08	1 ± 0.08
IAA 50 µM	1.6 ± 0.08	1.4 ± 0.08	1.4 ± 0.08	1.8 ± 0.08	0.9 ± 0.08	1.6 ± 0.08
IAA 10 µM	1.2 ± 0.08	1.7 ± 0.08	1.5 ± 0.08	1.6 ± 0.08	1.1 ± 0.08	1.9 ± 0.08
IAA 100 µM	1.1 ± 0.08	1 ± 0.08	1 ± 0.08	0.9 ± 0.08	1 ± 0.08	0.6 ± 0.08
IAA 200 µM	0.6 ± 0.08	0.6 ± 0.08	0.7 ± 0.08	0.85 ± 0.08	1 ± 0.08	0.9 ± 0.08

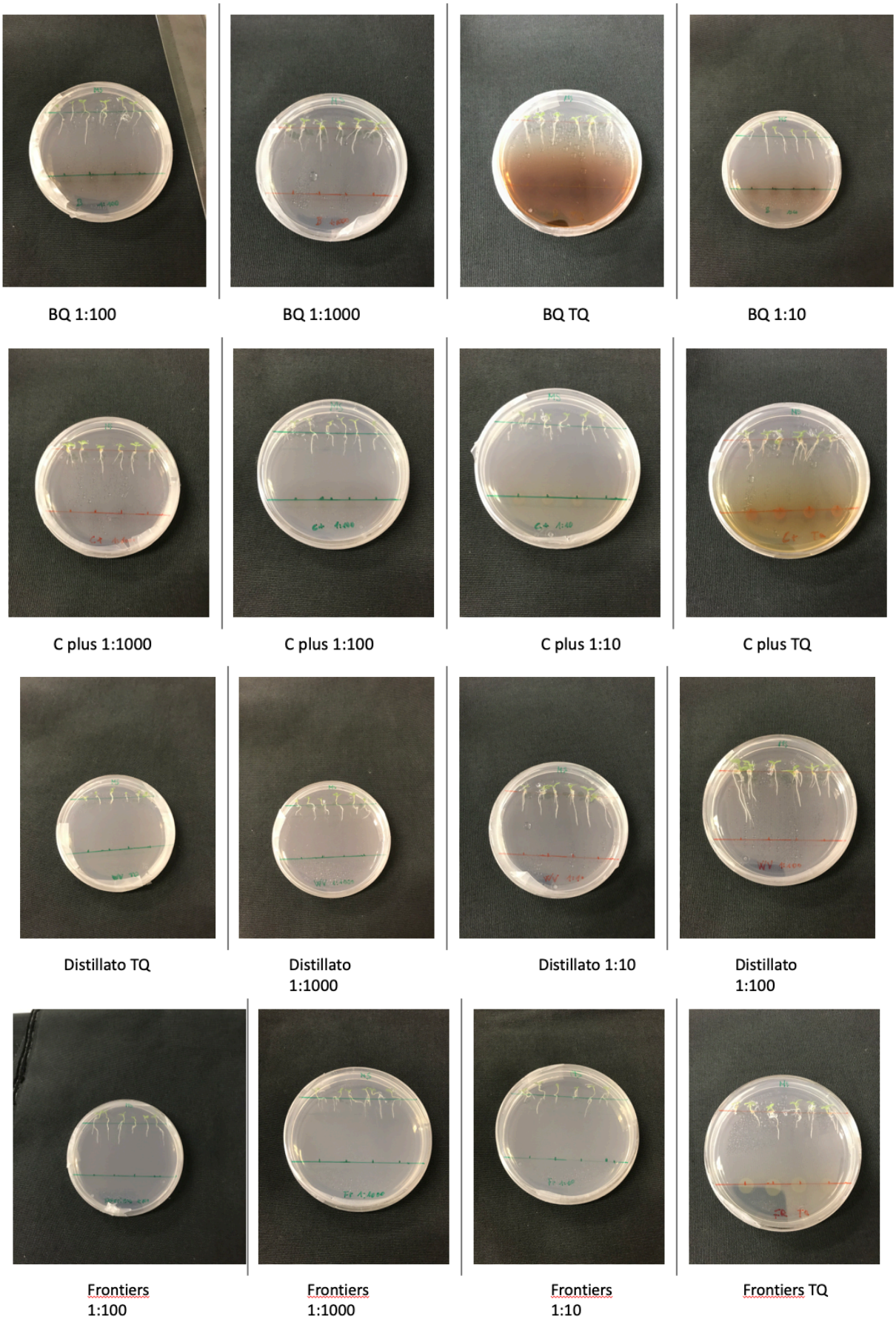

Figure 13. Plates with *N. tabacum* after 5 days of root growth. "Distillato di Legno" is reported as "Distillato and "Frontiere 2.0" as "Frontiers"

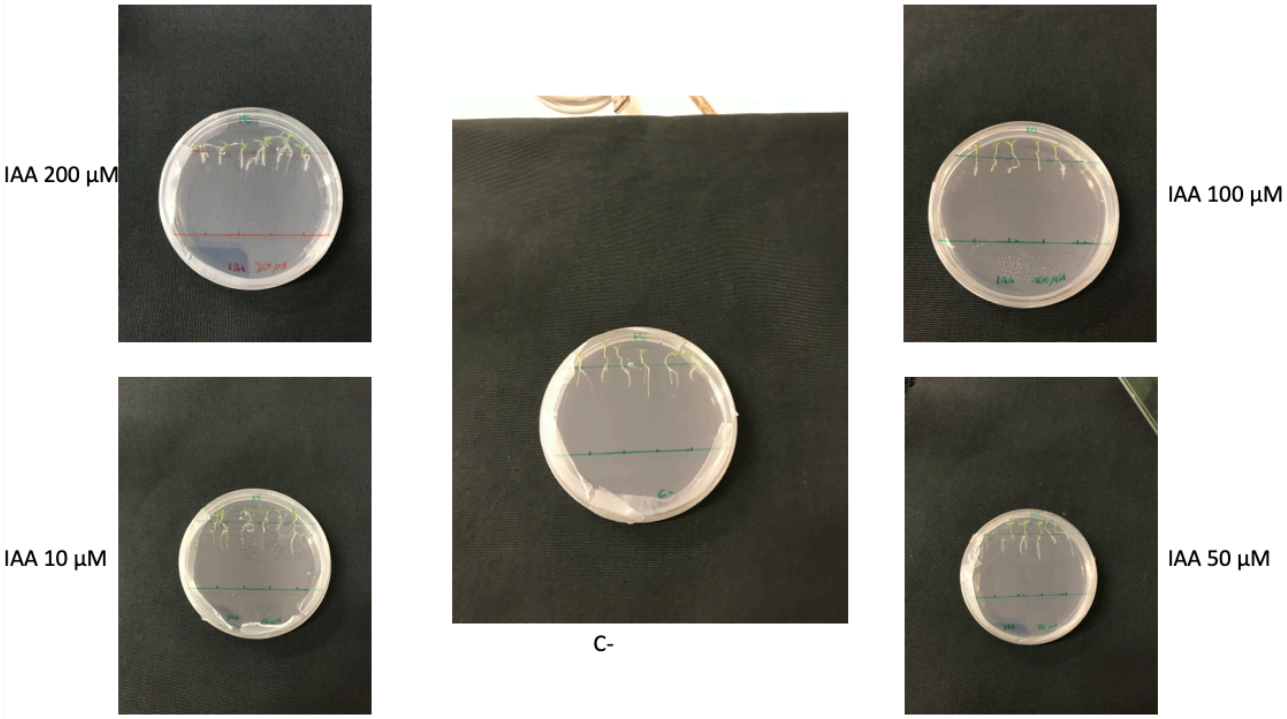

Figure 14. C- and IAA plates with *N. tabacum* after 5 days of root growth

Table 15. *N. tabacum* roots measures in cm ± standard error at 10th day. "Distillato di Legno" data is reported as "D" and "Frontiere 2.0" data as "FR"

	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6
D as such	0	0.7 ± 0.23	0.9 ± 0.23	0.4 ± 0.23	0.7 ± 0.23	0.4 ± 0.23
D 1:10	2.2 ± 0.23	2.1 ± 0.23	3 ± 0.23	3.15 ± 0.23	3.4 ± 0.23	2.9 ± 0.23
D 1:100	4.5 ± 0.23	2.55 ± 0.23	2.1 ± 0.23	2.9 ± 0.23	2.1 ± 0.23	2.3 ± 0.23
D 1:1000	1 ± 0.23	1.9 ± 0.23	1.9 ± 0.23	3.1 ± 0.23	1.9 ± 0.23	1.6 ± 0.23
FR as such	0.6 ± 0.16	1.4 ± 0.16	2.45 ± 0.16	1 ± 0.16	2.5 ± 0.16	1.6 ± 0.16
FR 1:10	1.8 ± 0.16	2 ± 0.16	2.2 ± 0.16	0.5 ± 0.16	2.2 ± 0.16	2.55 ± 0.16
FR 1:100	2.5 ± 0.16	2.1 ± 0.16	2.6 ± 0.16	1 ± 0.16	1.4 ± 0.16	2.5 ± 0.16
FR 1:1000	1.95 ± 0.16	2.2 ± 0.16	3.35 ± 0.16	1.7 ± 0.16	3.3 ± 0.16	3.45 ± 0.16
C-plus as such	1.3 ± 0.08	1 ± 0.08	0.5 ± 0.08	1.4 ± 0.08	1.6 ± 0.08	1.5 ± 0.08
C-plus 1:10	1.2 ± 0.08	0.8 ± 0.08	1.45 ± 0.08	0.8 ± 0.08	1.1 ± 0.08	0.9 ± 0.08
C-plus 1:100	1.2 ± 0.08	1.9 ± 0.08	1.1 ± 0.08	1.2 ± 0.08	1.6 ± 0.08	1.45 ± 0.08
C-plus 1:1000	1.5 ± 0.08	1.2 ± 0.08	1.9 ± 0.08	1.8 ± 0.08	2 ± 0.08	1.9 ± 0.08
BQ as such	1.6 ± 0.15	1.5 ± 0.15	1.5 ± 0.15	1 ± 0.15	2.1 ± 0.15	1.2 ± 0.15
BQ 1:10	0.5 ± 0.15	2.4 ± 0.15	2.1 ± 0.15	2 ± 0.15	2.9 ± 0.15	1.5 ± 0.15
BQ 1:100	2.1 ± 0.15	1.3 ± 0.15	3.2 ± 0.15	1.8 ± 0.15	2.2 ± 0.15	1.6 ± 0.15
BQ 1:1000	1.95 ± 0.15	1.7 ± 0.15	2.8 ± 0.15	2.2 ± 0.15	3.5 ± 0.15	3.1 ± 0.15
C-	2.3 ± 0.15	2.1 ± 0.15	1.9 ± 0.15	2.6 ± 0.15	1.8 ± 0.15	1.6 ± 0.15
IAA 50 µM	1.6 ± 0.11	1.4 ± 0.11	1.4 ± 0.11	1.8 ± 0.11	0.9 ± 0.11	1.6 ± 0.11
IAA 10 µM	1.9 ± 0.11	1.5 ± 0.11	1.7 ± 0.11	2.2 ± 0.11	1.5 ± 0.11	3.5 ± 0.11
IAA 100 µM	1.7 ± 0.11	1.5 ± 0.11	1.6 ± 0.11	1.3 ± 0.11	2.7 ± 0.11	1 ± 0.11
IAA 200 µM	0.8 ± 0.11	1.1 ± 0.11	1.3 ± 0.11	1.25 ± 0.11	1.1 ± 0.11	1.2 ± 0.11

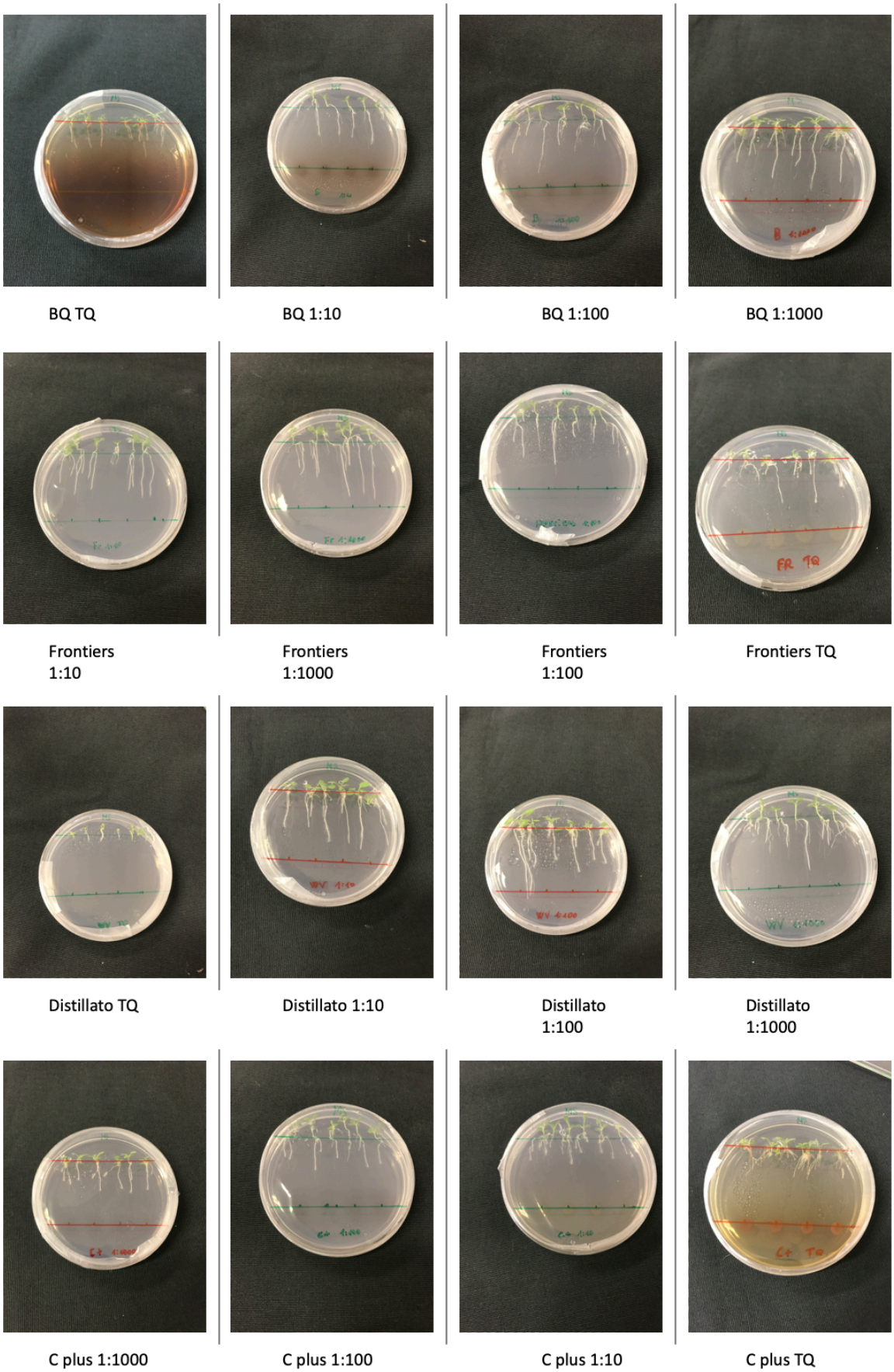

Figure 15. Plates with *N. tabacum* after 10 days of root growth. "Distillato di Legno" is reported as "Distillato and "Frontiere 2.0" as "Frontiers"

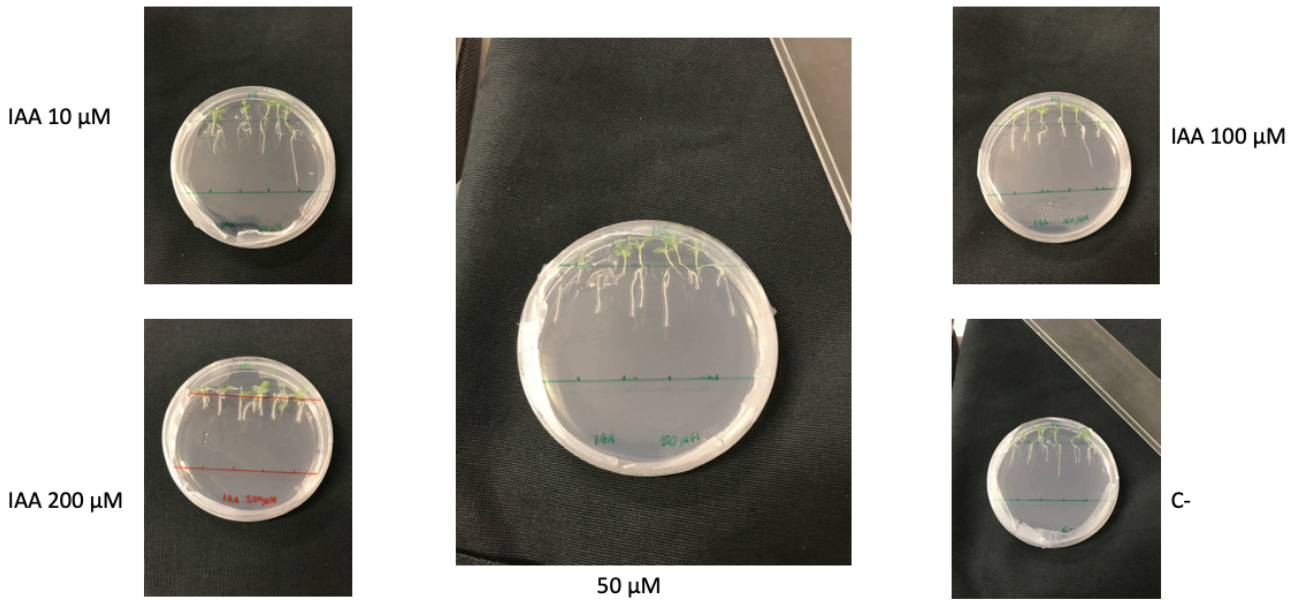

Figure 16. C- and IAA plates with *N. tabacum* after 10 days of root growth

Table 16. *N. tabacum* roots measures in cm ± standard error at 15th day. "Distillato di Legno" data is reported as "D" and "Frontiere 2.0" data as "FR"

