LIFE Project Number

LIFE13 ENV/IT/000461

FINAL Report

Covering the project activities from 01/10/2014 to 30/09/2016

Reporting Date

31/12/2016

LIFE+ PROJECT NAME or Acronym

EVERGREEN

Project Data

Project location	Italy – Spain
Project start date:	01/10/2014
Project end date:	30/09/2016
Total Project duration (in months)	24 months
Total budget	1,273,407
EU contribution:	631,747
(%) of eligible costs	49.61%

Beneficiary Data

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1. List of contents

2. List of abbreviations, acronyms and symbols.

DGGE, Denaturation Gradient Gel Electroforesis;
E. coli, Escherichia coli;
gfp, green fluorescent protein*;*
HRMA, High Resolution Melting Analysis;
Luc, luciferase*;*
PLFAs, Phospholipid Fatty Acids
Psa, *Pseudomonas syringae* pv. (pathovar) *actinidiae;*
Psv, Pseudomonas savastanoi pv. *savastanoi;*
SERCA, sarco/endoplasmic reticulum (SR) Ca2+-ATPase;
SSM, solid supported membrane;
T3SS, Type Three Secretion System;
T3Es, Type Three Effectors.
AI – Active ingredient
CHT - sweet CHestnut (*Castanea sativa* Mill.) biomass extracted Tannins
CP – Commercial product
DAT – Days After Transplanting
MG - Microgranules
PT – Pre-Transplant
TMDr - Tissue Mass Density of roots
WG – Wettable granules
WP – Wettable powder

3. Executive summary

This report deals with the activities carried out under the project LIFE+ “Environmentally friendly biomolecules from agricultural wastes as substitutes of pesticides for plant diseases control - EVERGREEN“ (LIFE13 ENV/IT/000461) over the period 01/10/2014 - 30/09/2016.

Although to the management of the plant diseases caused by phytopathogenic bacteria and nematodes can also contribute several agronomical and cultural practices, still farmers mainly rely on the use of synthetic agrochemicals, with severe environmental impact. Bacterial plant diseases, albeit less represented than those caused by fungi, very often result in sudden, devastating financial losses to farmers because they are extremely difficult to control. To give just an example, it was estimated that the economic losses due to the attack of kiwifruit by *Pseudomonas syringae* pv. *actinidiae* have reached in the last two years 2 million Euros just in Italy. The control of bacterial diseases of plants is a worldwide considerable challenge because of the limited availability of bactericides, which essentially are copper and antibiotics, the latter not allowed to be used as plant protection products within the EU Member States. Therefore, the control of bacterial diseases of plants is largely based on the use of copper salts, which are among the few chemicals still allowed in EU also in organic agriculture, despite the fact that copper cannot be degraded or destroyed in the environment. Thus, treatments with copper derivatives against plant pathogenic bacteria, and fungi as well, contribute to its accumulation in soils more than any other agricultural activity, posing a serious threat to a wide range of organisms and microorganisms, and to terrestrial and aquatic ecosystems. In fact, high concentrations of copper dangerously affect physiological and biochemical processes in microorganisms, algae and higher organisms. In addition to its negative eco-toxicological profile, a great concern is given by the residual copper in vegetables and fruits for human consumption.

Similarly, plant parasitic nematodes are worldwide responsible of heavy crop losses (up to 12%) and the diseases they cause are difficult to be controlled. Traditionally, management of nematodes has been achieved with the utilization of plant resistance, crop rotation, biological control, and cultural practices, including solarisation and various organic amendments, but the use of agrochemicals have been always preferred by farmers. Mainly, since 1950 two groups of chemical nematicides have been in use: low molecular weight soil fumigants and carbamates or organophosphates. As far as methyl bromide is concerned, it was banned from EU because it is considered to be a significant ozone depleting substance.

Presently, most nematicides and nemastats are no more included among the labeled agrochemicals for crops under Good Agricultural Practices because their belonging to high risk classes. Moreover, the development of resistance phenomena and the potential for adverse ecological impact from most of the nematicides still in use create a continuous need for new products and alternative control strategies for these plant parasites.

The adoption of sustainable agricultural practices can be essential to help farmers to achieve these goals, by lowering production costs and inputs, related to the use of pesticides and agrochemicals, fuel consumption, and waste and non-food biomass disposal, that ultimately impact also the environment with accumulation of toxic substances in water and soil and greenhouse gas emission in the air. In a regime of sustainable and cost-effective use of resources in agriculture, it is therefore mandatory to reduce the levels of chemicals used, such as pesticid, and to develop environmentally friendly, added value management of agricultural wastes and biomasses.

In this connection it has been calculated that globally 140 billion tonnes of agricultural biomasses and wastes are produced annually (UNEP 2009) and a 42.5% increase could be generated in 2020 compared to 1995 if no specific management strategies will be applied (Joint Research Centre Data). Most of these are agricultural residues are left to rot into the fields or they are simply burnt. As far as Europe is concerned, about 31% of the total wastes including also those from agricultural industry are land filled, 42% is recycled, 6% is incinerated with energy recovery and 21% is unaccounted for. Agricultural biomasses and wastes disposal have very high costs, which can reach

even 58% of the total production costs. Therefore, land filling and disposal by on-site burning are generally preferred, compatibly with the provisions of the current EU and national legislation. This management does not only contribute to pollution of the air, water and soil but also represents a huge loss of a valuable and renewable resources, in the form of both materials and energy. In EU, agriculture is the most important land use in geographic terms, with 40% (corresponding to about 130 million ha) of the total land area (323 million ha).
Very recently (May 2013) the European Commission adopted a package of measure to modernise, simplifying and strengthen the EU agrofood sector (REF). Among its four main bullet points there is a proposal for a new regulation on protective measures to be applied against plant pests, because plant health is considered a key factor for a sustainable and competitive agriculture, and in this respect the EU CR N.284/2013 was published, setting out the new data requirements for plant protection products, in accordance with EC R N.1107/2009 and with the Directive 2009/128/EC on the sustainable use of pesticides. Among the plant pathogens that are most difficult to control there are nematodes and bacteria, mainly because of the unavailability of safe and effective plant protection products.
Traditionally, farmers have used agricultural practices, such as crop rotations, field scouting and selection for resistant varieties try to control nematode populations. Currently nematode management strategies are essentially dependent upon highly toxic nematicides, which are harmful to the environment, and damaging on soil biodiversity by their non-target action/effects. Moreover, nematicides are readily transported in soil water and attached to colloidal soil particles, and thus heavily pollutant for water and soil. Nevertheless, it was estimated that European farmers still apply up to 10,000 tonnes of nematicides each year and this amount could be forced to increase: in Europe, root knot nematodes are becoming increasingly important and several highly aggressive and quarantine nematodes reported in the EPPO alert list are at risk of introduction as a result of both climate changes, which extend their geographical range, and for the globalization of trades. Currently the annually nematicide market is estimated at around 1 billion Euros, with a trend that appears to remain robust and it remains an opportunity for industry players, because the phasing-out of the ozone depleting leading nematicide product, methyl bromide, has forced to look for safer alternatives.
As far as the control of bacterial diseases of plants is concerned, this is another major challenge for EU agriculture and, similarly to plant parasitic nematode, because of the limited availability of effective bactericides, mainly copper salts and antibiotics, the latter not allowed to be used within EU Member States. Although the amount of antibiotics used on plants is negligible compared to medical and veterinary uses, undesirable effects either for the environment or for human and animal health have been observed. In particular, periodical applications of antibiotics in agriculture can select for bacteria resistant to these drugs, and thus increase the frequency in the environment of antibiotic resistance genes that can eventually be transferred into medically important bacteria.
Therefore, there is a worldwide need for the reduction or replacement in agriculture of pesticides such as nematicides and copper, with the development of alternative compounds and/or methods to be used in the frame of the integrated management of resources that is characteristic of sustainable agriculture.
EVERGREEN consisted of several actions related to each other able to demonstrate the validity and effectiveness of innovative and standardised preparation of high quality polyphenolic-based molecules from agricultural vegetable biomass and wasteplant pathogenic bacteria and nematodes and to control the diseases they cause on plants.
EVERGREEN also perfectly met the EU vision about sustainable agriculture, with the use of renewable resources, such as vegetable not edible biomass and waste, to obtain environmentally friendly substitutes for traditional pesticides against plant pathogenic bacteria and nematodes, avoiding environmental pollution and lowering the costs deriving from both the use of conventional pesticides and the disposal of these wastes.

At the end of the EVERGREEN project, the following specific technical objectives were achieved:

- Green chemistry extraction of high quality and standardised polyphenolic fractions and molecules from not edible vegetable biomass/waste of chestnut, olive, artichoke and grapevine, and process optimization at laboratory scale (100% - Action B2).
- The EVERGREEN polyphenolic fractions and molecules are biologically and chemically stable, using water as the most performing and ecofriendly solvent, as demonstrated at laboratory level (100% - Action B3).
- The EVERGREEN high quality polyphenolic extracts are active against plant pathogenic bacteria and nematodes *in planta* at laboratory scale, using concentrations in the range 1-100µM, as demonstrated by traditional pathogenicity assay and molecular tests (100% - Action B4).
- The *in planta* biological activity of the EVERGREEN high quality polyphenolic extracts is comparable to that of copper-based and traditional nematicides (100% - Action B1, B4 and C1).
- The EVERGREEN high quality polyphenolic extracts do not possess any toxicity on organisms and microorganisms commonly used for acute and chronic toxicity tests, and on universally conserved subcellular targets such as Ca^{2+}-ATPase (100% -Actions B6 and C2).
- The EVERGREEN high quality polyphenolic extracts do not cause any direct selection towards the emergence of bacteria resistant to the polyphenolic molecules themselves, as well as any cross-selection of copper- and antibiotic-resistant bacteria (100% -Action C3 and C6).
- The Kilo-scale green extraction of the EVERGREEN polyphenolic fractions and molecules recovered from vegetable not edible biomass/waste was optimised (80% - Action B5).
- The EVERGREEN polyphenolic extracts are active in plant protection against phytopathogenic Gram negative bacteria and nematodes, as demonstrated by 4 optimised formulations on model systems at pilot and field screening, with beneficial effects on soil microflora (100% - Action B7 and C4).
- The spent vegetable biomass, at the end of the extraction of the EVERGREEN standardised polyphenolic fractions/molecules, can be recycled for energetic purposes and as fertilizers, as demonstrated for chestnut tannin, olive pomace and grape marc (80% - Action C5)**.**
- The EVERGREEN approach is an ecofriendly and sustainable solution in plant protection in the frame of circular economy, as demonstrated by the LCA carried out on the processes concerning the most active EVERGREEN formulations (100% - Action C7).

EVERGREEN has a high Community added value, as a result of its high environmental and transnational character, thanks to the extensive use of advanced technology, which assists in overcoming the geographical and cultural barriers preventing technical progress in the agricultural sector.
The Water Framework Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishes the legal framework for an innovative and unitary approach in water management across Europe, to protect and to restore clean water and to guarantee its sustainable use in a long-time period. It also sets specific deadlines for EU countries to adopt measures to protect aquatic ecosystems, together with several economic approaches for water management and services. In the Annexes of WFD and of Directive 2008/105/EC several priority hazardous substances whose presence in water has to be excluded or reduced have been reported, together with other pollutants such as selected existing chemicals, plant protection products, biocides, metals and other groups. In fact, concerning the control of emissions of chemicals into water, the WFD is also supported by other EU environmental legislation. In this frame environmental standards for copper and other metal contaminants in water were set out in WFD and other several EU Directives, to reduce or eliminate the risks posed by metals to the aquatic environment.

This EVERGREEN project is one of the most obvious examples of the tight and important link existing between WFD and the EU Thematic Strategy for Soil Protection, aimed to several actions concerning soil, such as to prevent its degradation, to preserve its ecological and social functions, and also to remediate polluted and degraded soils. In fact, although the framework Directive of EU Thematic Strategy for Soil Protection defines as "soil" everything that is between the earth surface and bedrock, it is obvious that the reductions of soil contamination by several pollutants such as copper, that can be easily runoff into water from soil, would at the end result in the protection of aquatic ecosystems, as asked by WFD.

EVERGREEN perfectly responds to the objectives of both the Water Framework Directive 2000/60/EC and of The EU Thematic Strategy for Soil Protection, offering an innovative strategy to control plant diseases caused by Gram negative phytopathogenic bacteria that also meets EU standards in terms of environmental protection of water and soil ecosystems. The use of these antiinfective substances are a reliable and efficient alternative to the application of chemicals as bactericides against plant pathogenic bacteria, that will contribute to the reduction of chemicals contamination into agricultural soils and water. Consequently, an increase in soil fertility will be also be obtained, as well as an important reduction in the percentage of chemicals resistant bacteria in the soil microflora, that are also a dangerous reservoir of antibiotic-resistant bacteria for humans and animals.

In the EVERGREEN project we demonstrated on a pilot field scale the effectiveness and the potential benefits for the environment of the use of these anti-virulence peptides, in the respect the principles of the Directive 91/414/CEE, concerning research field trials with novel and unregistered products and in compliance with good experimental practice.

The Final Report explained the technical and administrative topics and the organisation of the project in chapter 4 and 5 and the technical and financial results in chapter 6 and 7.

4. Introduction

Environmental problem/issue addressed

The goal of the project was the demonstration of an environmentally friendly, sustainable and integrated strategy for the control of bacterial and nematode diseases of plants, in the view of an economically viable and environmental respectful reduction or replacement of the traditional pesticides used in conventional and organic agriculture. The EVEGREEN approach perfectly matches the restrictions established within the EU Member States concerning the use and the placing on the market of plant protection products and the main aim of the landmark package very recently proposed by the European Commission to modernise, simplify and strengthen the agri-food chain in Europe, which is definitely oriented towards a holistic vision of a sustainable and more competitive agriculture. Furthermore, EVERGREEN project was also aim at demonstrating how several simple and inexpensive extraction processes can allow a cost-effective exploitation of plant biomass and non-edible portion of crops. This allows greater short and long term environmental benefits in comparison with other current approaches concerning the management of these wastes. Due to their natural origin, these extracts can be properly used without any damaging side effects for humans, animals, plants and any ecosystem. More importantly, in addition to the phenolic and polyphenolic compounds, these extracts contain other components whose presence have been analytically tested and standardised and which reinforce their biological activity against bacteria and nematodes of plants, if compared with the results obtained when using the same single purified chemical molecules.

Outline the hypothesis demonstrated by the project.

EVERGREEN aimed at innovating the approach towards plant disease control, reducing conventional chemical pesticides with environmentally friendly and highly bioactive molecules recovered from agricultural vegetable wastes. The project had a double goal to simultaneously improve the safety profile of plant disease control products for environmental compatibility and to support the sustainability of plant production to pursue cost-effective strategies.

EVERGREEN demonstrated in vitro and in vivo efficacy and reliability of the polyphenolic-based biomolecules extracted from agricultural non-food biomasses and wastes as disease control products against phytopathogenic bacteria and nematodes, for replacing current commercial pesticides and application of copper salts in conventional and organic agriculture.

Optimised field treatments were carried out on several plants and crops having a high commercial value and here used as a model (*e.g.* Olive, Kiwi, Tobacco).

Description of the technical solution.

EVERGREEN consisted on actions aiming to:

1. demonstrate the negative environmental impact of the use of conventional chemical pesticides for the control of bacterial and nematode diseases of plants.
2. demonstrate the performances as plant disease control products of the polyphenolic-based molecules extracted from agricultural vegetable wastes, at laboratory, pilot and field scale.
3. demonstrate the efficiency of tailored formulations of these polyphenolic-based molecules as plant disease control products, to achieve the highest activity on the different plant pathogens.
3. demonstrate the reliability and the compliance with REACH document of EU legislation of the extraction processes for these polyphenolic-based molecules, from the lab-scale to the kilo-lab scale.
4. demonstrate the project technical validity for optimising the up-scaling of treatments with these polyphenolic-based molecules recovered from agricultural vegetable wastes, from laboratory to pilot scale and to semi-industrial scale.

5. demonstrate the more efficient and ecotoxicologically compatible profile of these bioactive molecules than conventional pesticides, at laboratory, pilot and field scale.
6. demonstrate the absence of any side unexpected effect of the bioactive polyphenolic-based molecules on common molecular targets of living organisms, at laboratory, pilot and semi-industrial scale.
7. demonstrate the absence of any direct or cross-acting selection operated by the bioactive polyphenolic-based molecules towards the emergence of unwanted resistance phenomena in their respect or for conventional pesticides, copper or even antibiotics, at laboratory, pilot and semiindustrial scale.
9. demonstrate the short term environmental benefits and the economic advantages from the use of polyphenolic-based molecules recovered from agricultural vegetable wastes in the control of biotic plant diseases.

Expected results and environmental benefits.

The project had the following technical results:
• Long term reduction (80-100%) of pollution in agricultural soils given by conventional pesticides used against plant pathogenic bacteria and nematodes, following their replacement with the project polyphenolic-based bioactive molecules recovered from agricultural non-food vegetable biomasses and wastes.
• Increased performances in the control of the bacterial and nematode diseases of plants (55%) for the wider temporal application of the project polyphenolic-based bioactive molecules in comparison with conventional pesticides, even in period of plant life cycle which are critical for infections but during which traditional chemical plant protection treatments are not allowed.
• Reduction (80-100%) of point-source and diffuse pollution from the disposal of agricultural vegetable wastes in the environment.
• Reduction (45%) of costs for disposal of agricultural non-food vegetable biomasses and wastes.
• Reduction (20%) of energy consumption used for remediation processes of pesticides-contaminated soils
• Improved soil fertility (70%) following the reduction/replacing of conventional agrochemicals products for plant diseases control with the project polyphenolic-based bioactive molecules.
• Increased soil microbial diversity (65%) with positive impact on soil biology and on the transformation and dynamic of nutrients.
•Increased (45%) natural competence in agrosystems for suppression against plant pathogenic bacteria and fungi.
• Long term reduction (90%) of pesticides pollution in watercourses.
• Reduction (80%) of toxicological impact of pesticides pollution on terrestrial, aerial and aquatic fauna.
• Short term reduction (85%) of pesticide- and drug-resistant bacteria and nematodes in agricultural sites.
• Long term reduction (60%) of the reservoir of environmental antibiotic-resistant bacteria and of their spread with reduction of the risks for the health of humans and animals.
• Reduction (90%) of pesticides residues on fruit and vegetable for human and animal consumption.
• Increased quality (40%) of food and feed from vegetable origin with benefits on human and animal health.

Expected long-term results.

EVERGREEN perfectly meets the EU vision about sustainable agriculture, with the use of renewable resources, such as vegetable not edible biomass and waste, to obtain environmentally friendly substitutes for traditional pesticides against plant pathogenic bacteria and nematodes, avoiding environmental pollution and lowering the costs deriving from both the use of conventional pesticides and the disposal of these wastes. EVERGREEN project is one of the most obvious examples of the tight and important link existing between WFD and the EU Thematic Strategy for Soil Protection, aimed to several actions concerning soil, such as to prevent its degradation, to preserve its ecological and social functions, and also to remediate polluted and degraded soils. In fact, although the framework Directive of EU Thematic Strategy for Soil Protection defines as "soil" everything that is between the earth surface and bedrock, it is obvious that the reductions of soil contamination by several pollutants such as copper, that can be easily runoff into water from soil, would at the end result in the protection of aquatic ecosystems, as asked by WFD. The use of the EVERGREEN antiinfective substances will be a reliable and efficient alternative to the application of chemicals as bactericides against plant pathogenic bacteria, that will contribute to the reduction of chemicals contamination into agricultural soils and water. Consequently, an increase in soil fertility will be also be obtained, as well as an important reduction in the percentage of chemicals resistant bacteria in the soil microflora, that are also a dangerous reservoir of antibiotic-resistant bacteria for humans and animals.

5. Administrative part

5.1 Description of the management system

The EVERGREEN project proceeded smoothly and all actions were completed as foreseen. During all project phase, the EVERGREEN project has benefited from close collaboration between all beneficiaries and has maintained close contact with all through different media: emails, telephone, meetings, etc.

The EVERGREEN management process needed daily work to maintain a permanent flow of action with the aim of achieving the objectives set. The specific management activities carried out were:

- Preparation of the Partnership Agreement (sent with the Inception Report)
- Organisation of Coordination meetings
- Organisation of Monitoring meetings
- Organisation of different phone and web meetings between some beneficiaries in order to plan and monitor the project technical activities
- Continuous contact between all project beneficiaries for monitoring project activities
- Preparation of material for meetings and dissemination events.
- General actions and activities for the coordination of the project.
- Management of the financial aspects of the project.
- Monthly reports to the LIFE external team monitor on the evolution of the project.

The management of the project was carried out in compliance with what was established in the proposal approved by the European Commission, with all partners acting in compliance with the Common Provisions and the Partnership Agreement.

The project management structure is very simple as only 5 beneficiaries, plus the EC and the LIFE external team. The following diagram provides information about the general management structure:

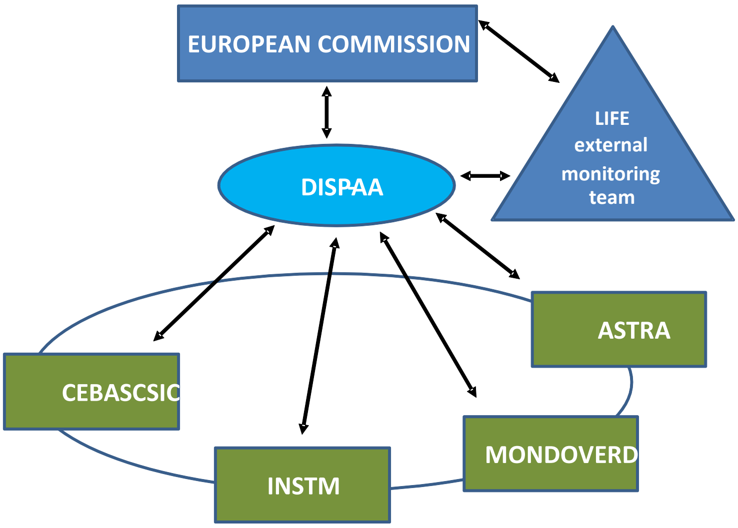


Figure EVERGREEN management structure.

In particular, the EVERGREEN beneficiaries defined the following two management structures:

- **Technical Committee:**
  - DISPAA: Stefania Tegli
  - ASTRA: Vanni Tisselli
  - CEBAS: Carlos Garcia Izquierdo
  - INSTM: Sergio Miele
  - MONDOVERDE: Enrico Banci and Bernardo Banci

- **Administrative Committee:**
  - DISPAA: Silvia Borselli
  - ASTRA: Elisabetta Baldassarri
  - CEBAS: Maria Teresa Hernandez
  - INSTM: Daniele Consigli
  - MONDOVERDE: Enrico Banci and Bernardo Banci

Project beneficiaries have carried out different meetings in order to organize, coordinate and develop the project. The following coordination and monitoring meetings were organized

- Progress and Coordination meetings:
  - Kick-off meeting, 23th October 2014, at the coordinator partner DISPAA premises in Sesto Fiorentino (Florence), Italy;
  - Progress and Coordination 6 month meeting, 28th April 2015, at the coordinator partner DISPAA premises in Sesto Fiorentino (Florence), Italy;
  - Progress and Coordination 12 month meeting, at the coordinator partner DISPAA premises in Sesto Fiorentino (Florence), Italy on 5th November 2015.
  - Progress and Coordination 18 month meeting, at the associated beneficiary ASTRA premises in Imola, Italy, Italy on 13rd May 2016.
  - Progress and Coordination 24 month meeting, at the associated beneficiary Mondoverde premises in Scarperia e San Piero (Florence), Italy on 23th September 2016.

- Monitoring meetings with LIFE's External Assistance Team:
  - Monitoring meeting during the Progress 6 month meeting, 28th April 2015: Dr. Carlo Ponzio.
  - Monitoring meeting during the Progress 18 month meeting, 13rd May 2016: Dr. Carlo Ponzio.

5.2 Evaluation of the management system

During this project period, the 4 associated beneficiaries ASTRA, CEBAS, INSTM and MONDOVERDE participated in project management activities keeping in smooth contact with the project coordinator. In this sense, they prepared and attended the project management meetings and collaborated with the project coordinator (DISPAA) in the preparation of the Inception Report and of this Final Report, as set out in the project proposal.
During all project phase, monitoring tasks have been carried out for each action, in particular:

- DISPAA, as coordinating beneficiary, has had continuous contact with all project beneficiaries for monitoring project activities;
- DISPAA, as coordinating beneficiary, has prepared and sent a monthly indication of operative activities to be done to all the partners;
- DISPAA, as coordinating beneficiary, has sent a monthly report to the monitor of LIFE's External Assistance Team on the progress of the project, allowing to follow-up of the EVERGREEN project.

As shown in the previous diagram reported above, the EVERGREEN beneficiaries added values were:

- DISPAA, project coordinator, a University Department specialist and responsible of plant analysis
- MONDOVERDE, an Italian company specialized in the production and trading of products for gardening, home and outdoors life

- ASTRA, an Italian company expert in soil and plant technologies and products and responsible for the field trials in Italy.
- CEBASCSIC, an Institute of Spanish National Research Council, expert in soil and plant and responsible for the field trials in Spain.
- INSTM an Italian university consortium specialized in innovative soil and agriculture materials and technologies

6. Technical part.

6.1. Technical Actions

6.1.1. Action B.1. Demonstration of the performances of traditional pesticides for the control of bacterial and nematode diseases of plants important for the EU

Starting date foreseen: 1st of October 2014
Actual start date: 1st of May 2014
End date foreseen: 31st of March 2015
Actual end date: 30th of October 2015

The results of the activities carried out in Action B.1 are defined in the following project Deliverable, foreseen at the end of the 6th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 1. Deliverable Action B1: Demonstration of the use of copper compounds for the control of bacterial diseases of plants important for the EU.

Activities carried out
Action B1 was delegated to DISPAA-UNIFI, CEBAS CSIC and INSTM, and was completed by 100%. A longer time than that foreseen was spent on this Action, but absolutely without any cost increase. This choice was essential to have a longer, and thus more reliable, reference period to evaluate field-applied nematicide products on Tobacco, whose growing season in central Italy begins in May and usually ends in October. Therefore, already informed about the EVERGREEN project approval, starting from May 2014 INSTM worked on activities producing data useful also for fullfilling demonstration for B1 Action. Then, as already planned and as also asked from EU Commission following the IInd monitoring visit, other trials were carried out by INSMT on Tobacco in 2015 season.
This Action had to demonstrate the performances of traditional copper compounds and nematicides for the control and the management of diseases caused on plants by phytopathogenic bacteria and nematodes, respectively. As planned in several following Actions, these data had then to be compared with those obtained using the EVERGREEN polyphenolic-based standardised preparations extracted from agricultural vegetable no food/feed biomasses.
For B1 demonstration activities, three important plant pathogens for EU Mediterranean Countries were selected as a model: *Pseudomonas savastanoi* (*Psv*), *P. syringae* pathovar (pv.) *actinidiae* (*Psa*) and the plant parasitic nematodes *Meloidogyne* spp. In particular, *Psv* and *Psa* are the causal agents of Olive knot disease and of the emerging and highly destructive bacterial canker of Kiwi tree, respectively, while the nematode belonging to the genus *Meloidogyne* are among the most destructive pests on Tobacco and most vegetable crops.

Fig.1. Disease symptoms caused by *Psv, Psa* and *Meloidogyne* on olive, kiwifruit and tobacco, respectively (from left to right).

Tests were carried out on a laboratory/greenhouse and field scale, on plants artificially inoculated and grown in Italy and Spain, previously treated or not with several copper or nematicide compounds as traditional control means against bacterial phytopathogens and nematodes of plants. Indoor scale pathogenicity trials were carried out at CEBAS CSIC (olive and kiwifruit) (Figures 2 and 3, respectively), while experimental outdoor plots were prepared by INSTM (Tobacco) (Figures 4). Chemical and microbiological analysis were carried out by DISPAA-UNIFI and CEBAS CSIC.

Olive negative control ($CuSO_4^-$ Psv^-)	Olive ($CuSO_4^-$ Psv^+)	Olive ($CuSO_4^+$ Psv^+)

Fig. 2. Olive 4 years potted plants, artificially inoculated with *P. savastanoi Psn23* strain, treated or not with Cu^{2+} (500 µM x 3 applications)

Kiwifruit negative control ($CuSO_4^-$ Psa^-)	Kiwifruit ($CuSO_4^-$ Psa^+)	Kiwifruit ($CuSO_4^+$ Psa^+)

Fig. 3. Kiwifruit 4 years potted plants, artificially inoculated with *P. syringae* pv. *actinidiae*, treated or not with Cu^{2+} (500 µM x 3 applications)

Fig. 4. Tobacco plants grown in pots on steam-sterilised soil previously inoculated with *M. incognita*, then transplanted in soils treated with synthetic nematicides (MOCAP and OIKOS).

The detailed description of EVERGREEN Action B1 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 1 Deliverable B1. Firstly, phytosanitary services in Italy and Spain were asked by DISPAA-UNIFI, CEBAS CSIC, INSTM, but also by the beneficiaries not directly involved in this Action B1 ASTRA and Mondo, to suggest which copper compounds and nematicides have to be used for EVERGREEN experimental purposes. Copper sulphate based commercial formulations were selected and then used throughout the Action B1 activities. *In vivo* artificial bacterial infections were carried out seven days after performing copper spraying treatments (500 µM at copper ions x 3 times). Control plants were not treated and used for comparison. Performances of copper treatments were evaluated after three and four months from infection.
As nematicides, MOCAP (Etoprofos 10% MG), OIKOS (Azadirachtine 2.4% WG), Vydate (Oxamyl 5% MG) were used Tobacco cv. Virginia Bright. For comparison, untreated plants were considered, as well as plants treated with a commercial biological preparation based on *Bacillus firmus* (FlocteR Bayer CropScience). Nematode infestation on the roots, and plant growth were then determined at 20 and 40 days after treatments.

Problems encountered
No particular problems have been encountered during this Action B1, and this action was completed by 100%, as scheduled.

Verification of the expected results actually achieved
The detailed description of EVERGREEN Action B1 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 1 Deliverable Action B1.
The expected results were:
• Study on the performances and efficay of copper-based compounds and of traditional nematicides (*e.g*. MOCAP-etoprophos, AZA-NEMA-azadirachtine) for the control of *Psv*, *Psa* and *Meloidogyne* spp, respectively.
• Evaluation of the negative effects of copper-based products on the epiphytic populations of *Psv* and *Psa*, related to the emergence of copper- and antibiotic-resistant strains.
The expected results were fully achieved, as described in the Annex X Deliverable Action B1.

The performances of copper efficacy in controlling bacterial diseases of plants was demonstrated and confirmed after *in vivo* artificial inoculation with *Psv* and *Psa* on olive and kiwifruit, respectively. Inoculation were carried out after copper spraying treatments (500 µM at copper ions x 3 times), and control plants were not treated and used for comparison. Data were collected 3 and 4

months post-infectionat Performances of copper treatments were evaluated after three and four months from infection.
Following chemical analysis on soil and/or leaves of potted plants, no relevant differences were found concerning microelements, both in Olive and Kiwifruit leaves. As far as soil enzymatic activities and biodiversity are concerned, an increase in total monounsaturated PLFAs and Gram negative bacteria, as well as a decrease in fungal soil population, was demonstrated in copper-treated and in infected plants. In particular, also the variability of bacterial plant pathogenic populations was altered by copper treatments, with a decrease of biodiversity in antibiotic-resistant populations and an increase in those resistant to copper.
Concerning plant parasitic nematodes, a reduction of nematode count was demonstrated to occur on Tobacco as a consequence of the treatments with the ordinary nematicides, as well as with the biological control agent *B.firmus* I-1582.

Perspectives for continuing the action after the end of the project
During the project and just after its conclusion, both the coordinator as well as the other beneficieries have been contacted by stakeholders interested to monitor the effects deriving from copper pollution in agroecostystems, such as Confagricoltura Toscana, with the perspective to be part of operative groups working on this topic at national at internation level.

6.1.2. Action B.2 Demonstration of the qualitative and quantitative yields of extraction process for the recovery of high quality polyphenolic molecules from not edible vegetable biomass and waste at laboratory scale

Starting date foreseen: 1st of January 2015
Actual start date: 1st of January 2015
End date foreseen: 31st of December 2015
Actual end date: 31st of December 2015

The results of the activities carried out in Action B.2 are defined in the following project Deliverable, foreseen at the end of the 15th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 2 Deliverable Action B2: Demonstration of the qualitative and quantitative yields of extraction process for the recovery of high quality polyphenolic molecules from not edible vegetable biomass and waste at laboratory scale.

Activities carried out
This demonstration was delegated to DISPAA-UNIFI, INSTM and CEBAS CSIC, and was completed by 100%.
This Action has to demonstrate, at laboratory level, the efficiency of the extraction process here developed for the recovery of high quality polyphenolic molecules from agricultural not edible vegetable biomass and waste, in terms of qualitative and quantitative yields.
Every year, agricultural industry produces not edible biomass for billions of metric tons worldwide, which have to be considered one of the most important and renewable resources available on earth. According to the definitions from the Waste Framework Directive (EU 2006), these residues fall into two main categories, that are i) crop residues, generated into the farms, and ii) agro-industrial residues, generated starting from any post-harvest process. Crop residues are primary biomass residues, which are abundant and cheap, and that can be heavily environmentally pollutant when not properly managed. Up to now, agricultural residues have been used as such, or converted by physical, chemical or biochemical processes, or fractionated into their components, in very different sectors, for instance to produce fuels, energy, several fibre-based products, and a wide range of high value chemical products (Figure 5).

