EVERGREEN
Manual

Ecofriendly control plant pathogenic bacteria and nematode by plant polyphenolic extracts

LIFE13 ENV/IT/000461

EVERGREEN

The PARTNERS

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ASTRA
INNOVAZIONE E SVILUPPO
IN AGRICOLTURA

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Introduction

Agriculture of the 21st century is characterized by a more carefull and rational use of any technical approach ensuring production: while after the 2nd World War, the main aim of agriculture was to obtain the highest yields, today more attention is given to the negative impact that an indiscriminate use of pesticides, herbicides and fertilizers could have sometimes on the environment and on human health. This change of perspective is certainly due to the growing general awareness on the need to preserve the environment, but also to other important challenges posed to agriculture, such as those related to the ongoing global climate changes. In other words, a general awareness and a strong pressure are widely diffuse about the use of environmental-friendly methods to control plant diseases, possibly by sustainable strategies that minimise pollutant emissions and maximise the use of renewable resources. Moreoever, according to the guidelines of the European 10-year strategy "Europe 2020" and to the Common Agricultural Policy (CAP) "The CAP towards 2020”, agriculture is definitely considered as a strategic sector for Europe, able to successfully contribute to generate european economic growth by increasing the context of food security, through the application of standards of quality and safety of products that have to be high, uniform, and that they can competitively satisfy on a global scale the expectations of the internal market and outside Europe. The success of agriculture of the 21st century in Europe relies on a model of sustainable development, where essential issues such as environmental protection and protection of natural resources, as well as of rural areas, have to be the respect together with rural populations and human resources. Therefore, the management and the control of plant diseases urgently need the development and introduction of innovative control strategies aimes to reduce the use of synthetic pesticides, while maintaining unaltered income and production, as also required as mandatory by the most recent European legislation on this matter. To get these goals, in recent years the European Union has strongly fostered research on innovative and effective plant protection compounds and pesticides, as well as information and training of users when their positive performances in plant defense were demonstrated.

Copper in plant protection: use and issues

Copper derivatives are used since 150 years ago as fungicides and bactericides for the control of most plant diseases, and are among those phytoiatric compounds whose reduction was planned by the current European legislation, in conventional and organic agriculture as well. Copper containing fungicides and bactericides are generally applied as a chemical spray, and thus in part treatments miss their targets and much of this lost copper enters the soil surface, where copper can persist for very long period of time and potentially migrate off-site due to leaching and/or runoff. Although copper is an enzymatic cofactor in several metabolic processes of any living organism, as well as an essential trace element for crop growth at low concentrations, copper accumulation within topsoil causes adverse and heavy eco-toxicological effects, which are often not limited to the agroecosystems. As a conseqeunce, European Union recently restricted the amount of copper used in conventional and organic agriculture against plant pathogenic bacteria and fungi within the EU Member States up to 6 kg/ha/year, with the possibility to make an average over 5 years in perennial crops (Council Regulation 2015/229/EU; Directive 2009/37/EC; Council Regulation No 834/2007/EC; Council Directive 91/414/EEC). Further reductions have been also established in some EU countries: copper is forbidden in organic agriculture (*e.g.* NL, DK), and in other countries there is a lower quantitative limit (*e.g.* 3 kg/ha/year in Germany). Actually, copper is the most effective substances and the only chemical allowed to control phytopathogenic bacteria and fungi in organic farming. Nevertheless, the substitution of copper compounds is a declared priority in the EU organic legislation (EC Reg. 473/2002), and according to the current EC regulation 473/2002 the annual dose of 6 kg Cu/Ha should correspond to an annual accumulation of about 5 mg Cu/Kg soil in the top 10 cm assuming no losses.

Copper (Cu) is a transition metal with three oxidation states: zero (Cu0, solid metal); +1 [Cu(I), cuprous ion]; and +2 [Cu(II), cupric ion]. Also, Cu is classified as a heavy metal, with a density greater than 5 g cm^{-1}. Cu-based biocides are widespread chemical controls for both fungal and bacterial diseases in crop fields. They showed that, Cu ions at a concentration of 100 μM enhance t-butyl hydroperoxide (tBOOH) and hydrogen peroxide (H_2O_2) killing microrganisms through different mechanisms. Cu is required as a cofactor for a variety of enzymes, such as terminal oxidases, monooxygenases, and dioxygenases. An excess of Cu in aerobic cells generates ROS (Reactive Oxygen Species) through a Fenton-like reaction, in which Cu (I) ions react with hydrogen peroxide to form hydroxyl radicals. Nonetheless, the precise mechanisms by which Cu ions exert lethal effects on bacterial cells remain ambiguous. A study in Escherichia coli revealed that membrane injury caused by lipid peroxidation is one of the factors responsible for Cu-induced cell death. Intracellular Cu failed to catalyse the formation of oxidative DNA damage.