	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6
D as such	0	0.7 ± 0.28	0.9 ± 0.28	0.4 ± 0.28	0.7 ± 0.28	0.4 ± 0.28
D 1:10	2.6 ± 0.28	2.2 ± 0.28	3.1 ± 0.28	3.4 ± 0.28	3.7 ± 0.28	3.2 ± 0.28
D 1:100	4.6 ± 0.28	2.6 ± 0.28	2.2 ± 0.28	2.9 ± 0.28	2.2 ± 0.28	2.3 ± 0.28
D 1:1000	1.1 ± 0.28	2.9 ± 0.28	3.2 ± 0.28	5.5 ± 0.28	3.3 ± 0.28	2.25 ± 0.28
FR as such	0.9 ± 0.23	1.4 ± 0.23	2.5 ± 0.23	1 ± 0.23	2.7 ± 0,23	1.8 ± 0.23
FR 1:10	3.5 ± 0.23	3.6 ± 0.23	3.4 ± 0.23	2 ± 0.23	4,3 ± 0.23	4.65 ± 0.23
FR 1:100	2.5 ± 0.23	1.8 ± 0.23	3.2 ± 0.23	1.2 ± 0.23	1.4 ± 0.23	2.5 ± 0.23
FR 1:1000	2.5 ± 0.23	2.85 ± 0.23	4.3 ± 0.23	2 ± 0.23	4 ± 0.23	4.2 ± 0.23
C-plus as such	1.4 ± 0.08	1 ± 0.08	0.5 ± 0.08	1.5 ± 0.08	1.6 ± 0.23	1.5 ± 0.08
C-plus 1:10	1.2 ± 0.08	0.8 ± 0.08	1.6 ± 0.08	0.9 ± 0.08	1.2 ± 0.08	1.1 ± 0.08
C-plus 1:100	1.3 ± 0.08	2.1 ± 0.08	1.25 ± 0.08	1.2 ± 0.08	1.7 ± 0.08	1.5 ± 0.08
C-plus 1:1000	1.5 ± 0.08	1.2 ± 0.08	1.9 ± 0.08	1.8 ± 0.08	2.1 ± 0.08	2 ± 0.08
BQ as such	1.7 ± 0.15	1.8 ± 0.15	1.5 ± 0.15	1 ± 0.15	2.1 ± 0.15	1.25 ± 0.15
BQ 1:10	0.6 ± 0.15	2.5 ± 0.15	2.3 ± 0.15	2 ± 0.15	2.9 ± 0.15	1.55 ± 0.15
BQ 1:100	2.4 ± 0.15	1.4 ± 0.15	3.3 ± 0.15	2.2 ± 0.15	2.2 ± 0.15	2.1 ± 0.15
BQ 1:1000	2.1 ± 0.15	1.7 ± 0.15	3.2 ± 0.15	2.6 ± 0.15	3.6 ± 0.15	3.4 ± 0.15
C-	2.3 ± 0.20	2.1 ± 0.20	1.9 ± 0.20	3.2 ± 0.20	2 ± 0.20	2 ± 0.20
IAA 50 µM	2.2 ± 0.15	2 ± 0.15	2 ± 0.15	2.4 ± 0.15	1 ± 0.15	1.9 ± 0.15
IAA 10 µM	1.1 ± 0.15	2.5 ± 0.15	1.5 ± 0.15	2.6 ± 0.15	2 ± 0.15	4.35 ± 0.15
IAA 100 µM	2.1 ± 0.15	1.8 ± 0.15	1.8 ± 0.15	2 ± 0.15	2.8 ± 0.15	1.1 ± 0.15
IAA 200 µM	0.8 ± 0.15	1.2 ± 0.15	1.7 ± 0.15	1.3 ± 0.15	1.6 ± 0.15	1.3 ± 0.15

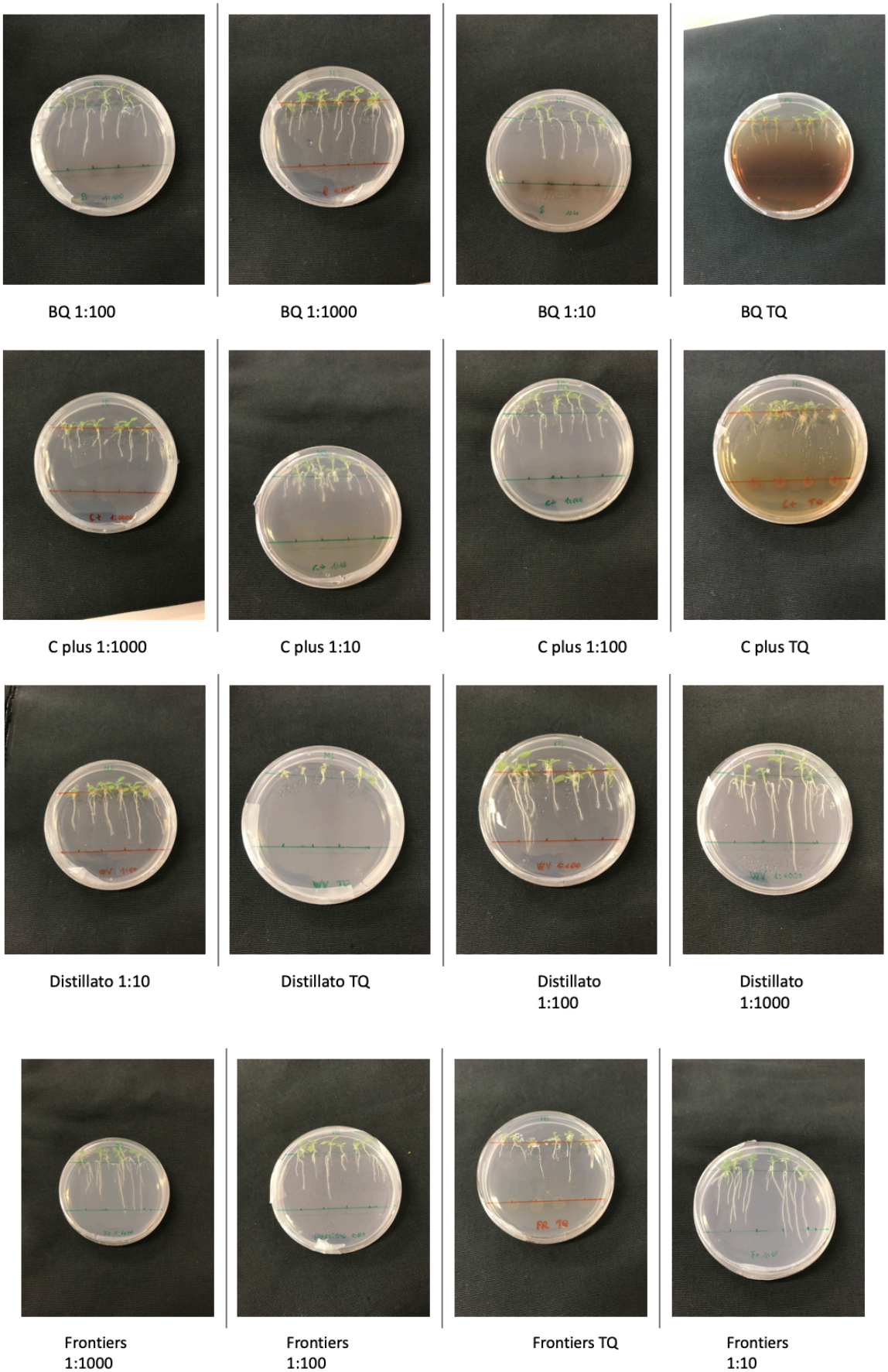

Figure 17. Plates with *N. tabacum* after 15 days of root growth. "Distillato di Legno" is reported as "Distillato and "Frontiere 2.0" as "Frontiers"

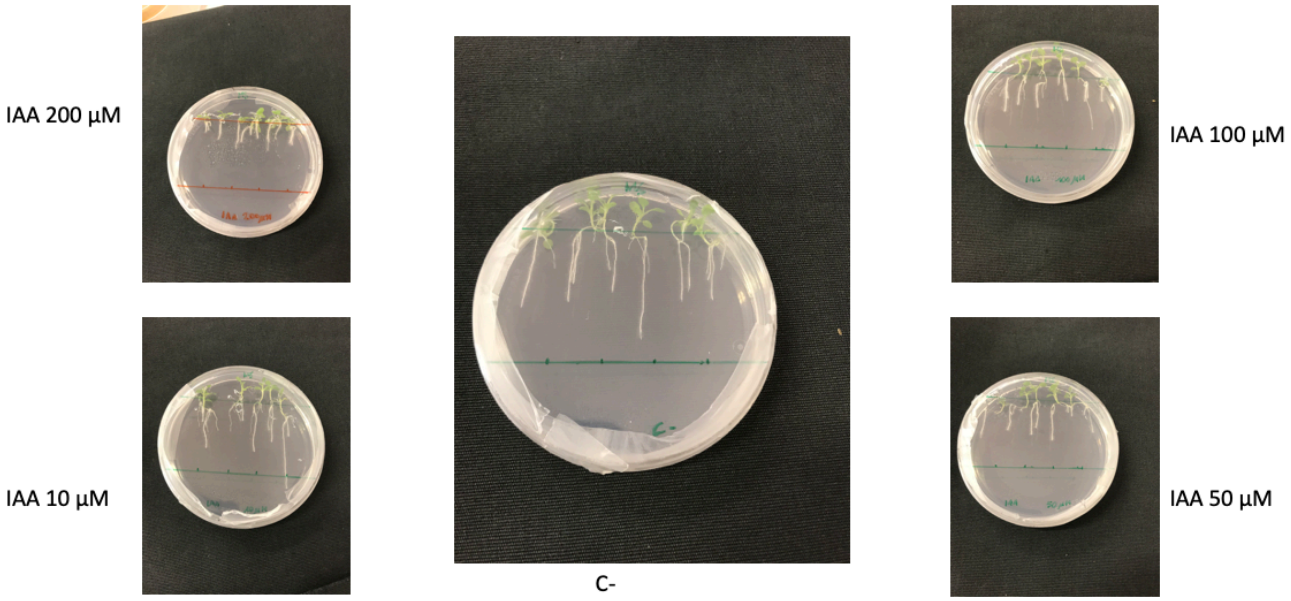

Figure 18. C- and IAA plates with *N. tabacum* after 15 days of root growth

"Distillato di Legno" in plate WV1 (Figure 9-B) shows that the most concentrate dilution, that is 1:10, works similarly to auxinic herbicides, thus causing a poor root development (sample 5, 6 and 7) and even death of the plant (sample 6).

More diluted concentrations, 1:100 and 1:1000, show a similar positive influence on root growth of plant 3 and 4 (respectively 3.8 cm and 4.2 cm), more than plant 1 and 2 that are on C- spot.

This trend is partially confirmed in WV2 (Figure 9-A) where plant 4 and 6 (6.5 cm and 6 cm) are positively influence by 1:100 and 1:1000 spots; plant 5 doesn't show any activities.

Plant 3 shows the same result saw in WV1 plate where 1:10 dilution spot seems to have an herbicide activity, while plant 1 and 2 on C- spot grow less than 4 and 6 and more than 3 and 5.

Silvateam Agritan C-plus (Figure 11-B) plate shows an interesting result. 1:100 dilution spot influence root growth on all the plants and plant 7 seems to be directing the roots in that direction, while more concentrated 1:10 spot inhibits root growth and 1:1000 and C- spots don't have any effects.

Silvateam Agritan BQ (Figure 11-A) plate show that the 1:100 and 1:1000 spots attract plant 4 and 6 while 3 seems be inhibited by 1:10 spot. Plant 2 near C- spot grows less than the 4 and 6 but it starts to be directing to the 1:10 spot for presumably reach the 1:100 spot.

"Frontiere 2.0" (Figure 12) plate has the most representative result: all the plant, expect plant 6, are completely inhibit in particular from 1:10 spot. Roots seems to direct in other directions to avoid 1:10 spot and only plant 6 in direction of 1:1000 shows a positive influence in term of roots growth.
IAA 1 (Figure 10-A) and IAA 2 (Figure 10-B), the positive controls, show different measures. In the first hormones completely inhibit plant growth expect for plant 5 and 7 that are directed to 10 µM spot, while the second show a more complex result.
Spot with IAA 10 µM attracts plants 1, 2 and 3 (4.55 cm, 5.5 cm and 5.8 cm) but, conversely to IAA 1 plate, plants 5 and 7 (both with a length of 5.6 cm) also grow in direction of 100 and 200 µM spots. Plants 4 and 6 don't show a statistical relevant result.

Test on *N. tabacum* root elongation shows a more complex situation. Starting from Distillato di Legno plates, the results are reported as average root length ± standard error compared to the negative control in Table 17.
Undiluted product acted like an herbicide on the plants of tobacco with a complete inhibition on the root growth in all the three time points. Distillato di Legno 1:10 and 1:100 dilutions have a similar effect on the plant eradication, generally more than C-, while 1:1000 dilution have a lower influence than control at 5 and 10 days. However, Distillato 1:1000 increased drastically at 15 days, overcome all the others and maintaining similar to 1:10 concentration.

Table 17. Comparison of the average in cm ± standard error of different dilutions of "Distillato di Legno" and C- on *N. tabacum* in 5, 10 and 15 days

	5 days	**10 days**	**15 days**
C-	1.17 ± 0.08	2.25 ± 0.15	2.25 ± 0.20
"Distillato di Legno" as such	0.42 ± 0.14	0.52± 0.23	0.52 ± 0.28
"Distillato di Legno" 1:10	1.75 ± 0.14	2.8 ± 0.23	3.03 ± 0.28
"Distillato di Legno" 1:100	1.93 ± 0.14	2.74 ± 0.23	2.8 ± 0.28
"Distillato di Legno" 1:1000	1.12 ± 0.14	1.9 ± 0.23	3.04 ± 0.28

"Frontiere 2.0" has a different effect on root elongation than Distillato di Legno. At 5 days, only 1:100 dilution shows a better influence on root elongation than C- plate as far as 1:100 and 1:1000

dilutions are concerned, these treatments have a similar effect, with a positive influence on root growth in comparison to C-, while an inhibitory effect is due to “Frontiere 2.0” as such and to dilution 1:10 but just at 5 days.

Table 18. Comparison of the average in cm ± standard error of different dilutions of “Frontiere 2.0” and C- on *N. tabacum* in 5, 10 and 15 days

	5 days	**10 days**	**15 days**
C-	1,17 ± 0,08	2,25 ± 0,15	2,25 ± 0,20
“Frontiere 2.0” as such	0,99 ± 0,10	1,72 ± 0,16	1,72 ± 0,16
“Frontiere 2.0” 1:10	0,59 ± 0,10	3,57 ± 0,16	3,57 ± 0,16
“Frontiere 2.0” 1:100	1,28 ± 0,10	2,1 ± 0,16	2,1 ± 0,16
“Frontiere 2.0” 1:1000	0,74 ± 0,10	3,31 ± 0,16	3,31 ± 0,16

Silvateam Agritan C-plus generally show a low capacity to influence roots growth (Table 19). Indeed, each dilution and at all the time points considered was not effective to promote root elongation in Tobacco, in comparison to the negative control.

Table 19. Comparison of the average in cm ± standard error of different dilutions of Silvateam Agritan C-plus and C- on *N. tabacum* in 5, 10 and 15 days

	5 days	**10 days**	**15 days**
C-	1.17 ± 0.08	2.25 ± 0.15	2.25 ± 0.20
C-plus as such	1.06 ± 0.10	1.22 ± 0.08	1.25 ± 0.08
C-plus 1:10	0.58 ± 0.10	1.04 ± 0.08	1.13 ± 0.08
C-plus 1:100	0.97 ± 0.10	1.41 ± 0.08	1.51 ± 0.08
C-plus 1:1000	1.23 ± 0.10	1.72 ± 0.08	1.75 ± 0.08

Plates treated with Silvateam Agritan BQ (Table 20) show that at 5th day every dilution has a positive effect on root elongation. At 10 days, only 1:1000 dilution spot has a better effect than negative control differently to as such, 1:10 and 1:100, while at 15 days also 1:100 have a positive effect, although the difference is low. However, 1:1000 always at 15 days have the best positive influence respect to the others.

Table 20. Comparison of the average in cm ± standard error of different dilutions of Silvateam Agritan BQ and C- on *N. tabacum* in 5, 10 and 15 days

	5 days	10 days	15 days
C-	1.17 ± 0.08	2.25 ± 0.15	2.25 ± 0.20
BQ as such	1.4 ± 0.08	1.48 ± 0.15	1.56 ± 0.15
BQ 1:10	1.3 ± 0.08	1.9 ± 0.15	1.98 ± 0.15
BQ 1:100	1.4 ± 0.08	2.03 ± 0.15	2.27 ± 0.15
BQ 1:1000	1.39 ± 0.08	2.54 ± 0.15	2.77 ± 0.15

At last, IAA was used as positive control and compared to water, used as negative control. Treatment with IAA at 10 and 50 µM, as expected, don't inhibit drastically at all the time points. In particular, 10 µM stimulate eradication at 10 and 15 days in comparison to water. Conversely, at all the time points IAA at 100 and 200 µM inhibit Tobacco root elongation.

Table 21. Comparison of the average in cm ± standard error of different concentrations of IAA and C- on *N. tabacum* in 5, 10 and 15 days

	5 days	10 days	15 days
C-	1.17 ± 0.08	2.25 ± 0.15	2.25 ± 0.20
IAA 10 µM	1.5 ± 0.08	2.34 ± 0.11	2.34 ± 0.15
IAA 50 µM	1.45 ± 0.08	1.45 ± 0.11	1.92 ± 0.15
IAA 100 µM	0.93 ± 0.08	1.63 ± 0.11	1.93 ± 0.15
IAA 200 µM	0.78 ± 0.08	1.13 ± 0.11	1.32 ± 0.15

The results of *A. thaliana* and *N. tabacum* root elongation are similar, in particular data at 15th day are definitely comparable.

Silvateam Agritan C-plus acts as herbicide in every dilution tested differently from Silvateam BQ. This last act like an herbicide only at such and 1:10 concentration, while 1:100 partially have positive influence on root growth and 1:1000 have a positive influence.

"Distillato di Legno" has a positive influence at 1:10, 1:100 and 1:1000 concentration, but not the as such concentration who plays the role of herbicide in a major way than IAA hormones at 10, 50, 100 and 200 µM.

For last, “Frontiere 2.0” acts like an herbicide at as such and in little part at 1:100 concentration while at 1:10 and 1:1000 show the highest influence on root eradication.

4.2 Ion Leakage

Ion leakage on *N. tabacum* cv. *Kentucky* has been tested by induced penetration of Distillato di Legno and Frontiere 2.0 in foliar disks of 0.7 mm of diameter. This test has the role to answer about the possibility that the HR is activated or not by these two products. Negative control (C-) is represented by demineralized water, while positive control (C+) by *Pseudomonas syringae* pv. *tomato,* essential to compare the HR activation.

Data have been collected at 0, 3, 6, 24 and 48 hours post infiltration, and they are reported in Table 22, and expressed as electric conductibility (µS/cm ± standard error).