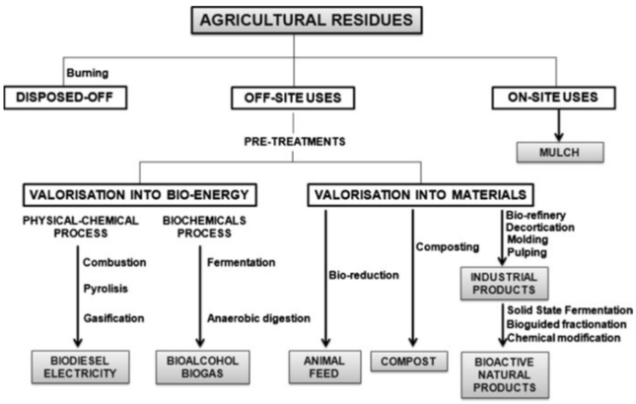


Fig. 5. Scheme of main processes of agricultural biomass conversions (@Santana-Meridas *et al.*, 2012)

In the last two decades, the chemicals derived from agricultural biomass have been mainly studied for their antioxidant and/or pharmacological properties for humans. Conversely, only in very recent times these products started to be exploited also as potential natural biopesticides or plant disease control product, although a quite significative lack of knowledge still exists on this regard. Among the most bioactive components obtained from plants, there are aromatic secondary metabolites, such as phenolics and polyphenols, including flavonoids, quinones, tannins, and coumarins, which in nature are essential for enhancing plant fitness and defense against pathogens and predators. Optimization of sample preparation and extraction processes are pivotal to get polyphenolic preparations from agricultural residues having the highest yields and bioactivity, as well as a well-defined and constant composition.

According to the project description, the vegetable residues to be here used as a model for lab-scale optimization of polyphenolics extraction were *Olea europaea* L., *Cynara scolymus* L., *Castanea sativa* M., and *Vitis vinifera* L.. These plants are particularly rich in polyphenolic secondary metabolites, such as secoiridoids, tyrosol and hydroxytyrosol, lignans, hydroxycinnamic derivatives, flavonoids and hydrolysable tannins.

The detailed description of the EVERGREEN Action B2 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex X Deliverable Action B2.

Briefly, plant materials were processed over the conditions of the so called "green chemistry", that is using only water as solvent, combined with membrane separation technology systems to fractionate the extracts. In the frame of the EVERGREEN project, these procedures were essential to ensure the energetic, ecological and economic sustainability of the whole process.

The extracts were analyzed for their polyphenol content, as characterization and quantification by HPLC/DAD/MS methods and techniques (Fig. 6, 7, 8 and Table 1). Then, each polyphenol fraction was evaluated for its antiradical, antioxidant and antimicrobial properties.

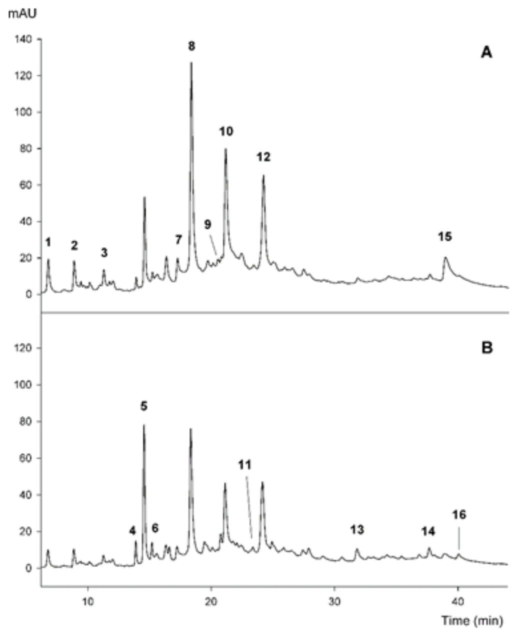


Fig. 6. Chromatographic profile of the liquid fraction of *C. sativa* (fraction 6), registered at 254 nm (A) and 280 nm (B). Peaks: 1. Vescalin; 2. Castalin; 3. Pedunculagin I; 4. Monogalloyl glucose I; 5. Gallic acid; 6. Monogalloyl glucose II; 7. Roburin D; 8. Vescalagin; 9. Dehydrated tergallagic-C-glucoside; 10. Castalagin; 11. Digalloyl glucose; 12. O-galloyl-castalagin isomer; 13. Trigalloyl glucose; 14. Tetragalloyl glucose; 15. Ellagic acid; 16. Pentagalloyl glucose.

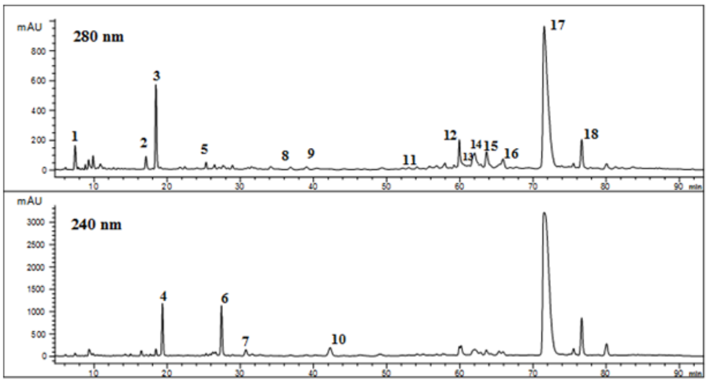


Fig. 7. Chromatograms of *O. europea* extract (PHENOLEA fraction). Peaks: 1. Hydroxytyrosol derivative; 2. Hydroxytyrosol; 3. Hydroxytyrosol glucoside; 4. Oleoside; 5. Esculin; 6. Demetyl elenolic acid diglucoside; 7. Elenolic acid glucoside; 8. Olivile; 9. Hydroxycinnamic derivative; 10. Elenolic acid glucoside derivative; 11. β-OH-verbascoside; 12. Verbascoside; 13. Luteolin 7-O-glucoside; 14. Pinoresinol; 15. Verbascoside isomer; 16. Acetoxypinoresinol; 17. Oleuropein; 18. Oleuropein isomer.

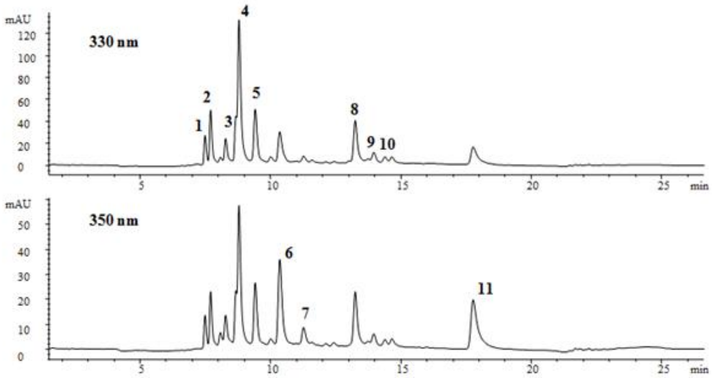


Fig. 8. Chromatograms of *C. scolymus* extract (CYNARA_SOL fraction). Peaks: 1-O-caffeoylquinic acid; 2. 3-O-caffeoylquinic acid; 3. caffeoylquinic acid; 4. chlorogenic acid; 5 cynarin; 6. luteolin 7-O-rutinoside; 7. luteolin 7-O-glucoside; 8. dicaffeoylquinic acid; 9. dicaffeoylquinic acid; 10. dicaffeoylquinic acid; 11. luteolin.

Dried pomace	mg/g	mmol/Kg	%
Delphinidin-3-glucoside	0.040	0.086	0.7
Cyanidin-3-glucoside	0.079	0.175	1.3
Petunidin-3-glucoside	0.064	0.134	1.0
Peonidin-3-glucoside	0.265	0.530	4.0
Malvidin-3-glucoside	0.249	0.505	3.8
Cyanidin-3-acetyl glucoside	0.007	0.014	0.1
Malvidin-3-acetyl glucoside	0.078	0.145	1.1
Malvidin-3-caffeoyl glucoside	0.012	0.018	0.1
Petunidin-3-cumaroyl glucoside	0.016	0.025	0.2
Peonidin-3-cumaroyl glucoside	0.022	0.034	0.3
Malvidin-3-cumaroyl glucoside	0.098	0.152	1.2
Delphinidin aglycone	0.287	0.867	6.6
Total anthocyanins	**1.215**	**2.685**	**20.4**
Gallic acid	0.019	0.112	0.9
Catechin	0.030	0.102	0.8
Epicatechin	0.032	0.112	0.9
ECG dimers	0.891	1.010	7.7
catechin/epicatechin trimers digallated	10.661	9.112	69.4
Total tannins	**11.633**	**10.448**	**79.6**
Total polyphenols	**12.848**	**13.133**	**100.0**

Table 1. Quali-quantitative HPLC/DAD/MS analysis of grape dried pomace.

Problems encountered
No particular problems have been encountered during this Action B2, and this action was completed by 100%, as scheduled.

Verification of the expected results actually achieved
The detailed description of EVERGREEN Action B2 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 2 Deliverable Action B2.
The expected results were:
• Demonstration of the extraction procedure for the recovery of high quality polyphenolic molecules from agricultural not edible vegetable biomass and waste at laboratory scale.
• Demonstration of the extraction procedure in compliance with REACH regulations.
• Demonstration of the outputs from extraction procedures carried out on different batches of the same vegetable biomass, at laboratory scale
• Demonstration of the purity assessment procedure for these high quality polyphenolic molecules through chromatographic, spectrophotometric and spectrometric (HPLC/DAD/ESI-MS) methods.
The expected results were fully achieved, as described in the Annex 2 Deliverable Action B2.

The activities carried out in the EVERGREEN Action B2 succesfully allowed the design of the experimental procedures for the "green chemistry" extraction of polyphenolic compounds and fractions from the plant species here used as a model, at laboratory scale. Moreover, the chromatographic, spectrophotometric and spectrometric (HPLC/DAD/ESI-MS) analysis carried out on extracts obtained from different batches of the same vegetable biomass demonstrated the reproducibility of these optimised and standardised protocols.

Perspectives for continuing the action after the end of the project
In the frame of this Action B2, and as potential follow up of the results of EVERGREEN project on different vegetable biomass and waste, CEBAS CSIC carried out a detail investigation on the vegetable residues produced in Spain and useful for the future application of the EVERGREEN approach.

6.1.3. Action B.3 Demonstration of the biological and of the chemical stability of the crude polyphenolic extracts and of their fractions, recovered from not edible vegetable biomass and waste, at laboratory scale

Starting date foreseen: 1st of January 2015
Actual start date: 1st of January 2015
End date foreseen: 31st of December 2015
Actual end date: 31st of December 2015

The results of the activities carried out in Action B.3 are defined in the following project Deliverable, foreseen at the end of the 15th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 3 Deliverable Action B3: Demonstration of the biological and of the chemical stability of the crude polyphenolic extracts and of their fractions, recovered from not edible vegetable biomass and waste, at laboratory scale.

Activities carried out
This demonstration was delegated to DISPAA-UNIFI, INSTM and CEBAS CSIC, and was completed by 100%.
This Action has to demonstrate the chemical and biological stability of the EVERGREEN crude polyphenolic extracts and of their fractions, as well as the influence of several extraction parameters on these values.
The optimization of sample preparation/extraction is a challenging step, not only to maximize yields, but in particular to maintain as optimal both the chemical and biological stability of the beneficial profile of the bioactive phytochemicals of interest. Several extraction parameters, such as solvent composition, extraction time, particle size, flush volume, temperature, and pressure, play an essential role in this critical step. Moreover, accurate quantification and evaluation analysis are needed to properly and correctly ascertain these parameters and to optimize the process. Chemical-based methods (*e.g.* HPLC/DAD/MS) are useful for a first screening of a large number of samples due to the speed of execution, and their low cost. Moreover these tests can be combined with other specific assays, in order to early exclude those products and/or fractions with neither an antioxidant nor a radical scavenger activity, which are at the basis of the biological properties of polyphenols. Here, by DISPAA-UNIFI, INSTM and CEBAS CSIC the antioxidant and antiradical activities were evaluated for each polyphenolic fraction of interest by *in vitro* tests using the Folin-Ciocalteu reagent and stable radical DPPH• (1,1-diphenyl-2-picrylhydrazyl), respectively. An in-depth antioxidant activity estimation with the popular ORAC test was also performed on selected and already marketed fractions (liquid and spray dried). The results of the Folin-Ciocalteu assay were expressed as total phenol content, measured as GAE (Gallic Acid Equivalents), which is considered to have an excellent correlation with the *in vitro* antioxidant activity, as confirmed by comparisons with other *in vitro* assays based on electron transfer reactions (*e.g.* FRAP, TEAC and ORAC). For the fractions under study, in view of the extraction method and the initial vegetal matrices, it can be assumed that there are no other compounds, such as proteins containing tyrosine or non-phenolic compounds, which interfere with the electron-transfer reaction between the samples under examination and the Folin-Ciocalteu reagent. The assay with DPPH• gives a measure of the antiradical activity of the samples, expressed as their EC50 (polyphenolic concentration inhibiting DPPH• activity to 50%). In the ORAC test, the antioxidant capacity is measured as Trolox Equivalents (TE) through the area under the curve (AUC) of the kinetic profiles of a target molecule (fluorescein) consumption, oxidized by free radicals produced by the free radical initiator AAPH (2,2’-azobis(2-amidinopropane) dihydrochloride) in the presence of the sample. The AUC value obtained is compared with that obtained with Trolox in order to evaluate the antioxidant activity.

Then, the chemical stability of the lab-scale extracts was also evaluated by monitoring by HPLC/DAD analysis the chemical composition of each sample stored at room temperature for 12 months. Moreover, the chemical stability of the EVERGREEN polyphenolic extracts to pH and temperature changes were also tested, using solutions at 0.1 g/l concentration). Changes were assessed by measuring water soluble C and N, which are the most labile component for both C and N. Tests were carried out by varying pH within the range 4-10, and by varying incubation temperature in the range 25°-50°C.
Tests concerning the stability of the bioactivity of EVERGREEN extracts and their fractions were carried out *in vitro* on *Psv* and *Psa* as model bacteria by DISPAA-UNIFI and CEBAS CSIC, and on *Meloidogyne* spp. for nematodes by INSTM, by using several preparations obtained at different times and by different batches of vegetable biomass.
In particular, on *Psv* and *Psa* was demonstrated the inhibitory activity of specific polyphenolic extracts and/or fractions by using a biotechnological tool already set up and optimised by DISPAA-UNIFI: *Psv* and *Psa* strains were transformed with a recombinant plasmid, called pT3GFP, carrying a reporter gene whose activity is directly correlated to the expression of their master pathogenicity/virulence system (Type Three Secretion System, TTSS), which in turn allows the synthesis of the "Green Fluorescent Protein" (GFP), and thus the emission of fluorescence, that is a signal easily detectable and quantitatively evaluable. The TTSS inhibition induced by different batches and preparations of the EVERGREEN extracts and fractions was evaluated, as indicative of their biological activity. At the same time, the impact of the EVERGREEN extracts and fractions on *in vitro* bacterial growth was also evaluated, by measuring "optical density" (OD) at 600 nm.
The detailed description of the EVERGREEN Action B3 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 3 Deliverable Action B3.
The results obtained demonstrated that chemical stability is strictly dependent on the dilution of the EVERGREEN extract, with concentrated and/or dry fractions highly stable up to 12 months at room temperature. In fact, a decrease in polyphenols content was occurring during the time in the EVERGREEN diluted fractions, and this was an important finding in the view to obtain stabilized and standardized solutions to be produced at industrial scale. As an example, in Figure 9 the results obtained with *C. sativa* diluted and concentrated extracts are reported: a clear decrease of castalagin and vescalagin was costantly shown, with an increase in related derivatives of hydrolysis (*i.e.* gallic acid, pedunculagin, castalin, vescalin).

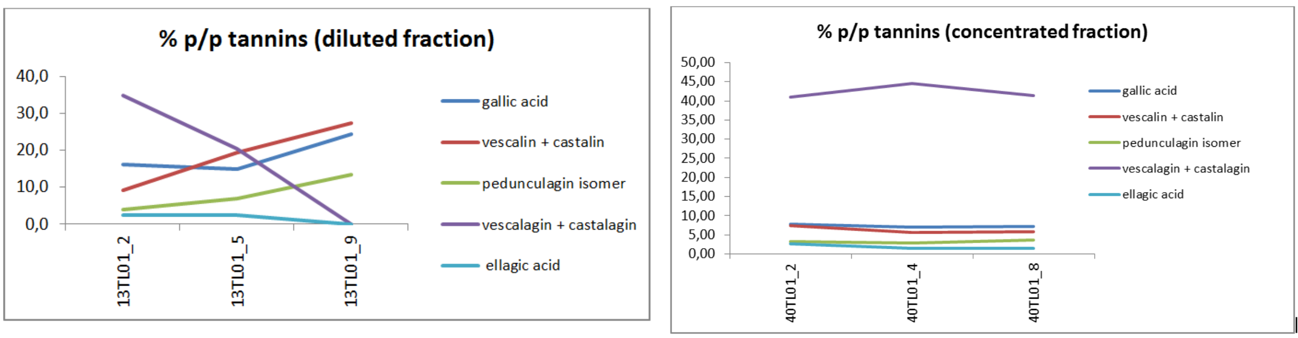


Fig. 9. Variations in polyphenolic composition of the diluted and the concentrated liquid Sweet Chestnut fractions up to 12 months from extraction.

The quantitative analysis carried out on the stability of antioxidant and radical scavenging activity of the EVERGREEN polyphenolic preparations confirmed the data from HPLC/DAD/MS analysis reported above. Moreover, the changes occurring on the EVERGREEN polyphenolic extracts/fractions treated at different pHs and temperatures are neglegible, and this further demonstrated the chemical stability of the polyphenols extracted in the project EVERGREEN (Table 2).

Polyphenol	Temp. °C	ppm Cs	ppm Ns	pH	ppm Cs	ppm Ns
TC	25	287,74	0	4	254,50	0
	30	287,16	0	5,5	233,30	0
	37	304,20	0	7	232,20	0
	45	300,78	0	8,5	232,55	0
	50	299,66	0	10	232,32	0
TCO	25	276,25	0	4	288,20	0
	30	277,14	0	5,5	292,40	0
	37	278,95	0	7	290,30	0
	45	287,53	0	8,5	291,98	0
	50	294,85	0	10	289,29	0
TCC	25	315,49	1,48	4	239,80	0,53
	30	336,38	1,40	5,5	240,50	0,40
	37	338,65	1,36	7	238,90	0,22
	45	347,65	1,33	8,5	239,52	0,31
	50	326,63	1,39	10	221,66	0,34
TAN	25	307,04	0	4	286,20	0
	30	310,77	0	5,5	288,50	0
	37	311,25	0	7	286,30	0
	45	319,18	0	8,5	289,80	0
	50	323,70	0	10	284,17	0
PV	25	289,90	0	4	318,40	0
	30	295,19	0	5,5	327,60	0
	37	296,33	0	7	338,00	0
	45	308,27	0	8,5	337,53	0
	50	309,06	0	10	339,69	0
EPV	25	372,73	2,41	4	344,50	2,24
	30	375,74	2,35	5,5	348,30	2,13
	37	357,01	2,64	7	353,80	1,79
	45	384,92	2,33	8,5	354,03	2,15
	50	385,36	2,33	10	352,74	2,26
PFV	25	241,24	0	4	210,90	0
	30	239,13	0	5,5	209,60	0
	37	238,11	0	7	209,30	0
	45	249,01	0	8,5	212,81	0
	50	247,41	0	10	213,45	0

Table 2. Variations occurring on C and N water soluble into the EVERGREEN extracts treated at different pHs and temperatures.

As far as the biological stability is concerned, a biotechnological tool already set up and optimized by DISPAA-UNIFI was adopted to monitor *in vitro* the anti-infective bioactivity of different batches of the EVERGREEN polyphenolic preparations. Representative strains from *Psv* and *Psa* (Psn23 and Psa21, respectively), transformed with the recombinant plasmid pT3GFP, were demonstrated to be inhibited in the activation of their T3SS by specific polyphenolic extracts and/or fractions, with no relevant differences with concentrated preparations mantained up to 12 months at room temperature. As an example, in Figure 10 data obtained on Psn23 are reported. Bacteria were grown for 16 or 22 h on MM, amended or not with the EVERGREEN polyphenolic extract. In this case Sweet Chestnut preparations have been used, named OP1, OP2 and OP3, corresponding to those previously reported in the paragraph about chemical analysis, and at concentration of 100 µM. For comparison and as a positive control, the antibiotic kanamicin was also used, at concentration of 50 mg/L: as expected, there was a null T3SS activity, but just as a consequence of the bacterial cell death. Therefore, as further positive control tryptophan was also used, at concentration of 50 and 500 µM, and a partial T3SS inhibition was obtained as expected. The results obtained using three different polyphenolic batches produced at different times were coherent and highly similar.

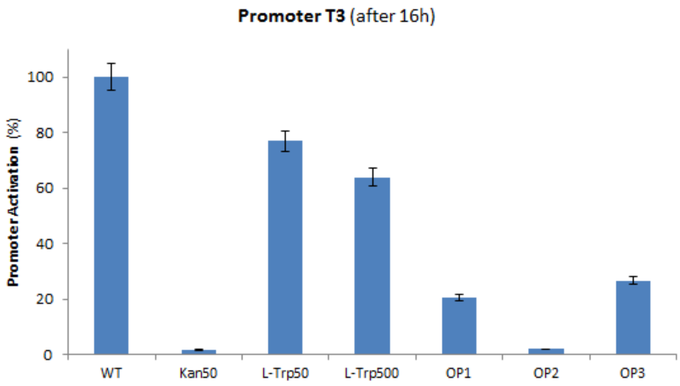


Figure 10. pT3 activity in *Psv* strain Psn23 following treatments with fractions from sweet chestnut (OP1, OP2, OP3), expressed as a percentage of the results obtained on the untreated samples (wt). For comparison known pT3 inhibitors were examined, specifically L-Trp and the antibiotic kanamicin.
The results shown are the average of three indipendent experiments carried out with three different polyphenolic batches produced at different times.

Problems encountered
No particular problems have been encountered during this Action B3, and this action was completed by 100%, as scheduled.

Verification of the expected results actually achieved
The detailed description of EVERGREEN Action B3 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 3 Deliverable Action B3.
The expected results were:
• Demonstration of the most performing solvents to be used for the recovery and the further *in vivo* application of the high quality polyphenolic molecules.
• Demonstration of the chemical and biological stability of the innovative high quality polyphenolic molecules.
• Demonstration of antibacterial activity of single compounds and of mixture of different standardized extracts, evaluating their synergistic effect, in comparison with synthetic traditional antibiotics.
• Demonstration of possible nematicide/nemastat action of the extracts under test.
• Demonstration of antioxidant and radical-scavenging activities of the high quality polyphenolic molecules.
• Demonstration of antioxidant and radical-scavenging synergistic effect of mixtures of different standardised extracts.
The activities carried out in the EVERGREEN Action B3 demonstrated the experimental “green chemistry” procedure using water and filtration as core steps for the standardised extraction of polyphenolic compounds and fractions from the plant species here used as a model, at laboratory scale. Moreover, when used as concentrated extracts, the EVERGREEN optimised polyphenolic preparations were demonstrated to be chemically stable, in their composition as well as in their antioxidant and radical-scavenging activities, even when subjected to pH and temperature changes.

Similarly, the stability of the antivirulence bioactivity of EVERGREEN extracts on plant pathogenic bacteria was also demonstrated, still related to concentrated fractions.

Perspectives for continuing the action after the end of the project

In the frame of Action B3, DISPAA-UNIFI, INSTM and CEBAS CSIC were asked to partecipate to a H2020 call (SFS-17-2017) concerning innovation in plant protection, with application of the EVERGREEN approach.

6.1.4. Action B.4 Demonstration of the biological activity of the high quality polyphenolic extracts recovered from not edible biomass and waste, against plant pathogenic bacteria and nematode, *in planta*

Starting date foreseen: 1st of April 2015
Actual start date: 1st of April 2015
End date foreseen: 31st of December 2015
Actual end date: 31st of December 2015

The results of the activities carried out in Action B.4 are defined in the following project Deliverable, foreseen at the end of the 15th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 4 Deliverable Action B4: Demonstration of the biological activity of the high quality polyphenolic extracts recovered from not edible biomass and waste, against plant pathogenic bacteria and nematode, *in planta*.

Activities carried out
This demonstration was delegated to DISPAA-UNIFI, INSTM and CEBAS CSIC, and was completed by 100%.
This Action has to demonstrate the biological *in planta* activity of the EVERGREEN polyphenolic extracts, from vegetable not edible biomass, in plant protection and against the phytopathogenic bacteria and nematodes here used as a model, by pathogenicity tests carried out at laboratory/pilot scale level on plants artificially inoculated.
The *in planta* anti-virulence and antibacterial activities have been assessed by comparing the results obtained on plants artificially inoculated with representative *Psv* and/or *Psa* strains, and treated at different times with the EVERGREEN high-quality polyphenolic extracts or their fractions. In particular, the appearance of microscopic and macroscopic symptoms was evaluated, together with *in planta* bacterial growth. Moreover, laboratory tests have been also carried out, to detect the suppression/inhibition of TTSS, the master pathogenicity system for bacteria, by using the biotechnological tool pT3GFP developed by DISPAA-UNIFI, for a GFP-based transcriptional screening relying on spectrofluorometric measures, as already described above in Action B3. Accordingly, TTSS inhibition by the EVERGREEN polyphenolic extracts was also evaluated by analysing the suppression of the Hypersensitive Response (HR) induced on Tobacco by *Psv* and/or *Psa21*.

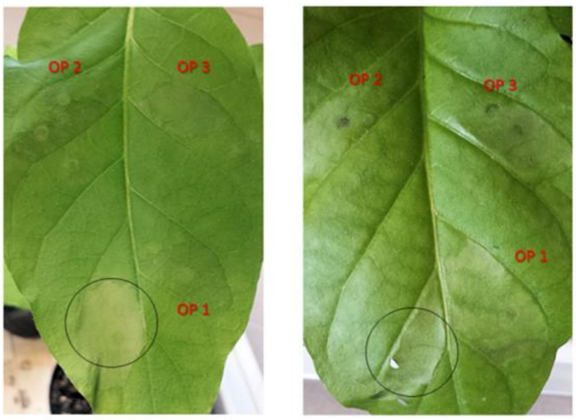


Fig. 11. HR on Tobacco at 48 hours post inoculation (hpi) challenged by *Psn23*, with or without treatment with the EVERGREEN polyphenolic extracts OP1, OP2 and OP3.

The detailed description of the EVERGREEN Action B4 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex X Deliverable Action B4.
As an example, here the inhibition by several EVERGREEN polyphenolic extracts and fractions of the HR induced by *Psn23* is reported in Figures 11 and 12.

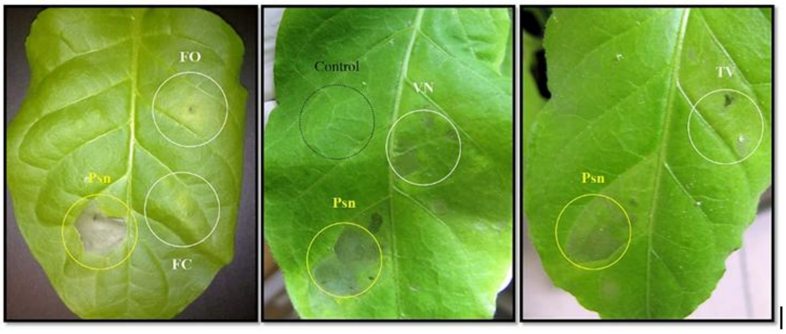


Fig. 12. HR on Tobacco at 48 hpi challenged by *Psn23* and co-infiltration with EVERGREEN extracts from olive leaves (FO), artichoke leaves (FC), grape seeds (VN), or green tea (TV) (white rings). As control, sterile physiological solution was used (black ring).

These extracts were also demonstrated able to decrease symptoms appearance, as here shown for *Psn* on Oleander cuttings as an example, although to a different extent and with catechins/epicathechins as the most active fractions, as confirmed by epigallocatechin gallate from green tea, which was used as control for its known bioactivity on human pathogenic bacteria (Figure 13).

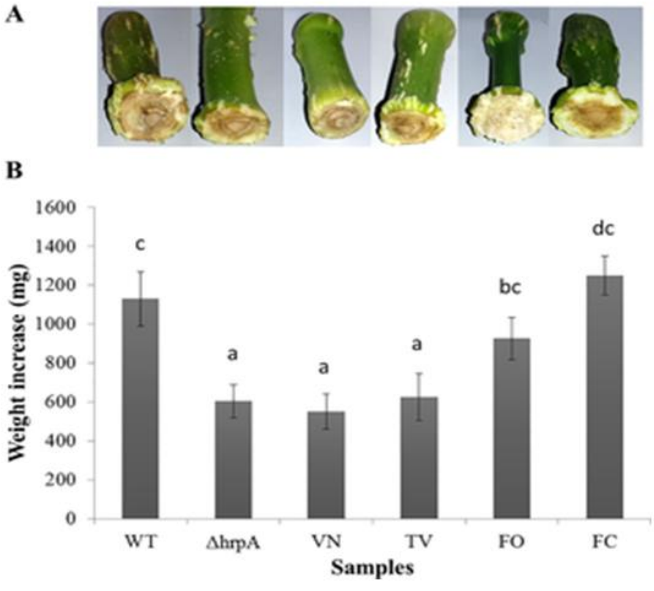


Fig. 13. Pathogenicity test with *Psn23* on Oleander explants, following treatment with the EVERGREEN polyphenolic extracts VN, TV, FO, FC extracts (100µM). As negative control the no pathogenic mutant Δ*hrpA* was used. (A) Development of hyperplastic knots at 21 dpi (days post inoculation) with (from left to right); *Psn23*, Δ*hrpA, Psn23*+VN, *Psn23*+TV, *Psn23*+FO, *Psn23*+FC. The symptoms are detectable as swelling at the inoculated end of oleander explants. (B) Normalized weight increase of oleander explants at 21 dpi inoculated with (from left to right): *Psn23*, Δ*hrpA, Psn23*+VN, *Psn23*+TV, *Psn23*+FO, *Psn23*+FC. Values are means ±SD of nine replicates for each treatment. Different letters indicate significant differences among means at $P < 0.05$, according to Tukey's test.

Moreover, these extracts caused a decrease of bacterial growth *in planta* (Fig. 14) but also a strong inhibition of TTSS (Table 3), as already reported for their *in vitro* bioactivity in Action B3, to confirm their anti-virulence and anti-bacterial properties.

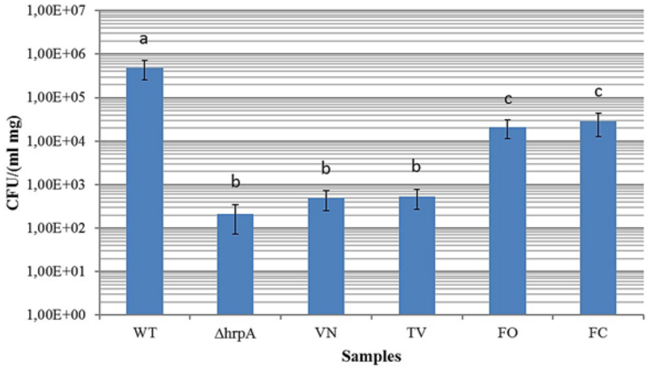


Fig. 14. ***In planta*** **bacterial growth at 21 days post inoculation (dpi) for** ***Psn23*** **following treatments with polyphenolic extracts VN, TV, FO or FC. Values are means ± SD of nine replicates for each treatment. Different letters indicate significant differences among means at** ***P*** **< 0.05, according to Tukey's test.**

Extract	Vegetable matrix/main molecule	Bacterial growth[§]	*hrpA* promoter[§]
VN	**Grape seeds**	**1.05 ± 0.30[a]**	**0.52 ± 0.11[ab]**
	Catechin	1.02 ± 0.22[a]	1.08 ± 0.11[a]
	Epicathechin	0.99 ± 0.19[a]	1.12 ± 0.13[a]
TV	**Green tea leaves**	**1.01 ± 0.16[a]**	**0.46 ± 0.13[b]**
	Epigallocatechin gallate	1.00 ± 0.17[a]	0.59 ± 0.24[ab]
FO	**Olive leaves**	**1.33 ± 0.16[a]**	**0.75 ± 0.18[a]**
	Oleuropein	1.00 ± 0.15[a]	1.24 ± 0.18[a]
	Hydroxytyrosol	0.98 ± 0.18[a]	0.49 ± 0.17[b]
	Luteolin 7-*O*-glucoside	1.32 ± 0.12[a]	1.18 ± 0.10[a]
FC	**Artichoke leaves**	**1.45 ± 0.18[a]**	**0.81 ± 0.17[a]**
	Caffeic acid	1.16 ± 0.13[a]	0.99 ± 0.10[a]
	Chlorogenic acid	1.13 ± 0.15[a]	1.02 ± 0.12[a]
	Cynarine	1.22 ± 0.10[a]	1.10 ± 0.32[a]
	Kanamycin[^]	**0.46 ± 0.18[b]**	**0.21 ± 0.18[b]**
	p-Coumaric acid[^]	**0.92 ± 0.15[a]**	**1.03 ± 0.22[a]**

Table 3. ***In planta*** **bacterial growth and TTSS inhibition for** ***Psn23*** **on Oleander cuttings, following treatments with polyphenolic extracts VN, TV, FO or FC and their main constituents. § OD_{600} was recorded after 24h and data are calculated as GFP Abs (Ex. 485nm; Em, 535 nm)/ Abs $_{(600nm)}$ ± SD, and as normalized fold versus untreated bacterial cultures. ^ Kanamycin and p-coumaric acis (PCA) were used as negative and positive control, respectively. Common letters in correspondence of each chemica compound indicate differences not statistically significant at P< 0.05 according to Tukey's test. Values are means ± SD of nine replicates for each treatment, and for three indipende runs.**

Experiments carried out with potted plants showed differences between the effects obtainde on Olive and Kiwifruit with the different EVERGREEN compounds and fractions. In particular, polyphenol extracts from grapevine (*e.g.* EPV, PV and PVF) have the lowest protective effect, while polyphenols derived from sweet chestnut (*e.g.* TC, TCO, TCC, and TAN) were demonstrated more effective (Figures 15 and 16).
The chlorophyll content determined in leaves with inoculation of pathogenic bacteria are lower than in control plants, clearly indicating the stress of such plants. Such stress is manifested to a lesser extent when polyphenols are introduced into the soil along with the bacteria.

