Chapter

Hydrolysable Tannins in Agriculture

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Abstract

Hydrolysable tannins, water-extracted from sweet chestnut (*Castanea sativa* Mill.) (CHT) and membrane concentrated, have several effects as antioxidant, antimicrobial, and metal complexing agents. Some patents described their use as nitrogen release modulators and iron complexing agent to fight plant chlorosis and to control legume seed-borne disease and nitrosamine and mycotoxin during plant and food processing. Biostimulating activity of raw CHT, placed near seed or transplant seedlings, was assessed on early plant growth (starter effect) and found related to earlier production of a larger plant fine root mass, with greater P early uptake. Increased resistance to nematodes, with CHT applications on tobacco, was investigated. Recent process stream fractioning permitted to identify some CHT fractions with antimicrobial and antioxidant effects which were tested for their potential in promoting selected aspects of plant yield, quality, and protection and maintaining and improving feed and food quality during processing. EU Life+2013 Evergreen found a method of application of a CHT fraction to protect tobacco and carrot plants in nematode-infested fields. A protective effect of CHT on some bacterial diseases of olive tree and kiwi was disclosed. Environmental and soil toxicities were also investigated finding very low impacts and the possibility to reduce Cu use in agriculture.

Keywords: plant biostimulant, hydroculture, gall nematodes, TSNA, mycotoxins

1. Introduction

Polyphenols, a complex group of phytochemicals derived from phenylalanine, are characterized by an aromatic ring with a reactive hydroxyl group. They include phenolic acid derivatives, called hydrolysable tannins (HTs). They present a carbohydrate molecule (generally ∂-glucose), esterified at various levels with gallic or ellagic acids. These gallotannins and ellagitannins are hydrolyzed by weak acids or bases and are more prone to oxidation than condensed tannins (CTs) which are characteristics of oak wood and grapeseed extracts. Most HTs are typical of Mediterranean plants, but only tannins from water-extracted sweet chestnut (*Castanea sativa* Mill.) biomass (CHT) have been industrially exploited in agriculture as a corrective, a fertilizer chemistry modifier, and/or a biostimulant product, with a protective activity against nematodes and some microbial strains. Another important agricultural use, as a feed ingredient, is in expansion [1], while some technological applications in food processing and supplement production are just at their beginning [2, 3]. Other Mediterranean HTs "competitors" of CHT, i.e., those

of myrtle (*Myrtus communis* L.) and pomegranate (*Punica granatum* L.), have found so far a much larger use in herbal medicine and supplement production [4].

A previous paper dealt with water-extracted CHT chemistry, in particular with the different fractions that can be separated by membrane concentration and reverse osmosis [5]. These fractions can be industrially mixed to maintain a fairly consistent composition in the final product, with better consistency of the agronomic results.

CHT have several remarkable effects as antioxidant, radical scavenging, antimicrobial, and metal complexing agents. Typically, tannins are renowned for their capacity to precipitate proteins, pectins, and cellulose. So they can inhibit many enzymes [6]. This is considered one of the major causes that reduces urea losses for volatilization and—in general—modifies nitrogen release in soluble forms.

Due to their fraction of non-tannins, and to the chemical structure of the tannins, they easily form complexes with several polyvalent ions [7]. Those with iron, manganese, and zinc are retained particularly interesting under the agronomic standpoint.

However, the single, most interesting effect of CHT on plants is related to their biostimulant effect on plant early rooting, which determines an increased resilience to abiotic stress (water and nutrient shortage in the soil) and some biotic stress also, e.g., soilborne disease and nematodes [8].

2. Effect of CHT in plant fertilization

2.1 Effect of CHT on water and fertilizer acidification

The first characteristic of CHT our research team has investigated, since 1999, was related to the acidifying effect of these tannins when applied to water and fertilizers. For organic farming, which relies almost exclusively on unacidified phosphate rock as a P_2O_5 source, a microgranulated (0.5–0.8 mm ø) 5.14.0–15 SO_3–28 C fertilizer, for local application at crop planting (rates: 35–50 kg/ha), was developed, made of phosphate rock, CHT, sulfur, and blood meal. In comparative tests, this fertilizer performed equally or better than ammonium phosphate, applied at much larger rates (150–200 kg/ha). In particular, co-granulation of 5–10% dry CHT with powdery phosphate rock increased 2.6–6 times the water and neutral ammonium citrate solubility [9].