Table 22. Measures in time bends of electric conductibility (µS/cm ± standard error) of the demineralized water where leaf disks have been suspended

	T_0	T_3	T_6	T_{24}	T_{48}
“Distillato di Legno” as such	25.6 ± 16.02	100.2 ± 16.02	102.6 ± 16.02	108.1 ± 16.02	110 ± 16.02
“Distillato di Legno” 1:10	11.4 ± 8.95	43 ± 8.95	49.8 ± 8.95	58.7 ± 8.95	61.1 ± 8.95
“Distillato di Legno” 1:100	10.45 ± 2.35	15.6 ± 2.35	16.9 ± 2.35	20.9 ± 2.35	24.2 ± 2.35
“Distillato di Legno” 1:500	10.34 ± 2.42	14.5 ± 2.42	15.8 ± 2.42	20 ± 2.42	24.5 ± 2.42
“Distillato di Legno” 1:1000	10.3 ± 2.6	14.9 ± 2.6	16.2 ± 2.6	20.8 ± 2.6	25.5 ± 2.6
“Frontiere 2.0” as such	41.1 ± 25.87	161.3 ± 25.87	168.4 ± 25.87	175.3 ± 25.87	174.3 ± 25.87
“Frontiere 2.0” 1:10	12.5 ± 6.98	32.5 ± 6.98	37.5 ± 6.98	46.1 ± 6.98	53.5 ± 6.98
“Frontiere 2.0” 1:100	10.4 ± 4.09	16.6 ± 4.09	18.9 ± 4.09	25.9 ± 4.09	34.2 ± 4.09
“Frontiere 2.0” 1:1000	10.4 ± 4.73	17.5 ± 4.73	21.7 ± 4.73	29.1 ± 4.73	37.8 ± 4.73
C+	10.2 ± 9.37	17.9 ± 9.37	27.9 ± 9.37	51.8 ± 9.37	58.1 ± 9.37
C-	10.75 ± 2.30	15.55 ± 2.30	17.25 ± 2.30	23.2 ± 2.30	30.25 ± 2.30

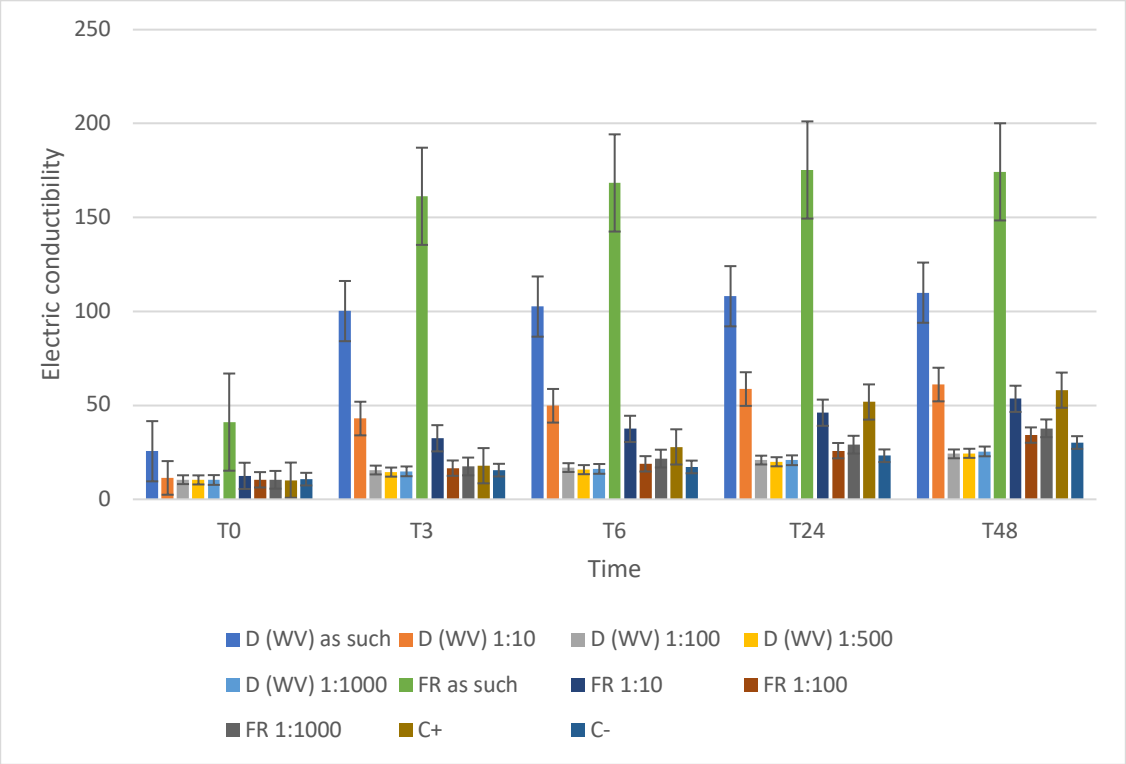

Figure 20. Comparison between the results of Ion Leakage theses

Results of this test are also graphically represented in Figure 7. As expected, *P. syringae* pv. *tomato* induces the HR with a gradually release of ions in the suspension water, differently from what happened to the other treatments. "Frontiere 2.0" and "Distillato di Legno" as such, compared to C- and C+, show an evident ion release at T_0 that rapidly grow at T_3 and T_6 to then remain stable at T_{24} and T_{48}. The reason isn't the HR activation, but the necrosis induced by the two products as such that are causing the break of cellular wall and the complete ions release just at tempo 0. "Distillato di Legno" 1:10 and "Frontiere 2.0" 1:10 confirm what has been just said. Ions release increase drastically between T_0 and T_3 and between T_6 and T_{24}, maintaining at T_{48} a constant value, although Distillato di Legno 1:10 conductibility is a little bit superior than positive control.

Same situation is shown for all the other dilutions "Distillato di Legno" 1:100, 1:500, 1:1000 and "Frontiere 2.0" 1:100 and 1:1000. All these data are similar and show low value of electric conductibility due to not activation of hypersensitive response and necrosis induction on leaf disks.

Necrosis induction on leaf disks by as such products is also viewable in Figure 21 and 22. Indeed, disks in the "Distillato di Legno" and "Frontiere 2.0" as such spots clearly show necrosis differently from the positive control by *P. syringae.*

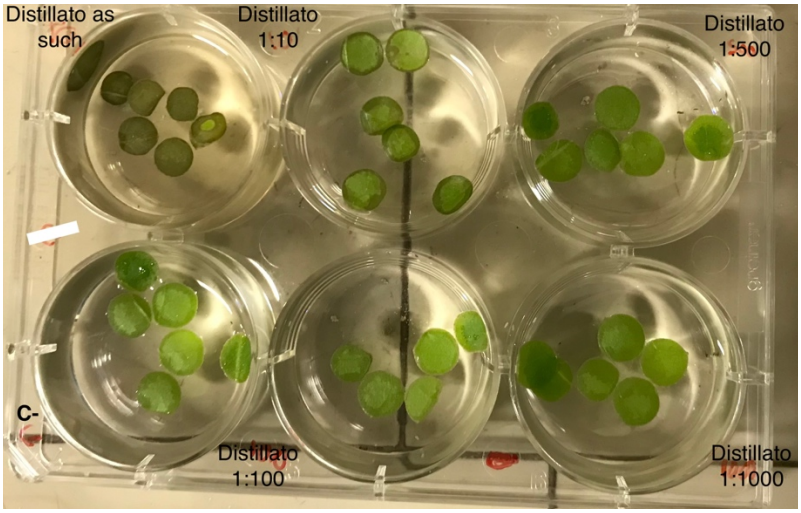

Figure 21. Leaf disks after 48h ("Distillato di Legno" plate)

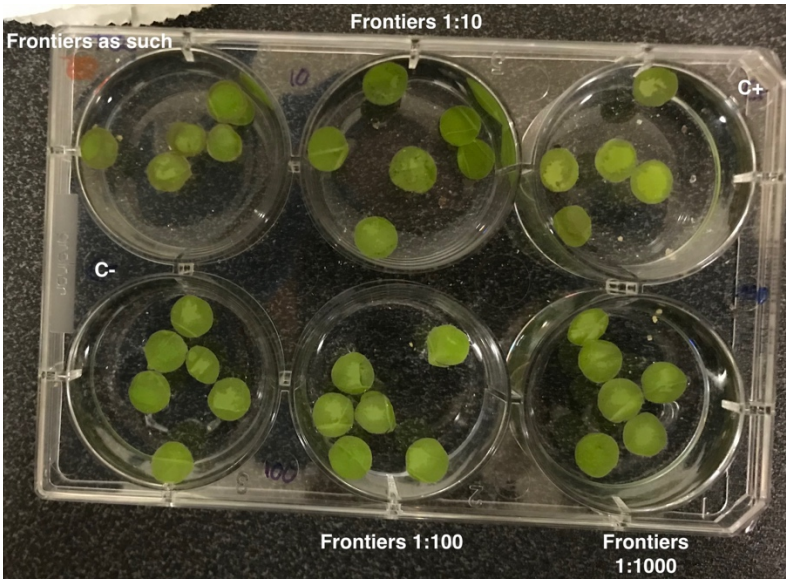

Figure 22. Leaf disks after 48h ("Frontiere 2.0" plate)

4.3 HR test on *N. tabacum*

Inoculation on *N. tabacum* cv. Kentucky leaf of dilutions of "Distillato di Legno" (1:500 and 1:1000) and "Frontiere 2.0" (1:500 and 1:1000) plus a positive control represented by *P. syringae* pv. *tomato* is complementary to the results of ion leakage (4.2).

In this test the hypersensitive response induction is directly viewable on the inoculate leaf.

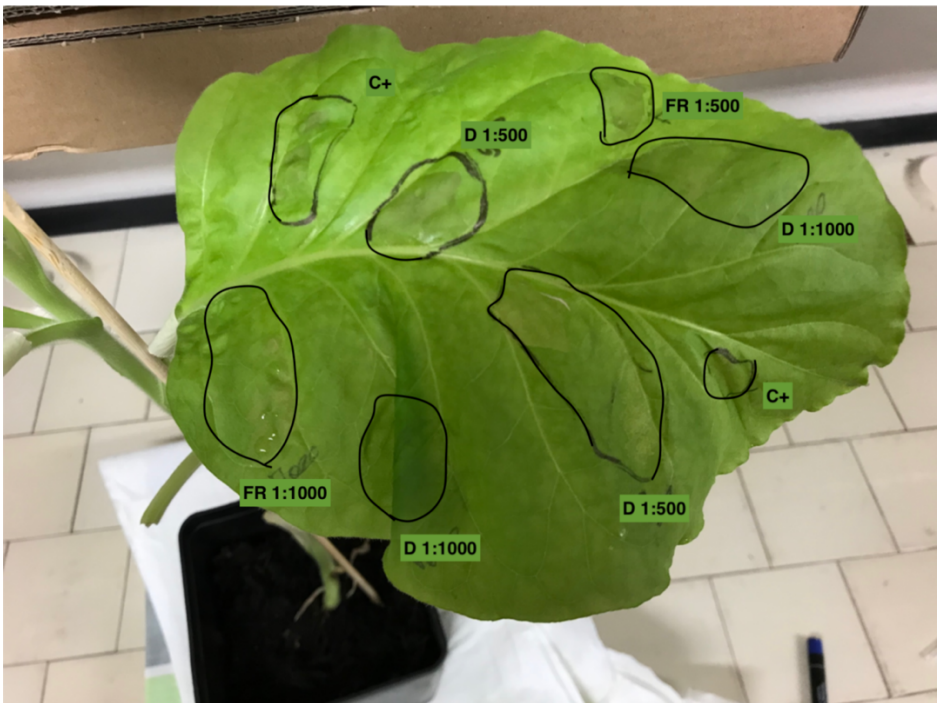

Figure 23. Tobacco leaf after the inoculation of the products and the bacteria at T_0. "Distillato di Legno" is reported as "D" and "Frontiere 2.0" as "FR"

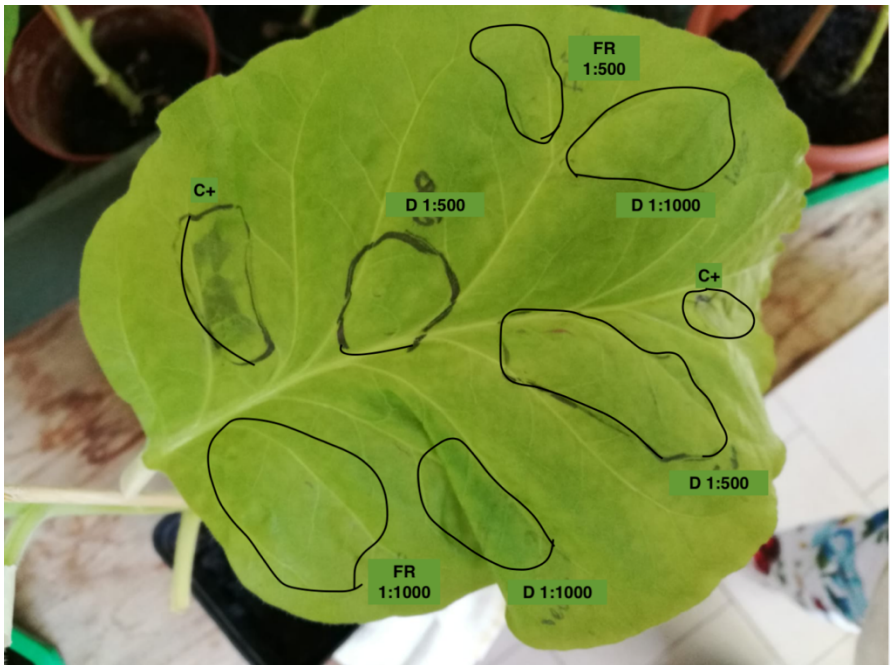

Figure 24. Tobacco leaf after the inoculation of the products and the bacteria at T_{24}. "Distillato di Legno" is reported as "D" and "Frontiere 2.0" as "FR"

The results reported in the Figure 24 show that *P. syringae* pv. *tomato* (C+) induces the hypersensitive response by formation of necrosis in the area of the inoculation of the bacterium, while both

Distillato di Legno and Frontiere 2.0 no. These two products don't activate HR as defense mechanism in the plant, confirming as reported in the ion leakage results part (4.2).

4.4 Antimicrobial activity on plant pathogenic fungi and bacteria

4.4.1 *In vitro* fungal growth inhibition

The tested products, "Distillato di Legno", "Frontiere 2.0", Silvateam Agritan BQ and C-plus, show different influence on growth inhibition on the different fungi. Data has been collected by measuring the radius of the fungus starting from the center of the plate and comparing the measures to a negative control represented by sterilized water.

S. rolfsii has been tested in a different way than the other fungi: dilutions used has been two replicates at 1:10 concentration, two at 1:100 concentration and two at 1:1000 concentration in the same plate, plus a negative control.

The other fungi have been tested with products as such plus a negative control in the same plate.

Data has been collected at the 6th day of growth for each fungus tested and results are showed in Table 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35 and 36 as value ± standard error.

S. rolfsii

Table 23. Inhibition *in vitro* of *S. rolfsii* by the 4 botanicals at different concentrations estimated as radius in cm ± standard error. Data reported are the average of the two replicates

	S. rolfsii								
	"Distillato di Legno"	**"Distillato di Legno" disks**	**"Frontiere 2.0"**	**"Frontiere 2.0" disks**	**C-plus**	**C-plus disks**	**BQ**	**BQ disks**	**C-**
1:10	5.45 ± 0.076	4.7 ± 0.14	4.8 ± 0.11	4.75 ± 0.13	4.2 ± 0.26	3.9 ± 0.20	4.5 ± 0.07	4.6 ± 0,.9	6.3 ± 0.15
1:100	5.2 ± 0.076	5 ± 0.14	4.9 ± 0.11	4.9 ± 0.13	5.4 ± 0.26	4.65 ± 0.20	4.75 ± 0,07	4.85 ± 0.19	5.7 ± 0.15
1:1000	5.4 ± 0.076	4.75 ± 0.14	5,35 ± 0.11	4.5 ± 0.13	5.4 ± 0.26	4.65 ± 0.20	4,8 ± 0,07	5.55 ± 0.19	5.8 ± 0.15

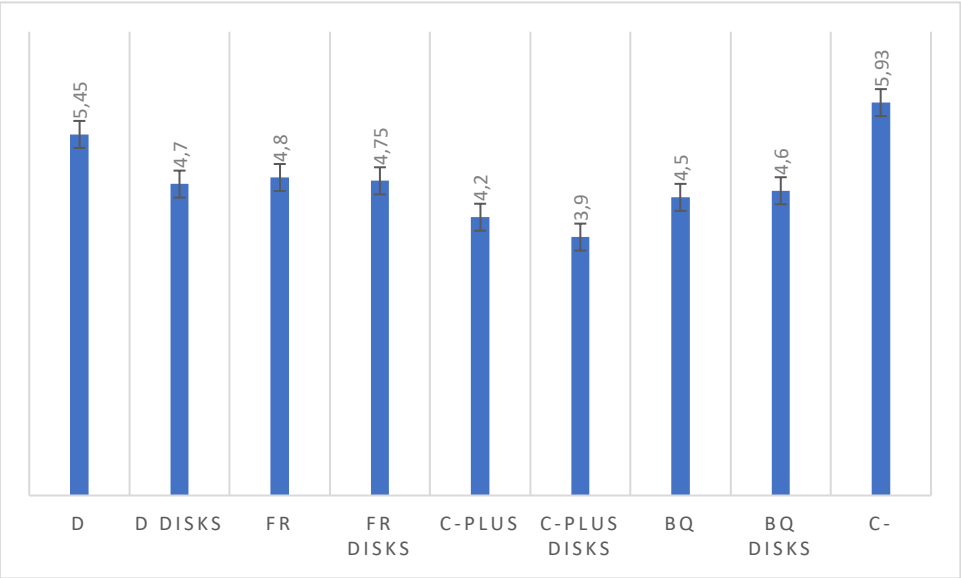

Figure 25. Comparison between average of 1:10 dilutions of every product and negative control

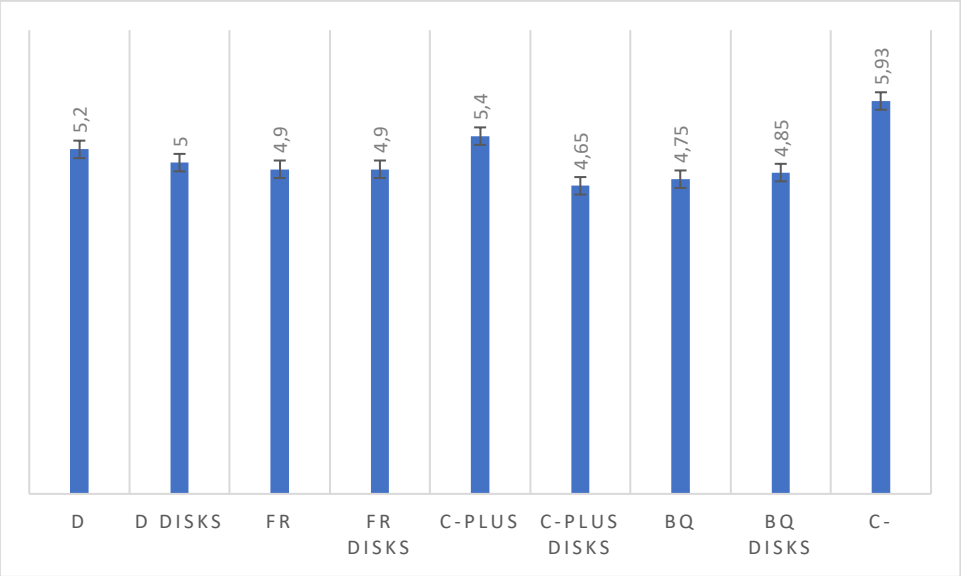

Figure 26. Comparison between average of 1:100 dilutions of every product and negative control