Fig. 15. Pathogenicity test with *Psa21* on Kiwifruit potted plants, following treatment with the EVERGREEN polyphenolic extracts from grapevine and sweet chestnut.

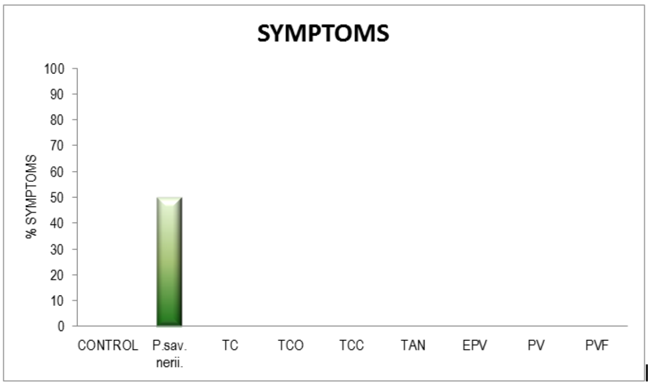


Fig. 16. Data from pathogenicity test with *Psn23* on Olive potted plants, following treatment with the EVERGREEN polyphenolic extracts from grapevine and sweet chestnut.

The biological activity of the EVERGREEN polyphenolic extracts against nematodes were carried out by INSTM and CEBAS CSIC on laboratory potted plants, in Italy and Spain, respectively. As controls, no treatment, just nematode and traditional nematicides were used (*e.g.* Mocap, Etoprofos, and Neemazal) (Figures 17, 18 and 19). As already assessed in B1 Action, the effectiveness of traditional nematicides was confirmed. Highly effective were also demonstrated the EVERGREEN polyphenols from sweet chestnut (*e.g.* TC and TCO). The positive effect in controlling nematode infection and to promote plant growth (epigeal and hypogeal parts) was also demonstrated (Table 4, and Figures 20 and 21).

Fig. 17. Inoculation of *M. incognita* on Tobacco potted plants, and treatment with several EVERGREEN polyphenolic extracts.

Fig. 18. Results of *M. incognita* inoculation on Tobacco potted plants, in presence or not of treatments with several EVERGREEN polyphenolic extracts.

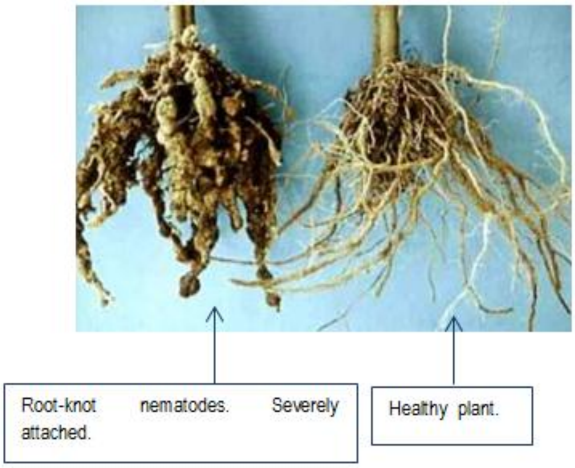


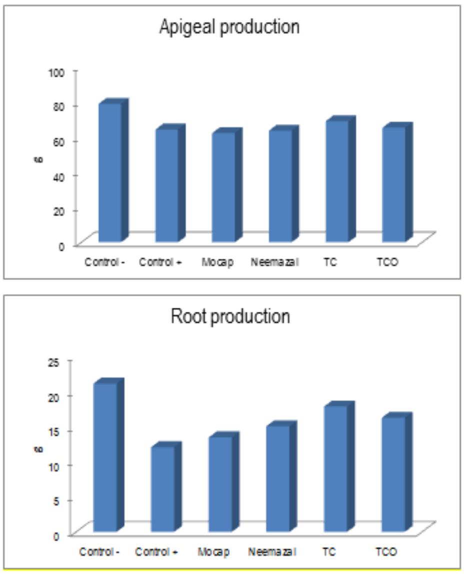
Fig. 19. Effects of *M. incognita* inoculation on Tobacco roots.

Treatm.No.	Nematode count No/200	TMDr (g/cm^3)	Fine roots (%)	Barker grading[a]		DM epigeal yield (g/pot)	
	40 DAT	40 DAT	40 DAT	20 DAT	40 DAT	20 DAT	40 DAT
Non infested control	NI	0.20 a	73.5 a	NI	NI	8.5 a	12.3 ab
Infested control	845 a	0.08 c	53.0 b	2.9 a	3.9 a	3.8 c	5.1 d
Etoprofos	102 c	0.14 b	67.5 a	0.2 b	1.6 b	8.0 ab	9.8 c
Azadirachtine	154 c	0.16 b	64.0 b	0.4 b	1.3 b	7.4 b	10.4 bc
CHT	486 b	0.21 a	76.0 a	0.6 b	1.0 b	9.2 a	12.7 a

[a] 0=0-10%; 1=11-20%; 2=21-50%; 3=51-80%; 4=81-90%; 5=91-100% [11]
Means within a column followed by the same letters are not significantly different (P = 0.05).

Tab. 4. Nematode root gall index and plant epigeal DM determinations at 20 and 40 DAT, on potted Tobacco plants. (Soil for nematode count, Tissue Mass Density of roots (TMDr), and Fine (<0.5 mm ø). Roots percentage was sampled at 40 DAT).

Fig. 20. Effects of treatments with the EVERGREEN polyphenols on the growth of Tobacco potted plants, after *M. incognita* inoculation.



	Apigeal	Root
Control -	79,0 b	21,2 c
Control +	64,3 a	12,1 a
Mocap	62,3 a	13,5 ab
Neemazal	63,7 a	15,1 ab
TC	69,1 ab	17,9 bc
TCO	65,4 a	16,3 ab

Fig. 21. Effects of treatments with the EVERGREEN polyphenols on the growth of aerial and epigeal parts of Tobacco potted plants after *M. incognita* inoculation.

Problems encountered

No particular problems have been encountered during this Action B4, and this action was completed by 100%, as scheduled.

Verification of the expected results actually achieved

The detailed description of EVERGREEN Action B4 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 4 Deliverable Action B4.

The expected results were:

• Demonstration of the most performing combinations of the high-quality polyphenolic-based molecules to be used for the *in planta* application of these bioactive compounds.

• Demonstration of the chemical and biological in planta stability of the innovative high-quality polyphenolic based preparations.

The activities carried out in the EVERGREEN Action B4 demonstrated the effectiveness *in planta* of the EVERGREEN polyphenol extracts and of their fractions against phytopathogenc bacteria and nematodes, both by traditional pathogenicity assays and molecular tests. The EVERGREEN extracts, all solubilised in water, were differently effective on the different pathosystems, but constantly they caused a decrease in symptoms appearance and infection, as well as sometimes they promote a more vigorous plant growth, as occurring with the EVERGREEN polyphenols from sweet chestnut. These results of these tests, carried out in a temporal range of about 6 months, further confirmed the chemical stability of the EVERGREEN formulations, in particular where extracts having antioxidant properties were combined with those have much higher antivirulence properties (*e.g.* olive extracts and chestnut tannins).

Perspectives for continuing the action after the end of the project

In the frame of Action B4, DISPAA-UNIFI, INSTM and CEBAS CSIC were asked to partecipate to a H2020 call (SFS-17-2017) concerning innovation in plant protection, with application of the EVERGREEN approach.

6.1.5. Action B.5 Demonstration of Kilo-scale extraction of the high quality poly-phenolic bioactive molecules recovered from vegetable not edible biomass and waste.

Starting date foreseen: 1st of July 2015
Actual start date: 1st of July 2015
End date foreseen: 31st of March 2016
Actual end date: 31st of March 2016

The results of the activities carried out in Action B.5 are defined in the following project Deliverable, foreseen at the end of the 18th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 5 Deliverable Action B5: Demonstration of Kilo-scale extraction of the high quality poly-phenolic bioactive molecules recovered from vegetable not edible biomass and waste.

Activities carried out
This demonstration was delegated to DISPAA-UNIFI, INSTM and CEBAS CSIC, and was completed by 100%.
This Action has to demonstrate the effectiveness of newly developed technology for large-scale industrial recovery of high quality and standardised polyphenolic bioactive preparations from agricultural vegetable not edible biomass, to be used in plant protection.
Polyphenolic secondary metabolites, found abundantly in plants and their residues, are mainly involved in various mechanisms for protecting plants against pathogens, fungi, insects and herbivorous animals. When extracted from plants they are known for many specific biological properties, such as antioxidant, radical scavenging and antimicrobial agents, and more recently also as anti-inflammatory, antitumoral, cholesterol-lowering, antiviral, and nematostatic compounds. Unfortunately, polyphenols sometimes are difficult to study because, as occurring in particular with hydrolysable tannins, they can have complex and unstable chemical structures and properties. This low stability can affect the analytical phase, but even more the development of extraction processes suitable for the standardization of their products. The use of modern high performance chromatography tools (*e.g*. HPLC), associated with DAD-MS and DAD-MS-MS detectors, has recently allowed to carry out more detailed analysis during these extraction processes. In particular they are essential for in-depth studies on those fractions obtained from the total extract which need to be isolated and stabilized, because preliminarily identified as the most promising for their biological activity.
In this B5 Action, Kilo-scale extraction of the EVERGREEN high quality polyphenolic fractions/molecules was optimised and demonstrated on biomass from Sweet Chestnut, Olive, Artichoke and *Vitis* spp. For any of the vegetable extracts here obtained, the fractions were always characterised and quantified by HPLC/DAD-ESI/MS. The detailed description of the EVERGREEN Action B5 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 5 Deliverable Action B5.
Briefly, *C. sativa* bark and wood residues were subjected to a hot water-extraction process coupled with a membrane separation technology system, by using an industrial plant located in Radicofani, Siena (Mauro Saviola Group company). The use of only water as solvent makes ecologically and economically sustainable the described extraction and purification method, since the industrial processes are usually based on purification methods by extraction with organic solvents. The main operating steps are shown in Fig. 22, where the fractions obtained are numbered as 1) filtered tannin broths, 2) permeate from nanofiltration step-1, 3) concentrate from nanofiltration step-1, 4) concentrate from nanofiltration step-1 and after cooling, 5) permeate from nanofiltration step-2, 6)

concentrate from nanofiltration step-2, 7) osmosis permeate, 8) osmosis concentrate, 9) settled fraction from clarification step, and 10) spray-dried obtained from fraction 6.

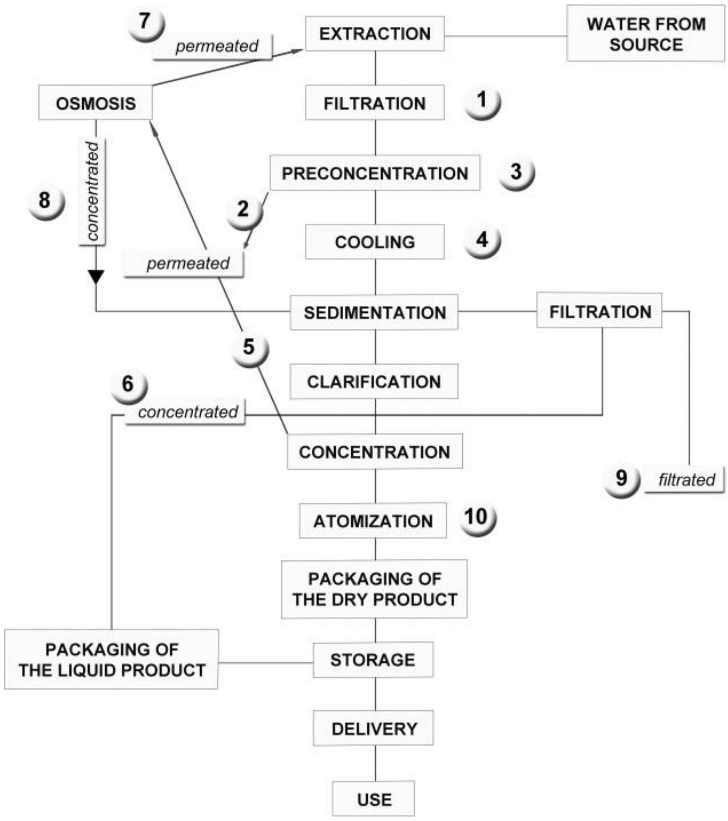


Fig. 22. Operating diagram for extraction and fractionation processes of the EVERGREEN Sweet Chestnut polyphenols.

In Table 5, the quali-quantitative composition of *C. sativa* bark extract from the EVERGREEN pilot plant analysis was reported, as assessed by HPLC/DAD.

Bark extract from pilot plant	**mg/mL**
Gallotannins*	0.024
Ellagitannins with ellagic acid units**	0.014
Ellagitannins with HHDP- or NHTP- units**	0.014
Procyanidins***	0.036
Ellagic acid	0.032
Total tannins	**0.118**

Table 5. Quali-quantitative analysis HPLC/DAD of *C. sativa* bark extract from the EVERGREEN pilot plant. *Calibrated as gallic acid; ** Calibrated as ellagic acid; * Calibrated as catechin.**

The polyphenols from *O. europaea* and Artichoke biomass (mainly leaves) were obtained by a water extraction followed by selective fractionation in five steps, as shown in Figure 23. These steps are i) physicochemical pre-treatment with enzymes and acidifying substances (pre-filtration); ii) microfiltration (MF); iii) ultrafiltration (UF); iv) nanofiltration (NF) and v) reverse osmosis (RO). To this aim, free support was obtained by the industrial plants VitaSafer (Montecatini, PT) and Pollipoli srl (Fasano, BA), respectively. The kilo-scale green-extraction of Olive and Artichoke biomass were performed in a Rapid Extractor Timatic series, using a solid-liquid extraction technology. The extraction was performed with water, in a stainless steel basket at a temperature of 60°C. The separation process was based on physical technologies, in particular using tubular ceramic membranes in titanium oxide and spiral wound module membranes in polyethersulfone (PES).

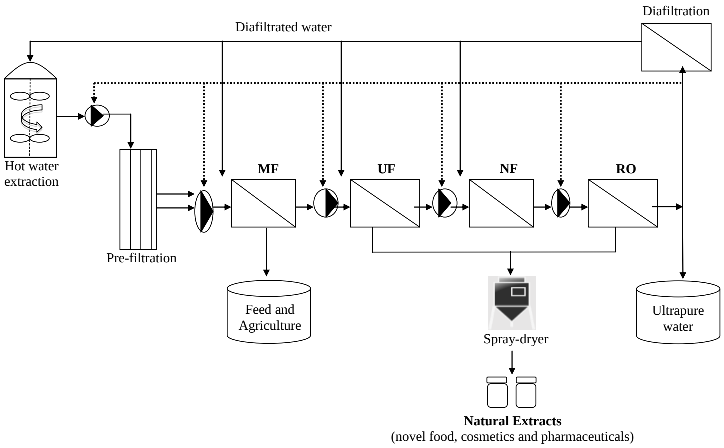


Fig. 23. Operating diagram for extraction and fractionation processes of the EVERGREEN polyphenols from Olive and Artichoke.

In Tables 6 and 7, the HPLC/DAD quali-quantitative analyses of Olive and Artichoke extracts and their bioactive fractions, obtained from the EVERGREEN pilot plants, were reported.

	PHENOLEA S mg/L	PHENOLEA F mg/L	PHENOLEA OH-TYR mg/L	PHENOLEA RED mg/L	PHENOLEA S SPRAY DRIED mg/g	PHENOLEA F SPRAY DRIED mg/g
Hydroxytyrosol der.	4970.28	5566.87	28846.90	6783.22	15.30	23.57
Secoiridoid der.	5320.60	34536.40	0	0	33.33	89.99
Elenolic acid der.	1729.83	5201.12	2248.80	1458.75	12.46	17.81
Hydroxycinnamic der.	1041.90	1237.20	424.16	996.90	4.45	6.14
Flavonoids	705.98	579.57	0	72.56	3.83	5.53
Coumarins	73.60	149.80	0	0	0.22	0.30
Lignans	traces	2355.94	0	0	4.34	7.92
TOTAL POLYPHENOLS	**13842.19**	**49626.90**	**31519.86**	**9311.43**	**73.93**	**151.26**

Table 6. Quali-quantitative analysis HPLC/DAD of Olive extract from the EVERGREEN pilot plant.

	CUF Cynara GL mg/L	CRO Cynara GL mg/L	Cynara GL Soft extract mg/g	Cynara GL Spray Dried mg/g
MCC	1.07 ± 0.58	65.19 ± 13.28	6.61± 1.34	14.23± 0.48
DCC	2.81 ± 1.19	3.96 ± 5.60	7.64± 0.69	7.63± 0.20
Chlorogenic acid	2.04 ± 0.47	34.00 ± 7.38	11.93± 1.72	12.36± 0.03
Cynarin	0.50 ± 0.43	28.94 ± 14.89	1.62± 0.01	4.41± 0.34
Flavonols	0.23 ± 0.06	10.11 ± 5.39	1.09± 0.27	3.48± 0.56
Total Polyphenols	**6.57 ± 1.92**	**142.21 ± 9.58**	**28.90± 4.02**	**42.10± 0.42**

Table 7. Quali-quantitative analysis HPLC/DAD of Artichoke extract from the EVERGREEN pilot plant.

As far as *V. vinifera* is concerned, its polyphenols consist mainly of anthocyanins, catechins, flavonols and stilbenes, which here were obtained from grape seeds and pomaces, obtained after wine production and mechanical extraction of oil, notably without to use any organic solvent (from Signae-Cesarini, Bastardo, PG; Cantina Cesarini Sartori, PG). Grape seeds were dried and used for a mechanical, solvent free, extraction of oil, containing 70% linoleic acid, 16% oleic acid, 7% palmitic acid, 4% stearic acid and small portion of tocopherols and vitamins. Then, the extruded material obtained by mechanical pressing for the extraction of oil was the grape seeds by-product useful for the extraction of the EVERGREEN polyphenols (Figure 24), with a pilot "green" process carried out for free at Bracco S.r.l. (BG).

Fig. 24. Grape seed residue post-mechanical extraction of oil, used for "green" extraction of the EVERGREEN polyphenols.

As shown in Table 8, the aqueous "green" extraction from grape seeds allowed for a lower recovery (46.5%) than that which is possible to obtain using the acidified mixture of EtOH/H_2O, given the chemical nature of grape chemical compounds such as catechins. However, as assessed in other Actions of this project, it was verified the conservation of the biological properties of interest, by specific tests carried out *in vitro* and *in vivo*.

	RPE H	RPE A
Gallic acid	85.1	973.1
Catechin dimer B3	89.7	90.1
Catechin	123.0	78.9
Catechin trimer	-	-
Catechin dimer B6	51.4	59.5
Catechin dimer B2	100.2	124.1
Epicatechin	152.6	101.6
Catechin trimer	82.6	0.0
ECG dimers	89.1	64.1
Catechin oligomers quantified as tetramers	65.0	36.2
ECG dimers	70.8	32.2
catechin/epicatechin trimers digallated	63.9	34.2
catechin/epicatechin trimers digallated	149.5	199.4
TOTAL	**72.1**	**46.5**

Table 8. Recovery (%) of single tannins and total tannin compounds from the tested grape seeds after mechanical oil extraction and subsequent residue extraction with hydroalcoholic (RPE H) or aqueous (RPE A) solvent. The percentages are expressed with respect to the tannins amounts found in the seeds pre-oil extraction.

According to analysis from extraction pilot plants and to data about bioactivity of these polyphenolic fractions, four different formulations were developed and optimised, using tannins /fraction 6) from Sweet Chestnut (TC), Soft Extract from Olea (O), and grape seeds by-products (V), as following:
1) Concentrated formula 1:10, water based, TC 2%, O,1% (1L)
2) Concentrated formula 1:10, water based, TC 1,5%, O,1%, V 0,3% (2L)
3) Gel formula, ready to use, TC 0,2%, O 0,1% + hydrophilic polymer (1G)
4) Gel formula, ready to use, TC 0,15%, O 0,1%, V 0,03% + hydrophilic polymer (2G)
Samples for these formulation tests had small volume (*i.e.* two litres formulas), up to 75 litres per formula.
On these formulations, the chemical stability was evaluated by an “accelerated aging” protocol, as reported in the International Conference on Harmonisation (ICH) guidelines. In particular, these formulations were keept at 40±2°C, then 5.0 mL of each sample were taken at different times [T0, every 7 days for the first month in oven, then every 14 days (T1-T6) and every month (T7-T9)]. Each sample was then spettrophotometrically evaluated by Folin-Ciocalteu assay, to monitor its phenolic content and antioxidant activity. The results of Folin-Ciocalteu assay during the accelerated aging test are reported in Table 9, which show that both the concentrated formulations are stable, with regards to phenolic content and antioxidant activity, within the first 3 months at 40°C.

	mg/mL GAE									
	T0	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**
Form1	154.56	149.64	156.67	151.87	154.71	153.26	155.60	154.12	152.32	148.59
Form2	10.286	11.276	11.545	11.162	11.160	11.580	11.161	11.151	11.285	11.52

Table 9. Data from Folin-Ciocalteu assay on the concentrated EVERGREEN formulations 1 and 2, as such (T0) and during accelerated aging at 40°C (T1-T7). The results are expressed as mg/mL GAE (Gallic Acid Equivalents). Measures: T0 nov 27, 2015; T1 dec 4, 2015; T2 dec 11, 2015; T3 dec 18, 2015; T4 dec 23, 2015; T5 jan 7, 2016; T6 jan 14, 2016; T7 feb 15, 2016: T8 mar 15, 2016; T9 apr 15, 2016.

Problems encountered

No particular problems have been encountered during this Action B5, and this action was completed by 100%, as scheduled.

Verification of the expected results actually achieved
The detailed description of EVERGREEN Action B5 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 5 Deliverable Action B5.
The expected results were:
• Demonstration of the most performing conditions for the pilot scale production of high quality and standardised polyphenolic bioactive preparations from agricultural vegetable not edible biomass and waste.
• Optimization of the process for the pilot scale recovery of high quality and standardised polyphenolic bioactivemolecules.
The activities carried out in the EVERGREEN Action B5 demonstrated the effectiveness of the Kilo-scale extraction processes developed for the EVERGREEN polyphenol extracts and of their fractions. These pilot systems were all based on environmentally friendly approaches and technologies, such as the use of water as only solvent and filtration steps. Four formulations were also set up, according to those fractions demonstrated to be more active in plant protection, and on these new formulations tests of accerated aging were carried out, demonstrating their chemical stability.

Perspectives for continuing the action after the end of the project
According to the results obtained in Action B5, DISPAA-UNIFI, INSTM and CEBAS CSIC were asked to partecipate to a H2020 call (SFS-17-2017) concerning instruments to be developed to control emerging diseases of plants, also by using the EVERGREEN extracts and fractions.

6.1.6. Action B.6 Demonstration of the null toxicity profile of the high quality poly-phenolic bioactive molecules recovered from vegetable not edible biomass and waste, on model organisms and microorganisms.

Starting date foreseen: 1st of April 2015
Actual start date: 1st of April 2015
End date foreseen: 31st of March 2016
Actual end date: 31st of March 2016

The results of the activities carried out in Action B.6 are defined in the following project Deliverable, foreseen at the end of the 18th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 6 Deliverable Action B6: Demonstration of the null toxicity profile of the high quality poly-phenolic bioactive molecules recovered from vegetable not edible biomass and waste, on model organisms and microorganisms.

Activities carried out
This demonstration was delegated to DISPAA-UNIFI and CEBAS CSIC, and was completed by 100%.
This Action has to demonstrate the absence of any toxicity of the EVERGREEN high quality polyphenolic extracts and fractions, by applying several *in vitro* tests, already validated and accepted by international regulatory authorities. In fact, despite the highly amount of favourable data supporting the virtues of these molecules, it was essential to test the toxicological profile of the EVERGREEN polyphenolic extracts, because it is mandatory to know what is going to occur when administered at higher doses than those present in nature, and also following its accumulation.
The detailed description of the EVERGREEN Action B6 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 6 Deliverable Action B6.
The experimental scheme was succesfully optimised on *Daphnia magna* and *Artemia salina* (Figure 25), by using Epigallocatechin gallate (EGCG), which is the main (up to 95%) polyphenolic component of green tea. On the same organisms no toxicity was revealed up to 2,000 µM for each EVERGREEN polyphenol extract tested (Tables 10A and 10B).

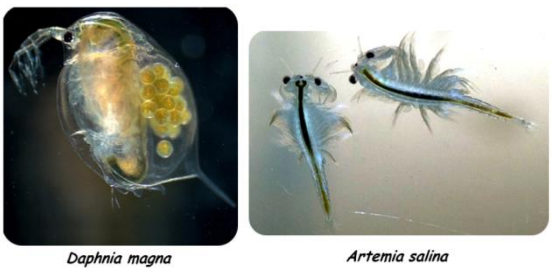


Fig. 25. *Daphnia magna* and *Artemia salina*, internationally and officially used eukariotic organisms for *in vitro* toxicity tests.

Extract	EC50 24h (µM)	EC50 48h (µM)
Chestnut tannins OP2	25.6	25.6
EGCG	25.6	25.6
[a]$K_2Cr_2O_7$	4,55	4,55

(a) Toxicity positive control

Table 10A. *Daphnia magna* acute toxicity *in vitro* test, with EGCG and OP2 tannins.

Extract	EC50 96h (µM)
Chestnut tannins OP2	26.2
EGCG	26.2

Table 10B. *A. salina* acute toxicity *in vitro* test with EGCG and OP2 tannins.

Then, experiments were also carried out on several model bacteria, such as *E. coli*, *Bacillus cereus* and *Staphylococcus aureus*, and fungi and yeasts (*Candida albicans* and *Saccaromyces cerevisiae*), and the EVERGREEN showed heavy antibiotic activity far 1,000 µM (Figures 26 and 27).

Fig. 26. Toxicity test on *E. coli*, performed on solid LB and KB media, ammended with the EVERGREEN polyphenols (here showed from 10 µM to 2 mM), and using as positive control copper sulphate (10 µM to 6 mM).

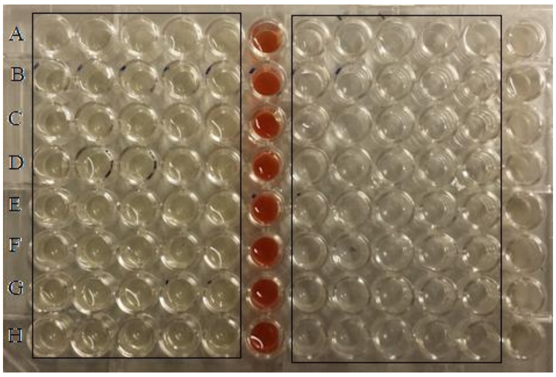


Fig. 27. Toxicity test carried out on model bacteria and yeasts, by using Microliter 96 well plates amended with the EVERGREEN polyphenols (10 µM to 2 mM).

An acute toxicity test was also carried out by using luminescent bacteria (Microtox®). Here, the inhibition of the luminescence of *Vibrio fischeri* was measured using a luminometer after adding extracts of the samples to be assayed. Microtox® test uses a suspension of the luminescent bacterium *V. fischeri* as bioassay organism for measuring acute toxicity in aqueous extracts. *V. fischeri* are non-pathogenic, marine, bacteria that emit light as a natural part of their metabolism. When exposed to a toxic substance, the respiratory process of the bacteria is disrupted, reducing light output. *V. fischeri* has been demonstrated to be high sensitive across a wide variety of toxic substances, and a strong response to toxicity is observed as a change in luminescence, which is a by-product of cellular respiration. This change can be used to calculate a percent inhibition of *V. fischeri* natural luminescence, that directly correlates to toxicity of the compound tested. According to the official guidelines, a substance is to be considered toxic when giving EC50 (15 minutes at

15ºC) under 3,000 mg/L by using Microtox®. Ecotoxicity assays were here carried out with the EVERGREEN polyphenols from sweet chestnut, olive, artichoke and grape at two different concentrations (1g/L and 0.1g/L). As shown in Table 11, results from Microtox® test indicate that no toxic effects were produced by the following EVERGREEN polyphenols: TC (chestnut), TCO (chestnut + olive), TCC (chestnut + artichoke), PFV (grape leaves powder), EPV (grape seeds extracts) and PV (grape seeds powder).

Reference	Ecotoxicity (mg/l)
TC 1g/l	351110
	292600
TC 0,1 g/l	6990000
	5120000
TCO 1g/l	343350
	269280
TCO 0,1g/l	4720000
	5140000
TCC 1g/l	431120
	624830
TCC 0,1g/l	3790000
	5820000
TAN 1g/l	366810
	349200
TAN 0,1g/l	5290000
	6010000
PV 1g/l	239570
	249100
PV 0,1g/l	447948110
	316232270
EPV 1g/l	83990
	80680
EPV 0,1g/l	295670
	406140
PFV 1g/l	5190000
	3800000
PFV 0,1g/l	7480000
	8380000

Table 11. Toxicity of the EVERGREEN polyphenols evaluated by Microtox® test. TC (chestnut), TCO (chestnut+olive), TCC (chestnut), TAN (chestnut+artichoke), PFV (grape leaves powder), EPV (grape seeds extract), PV (grape seeds powder).

In view of their application into the fields for plant protection, the EVERGREEN polyphenols were also tested to ascertain if they had some phytotoxic activity, that could make them inadvisable for agronomic use. To this aim, germination tests using seeds of *Lepidium sativum* seeds were carried out, in laboratory growth chambers with control of temperature, humidity, and light conditions (Figure 28). The potential phytotoxicity was established by seed germination test on filter paper. After germination, the number of germinated seeds was recorded and the length of the seedling roots and shoots was measured. A direct inhibiroty effect on seeds germination was observed with some polyphenols, such as TAN, TCC, PV, when applied on filter paper (Figure 29), while this effect was not evident when polyphenols were added to microcosms as described below.

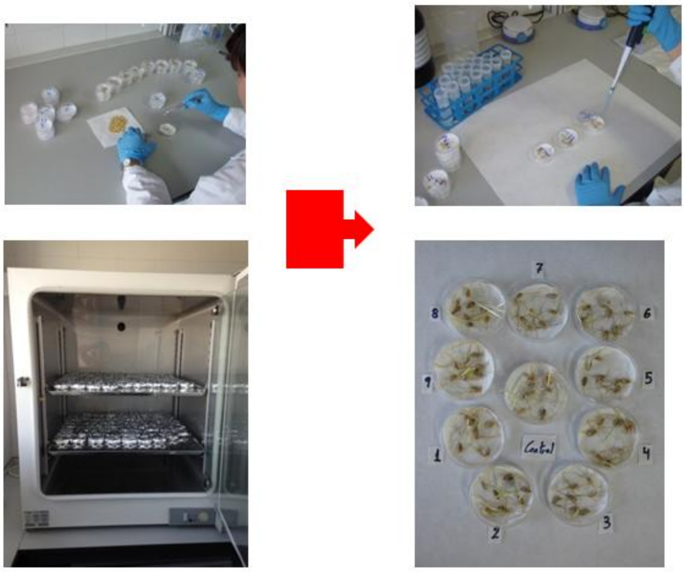


Fig. 28. Procedures for phytotoxicity tests on the EVERGREEN polyphenols.

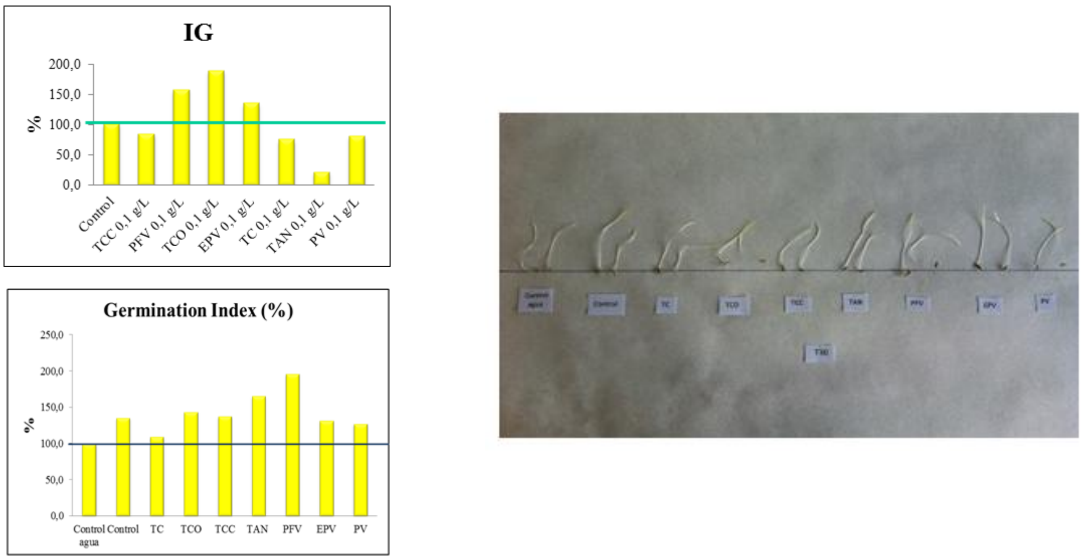


Fig. 29. Data from phytotoxicity test on the EVERGREEN polyphenols.

To this aim, more tests were then carried out on rye grass and on Tobacco.
Concerning rye grass, the results obtained are shown in Figure 30 and Table 12. A negative effect was found at 0.1 g/L, which was less pronounced when polyphenols extracted from sweet chestnut were used. At 2 g/L of polyphenols, the extract PV from grape (PV) caused a plant development

similar to that of the the control plant, without any phytotoxic effect which conversely was found with the pther polyphenols.