Although Cu is an essentialmicroelement, at elevated levels Cu becomes toxic. Therefore, Cu levels in natural environments, as well as its biological availability, are very important parameters to be evaluated. To be available for biological systems, Cu has to be present in a readily soluble form. Some toxic elements are biologically unavailable because they are rare or highly insoluble in the environment. Cu is a relatively abundant in the Earth's crust and moderately soluble. The form taken by the metal (*i.e.* ionic, complexed, precipitated), and hence its bioavailability, depends on several environment factors such as pH, redox potential, soil and sediments type, water hardness, and organic content. These factors vary in the environment, giving rise to possible conditions of Cu deficiency or toxicity.

First records of the agricultural use of Cu compounds date back to 1761, when the discovery of the antibacterial effects of copper sulphate preparations used on seed grains set up further standards in cultivation practices for the following decades. But the most important breakthrough of copper use in viticulture though was undoubtedly in 1880, when the French botanist and mycologist Pierre-Marie Alexis Millardet from the Bordeaux district in France noticed that those vines, which had been daubed with a paste of copper sulphate and lime in water, in order to make the grapes unattractive to passers-bys and as well as animals, appeared less affected by downy mildew. Only five years later in 1885 Millardet announced his discovery to the world, a cure for the dreaded mildew through the application of the mixture of copper sulphate, lime and water, up to the present day called the Bordeaux mixture. Additionally to the Bordeaux mixture, but including copper sulphate and sodium carbonate (*i.e.* soda crystals) the so called Burgundy mixture appeared few years later. At that time the Bordeaux and Burgundy mixtures became indispensable fungicides against various fungus diseases of plants, where the prevention enhanced with the proper application, means an appropriate timing and correct use of the fungicide. Consequently, as standing for a successful plant protection method up to the present days, many thousands of tons of copper are used annually in agriculture all over the globe. The days of prosperity of the production of fungicides based on copper compounds were in the middle of 20th century when many different chemical combinations with copper were applied. In the last decades and these days pharmaceutical corporations have been fabricating copper based fungicides in soluble forms of sulphates, oxychlorides, acetates, carbonates, oleates, silicates, ohydroxides etc. Most compounds of copper adopt the oxidation states Cu^+ and Cu^{2+}, respectively called cuprous and cupric. Their efficiency against fungal and bacterial infections is mostly reflected in their capability to retain on the plant surface, but not by the number of applications or the concentration of the agens in the fungicide. Nowadays a considerable number of phytochemical companies offer numerous and different classes of fungicides, where copper fungicides according to their antifungal and antibacterial effects play an important role. The efficiency of copper fungicides, particularly aggressive in moist media, is caused by denaturation of protein structures (secondary and tertiary) of fungi and bacteria, and consequently the interruption of their functions. Copper forms compounds in the oxidation states of cuprous and cupric, but trivalent copper in aqueous solution splits rapidly.

Classification of copper fungicides and bactericides, according to their chemical structure:

- copper sulphate (cupric sulphate; $CuSO_4$),
- copper acetate (cupric acetate; $Cu(OAc)_2$),
- cuprous oxide (copper(I) oxide; Cu_2O),
- cupric chloride (copper(II) chloride; $CuCl_2$),
- copper oxychloride (copper(II) oxychloride; $CuC_{l2}\cdot 3Cu(OH)_2$),
- cuprous chloride (copper(I) chloride; CuCl),
- cupric nitrate (copper(II) nitrate; $Cu(NO_3)_2$),
- copper cyanide (copper(I) cyanide; CuCN),
- copper soap (solution of copper octanoate),
- copper naphthenate.