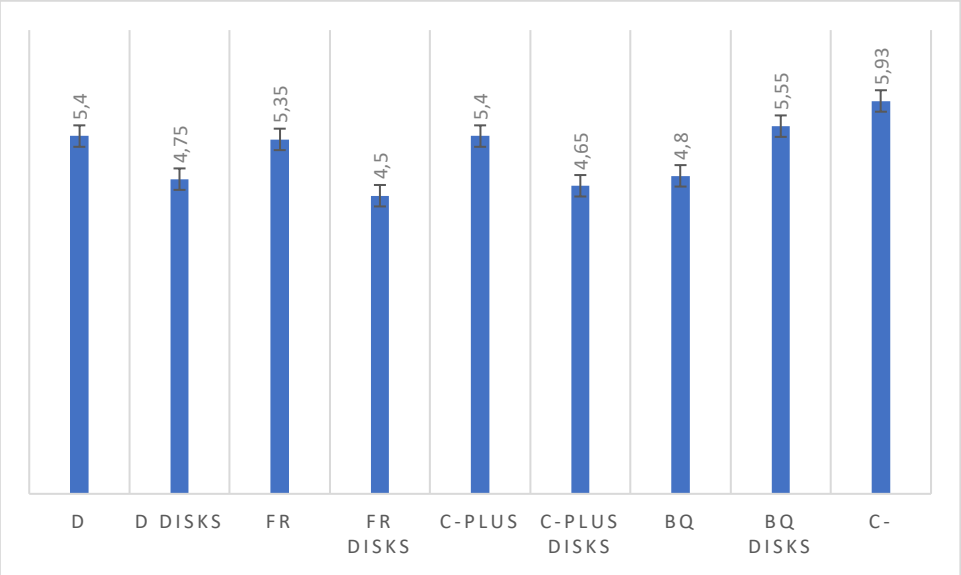

Figure 27. Comparison between average of 1:1000 dilutions of every product and negative control

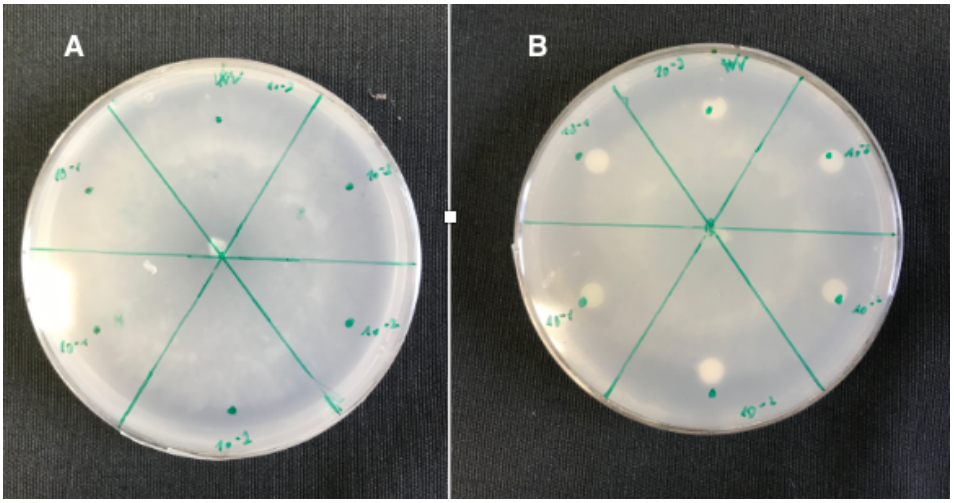

Figure 28. *S. rolfsii* treated by "Distillato di Legno" directly on agar (A) and by soaked disks (B)

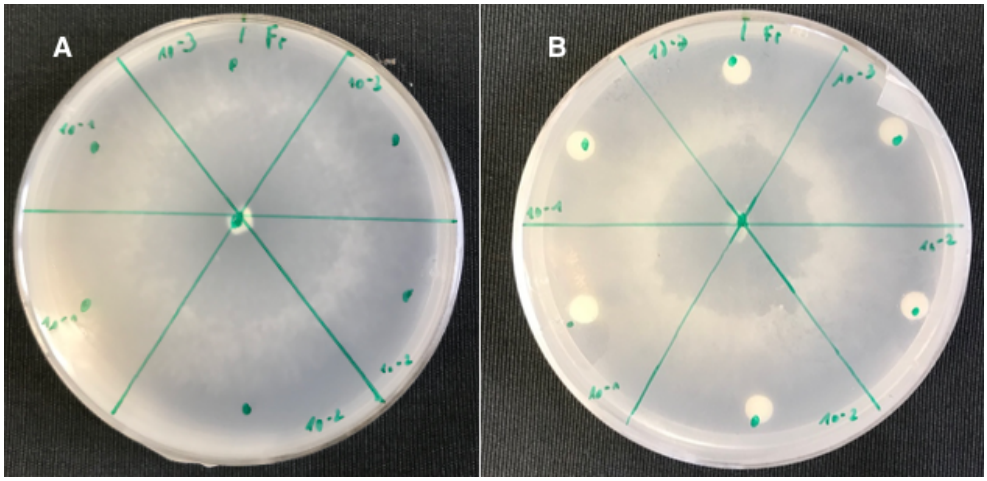

Figure 29. *S. rolfsii* treated by "Frontiere 2.0" directly on agar (A) and by soaked disks (B)

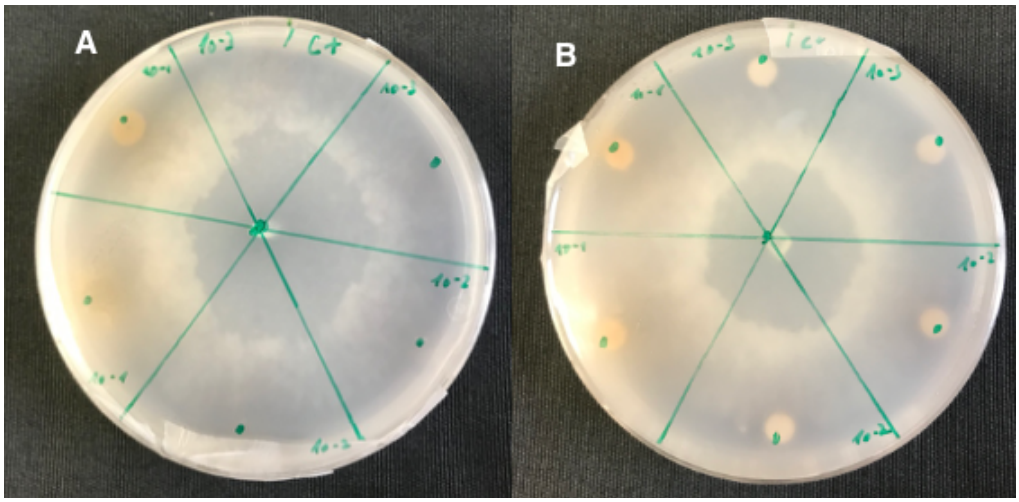

Figure 30. *S. rolfsii* treated by Silvateam Agritan C-plus directly on agar (A) and by soaked disks (B)

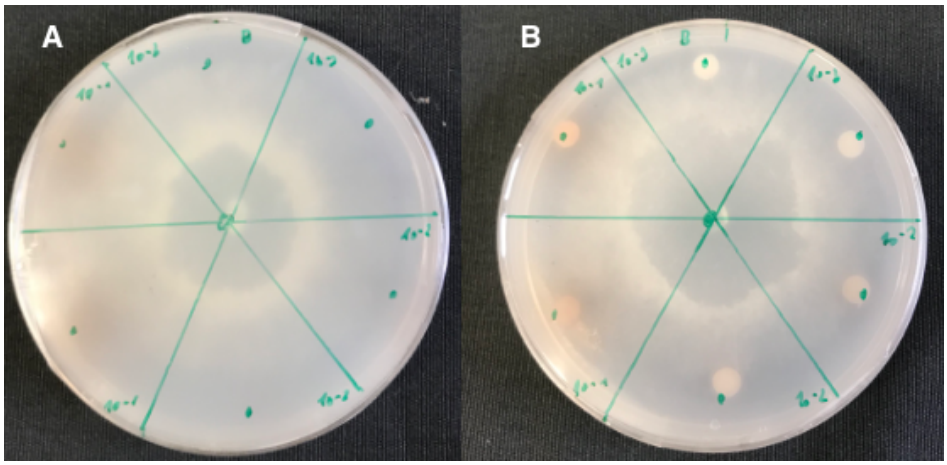

Figure 31. *S. rolfsii* treated by Silvateam Agritan BQ directly on agar (A) and by soaked disks (B)

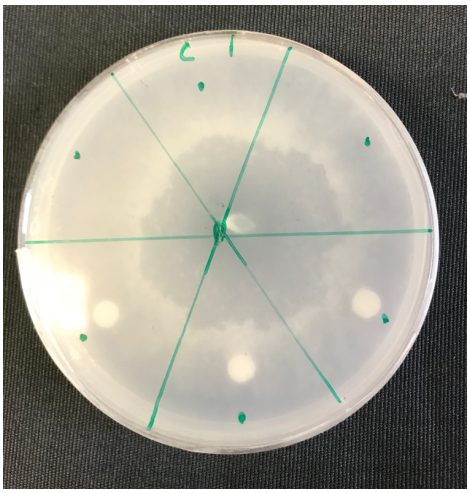

Figure 32. *S. rolfsii* treated with sterilized water

Table 24. Percentage of inhibition of *S. rolsfii* compared to negative control

	S. rolfsii							
	"Distillato di Legno"	**"Distillato di Legno" disks**	**"Frontiere 2.0"**	**"Frontiere 2.0" disks**	**C-plus**	**C-plus disks**	**BQ**	**BQ disks**
1:10	8,09%	20,74%	19,06%	19,90%	29,17%	34,23%	24,11%	22,43%
1:100	12,31%	15,68%	17,37%	17,37%	8,94%	21,59%	19,90%	18,21%
1:1000	8,94%	19,90%	9,78%	24,11%	8,94%	21,59%	19,06%	6,41%

F. graminearum

Table 25. Inhibition *in vitro* of *F. graminearum* by the botanicals estimated as radius in cm ± standard error of the fungi colony at 6 days of growth

	F. graminearum	
	Fungal growth (cm)	**Growth inhibition (%)**
"Distillato di Legno"	4.5 ± 0.01	0.00%
BQ	4.5 ± 0.01	0.00%
C-plus	4.5 ± 0.02	0.00%
"Frontiere 2.0"	4.5 ± 0.01	0.00%
C-	4.5 ± 0.03	0.00%

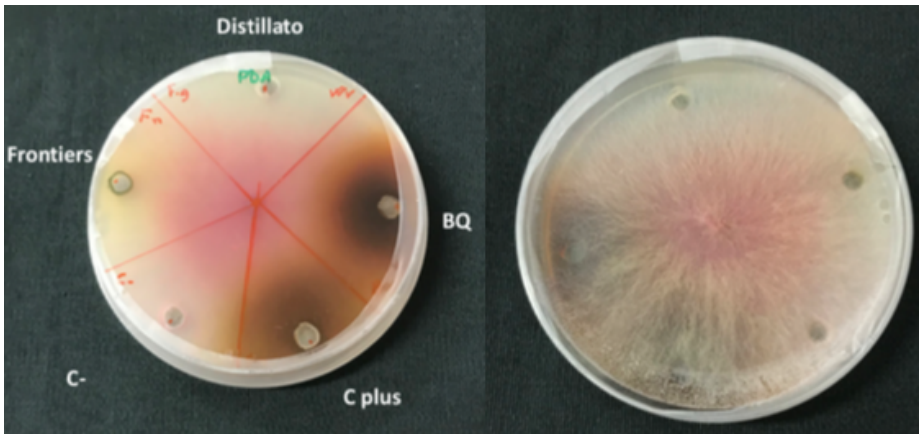

Figure 33. *F. graminearum* treated with products as such

F. poae

Table 26. Inhibition *in vitro* of *F. poae* by the botanicals estimated as radius in cm ± standard error of the fungi colony at 6 days of growth

	F. poae	
	Fungal growth (cm)	**Growth inhibition (%)**
"Distillato di Legno"	2.2 ± 0.02	18.18%
BQ	2 ± 0.01	10.00%
C-plus	1.5 ± 0.02	-20.00%
"Frontiere 2.0"	2.2 ± 0.01	18.18%
C-	1.8 ± 0.01	0.00%

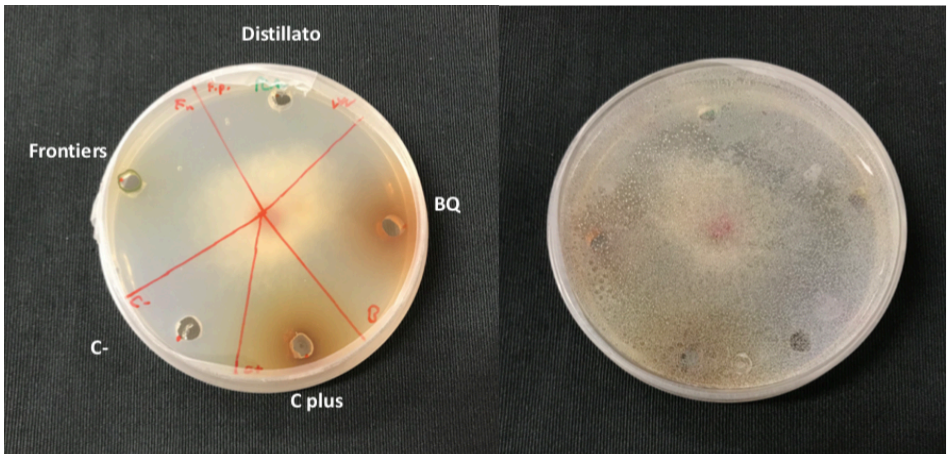

Figure 34. *F. poae* treated with products as such

F. avenaceum

Table 27. Inhibition *in vitro* of *F. avenaceum* by the botanicals estimated as radius in cm ± standard error of the fungi colony at 6 days of growth

	F. avenaceum	
	Fungal growth (cm)	**Growth inhibition (%)**
"Distillato di Legno"	2.7 ± 0.01	-5.56%
BQ	3 ± 0.02	5.00%
C-plus	2.6 ± 0.02	-9.62%
"Frontiere 2.0"	2.7 ± 0.01	-5.56%
C-	2.85 ± 0.03	0.00%

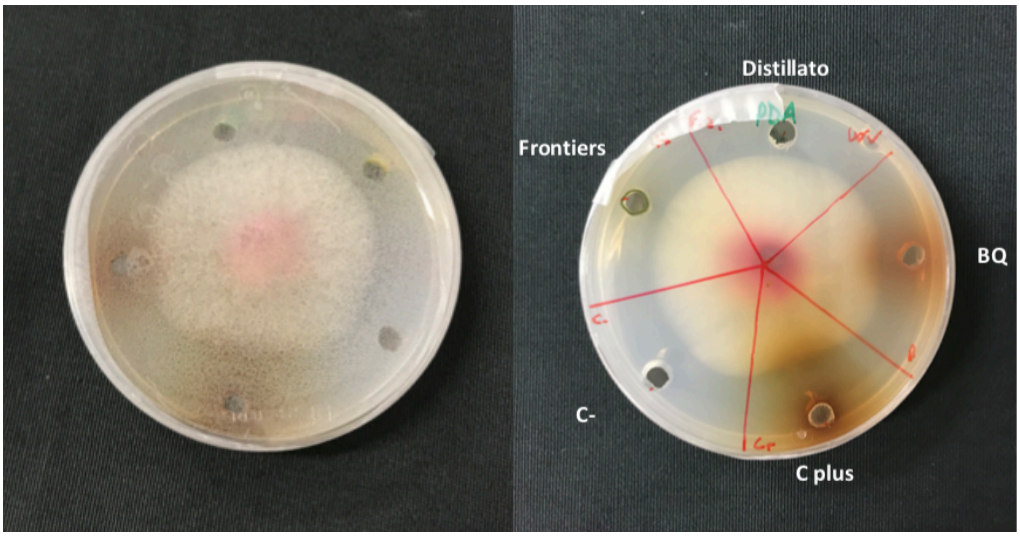

Figure 35. *F. avenaceum* treated with products as such

F. culmorum

Table 28. Inhibition *in vitro* of *F. culmorum* by the botanicals estimated as radius in cm ± standard error of the fungi colony at 6 days of growth

	F. culmorum	
	Fungal growth (cm)	**Growth inhibition (%)**
"Distillato di Legno"	4.5 ± 0.01	0.00%
BQ	4.5 ± 0.02	0.00%
C-plus	4.5 ± 0.01	0.00%
"Frontiere 2.0"	4.5 ± 0.01	0.00%
C-	4.5 ± 0.02	0.00%

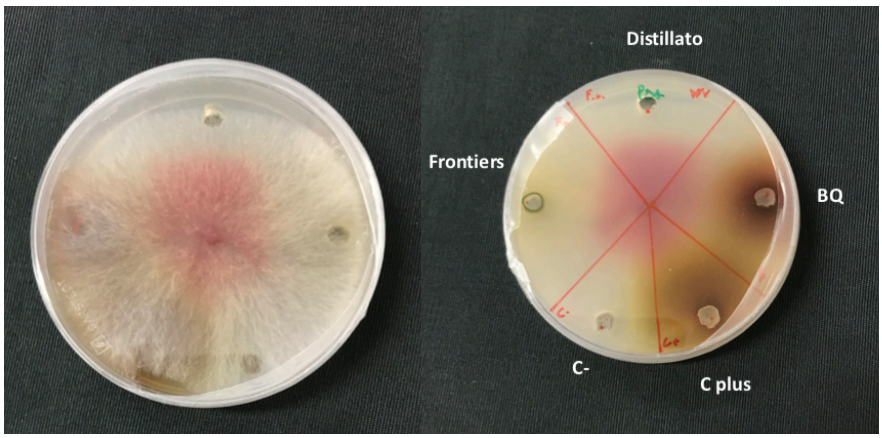

Figure 36. *F. culmorum* treated with products as such

A. alternata

Table 29. Inhibition *in vitro* of *A. alternata* by the botanicals estimated as radius in cm ± standard error of the fungi colony at 6 days of growth

	A. alternata	
	Fungal growth (cm)	**Growth inhibition (%)**
"Distillato di Legno"	2.45 ± 0.03	-47.76%
BQ	2.55 ± 0.01	-41.96%
C-plus	2.6 ± 0.01	-39.23%
"Frontiere 2.0"	3.15 ± 0.03	-14.92%
C-	3.62 ± 0.01	0,00%

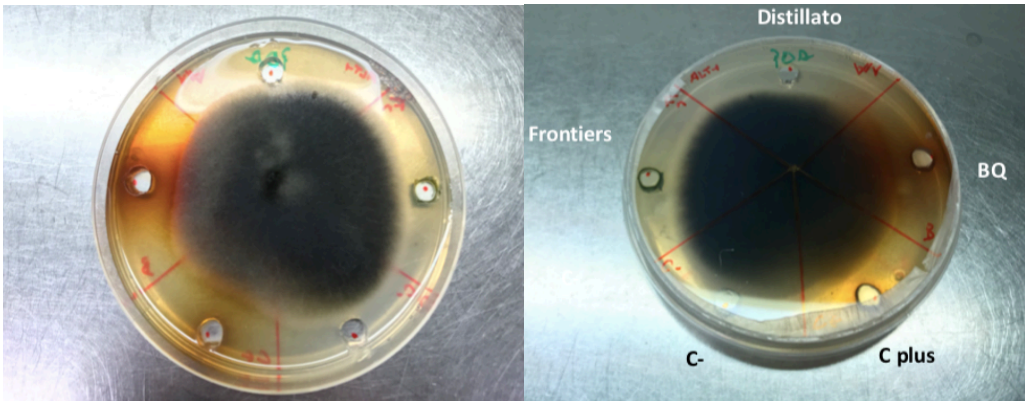

Figure 37. *A. alternata* treated with products as such

C. parasitica

Table 30. Inhibition *in vitro* of *C. parasitica* by the botanicals estimated as radius in cm ± standard error of the fungi colony at 6 days of growth