Fig. 30. Phytotoxicity test on rye grass in presence of the EVERGREEN polyphenols and grown in microcosms.

Dry weight	T30	Dry weight	T30
Control	0,299a	Control	0,299cd
TC	0,267cd	TC	0,226ab
TCO	0,170ab	TCO	0,177a
TCC	0,152a	TCC	0,219a
TAN	0,1793ab	TAN	$0,213^{a}$
PFV	0,134a	PFV	0,229ab
EPV	0,183ab	EPV	0,256abc
PV	0,220bc	PV	0,307c

Table 12. Data from phytotoxicity test on rye grass, in presence of the EVERGREEN polyphenols and grown in microcosms for 30 days. Mean dry weight values were evaluated at 0.1 (left) and 2 g/L (right) polyphenols

Therefore, tannins from sweet chestnut and polyphenols from grape were then tested also on Tobacco, grown in pot in controlled chamber and in hydroponic culture, with the polyphenols tested added to the soils or to the nutritive solution (0.1 and 2.0 g/L) (Figure 31).

Fig. 31. Phytotoxicity test on Tobacco, potteed plants and hydroponic culture, treated with the EVERGREEN polyphenols from sweet chastnut and grape.

	FRESH WEIGHT (g)	DRY WEIGHT (g)		FRESH WEIGHT (g)	DRY WEIGHT (g)
CONTROL	12,00 ab	1,21 a	**CONTROL**	40 a	2,35 a
TC 0,1 g/l	14,67 ab	1,50 a	**TC 0,1 g/l**	38 a	1,84 a
TC 2 g/l	10,67 a	0,96 a	**TC 2 g/l**	48 ab	2,52 a
TCO 0,1 g/l	14,67 ab	1,20 a	**TCO 0,1 g/l**	46,6 ab	2,44 a
TCO 2 g/l	18,00 b	1,43 a	**TCO 2 g/l**	74 b	4,45 b
EPV 0,1 g/l	16,00 ab	1,28 a	**EPV 0,1 g/l**	40,33 ab	2,06 a
EPV 2g/l	12,67 ab	1,18 a	**EPV 2g/l**	58 ab	2,91 a
Potted plants in growth chamber			**Plants in hydroponic culture**		

Table 13. Data from phytotoxicity test on rye grass, in presence of the EVERGREEN polyphenols and grown in microcosms for 30 days. Mean dry weight values were evaluated at 0.1 (left) and 2 g/L (right) polyphenols

Besides data referred to the increase in fresh and dry weight (Table 13), it was noticeable to observe that we tannins caused a biostimulating effect on root development, while the development of stem was favoured by TCO as well as by polyphenols from grape (*i.e.* EPV).

Problems encountered

No particular problems have been encountered during this Action B6, and this action was completed by 100%, as scheduled.

Verification of the expected results actually achieved

The detailed description of EVERGREEN Action B6 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 6 Deliverable Action B6.

The expected results were:

• Determination of toxicological profile of these high quality standardised polyphenolic preparations on microorganisms and organisms used as a model.

The activities carried out in the EVERGREEN Action B6 demonstrated the positive and beneficial profile of the EVERGREEN polyphenol extracts, of their fractions and even of their combinations. Bacterial and yeast model systems were used, but the phytotoxicity was also assessed on some plants such as Tobacco and rye grass, in view of the application of the EVERGREEN polyphenols into the field for plant protection. The data obtained confirmed the absence of any significant phytotoxic effect and revealed in some cases a biostimulant activity.

Perspectives for continuing the action after the end of the project

According to the results obtained in Action B6, DISPAA-UNIFI and CEBAS CSIC are developing formualtions active as biostimulant to be used in hydroponic culture, whose properties will be the subject of dissemination events they are going to organize.

6.1.7. Action B.7 Demonstration of the in vivo performances of the high quality poly-phenolic bioactive preparations, recovered from vegetable not edible biomass and waste, at pilot scale level in field screenings

Starting date foreseen: 1st July 2015
Actual start date: 1st July 2015
End date foreseen: 30th of September 2016
Actual end date: 30th of September 2016

The results of the activities carried out in Action B.7 are defined in the following project Deliverable, foreseen at the end of the 24th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 7 Deliverable Action B7: Demonstration of the in vivo performances of the high quality polyphenolic bioactive preparations, recovered from vegetable not edible biomass and waste, at pilot scale level in field screenings.

Activities carried out
This demonstration was delegated to DISPAA-UNIFI, INSTM and CEBAS CSIC, Mondo Verde and ASTRA, and was completed by 100%.
This multi-level Action B7 has to demonstrated the properties in plant protection of the EVERGREEN formulations at pilot and field scale. The detailed description of the EVERGREEN Action B7 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 7 Deliverable Action B7.
In 2015 several EVERGREEN polyphenol extracts were tested in pilot scale and fiedl screenings, in order to find those more active for plant protection against phytopathogenic bacteria and nematodes. On the basis of the results thus obtained and on those from the Actions B1, B3 and B4, four formulations were then set up and used in 2016 experiments, using the Kiloscale production set up in Action B5 (Figure 32). As model systems, Olive, Kiwifruit and Tobacco were used as foreseen, and Tomato, Carrot and Sugar Beet were also assayed for a more complete screening (Figures 33, 34 and 35). For comparison, copper sulphate was used against *Psv* and *Psa*, while two traditional nematicides were used, as shown in Figure 36.

1) Concentrated formula 1:10, water based, TC 2%, O 1% (1L)
2) Concentrated formula 1:10, water based, TC 1,5%, O 1%, V 0,3% (2L)
3) Gel formula, ready to use, TC 0,2%, O 0,1% + hydrophilic polymer (1G)
4) Gel formula, ready to use, TC 0,15%, O 0,1%, V 0,03% + hydrophilic polymer (2G)

Fig. 32. Formulations developed for the EVERGREEN project.

Fig. 33. Typical symptoms by *Psv* and *Psa*, on Olive and Kiwifruti, respectively.

Fig. 34. Typical symptoms on Tomato by *P. syringae* pv. *tomato* (*Pst*).

Fig. 35. Typical symptoms on Sugar beet and Carrot by *M. incognita*.

Fig. 36. Traditional nematicides used here for comparison with the EVERGREEN polyphenol formulations.

The detailed description of the methods adopted for inoculations and treatment application in this EVERGREEN Action B7 are fully described in the Annex 7 Deliverable Action B7, and here shown in Figures 37, 38 and 39.

Fig. 37. Inoculation of *Psv* and *Psa* on Olive and Kiwifruti, respectively, and treatments with the EVERGREEN polyphenols.

Fig. 38. Inoculation of *Pst* on Tomato, and treatments with the EVERGREEN polyphenols.

Fig. 39. EVERGREEN polyphenol treatment application on sugar beet and carrot against nematodes.

In Tables 14 and 15, and Figures 40 and 41, the results concerning the tests carried out on Kiwifruit in greenhouse and open field, after treatments with the four EVERGREEN formualtions are reported.

EVERGREEN formulations - 2016 greenhouse tests on Kiwifruit

Test without bacteria	Test with bacteria	CU SO4 without bacteria	CU SO4 with bacteria	Form 1 : Bacteria+ Form 1 spray 1 day before	Form 1. Bacteria+ Form 1 spray 1 day after	Form 2+bcteria+ Form 2 spray
0	4	1	1	4	3	2
0	3	0	2	0	4	3
1	3	2	0	2	3	2
0	4	0	3	3	4	4
0	3	0	2	2	2	0
0	5	1	2	4	3	1
1	3	0	2	3	5	2
0	5	0	0	2	2	1
1	2	0	2			2
0	6	0	3			3
0	4	1	4			1
0	5	0	1			3
1,5	4	0	2			2
0	3	0	1			2
0	2	0	3			2
0	5	1				3
0,28	3,81	0,38	1,75	1,25	1,63	2,06

Table 14. Data from tests on Kiwifruit in greenhouse, inoculated with *Psa* and treated with the EVERGREEN polyphenolic formulations.

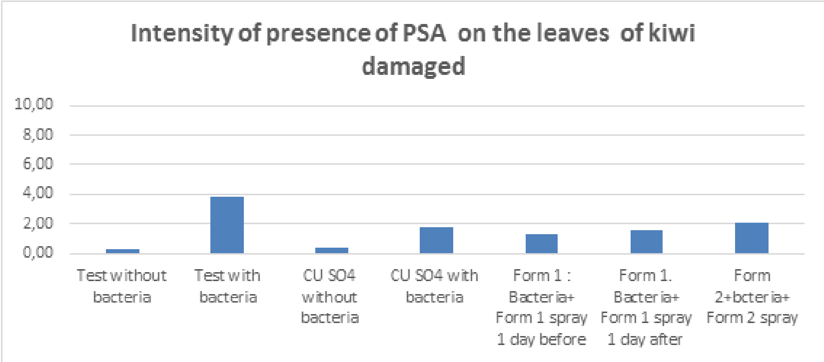


Fig. 40. Results on bacterial growth on Kiwifruit in greenhouse, inoculated with *Psa* and treated with the EVERGREEN polyphenolic formulations.

EVERGREEN formulations - 2016 field tests on Kiwifruit

Plants	Test without bacteria	Test with bacteria	CU SO4 without bacteria	CU SO4 with bacteria	Bacteria+ Form 1 spray 1 day before	Form 1. Bacteria+ Form 1 spray 1 day after	bcteria+ Form 2 spray 1 day before	Form 2+bcteria +Form 2 spray 1 day after
1	0	4	0	1	3	2	3	2
2	0	6	0	2	2	3	1	3
3	1	5	0	0	3	2	2	3
4	0	4	0	3	1	1	2	4
5	0	3	0	2	2	2	2	2
6	0	5	1	2	4	2	3	2
7	1	6	0	2	5	2	3	2
8	0	5	0	0	2	3	2	3
9	1	2	0	2				
10	0	6	0	3				
11	0	4	1	4				
12	0	5	0	1				
13	1	4	0	2				
14	0	5	0	1				
15	0	4	0	3				
16	0	6	1	2				
	0,25	4,63	0,19	1,88	2,75	2,13	2,25	2,63

Tab. 15. Data from tests on Kiwifruit in open field, naturally inoculated with *Psa* and treated with the EVERGREEN polyphenolic formulations.

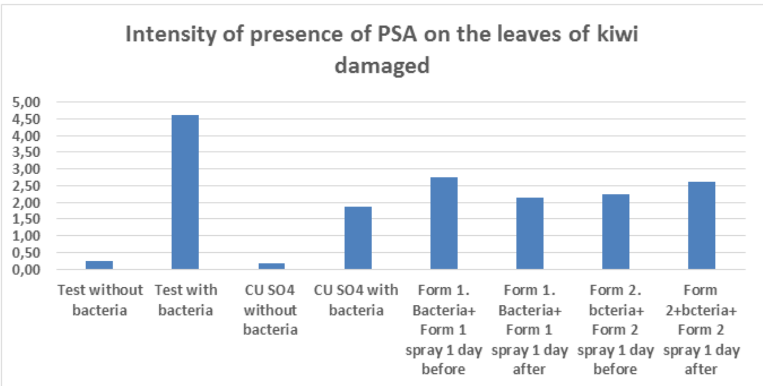


Fig. 41. Results on bacterial growth on Kiwifruit in open field, naturally inoculated with *Psa* and treated with the EVERGREEN polyphenolic formulations.

The effectiveness of the four EVERGREEN formulations was also confirmed by the resulst of experiments carried out on Olive and Tomato, inoculated with *Psv* and *Pst*, respectively, as shown in Table 16 and Figure 42, and in Table 17.

Plants	Control +: bacteria, no applications	Control -: no bacteria, no applications	Control CuSO4 - : no bacteria, applications with CuSO4	CuSO4: bacteria + applications with CuSO4	Form 3+1: Gel Form 3+bacteria + applications of Form 1(applied on 50% of the plants 1 day before inoculation	Form 3+1: Gel Form 3+bacteria + applications of Form 1(applied on 50% of the plants 1 day after inoculation	Form 4+2: Gel Form 4+ bacteria + application of Form2
1	3	0	0	2	1	2	2
2	1	0	0	1	2	1	1
3	3	0	0	0	1	2	2
4	0	0	0	1	0	1	2
5	3	0	0	0	2	1	0
6	2	0	0	1	2	2	1
7	1	0	0	2	1	2	2
8	2	0	0	2	2	1	1
9	2	0	0	2			1
10	3	0	0	1			3
11	2	0	0	1			1
12	1	0	0	2			0
13	0	0	0	2			2
14	1	0	0	0			2
15	3	0	0	3			2
16	1	0	0				1
	43,75	0,00	0,00	31,25	34,38	37,50	35,94

**Table 16. Data from tests on Olive in open field,
inoculated with *Psv* strain *Psn23* and treated with the EVERGREEN polyphenolic formulations.**

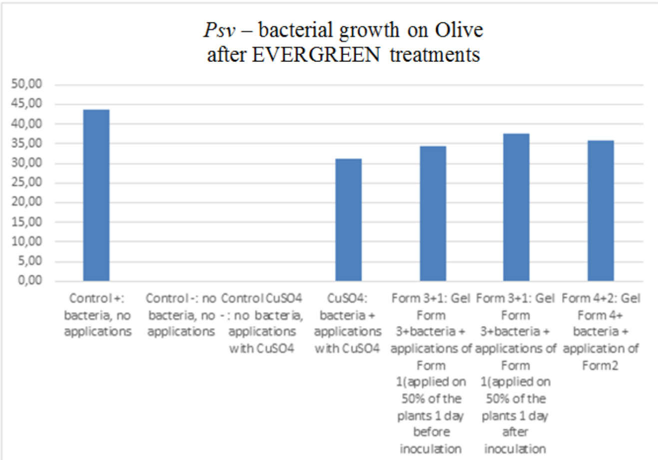


Fig. 42. Results on bacterial growth on Olive in open field, inoculated with *Psv* strain *Psn23* and treated with the EVERGREEN polyphenolic formulations.

Treatment	Description	N°Plants	average plant height (cm)	branches length (cm)	N ° of healthy berries	Number of infected berries	Total Number of berries	N ° of healthy berries/100 plants	Number of infected berries/100 plants	Total Number of berries/100 plants
1	Control +: bacteria, no treatments	65	31	19	45	16	61	69	25	94
2	Control -: no bacteria, no treatments	70	30	18,5	56	10	66	80	14	94
3	Control $CuSO_4$ - : no bacteria, treatments with $CuSO_4$	74	32,5	18,2	84	2	86	114	3	116
4	$CuSO_4$: bacteria + treatments with $CuSO_4$	80	34	19,4	95	2	97	119	3	121
5	$CuSO_{4(4X\ Concentration)}$: bacteria + treatments with $CuSO_4$	77	31	17,8	100	3	103	130	4	134
6	Form 4 (1 day after inoculation) +Form2	63	35	20,2	95	1	96	151	2	152
7	Form 4 (1 day before inoculation)+Form2	70	34	19,6	94	4	98	134	6	140
8	Form 3 (1 day after inoculation) +Form1	72	33,5	19,3	113	3	116	157	4	161
9	Form 3 (1 day before inoculation) +Form1	71	34	20,4	115	5	120	162	7	169

Tab. 17. Data from tests on Tomato in greenhouse, inoculated with *Pst* strain DC3000 and treated with the EVERGREEN polyphenolic formulations.

Similarly, the EVERGREEN polyphenolic formualtions were demonstrated effective to control nematodes on Tobacco and Carrot, as shown in Table 18.

	Thesis		Rip.	Tot.root	Infestation	root
				N°	(%)	average weigh
			a	30	60,00	35
			b	25	63,00	38
TEST	No treatment	**3**	**med.**	**27,5**	**61,5**	**36,5**
			c	26	55,00	33
			d	28	60,00	31
	No treatment	**6**	**med.**	**27,00**	**57,50**	**32**
			a	32	48,00	28
			b	27	53,00	31
Form liquic	4 (Form 1)	**4**	**med.**	**29,5**	**50,5**	**29,5**
			c	28	50,00	35
			d	25	47,00	31
	5 (form 2)	**5**	**med.**	**26,50**	**48,50**	**33**
			a	28	51,00	25
			b	27	56,00	33
Gel + Form	2 (Gel 3)+Fc	**2**		**27,5**	**53,5**	**29**
			c	31	52,00	28
			d	27	38,00	24
	1 Gel 4+For	**1**	**med.**	**29,00**	**45,00**	**26**

Table 18. Data from tests on Carrot in open filed, naturally infested by nematodes, and treated with the EVERGREEN polyphenolic formulations.

Problems encountered

No particular problems have been encountered during this Action B7, and this action was completed by 100%, as scheduled.

Verification of the expected results actually achieved

The detailed description of EVERGREEN Action B7 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 7 Deliverable Action B7.

The expected results were:

• Demonstration of the best *in vivo* conditions for the maximum efficiency of the high-quality standardised polyphenolic preparations against phytopathogenic bacteria and nematodes.

• Demonstration of the process for the *in vivo* application of the high-quality standardised polyphenolic preparations for the control of plant diseases caused by bacteria and nematodes.

According to the data obtained during Action B7, tests demonstrated that the EVERGREEN polyphenolic preparations here developed and optimised can be an effective ecofriendly approach for the control of phytopathogenic bacteria and nematodes. The activities carried on 2015 had suggested that the more efficient polyphenols were those form sweet chestnut and from grape, with those from Olive useful to stabilize the formulations. Therefore in 2016, four formualtions were set up, as liquid and gel preparations. The gel formualtion was mainly used added to the soil, to develop the natural resistance of the plants, while liquid formalations were used as spray and/or for irrigation. The results of the combined liquid+gel applications were really encouraging, and all tests put in evidence the positive impact of the EVERGREEN polyphenols for the control of phytopathogenic bacteria as a substitute of copper. Of course, these preparations were demonstrated more effective in controlled conditions, but their effectiveness and performances were also confirmed in open field. In the next future, more field and long-term experiments are needed to be performed on a larger scale, to define a strategy for using the EVERGREEN polyphenols in different conditions (*e.g.* preventive treatments or not, periodical treatments, *etc*).

Perspectives for continuing the action after the end of the project
According to the results obtained in Action B7, Mondo Verde srl is developing other formulations based on the EVERGREEN polyphenols, together with DISPAA-UNIFI, CEBAS CSIC, INSTM and ASTRA srl, to increase their catalogue for plant protection innovative products.

6.1.8. Action C.1 Monitoring on the environmental impact of copper compounds and nematicides for the crop defence against phytopathogenic bacteria and nematodes

Starting date foreseen: 1st of October 2014
Actual start date: 1st of October 2014
End date foreseen: 31st of March 2015
Actual end date: 31st of March 2015

The results of the activities carried out in Action C.1 are defined in the following project Deliverable, foreseen at the end of the 6th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 8 Deliverable Action C1: Monitoring on the environmental impact of copper compounds and nematicides for the crop defence against phytopathogenic bacteria and nematodes.

Activities carried out
This demonstration was delegated to DISPAA-UNIFI, INSTM and CEBAS CSIC, and Mondo Verde, and was completed by 100%.
The main objective of this Action C1 was to demonstrate the environmentally negative impact on soil microbiological communities of copper compounds and traditional nematicides used in plant protection, by examining at what extent soil microbiological activities and composition are affected. The detailed description of EVERGREEN Action C1 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 8 Deliverable Action C1. Firstly, some laboratory experiments were carried out on a sandy loam soil (fine sand 65.2%, clay 16.1% and silt 18.7 %), whose main feature were assessed before to start any experiment. The influence of copper sulphate and of three traditional non fumigant nematicides (Nemacur®, Mocap®, Vydate®) as well as of the agrochemical NeemAzal® were monitored on "microcosms" (*i.e.* plastic containers with 100 g of bulked soil/each) treated and untreated with the compounds of interest. The microcosms were placed in a growth chamber, set at 16h photoperiod, with a day/night temperature regime of 24/15ºC (Figure 43).

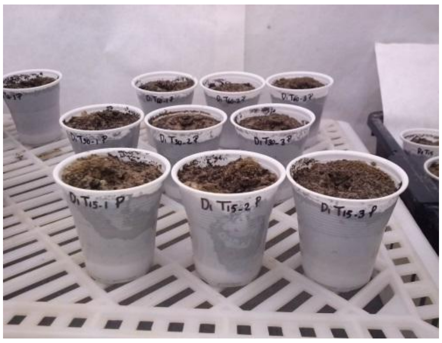


Fig. 43. Microcosms used to demonstrate the negative impact of antibacterial copper salts and several nematicides on soil microflora.

For each treatment, two different concentrations were used, added in a single application, in the effort to simulate what occurring in nature and considering the limits established by EU Regulation on Organic Production and Labeling of Organics Products. The experiment was realized during 60 days, and samplings were established at initial point (0 days) and 15, 30 and 60 days post-treatment, unless differently specified. For each treatment, three replicates were evaluated, placed in a random design into the growth chamber. This experiment was repeated twice, in independent runs. The data obtained were submitted to one-way ANOVA, in order to determine pair-wise difference by post-hoc test (Tuckey's method). The software used for the statistical analysis was SPSS 15.0.

Firstly, some biochemical parameters depending on the compostition of soil microflora were evaluated. On these samples, the soil activities for dehydrogenase, urease, β-glucosidase, phosphatase were determined, as well as basal respiration. Moreover, water-soluble carbon, total organic carbon and total nitrogen were assessed, as well as pH and conductance values, and micro- and macronutrients (Figure 44).

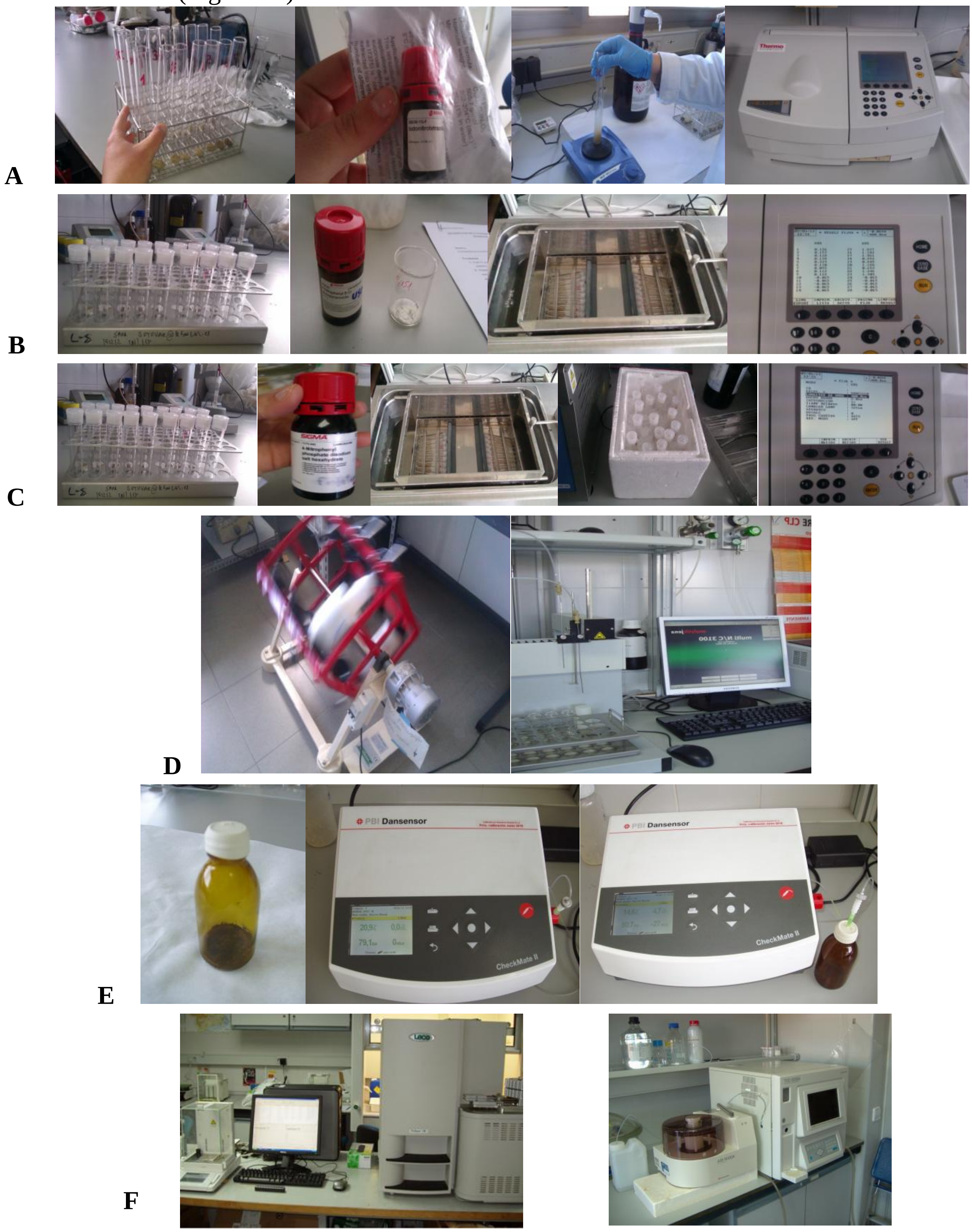


Fig. 44. Operational sequences (from left to right) to determine soil dehydrogenase (A), β-glucosidase (B) and phosphatase (C) enzyme activities, and for water soluble carbon determination (D), basal respiration analysis (E), and TOC and Nt evaluation (F).

Soil pH is a measure of the alkalinity or acidity of the soil, and affects the availability of nutrients for plants as well as enzymatic activities of soil microflora. Here, the soil pH ranged from 7.47-7.99 and these values were maintained after any treatment. Conversely, conductance appeared to be affected by treatments, particularly at the beginning following treatments, likely for the increase in salts which are present into these solutions. However, no significant changes in “ionome” composition were observed in comparison to the controls, unless an obvious increase in copper following its treatments.

Briefly, no particular effects were also to derive from treatments on β glucosidase activity, which catalyzes the final step of cellulose degradation, unless when copper was administrated at the higher concentration and after 60 days. Similarly, phosphatase activity was impaired by copper and Vydate, while urease was negatively affected by Mocap and copper sulphate. High doses of NeeMazal increased dehydrogenase activity as well as of the organic carbon content, maybe for a secondary effect still unknown, related to its use as substrate for soil microorganisms. NeemAzal, together with Mocap and Vydate, also caused a negative effect on water soluble N when added to the soil. All the data here summarised are fully reported in the Tables of the Annex 8 Deliverable Action C1.

On the same samples, Phospholipid Fatty Acids (PLFAs) and Fatty Acid Methyl Esters (FAMEs) analysis were carried out, together with traditional microbiological tests and and molecular assays related to the direct monitoring of variations occurring on the composition of soil micorflora following treatments. Firstly, total PLFAs decreased in the soil when nematicides and copper were added, particularly 60 days after treatments with copper and Nemacur. This effect was mainly attributable to a decrease on fungal population in comparison to those from bacteria (Fig. 45).

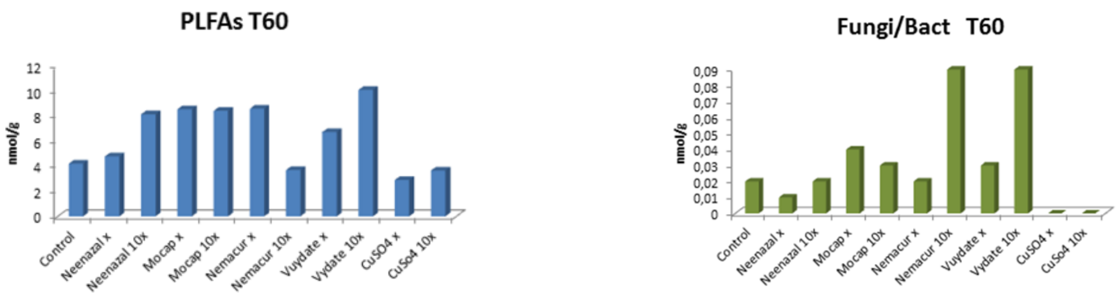


Fig. 45. PFLAs analysis after treatments with copper and nematicides on microcosms (60 days).

And this effect was quite obvious and attributable to an increase/emergence of strains resistant to copper and, as an indirect consequence, of antibiotic-resistant strains after copper sulphate treatments (Fig. 46).

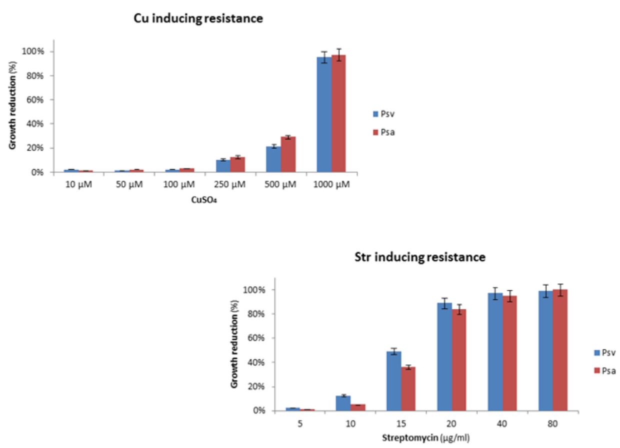


Fig. 46. Resistances after *in vitro* copper treatments on *Psv* and *Psa*.

Soil samples deriving from pilot/field experiments with copper and nematicides treatments (from Action B1) were also analysed.
An increase in copper- and antibiotic resistant strains was also found to occur on soil microflora into the fields, although to a lesser extent, as shown in Figure 47 when the equivalent of 10 or 20 kg/y/ha were adminstered into soils of Olive and Kiwifruit plants. It could be hypothesised that onto soil a sort of “buffer” effect exists in nature, limiting during the time the increse of these resistant bacteria, also driven by plant species radical exudates.

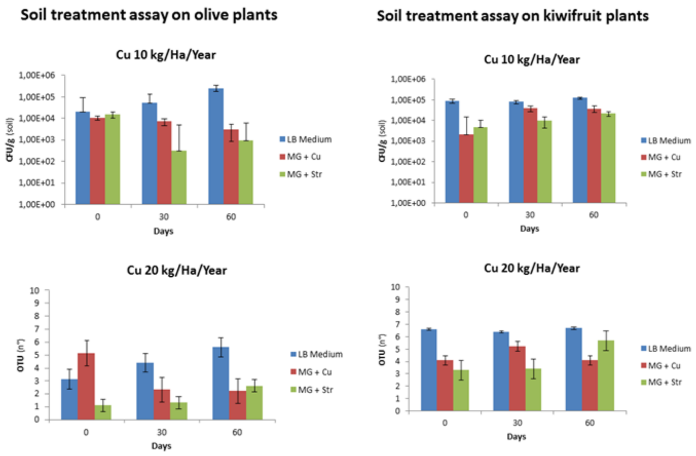


Fig. 47. CFU and variability of soil bacterial populations (OTUs) following copper treatments (60 days).

At the end, a PCR screening on nematicide-degrading bacteria, present into the soil treated with traditional nematicide into Tobacco fields, was carried out according to (Meyer *et al*., 1999). As shown in Figure 48, data confirmed the emergence of nematicide-degrading bacteria, in particular after tretments with Mocap and Vydate.

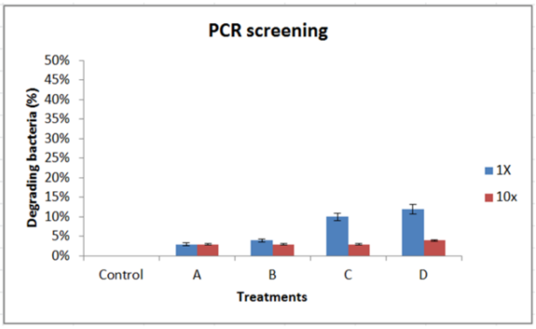


Fig. 48. Emergence of nematicide-degrading bacteria in soil microflora, following treatments (60 days) with (A) NeemAzal®, (B) Nemacur®, (C) Mocap®, (D) Vydate®.

Problems encountered
No particular problems have been encountered during this Action C1, and this action was completed by 100%, as scheduled.

Verification of the expected results actually achieved
The detailed description of EVERGREEN Action C1 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 8 Deliverable Action C1.
The expected results were:

• Monitoring and evaluation of the environmental impact of copper salts and nematicides on soil microflora, related to the emergence of copper- and antibiotic-resistant strains, and of nematicide degrading bacteria.
• Monitoring and evaluation of the environmental impact on the composition of bacterial microflora in copper and nematicide contaminated soils.
This EVERGREEN monitoring C1 activity further demonstrated the urgent need to develop alternative and environmentally safe plant disease control products against phytopathogenic bacteria and nematodes. In fact, the data here briefly reported show the heavy negative effects on soil microflora of traditional nematicides and copper salts, according to chemical and microbiological tests, as well as to phenotypic and genotypic characterization. In particular, it was demonstrated the increase/emergence of resistance bacteria, towards copper and antibiotics, as well as of nematicide-degrading bacteria and nematicides. These consequences are a risk for both environment and human health, besides to determine a reduction of the effective use of these compounds in plant protection.

Perspectives for continuing the action after the end of the project
During the project and just after its conclusion, both the coordinator as well as the other beneficieries have been contacted by stakeholders interested to monitor the effects deriving from copper and nematicids pollution in agroecostystems, such as Confagricoltura Toscana, with the perspective to be part of operative groups working on this topic at national at internation level.

6.1.9. Action C.2 Monitoring of the absence of side effects for the high quality standardised polyphenolic preparations on common targets of any living organism at laboratory level

Starting date foreseen: 1st January 2015
Actual start date: 1st January 2015
End date foreseen: 31st of December 2015
Actual end date: 31st of December 2015

The results of the activities carried out in Action C.2 are defined in the following project Deliverable, foreseen at the end of the 15th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 9 Deliverable Action C2: Monitoring of the absence of side effects for the high quality standardised polyphenolic preparations on common targets of any living organism at laboratory level.