Unfortunately copper does not degrade into the environment and a high likelihood of soil copper accumulation results in particular from annual applications made on perennial cropping systems. Despite its negative eco-toxicological profile, the use of copper is still tolerated because of its properties as a wide-spectrum fungicide and bactericide. Copper compounds are the only chemicals allowed in organic agriculture, where strong disputes raised up since copper is more toxic and persistent than other synthetic pesticides. Although applications are made less frequently, copper is widely used also on annual crops. Therefore, the serious environmental concerns about the irreversible and cumulative effects of agronomic treatment with copper compounds prompted the EU and worldwide governments to set copper limits. Copper is also biostatic which means that bacteria cannot grow on surfaces treated with it.

Intensive use of copper compounds in plant protection in the last 200 years lead to copper accumulation in soils, especially in traditional horticultural areas, where more than 150 mg copper per kg are reached in thhe upper soil layers (< 20 cm) after 30 years of permanent Cu usage. The accumulation of exceeded concentrations of Cu in soils leads to the perish of flora and fauna in the soils, and the greatest concerns are residuals of Cu in food for humans (*i.e.* vegetables and fruits). The restrictions imposed within the EU on copper applications for plant disease control in agriculture stimulated the development of strategies to lower copper load into the topsoil, the optimisation of copper use, and the research of realistic and efficient alternative strategies.
Several remediations of copper contaminated soils were explored:

- copper immobilization through pH variations, or organic matter addition,
- direct removal through acid washing and incineration,
- scavenging/sequestration through exopolymers,
- active mixing of top and deep soils to dilute copper contamination, to end with plant uptake or phytoextraction.

Unfortunately no one of those methods was proven practical in agronomic ecosystems. In a view of an optimization of the use of copper, it is essential to specify that the biological activity of copper is strictly dependent on its ability to exist in a “free” or “ionic” state. In the latter condition copper is very reactive against a broad spectrum of plant pathogenic fungi and bacteria. The amount of copper in the ionic state greatly increases at pH values lower than 6.5, and so does its mobility, as well as its fungicidal and bactericidal activity. Unfortunately, this reactiveness is also responsible for the phytoxicity of copper and thus, the challenging goal is to reach a balance between these two extremes. Several copper formulations with low total copper content have been developed in the last years to maximize the control per unit of metallic copper and minimize copper accumulation. These preparations show a favourable rate of metallic copper, which is responsible for the efficacy of copper-based plant protection compounds; often they contain relatively small amounts of metallic copper and specific ingredients to increase copper solubility, adsorption and distribution on the plant surface. Among these novel products there are copper sulphate and copper oxychloride dispersions, and copper gluconate and copper amino/peptidate chelated formulations. Obviously the reduction of copper accumulation strongly relies on tailored application of copper compounds, which can be achieved with sophisticated software programs, taking into account several environmental conditions. The presence of copper into the soil copper is almost exclusively in the ionic divalent form Cu^{2+}, and it is naturally attracted by negatively charged clay minerals, anionic salts and organic matter producing several metal-coordination compounds: the complete decomposition and mineralization of organic matter is indirectly prevented by copper, causing severe ecological imbalances in nutrient recycling. More important, as already said, the mechanism of action of copper is not selective, so it acts on all forms of life. The biocidal action of this element results in a reduction of the microbial flora and fungal, and its accumulation in the soil lead to a ground depleting and even more aquatic ecosystems. It is well known that a soil rich in biodiversity is a healthy soil, able to defend itself and defend agricultural crops; a good balance of the soil is the key to a successful agricultural practice, making crops more resistant. In addition, it is known that the use of copper leads to a decrease of the soil organic matter, reducing the quality of soil. This metal has a negative effect on most species of the soil (the micro and macro-fauna), leading to a decrease in biodiversity of the agricultural environment, an effect that increases over time due to accumulation of copper. The presence of bacteria and fungi in the soil, but also on the aerial parts of the plant, can be a particularly effective defense mechanism against parasites microscopic plants and enrich the soil of minerals, essential for crops; animals sensitive to copper, such as earthworms and snails, can improve soil texture, creating channels for aeration of the roots and promote the decomposition of organic matter, improve soil quality. It is known that the bacteria are important agents in determining the form and distribution of metals in the environment. They play a major part in the modification, activation and detoxification of heavy metals. However, they may themselves be subject to metal toxicity. Copper also has a documented action insecticide and its accumulation in the environment could lead to removal of spider beetles and pollinators.