	C. parasitica	
	Fungal growth (cm)	**Growth inhibition (%)**
"Distillato di Legno"	3.1 ± 0.01	-16.13%
BQ	3.2 ± 0.02	-12.50%
C-plus	3.3 ± 0.01	-9.09%
"Frontiere 2.0"	3.3 ± 0.03	-9.09%
C-	3.6 ± 0.02	0.00%

P. cinnamomi

Table 31. Inhibition *in vitro* of *P. cinnamomi* by the botanicals estimated as radius in cm ± standard error of the fungi colony at 6 days of growth

	P. cinnamomi	
	Fungal growth (cm)	**Growth inhibition (%)**
"Distillato di Legno"	2.9 ± 0.02	-51.72%
BQ	4.4 ± 0.01	0.00%
C-plus	4.4 ± 0.03	0.00%
"Frontiere 2.0"	4 ± 0.02	-10.00%
C-	4.4 ± 0.01	0.00%

G. castaneae

Table 32. Inhibition *in vitro* of *G. castaneae* by the botanicals estimated as radius in cm ± standard error of the fungi colony at 6 days of growth

	G. castaneae	
	Fungal growth (cm)	**Growth inhibition (%)**
"Distillato di Legno"	3.15 ± 0.01	-23.81%
BQ	3.4 ± 0.02	-14.71%
C-plus	3.55 ± 0.01	-9.86%
"Frontiere 2.0"	3.9± 0.02	0.00%
C-	3.9 ± 0.02	0.00%

M. fructigena

Table 33. Inhibition *in vitro* of *M. fructigena* by the botanicals estimated as radius in cm ± standard error of the fungi colony at 6 days of growth

	M. fructigena	
	Fungal growth (cm)	**Growth inhibition (%)**
"Distillato di Legno"	4.5 ± 0.01	0.00%
BQ	4.5 ± 0.01	0.00%
C-plus	4.5 ± 0.02	0.00%
"Frontiere 2.0"	4.5 ± 0.01	0.00%
C-	4.5 ± 0.02	0.00%

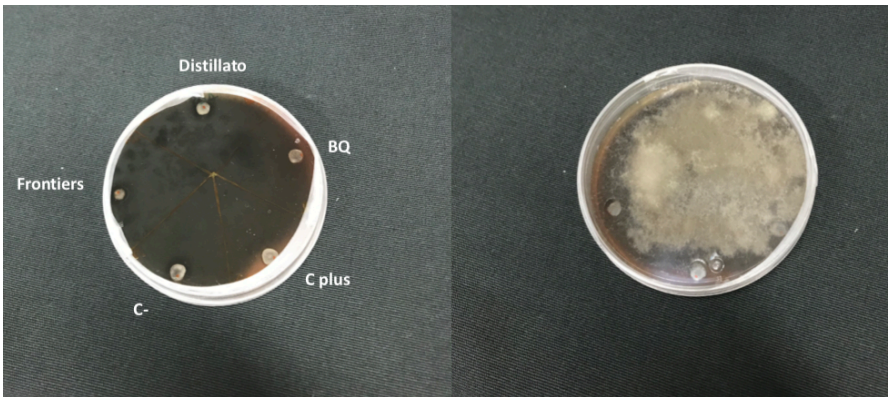

Figure 38. *M. fructigena* treated with products as such

P. infestans

Table 34. Inhibition *in vitro* of *P. infestans* by the botanicals estimated as radius in cm ± standard error of the fungi colony at 6 days of growth

	P. infestans	
	Fungal growth (cm)	**Growth inhibition (%)**
"Distillato di Legno"	4.5 ± 0.02	0.00%
BQ	4.5 ± 0.02	0.00%
C-plus	2.3 ± 0.01	-95.65%
"Frontiere 2.0"	2.6 ± 0.01	-73.08%
C-	4.5 ± 0.01	0.00%

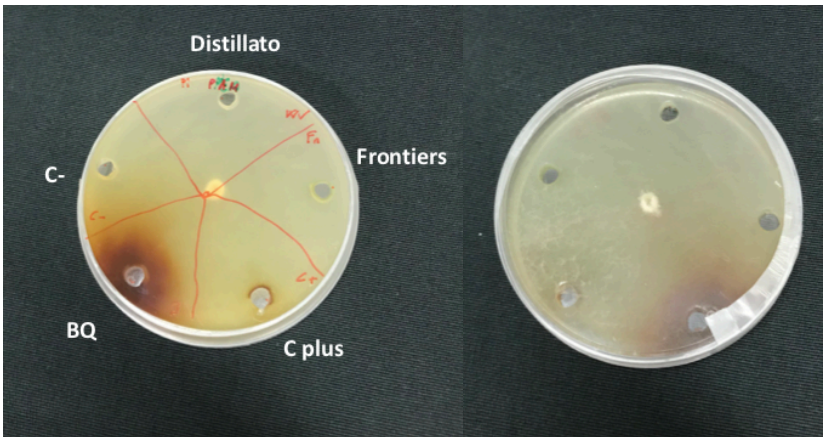

Figure 39. *P. infestans* treated with products as such

S. sclerotiorum

Table 35. Inhibition *in vitro* of *S. sclerotiorum* by the botanicals estimated as radius in cm ± standard error of the fungi colony at 6 days of growth

	S. sclerotiorum	
	Fungal growth (cm)	**Growth inhibition (%)**
"Distillato di Legno"	4.5 ± 0.01	0.00%
BQ	4.5 ± 0.02	0.00%
C-plus	4.5 ± 0.01	0.00%
"Frontiere 2.0"	4.5 ± 0.02	0.00%
C-	4.5 ± 0.02	0.00%

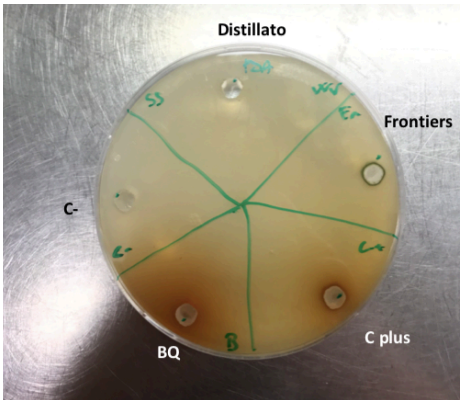

Figure 40. *S. sclerotiorum treated with products as such*

V. longisporum

Table 36. Inhibition *in vitro* of *V. longisporum* by the botanicals estimated as radius in cm ± standard error of the fungi colony at 6 days of growth

	V. longisporum	
	Fungal growth (cm)	**Growth inhibition (%)**
"Distillato di Legno"	4 ± 0.03	25.00%
BQ	2.8 ± 0.01	-7.14%
C-plus	2.3 ± 0.02	-30.43%
"Frontiere 2.0"	3.6 ± 0.01	16.67%
C-	3 ± 0.02	0.00%

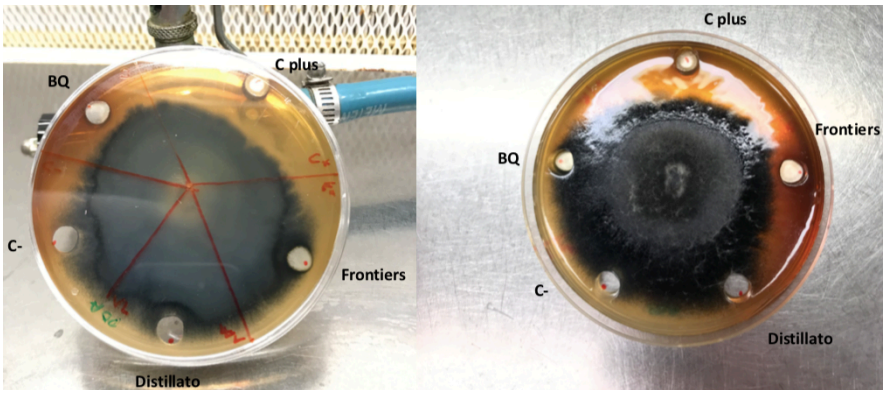

Figure 41. *V. longisporum* treated with products as such

Generally, "Distillato di Legno" has the capacity to inhibit the growth of the most part of the fungi here tested, in particular when used as such. Similarly, the other botanicals here tested, when active they retain this ability when undiluted.

On *S. rolfsii* the average data of the replicates of each tested product compared to negative control show the capacity to inhibit its growth in all the dilutions tested (Table 22). "Distillato di Legno" without cellulose disks inhibit the growth of 8.09% in concentration 1:10, of 12.31% in concentration 1:100 and of 8.94% in concentration 1:1000, while with the application of soaked cellulose disks inhibition percentage is respectively 20.74% (1:10), 15.68% (1:100) and 19.90% (1:1000).

Frontiere 2.0" inhibits with a similar percentage at concentrations 1:10 and 1:100 for both directly application and by soaked cellulose disks. 1:10 concentrations inhibit for 19.06% for the first and 17.37% for the second, while for 1:100 the inhibition percentage, 17.37%, is identical for both. 1:1000 soaked disks and spots on agar have different capacity of inhibition: 24.11% the first and 9.78% the second.

Silvateam Agritan C-plus at concentration 1:10 have the most significant inhibition capacity: 29.17% by direct application and 34.23% by soaked disks. 1:100 and 1:1000 dilutions directly applicated on agar both show an inhibition of 8.94% and by soaked disks both 21.59%. These lasts are the highest values of inhibition comparing all the 1:100 concentrations.

The other Silvateam product, Agritan BQ, show similar inhibition percentages for 1:10 concentrations in both ways of application of the product: 24.11% for direct application and 22.43% for application by soaked disks. Same thing for 1:100 with 19.90% of inhibition for the first and 18.21% for the second, while for 1:1000 concentrations there is a substantial difference between disks and without them. With the first the inhibition percentage is 6.42% and for the second 19.06%.

The other fungi have been tested with the products as such and compared to negative control.
F. poae growth is not inhibited by "Distillato di Legno" and "Frontiere 2.0" (Table 26). Indeed, it seems to be positive influenced by both with the same increment of 18.18%, and also Silvateam Agritan BQ doesn't inhibit the fungus; growth is for 11.11% greater than negative control. Silvateam Agritan C-plus is the only one that has effect on inhibition with a reduction of 20%.

F. avenaceum is inhibited by "Distillato di Legno" and "Frontiere 2.0" both for 5.56% and by Silvateam Agritan C-plus for 9.62%. Agritan BQ doesn't have any influence and also seems to promote the growth of 5% compared to negative control (Table 27).

A. alternata is inhibits by "Distillato di Legno" for 47.76% and by "Frontiere 2.0" for 14.92%. The two Silvateam have both the capacity of inhibits growth but in different percentage: Agritan BQ for 41.96 and Agritan C-plus for 39.23% (Table 29).

C. parasitica shows a growth reduction of 16.13% by "Distillato di Legno", while "Frontiere 2.0" of 9.09%. Silvateam Agritan BQ and C-plus show no inhibition on the fungus; on the contrary, they seem to positively influence its growth respectively of 12.50% and 9.09% (Table 30).

P. cinnamomi is inhibited by "Distillato di Legno" for 51.72% and "Frontiere 2.0" for 10%. The two Silvateam products seem to produce no effect and growth is identical to negative control (Table 31).

"Distillato di Legno" and Silvateam Agritan BQ and C-plus have capacity to inhibit growth on *G. castaneae* respectively for 23.81%, 14.71% and 9.86% while "Frontiere 2.0" doesn't have any effect on the fungus growth (Table 32).

P. infestans (Table 34) is not inhibited by "Distillato di Legno" and Silvateam Agritan BQ but it's inhibited by Agritan C-plus (95.65%) and "Frontiere 2.0" (73.08%) (Table 34).

V. longisporum growth it's influenced by all the products but in different way. "Distillato di Legno" and "Frontiere 2.0" don't inhibit compared to negative control but they increase the growth of 25% and 16.67%. The two Silvateam Agritan BQ and C-plus inhibit the fungus for 7.14% and 3.43% (Table 36).

Inhibition data collected on *F. graminearum* (Table 25)*, F. culmorum* (Table 28)*, M. fructigena* (Table 33) and *S. sclerotiorum* (Table 35) show an identical result: all the products have no capacity to inhibit these 4 fungi. Indeed, their growth seem to be identical if compared to negative control.

4.4.2 *In vitro* bacterial growth inhibition

P. syringae pv. *tabaci*

Table 37. Inhibition of *in vitro* growth of *P. syringae* pv. *tabaci* by the botanicals here tested at 6 days

	P. syringae* pv. *tabaci
	Inhibition halo (cm)
"Distillato di Legno" as such	0.91 ± 0.02
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.15 ± 0.03
Wood Vinegar 1:10	0.6 ± 0.05
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

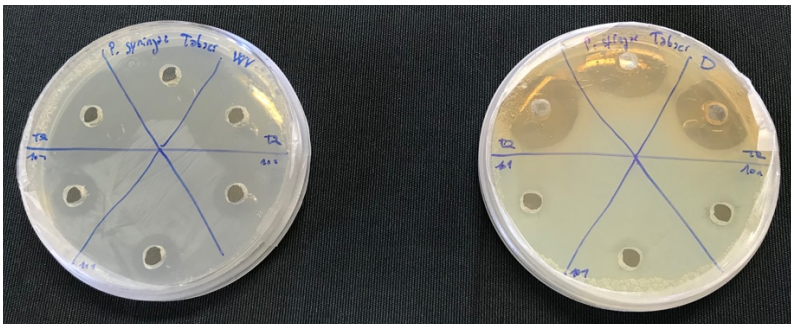

Figure 42. *P. syringae* pv. *tabaci* treated with products as such and at 1:10 concentration

P. syringae pv. *phaseolicola*

Table 38. Inhibition of *in vitro* growth of *P. syringae* pv. *phaseolicola* by the botanicals here tested at 6 days

	P. syringae* pv. *phaseolicola
	Inhibition halo (cm)
"Distillato di Legno" as such	1.33 ± 0.07
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.26 ± 0.07
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

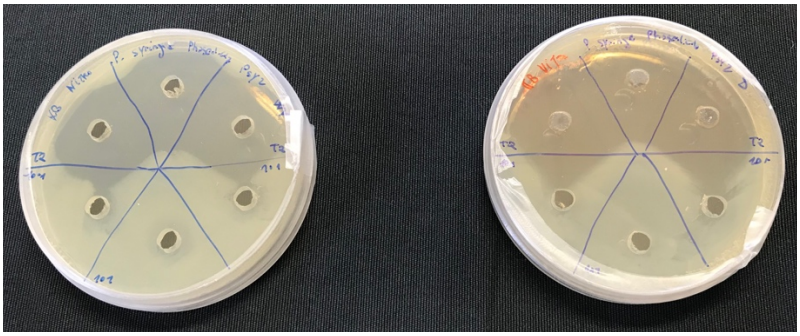

Figure 43. *P. syringae* pv. *phaseolicola* treated with products as such and at 1:10 concentration

P. syringae pv. *tomato* strain DC3000

Table 39. Inhibition of *in vitro* growth of *P. syringae* pv. *tomato* DC3000 by the botanicals here tested at 6 days

	***P. syringae* pv. *tomato* DC3000**
	Inhibition halo (cm)
"Distillato di Legno" as such	0.92 ± 0.03
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.07 ± 0.02
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

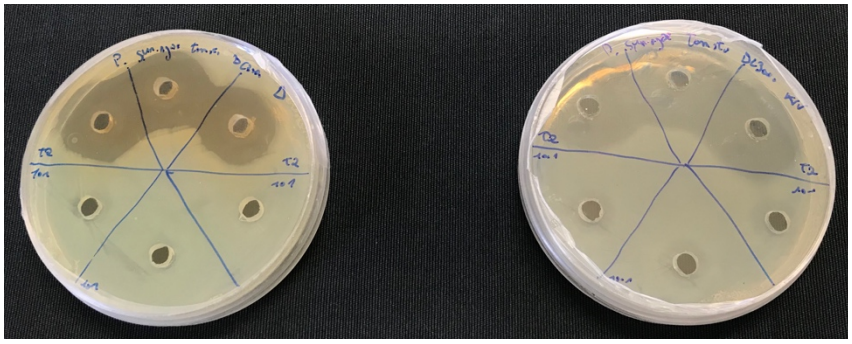

Figure 44. *P. syringae* pv. *tomato* DC3000 treated with products as such and at 1:10 concentration

P. syringae pv. *actinidiae*

Table 40. Inhibition of *in vitro* growth of *P. syringae* pv. *actinidiae* by the botanicals here tested at 6 days

	P. syringae* pv. *actinidiae
	Inhibition halo (cm)
"Distillato di Legno" as such	1.1 ± 0.04
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.19 ± 0.06
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

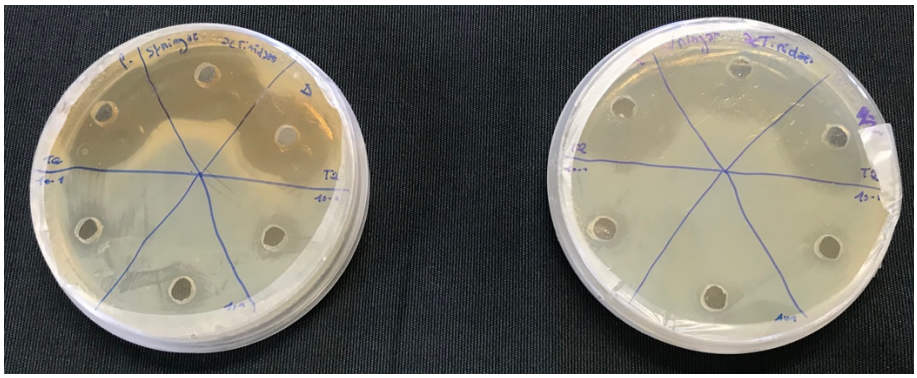

Figure 45. *P. syringae* pv. *actinidiae* treated with products as such and at 1:10 concentration

P. savastanoi pv. *nerii* strain Psn23

Table 41. Inhibition of *in vitro* growth of *P. savastanoi* pv. *nerii* Psn23 by the botanicals here tested at 6 days

	***P. savastanoi* pv. n*erii* Psn23**
	Inhibition halo (cm)
"Distillato di Legno" as such	1.11 ± 0.05
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.27 ± 0.04
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

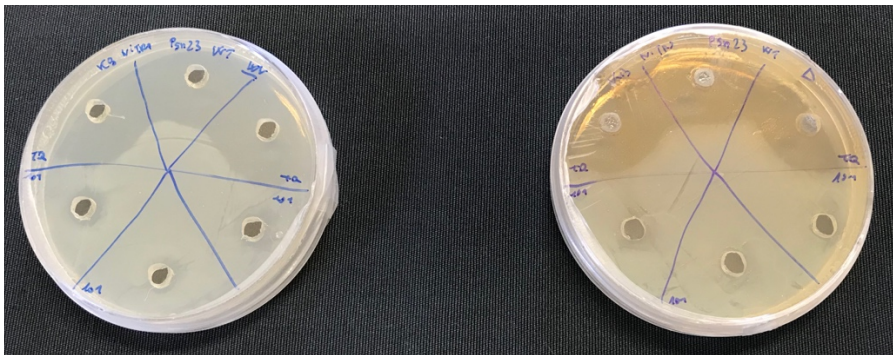

Figure 46. *P. savastanoi* pv. *nerii* strain Psn23 treated with products as such and at 1:10 concentration