Activities carried out
This demonstration was delegated to DISPAA-UNIFI and CEBAS CSIC, and was completed by 100%.
This Action has to monitor, at laboratory level, the absence of any toxicity of the EVERGREEN polyphenolic extracts and fractions on subcellular targets universally present in any living organism, in order to demonstrate the ecofriendly toxicological profile of these molecules, recovered from agricultural vegetable not edible biomass.
The development of molecules and formulates for plant disease control is time- and cost-intensive. Therefore, such as occurring for the development of drugs for human and animal health, is essential to perform as early as possible specific assays to evaluate the risk of any short- and long-term toxicity associated to the use of a potential drug, to avoid to make this long process inefficient. As a consequence of their results, these tests are able to identify the potential for failure with high sensitivity, generally related to adverse drug reactions. Model *in vitro* systems are currently used for high-throughput screening of drugs, also in plant pathology. Among the most important targets to be used to verify the occurence/the absence of any undesiderable side effect on a wide range of living organisms, there are the so-called P-type ATPases, which are a large group of ubiquitous evolutionarily related molecular pumps present into any cellular membrane, and that are highly conserved in eukaryotes and prokaryotes. Most members of this family of transporters are specific for pumping into the cell a wide array of ions (mostly cations), but there arealso some ATPases involved in the maintenance of the asymmetric nature of the biomembrane. In bacteria, several ATPases have been shown to be essential for protection from extreme environmental stress conditions. The P-type ATPases are targets for many toxic compounds (e.g mercury, toxins from algae, fungi, bacteria, etc), that are thus able to dramatically impact on cell homeostasis. Many potential drugs in human medicine were excluded for further development for important adverse side effects just on important P-type ATPases, such as the calcium ATPase of sarco-endoplasmic reticulum (indicated as SERCA). SERCA couples the hydrolysis of a molecule of ATP to the active transport of two Ca^{2+} ions from the cytoplasm to the lumen of the sarco-endoplasmic reticulum, thereby promoting muscle relaxation. The functionality of this calcium pump can be assessed *in vitro* by electrochemical methods. These procedures measure the electrogenicity of SERCA pump and consists in carrying out concentration jumps of activating substances on proteoliposomes adsorbed on a mixed alkanethiol/phospholipid bilayer supported by a gold electrode (the solid supported membrane, SSM). The concentration jump induces the flow of a capacitive current that is recorded against time. The resulting current transient exhibits a relatively steep rising section, followed by a current peak and by a descending branch. Integration of the current transient measures the charge moved by the activating substance. Briefly, any substance/molecule reducing

calcium transport by this kind of calcium pumps has to be considered highly and widely toxic on any living organism.

The detailed description of the EVERGREEN Action C2 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 9 Deliverable Action C2.

As a positive control, the inhibitory effect of Cu^{2+} ions on Ca^{2+}-ATPase was firstly investigated. To this aim, we performed current measurements on SR vesicles adsorbed on a SSM. A 100μM ATP concentration jump was carried out in the presence of $CaCl_2$ (10μM) and in the absence of $CuCl_2$. The current signal observed following the ATP jump was taken as a control measurement (Figure 49. It is worth mentioning that the charge obtained by numerical integration of the ATP-dependent current signal is attributed to an electrogenic event corresponding to translocation of bound Ca^{2+} through the ATPase after utilization of ATP. The ATP concentration jump was then performed in the presence of 10μM $CaCl_2$ and 1μM $CuCl_2$ and the corresponding ATP-induced current signal was compared to the control measurement. We found that Cu^{2+} ions suppress the ATP-induced current signal and the associated displaced charge both at 0.1μ and 1μM concentration (Figure 49). Therefore, it could be concluded that sub-micromolar copper exerts a strong inhibitory effect on Ca^{2+}-ATPase by interfering with ATP-dependent calcium translocation through the enzyme. Briefly, then the experimental scheme was succesfully optimised by using the polyphenol Epigallocatechin gallate (EGCG) in comparison to copper salts (Figure 49), demonstrating the positive toxicological profile of EGCG up to 1 μM.

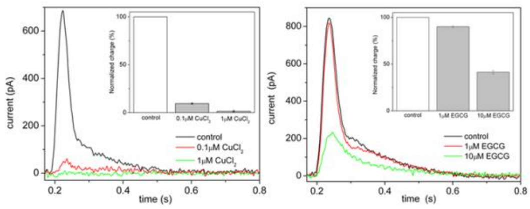


Fig. 49. Effect of copper salts and EGCG on ATP-dependent currents genrated by Ca-ATPase.

Similarly, the interaction of SERCA with other EVERGREEN polyphenols was investigated. In particular, the polyphenols which were the representative molecules for those EVERGREEN extracts demonstrated to be the most biologically effective in plant protection in the other EVERGREEN Actions were deeply analysed. In Figure 50, the results for Catechin, Hydroxytyrosol, Oleuropein and Chlorogenic acid are reported, assuming these molecules as a standard for the EVERGREEN polyphenolic-based extracts from Grape, Olive, Artichoke and Sweet Chestnut. As previously assessed for Cu^{2+} ions, we compared the current signals generated by the ATPase following 100μM ATP concentration jumps in the absence of polyphenol molecules (control measurement) and in the presence of each polyphenol molecules. These polyphenol-based molecules have minor, if any, effects on the ATP-induced current signal over a concentration range from 1 to 10 μM, where more pronounced were inhibitory effects when using EGCG. In fact, in the case of EGCG a significant reduction of the current amplitude was recorded at 10 μM EGCG. Such an interference with ATP-dependent Ca^{2+} translocation in the presence of a high EGCG concentration has been reported in recent biochemical studies, where EGCG was found to inhibit both Ca^{2+}uptake rate and ATPase activity with half-maximal effects observed at ~12 μM and ~16 μM. In these studies, however, no inhibitory effect of EGCG on SR Ca^{2+-}ATPase activity was reported in the concentration range between 0.1 and 1 μM. Therefore, our results indicate that as compared to copper, the EVERGREEN polyphenolic extratcs do not affect the SR Ca^{2+-}ATPase transport activity in the sub-micromolar concentration range.

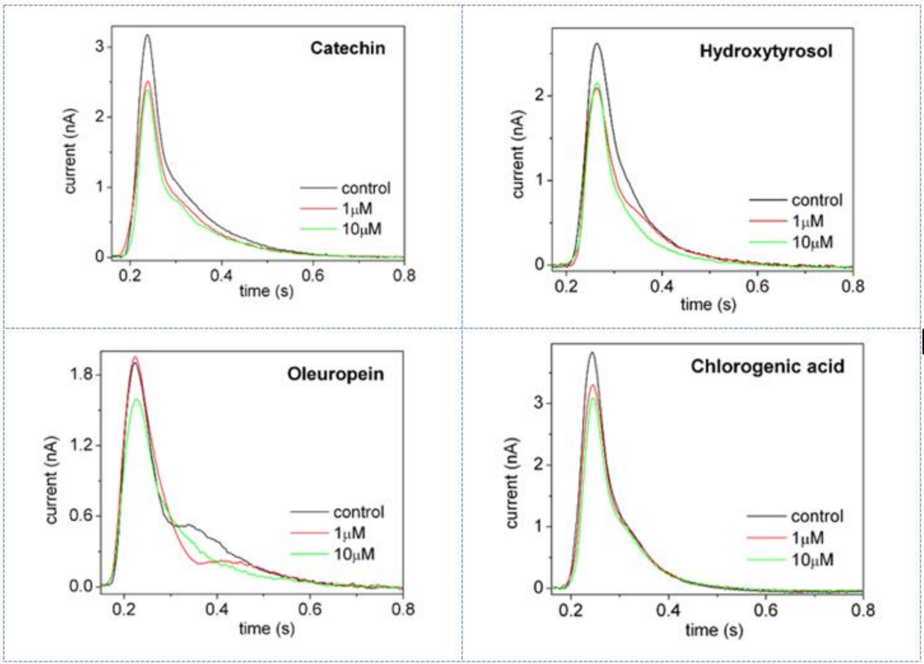


Fig. 50. Current measurements on SR vesicles adsorbed on a SSM. Current signals induced by 100 µM ATP concentration jumps in the presence of 10 µM Ca^{2+} and in the absence (black curve, control measurement) or of $CuCl_2$ (red curve), or of Catechin, Oleuropein, Hydroxytyrosol and Chlorogenic acid (green curves).
Charges are normalized with respect to the value measured in the absence of copper ions and polyphenols.

Problems encountered
No particular problems have been encountered during this Action C2, and this action was completed by 100%, as scheduled.

Verification of the expected results actually achieved
The detailed description of EVERGREEN Action C2 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 9 Deliverable Action C2.
The expected results were:
• Monitoring of the biological activity of the high quality and standardised polyphenolic molecules and preparations on P-type ATPases in membrane model systems by electrochemistry.
The so-called P-type ATPases are among the most important targets to monitor and assess the subcellular toxicity of a drug, because they are ubiquitous and evolutionarily conserved into any cellular membrane of organisms and microorganisms. The absence of any toxicity of the EVERGREEN polyphenolic-based extracts was here monitored, to demonstrate the friendly ecotoxicological profile of these high molecules recovered from agricultural vegetable not edible biomass and waste. The experimental set up was optimized by using as a positive control Cu^{2+} ions, as well as EGCG as a model for polyphenols. Cu^{2+} ions are known to exert a strong inhibitory effect on Ca^{2+}-ATPase at sub-micromolar concentrations, as here confirmed. Conversely, no inhibitory effect of EGCG on the ATP-induced current signal was found at a concentration of 1μM, while a partially suppression was demonstrated to occur at a concentration of 30μM. Conversely no significant inhibitory effects were found with the other polyphenols which are standards for the EVERGREEN extracts and preparations at concentration up of 1 and even 10 μM.

Perspectives for continuing the action after the end of the project
According to the activities and the results obtained in Action C2, UNIFI-DISPAA and CEBAS CSIC as well as the project coordinator were contacted to partecipate to national and EU projects

related to the testing of substances and formulation to be potentially used in plant protection, by electrochemistry cutting-edge techniques.

6.1.10. Action C.3 Monitoring of the absence of a direct selection operated by the polyphenolic preparations towards the emergence of bacteria resistant to the polyphenolic molecules themselves, at laboratory level

Starting date foreseen: 1st of Aprile 2015
Actual start date: 1st of April 2015
End date foreseen: 31st of March 2016
Actual end date: 31st of March 2016

The results of the activities carried out in Action C.3 are defined in the following project Deliverable, foreseen at the end of the 18th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 10 Deliverable Action C3: Monitoring of the absence of a direct selection operated by the polyphenolic preparations towards the emergence of bacteria resistant to the polyphenolic molecules themselves, at laboratory level.

Activities carried out
This demonstration was delegated to DISPAA-UNIFI and CEBAS CSIC, and was completed by 100%.
This Action has to monitor, at laboratory level, the absence of the direct emergence of bacteria resistant to the polyphenolic extracts following repeated treatments with these molecules. This action has the objective to evaluate a phenomenon usually occurring on bacterial populations when treated with anti-bacterial molecules, having a selective toxicity means that inhibits or kills the pathogen of interest without damaging the host. Among the most important antimicrobial chemotherapeutic agents there are antibiotics, which are metabolic products of microorganisms inhibiting or killing other microorganisms. Also in nature, bacteria can become resistant to antibiotics by several mechanisms coded by genes that are present in the mobile and easily transmissible parts of the bacterial genome, called plasmids. As far as plant protection is concerned, copper treatment induce resistance on bacterial population, and generally this leds to the simultaneous emergence of resistance to antibiotics, because of a cross-resistance mechanism based on the presence on the same plasmids of both the genes for copper- and antibiotic-resistance. According to the results obtained in other EVERGREEN Actions, the inhibitory/antivirulence effect of the EVERGREEN molecules, extracts and fractions on TTSS had been demonstrated and recently published (Biancalani *et al*., 2016). This was strobngly suggested that the selective pressure generated by the EVERGREEN polyphenol extracts application would be very low and thus a null or neglegible risk of resistance could be hypothesized. Nevertheless, the activities carried out on thsi Action C3 were focused on monitoring and demonstrate this hypothesis, concerning the absence of any selection for bacterial resistance to polyphenols themselves.
The detailed description of the EVERGREEN Action C3 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 10 Deliverable Action C3.
As foreseen, *in vitro* tests have been carried out on type strains and/or representative strains of the bacteria *Psv* and *Psa***,** here used as models. The *in vitro* TTSS inhibition of *Psv* and *Psa* by the EVERGREEN high quality phenolic molecules was compared with the same values found in these bacteria previously treated with the EVERGREEN extracts *in vitro* for several generations and quantitatively assessed by expression studies by RealTime PCR on the master genes of the TTSS of these bacteria, molecular tools already developed by DISPAA-UNIFI (*e.g.* pT3GFP, already described in other previous Actions, such as Action B3), and *in planta* inoculation studies of *Psv* and *Psa*.

Besides some preliminary tests on crude EVERGREEN extracts, most of the demonstration activities were then focused on the use of the EVERGREEN polyphenolic formulations developed in the frame of this project, and which were: Formulation 1 (hereafter indicated as 1L): Sweet chestnut tannin 2% w/v (total volume), Olive extract 1% w/v (total volume); Formulation 2 (hereafter indicated as 2L): Sweet chestnut tannin 1,5% w/v (total volume), Olive extract 1% w/v (total volume), by-product grape seeds post-oil extraction 0,3% w/v (total volume). Formulations 1L and 2L were added as such (therefore, ten times more concentrated than when used in field experiments in the Action B7), in a flask of 100 ml with bacteria at a starting inoculum of 0.5 OD (600 nm). Every 12 hours, a sample of 1 ml was collected from each bacterial preparation in order to evaluate its capability to reduce HrpA promoter activation and TTSS gene expression, as well as HR response on Tobacco leaves. The same bacterial species/strains were used as untreated, as a positive control. The EVERGREEN polyphenolic treatments produced 20 (20^{th}), 40 (40^{th}), 60 (60^{th}) and 80 (80^{th}) bacterial generation at 12, 24, 36 and 48 hours of incubation, respectively. These samples were then used in the experimental tests described above, to identify differences between generations treated and untreated.

Firstly, both *Psv* and *Psa* grew *in vitro* similarly when treated or not with the EVERGREEN formulations, both in a rich medium (KB) or in a medium resembling apoplast conditions (MM) (Figure 51).

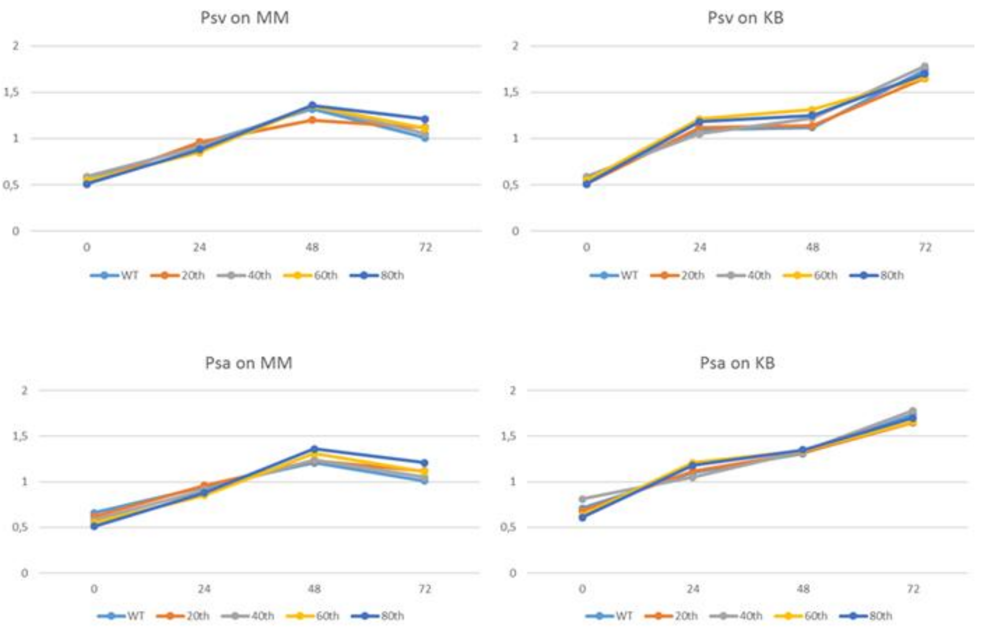


Fig. 51. *In vitro* growth of *Psv* and *Psa* on KB and MM, after treatmens for several generations (20, 40, 60, 80) with the EVERGREEN formulation 2L or not (wt).

When transformed with pT3GFP and then grown in MM, with or without adding the EVERGREEN formulations, both *Psv* and *Psa* were spectophotometrically evaluated for GFP fluorescence activity. This was given by the activation of pT3 promoter and, as shown for *Psv* in Figure 52, no any difference was observed in the pT3 activation/GFP fluorencesce between treated and untreated bacteria.

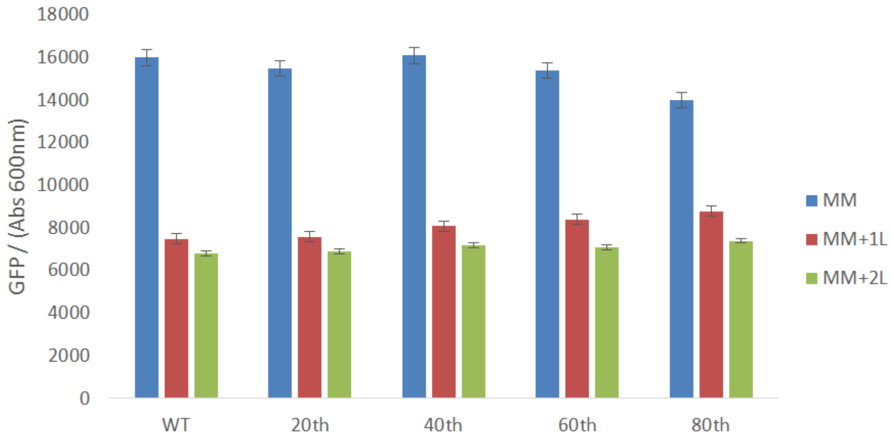


Fig. 52. GFP fluorescence emitted by *Psv* transformed with pLPVM-pT3-GFP recombinant plasmid, when grown in MM, after treatments for several generations (20, 40, 60, 80) with the EVERGREEN formulation 1L and 2L, or not (wt).

Similarly, no differences were ever observed in TTSS genes expression analysis by RealTime PCR, both in *Psv* and *Psa* when treated or not with the EVERGREEN extracts (Figure 53).

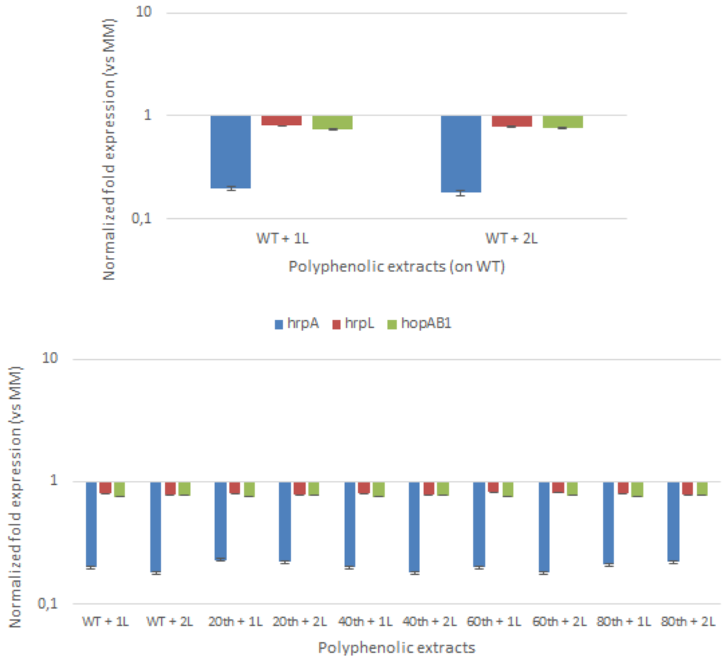


Fig. 53. RealTime PCR gene expression analysis on *Psv* grown in MM, treated for several generations (20, 40, 60, 80) with the EVERGREEN formulation 1L and 2L, or not (wt).

HR induced on Tobacco is a defence response induced by the infirltration of phytopathogenic Gram negative bacteria having a functional TTSS. As demonstrated previously in Action C3, the EVERGREEN polyphenolic extracts are able to inhibit, with a result comparable to that obtained with TTSS-impaired mutants such as Δ*hrpA*. *Nicotiana tabacum* var. Burley White leaves were infiltrated with *Psv* and *Psa* bacterial cultures (resuspended in SPS to an 0.5 OD_{600}), with six replicates/thesis and in three independent experiments, and the HR development was assessed according to the macroscopic tissue collapse at 24 h post-inoculation. Both bacterial *Psv* and *Psa* wild type and polyphenolic extracts-treated strains were inoculated into Tobacco leaves. As here shown for *Psv* (Figure 54), both all the bacteria were demonstrated equally able to triggered HR, and these data further confirmed that no resistance to the EVERGREEN extracts was developed by treated bacteria.

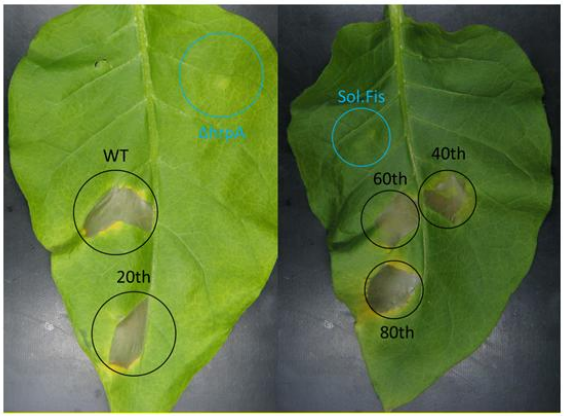


Fig. 54. HR induced on Tobacco leaves by *Psv*, after treatments for several generations (20, 40, 60, 80) with the EVERGREEN formulation 1L and 2L, or not (wt). For comparison and as negative controls, Δ*hrpA* and physiological solution were also used.

Problems encountered

No particular problems have been encountered during this Action C3, and this action was completed by 100%, as scheduled.

Verification of the expected results actually achieved

The detailed description of EVERGREEN Action C3 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 10 Deliverable Action C3.

The expected results were:

• Monitoring of the absence of polyphenolic-based molecules resistant bacteria following treatments with these compounds.

• Monitoring of the low selective pressure strategy proposed for reducing/replacing traditional pesticides.

The activities performed in this Action C3 had the objective to monitor and demonstrate that repeated treatments with the EVERGREEN polyphenolic extracts/formulations did not decrease or their inhibitory activity on T3SS of the *Pseudomonas* species here used as a model, thus confirming the absence of a selection for resistance to the EVERGREEN polyphenolic extracts themselves. This resistance phenomenon usually occurs on bacterial populations when treated with antibiotics. Conversely, the EVERGREEN polyphenolic extracts here used against *Pseudomonas* species, until 80 generations, did not induce any type of resistance, as monitored and demonstrated by several reliable tests referred to TTSS inhibition. In fact, bacteria treated with the polyphenolic extracts have been shown to have the same behaviors of type strains, in all the tests performed. Based on these results we can affirm that the polyphenolic extracts designed, synthesized and used in this demonstration study not induce resistance phenomena in bacterial Gram negative phytopathogens and thus they will extremely useful for plant diseases control.

Perspectives for continuing the action after the end of the project

According to the activities and the results obtained in Action C3, DISPAA-UNIFI and CEBAS CSIC were asked to be part of a team for a H2020 call (SFS-17-2017) concerning innovation in plant protection, with application of the EVERGREEN approach.

6.1.11. Action C.4: Monitoring of the short term environmental benefits from the use of the high quality standardised polyphenolic preparations in plant disease control at pilot scale level in field screenings

Starting date foreseen: 1st of July 2015
Actual start date: 1st of July 2015
End date foreseen: 30th of September 2016
Actual end date: 30th of September 2016

The results of the activities carried out in Action C.4 are defined in the following project Deliverable, foreseen at the end of the 24th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 11 Deliverable Action C4: Monitoring of the short term environmental benefits from the use of the high quality standardised polyphenolic preparations in plant disease control at pilot scale level in field screening.

Activities carried out
This demonstration was delegated to UNIFI-DISPAA and INSTM, and was completed by 100%.
Pesticide and agrochemicals, including copper salts and nematicides, have many negative environmental impacts, included a heavy alteration of the ecological interactions taking place into agroecosystems. The activities carried out in this Action C4 had the objective to monitor one of the most important short term environmental benefits deriving from the use of the EVERGREEN polyphenolic extracts and formulations for plant disease control, that is they do not negatively affect the structure of the microbial communities naturally resident in soil and as a consequence also basal plant health and vigor.
The detailed description of EVERGREEN Action C4 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 11 Deliverable Action C4. Firstly, some preliminary tests on several crude EVERGREEN polyphenolic extracts were carried out to monitor their effect on soil microflora, as described also in other Actions. Then, according to the data obtained, four polyphenolic-based formulations were developed and optimised during the EVERGREEN project, and here also used (Figure 32):

- Form 1 (liquid): Chestnut polyphenol 2%, Olive polyphenol 1% in water (1:10)
- Form 2 (liquid): Chestnut polyphenol 1,5%, Olive polyphenol 1%, and Grape seeds 0,3% in water (1:10)
- Form 3 (gel): Chestnut polyphenol 0,2% and Olive 0,1% in water
- Form 4 (gel): Chestnut polyphenol 0,15%, Olive polyphenol 0,1% and Grape seeds 0,03% in water.

For comparison, $CuSO_4$ (6 k Cu^{2+}/y/ha equivalent), and Mocap and Neemazal were used as traditional bactericide and nematicides, respectively. In particular, Olive, Kiwifruit and Tobacco plants were treated as fully describe in Material and Methods of Action B4 with the EVERGREEN polyphenolic extracts/formulations, then inoculate or not with *Psv*, *Psa* and *M. incognita.* These samples were analysed in Action B4 for symptoms development, to demonstrate the effectiveness of the EVERGREEN extracts/formulations in plant protection, while here the short term beneficial environmental deriving from their use was monitored on soil and leaves, by chemical and microbiological tests, as well as by collecting plant biometric data. In particular, the parameters analysed in leaves and soil samples are reported in Table 20.

Parameters	Plant	Leaves	Soil
Macro and micronutrients		X	X
Total Carbon and total nitrogen		X	X
Chlorophyll		X	
Height	X		
Trunk diameter	X		
Enzyme Activities			X
Microrespiration			X
PLFAs			X

Table 20. Parameters assessed for Action C4 following treatments with the EVERGREEN extracts/formulations.

Samples were analysed at T=0, just after treatments with the EVERGREEN extracts/formulations and Tf=6 months after treatment, unless differently specified, to monitor changes occurring on the parameters here evaluated.

The main conclusions, according to the data obtained, start from the evidence that the use of the EVERGREEN polyphenols can be generally regarded as positive, since they are able to prevent bacterial diseases on crops such as kiwi or olive, as well as diseases caused by nematodes in crops (see Action B4 and B7). This confirms the fact that we could point to the use of polyphenols as "biopesticides". The use of polyphenols as biopesticides will allow to avoid repeated use of salts of Cu (thus avoiding the accumulation of a heavy metal such as Cu in soils and its possible translocation to plants in amounts that could be problematic). Similarly, the use of polyphenols could prevent the widespread use of nematicides, which would certainly be a benefit to many ecosystems involved. Moreover, according to the main aims of Action C4, the use of the EVERGREEN polyphenolic formulation does not imply problems on crops (*e.g.* macro- and micro-elements in leaves, plant height, *etc*). It is worth to mention that the effects caused by polyphenols on soils were linked to the type of crop: thus, the effects of polyphenols on soils under olive cultivation differed from those under cultivation of kiwi. An example of this can be checked with the enzymatic activities of cycles of P, C and N, these cycles were affected with the use of polyphenols in soils under olive cultivation, while they were not affected in soils under kiwi cultivation. This highlights the importance of using sensitive parameters such as enzyme activities, to determine the effect of organic compounds such as polyphenols on the quality of soil where they are applied. However, no negative effects were never found on the microflora composition that could be deleterious for plant vigor and health (Figures 55 and 56).

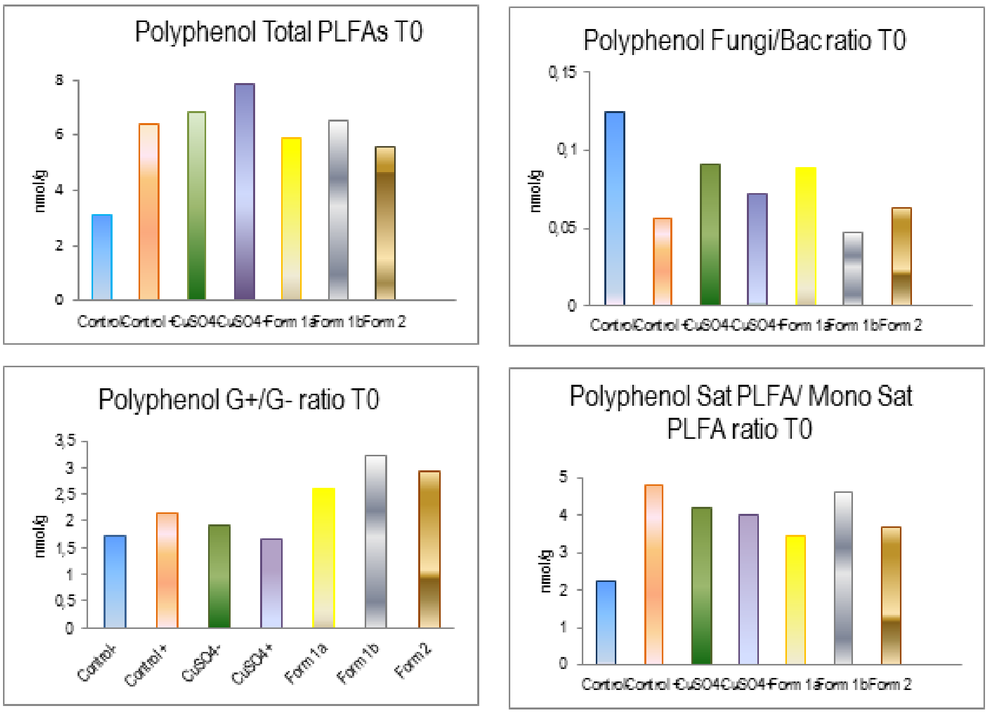


Fig. 55. Total PLFA and bacteria/fungi, Gram$^+$/Gram$^-$ and monounsatured/satured ratios in kiwifruit soils T0, following treatments with the EVERGREEN extracts/formulations.

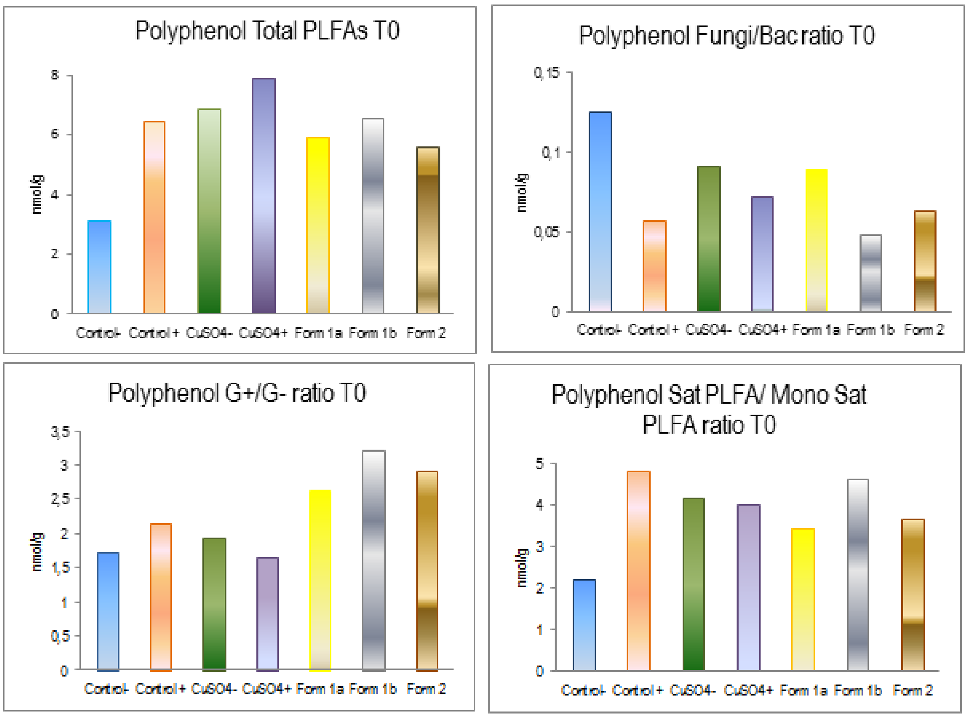


Fig. 56. Total PLFA and bacteria/fungi, Gram$^+$/Gram$^-$ and monounsatured/satured ratios in Olive soils T0, following treatments with the EVERGREEN extracts/formulations.

Problems encountered

No particular problems have been encountered during this Action C4, and this action was completed by 100%, as scheduled.

Verification of the expected results actually achieved

The detailed description of EVERGREEN Action C4 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 11 Deliverable Action C4.

The expected results were:

• Monitoring of the beneficial effect of the polyphenolic based molecules and preparations on microbial community structure, in comparison to that of copper and nematicide treatments for control of plant diseases caused by bacteria and nematodes, respectively.

In addition to demonstrate that the EVERGREEN polyphenols able to prevent bacterial and nematode diseases on the pathogentic systems here used as a model (see Action B4 and B7), in thsi C4 Action the positive impact on plant soil activities was also experimentally demonstrated. By sensitive parameters, such as soil enzymatic activities and PFLAs, it was monitored the effect of the EVERGREEN polyphenolic extracts/formulations on the quality of the soils where they have been applied. No significant negative effects were ever found on the microflora composition that could be deleterious for plant vigour and health, as occurring with copper salts and traditional nematicides.