For organic farming, CHT were used to acidify water used as a carrier of azadirachtin, a soil-applied natural insecticide-nematicide, which is stable only at acid water pH. This contributed to achieve more consistent results of azadirachtin vs. an ordinary treatment with citric acid.

2.2 Effect of CHT on nitrogen, iron, other microelements, and proposed fertilizer products

First studies on the inhibiting effect of purified tannins, both of sweet chestnut and Australian *Acacia* (*Acacia mearnsii* De Wild.), on ammonium sulfate nitrogen indicated they are not toxic to microbial growth; however, they reduce nitrification rate of some 20–30% at concentrations as low as 0.5–1.0% in the first weeks after application, and this effect remains for 12 weeks. This effect does not indicate a direct toxicity of tannins to nitrification bacteria [10]. The author of this research speculates also a reduction in soil-free ammonium nitrogen after ammonium application with CHT, due to the growth of heterotrophic microorganism on the carbon-rich substances (sugars, acids, etc.) usually present in commercial CHT.

Later, different mechanisms were proposed, inhibition of extracellular enzymes, iron deprivation, etc. [11], and more recently, this effect was explained on the basis of chemically combining tannins with extracellular enzymes and with an anti-infective action [12]. Interactions between CHT and chemistry of nitrogen forms were in part disclosed by a patent, dealing with the reduction of tobacco-specific nitrosamines by the use of field and/or post-harvest treatments [13].

An interesting point of view on this matter was carried out by the results of a paper dealing with condensed tannins of poplar species: small tannins (tetramer and smaller molecules) have a direct biological effect, as they act more often as substrata and sometimes as toxins, while highly polymerized tannins determine a reduction of net mineralization through substrate binding, with otherwise limited effects on overall C cycling or microbial communities directly [14]. Similar researches should be done also in the case of CHT.

Concerning the anti-urease activity, the so-called tannic acid (gallotannins) was found to determine a marked inhibition of urease activity (the inhibition constant was Ki = 0.040). The concentration of 0.1 mM reduces the urease activity of 72.4%. Apparently, the kinetic parameters (V_{max} and K_m) depend upon the concentration of "tannic acid," therefore suggesting the presence of a noncompetitive, pure inhibition mechanism. Soluble and insoluble tannin-urease complexes are formed both the longer the time of their contact and the higher the ratio "tannic acid to urease." The mechanism is related to the formation of reversible and nonreversible bonds with the substrate. Oxidative polymerization of tannins, which becomes evident by their browning, makes the enzyme progressively less accessible [15].

Low concentrations of CHT (0.8–1%) in a nitrogen-urea solution (18–20% N) determine a releasing curve which is comparable to that of a granular methylene urea. Lab tests carried out according to the Stanford and Smith incubation and leaching methodology, at equal N rate, indicated that a urea solution 20% with 1% CHT had a potential total nitrogen efficiency of 65–70% vs. 50% of urea (control), which releases completely in 28 days (**Figure 1**) [16].

Tests on corn and wheat consistently demonstrated that this technology is permitted to maintain yields with a reduction of the nitrogen rate up to 25%. In the case of wheat topdressing and topsoil applications on alkaline and subalkaline soils, reduction in volatilization losses represents the major mechanism of action for the increased efficiency.

Complexes of condensed tannins of *Quercus falcata*, *Salix sieboldiana*, and most of the Japanese conifers were studied as adsorbents of heavy metal ions, among which Cr^{6+}, Cd^{2+}, but also Cu^{2+}, and Fe^{2+} [18]. Later, *Larix* spp. condensed tannins

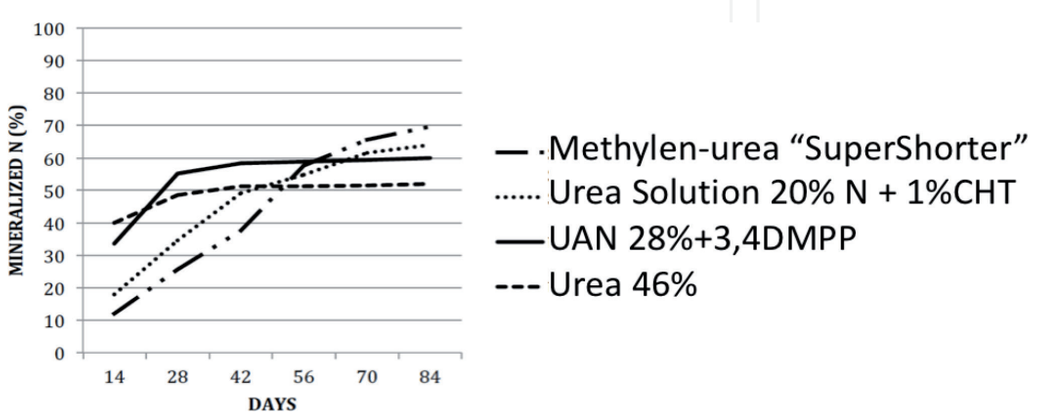

Figure 1.
Nitrogen mineralization determined as nitrates in the leachates after incubation in standard conditions (medium, temperature, moisture) according to Stanford and Smith method as modified by [17].