P. savastanoi pv. *savastanoi* strain Psv5

Table 42. Inhibition of *in vitro* growth of *P. savastanoi* pv. *savastanoi Psv5* by the botanicals here tested at 6 days

	***P. savastanoi* pv. *savastanoi* Psv5**
	Inhibition halo (cm)
"Distillato di Legno" as such	1.13 ± 0.05
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.37 ± 0.12
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

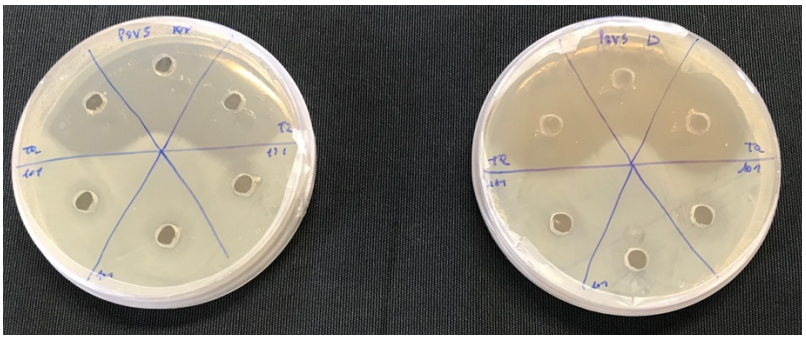

Figure 46. *P. savastanoi* pv. *savastanoi* strain Psn23 treated with products as such and at 1:10 concentration

X. axonopodis pv. *euvesicatoria*

Table 43. Inhibition of *in vitro* growth of *X. axonopodis* pv. *euvesicatoria* by the botanicals here tested at 6 days

	X. axonopodis* pv. *euvesicatoria
	Inhibition halo (cm)
"Distillato di Legno" as such	0
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	0.47 ± 0.02
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

X. axonopodis pv. *gardneri*

Table 44. Inhibition of *in vitro* growth of *X. axonopodis* pv. *gardneri* by the botanicals here tested at 6 days

	X. axonopodis* pv. *gardneri
	Inhibition halo (cm)
"Distillato di Legno" as such	0.97 ± 0.02
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	0.14 ± 0.01
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

X. axonopodis pv. *perforans*

Table 44. Inhibition of *in vitro* growth of *X. axonopodis* pv. *perforans* by the botanicals here tested at 6 days

	X. axonopodis* pv. *perforans
	Inhibition halo (cm)
"Distillato di Legno" as such	0.53 ± 0.02
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	0.32 ± 0.03
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

X. axonopodis pv. *pelargoni*

Table 45. Inhibition of *in vitro* growth of *X. axonopodis* pv. *pelargoni* by the botanicals here tested at 6 days

	X. axonopodis* pv. *pelargoni
	Inhibition halo (cm)
"Distillato di Legno" as such	0.57 ± 0.03
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	0
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

X. axonopodis pv. *phaseoli*

Table 46. Inhibition of *in vitro* growth of *X. axonopodis* pv. *phaseoli* by the botanicals here tested at 6 days

	X. axonopodis* pv. *phaseoli
	Inhibition halo (cm)
"Distillato di Legno" as such	0.97 ± 0.05
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	0
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

X. axonopodis pv. *alpha-alpha*

Table 47. Inhibition in vitro of *X. axonopodis* pv. *alpha-alpha* by the botanicals here tested at 6 days

	X. axonopodis* pv. *alpha-alpha
	Inhibition halo (cm)
"Distillato di Legno" as such	1.23 ± 0.03
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.17 ± 0.04
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

X. axonopodis pv. *vesicatoria*

Table 48. Inhibition of *in vitro* growth of *X. axonopodis* pv. *vesicatoria* by the botanicals here tested at 6 days

	X. axonopodis* pv. *vesicatoria
	Inhibition halo (cm)
"Distillato di Legno" as such	0.64 ± 0.04
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.17 ± 0.07
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

X. axonopodis pv. *juglandi*

Table 49. Inhibition of *in vitro* growth of *X. axonopodis* pv. *juglandi* by the botanicals here tested at 6 days

	X. axonopodis* pv. *juglandi
	Inhibition halo (cm)
"Distillato di Legno" as such	0
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	0.51 ± 0.01
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

C. flaccumfaciens pv. *poinsettiae*

Table 50. Inhibition of *in vitro* growth of *C. flaccumfaciens* pv. *poinsettiae* by the botanicals here tested at 6 days

	C. flaccumfaciens* pv. *poinsettiae
	Inhibition halo (cm)
"Distillato di Legno" as such	0.6 ± 0.03
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.43 ± 0.05
Wood Vinegar 1:10	0.53 ± 0.04
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

C. flaccumfaciens pv. *ortii*

Table 51. Inhibition of *in vitro* growth of *C. flaccumfaciens* pv. *ortii* by the botanicals here tested at 6 days

	C. flaccumfaciens* pv. *ortii
	Inhibition halo (cm)
"Distillato di Legno" as such	1.13 ± 0.02
"Distillato di Legno" 1:10	0.34 ± 0.01
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.57 ± 0.06
Wood Vinegar 1:10	1.23 ± 0.06
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

C. flaccumfaciens pv. *betae*

Table 52. Inhibition of *in vitro* growth of *C. flaccumfaciens* pv. *betae* by the botanicals here tested at 6 days

	C. flaccumfaciens* pv. *betae
	Inhibition halo (cm)
"Distillato di Legno" as such	1.14 ± 0.04
"Distillato di Legno" 1:10	0.24 ± 0.02
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.38 ± 0.09
Wood Vinegar 1:10	0.5 ± 0.02
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

C. flaccumfaciens pv. *flaccumfaciens*

Table 53. Inhibition of *in vitro* growth of *C. flaccumfaciens* pv. *flaccumfaciens* by the botanicals here tested at 6 days

	C. flaccumfaciens* pv. *flaccumfaciens
	Inhibition halo (cm)
"Distillato di Legno" as such	0.35 ± 0.03
"Distillato di Legno" 1:10	0
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.41 ± 0.08
Wood Vinegar 1:10	0.6 ± 0.02
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

C. flaccumfaciens pv. *ilicis*

Table 54. Inhibition of *in vitro* growth *C. flaccumfaciens* pv. *ilicis* by the botanicals here tested at 6 days

	C. flaccumfaciens* pv. *ilicis
	Inhibition halo (cm)
"Distillato di Legno" as such	1.09 ± 0.04
"Distillato di Legno" 1:10	0.27 ± 0.02
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.31 ± 0.04
Wood Vinegar 1:10	0.48 ± 0.01
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

C. michiganensis pv. *michiganensis* strain NCPB 382

Table 55. Inhibition of *in vitro* growth *C. michiganensis* pv. *michiganensis* NCPB 382 by the botanicals here tested at 6 days

	***C. michiganensis* pv. *michiganensis* NCPB 382**
	Inhibition halo (cm)
"Distillato di Legno" as such	1.17 ± 0.04
"Distillato di Legno" 1:10	0.43 ± 0.01
"Distillato di Legno" 1:100	0
"Distillato di Legno" 1:1000	0
Wood Vinegar as such	1.19 ± 0.06
Wood Vinegar 1:10	0
Wood Vinegar 1:100	0
Wood Vinegar 1:1000	0

In conclusions, the data obtained and here reported are coherent among the two treatments and the bacteria tested, that is no any inhibitory activity was found at dilutions of 1:100 and 1:1000. Conversely, the two botanicals showed inhibitory properties when tested as such, or at a certain extent also at 1:10 dilution although not constantly.

The "Distillato di Legno" as such inhibits all the bacteria tested, except *X. axonopodis* pv. *euvesicatoria* and pv. *juglandi*. The 1:10 dilution 1:10 of "Distillato di Legno" has the capacity to inhibit five bacteria, that are *P. syringae* pv. *tabaci*, *C. flaccumfaciens* pv. *ortii*, *betae* and *ilicis* and *C. michiganensis* pv. *michiganensis* strain NCPB 382.

The Wood Vinegar as such, like "Distillato di Legno" as such, doesn't inhibit all the bacteria. Indeed, two bacteria, *P. syringae* pv. *phaseolicola*, *X. axonopodis* pv. *pelargoni* and *phaseoli*, seems to don't be influenced by the product, differently from all the other bacteria.

The Wood Vinegar has a greater inhibition effect than "Distillato di Legno" when used at 1:10 dilution. The bacteria inhibited by this dilution are *P. syringae* pv. *tabaci*, pv. *tomato* strain DC3000, *C. flaccumfaciens* pv. *poinsettiae*, pv. *ortii*, pv. *betae*, pv. *flaccumfaciens* and pv. *ilicis*.

Overall these data show that "Distillato di Legno" has lower antibacterial activity in comparison to Wood vinegar when both are used as such. Conversely, at dilution 1:10 Wood Vinegar conserved a higher inhibitory activity than "Distillato di Legno".

4.5 Anti-infective activity on *P. savastanoi* pv. *nerii* strains Psn23 and Psn23 pT3-GFP

The results here reported are referred to the inhibitory activity on the pathogenicity of *P. savastanoi* pv. *nerii* strain Psn23, evaluated and expressed as inhibition of the activation of its TTSS. To this aim the construct pT3-GFP was used to transform *Psn23* wild type, to give the mutant *Psn23* pT3-GFP, to be then tested with "Distillato di Legno" at dilutions 1:10, 1:100 and 1:1000 in liquid MM medium. As positive control, IAA was used at concentration 200 µM (Cerboneschi *et al.*, 2016), while as negative control just bidistilled water was used.

The increase of the relative fluorescence on MM of *Psn23* pT3-GFP, in comparison to *Psn23* and to the negative control, was calculated by using the formula

$$\frac{Fluorescence\ (485nm/535nm)}{Adbsorbance\ (600nm)}$$

Each measure is represented by the normalized values ± standard error (Table 56) obtained in MM).

Table 56 Normalized ratio fluorescence/absorbance on *P. savastanoi* pv. *nerii* Psn23 pT3-GFP, treated with "Distillato di Legno" in MM and at different time points

	***P. savastanoi* pv. *nerii* Psn23 pT3-GFP**			
	T_0	**T_2**	**T_{24}**	**T_{48}**
Psn23 pT3 GFP	0.97 ± 0.01	0.96± 0.01	1.51 ± 0.03	1.73 ± 0.03
Psn23 pT3 GFP + IAA µM	1.17 ± 0.02	0.96 ± 0.01	1.03 ± 0.02	0.93 ± 0.01
Psn23 pT3 GFP + WV 1:100	-	-	1.5± 0.02	1.2 ± 0.02
Psn23 pT3 GFP + WV 1:1000	-	-	0.83 ± 0.01	0.62 ± 0.01

The comparison between these treatments show a significant result. The dilutions of "Distillato di Legno" 1:100 and 1:1000 show the capacity to inhibit the TTSS of Psn23.

In Table 57, the same data are reported as percentage in comparison to the control.

Table 57. Percentage of variation obtained by the comparison between the four theses *P. savastanoi* pv. *nerii* Psn23 pT3-GFP treated by "Distillato di Legno" in MM

	***P. savastanoi* pv. *nerii* Psn23 pT3-GFP**			
	T_0	**T_2**	**T_{24}**	**T_{48}**
Psn23 pT3 GFP	0%	0%	0%	0%
Psn23 pT3 GFP + IAA µM	0%	0%	46%	32%
Psn23 pT3 GFP + WV 1:100	0%	0%	13%	21%
Psn23 pT3 GFP + WV 1:1000	0%	0%	45%	64%

Generally, at T_0 and T_3 no significant differences have been found, while a significant decrease in TTSS activation was observed at T_{24} and T_{48} following the treatment with "Distillato di Legno" in particular, "Distillato di Legno" used at 1:100 dilution inhibits for 13% at T_{24}, and for 21% at T_{48}, while the dilution 1:1000 gives an inhibition for 45% and 64% at T_{24} and at T_{48}, respectively.

4.6 Tobacco in field

N. tabacum cultivars Virginia and Kentucky data about height, photosynthetic efficiency and number of leaves of the plants have been collected during an experiment carried out on summer 2019.

4.6.1 Virginia and Kentucky plants height

Measures of plant height have been directly collected in field and data are divided by cultivars and show as value in mt ± standard error in Table 39 and 41, while in Table 40 and 42 are reported the averages.

Figure 48. Plants of *N. tabacum* cv. Virginia

Virginia

Table 58. Comparison between effects of different treatments on height by "Distillato di Legno" and "Frontiere 2.0" on cultivar Virginia. Measures of height are reported in cm ± standard error

***N. tabacum* cv. Virginia height (cm)**			
"Frontiere 2.0" foliar	**"Distillato di Legno" fertirrigation**	**"Distillato di Legno" foliar**	**Control (no treatment)**
1.73 ± 0.12	1.4 ± 0.04	1.49 ± 0.08	1.36 ± 0.08
1.3 ± 0.12	1.52 ± 0.04	1.46 ± 0.08	1.95 ± 0.08
1.52 ± 0.12	1.63 ± 0.04	1.74 ± 0.08	2.06 ± 0.08
1.15 ± 0.12	1.65 ± 0.04	1.65 ± 0.08	1.89 ± 0.08
1.05 ± 0.12	1.34 ± 0.04	1.76 ± 0.08	1.85 ± 0.08
	1.37 ± 0.04	1.29 ± 0.08	1.61 ± 0.08
	1.62 ± 0.04	1.12 ± 0.08	1.95 ± 0.08
	1.58 ± 0.04	1.09 ± 0.08	2.14 ± 0.08
	1.55 ± 0.04	1.67 ± 0.08	1.54 ± 0.08
	1.73 ± 0.04	1.53 ± 0.08	1.74 ± 0.08

Table 59. Comparison between averages of the different treatments on cultivar Virginia. Measures are reported in cm ± standard error with variation in percentage compared to not treated

	***N. tabacum* cv. Virginia**			
	"Frontiere 2.0" foliar	**"Distillato di Legno" fertirrigation**	**"Distillato di Legno" foliar**	**Control (no treatment)**
Averages height (cm)	1.35 ± 0.12	1.54 ± 0.04	1.48 ± 0.08	1.81 ± 0.08
Difference (%)	-34.07%	-17.53%	-22.30%	0.00%

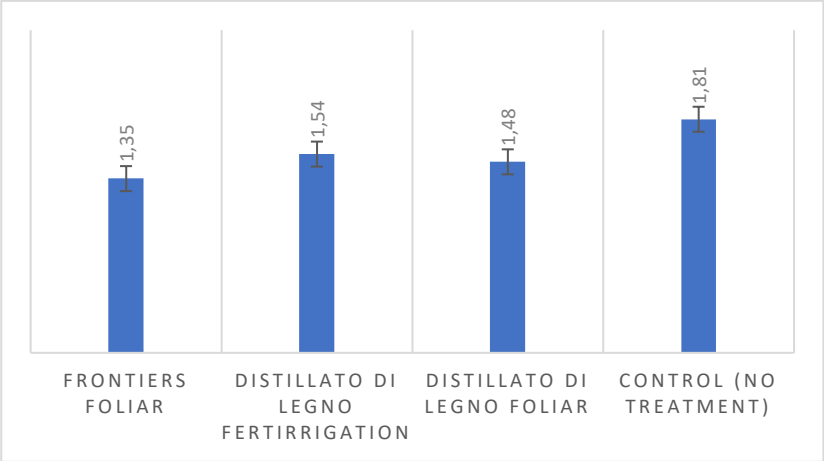

Figure 49. Comparison between height averages of treated and not treated Virginia plants. "Frontiere 2.0" treatments are reported as "Frontiers"

Kentucky

Table 60. Comparison between effects of different treatments on height by "Distillato di Legno" and "Frontiere 2.0" on cultivar Kentucky. Measures of height are reported in cm ± standard error

***N. tabacum* cv. Kentucky height (cm)**			
"Frontiere 2.0" foliar	**"Distillato di Legno" fertirrigation**	**"Distillato di Legno" foliar**	**Control (no treatment)**
2.03 ± 0.01	1.74 ± 0.13	1.63 ± 0.09	2.52 ± 0.1
1.97 ± 0.01	1.71 ± 0.13	1.69 ± 0.09	3.01 ± 0.1
2.03 ± 0.01	2.3 ± 0.13	1.39 ± 0.09	2.52 ± 0.1
2.01 ± 0.01	2.6 ± 0.13	1.61 ± 0.09	2.65 ± 0.1
1.98 ± 0.01	1.59 ± 0.13	1.24 ± 0.09	2.08 ± 0.1
	2.8 ± 0.13	2.18 ± 0.09	2.63 ± 0.1
	1.92 ± 0.13	1.36 ± 0.09	2.04 ± 0.1
	1.88 ± 0.13	1.73 ± 0.09	2.59 ± 0.1
	1.98 ± 0.13	2.04 ± 0.09	2.48 ± 0.1
	1.87 ± 0.13	1.82 ± 0.09	3.02 ± 0.1

Table 61. Comparison between averages of the different treatments on cultivar *Kentucky.* Measures are reported in cm ± standard error with variation in percentage compared to not treated

	***N. tabacum* cv. Kentucky**			
	"Frontiere 2.0" foliar	**"Distillato di Legno" fertirrigation**	**"Distillato di Legno" foliar**	**Control (no treatment)**
Averages height (cm)	2.00 ± 0.01	2.04 ± 0.13	1.67 ± 0.09	2.55 ± 0.1
Difference (%)	-27.50%	-25.00%	-52.69%	0.00%

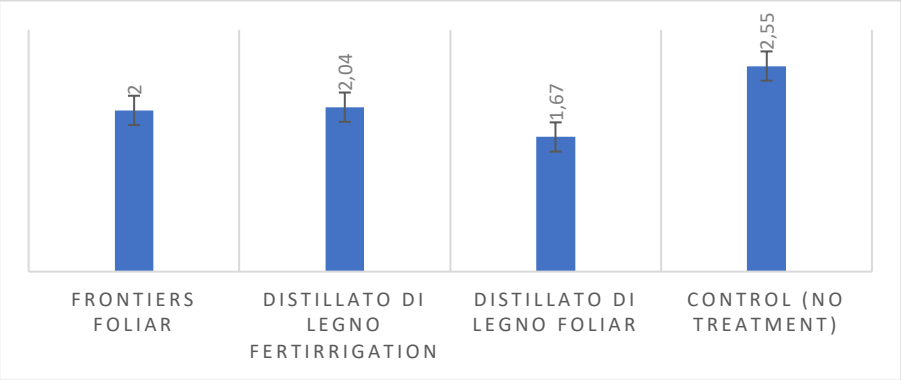

Figure 50. Comparison between height averages of treated and not treated Kentucky plants. "Frontiere 2.0" treatments are reported as "Frontiers"

Data collected for each plant show significant differences between plants treated with "Distillato di Legno" and "Frontiere 2.0" and those untreated. These last plants are higher than the others, both for Kentucky and Virginia cultivars. These data are also confirmed in Table 40 and 42, by comparing the averages of the 4 theses for cultivar.

Plants treated by "Frontiere 2.0" show a height reduction of 34.07% for Virginia and 27.50% for Kentucky, while "Distillato di Legno" treatments by fertirrigation show a reduction of 17.53% for Virginia and 25.00% for Kentucky. "Distillato di Legno" foliar treatments show the most significant results on Kentucky with a reduction in height of 52.69% compared to no treated plants, beside Virginia data that show a reduction of 22.30%.