Perspectives for continuing the action after the end of the project

According to the activities and the results obtained in Action C4, the coordinator and the other EVERGREEN beneficiaries were asked to participate to a H2020 call (SFS-17-2017) concerning instruments to be developed to control emerging diseases of plants, by using the EVERGREEN extracts and fractions.

6.1.12. Action C.5. Monitoring of the economic benefits deriving from the recycling of the spent vegetable biomass after the extraction of the high quality standardised polyphenolic molecules at laboratory level

Starting date foreseen: 1st of April 2015
Actual start date: 1st of April 2015
End date foreseen: 30th of September 2016
Actual end date: 30th of September 2016

The results of the activities carried out in Action C.5 are defined in the following project Deliverable, foreseen at the end of the 24th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 12 Deliverable Action C5: Monitoring of the economic benefits deriving from the recycling of the spent vegetable biomass after the extraction of the high quality standardised polyphenolic molecules at laboratory level.

Activities carried out
This demonstration was delegated to INSTM and Mondo Verde, and was completed by 80%.
This Action has to monitor the sustainability of the integrated use of the spent vegetable not edible biomass, obtained after the extraction of the high-quality polyphenolic compounds, for bioenergy production and of the recycled digested phase as a soil amendment.
The detailed description of EVERGREEN Action C5 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 12 Deliverable Action C5.
European regulations on fertilizers are currently including both inorganic fertilizers and virtually all fertilizers produced from organic materials, such as animal or other agricultural byproducts, or recycled bio-waste from the food chains. However, new fertilizing materials can be produced as a consequence of domestically sourced resources, as those created by some opportunities of circular economy and green chemistry, related to locally exploiting biomass by previous extraction of useful botanicals. Research on agricultural production chains have been contributing to the development of a zero-waste agriculture concept, due to the network of related industries that can use spent biomass as a feedstock for materials and energy, according to a scheme of an after-process circular economy, therefore avoiding direct land disposal or disposal as landfill waste, that potentially cause non-point pollution, with related nitrate losses, eutrophication and GHG emissions.
There are presently two major routes to treat waste biomass with a relatively high water concentration (>20%), especially after a REACH-compliant extraction. The most diffuse and established one is anaerobic digestion. Then, a new technology has been recently proposed, initially developed for urban waste treatment, that is hydrochar and biochar production. Starting from fresh field and vegetable crop residues it is possible to produce hydrochar, while starting from tree-crops and forestry pruning and residues biochar is the main product. The use of both these products into the field is beneficial for soil microorganisms, and can be used as substitutes of ordinary fertilizers and also to fight desertification and many effects of climate change.
As far as evaluations of the EVERGREEN spent biomass are concerned, residues from extraction from sweet chestnut, grape marc and olive pomace have been here used and evaluated.
Chipping, as well as breaking and shredding, typically consumes 15 kWhe/t input. However, it is a necessary step for extraction and should be considered in the LCA of tannins. In case of chipped chestnut tannin with 7% wood water content, the Heating Value is estimated 17.5 MJ/kg. The energy is in part used to reduce the water content of the extracted shredded wood from 60% to <30%. CHP plants are usually used (Figure 57).

Fig. 57 Biomass from sweet chestnut tannins extraction from INSTM.

In particular, 95% of the ash produced is the bottom ash, consisting of slags, sand and unburned wood; whereas the rest is fly ash, with most of the heavy metals of the biomass. Returning ashes to the environment where biomass was grown permits to return those mineral components to a place where it originated, and therefore closes the mineral cycles, and it is the most sustainable for of ash utilization, and it is possible with small plants (<3 MWh). However, plant minerals returning to soils are mainly represented by calcium oxide (30-40%) and magnesium oxide (3-6%), with low concentrations of fertilizer K_2O (2-6%), and P_2O_5 (0.9-2.5). Therefore, effective benefits are here gained by adding these residues to agricultural compost.

Grape marc is usually energetically exploited by anaerobic digestion; however, as it is strictly season-available, it is co-digested with other feedstocks. Concentrations in the range of 2.5-3% in the feedstock mixture are used, to have a stable digestor "diet". Pressed grape marc yields 150 Nm^3/t 55% methane biogas. Therefore, for 1 MWh/y (a typical farm-based plant) actual energy production from grape marc is 2.5-3.5%. Residual digested phase characteristics are therefore dependent more upon the other major feedstocks. Typically, a maize-barley silage complement is used, as previously indicated. Digestion phase is 58% w/w of the input feedstock, and is typically 44% w/w in liquid form and 14% w/w in solid form, with a N concentration of 0.5 and 0.4%, respectively. Therefore, not only energy was produced by exploiting spent biomass (2.5-3.5% of a 1 MWh/y plant), but also a proportional percentage of the actual Nitrogen of the digested phase applied to the soil (Figure 58).

Fig. 58. Utilization of spent biomass from grape marc from INSTM (at Banfi, Montalcino, SI, and Fattoria autonoma Tabacchi).

Then Olive pomace was successfully used as a preplant composted amendment in combination with fossil fertilizers on wheat, with a corresponding reduction of the applied chemical fertilizers (reference element for calculation is Nitrogen concentration). In Table 21, the total macro and micronutrients on a dry weight basis are reported.

pH	6.8	**Mg (%)**	0.96	**N org. (%)**	1.1	**Cu (ppm)**	13.3
EC (dS/m)	1.2	**Na (ppm)**	180.0	**C/N**	32.2	**Mn (ppm)**	66.7
OM (%)	60.3	**K (%)**	0.29	**P (%)**	0.03	**Zn (ppm)**	9.8
OC. (%)	35.5	**Fe (ppm)**	2.6	**Ca (%)**	1.2	**Humidity (%)**	49.6

Table 21. Macro- and micronutrinets from olive pomace from INSTM.

When the spent olive pomace and its fractions were used in anaerobic digestion, using a mixing technique similar to that indicated for grape marc, the biochemical methane potential (BMP) was as shown in Table 22.

Character	Denutted Pomace	Pulp (Paté)	Fibre
Dry Matter (DM) %	27.8	19.9	34.5
Organic Matter (% DM)	93.6	82.2	97.0
BMP – Methane (Nm^3/T as it is)	81.4	70.6	60.3
Methane in biogas (%)	64.7	63.9	61.9
Maize silage equivalency *	0.73	0.64	0.54
(*) 33% total solids, 4% ashes, and BMP = 350 m^3 CH_4/volatile solids (110.9 m^3 CH_4/T as it is)			

Table 22. BMP from olive pomace and its fractions from INSTM

A bright example of the importance of a correct recycling of spent and waste biomass was thus developed by INSTM at by Fattoria Autonoma Tabacchi, where some of the EVERGREEN experiments on the effect of polyphenols for nematode control were also carried out on Tobacco. Here, three anaerobic digestion plants were demonstrated to produce 21,968,000 kW/year (Figure 59).

Fig. 59. Anaerobic digestion plants used from INSTM at Fattoria Autonoma Tabacchi.

Most of the energy thus obtained was used in the frame to develop new circular economy activities and social benefits. In particular, the hot water produced in the anaerobic digestion plants was and still is used to heat greenhouses during wintertime to grow vegetables all the year around (Figure 60).

Fig. 60. Energy produced by spent biomass useful for green house heating at Fattoria Autonoma Tabacchi.

Besides the spent biomass from the EVERGREEN project, the INSTM C5 monitoring activity carried out at Fattoria Autonoma Tabacchi showed that the total energy from renewable energy (anaerobic digestion, combustion, and photovoltaics) contributes for 41.74% (51,510,400 kWh) to the annual energy requirement, keeping energy cost sustainable for some of the most energy-intensive farming operations. Less CO_2 emissions contribute to reduce tobacco and vegetables carbon footprint, where in particular in 2015-2016 savings were in the range of 16,700 T/year CO_2.

Problems encountered
No particular problems have been encountered during this Action C5, and this action was completed by 80%, as scheduled.

Verification of the expected results actually achieved
The detailed description of EVERGREEN Action C5 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 12 Deliverable Action C5.
The expected results were:
• Monitoring of the economical beneficial effects of the use of the spent biomass for bioenergy production and of the recycled digested phase as a soil amendment.
The activities carried out in this Action C5 had the objective to monitor how the use of the spent biomass from the extraction of the EVERGREEN polyphenols is in the frame of what requested by the Circular Economy Package proposal by EU. Here it was monitor, using already existing plants, how spent biomass can represent a valuable source of material and energy, with new opportunities for local investments and activities.

Perspectives for continuing the action after the end of the project
According to the activities and the results obtained in Action C5, INSTM was already involved several EU projects and calls (*e.g.* LIFE16 CCM/IT/000079; *SuN-MaBios*, Fondazione CARIPLO 2016 Call on Integrated Research on Industrial Biotechnology) and several similar applications on H2020 calls are in progress.

6.1.13 Action C.6: Monitoring of the absence of a selection on the polyphenolic preparations on copper and antibiotic resistant bacteria, on plant and in soil, from laboratory to in field screenings.

Starting date foreseen: 1st of July 2015
Actual start date: 1st of July 2015
End date foreseen: 30st of September 2016
Actual end date: 30st of September 2016

The results of the activities carried out in Action C.6 are defined in the following project Deliverable, foreseen at the end of the 15th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 13 Deliverable Action C6: Monitoring of the absence of a selection on the polyphenolic preparations on the selection of copper and antibiotic resistant bacteria, on plant and in soil, from laboratory to in field screenings.

Activities carried out
This demonstration was delegated to UNIFI-DISPAA and CEBAS CSIC, and was completed by 100%.
Pesticides, such as copper derivative and nematicide compounds, are going to accumulate in the soil environment as a consequence of their use in agriculture against plant pathogenic bacteria and nematodes. Changes in bacterial community structure to adapt, directly or indirectly, to the stress given by the presence of pesticides residues have been demonstrated, and horizontal transfer of several resistance genes (e.g. for antibiotic or copper) is occurring as well, with an increasing and huge risk for the humans and animals health.
This Action has to monitor the absence of an indirect selection operated by the polyphenolic-based molecules here used for plant disease control on copper- and antibiotic-resistant bacteria, to assess the further benefits deriving from the use of these innovative plant disease control preparations in comparison to conventional pesticides against plant pathogenic bacteria and nematodes.
According to experiments carried out in Actions B.1 and C.1 (on epiphytic and soil microflora, respectively), the same analitical methods were used for kiwifruit, olive and tobacco plants, to monitor the absence of a selection on the polyphenolic preparations on the selection of copper and antibiotic resistant bacteria.
As reported in Deliverable B.7, field experiments have been carried out on representative strains of the bacteria Psv (Olive) and Psa (Kiwifruit). While *Meloidogine incognita* was used to induce in Tobacco roots shortening and deformation as well as a decrease of root efficiency of plant. According to Deliverable B.7, activities were focused on the use of the EVERGREEN polyphenolic formulations developed in the frame of this project: Form 1 (1L): Sweet chestnut tannin 2% w/v (total volume), Olive extract 1% w/v (total volume); Form 2 (2L): Sweet chestnut tannin 1,5% w/v (total volume), Olive extract 1% w/v (total volume), by-product grape seeds post-oil extraction 0,3% w/v (total volume). All informations related to application of EVERGREEN polyphenols and positive controls (copper and nematicides) were reported in Deliverable B.7.
In the last years there has been an increase in molecular-based methods for profiling microflora. One of the most adopted in soil microbiology is the Phospholipid Fatty Acids (PFLAs) analysis, which represents changes occurring in the soil microbial community structure by their PFLAs signatures. This approach can be combined with DNA-based fingerprinting techniques, such as polymerase chain reaction (PCR) amplicons of 16S rDNA sequence of bacteria, and more recently also High Resolution Melting Analysis (HRMA), which is a very informative and cutting edge PCR-based method.

According to the sampling protocol defined in Deliverable C.6, Phospholipid Fatty Acids (PLFAs) and Fatty Acid Methyl Esters (FAMEs) analysis were carried out, together with traditional microbiological tests and molecular assays related to the direct monitoring of variations occurring on the composition of soil and epiphytic micorflora following treatments.
Total PLFAs didn't decrease in epiphytic and soil microflora when EVERGREEN formulations were added, otherwise when copper and nematicides were applied as positive control strategy (Figure 61A).

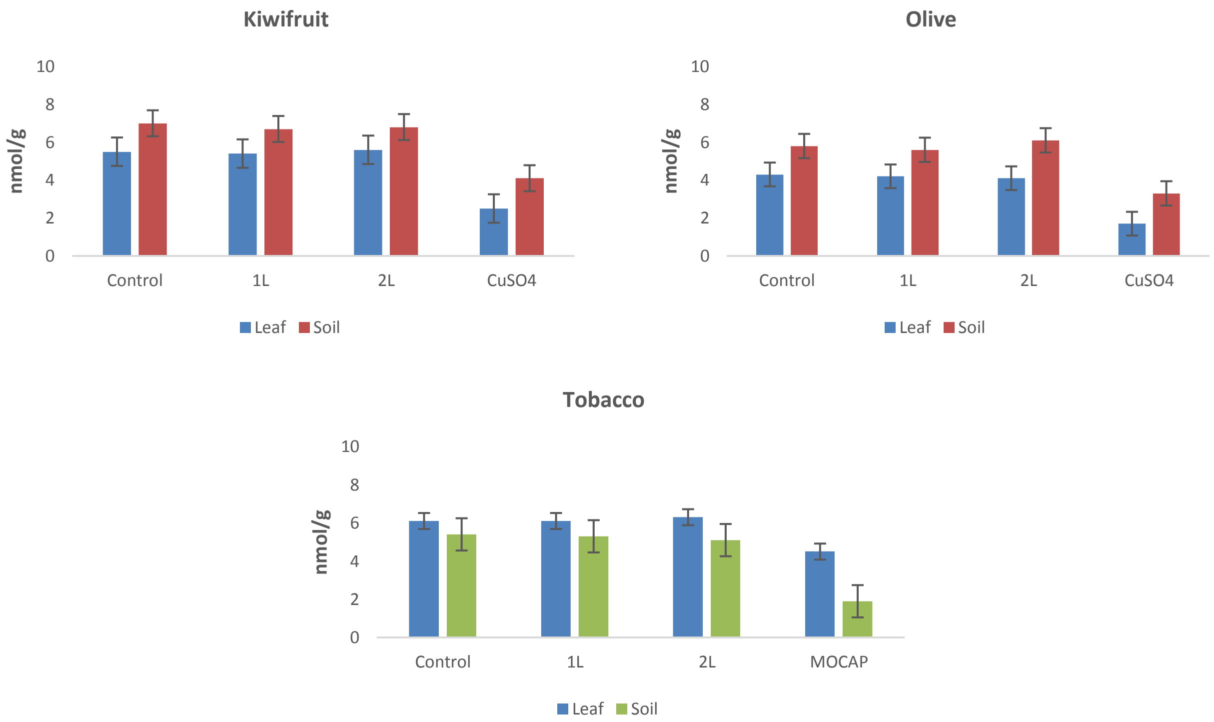
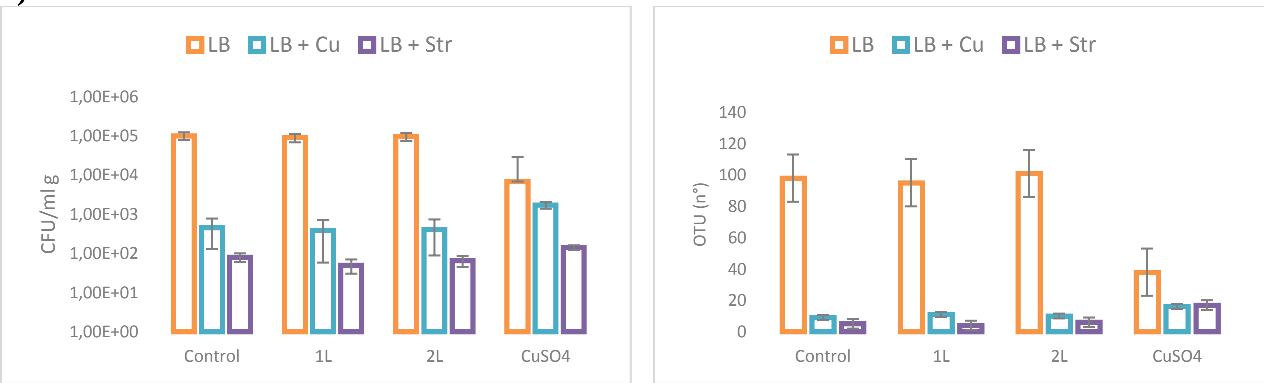


Fig. 61A. PFLAs analysis after treatments with EVERGREEN formulations (1L and 2L), copper and nematicide (Mocap), on soil and epiphytic microflora after one month.

These microbiological statements were verified by a genetic analisys, HRMA-based, to evaluate a possible increase/decrease of bacterial strains resistant to copper and, as an indirect consequence, of antibiotic-resistant strains after field treatments. To this purpose, only leaf surfaces of kiwifruit and olive plants were analised and results reported in Figure 61B, in order to assess the improvement/worsening in environmental biodiversity.

a)



b)

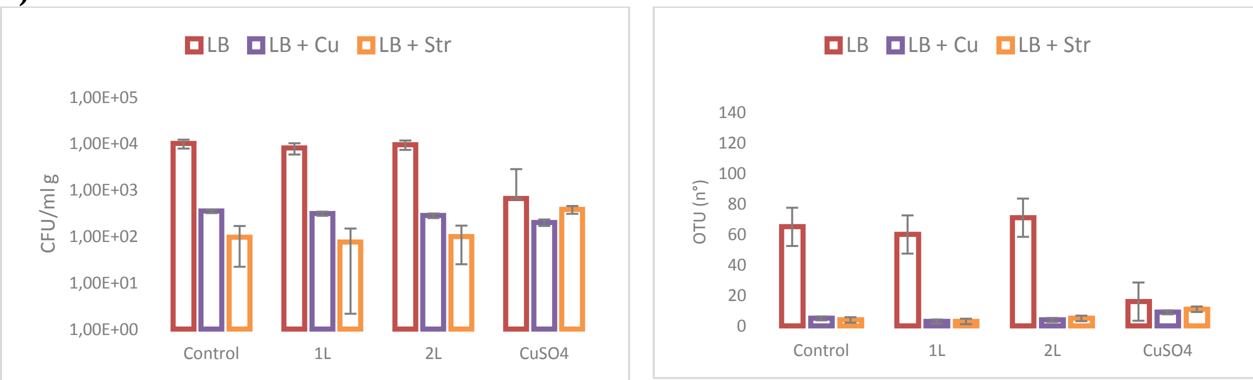


Fig. 61B. Bacterial microflora on leaf surfaces of kiwifruit and olive plant evaluated by HRMA. Bacterial microflora (as UFC/ml) and variability (as OTU) on Kiwifruit (a) and Olive (b) treated with 1L and 2L (polyphenolic formulations), copper ($CuSO_4$) and untreated (control) after one month.

An increase in copper- and antibiotic resistant strains was observed to occur on epiphytic microflora into the fields, when plants were treated with copper, conversely when polyphenolic forms (1L and 2L) were used to reduce bacterial diseases, belonging to EVERGREEN approach.

Problems encountered

No particular problems have been encountered during this Action C.6, and this action was completed by 100%, as scheduled.

Verification of the expected results actually achieved

The detailed description of EVERGREEN Action C.6 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 13 Deliverable Action C.6.

The expected results were:

- Monitoring of the absence of the indirect emergence of copper- and antibiotic-resistant bacteria following treatments with the polyphenolic natural preparations.
- Monitoring of null indirect selective pressure strategy proposed for replacing traditional pesticides in the control of plant diseases caused by bacteria and nematodes.

This EVERGREEN monitoring C.6 activity demonstrated the urgent need to develop alternative and environmentally safe plant disease control products against phytopathogenic bacteria and nematodes. According to Deliverable C.1, data here reported showed the heavy negative effects on epiphytic and soil microflora of traditional nematicides and copper salts, according to chemical and microbiological tests, as well as to phenotypic and genotypic characterization. In particular, it was demonstrated the increase/emergence of resistance bacteria, towards copper and antibiotics (streptomycin), as well as nematicides. These consequences are a risk for both environment and human health, besides to determine a reduction of the effective use of these compounds in plant protection.

Perspectives for continuing the action after the end of the project
As reported also for other EVERGREEN Actions, during the project and just after its conclusion, both the coordinator as well as the other beneficieries have been contacted by stakeholders interested to monitor the effects deriving from copper and nematicids pollution in agroecostystems, such as Confagricoltura Toscana, with the perspective to be part of operative groups working on this topic at national at internation level.

6.1.14. Action C.7: Monitoring of technical-socio-economic assessment of the EVERGREEN project

Starting date foreseen: 1st of April 2015
Actual start date: 1st of April 2015
End date foreseen: 30st of September 2016
Actual end date: 30st of September 2016

The results of the activities carried out in Action C.7 are defined in the following project Deliverable, foreseen at the end of the 18th month of the project, and here attached in its final version as annex to this Final Report:
►Annex 14 Deliverable Action C.7: Monitoring of technical-socio-economic assessment of the EVERGREEN project.

Activities carried out
Action C.7 was carried out by CEBAS CSIC, UNIFI-DISPAA, and ASTRA as foreseen, and completed by 75%. The aim of this Action was to monitor that polyphenolic extracts comply with quality standards and were socio-economically viable, increasing in particular the social awareness and acceptance of the benefits of protecting the environment. The limit to properly carry out this Action was given by partial results obtained in field trials carried out during summer 2015. In this first season, some problems have occurred about polyphenolic treatments, mainly due to their in-field application on the tested plants. Nevertheless, it was possible to develop a Life Cycle Analysis (LCA), comparing EVERGREEN formulations with traditional pesticides - $CuSO_4$ and Mocap - in Kiwifruit, Olive and Tobacco.
Life Cycle Analysis (LCA) should be used for the quantitative assessment of the environmental (mainly related to soil and crops), economic and social impacts during the life cycle of a product or a process. LCA may be implemented in various processes, such as the production of polyphenols from agricultural wastes.
The full life cycle of a product/process includes the phases of polyphenols (raw materials) extraction, processing, production, utilization and disposal of the final (biopesticide) products. The assessment of the impacts of selected or all the phases of the cycle should allow the identification of the most important ones and indicates the best management practices.
An integrated approach, including LCA and risk analysis in agriculture, may contribute to the establishment of guidelines for the controlled use of biopesticides as polyphenols to avoid the massive use of different agrochemicals (Cu salts and nematicides) to control several bacterial diseases. It is important for soils, crops, environment and farmers as an entire system whose components are interrelated.
Parameters to be taken into account include:
- Implementation of an integrated scenario for sustainable cultivations, including strategies for the monitoring of bacteria disease, soil and crop (soil-plant system).
- Quantification of the most important parameters/indicators affecting agricultural sustainability:
    a. Soil quality (pH, organic matter, N, P, K micro-nutrients and heavy metal content, soil biodiversity).
    b. Crop yield, biometric data and biomass production.
    c. Disease symptoms of crops.
- Minimization of the use of conventional agrochemical products (Cu salts, nematicides)
- Minimization of the cost (operating, production and labor) as well as maximization of the profits (e.g. from polyphenols sales or increased plant yield)
- Environmental benefits such as reduction of possible soil and environmental pollution

Problems encountered

During this Action, we have encountered some problems related to the evaluation of some parameters related to the energy used for the various steps of the global EVERGREEN process, given the short period for collecting data (less than 2 years). Due to these technical issues, several data are then to be considered less reliable to make a definitive assessment, and this Action can be completed by 75%,

Verification of the expected results actually achieved

The detailed description of EVERGREEN Action C.7 activities, the experimental details and the results obtained (included plots, pictures and diagrams) are reported and fully described in the Annex 14 Deliverable Action C7.

The expected results were:

- Proof of agricultural production quality and economic viability.
- Elaboration and analysis of data in terms of socio-economic impact on the local economy and population.

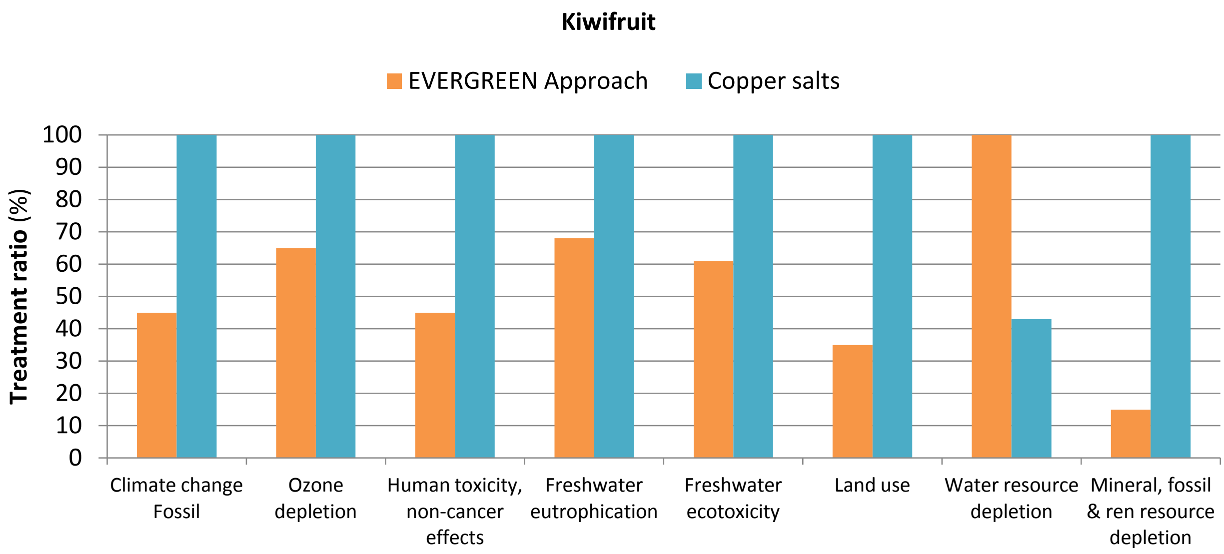
Life-cycle assessment is a technique to assess environmental impacts associated with all the stages of a product's life from raw material extraction, through materials processing, manufacture, distribution, use, and disposal or recycling. This approach was used to evaluate environmental impacts of EVERGREEN formulations against traditional pesticides (copper salts and Mocap). LCA developed in this Action allowed to produce important informations about great benefits related to polyphenolic extracts application to control plant diseases in agroecosystems.

Parameters taken into account for this environmental analysis were the following:

✓ Climate change Fossil
✓ Ozone depletion
✓ Human toxicity, non-cancer effects
✓ Freshwater eutrophication
✓ Freshwater ecotoxicity
✓ Land use
✓ Water resource depletion
✓ Mineral, fossil & ren resource depletion

As reported in Figure 62, the main benefits produced by EVERGREEN formulations, both on kiwifruit and olive plants, are related to all parameters evaluated, excepting for water resource depletion, where there is a large consumption of water for polyphenol extraction from spent biomass.

The same scenario was observed in the case of tobacco plants, as reported in Figure 63, where there was a greater reduction of the scores of all environmental parameters.



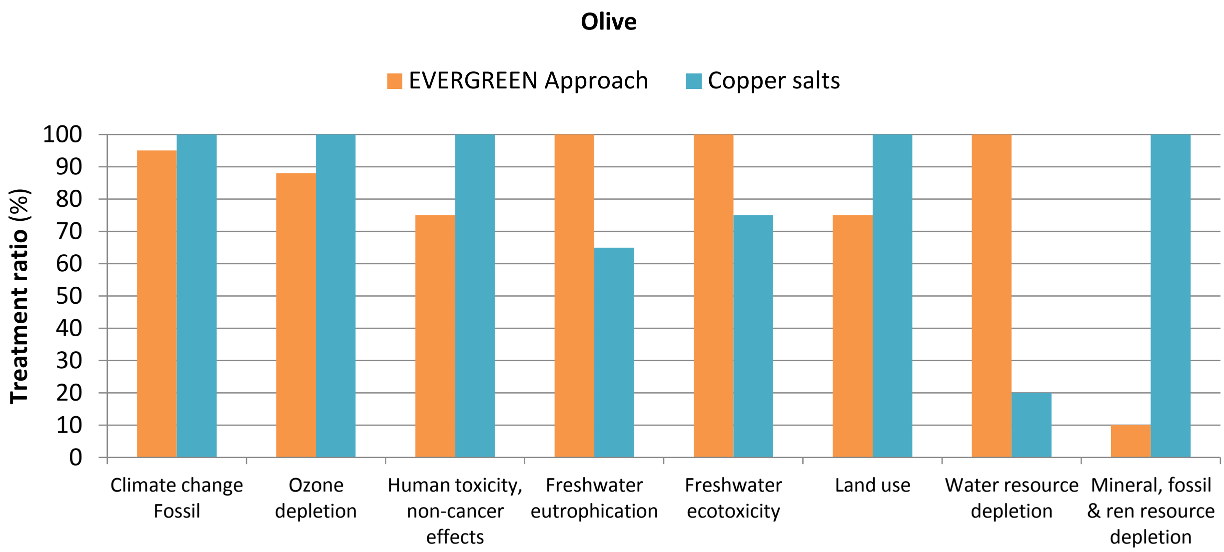


Fig. 62. LCA associated to EVERGREEN formulations (compared to traditional copper salts) to control bacterial diseases on Kiwifruit and Olive.

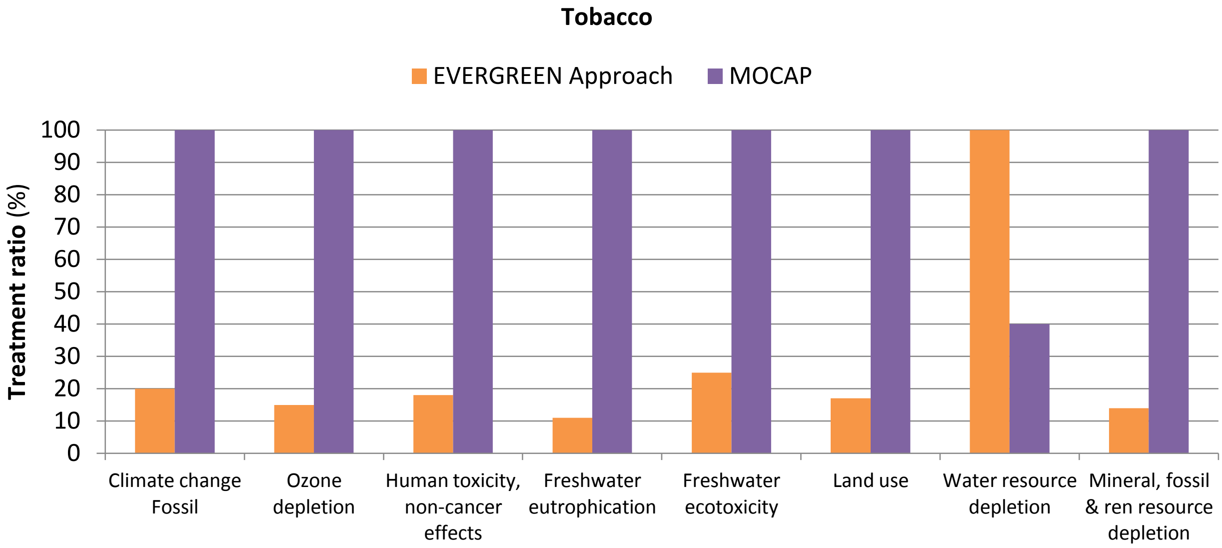


Fig. 63. LCA associated to EVERGREEN formulations (compared to Mocap) to control root-knot diseases induced by nematodes on Tobacco.

The control strategy based on the polyphenolic extracts still has today higher cost compared to that of traditional copper compounds and nemeticides, it must be taken into account that this approach provides benefits for agrosoil, with a lower need of mineral fertilization. Moreover, the EVERGREEN strategy has the potential to be widely applied with the same success whatever is the plant species tested. At least but not the last, priceless are the results about the total null toxicity of polyphenolic extracts and the recycling of spent vegetable biomass, and these data go definitely beyond the immediate economic convenience. This is finally a policy issue, which must be considered as a pivotal investment at EU level to maintain a safer and more fertile agroecosystem for the future generations and to implement recycling strategies with the aim to reduce social costs of waste disposal.

Perspectives for continuing the action after the end of the project

During and after the end of the EVERGREEN project, the coordinator has been contacted by companies, stakeholders and international research groups, interested in the effectiveness in plant protection and in the socio-economic impact of the EVERGREEN polyphenolic extracts.

6.2 Dissemination actions

6.2.1 Objectives

All the project beneficiaries made sure that the EVERGREEN project gained maximum visibility at a European level. During all the project life various dissemination materials were produced and distributed in fairs, conferences, workshops and so on. A logo was designed together with brochures, gadgets and various other items which allowed for a full dissemination of the project aim and results. 29 articles were also edited on newspapers, a project website and a Facebook page were created and published online within the second month of the project.
The tight, fruitful cooperation among EVERGREEN beneficiaries gave very positive results, feasible in the practice and also in dissemination activities.
Among the actions started, dissemination activities and material are fundamental in order to show the role of Life+ projects, in general, and the importance of EVERGREEN project, in particular, to people working in the soil, plant and agricultural sectors. EVERGREEN has been presented in different events, in a specific web site, in notice boards, in gadgets and in brochures. In particular, the EVERGREEN dissemination Actions (Actions D.1 to D.13) has been performed during all the project life detailing the type of EVERGREEN innovative technology and product.
All the beneficiaries were involved in the development and implementation of the following main EVERGREEN dissemination activities from the start of the project (01/10/2014) until the end of the project (30/09/2016):

- Development of the web site: DISPAA registered, developed and updated the website www.life-evergreen.com and the project Facebook page
- Notice board: DISPAA produced 10 EVERGREEN notice boards, which were sent to all the partners and displayed in visible spots and accessible places to the public on the partners' premises.
- Dissemination material:
    - project logo, 13,200 brochures/leaflets in Italian and in English, 1,500 pendrives, 1,000 notebooks, 500 cups and 100 pens as project gadgets, 12 posters and 3 project presentations (in Italian, in Spanish and in English)
- Events: EVERGREEN project was presented in 8 specific workshops and 13 events and fairs
- Articles and press release: 29 press articles

The target groups of the EVERGREEN dissemination material were:

- soil, plant and agricultural SMEs, Soil and Plant Associations and Institutions, technology transfer organisations managers, European environmental authorities and organizations, general public authorities;
- all the public and consumers.