For these reasons the European Union has issued a directive that regulates the use in agriculture (91/414 / EEC). Copper is also a close ally of the biological agriculturist and the EC Reg. 889/2008 establishes the limit of use of the metallic element to 6 kg per hectare per year for up to 36 kg in six years, of course, unless exceptions .

Nematocides in plant protection

As far as the management of plant parasitic nematodes is concerned, undoubtedly its control starts with the adoption of preventive measures, such as a deep soil analysis to detect and quantify plant parasitic nematodes possibly present prior to select a field for vegetable production. Then, the other most frequently used strategies for managing nematode in agriculture include crop rotation, resistant cultivar when and if available, and at the end the use of nematicides. Concerning chemical control, soil-applied fumigants and non-fumigant nematicides can help to prevent serious crop losses, in particular when used in conjunction with long-term management practices. However, nematodes live in the soil a produce several generations in a very short time, therefore chemical control requires applications of large amounts of chemicals with specialized equipment, and thus these methods are really costly. Moreover, nematicides have a very negative environmental impact. and fumigant nematicides have been banned, phased-out, or their use has been heavily restricted. For instance, methyl bromide was banned out, under the Montreal Protocol, because of its ability to deplete stratospheric ozone (Martin, 2003). Among the most used non-fumigant nematicides there are Nemacur® (ADAMA-AMVAR, active principle fenamiphos), Mocap® (Bayer Crop Science, active principle ethoprophos), Vydate® (Du Pont, active principle oxamyl). The organophosphates Mocap® and Nemacur®, as well as the carbamate Vydate®, act as acetylcholinesterase inhibitors on the primitive nervous system of nematode. Concerning their toxicological profile, non fumigant nematicides can also be very environmentally impactant. Presently there are only few commercial nematicides left in use, and their repeated applications lead to the enhancement of biodegradation mechanisms in soil and the development of pest resistance, both expressed as a lack of efficacy under field conditions.

Plant protection: issues and current European legislation

The replacement of copper with more favourable alternatives is a high priority of European agricultural policy. The current annual permitted applied dose of 6 kg copper per ha (Regulation 473/2002/EC) should correspond to an annual accumulation of about 5 mg copper kg^{-1} soil in the top 10 cm. Unfortunately, these data are purely theoretical and are actually known to be highly variable according to the composition and pH of the different soils. Reducing the use of copper as pesticide would be good not only for the environment but also for human and animal health. Indeed, in addition to the direct toxicity due to the bioaccumulation of the metal in soil, the repeated use of copper in agriculture as fungicides and bactericides has a side effect that is absolutely not to be underestimated. There has been a corresponding increased resistance of pests to the pesticide poisons. This resistance of pests could link with bacteria resistance to human antibiotics. The fraction of resistant bacteria to antibiotics constitute a sort of reservoir of genes for antibiotic resistance. These genes are present on movable elements of their genome, plasmids, which can be transmitted easily even to pathogenic bacteria of man and animals, thus making them resistant to antibiotics, failing the action of therapeutic in human and veterinary medicine.

The EU has established several rules for the sustainable use of pesticides, included into the Directive 2009/128/EC, in order to reduce the risks and impacts deriving from their use on people's health and on the environment. According to this Directive, EU Member States have to promote low pesticide-input pest management, whose implementation has to be ensured by principles, conditions and measures established in each Country by specific National Action Plans on this matter. In the frame to meet the requirements of the Sustainable Use of Pesticide Directive, new plant protection molecules and technologies need to be developed, as also foreseen by the Regulation 1107/2009/EC, which established a list of substances identified as “candidates for substitution”, that are those plant protection products that have to be replaced by other effective alternative adequate solutions, both chemical and non-chemical. Basically, Regulation 1107/2009/EC lays down the rules and procedures concerning the placing on the market of new plant protection products, included more effective requirements for the approval process, and which repealed Directives 79/117/EEC and 91/414/EEC starting from the 14th of June 2011.

Furthermore, to implement Regulation 396/2005/EC, a coordinated multiannual control programme for 2017, 2018 and 2019 was recently established by the EU Commission with Regulation 2016/662, to ensure compliance with maximum residue levels of pesticides and to assess the consumer exposure to pesticide residues in and on food of plant as well as of animal origin.

Detailed and updated information on EU legislation concerning plant protection products and pesticides are available online at http://ec.europa.eu/food/plant/pesticides_en

What alternatives for reducing copper?