Generally, the data collected show a substantial reduction on "Frontiere 2.0" treated plants both for Virginia and Kentucky, followed by fertirrigation by "Distillato di Legno" and foliar treatment with the exception represented by this last on Kentucky.

Nevertheless, it's possible to observe a significant difference between "Distillato di Legno" treated plants and the other two theses comparing the single values reported in Table 58 and 60. Plants treated by fertirrigation and foliar treatments by "Distillato di Legno" have similar measures for

each one despite some exceptions, in particular for the Virginia cultivar, showing a synchronization not present on the "Frontiere 2.0 " treated plants and not treated. This is viewable in the Figure 45.

Figure 51. Tobacco plants treated with "Distillato di Legno"

4.6.2 Virginia and Kentucky leaves

Number of leaves has been collected at 90th day after planting directly in field. Data are reported in Table 43 and 45 as number of leaves ± standard error plus the averages in Table 44 and 45.

Virginia

Table 62. Comparison between effects of different treatments on height by "Distillato di Legno" and "Frontiere 2.0" on cultivar Virginia. Measures are reported as number of leaves ± standard error

***N. tabacum* cv. Virginia**			
"Frontiere 2.0" foliar	**"Distillato di Legno" fertirrigation**	**"Distillato di Legno" foliar**	**Control (no treatment)**
75 ± 11	26 ± 2	23 ± 1	51 ± 8
32 ± 11	34 ± 2	21 ± 1	70 ± 8
29 ± 11	35 ± 2	23 ± 1	108 ± 8
15 ± 11	24 ± 2	22 ± 1	65 ± 8
14 ± 11	25 ± 2	24 ± 1	69 ± 8
	21 ± 2	28 ± 1	40 ± 8
	23 ± 2	19 ± 1	31 ± 8
	36 ± 2	19 ± 1	82 ± 8
	24 ± 2	25 ± 1	23 ± 8
	25 ± 2	24 ± 1	65 ± 8

Table 63. Comparison between averages of the different treatments on cultivar Virginia.
Measures are reported as number of leaves ± standard error with variation in percentage compared to not treated

	***N. tabacum* cv. Virginia**			
	"Frontiere 2.0" foliar	**"Distillato di Legno" fertirrigation**	**"Distillato di Legno" foliar**	**Control (no treatment)**
Averages height (cm)	33 ± 11	27 ± 2	23 ± 1	60 ± 8
Reduction (%)	-45%	-55%	-61.7%	0.00%

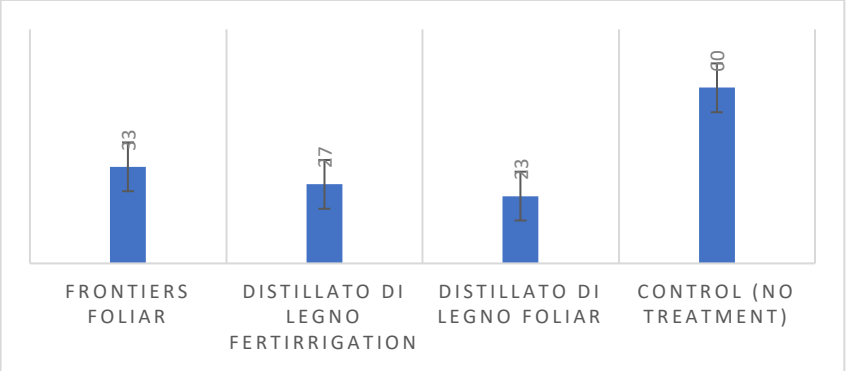

Figure 52. Comparison between averages of number of leaves of treated and not treated Virginia plants.
"Frontiere 2.0" treatments are reported as "Frontiers"

Kentucky

Table 64. Comparison between effects of different treatments on height by "Distillato di Legno" and "Frontiere 2.0" on cultivar Kentucky. Measures are reported as number of leaves ± standard error

***N. tabacum* cv. Kentucky**			
"Frontiere 2.0" foliar	**"Distillato di Legno" fertirrigation**	**"Distillato di Legno" foliar**	**Control (no treatment)**
54 ± 3	50 ± 4	26 ± 1	96 ± 7
50 ± 3	23 ± 4	26 ± 1	69 ± 7
49 ± 3	43 ± 4	23 ± 1	74 ± 7
65 ± 3	53 ± 4	24 ± 1	95 ± 7
45 ± 3	25 ± 4	21 ± 1	52 ± 7
	33 ± 4	25 ± 1	81 ± 7
	26 ± 4	16 ± 1	36 ± 7
	26 ± 4	21 ± 1	58 ± 7
	42 ± 4	24 ± 1	92 ± 7
	25 ± 4	21 ± 1	92 ± 7

Table 65. Comparison between averages of the different treatments on cultivar Kentucky. Measures are reported as number of leaves ± standard error with variation in percentage compared to not treated

	***N. tabacum* cv. Kentucky**			
	"Frontiere 2.0" foliar	**"Distillato di Legno" fertirrigation**	**"Distillato di Legno" foliar**	**Control (no treatment)**
Averages height (cm)	53 ± 3	35 ± 4	23 ± 1	75 ± 8
Reduction (%)	-29.3%	-53.3%	-69.3%	0,00%

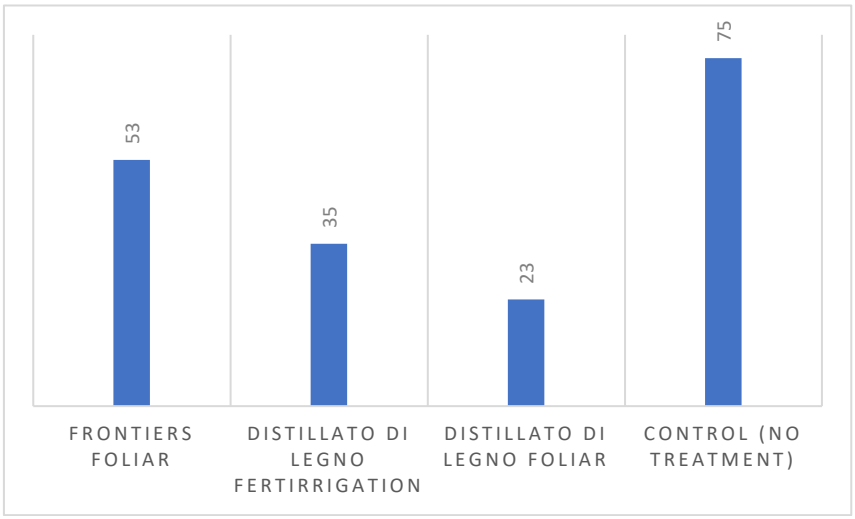

Figure 53. Comparison between averages of number of leaves of treated and not treated Kentucky plants. "Frontiere 2.0" treatments are reported as "Frontiers"

For both Tobacco cultivars, the total number of leaves is decreasing when plants have been treated with "Distillato di Legno" in comparison to those untreated or treated with "Frontiere 2.0".

When Tobacco cv. Virginia and Kentucky plants have been treated by fertirrigation with "Distillato di Legno", a reduction of 55% and 53.3% has been observed, respectively, in comparison to the negative control (Table 44 and 46). Plants treated with "Distillato di Legno" have the most significant reduction in both Tobacco cultivars: 61.7% for Virginia and 69.3% for Kentucky. Similarly, "Frontiere 2.0" treated plants have a reduction of 45% for Virginia and 29.3% for Kentucky in comparison to the negative controls.

4.6.3 Photosynthetic efficiency

Different parameters have been collected to analyze the possible interference of "Distillato di Legno" and "Frontiere 2.0" on photosynthetic efficiency.

The parameters Φ_{P0}, F_0, F_m, F_v, PI_{abs} and Ψ_0, are here reported as averages. For each Tobacco cultivar values are compared among differently positioned leaves (*i.e.* at the top or at the bottom of the plant).

Φ_{P0}

Table 66. Comparison of Φ_{P0} averages of Virginia and Kentucky leaves reported with standard error

	Virginia Φ_{P0}	**Kentucky Φ_{P0}**
"Distillato di Legno" fertirrigation (Top leaf)	0.80 ± 0.01	0.8 ± 0.01
"Distillato di Legno" fertirrigation (Bottom leaf)	0.81 ± 0.01	0.78 ± 0.02
"Distillato di Legno" foliar (Top leaf)	0.76 ± 0.02	0.7 ± 0.02
"Distillato di Legno" foliar (Bottom leaf)	0.74 ± 0.01	0.78 ± 0.01
"Frontiere 2.0" foliar (Top leaf)	0.79 ± 0.01	0.72 ± 0.02
"Frontiere 2.0" foliar (Bottom leaf)	0.82 ± 0.01	0.79 ± 0.01
Control (Top leaf)	0.8 ± 0.01	0.71 ± 0.05
Control (Bottom leaf)	0.75 ± 0.003	0.81 ± 0.02

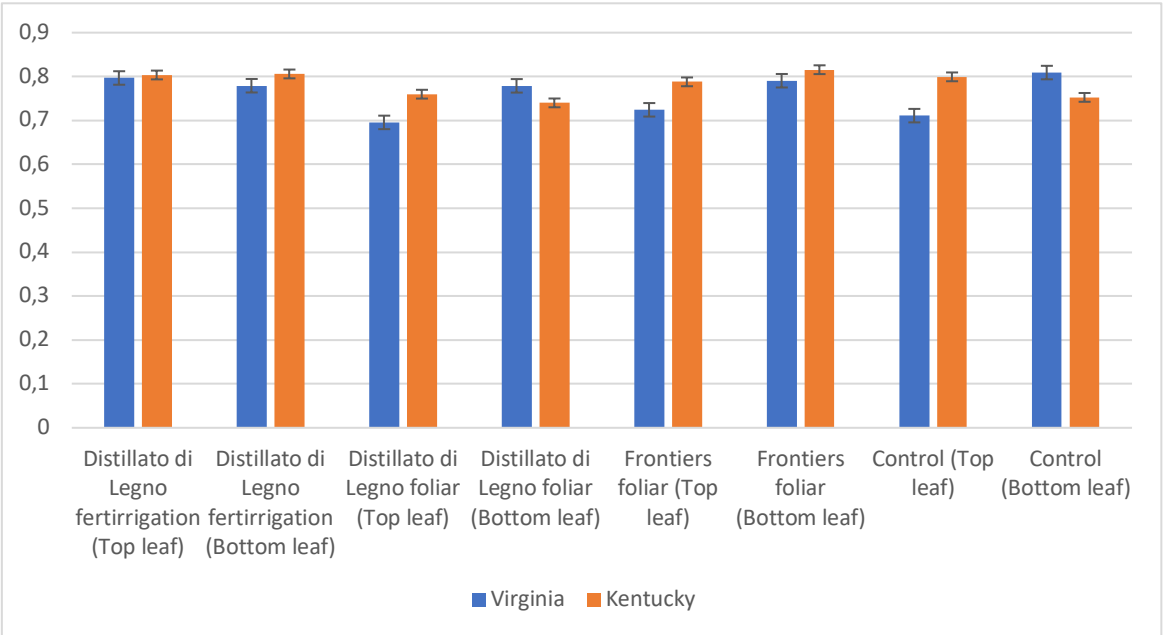

Figure 54. Comparison between Φ_{P0} averages of Virginia and Kentucky leaves. "Frontiere 2.0" treatments are reported as "Frontiers"

F_0

Table 67. Comparison of F_0 averages of Virginia and Kentucky leaves reported with standard error

	Virginia F_0	**Kentucky F_0**
"Distillato di Legno" fertirrigation (Top leaf)	265.63 ± 4.8	273 ± 6.7
"Distillato di Legno" fertirrigation (Bottom leaf)	297.86 ± 14.96	295.38 ± 7.91
"Distillato di Legno" foliar (Top leaf)	321.25 ± 11.95	311.38 ± 33.12
"Distillato di Legno" foliar (Bottom leaf)	284.5 ± 14.05	335.75 ± 13.84
"Frontiere 2.0" foliar (Top leaf)	316.5 ± 6.5	293 ± 5.51
"Frontiere 2.0" foliar (Bottom leaf)	290.67 ± 22.45	300.25 ± 12.57
Control (Top leaf)	365.88 ± 79.26	286 ± 7.43
Control (Bottom leaf)	274.14 ± 10.06	321.5 ± 24.05

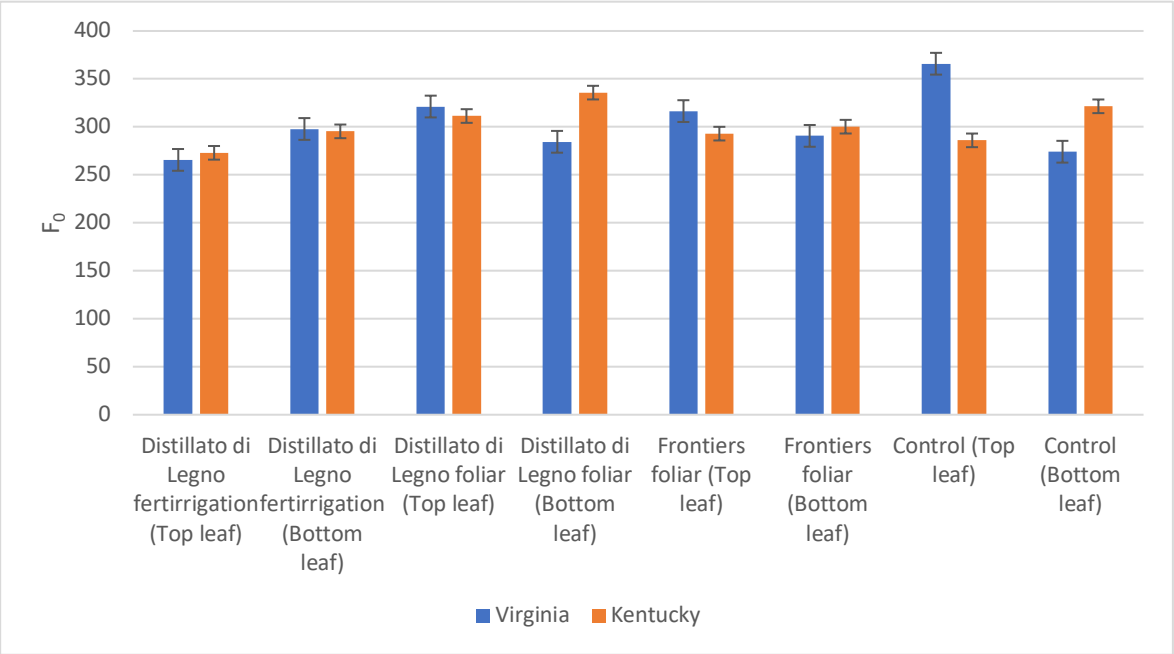

Figure 55. Comparison between F_0 averages of Virginia and Kentucky leaves. "Frontiere 2.0" treatments are reported as "Frontiers"

F_m

Table 68. Comparison of F_m averages of Virginia and Kentucky leaves reported with standard error

	Virginia Fm	Kentucky Fm
"Distillato di Legno" fertirrigation (Top leaf)	1592.88 ± 41.71	1612.29 ± 63.11
"Distillato di Legno" fertirrigation (Bottom leaf)	1683.43 ± 85.19	1763.75 ± 61.9
"Distillato di Legno" foliar (Top leaf)	1289.5 ± 76.91	1527.5 ± 44.35
"Distillato di Legno" foliar (Bottom leaf)	1623.63 ± 73.87	1515.38 ± 16.44
"Frontiere 2.0" foliar (Top leaf)	1384.5 ± 105.16	1641.33 ± 143.63
"Frontiere 2.0" foliar (Bottom leaf)	1637.67 ± 54.74	1882.5 ± 52.9
Control (Top leaf)	1468.5 ± 55.57	1665 ± 81.15
Control (Bottom leaf)	1737.14 ± 49.6	1567 ± 95.04

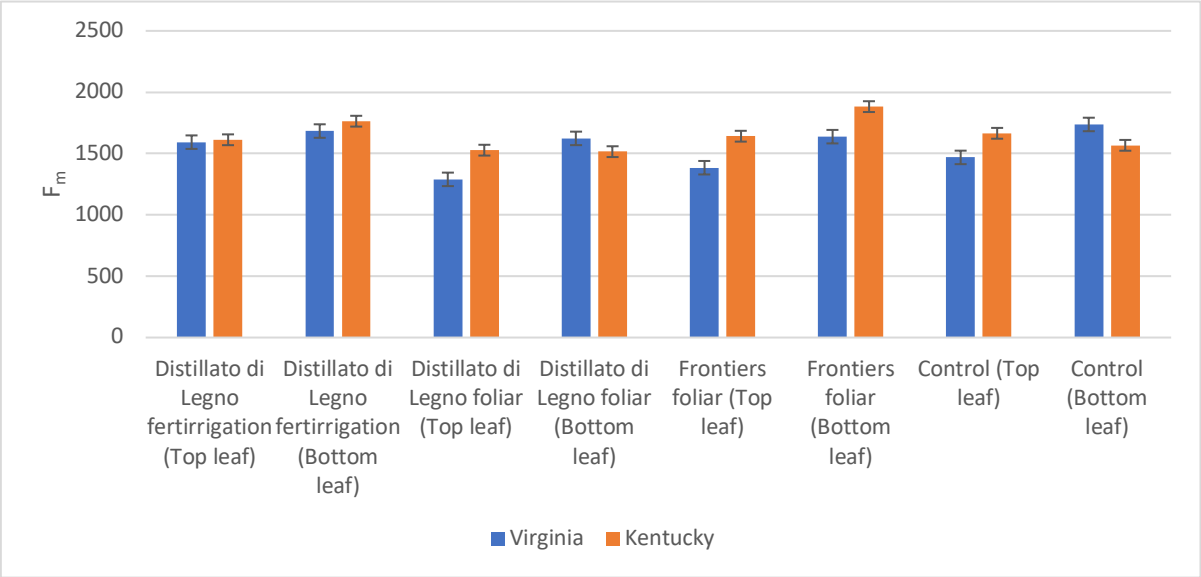

Figure 56. Comparison between F_m averages of Virginia and Kentucky leaves.
"Frontiere 2.0" treatments are reported as "Frontiers"

F_v

Table 69. Comparison of F_v averages of Virginia and Kentucky leaves reported with standard error

	Virginia F_v	**Kentucky F_v**
"Distillato di Legno" fertirrigation (Top leaf)	1327.25 ± 39.83	1339.29 ± 63.53
"Distillato di Legno" fertirrigation (Bottom leaf)	1385.57 ± 97.37	1468.38 ± 59.72
"Distillato di Legno" foliar (Top leaf)	968.25 ± 84.84	1216.13 ± 57.77
"Distillato di Legno" foliar (Bottom leaf)	1339.13 ± 81.89	1179.63 ± 25.81
"Frontiere 2.0" foliar (Top leaf)	1068 ± 101.14	1348.33 ± 148.06
"Frontiere 2.0" foliar (Bottom leaf)	1389 ± 89.25	1582.25 ± 44.68
Control (Top leaf)	1102.63 ± 107.27	1379 ± 80.93
Control (Bottom leaf)	1463 ± 44.31	1245.5 ± 95.89