The following table summarises and compares for each project Action which dissemination activity and material was foreseen in the project and which was the final real results.

DISSEMINATION PRODUCTS

Number of the Action	Name of the Action	Deadline	Expected results	Results at month 30
Action D.1	Website creation	3 months	Project web site	Project web site and continuous updating to general public
Action D.2	Notice boards	3 months	10 notice boards	10 notice boards displayed in beneficiary public places to general public visiting the beneficiary premises
Action D.3	Demonstration workshop in Italy	24 months	EVERGREEN workshops in Italy	DISPAA, ASTRA, INSTM e Mondoverde organised 6 workshops with the participation of soil, plant and agricultural experts, policy makers and business people
Action D.4	Demonstration workshop in Spain	24 months	EVERGREEN workshops in Spain	CEBAS organised 2 workshops with the participation of soil, plant and agricultural experts, policy makers and business people
Action D.5	Diffusion material preparation	24 months	Logo definition 25 posters 10,000 brochures/leaflets 2,500 various items	Logo definition 13,200 brochures/leaflets 12 posters 1,500 pendrives as gadget 1,000 notebooks as gadget 500 cups as gadget 100 pens as gadget 3 project presentations Distributed in workshops and project events to general public
Action D.6	Layman's report	24 months	Layman's report	1,000 copies of Layman's report to general public
Action D.7	Articles and press releases	24 months	30 articles	29 articles to general public
Action D.8	Networking	24 months	Clusters with 10 projects	Clusters with 10 projects and participation at 2 specific networking events
Action D.9	Technical manual	24 months	EVERGREEN manual	1,000 copies of the EVERGREEN manual

				sent to soil, plant and agricultural SMEs
Action D.10	International conferences and fairs	24 months	Participation at 6 fairs and international events	Participation at 13 fairs and international events with contacts and material distribution to soil, plant and agricultural managers and technicians, Soil and Plant Associations and Institutions, technology transfer organisations managers, European environmental authorities and organizations, general public authorities
Action D.11	Dissemination to institutions and policy makers	24 months	Successful communications	Contacts with 15 Institutions and policy makers during project events
Action D.12	After-LIFE Communication Plan	24 months	After-LIFE Communication Plan	After-LIFE Communication Plan
Action D.13	Digital supports for international diffusion	24 months	1,000 copies of 1 project video in English, Italian and Spanish	1,000 copies of EVERGREEN video in English, Italian and Spanish sent to soil, plant and agricultural managers and technicians

6.2.2 Dissemination: overview per activity

6.2.2.1 Action D.1Website creation

- Starting date foreseen: 01/10/2014 Actual start date: 01/11/2014
- End date foreseen: 31/12/2014 Actual end date: 30/09/2016

During November 2014 the web site www.life-evergreen.com and the project Facebook page were published and they are network-accessible in English, Italian and Spanish language. The site was periodically updated and it contains, in its public or reserved areas, all the documents produced during the project's activities, in particular:

- Visit counter;
- Link to LIFE+,
- Link to each beneficiary website;
- Results update;
- News update;
- Coming up;
- Reserved area;

- Link with the Facebook page for event booking/registration.

DISPAA was the responsible of the creation of the EVERGREEN web site and the project Facebook page. The project web site and Facebook page created are clearly and visibly marked with Life logo. In September 2016 the website visitors are 134,165 and the Facebook friends are 46.

6.2.2.2 Action D.2 Notice boards

- Starting date foreseen: 01/10/2014 Actual start date: 01/11/2014
- End date foreseen: 31/12/2014 Actual end date: 31/12/2014

During the first period of the EVERGREEN project DISPAA created the structure of the project Notice Board and produced 10 EVERGREEN Notice Boards, which were sent to all partners and displayed in visible spots and accessible places to the public on the partners' premises. The results of the activities carried out in Action D.2 were defined in the dedicated deliverable report, which was foreseen at the end of December 2015 and annexed to the Inception Report:

  - Deliverable Action D2: EVERGREEN Notice boards.

6.2.2.3 Action D.3 Demonstration workshop in Italy

- Starting date foreseen: 01/10/2015 Actual start date: 13/11/2015
- End date foreseen: 30/09/2016 Actual end date: 30/09/2016

The following 6 EVERGREEN workshops were organised in Italy:

- DISPAA organised two workshops:
  - on 13 November 2015 in Accademia dei Georgofili in Florence
  - on 29 September 2016 in DISPAA premises
- Mondoverde organised two workshops:
  - on 3 March 2016 in a Hotel in Borgo San Lorenzo (FI)
  - on 16 September 2016 in a Hotel in Borgo San Lorenzo (FI)
- ASTRA organised two workshops:
  - on 26 September 2016 in ASTRA premises in Cesena
- INSTM organised two workshops:
  - on 9 September 2016 in INSTM premises in Florence

The details and photos of the activities carried out in Action D.3 are defined in ANNEX 18: Annex Dissemination activities sent as annex to this Final Report.

6.2.2.4 Action D.4 Demonstration workshop in Spain

- Starting date foreseen: 01/10/2015 Actual start date: 12/11/2014
- End date foreseen: 30/09/2016 Actual end date: 30/09/2016

CEBASCSIC organised the following 2 EVERGREEN workshops in Spain:

- on 12 November 2014 in the University of Murcia in Salón de Grados
- on 28 September 2016 in CEBASCSIC premises in Murcia

Soil, plant and agricultural experts, policy makers and business people attended the two workshops.
The details and photos of the activities carried out in Action D.4 are defined in ANNEX 18: Annex Dissemination activities sent as annex to this Final Report.

6.2.2.5 Action D.5 Diffusion material preparation

- Starting date foreseen: 01/10/2014 Actual start date: 01/10/2014
- End date foreseen: 30/09/2016 Actual end date: 30/09/2016

During all the project period, all partners prepared various dissemination materials to be used in fairs, conferences, newsletters, etc, in particular:

- Logo definition and design performed. An EVERGREEN logo was created for the project, which was shown on all dissemination documents of the project;
- 12 EVERGREEN posters

- 10,000 EVERGREEN general brochures
- 200 EVERGREEN workshop brochures
- 3,000 EVERGREEN general plastic flyers
- 1.500 EVERGREEN pendrives as project gadget (two types)
- 1.000 EVERGREEN notebooks as project gadget
- 500 EVERGREEN cups as project gadget
- 100 EVERGREEN pens as project gadget
- 3 project presentations (In English, Italian and Spanish)

The details and photos of the activities carried out in Action D.5 are defined in ANNEX 18: Annex Dissemination activities sent as annex to this Final Report.

6.2.2.6 Action D.6 Layman's report

- Starting date foreseen: 01/04/2016 Actual start date: 01/04/2016
- End date foreseen: 30/09/2016 Actual end date: 30/09/2016

At the end of the project DISPAA created and produced 1,000 copies of the EVERGREEN Layman's report.

The results of the activities carried out in Action D.6 are defined in the following project Deliverable foreseen at the end of the project and attached as annex to this Final Report:

- ANNEX 11: Deliverable Action D6: EVERGREEN Layman's report

6.2.2.7 Action D.7 Articles and press releases

- Starting date foreseen: 01/10/2014 Actual start date: 01/10/2014
- End date foreseen: 30/09/2016 Actual end date: 30/09/2016

During all the project period, the project beneficiaries produced:

- 3 articles (Italian, Chinese and English) in Platinum journal
- 18 articles related to the LIFE event of ENEA and ASTRA in Faenza, on 22 January 2015 in local and web newspaper
- 1 article on Acta Horticulturae from the oral presentation at the "2nd World Congress on the Use of Biostimulants in Agriculture", Firenze (Italy), Nov. 16---19, 2015.
- 2 abstracts for Malaga conference, 2-5 June 2015
- Abstract to ICP-2016 Wien - - July 11-15, 2016 registration No. 280
- 4 web articles related to the project end

The documents of Action D.7 are defined in the ANNEX 19: full copy of all the project press articles.

6.2.2.8 Action D.8 Networking

- Starting date foreseen: 01/10/2014 Actual start date: 01/10/2014
- End date foreseen: 30/09/2016 Actual end date: 30/09/2016

During all the project, all the project beneficiaries were responsible of the following networking activities and have presented the project in different national and networking events, as listed below:

- Cluster with After-Cu, RESAFE, CLEANSED, BIOREM, AIS LIFE, SEMENTE, HORTISED, BIOBALE and COBRA (LIFE 11-12-13 projects)
- Horizon 2020 "From Biodiversity to Chemodiversity: Novel Plant Produced Compounds with Agrochemical and Cosmetic interest (AGROCOS)" KBBE
- Networking contacts at the UNIFI LIFE event at Firenze on 24th October 2014
- Networking contacts at the LIFE event of ENEA and ASTRA in Faenza, on 22 January 2015

The details and photos of the activities carried out in Action D.8 are defined in ANNEX 18: Annex Dissemination activities sent as annex to this Final Report.

6.2.2.3 Action D.9 Technical manual

Starting date foreseen: 01/01/2016 Actual start date: 01/01/2016

- End date foreseen: 30/09/2016 Actual end date: 30/09/2016

At the end of the project ASTRA and MONDOVERDE, with the collaboration of all the beneficiaries, have prepared the EVERGREEN technology manual, which was produced in 1,000 copies of the EVERGREEN manual and distributed and sent to soil, plant and agricultural SMEs.

The results of the activities carried out in Action D.9 are defined in the following project Deliverable foreseen at the end of the project and attached as annex to this Final Report:

- ANNEX 12: Deliverable Action D9: EVERGREEN technical manual

6.2.2.10 Action D.10 International conferences and fairs

- Starting date foreseen: 01/10/2014 Actual start date: 01/10/2014
- End date foreseen: 30/09/2016 Actual end date: 30/09/2016

All the project beneficiaries organised and presented EVERGREEN in the following events:

- "TOSCANA & AMBIENTE La sfida europea dei progetti LIFE" UNIFI LIFE event at Firenze on 24th October 2014
- LIFE event of ENEA and ASTRA "Dal confronto di esperienze diverse lo sviluppo di tecniche sostenibili", Faenza, 22/01/2015
- CORESTA Congress, October 12-16/10/2014, Quebec City (Canada)
- AOCS Annual Meeting, Orlando (USA), May 2-6/05/2015,
- Conference Psyringae 2015, Málaga, 2-5 June 2015
- EXPO Tuscany stand, Milan, 25 June 2015-06-26
- ECOMONDO, Rimini, 4 November 2015
- AOCS 2016 Salt Lake City (UT), USA, May 1-4, 2016, Session May 4, 2016
- Oral presentation at 47th Tobacco Workers' Conference Nashville (TN), USA, Jan. 11-14, 2016
- ICP-2016 Wien – July 11-15, 2016
- CORESTA Congress 2016 Berlin – Oct 9-13, 2016
- VI Food Bionergy Congress – Cremona 21 April 2016
- UNIFI LIFE event – Firenze 27 May 2016

In all the above events EVERGREEN project was presented and informative materials were distributed. The details and photos of the activities carried out in Action D.10 are defined ANNEX 18: Annex Dissemination activities sent as annex to this Final Report.

6.2.2.11 Action D.11 Dissemination to institutions and policy makers

- Starting date foreseen: 01/10/2014 Actual start date: 01/10/2014
- End date foreseen: 30/09/2016 Actual end date: 30/09/2016

Since the start of the project, all beneficiaries began to have some contacts with different Institutions and policy makers in order to organise specific project meetings and dissemination events. The following contacts and events with institutions have been taken:

- Environmental Department of Murcia Area, Regional Ministry of Agricultural of the Comunidad autónoma of Murcia (Dr. Francisco Javier of Murcia, Dra. Encarna of Molina – Spain (CEBAS workshop 2014)
- Firenze university rector, Georgofili Director (DISPAA and INSTM workshop 2015)
- Senator of the Italian Government - Italy (UNIFI event)
- Assessorato Agricoltura Regione Emilia-Romagna, ARPA Environmental Institution, Italian business center CENTURIA – Italy (ASTRA event 2015)

- Regione Umbria PSR funded Project QualiTaba (Traced quality of tobacco in a context of sustainable production in Central Italy) → double check in the year after this Project end (August 11, 2015)
- UNITAB (Union of European Tobacco Growers), CIA (Italian Confederation of Farmers), CONFAGRICOLTURA (General Confederation of Agriculture), FEDAGRI-Confcooperative, OPTA, and OPIT Meetings to prepare the proposal for the 2016-2020 Agro-environmental measures for tobacco in Regione Umbria, Toscana, Veneto and Campania
- Regione Umbria, Fattoria Autonoma Tabacchi, OPTA and OPIT Project for Certified Organic Tobacco from nursery to cured leaf starting in February 2016
- UniMI (coordinator): Proposal for an AGER Project "From OLive oil proDuction wastes to added-value compounds and biomaterials: an integrated BiOrefinerY – OLDBOY"
- Politecnico di Milano, UniPR and UniPI: proposal for a PRIN Project: "*Zero-Waste Agriculture for a circular bioeconomy in the production chains of tomato and tobacco*": from crop residues pharma and biostimulants/crop protectant
- Consortium Italbiotec and Politecnico di Milano Proposal for a Fondazione CARIPLO funded Project: "*Sustainable Nanostructured Materials to Formulate Innovative Living Biostimulants (SuN-MaBios)"*
- Univ. of Kentucky (USA) dr. Mihaylova-Kroumova, Jan. 2016: sclareol will be the best candidate for future studies due to its antifungal properties and availability.
- Meeting of April 14, 2016 at SIPCAM-OXON Central Office at Pero (MI) with the General Manager for Biostimulants Sandro Secco, CEO Giovanni Affaba, the President of the Administrative Board Nadia Gagliardini.
- Lab de Grasas y Aceites Dept. de Ciencia y Tecnoloìa de los Alimentos, Facultad de Quimica. Montevideo. Uruguay, Dr. Ignacio Vieitez Osorio co-Chairman at AOCS 2016
- Public Roundtable at Sansepolcro (Apr. 23, 2016) with the Candidate Mayor Mauro Cornioli and all the Kentucky growers of Tuscan Valtiberina, and the presence of about 100 citizens, included the principal entrepreneurs of the area
- Congress "Verde urbano sostenibile" 17/06/2016 Pisa with ODAF and CONAF representants

The details and photos of the activities carried out in Action D.11 are defined in ANNEX 18: Annex Dissemination activities sent as annex to this Final Report.

6.2.2.12 Action D.12 After-LIFE Communication Plan

- Starting date foreseen: 01/07/2016 Actual start date: 01/07/2016
- End date foreseen: 30/09/2016 Actual end date: 30/09/2016

At the end of the project, DISPAA created and produced the EVERGREEN After-LIFE Communication Plan.

The results of the activities carried out in Action D.12 are defined in the following project Deliverable foreseen at the end of the project and attached as annex to this Final Report:

- ANNEX 9: Deliverable Action D12: EVERGREEN After-LIFE Communication Plan

6.2.2.13 Action D.13 Digital supports for international diffusion

- Starting date foreseen: 01/10/2014 Actual start date: 01/10/2014
- End date foreseen: 30/09/2016 Actual end date: 30/09/2016

At the end of the project DISPAA collected all the video of each beneficiary and produced the final EVERGREEN video in 3 languages: English, Italian and Spanish. DISPAA produced 1,000 copies of the project video DVD which were sent and distributed after the project end to soil, plant and agricultural managers and technicians. The project beneficiaries decided to produce a standard

normal video with some subtitles in order to have an easier and more direct video to understand project activities and results for soil, plant and agricultural managers and technicians.
The results of the activities carried out in Action D.12 are defined in the DVD attached as dissemination annex to this Final Report:

6.3 Evaluation of project implementation.

The project coordination actions needed daily work to maintain a permanent flow of action with the aim of achieving the objectives set. The actions carried out were:

- Preparation of the Partnership Agreement
- 5 Coordination meetings
- 2 Monitoring meetings
- Organisation of different phone and web meetings between some partners in order to plan and monitor the project technical activities
- Continuous contact between all project partners for monitoring project activities
- General actions and activities for the coordination of the project.
- Management of the financial aspects of the project.
- Monthly reports to the LIFE external team monitor on the evolution of the project.
- DISPAA, as project coordinator, prepared and sent a monthly indication of operative activities to be done to all the partners
- DISPAA, as project coordinator, prepared and sent a monthly summary of the project activities carried out to monitoring representant and to all the partners

The following table compares through quantitative and qualitative information the results achieved at the end of the EVERGREEN project against the objectives of the proposal:

Action	Foreseen in the revised proposal	Achieved at project end	Evaluation
B.1 Demonstration of the performances of traditional pesticides for the control of bacterial and nematode diseases of plants important for the EU	• Study on the performances and efficacy of copper-based compounds and of the nematicides MOCAP (etoprophos) and AZA-NEMA (azadirachtine) for the control of Psv, Psa and Meloidogyne spp.. • Evaluation of the negative effects of copper-based products on the epiphytic populations of Psv and Psa, related to the emergence of copper- and antibiotic-resistant strains.	Report on performance of traditional pesticides and on their negative effects	No particular technical problems occurred during this period.
B.2 Demonstration of the qualitative and quantitative yields of extraction process for the	• Demonstration of the extraction procedure for the recovery of high quality polyphenolic molecules from agricultural not edible vegetable biomass and waste at laboratory scale.	Green chemistry extraction of high quality and standardised polyphenolic fractions and molecules from not edible vegetable biomass/waste of	No particular technical problems occurred during this period.

recovery of high quality polyphenolic molecules from not edible vegetable biomass and waste at laboratory scale.	• Demonstration of the extraction procedure in compliance with REACH regulations. • Demonstration of the outputs from extraction procedures carried out on different batches of the same vegetable biomass, at laboratory scale • Demonstration of the purity assessment procedure for these high quality polyphenolic molecules through chromatographic, spectrophotometric and spectrometric (HPLC/DAD/ESI-MS) methods.	chestnut, olive, artichoke and grapevine, and process optimization at laboratory scale	
B.3 Demonstration of the biological and of the chemical stability of the crude polyphenolic extracts and of their fractions, recovered from not edible vegetable biomass and waste, at laboratory scale.	• Demonstration of the most performing solvents to be used for the recovery and the further in vivo application of the high quality polyphenolic molecules. • Demonstration of the chemical and biological stability of the innovative high quality polyphenolic molecules. • Demonstration of antibacterial activity of single compounds and of mixture of different standardized extracts, evaluating their synergistic effect, in comparison with synthetic traditional antibiotics. • Demonstration of possible nematicide/nemastat action of the extracts under test. • Demonstration of antioxidant and radical-scavenging activities of the high quality polyphenolic molecules. • Demonstration of antioxidant and radical-	The EVERGREEN polyphenolic fractions and molecules are biologically and chemically stable, using water as the most performing and ecofriendly solvent, as demonstrated at laboratory level	No particular technical problems occurred during this period.

	scavenging synergistic effect of mixtures of different standardised extracts.		
B.4 Demonstration of the biological activity of the high quality polyphenolic extracts recovered from not edible biomass and waste, against plant pathogenic bacteria and nematode, in planta.	• Demonstration of the most performing combinations of the high-quality polyphenolic-based molecules to be used for the in planta application of these bioactive compounds. • Demonstration of the chemical and biological in planta stability of the innovative high-quality polyphenolicbased preparations.	The EVERGREEN high quality polyphenolic extracts are active against plant pathogenic bacteria and nematodes *in planta* at laboratory scale, using concentrations in the range 1-100μM, as demonstrated by traditional pathogenicity assay and molecular tests	No particular technical problems occurred during this period.
B.5 Demonstration of Kilo-scale extraction of the high quality polyphenolic bioactive molecules recovered from vegetable not edible biomass and waste.	• Demonstration of the most performing conditions for the pilot scale production of high quality and standardised poly-phenolic bioactive preparations from agricultural vegetable not edible biomass and waste. • Optimization of the process for the pilot scale recovery of high quality and standardised poly-phenolic bioactive molecules.	The Kilo-scale green extraction of the EVERGREEN polyphenolic fractions and molecules recovered from vegetable not edible biomass/waste was optimised	No particular technical problems occurred during this period.
B.6 Demonstration of the null toxicity profile of the high quality polyphenolic bioactive molecules recovered from vegetable not edible biomass and waste, on model organisms and microorganisms	• Determination of toxicological profile of these high quality standardised poly-phenolic preparations on microorganisms and organisms used as a model.	Report on the toxicological profile of high quality and standardised polyphenolic molecules on microorganism and organism	No particular technical problems occurred during this period.
B.7 Demonstration	• Demonstration of the best in vivo conditions for the	Demonstration of 4 optimised formulations	No particular technical

of the in vivo performances of the high quality polyphenolic bioactive preparations, recovered from vegetable not edible biomass and waste, at pilot scale level in field screenings.	maximum efficiency of the high-quality standardised polyphenolic preparations against phytopathogenic bacteria and nematodes. • Demonstration of the process for the in vivo application of the high-quality standardised polyphenolic preparations for the control of plant diseases caused by bacteria and nematodes.	on model systems at pilot and field screening, with beneficial effects on soil microflora	problems occurred during this period.
C.1 Monitoring on the environmental impact of copper compounds and nematicides for the crop defence against phytopathogenic bacteria and nematodes.	• Monitoring and evaluation of the environmental impact of copper salts and nematicides on soil microflora, related to theemergence of copper- and antibiotic-resistant strains, and of nematicide degrading bacteria. • Monitoring and evaluation of the environmental impact on the composition of bacterial microflora in copperand nematicide contaminatedsoils.	The *in planta* biological activity of the EVERGREEN high quality polyphenolic extracts is comparable to that of copper-based and traditional nematicides	No particular problems occurred during this period.
C.2 Monitoring of the absence of side effects for the high quality standardised polyphenolic preparations on common targets of any living organism at laboratory level.	• Monitoring of the biological activity of the high quality and standardised polyphenolic molecules and preparations on P-type ATPases in membrane model systems by electrochemistry. • Monitoring of the biological activity of the anti-virulence peptides on p-type ATPases from several model microorganisms and organisms by electrochemistry cutting-edge techniques.	The EVERGREEN high quality polyphenolic extracts do not possess any toxicity on organisms and microrganisms commonly used for acute and chronic toxicity tests, and on universally conserved subcellular targets such as Ca^{2+}-ATPase	No particular problems occurred during this period.
C.3 Monitoring of the absence of a direct selection operated by the polyphenolic	• Monitoring of the absence of polyphenolic-based molecules resistant bacteria following treatments with these compounds. • Monitoring of the low	The EVERGREEN high quality polyphenolic extracts do not cause any direct selection towards the emergence of bacteria	No particular problems occurred during this period.

preparations towards the emergence of bacteria resistant to the polyphenolic molecules themselves, at laboratory level.	selective pressure strategy proposed for reducing/replacing traditional pesticides against plant pathogenic bactyeria and nematodes.	resistant to the polyphenolic molecules themselves, as well as any cross-selection of copper- and antibiotic-resistant bacteria	
C.4 Monitoring of the short term environmental benefits from the use of the high quality standardised polyphenolic preparations in plant disease control at pilot scale level in field screenings.	• Monitoring of the beneficial effect of the polyphenolic based molecules and preparations on microbial community structure, incomparison to that of copper and nematicide treatments for control of plant diseases caused by bacteria and nematodes, respectively.	The EVERGREEN polyphenolic extracts are active in plant protection against phytopathogenic Gram negative bacteria and nematodes	No particular problems occurred during this period.
C.5 Monitoring of the economic benefits deriving from the recycling of the spent vegetable biomass after the extraction of the high quality standardised polyphenolic molecules at laboratory level	• Monitoring of the economical beneficial effects of the use of the spent biomass for bioenergy production and of the recycled digested phase as a soil amendment.	The spent vegetable biomass, at the end of the extraction of the EVERGREEN standardised polyphenolic fractions/molecules, can be recycled for energetic purposes and as fertilizers, as demonstrated for chestnut tannin, olive pomace and grape marc	No particular problems occurred during this period.
C.6 Monitoring of the absence of a selection on the polyphenolic preparations on the selection of copper and antibiotic resistant bacteria, on plant and in soil, from laboratory to in field screenings.	• Monitoring of the absence of the indirect emergence of copper- and antibiotic-resistant bacteria following treatments with the polyphenolic natural preparations. • Monitoring of null indirect selective pressure strategy proposed for replacing traditional pesticides in the control of plant diseases caused by bacteria and nematodes.	Report on the monitoring of the indirect selection absence on plant and in soil, from laboratory to in field screening	No particular problems occurred during this period.
C.7 Monitoring	- Proof of agricultural	The EVERGREEN	During this

of technical-socio-economic assessment of the EVERGREEN project	production quality and economic viability - Elaboration and analysis of data in terms of socio-economic impact on the local economy and population	approach is an ecofriendly and sustainable solution in plant protection in the frame of circular economy, as demonstrated by the LCA carried out on the processes concerning the most active EVERGREEN formulations	Action, we have encountered some problems related to in-field application of EVERGREEN formulations.
D.1 Website creation	Project web site	Project web site and continuous updating to general public	In line
D.2 Notice boards	10 notice boards	10 notice boards displayed in beneficiary public places to general public visiting the beneficiary premises	In line
D.3 Demonstration workshop in Italy	EVERGREEN workshops in Italy	DISPAA, ASTRA, INSTM e Mondo Verde organised 6 workshops with the participation of soil, plant and agricultural experts, policy makers and business people	In line
D.4 Demonstration workshop in Spain	EVERGREEN workshops in Spain	CEBAS organised 2 workshops with the participation of soil, plant and agricultural experts, policy makers and business people	In line
D.5 Diffusion material preparation	Logo definition 25 posters 10,000 brochures/leaflets 2,500 various items	Logo definition 13,200 brochures/leaflets 12 posters 1,500 pendrives as gadget 1,000 notebooks as gadget 500 cups as gadget 100 pens as gadget 3 project presentations Distributed in workshops and project events to general public	In line
D.6 Layman's report	Layman's report	1,000 copies of Layman's report to general public	In line
D.7 Articles and press releases	30 articles	29 articles to general public	In line
D.8 Networking	Clusters with 10 projects	Clusters with 10 projects and participation at 2 specific networking events	In line

D.9 Technical manual	EVERGREEN manual	1,000 copies of the EVERGREEN manual sent to soil, plant and agricultural SMEs	In line
D.10 International conferences and fairs	Participation at 6 fairs and international events	Participation at 13 fairs and international events with contacts and material distribution to soil, plant and agricultural managers and technicians, Soil and Plant Associations and Institutions, technology transfer organisations managers, European environmental authorities and organizations, general public authorities	More than expected
D.11 Dissemination to institutions and policy makers	Successful communications	Contacts with 15 Institutions and policy makers during project events	More than expected
D.12 After-LIFE Communication Plan	After-LIFE Communication Plan	After-LIFE Communication Plan	In line
D.13 Digital supports for international diffusion	1,000 copies of 1 project video in English, Italian and Spanish	1,000 copies of EVERGREEN video in English, Italian and Spanish sent to tanneries and leather managers and technicians	In line
E.1 Project management	Management of project activities	Continuous contact between all project partners and project meetings	Great beneficiaries collaboration
E.1 Project monitoring	Monitoring of project activities	monthly indication of operative activities and monthly summary of the project activities	Great help from monitoring team
E.3 Audit Report	Audit Report	Audit Report	In line

It is clearly evident from the above table that the work carried out during all the EVERGREEN project is perfectly in line with was expected in the EVERGREEN proposal.

6.4 Analysis of long-term benefit.

Environmental benefits

Agriculture of the 21st century is characterized by a more carefull and rational use of about any technical approach ensuring production. While after the 2nd World War, the main aim was to obtain the highest yield, today more attention is given to the negative impact that an indiscriminate use of pesticides, herbicides and fertilizers could sometimes have 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 at the same time it has been definitely and widely accepted that more generally many human activities, in addition to agriculture, are increasingly influencing worldwide changes such as those related to global climate. Temperature is projected to drammatically increase in the next decades, together with a much greater variability in climate with more extreme weather-related events. Obviously, these climate changes are going to impact differently on the various regions on the world, with major implications for practical processes such as those related to agriculture, included plant disease control and management. Climate variability and climate extremes always have had a strong impact on agricultural production systems, but future changes are associated to additional challenges. Actually, the impacts of the occurring climate changes can be theoretically positive, negative or neutral, depending on each region of the globe or the periodof time, by causing a decrease, an increase or having no impact on plant diseases. Additionally, these impacts will concern not just plant crops, but any other organism and microrganism present in agroecosystems. As shown by the so called "disease triangle", any plant disease is a dynamic process during which a host and a pathogen interact to each other, both under the influence of the environment. Briefly, the environment can affect host plant growth and susceptibility, or pathogen reproduction, dispersal, survival as well as activity and virulence, or directly host-pathogen interaction. Therefore, climate changes are going, and in some cases already are, to heavily alter the geographical and temporal distribution of many phytosanitary problems. Actually, this means that climate changes will impact on plant protection with challenges related to new emerging risks, but also with new opportunities, depending on the scenarios where host/pathogen/environment are simultaneosly acting and interacting. Accordingly, a deep forecasting analysis of the potential impacts of climate change on plant diseases is essential for the adoption of the best adaptation measures, case by case, in order to avoid more serious losses and risks and to get new opportunities if present. Some models are already available to simulate several different global climate change scenarios and their impacts on plant disease distribution, severity, *etc*, that could be essential for future decision making processes. Moreover, it is worth to mention that climate changes are able to affect chemical control of plant diseases as well: for instance, changes in temperature and precipitation can alter pesticides residue dynamics or the degradation of products can be modified, or enriched CO_2- atmosphere can indirectly affect the penetration, translocation and mode of action of systemic pesticides, *etc.* In other words, a new approach is needed to develop innovative pesticides whose effectiveness is not affected by climate changes. In addition, a general awareness and a strong pressure is 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. Although heavy metals are considered one of the major sources of soil pollution (Karaca *et al.*, 2010), causing long-term hazardous effects on soil ecosystems, and negatively influencing soil biological processes, there is a worldwide concern about copper pollution as well, as a consequence of its use in agricultural practices since the late 19th century. The continuative use of copper-based bactericides and fungicides in plant disease control has led to long-term accumulation of this element in the surface of some agricultural soils throughout the world (Mackie *et al.*, 2012). Copper containing fungicides and bactericides are generally applied as a chemical spray. Therefore, in part treatments miss its target 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 (Wightwick *et al.*, 2010). Copper is an enzymatic cofactor in several metabolic processes and an essential trace element for crop growth at low concentrations (Dewey *et al.*, 2012). However, when copper accumulates within topsoil following phytoiatric treatments (Pietrzak & McPhail, 2004; Rusjan *et al.*, 2007), its potential to cause adverse eco-toxicological effects on the environment is large. As far as European Union is concerned, its Member States have established the limits for several heavy metal in agricultural soils, which for Hg, Pb, and Zn are 1–1.5, 50–300, and 150–300 mg kg −1 dry soil, respectively (CEC, 1986). Traditional copper compounds applied in conventional and organic agriculture against plant pathogenic bacteria and fungi were recently restricted in their use within the EU Member States (Council Regulation 2015/229/EU; Directive 2009/37/EC; Council Regulation No 834/2007/EC; Council Directive 91/414/EEC). The process to minimize copper use in plant protection started in the year 2000, with a limit of 8 kg/ha, which was taken up by the EU regulation (EC Reg. 473/2002) establishing a limit of 8 kg/ha/year until 2005. Further reductions were established up to 6 kg/ha/year, with the possibility to make an average over 5 years in perennial crops. 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 compounds are 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 (Ruyters *et al.*, 2013).

Moreover, the regular and long term use of copper for the plant disease control caused the development and spread of bacterial strains resistant to copper, which make thus ineffective copper applications. Copper resistance mechanisms in bacteria are related to the occurrence into the bacterial genomes of genes encoding resistance to otherwise toxic concentrations of copper. The selection of resistant strains increases rapidly in the phytopathogen population, as an evolutionary surviving strategy following the copper sprays which thus become gradually less effective for disease control. These genes for copper-resistance have been found in bacteria from human, animal, plant, environment and food samples. They are often associated with another important feature, which is the antibiotic-resistance, coded by genes generally located on those mobile elements of the bacterial genomes called “plasmids”, easily exchangeable among bacteria, where also gene for copper–resistance. Therefore, the spread of copper resistant bacterial strains is often combined with antibiotic-resistance in the soil of agro-ecosystems, where resident bacteria can be potential reservoirs for antibiotic resistance genes with important consequences in clinical medicine (McManus, 2014). The fear of potential transfer of antibiotic resistance from plant to human pathogenic bacteria or other bacteria in nature drives the scrutiny on copper use for plant protection and aims to preserve antibiotic efficiency in human medicine (McManus, 2014). Similarly, nematicides are higly pollutant. Moreover, after the ban of the ozone depleter methyl bromide, the few synthetic nematicides remained tipically have a poor target specificity, and are still are a risk for soil and groundwater contamination, and more generally for the human and environmental safety. In addition, given their excessive use in the past, there has been an enhancement of biodegradation mechanisms that decrease the permanence of a bioactive concentration of nematicides in soil, with a lack of efficacy under field conditions.