with iron, manganese, and zinc were prepared and applied to correct nutrient deficiency in some crops, e.g., apple orchards.

Iron chlorosis is one of the major problems both for fruit orchards and greenhouse crops. The use of mineral iron fertilizers, i.e., ferrous sulfate, has a low agronomic impact, due to the rapid oxidation to Fe^{3+} in the soil, while only the Fe^{2+} is readily available for plants. This is the reason why synthetic chelates and complexes have received such a deep interest over the last years.

CHT-iron complex fertilizers may represent a "green" alternative to synthetic chelates. In the formulated fertilizers, ¾ w/w of the total iron is as complex, according to the Italian Official Test method, which uses a cation-exchange resin [19]. Field tests were carried out mainly on pear, kiwi, and vineyard to treat iron chlorosis. On pear cv. Abate, on Ba29 rootstock, three applications in micro irrigation at 100 L/ha (6% Fe from Fe sulfate heptahydrate) gave comparable or better results than the ordinary treatment of Fe(EDDHA) at 12 kg/ha in three applications. A longer shelf life (+18 days, compared with the control, at room temperature) and better refrigerator preservability (−16% incidence of softening at 60 days from harvest, as compared with the control) were the main quality improvements [20].

The Italian Decree 2010/75 concerning domestic fertilizers, complementary to the European law 2003/2003, considers the following products with tannins:

- Two water/soil correctives: one liquid plant extract with tannins 13% and one solid plant extract with tannins, minimum 75%

- A solution, minimum 20% nitrogen, fertilizer, with 0.8% minimum tannins, pH 4–6

- An iron complex fertilizer with plant extracts with tannins, 6% total Fe, 4.8% complex Fe

Only later, with the Decree 2018-07-18 related to the production and labeling of corroborants, to potentiate plant defense, an integral extract of sweet chestnut with tannins was included in this group, with the only requirements to indicate the tannin percent content, the use of water extraction, and physical means to concentrate the product.

2.3 CHT as a biostimulant

The raw CHT extract has found application in transplanted crops as a starter treatment, to boost early plant growth, rooting, and phosphate uptake and enhance plant resistance to nematodes. Some recent data of 2017–2018 indicated that the use of tannins in the hydroculture medium, where transplant seedlings were grown, was positive for early rooting and growing after their field transplanting, and differences maintain until harvest.

During early characterization, some mixes higher in gallic acid (HGA) or lower in gallic acid (LGA) were compared. Both tannin formulations decreased tobacco actual infestation (root gall index) at 30 DAT, but only the HGA-mix significantly reduced the J2 population vs. the infested control. In fact, for this parameter, there were no differences between the LGA-mix treatment and the infested control. However, tobacco plants treated with this same LGA-mix produced an epigeal DM yield not significantly different from the HGA-mix treated plants. This seems to indicate that products low in gallic acid, such as the LGA-mix and most commercial CHT extracts, have a predominant biostimulant activity, while a higher concentration of free gallic acid is associated with an increased biocidal activity, in agreement with the previous literature [21].

A recent paper reported a large part of the activity on tannin-nematode carried out during the European Project Life+2013 Evergreen [22]. For the first time, one of the CHT fractions was formulated as a microgranulated fertilizer and a fully dispersible powder for application at low rates in efficient, localized repeated placements along each transplant row. These experiments confirm that CHT acts as a biostimulant on plant root systems, enhancing indirectly plant resistance to nematodes. This mechanism could positively affect CHT efficacy in the medium long term, because it determines a lower selective pressure on nematode population than more aggressive a.i. (active ingredients). It should also be noticed that this sustainable approach was demonstrated to be coupled with a null toxicity profile on model organisms and microorganisms by researchers of the same project Evergreen.