The objective of reducing the use of copper in agriculture led the EU to fund several research projects and many researchers and the industries themselves have started studies based on partial substitution with other natural products. New formulations have been studied to reach better the target and to make a more efficient use of copper so as to be able to reduce the doses with the same effectiveness. Several agricultural machinery manufacturers have been studied and realized equipment to recover the excess distributed solution, avoiding that there are losses of product on the ground. Today there are already a lot of sprayers on the market that will certainly help to reduce the amount of product used. This improves distribution efficiency but does not cover the replacement of copper.

Genetic research of varieties resistant to bacterial and fungal diseases is a research than others has moved in the direction of search for varieties that do not require plant protection measures based on copper as long as such maintain this characteristic. Examples are for some bacterial diseases of tomato for grapevine downy mildew (resistant hybrid varieties) and other diseases. Unfortunately, history teaches us that the resistance will overcome in time and that this activity should always continue.

The EVERGREEN strategy

Plant polyphenols: valuable renewable resources in the frame of circular economy

The biological and chemical properties of polyphenols have been long investigated and also confirmed by many recent studies. These compounds are present in all vegetables and fruits where they have a role against parasites and pathogenic microbes. But they have further beneficial effects which make them suitable also for applications in cosmetics, medicine, agronomy and phytotherapy, such as to be antioxidant, active against inflammations, useful for the prevention of cancer and cardiovascular diseases, to end with their antimicrobial properties. The plant species, together with climatic conditions, harvesting stage, raw material freshness and other factors related to environment, such as soil characteristics, are the main factors which influence the plant polyphenol content.

The polyphenols from several *Olea europaea* L. matrices (*e.g.* olive oil by-products, leaves, branches and olive pulps), are known for the highly antioxidant properties, and protective biological and biomedical effects. The chemical characterization and the quantitative evaluation of these minor polar compounds can be useful to obtain active principles with important applications in agronomy, cosmetic and functional food products. The main constituent of olive leaves is a phenolic secoiridoid glycoside, oleuropein (Figure 1), which can be broken down to elenolic acid, a powerful anti-bacterial molecule, and hydroxytyrosol, which is known for its important antioxidant activity.

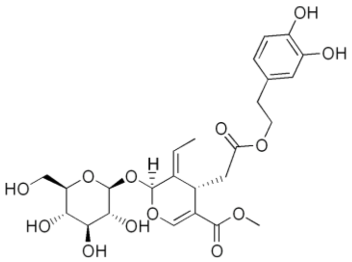

Figure 1. Structure of oleuropein.

Artichoke is an herbaceous perennial plant (*Cynara carduncul*us L.) belonging to the family of *Compositae* (*Asteraceae*) and cultivated especially in the Mediterranean area. Heads of the artichoke are worldwide known to be edible, whereas leaves are used since antiquity in popular medicine for their beneficial effects. On the other hand, extracts from such plants have been claimed to possess epatoprotective and antioxidant properties, due to their polyphenolic fraction. In particular, the presence of chlorogenic acid (Figure 2) and flavonoid glycosides has been observed.

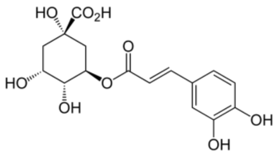

Figure 2. Structure of chlorogenic acid.

The hydrolysable tannins, water-extracted from Sweet Chestnut (*Castanea sativa* Mill.) biomass and membrane concentrated, containing both gallic and ellagic hydrolysable tannins (Figure 3).

These molecules have several remarkable effects as antioxidant, metal complexing agents, as well as antimicrobial.

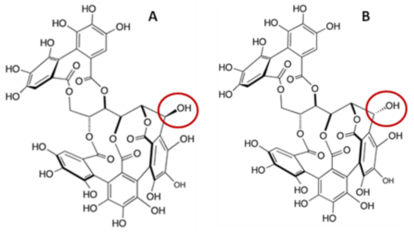

Figure 3. Structures of vescalagin (A) and castalagin (B),

The last decade has been characterized by a strong attention on the phenolic composition of grapes and wine, due to their potential health benefits. In fact, *Vitis vinifera* L. has an impressive phytochemical composition, in particular given by polyphenols consisting of anthocyanins, catechins, flavonols and stilbenes (Figure 4). The anthocyanins are located mainly in the riped fruit skin. Catechins are found in berries, seeds and peels, in varying amounts, depending on the cultivar, and are mainly represented by (+) catechin, (+) gallocatechin and (-) epigallocatechin, whose antimicrobial properties are known.