The EVERGREEN project provides an innovative effective alternative to reduce copper and nematicides use in plant protection, by an environmentally friendly, sustainable and integrated strategy based on a cost-effective exploitation of no food/feed vegetable biomassfor the “green chemistry” extraction of high-quality polyphenols used for the control of bacterial and nematode diseases of plants. Therefore, the EVERGREEN project fits perfectly into the concept of “circular economy”, and allows important short and long term environmental benefits concerning the management of vegetable agricultural wastes. Moreover, the EVERGREEN approach perfectly

matches the restrictions established within the EU Member States concerning the use and the placing on the market of plant protection products, as well as the package on food security recently proposed by the European Commission to modernise, simplify and strengthen the agri-food chain in Europe. Recently many directive and documents have been produced by EU about European policy on agriculture, which has currently a double aim, that is to maintain productivity and to reduce its impact on the environment at the same time. The Common Agricultural Policy (CAP) document “The CAP towards 2020” invite the EU to make highly targeted and strategic choices for the long-term future of EU agriculture and its rural areas. In particular, “The CAP towards 2020” recognizes agriculture as a strategic sector for Europe, able to generate economic growth and employment, for instance by increasing the potential of a pivotal context such as food security. To this aim, high quality standards for the safety of agri-food products have established as common all over Europe, which have to be respected in order to be able to competitively satisfy the expectations of the internal EU market and outside Europe. Moreover, “The CAP towards 2020” also aims to preserve the food production throughout the EU, in order to both guarantee long-term food security for EU and contribute to the growing world food demand.
These topics such as food safety, traceability and quality of agrifood products have to be are integrated transversely with other essential issues, such as environmental protection and protection of natural resources, as well as of rural areas, while respecting their populations and human resources. In fact, according to "The CAP towards 2020” the future success of modern agriculture in Europe must necessarily include a sustainable development model.
The results obtained by the EVERGREEN project offer strong basis for a positive impact on future EU policy and governance, primarily for the implementation of a EU common Action Plan to achieve sustainable use of pesticides. Moreover, the EVERGREEN project perfectly fullfills the objectives of many tematic Stategies and Framework Directives (*e.g.* Water Framework Directive 2000/60/EC, EU Thematic Strategy for Soil Protection, *etc*) and meets many EU standards in terms of environmental protection.
Accordingly, the EVERGREEN project had the following targets and objectives:
- to reduce the impact of copper and nematicides on the environment and on human health, by providing an effective protection of crops against Gram negative phytopathogenic bacteria and plant parasitic nematodes,
- by using “green chemistry” polyphenolic extracts from the recycling of agricultural no food/feed vegetable biomasses demonstrated to be effective in plant protection,
- having no short- and long-term toxicity on organisms, microrganisms and ecosystems,
- having null ability to cause the onset and the spread of any direct and cross-resistance,
- able to contribute to the reduction of greenhouse gas emissions given by the direct incineration of these vegetable biomasses,
- and to the reduction of the pollution of water and soil for their disposal in the environment,
- by their cost-efficient management, having a beneficial effect on farmer budgets, enabling a strong sustainable economic growth in the agricultural sector.

The validity of the EVERGREEN approach and of the achieved targets is sustained by the data fully reported in the Annexes related to the Deliverables of the EVERGREEN project.
These demonstration activities clearly show as the EVERGREEN approach and its high-quality polyphenolic extracts/formulations allow to get an effective protection against Gram negative bacteria and nematodes here used as a model, as demonstrated by a multi-level analysis (*i.e.* from laboratory, to pilot and even to field scale). Although several differences were obviously found to occur from country to country, data from the EVERGREEN project clearly provide evidence about the applicability and the high flexibility of the proposed alternative for copper and nematicides use in plant protection, as demonstrated in the 2 beneficiary countries (Italy and Spain), having different climate conditions.

Long-term benefits and sustainability

The long term qualitative environmental benefits of the project consist primarily in a lower pollution into agrosoils and water by copper and nematicides, as well as in a reduction of their residues into food/feed of vegetable origin. As here demonstrated, among the main consequences there is an improvement of soil biology, which in turn will improve basal plant health and vigour, and thus agricultural performance and crop yields. This will inevitably contribute to global economic benefits, also deriving directly from the EVERGREEN approach, based on the principles of the circular economy, and where a vegetable waste is here used as a precious source of high-quality bioactive extracts to be used in ecofriendly plant protection against phytopathogenic bacteria and nematodes. Furthermore, a sustainable solvent is used for their extraction and the spent biomass are also a renewable resource, to be then successfully used for bionergetic purposes.

Therefore, if applied on a wide scale the long term goals of the EVERGREEN project will be pivotal to achieve the objectives set out recently by EU Parliament and Commission through several Directives and Thematic Strategies, (such as the Water Framework Directive, and the Thematic Strategies on the Sustainable Use of Natural Resources, that on Soil, on the Prevention and Recycling of waste, *etc.*), to end with the fulfilling of the principles of the Action Plan for the Circular Economy published by the European Commission on the December 2015.

In particular, the long term goals of the EVERGREEN project are:

(1) to develop ecofriendly alternatives to chemical pest control of plants against bacteria and nematodes, to reduce copper and nematicide use;

(2) to develop effective alternatives also giving substantial economic and social advantages;

(3) to develop alternatives which are not toxic or harmful for any living organism and microrganism in the short and long term, as well as on biodiversity.

(4) to develop strategies and “green chemistry” protocols for obtaining these effective alternatives starting from a renewable resource that are vegetable no food/feed biomass;

(5) to further valorise the spent biomass after the extraction process for bioenergetic purposes;

(6) to develop effective ecofriendly alternatives to chemical control of phytopathogenic bacteria and nematodes which have an overall significant reduction in risks;

(7) to develop effective ecofriendly alternatives to chemical pest control which can contribute to reduce emissions by avoiding any remediation treatment on soils heavily contaminated by copper and nematicides.

Replicability, demonstration, transferability, cooperation

The EVERGREEN demonstrative approach is essential and even pivotal in its contents to strongly contribute to define new policy strategies and activities by government, at any level form local to European, finalized to promote the use of the EVEGREEN polyphenolic extracts/formulations as an effective and environmental friendly alternative to copper and nematicides against Gram negative phytopathogenic bacteria and nematodes, respectively.

Moreover, the results of the EVERGREEN project can also contribute to create innovative and appealing scenarios for the stakeholders involved in the agri-food sector, including new job opportunities which exploit the whole EVERGREEN process (*i.e.* from extraction of high-quality polyphenols from vegetable no food/feed biomass, to the use of spent biomass for bioenergy). The results of the EVERGREEN project thus contribute to develop and spread new perspectives in agriculture management, and thus are also of great interest for national and European policy makers, as well as to the general public. Obviously, the dissemination of the EVERGREEN results has been and will be in the after-plan essential to persuade policy makers, stakeholders and farmers on the effectiveness of the EVERGREEN approach, both for crops protection against bacteria and nematodes of plants and for the global preservation of the environment and its resources. Dissemination will be thus crucial also to guarantee the long-term sustainability of the

EVERGREEN results, by finding further companies interested to produce at industrial level and to market the EVERGREEN polyphenolic extracts and formulations, by improving processes to increase even more their economic sustainability.
In addition to the demonstration studies carried out so far during the EVERGREEN project, specific demonstration actions will be further implemented and sustained after the project-end-date, such as:
1) to develop other EVERGREEN formulations, in addition to those set up and tested so far, to widen the plant diseases controlled by the EVERGREEN approach, firstly by Mondo Verde supported by UNIFI-DISPAA, INSTM, CEBAS CSIC and ASTRA.
2) to develop a detailed business plan, essentially by Mondo Verde, INSTM and ASTRA, to identify and compare the costs related to the replacement of copper and nematicides with the EVERGREEN formulations, supported by experimental data produced by UNIFI-DISPAA, CEBAS CSIC and ASTRA in several years of sperimentation on those systems/models/plots already set up and used for the project implementation.
3) to create and promote a strong and systematic dissemination of the EVERGREEN results, which needs to be carried out by each project beneficiary with own resources, related to the presentation of those findings achieved during the EVERGREEN project, as well as of those deriving from the activities of the After LIFE period. To this aim, the production of technical and scientific papers, on scientific journal as well as on specialized agricultural magazines, and of thematic printed brochures, digital supports and videos, is foreseen. In this frame, the EVERGREEN project web site will be constantly updated with the new findings concerning any EVERGREEN aspect, which will be also periodically reported on the institutional web pages of any EVERGREEN beneficiary.

Best practice lessons

According to the demonstration and monitoring activity carried out in the EVERGREEN project, new solutions and best practice were implemented to meet in the most efficient way the current EU legislation and policies about innovation in plant protection. One of the most importan best practice lessons here given is the collaboration for free that was obtained by the EVERGREEN beneficiaries from several stakeholders, strongly interested to the EVERGREEN approach (*e.g.* for the use of plants for kiloscale polyphenolics production, or biofermentors, *etc*).
Moreover, another best practice lesson is the demonstration of the high potential for transferability within the EU. The implementation of the EVERGREEN project was located in Italy and Spain, which are two important Countries of the Mediterranean Basin where the socio-economic impact of agriculture is highly very significant within the EU. These features were essential to give an international dimension to the results obtained by the EVERGREEN project, whose conclusions could be easily extrapolated in the next future also to other EU countries facing the same problems related to copper and nematicides pollution for conventional plant protection treatments (*e.g.* Greece, France, Portugal). This will be possible according to the climatic differences existing between Italy and Spain, but in the AFTER plan will be essential to expand the application of the EVERGREEN approach also in EU and no EU Countries where climatic conditions are also more severe and extreme, as well as to different types of cultivations (*e.g.* hydroponic). Therefore, if promoted and applied on a wide scale, also as a consequence of actions as advisors carried EU policy makers, the results of the EVERGREEN could strongly support the EU to play an international leading role in innovative and ecofriendly plant protection, and to improve the competitiveness of its agro-food economy sector.

Innovation and demonstration value

The EVERGREEN project contributes to the many European Environmental Objectives, by reducing the impact and the pollution given by some chemical treatments applied in plant protection, essentially copper-based bactericides and traditional nematicides, as well as to avoid most of the environmental problems they cause. The EVERGREEN project is also based on the virtuous recycling of no food/feed vegetable biomass, that otherwise have been used for incineration of other pollutant disposals. Moreover, the valorization of these vegetable biomass/wastes is by an innovative “green chemistry” approach and their spent residues can be further used for bioenergetic purposes, even on-site.

The main features of the EVERGREEN project and its results are crucial for the many challenges that EU is facing. For instance, European farmers and especially those of Mediterranean countries have to cope with those serious problems, concerning the value of land and often the also yields of crops, which have been constantly decreasing over the last 20 years. In addition, more than 40% of these agricultural holdings in Italy and Spain have an income (Farm Net Value Added per Annual Work Unit) below 50% of the average FNVA/AWU in the region. Furthermore, EU has to face the dramatic impact deriving from the ongoing climate changes having strong consequences on the spread of several alien plant pathogens. All these challenges innovative technological opportunities such as those derived from the application of the EVERGREEN approach definitely boost the competitiveness of the agro-food sector, by:

1. Reducing the risk of pollution of agricultural land by copper and nematicides;
2. Preserving the soil fertility and biology of agricultural land, and as a consequence its productivity;
3. Preserving the soil biodiversity and more generally the agroecosystem;
4. Reducing any negative impact on human health given by copper and nematicides residues on food of plant origin
5. Reducing any negative impact deriving by the no-ecofriendly disposal of vegetable no food/feed biomass.

The objectives achieved by the EVERGREEN project are strongly connected to many European environmental policies and legislations, such as the Common Agricultural Policy which has progressively included more environmental requirements in recent times. Moreover, the EVERGREEN approach perfectly fulfills what asked as mandatory by EU policy makers, that is to implement as integrated actions beneficial for both environment and agricultural production.

The EVERGREEN project has identified the following target groups and actors:

1) Public stakeholders and policy makers, which are in charge for the management policies concerning agriculture, plant protection and environmental conservation. Innovative and more environmental friendly policies for a stronger sustainable agriculture have to be introduced and promoted at the local, municipal, regional, national, and particularly at EU level.
2) Farmers and agricultural associations, which have to adopt a high-value innovation for the environmental friendly protection of plants from phytopathogenic bacteria and nematodes, to increase soil quality and plant productivity, and to reduce the risk for human health related to high pesticides residues on vegetables and fruits.
3) Companies involved in the production and marketing of plant protection products, which will have economic benefits for the introduction of the innovative and low impact EVERGREEN solution.
4) Universities, researchers and students, which are involved and work at different levels in several sectors related to the core objectives of the project.
5) Public opinion and civil society, which have a strong interest in the development and application of low impact and safe technologies for a more sustainable agriculture, and for environment conservation and protection.

Long term indicators of the project success

The long-term success of the application of the EVERGREEN approach and its polyphenolic extracts/formulations needs of solid and significant parameters and indicators to be properly evaluated.

In the recent report (29th April 2016) on enhancing innovation and economic development in future European farm management, by the EU Committee on Agriculture and Rural Development, more efforts were clearly asked to develop and fully implement integrated plant protection management systems by supporting scientific research into non-chemical alternatives and low-risk measures, included more environmentally friendly pesticides, accordingly to the current and relevant EU legislation. Moreover, this official document stresses that innovations could be further encouraged by a new legislation for a smarted approval of new products, as well as gives a roadmap geared towards a more sustainable pest management system, where advisory services have a pivotal role together with organic agriculture and control strategies that may contribute to a better plant resilience. In this frame, the EVERGREEN approach is definitely useful to help the EU organic farmers, which at the end of 2018 have to face the consequences derived from the new EU legislation about the reduction of copper in plant protection more than those devoted to traditional agriculture. Therefore, organic agriculture has to be urgently to be supported by the introduction of effective innovations in plant protection, alternative to the use of copper which is the only chemical still allowed in this kind of agriculture. This need derived also from the growing economic impact in the EU of organic agriculture, which is one of the most dynamic sectors, and that in 2004 had an average it accounts for more than 3.5% of total agricultural lands. In recent years, the EU organic market has grown significantly (19,700 million of Euros, and a growth rate of 9% in 2011), driven by a steady increase in demand. During the period 2000-2012, the organic farming increased the total surface with an average annual increase of 6.7%. In addition, following the adoption in 2009 of a number of provisions to EU level, it is also seeing a rapid growth in the production of organic aquaculture. Spain is the EU country with the largest area under organic farming and one of the largest in the world (1.8 million hectares), followed by Italy (1.1 million hectares), and Germany (1 million hectares) (Source EU, 2012). According to the data of the Italian Ministry of Agriculture, Food and Forestry Policies (MIPAAF) available at December 2013, the number of organic operators in Italy reached 52,383 units, of which 41,513 were producers, 6,154 were processors (including companies engaged in retail), 4,456 were engaged in the production and processing, and 260 were importers. The MIPAAF also indicated that the number of "operators" has increased to more than 55,000 farmers, occupying thus the top positions in the EU together with Spain. Organic farming in Italy was thus confirmed as an important sector of the food industry, with more than € 3 billion turnover and an exponential growth in consumption. These results are very positive from an economical point of view, and are also symptom of the growing awareness of society towards a type of agriculture with less environmental impact, and practiced with methods that are less likely to use synthetic chemicals.

Concerning the application of the EVERGREEN approach, its wide spread in the next future necessarily relies on the implementation of industrial processes for the production of the EVERGREEN extracts/formulations, with a strong marketable value, as well as on the further valorization of the spent biomass following extraction.

The final considerations on the general and specific benefits deriving from the EVERGREEN strategy in plant protection are the following:

1. Although a disease control strategy based exclusively on the EVERGREEN polyphenols can have today higher costs compared to that of traditional pesticides, at a different extent accordingly to the crops examined, it must be taken into account that this approach provides strong benefits and advantages to agrosoil and plant vigour, with a lower need of mineral fertilization;

2. however, the EVERGREEN approach relies on the use of vegetable biomass otherwise subjected to disposal, that actually are a cost for farmers, and the spent biomass can be further exploited for bioenergy production;

3. at the end, the importance to apply and spread the EVERGREEN approach goes definitely beyond the immediate economic convenience, because it is an innovative and integrated low-impact

strategy that must be considered as a pivotal investment to maintain a safer and more fertile agroecosystem for a sustainable agriculture.

7. Comments on the financial report.

All the project beneficiaries define all the main cost deviations from foreseen budget providing the following explanations:

- DISPAA:
  - Personnel:
    - Stefania Tegli during year 2015 achieved a carrier advancement from researcher to associate professor: for this reason its cost is higher, compared to the budget forecast. This promotion was effective since November 2015. In attachment Tegli curriculum.
    - Carlo Viti during year 2014 achieved a carrier advancement from researcher to associate professor: for this reason its cost is higher, compared to the budget forecast. This promotion was effective since November 2014. In attachment Viti curriculum.
    - Stefano Biricolti during year 2015 achieved a carrier advancement from researcher to associate professor: for this reason its cost is higher, compared to the budget forecast. This promotion was effective since November 2015. In attachment Biricolti curriculum.
    - referring to the higher daily rate costs of Patrizia Bogani in the project, we underline that the involved person, not foreseen in the proposal phase due to unexpected project development and internal reorganisation, had specific technical capacities and knowledge necessary for the development the project itself. Patrizia Bogani, for her role, technical-scientific profile and experience is an important staff member, as demonstrated by her *curriculum* in respect of the project topics.
  - External Assistance:
    - The unforeseen external assistance by PRIMM srl was essential for the assessment in planta effectiveness of EVERGREEN polyphenol in order to satisfy the needs for pilot and field scale experiments.
- CEBAS CSIC
  - Personnel:
    - Carlos Garcia has a cost euro/hour higher than indicated in the initial proposal. However, we want to point out that his participation, not foreseen in the proposal phase due to foreseen participation of another professor of different level, is now particularly important for EVERGREEN project since he is now the coordinator of this project in Spain. The monitoring of the Spanish work during EVERGREEN project by Prof. Garcia is considered of paramount important for the successful development of the same. Also, the participation of the Dr. Garcia in various meetings justify some of the hours worked, which should be considered in the project. In attachment Garcia curriculum.
    - Teresa Hernandez and Jose Luis Moreno are members of the research group in Spain; it was considered necessary their inclusion in the project, despite being not initially considered. The participation of these researchers for EVERGREEN projects has been considered as necessary in order to carry out the enzymes analysis and microbiological works on some soils included in the project. The expertise of these researchers can see showed with the CV. The meetings carried out between the Spanish Group in order to know the project development justify some hours in the EVERGREEN project. In attachment Hernandez and Moreno curricula.
  - External Assistance:
    - In the EVERGREEN project, CEBAS-GROUP carried out the characterization (analysis) of different polyphenols (seven polyphenols); in addition, we considered interesting for the project to verify if the use of these polyphenols could have also bio-fertilizer effect (polyphenols as bio-fertilizers) since they are a source of C for microorganisms. For this reason, we asked BIOIILIBERIS to carry out different

experiments in order to know if the used C sources (polyphenols in our case), could be available for microorganisms that solubilize phosphate. It would have given an additional value to the use of these polyphenols: polyphenols as bio-fertilizers. However, the results in this sense were not conclusive.

- Also for the EVERGREEN Project, we requested to Murcia University (laboratory service), the analysis of the different anions in soils by ionic chromatography, in order to know the implication of the use of polyphenols on compounds such as phosphates and chlorides. It was considered interesting for the project to demonstrate that the use of polyphenols has not any risk on soil quality.

➢ INSTM:

- Personnel:
  - The cost/hour of Sergio Miele is little higher than that planned in the proposal due to the involvement of its specific experience, fundamental in order to reach the project objective in a due time, for the coordination and technical demonstration activities.

➢ ASTRA

- Personnel:
  - Referring to the personnel cost, ASTRA involved also three technicians (Paola Sgarbi, Massimo Scannavini and Angelo Sarti) not foreseen in the early project proposal with a small higher daily rate. Their involvement was necessary during the project in order to investigate in detail different plants with unforeseen specific diseases in different locations. They are three agronomists expert in variety trials and fertilizer test with a long experience on pest and disease control.
- External Assistance:

  Le spese non previste inizialmente nel progetto inquadrabili come External Assistance sono state in particolare: ASTRA conducted mainly tests to verify the efficiency of the polyphenols and nematodes in the control of bacterial diseases. This activity realized in the greenhouse and in full field resulted cultivation operations which are not classifiable not only as a personal commitment but also of machines, also in the case of evidence of efficacy against nematodes it was necessary to find fields with sandy soil where there was the presence of the nematode and pay the costs of management and non-farmer income. Unplanned External Assistance costs were in particular:
  - CRPV is the parent company of ASTRA representing 97.15% of the shares. CRPV expresses the management of ASTRA as Sole Director as management and coordination. All this is done transparently and traced by resolutions of the Board of CRPV council and from the minutes of the Assemblies of ASTRA members. ASTRA instead of hiring a director or other figures that support the coordination of its projects has an agreement with CRPV and agrees to reimburse to CRPV the costs they incurred for work co-ordination, management and technical support. In particular, the CRPV invoice refers to the role played by dr. Vanni Tisselli who always attended the project meeting and the coordination actions on ASTRA internal staff, reviewing experimental protocols, overseeing the administrative aspects. The CRPV bill also includes all expenses for trips made by Dr. Tisselli Vanni for his participation in project meetings and dissemination activities. It is believed that the role of project and technicians coordination determined minor expenses and allowed to carry out the work at a cost of lower against than originally planned. With regard to corporate relations and the role provided by CRPV was not possible to outsource this type of job or ask for three quotes in order to choose the cheapest. In addition, we confirm that the same type of costs was already accepted and funded by the EC to ASTRA in previous LIFE projects (RESAFE LIFE12 ENV/IT/356 and After-Cu LIFE12 ENV/IT/336).

- ASTRA has a long experience on the plant diseases monitoring but needed the unforeseen involvement of the external company Amek which is expert in persistence and relative environmental impact of copper use
- Mazzocchi Ulisse. Costs for land management where control tests were performed on carrot nematodes. Sowing, irrigation, weed control, loss of income had been unable to sell the product not uniform and partly damaged, eliminating carrots, plowing and placing terrain.
- Lucchini Idromeccanica is a company specialized in the construction and maintenance of greenhouses. To set the tests on tomato, kiwi and olive tree maintenance to the structures present at ASTRA was necessary to make to ensure functioning of the openings and the positioning of shading nets to reduce the heat during summer days.
- CAB. Giulio Bellini. unforeseen small cost for transportation of tomato plants.
- Scala Alberto is a company that performs services disposing of specific machines for tillage, for the settlement of land, for the mechanical weed control, the mechanical pruning etc. Within the project it was involved in the management of land machining operations where the test was carried out on full tomato field and on tillage of kiwi plant.
- DAIVES is a mechanical company which works in the maintenance and repair of agricultural machines and for the control of equipment for the execution of pesticide treatments. In the project has carried out maintenance of the equipment used by ASTRA for the execution of its activities.

- Mondo Verde:
  - Personnel:
    - referring to your exception about the higher daily rate costs of employers Enrico Banci, Bernardo Banci, Paola Bigazzi, Elsabetta Zanier, Riccardo Gemmi involved in the project, we underline that the involved persons, not foreseen in the proposal phase due to unexpected project development and internal reorganisation, have specific technical capacities and knowledge necessary for the development the project itself. These employers are important staff members and their level of remuneration is high because of their professional capacity and knowledge and so really different in spite of a standard employer. In attachment Enrico Banci, Bernardo Banci, Paola Bigazzi, Elsabetta Zanier, Riccardo Gemmi curricula.

7.1. Summary of costs incurred.

The following table concerning the incurred project costs from the start of the project 01/10/2014 until the end of the project 30/09/2016.

Budget breakdown categories	Total cost in €	Costs incurred from the start date to 30.09.2016 in €	% of total costs
1. Personnel	696,000	732,521.33	105.24
2. Travel and subsistence	41,600	28,985.46	69.67
3. External assistance	127,500	159,724.39	125.27
4. Durable goods			
Infrastructure			
Equipment			

Prototype			
5. Land purchase / long-term lease			
6. Consumables	325,000	274,006.84	84.30
7. Other Costs		529.31	
8. Overheads	83,307	83,703.71	100.47
TOTAL	1,273,407	1,279,471.04	100.47

7.2. Accounting system.

Each beneficiary has a specific payment responsible

- DISPAA: Stefania Tegli selects the project cost formally approved by department director or DISPAA council
- ASTRA: Vanni Tisselli as company manager selects and decides
- CEBAS: Carlos Garcia Izquierdo selects the project cost formally approved by CEBAS council
- INSTM: Sergio Miele as company manager selects and decides
- MONDOVERDE: Banci Enrico e Banci Bernardo as company managers select and decide

All beneficiaries have defined the following internal specific code (*codice commessa*) which identify the project and all costs and income related to the project:

- ➢ DISPAA: B12I15000010006
- ➢ ASTRA: EVER
- ➢ CEBAS: 201310
- ➢ INSTM: UELIFE1301
- ➢ MONDOVERDE: 1020100032, 1020200028, 3020600041

For DISPAA, CEBAS and INSTM VAT is a cost
All beneficiaries respect the procedure of the best value for money for selecting all the project costs.
All the beneficiaries approved only the costs:

- ➢ directly linked to, and necessary for, carrying out the EVERGREEN project;
- ➢ reasonable, justified and comply with the principles of sound financial management, in particular in terms of economy and efficiency;
- ➢ compliant with applicable tax and social legislation; and
- ➢ actually incurred during the lifetime of the project, as defined in the grant agreement, and which could be identifiable and verifiable

All the beneficiaries completed in the electronic way all the project financial documents before printing them for the original signatures.
All the beneficiaries charged to the project only invoices contain a clear reference to the EVERGREEN.

7.3. Partnership arrangements.

UNIFI, as coordinating beneficiary, carried out that all the appropriate EVERGREEN payments were made to the other beneficiaries without unjustified delay in accordance with the agreements concluded with the associated beneficiaries in the Partnership Agreement.
All the beneficiaries entered directly the information in the financial tables of the EVERGREEN project.

7.4. Auditor's report/declaration.

The selected auditor is Eugenio Presta, Via del Falcione, 9, 56017 Asciano (Pisa), Accountant and Statutory Auditor - N° 659 in the Pisa Register - Statutory Auditor N° 141192.

7.5 Summary of costs per action.

The following table presents the allocation of the incurred project costs per Action from the start of the project 01/10/2014 until the mid-term period of the project 30/09/2016.

Action no.	Short name of action	1. Personnel	2. Travel and subsistence	3. External assistance	6. Consumables	7. Other costs	TOTAL
B1	Demonstration of the use of copper compounds for the control of bacterial diseases of plants important for the EU.	32,200					**32,200**
B2	Demonstration of the qualitative and quantitative yields of extraction process for the recovery of high quality polyphenolic molecules from not edible vegetable biomass and waste at laboratory scale.	32,700					**32,700**
B3	Demonstration of the biological and of the chemical stability of the crude polyphenolic extracts and of their fractions, recovered from not edible vegetable biomass and waste, at laboratory scale.	35,600			26,000		**61,600**
B4	Demonstration of the biological activity of the high quality polyphenolic extracts recovered from not edible biomass and waste, against plant pathogenic bacteria and nematode, in planta.	83,900			25,000		**108,900**
B5	Demonstration of Kilo-scale extraction of the high quality polyphenolic bioactive molecules recovered from vegetable not edible biomass and waste.	32,400					**32,400**
B6	Demonstration of the null toxicity profile of the high quality polyphenolic bioactive molecules recovered from vegetable not edible biomass and waste, on model organisms and microorganisms.	43,600			26,500		**70,100**
B7	Demonstration of the in vivo performances of the high quality	31,300		11,360.59	113,006.84		**155,667.43**

	polyphenolic bioactive preparations, recovered from vegetable not edible biomass and waste, at pilot scale level in field screenings.						
C1	Monitoring on the environmental impact of copper compounds and nematicides for the crop defence against phytopathogenic bacteria and nematodes.	51,200					**51,200**
C2	Monitoring of the absence of side effects for the high quality standardised polyphenolic preparations on common targets of any living organism at laboratory level.	28,100		4,623.80	19,000		**51,723.80**
C3	Monitoring of the absence of a direct selection operated by the polyphenolic preparations towards the emergence of bacteria resistant to the polyphenolic molecules themselves, at laboratory level.	32,300					**32,300**
C4	Monitoring of the short term environmental benefits from the use of the high quality standardised polyphenolic preparations in plant disease control at pilot scale level in field screenings.	54,700			28,000		**82,700**
C5	Monitoring of the economic benefits deriving from the recycling of the spent vegetable biomass after the extraction of the high quality standardised polyphenolic molecules at laboratory level.	88,500			18,000		**106,500**
C6	Monitoring of the absence of a selection on the polyphenolic preparations on the selection of copper and antibiotic resistant bacteria, on plant and in soil, from laboratory to in field screenings.	42,500			18,500		**61,000**
C7	Monitoring of technical-socio-economic assessment of the EVERGREEN project	36,000					**36,000**
D1	Website creation	1,000		6,000			**7,000**
D2	Notice boards	1,000		3,000			**4,000**
D3	Demonstration workshop in Italy	6,000		25,000			**31,000**
D4	Demonstration workshop in Spain	3,000					**3,000**
D5	Diffusion material preparation	1,500		50,836.80			**52,336.80**
D6	Layman's report	2,500					**2,500**
D7	Articles and press releases	1,000		11,500			**12,500**
D8	Networking	4,000					**4,000**
D9	Technical manual	6,000					**6,000**
D10	International conferences and fairs	5,500	15,732.31	18,500		500.00	**40,232.31**

D11	Dissemination to Institutions and policy makers	4,500					**4,500**
D12	After-LIFE Communication Plan						
D13	Digital supports for international diffusion	2,000		27,000			**29,000**
E1	Project management	51,521.33	13,253.15			29.31	**64,803.79**
E2	Monitoring	19,000					**19,000**
E3	Audit			1,903.20			**1,903.20**
Over-heads							**83,703.71**
	TOTAL	**732,521.33**	**28,985.46**	**159,724.39**	**274,006.84**	**529.31**	**1,279,471.04**

8. Annexes.

8.1 Administrative annexes

The EVERGREEN Partnership agreement was already sent as attachment of the Inception Report.

8.2 Technical annexes

In attachment the following Deliverables foreseen in this project period:

- ANNEX 1: Report with requested Addendum to Action B1:Demonstration of the performance of traditional pesticides for the control of bacterial and nematode diseases of plants important for the EU
- ANNEX 2: Report on the laboratory extraction process of high quality polyphenolic molecules from not edible vegetable biomass and waste: Action B.2
- ANNEX 3: Report on the laboratory analysis of the chemical stability of the extracted polyphenolic molecules: Action B.3
- ANNEX 4: Report on the planta activity of the high quality and standardised polyphenolic molecules: Action B.4
- ANNEX 5: Report on the kilo-scale extraction of high quality polyphenolic molecules: Action B.5
- ANNEX 6: Report on the toxicological profile of high quality and standardised polyphenolic molecules on microorganism and organism: Action B.6
- ANNEX 7: Report on the field performances of the high quality and standardised polyphenolic molecules: Action B.7
- ANNEX 8: Report on the monitoring of the environmental impact of copper compounds and
- nematicides for the cropdefence against phytopathogenic bacteria and nematodes: Action C1
- ANNEX 9: Report on the biological activity of the high quality and standardised polyphenolic molecules: Action C.2
- ANNEX 10: Report on the laboratory tests and studies on the monitoring of the selective pressure applied by the treatments with polyphenolic-based preparations: Action C.3
- ANNEX 11: Report on the environmental benefits from the use of high quality polyphenolic molecules at pilot scale level: Action C.4
- ANNEX 12: Report on the economic benefits of the recycling of the spent vegetable biomass: Action C.5
- ANNEX 13: Report on the monitoring of the indirect selection absence on plant and in soil, from laboratory to in field screening: Action C.6
- ANNEX 14: EVERGREEN LCA document: Action C.7
- ANNEX 15: Audit report: Action E.3
- ANNEX 16: EVERGREEN "After-LIFE communication plan": Action D.12
- ANNEX 17: EVERGREEN Layman's report: Action D.6
- ANNEX 18: EVERGREEN technical manual: Action D.9

In attachment the following technical documents:

- ANNEX 19: Technical report Action E.2: Questionnaire for the statistic surveys in Europe

8.3 Dissemination annexes

In attachment the following dissemination documents:

- ANNEX 20: Annex Dissemination activities
- ANNEX 21: Answers to EC recommendations
- EVERGREEN video DVD

8.3.1 Other dissemination annexes

In attachment:

- A project brochure in English
- A project brochure in Italian
- A pendrive as project gadget
- ANNEX 22: full copy of all the project press articles

9. Financial report and annexes.

In attachment the following Financial report and annexes:

- ANNEX 23 Curricula:
  - Prof. Carlos Garcia, Dr. Teresa Hernandez and Dr. Jose Luis Moreno (CEBAS CSIC)
  - Enrico Banci, Bernardo Banci, Paola Bigazzi, Elsabetta Zanier, Riccardo Gemmi (Mondo Verde)
- ANNEX 24: EVERGREEN Final output indicators
- ANNEX 25: "Standard Payment Request and Beneficiary's Certificate" - duly signed original
- ANNEX 26: "Consolidated Cost Statement for the Project" - signed original
- ANNEX 27: "Financial Statement of the Individual Beneficiary" completed for each project beneficiary, signed, originals.
- Audit report: Action E.3 (ANNEX 8)
- ANNEX 28: Copies of salary slips, timesheets, proof of payment of social security and calculation of annual gross salary for:
  - UNIFI- DISPAA: Stefania Tegli, Carlo Viti, Stefano Biricolti, Patrizia Bogani
  - CEBAS CSIC: Carlos Garcia, Teresa Hernandez and Jose Luis Moreno
  - Mondo Verde: Enrico Banci, Bernardo Banci, Paola Bigazzi, Elsabetta Zanier, Riccardo Gemmi
  - ASTRA: Massimo Scannavini, Paola Sgarbi, Angelo Sarti