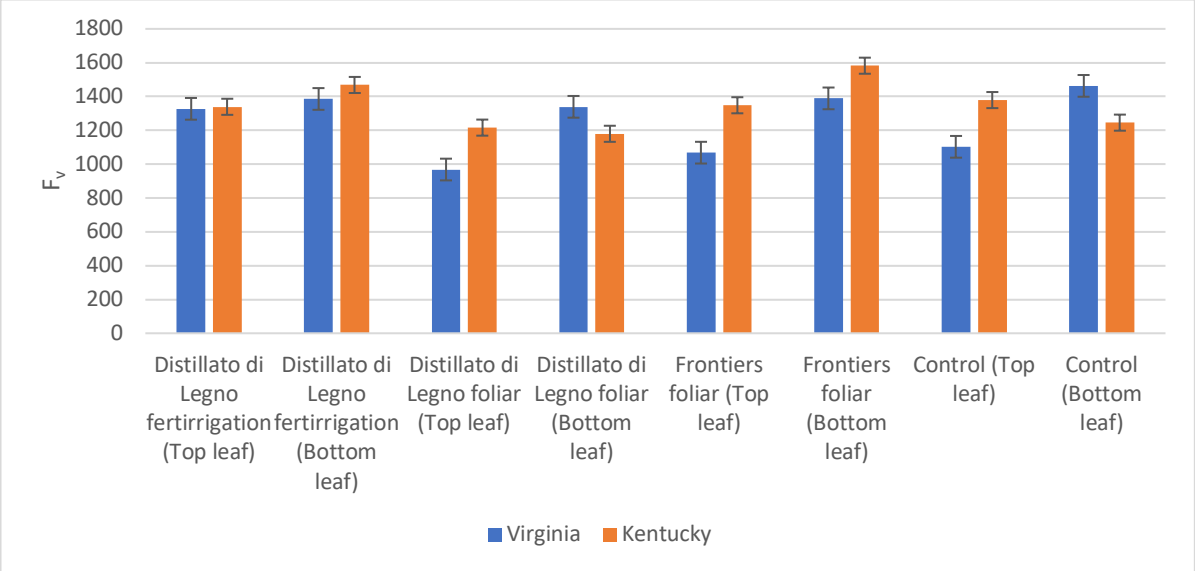

Figure 57. Comparison between F_v averages of Virginia and Kentucky leaves. "Frontiere 2.0" treatments are reported as "Frontiers"

PI_{abs}

Table 70. Comparison of PI_{abs} averages of Virginia and Kentucky leaves reported with standard error

	Virginia PI_{abs}	**Kentucky PI_{abs}**
"Distillato di Legno" fertirrigation (Top leaf)	22.19 ± 1.95	38.42 ± 5.74
"Distillato di Legno" fertirrigation (Bottom leaf)	24.92 ± 5.97	44.34 ± 6.61
"Distillato di Legno" foliar (Top leaf)	7.94 ± 2.22	22.07 ± 5.37
"Distillato di Legno" foliar (Bottom leaf)	21.15 ± 4.87	14.9 ± 3
"Frontiere 2.0" foliar (Top leaf)	13.04 ± 6.70	29.74 ± 13.23
"Frontiere 2.0" foliar (Bottom leaf)	29.52 ± 8.71	52.16 ± 10.11
Control (Top leaf)	15.71 ± 3.82	44.35 ± 9.26
Control (Bottom leaf)	32.27 ± 4.08	22.53 ± 5.61

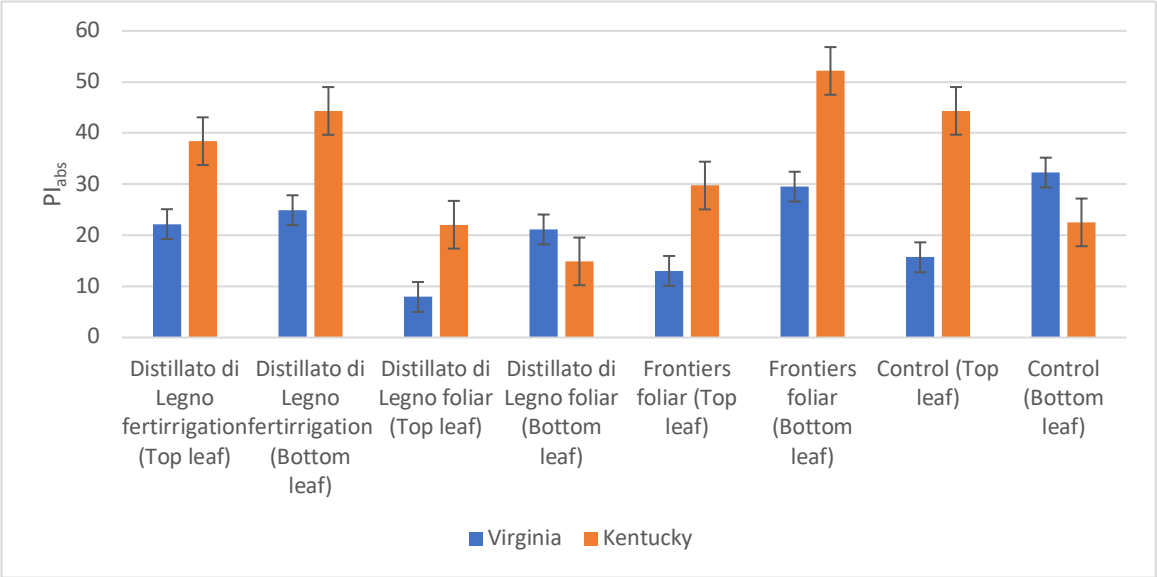

Figure 58. Comparison between PI_{abs} averages of Virginia and Kentucky leaves. "Frontiere 2.0" treatments are reported as "Frontiers"

Ψ_0

Table 71. Comparison of Ψ_0 averages of Virginia and Kentucky leaves reported with standard error

	Virginia Ψ_0	Kentucky Ψ_0
"Distillato di Legno" fertirrigation (Top leaf)	0.54 ± 0.01	0.61 ± 0.02
"Distillato di Legno" fertirrigation (Bottom leaf)	0.53 ± 0.05	0.65 ± 0.02
"Distillato di Legno" foliar (Top leaf)	0.40 ± 0.04	0.76 ± 0.03
"Distillato di Legno" foliar (Bottom leaf)	0.50 ± 0.04	0.74 ± 0.01
"Frontiere 2.0" foliar (Top leaf)	0.47 ± 0.07	0.56 ± 0.06
"Frontiere 2.0" foliar (Bottom leaf)	0.60 ± 0.06	0.68 ± 0.03
Control (Top leaf)	0.52 ± 0.02	0.64 ± 0.03
Control (Bottom leaf)	0.60 ± 0.03	0.53 ± 0.06

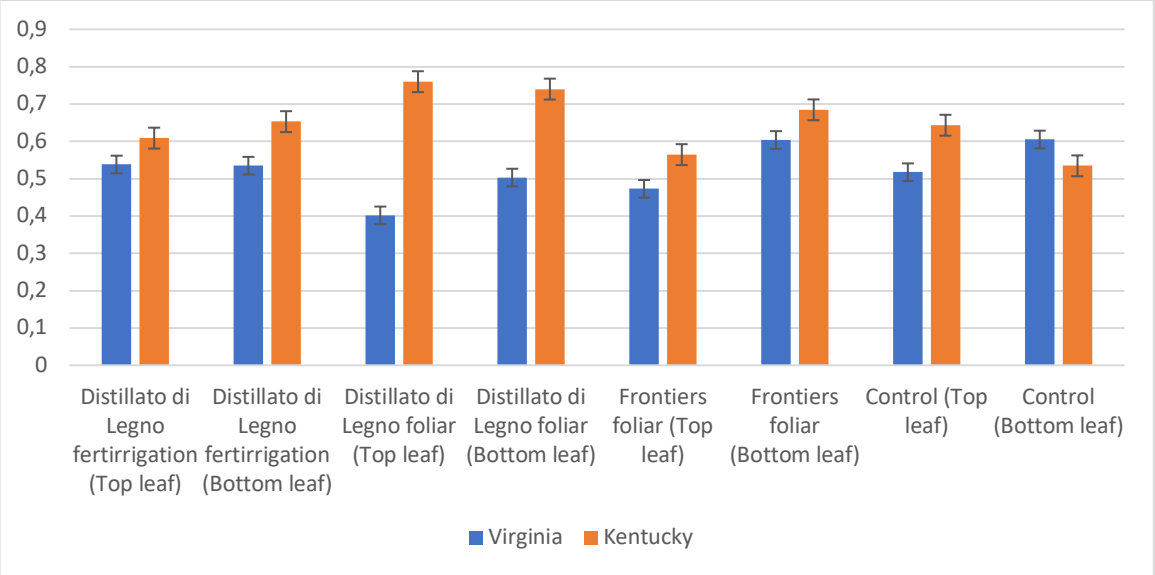

Figure 59. Comparison between Ψ_0 averages of Virginia and Kentucky leaves

Data of six the parameters have been analyzed by the statistical analysis techniques ANOVA and Tukey's test ($P < 0.05$) for every thesis.

Parameters Φ_{P0}, F_0, F_m, F_v and Ψ_0 don't give any significant result about the possible difference between treated plants and not treated plant, both for Virginia and Kentucky. Differently, the parameter related to the expression of the potential capacity of energy conservation that is PI_{abs} (Performance Index), gives an important result viewable in Table 51 and in Figure 33.

Both Tobacco cv. Virginia and Kentucky plants treated with Distillato di Legno foliar have a significantly decrease of the photosynthetic efficiency compared to the other treatments and to negative control. The difference in percentage is represented in Table 52.

Table 72. Variation of Performance Index (PI_{abs}) compared to negative control expressed as percentage for Virginia and Kentucky

	Variation PI_{abs} Virginia (%)	**Variation PI_{abs} Kentucky (%)**
"Distillato di Legno" fertirrigation (Top leaf)	41.2%	-13.4%
"Distillato di Legno" fertirrigation (Bottom leaf)	-22.8%	96.8%
"Distillato di Legno" foliar (Top leaf)	-49.5%	-2%
"Distillato di Legno" foliar (Bottom leaf)	-34.5%	-93.4%
"Frontiere 2.0" foliar (Top leaf)	-17%	-32.09%
"Frontiere 2.0" foliar (Bottom leaf)	-8.5%	131.5%
Control (Top leaf)	0.00%	0.00%
Control (Bottom leaf)	0.00%	0.00%

The "Distillato di Legno" foliar results for cv. Virginia evidence that top and bottom leaves measures are consistently lower than those of the negative control, respectively of 49.5% and 34.5%. The only exception consists in plants treated with "Distillato di Legno" by fertirrigation, that show an increment of 41.2% in terms of efficiency on top leaves, but a reduction of 22.8% on bottom leaves. Then, "Frontiere 2.0" shows an inhibition of 17% on top leaves and 8.5% on bottom leaves, that is lower than for "Distillato di Legno" foliar, although a reduction of performance index is still found.

The results obtained on cv. Kentucky are similar to those from cv. Virginia. The "Distillato di Legno" foliar treatments on top leaves produce a reduction of PI_{abs} of 2% and of 93.4% on bottom leaves. Conversely, "Distillato di Legno" fertirrigation treatments show a different result than in cv. Virginia: top leaves a decrease of 13.4% and bottom leaves an increment of 96.8%. Also, "Frontiere 2.0" treated leaves show a reduction of 39.2% on top leaves, but a very significant increment was found on bottom leaves.

5. Discussion

The use of “Distillato di Legno” or Wood Vinegar, or more generally of any compound related to what is define ed as “pyroligneous acid” could be an effective alternative to “traditional” copper-based compounds to step forward a more ecofriendly agriculture. However, there are some factors that still need to be better elucidated and studied. For instance, very often the composition of these products can be variable in different production times, and even among different batches. This could be related to the different biomass used as input, therefore with a great variability by the seasons. At this point, it would be important to be able to answer to the question of how this variability in composition can affect the effectiveness of wood vinegar. Roughly, its composition is represented by water and by phenolics (like catechol and 4-methylcatechol) and acetic acid, aldehydes, phenol and cresol (Suresh *et al.,* 2019), as assessed by gas chromatography-mass spectrometry (GC-MS) and, for big part, it. However, the composition it is rarely constant and stable. For example, a wood vinegar obtained from *Litchi chinensis* shows a composition given by 17 different chemical compounds: the most represented are 2,6-dimethoxyphenl for 29.54%, 2-methoxyphenol for 12.36% and 3,5-dimethoxy-4-hydroxytoluene for 11.07%, with also antioxidant with an activity similar to vitamin C and butylated hydroxyl toluene (Yang *et al.*, 2016).

Other examples of pyroligneous acids from different raw materials are that obtained from *Tectona grandis,* a waste material from industry, and from *Durio zibethinus*, a plant situated in countries like Indonesia, Thailand and Malaysia (Rahmat *et al*., 2014; Setiawati *et al.,* 2019).

The difference between the raw biomass used as input could be further valorized and exploited by creating a heterogeneous market, composed by many products having different botanical origins as well as different applicability and efficiency. These compounds could be then used as such or in prototyped blends, according to the different aims.

In this work some of these commercialized botanicals, maintaining a focus on “Distillato di Legno” and Wood Vinegar by BioDea, have been tested.

The first important result is about the role as plant growth stimulator, as assessed by the root elongation test carried out on *A. thaliana* and *N. tabacum*, and comparable results for these two species.

In particular, “Distillato di Legno” works as an herbicide if used as such, but it shows a positive effect on root elongation if applied at 1:10, 1:100 and 1:1000 concentrations. In particular if analyzed at 15th dpi, the 1:1000 dilution is probably the best concentration to be used for this purpose.

Another important part is represented by those tests for the assessment of the activation of plant defense mechanism, such as HR and ion leakage. In this part, “Distillato di Legno” has been compared to the effects of another commercial botanical, “Frontiere 2.0”, and to that of *P. syringae* pv. *tomato* strain DC3000*,* a bacterium knows as activator of this response. According to the results of both these tests any appreciable activation of plant defenses have been recorded.

Conversely, a remarkable antimicrobial activity was found for “Distillato di Legno”, particularly as inhibition effect on the plant pathogenic fungi here tested.

S. rolfsii is the only fungus tested with dilutions 1:10, 1:100 and 1:1000, showing the capacity of all the dilutions to inhibit the growth in different percentage compared to a negative control represented by sterile water. For the test done by cellulose disks soaked with the product inhibitions are for 20.74% at concentration 1:10, of 15.68% at concentration 1:100 and of 19.90% at concentration 1:1000, while for the test by the product directly applied on agar the inhibitions are respectively for 8.09%, 12.31% and 8.94%.

Other fungi have been tested with the product as such, compared to a negative control showing different results. Inhibition action is confirmed on *F. avenaceum* for 5.56%, on *A. alternata* for 47.76%, on *C. parasitica* for 16.13%, on *P. cinnamomi* for 51.72% and on *G. castaneae* for 23.81%, but in some cases seems to don’t have any effect. Indeed, on *P. infestans*, *F. graminearum*, *F. culmorum*, *M. fructigena* and *S. sclerotiorum,* “Distillato di Legno” doesn’t produce any inhibition. On two fungi, *F. poae* and *V. longisporum,* the product even acts like a promoter of the growth, reaching respectively an increase for 18.18% and 25%.

The inhibition tests carried on the plant pathogenic bacteria belonging to the genera *Pseudomonas, Xanthomonas, Curtobacterium* and *Clavibacter* have been done by using both “Distillato di Legno” and Wood Vinegar. No effects have been recorded at 1:100 and 1:1000 dilutions of both products on any of the bacteria here tested.

Differently, “Distillato di Legno” as such have inhibition effect on all the bacteria with exception on *X. axonopodis* pv. *euvesicatoria* and *juglandi,* while at concentration 1:10 it has the capacity to inhibit *P. syringae* pv. *tabaci*, *C. flaccumfaciens* pv. *ortii*, *betae* and *ilicis* and *C. michiganensis* pv. *michiganensis* strain NCPB 382. Wood Vinegar as such doesn’t inhibit all the bacteria, indeed the

exception are *X. axonopodis* pv. *pelargoni* and *phaseoli* that are not influenced by the product. As far as Wood Vinegar is concern, the dilution 1:10 inhibits *P. syringae* pv. *tabaci*, pv. *tomato* strain DC3000, *C. flaccumfaciens* pv. *poinsettiae*, *ortii*, *betae*, *flaccumfaciens* and *ilicis*. In conclusion, "Distillato di Legno" as such has a lower inhibition capacity than Wood Vinegar, that is active at the concentration 1:10.

The molecular test on the inhibition of pathogenicity of *P. savastanoi pv. nerii* strain Psn23 pT3-GFP show the capacity of "Distillato di Legno" to inhibits at concentrations 1:100 and 1:1000. Indeed, different percentage have been collected: 1:100 inhibits for 13% at T_{24} and for 21% at T_{48} and 1:1000 respectively for 45% and 64%.

At last, in summer 2019 several field tests have been carried out Using Tobacco as model plants treated with these compounds. Averages of height data show that tobacco treated by "Distillato di Legno" by fertirrigation have a reduction in height of 17.53% for cv. Virginia and 25% for cv. Kentucky compared to the not treated, while plant treated by the same product but foliar show a reduction of 52.69% for cv. Kentucky and 22.30% for cv. Virginia. Then, it is worth to mention that most of the Tobacco plants treated with "Distillato di Legno" showed a sort of "synchronization" of their height, especially for the cultivar Virginia.

The second data collected is the number of leaves and in this case Virginia and Kentucky treated by "Distillato di Legno" foliar and by fertirrigation showed a number lower than the others plant. The reduction is of 55% for Virginia and 53.3% for Kentucky for the foliar treatments, while 61.7% for Kentucky and 69.3% for Virginia for the fertirrigation treatments. The particular "synchronization" reported for the plant height is also viewable for the number of leaves: the single number of leaves of each plant treated by "Distillato di Legno" is very similar for both foliar and fertirrigation treatment, except for Kentucky.

One of the most significant and interesting data collected in field is about the photosynthetic efficiency. As reported in 4.6.3, the parameter PI_{abs} or Photosynthetic index shows a drastically reduction of the efficiency of the tobacco plants treated by "Distillato di Legno" foliar and by fertirrigation.

Virginia treated by "Distillato di Legno" foliar measures are lower than control respectively of 49.5% and 34.5%, while the same cultivar treated by "Distillato di Legno" fertirrigation show an increment of 41.2% on top leaves and a reduction of 22.8% on bottom leaves.

Kentucky treated by “Distillato di Legno” foliar on top leaves show a reduction of 2% and of 93.4% on bottom leaves, while fertirrigation treatments by the same product show that top leaves have a diminution of 13.4% and bottom leaves an increment of 96.8%.

In conclusion, the results reported in this work show a variable efficiency of “Distillato di Legno” and Wood Vinegar.
In *in vitro* tests, “Distillato di Legno” positively influenced root elongation on *A. thaliana* and *N. tabacum,* confirming its role as plant growth promoter. Still *in vitro*, its role as antimicrobial can be partially confirmed. Similar conclusions can be drawn for Wood Vinegar.
In field experiments, “Distillato di Legno” have the particular capacity to synchronize most of the treated plant, that have similar height and number of leaves. This can be an advantage for culture practices and not only, but, maybe, this and the other data are an important indication. However, more research is needed to confirm these results and to discover new information and to better exploit the potential of wood vinegar and Distillato di legno, for a future agriculture without copper compounds and antibiotic, for a future more sustainable not only for us, but in particular for the future generations.

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