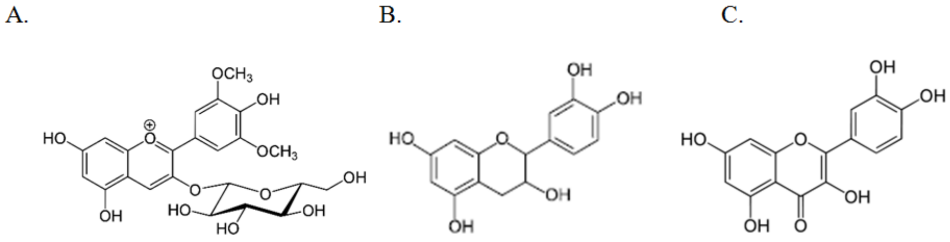

Figure 4. Structures of malvidin 3-glucoside (A), catechin/epicatechin (B) and quercetin (C).

Recent studies demonstrated that the byproducts and waste from the agro-industrial transformation of grape, in particular from the production of wine, are still rich in bioactive polyphenols, which could be used to obtain extracts and semi-finished products useful in agronomics, cosmetics, feed, food, nutraceuticals and pharmaceuticals. To this concern, to obtain extracts rich in polyphenols by aqueous extraction from vegetable not-edible waste matrices of the agro-industry allows to implement the modern principles of circular economy, to pursue the aim of a better protection of the environment in compliance with the most recent EU guidelines, and also bringing substantial economic benefits for companies and producers.

Circular economy integrates socio-economic aspects with and environmental systems. The main concept on which it is based is the thermodynamic law of mass conservation, according which material inputs into a system equal material outputs plus net accumulation. A sustainable system is characterized, among others, by a much reduced use of inputs (both renewable and non-renewable) and by the reuse and recycling of material outputs (*i.e.* waste is considered a resource). Hence, the circular economy model moves towards the closing loop, and also suggests the drastic reduction or even elimination of waste and dissipative losses.

The transition to a circular economy requires substantial changes throughout a value chains, included the product design to new business and market models, such as to find way to turn a waste into a valuable resource to end with new modes of consumer behaviour. To this concern, in 2011, EU Commission approaved the Roadmap to a Resource Efficient Europe (COM (2011) 571), which supports the shift towards an European sustainable growth via a resource-efficient and low-carbon economy. This Roadmap is one of the main building blocks of the resource-efficient Europe initiative, which outlined the structural and technological changes needed by 2050, including those milestones to be reached by 2020, according to the Europe 2020 - A strategy for smart, sustainable and inclusive growth (COM(2010) 2020). In view of these challenges, in July 2014 the EU Commission adopted a Circular Economy Package, including the Communication COM (2014) 398 "Towards a circular economy: a zero waste programme for Europe", where one of the main issues is to turn waste into resources, in line with the EU Waste Framework Directive

Based upon these perspectives, the exploitation of not-edible biomass, waste and by-products within the agroindustrial system and agri-food chain can be develop for the production of biomolecules usefull as active principles in agronomy, cosmetics, foods, feeds and pharmaceutical applications. Moreover, a similar scenario is ideal to promote a new agribusiness model, because more suitable to be tailored on a regional context, involving all the actors of the production chain, from farm to industry and service sectors.

Closing the circle: “green chemistry” for plant polyphenols extraction

The laboratory scale extraction and purification of bioactive polyphenols from plant material is usually performed by hydroalcoholic mixtures or other solvents such as ethyl acetate. The industrial processes, especially if aimed at obtaining cosmetic, food or pharmaceutical products, must be based on the use of solvents ecologically and economically eligible, and compatible with human health.

On an industrial scale, hot water extraction - coupled with the agro-industrial production process - has been here found to be an useful and suitable instrument to obtain high quality polyphenols from not-edible biomass and waste plant matrices. The inclusion of filtration elements, based on membrane technology, to concentrate and purify the crude extracts, allowed to obtain extracts enriched in specific polyphenolic subclasses, used so far mainly for cosmetics, food and nutraceuticals. Moreover, the exploitation of the post-extraction matrices for bioenergy purposes allows to further optimize the production process, whose environmental and economic sustainability is even stronger if the whole process is designed to take place within a single integrated platform, thus to be a closed loop industrial production according to the concept of circular economy.

The EVERGREEN plant polyphenols extracts and formulations

In the EVERGREEN project, “green extraction” and purification/concentration processes from agro-industrial not-edible biomass and by-products from *O. europea*, *C. scolymus*, *C. sativa* and *V. vinifera* have been standardized and optimized to obtain extracts enriched in specific polyphenolic subclasses, highly sustainable and suitable for eco-friendly agronomic formulations to reduce or eliminate the use of conventional pesticides against phytopathogenic bacteria and nematodes.

All the EVERGREEN extracts and formulations, obtained in compliance with the current EU legislation REACH 1907/2006, were chemically and biologically characterized and standardized, then tested to find the best ecofriendly formulations according to their effectiveness against plant pathogenic Gram negative bacteria and nematodes from the lab to the field. Moreover, the whole exploitation of plant biomass and by-products from the agri-food sector was realized through the optimization of industrial closed cycle platforms.

The EVERGREEN extracts and formulations: how to use it?

The control of plant diseases caused by phytopathogenic bacteria belonging to the genus *Pseudomonas* of Olive and Kiwifruit, and by the nematode *M. incognita* on Tobacco and other herbaceous species has foreseen the optimization of the “green extraction” process of high-quality polyphenols from no food/feed biomass, and the development of formulations whose activity in plant protection was here demonstrated at laboratory, pilot and field level during the EVERGREEN project, through direct and indirect artificial inoculations. The following indications concerning the application of the EVERGREEN formulations set up so far were drafted accordingly to the experimental data until now collected on the model systems here used.

✓ Formulation 1L (liquid):
Sweet Chestnut polyphenol extract 2%
Olive polyphenol extract 1%

✓ Formulation 2L (liquid):
Sweet Chestnut polyphenol extract 1.5%
Olive polyphenol extract 1%
Grape seeds extract 0,3%

✓ Formulation 1G (gel):
Sweet Chestnut polyphenol extract 0.2%
Olive polyphenol extract 0.1%

✓ Formulation 2G (gel):
Sweet Chestnut polyphenol extract 0.15%
Olive polyphenol extract 0.1%
Grape seeds extract 0.03%

Formulations 1L and 2L: dilute in water 1:10 at time of use.
Formulations 1G and 2G are water-based and ready for use

The digital version of the EVERGREEN manual is available on the project website

For further information, please ask to the EVERGREEN team at
http://life-evergreen.com/

Suggestions for the use

- Formulation 1L and 2L for spray treatments on plant aerial parts, applied at 10 days interval in high risk periods (*e.g.* against *P. syringae* pv. *actinidiae,* and pv. *tomato,* and *P. savastanoi*).

- Formulation 1G and 2G for direct applications to soil, near the plant crown (*e.g.* against plant parasitic nematodes at 30kg/ha equivalents, and against *P. syringae* to improve plant defense).

- Applications should take into account some important parameters and variables:
Temperature)
Rainfall (mm)
Phenological growth stage
Pruning
General health status
Epidemiological trend and aggressiveness of the disease

FORMULATION 1L. Polyphenol extracts in liquid solution

Storage and transport.: Store in a cool and dry place. Transport in suitable opaque containers.

General information:
Polyphenols are known to be natural secondary metabolites from plants. They are used to control of plant diseases caused by phytopathogenic bacteria belonging to the genus Pseudomonas of Olive and Kiwifruit, and by the nematode *M. incognita* on Tobacco. These products have several remarkable effects as antioxidant, metal complexing agents, as well as antimicrobial.

Indicative Doses for several model systems
Kiwi trees (according to plantation frame 4x5), irrigate through the fertirrigation system 150 L polyphenol/ha dispensed along three days (0, 7 and 15) and spraying aerial part of trees at the rate of 10 L polyphenol/ha in a single application.
Olive trees (according to plantation frame 6x3): irrigate through the fertirrigation system 167 L polyphenol/ha dispensed along three days (0, 7 and 15) and spraying aerial part of trees at the rate of 10l polyphenol/ha in several applications (0,7,15,30, 45 y 60 days).
Tobacco (according to plantation frame 0.9x1.20) irrigate through the fertirrigation system 25 L polyphenol/ha dispensed in a single application.
Tomato irrigate through the fertirrigation system 100 L/ha dispensed three times, for a total of 300 L/ha.
Carrot irrigate through the fertirrigation system 100 L/ha dispensed four times, for a total of 400 L/ha.

Polyphenols must be applied both in spring (March-April) after pruning and autumn (September-October) before the rains and frosts.

Product must be agitated before use.

Polyphenol liquid solution will be always diluted in water (1:10) at time of use.

When spraying will be performed avoid the hours of greater insolation when the stomatal closure occurs and therefore the leaf adsorption is reduced.

Incompatibilities: No incompatibilities have been detected.

FORMULATION 2L. Polyphenol extracts in liquid solution

Storage and transport.: Store in a cool and dry place. Transport in suitable opaque containers.

General information:
Polyphenols are known to be natural secondary metabolites from plants. They are used to control of plant diseases caused by phytopathogenic bacteria belonging to the genus Pseudomonas of Olive and Kiwifruit, and by the nematode *M. incognita* on Tobacco. These products have several remarkable effects as antioxidant, metal complexing agents, as well as antimicrobial.

Indicative Doses for several model systems
Kiwi trees (according to plantation frame 4x5), irrigate through the fertirrigation system 150 L polyphenol/ha dispensed along three days (0, 7 and 15) and spraying aerial part of trees at the rate of 10 L polyphenol/ha in a single application.
Olive trees (according to plantation frame 6x3): irrigate through the fertirrigation system 167 L polyphenol/ha dispensed along three days (0, 7 and 15) and spraying aerial part of trees at the rate of 10l polyphenol/ha in several applications (0,7,15,30, 45 y 60 days).
Tobacco (according to plantation frame 0.9x1.20) irrigate through the fertirrigation system 25 L polyphenol/ha dispensed in a single application.
Tomato irrigate through the fertirrigation system 100 L/ha dispensed three times, for a total of 300 L/ha.
Carrot irrigate through the fertirrigation system 100 L/ha dispensed four times, for a total of 400 L/ha.

Polyphenols must be applied both in spring (March-April) after pruning and autumn (September-October) before the rains and frosts.

Product must be agitated before use.

Polyphenol liquid solution will be always diluted in water (1:10) at time of use.

When spraying will be performed avoid the hours of greater insolation when the stomatal closure occurs and therefore the leaf adsorption is reduced.

Incompatibilities: No incompatibilities have been detected.

FORMULATION 1G.

Storage and transport.: Store in a cool and dry place. Transport in suitable opaque containers.

General information:

Polyphenols are known to be natural secondary metabolites from plants. They are used to control of plant diseases caused by phytopathogenic bacteria belonging to the genus *Pseudomonas* of Olive, Kiwifruit and Tomato, and against nematodes of Carrot. These products have several remarkable effects as antioxidant, metal complexing agents, as well as antimicrobial.

Indicative Doses for several model systems
Kiwi trees and Olive trees: apply directly on the wounds of plants helping with a syringe.
Olive tree: on the soil, at 500kg/ha
Tomato: on the soil, at 250kg/ha
Carrot: on the soil, at 500kg/ha
Polyphenols must be applied both in spring (March-April) after pruning and autumn (September-October) before the rains and frosts, and in high risk periods (wind storm).

Polyphenol gel is water-based and ready for use.

Incompatibilities: No incompatibilities have been detected.

FORMULATION 2G.

Storage and transport.: Store in a cool and dry place. Transport in suitable opaque containers.

General information:

Polyphenols are known to be natural secondary metabolites from plants. They are used to control of plant diseases caused by phytopathogenic bacteria belonging to the genus *Pseudomonas* of Olive, Kiwifruit and Tomato, and against nematodes of Carrot. These products have several remarkable effects as antioxidant, metal complexing agents, as well as antimicrobial.

Indicative Doses for several model systems
Kiwi trees and Olive trees: apply directly on the wounds of plants helping with a syringe.
Olive tree: on the soil, at 500kg/ha
Tomato: on the soil, at 250kg/ha
Carrot: on the soil, at 500kg/ha
Polyphenols must be applied both in spring (March-April) after pruning and autumn (September-October) before the rains and frosts, and in high risk periods (wind storm).

Polyphenol gel is water-based and ready for use.

Incompatibilities: No incompatibilities have been detected.