3. Potential and opportunities of tannins in the control of biotic plant diseases

The food demand is increasing worldwide for the constant growth of the global population, with a significant impact on natural resources, such as water, land, and biodiversity, that are already under pressure. Moreover, climate change is also further threatening food security, also by negatively affecting incidence, severity, and distribution of biotic plant diseases of plants. In this global scenario, where agriculture is pivotal as a source for both food commodities and income, more efficient, innovative, and sustainable production and control methods need to be urgently developed and adopted to prevent crop yield and quality losses. Other important challenges for future plant disease management are also to be able to preserve the environment, agroecosystems, and human health, as well as to reduce dependency from natural resources. In this regard, synthetic agrochemicals have been essential to determine a noteworthy increase in crop yields and food production during the last century, but their extensive use for the control of plant diseases resulted in an undeniable negative impact on the environment and ascertained risks for human health. In addition, the increasing demand for organic plant food has also inevitably determined the request for more environmentally friendly and safer pesticides, possibly from natural origin, to be used both in pre- and post-harvest disease management.

Safe and effective bioactive compounds useful as alternatives to synthetic pesticides for a sustainable plant disease control can be obtained by exploiting those secondary metabolites naturally produced by the plants also to provide protection against phytopathogens and pests, such as phenolic compounds. Plant phenolics form a class of chemically heterogeneous compounds, which include tannins, which occur in monocots, dicots, and ferns. Tannins were firstly classified into hydrolysable and condensed tannins; the former is absent in monocots, while their most recent classification is based on their structural characteristics and includes gallotannins, ellagitannins, complex tannins, and condensed tannins.

Tannins, traditionally used in the leather industries, have been widely investigated also for their beneficial effects on human health, including their antioxidant bioactivity and antimicrobial properties against some human pathogens. In the past, tannin-based extracts have been obtained from several medicinal plants, and used accordingly in traditional ethnomedicine of some countries, such as China, Japan, and Malaysia. In more recent times, tannins have been also studied to assess their potential as natural bactericides and fungicides in plant protection and management, to replace or reduce synthetic pesticides. Furthermore, the availability of green extraction and purification procedures to obtain raw or purified tannin extracts from agro-industrial waste makes these bioactive compounds highly attractive, contributing to the sustainability of agricultural practices in the frame of a circular economy [23].

The quali-quantitative yields obtained in the recovery of bioactive tannins are strictly related not just to the plant species used, as well as their specific parts or by-products and wastes, but also to the extraction procedures adopted. Microwave- and ultrasonic-assisted extraction of tannins are among the most innovative procedures adopted, to overcome the main limits of the traditional methods, by reducing extraction temperatures and times, as well as the amount of solvents used. As a general rule, tannins are not only soluble in water but also in several alcohols, ethyl acetate, and acetone, with differences in yield attributable to other factors such as the temperature and the extraction time and the ratio of liquid to plant solid matrix. These extracts, composed by several phytochemical constituents, are usually more active than the individually isolated compounds, because of synergistic effects [24]. In this frame, a clear and homogeneous standardization would be then desirable both for the extraction procedures adopted and for the *in vitro* and *in planta* tests to assess the antimicrobial effectiveness of tannin extracts in controlling biotic plant diseases [25]. Accordingly, the antimicrobial and anti-infectivity activities of standardised tannin extracts were deeply investigated and demonstrated in the EU LIFE 13 EVERGREEN project, coordinated by Prof. Stefania Tegli.

At last it is worth to mention that recently, formulations based on *Castanea sativa* and *Schinopsis lorentzii* tannins and on *Vitis vinifera* cane tannins have received a positive evaluation as "basic substances" for their use in plant protection by EFSA [26].

3.1 Tannins against plant pathogenic fungi

Fungi are important agents of pre- and post-harvest diseases of plants and of their products, causing heavy economic losses which can increase up to 50%, in developing countries and under highly severe conditions. Additionally, some phytopathogenic fungal species are known to produce mycotoxins, both under field conditions and mainly on harvested crops, causing considerable economic losses and high health risks for consumers.

To this concern, extracts from leaves of *Capsicum annuum* accession no. CGN 21526 (10 g plant material/100 ml solvents, to get a vacuum-dried powder at a final concentration of 50 mg/ml in water) have been proven effective against *Alternaria alternata* causing post-harvest infections on tomato [27]. The most abundant phenolic compound of all these extracts was gallic acid, whose concentration ranged from 23 to 64% of the total phenols. When added to the culture medium at the final concentrations of 5, 10, and 25 mg/ml, these extracts inhibited the mycelial growth *in vitro* from about 43 to 82% in comparison to the negative control, in a dose-dependent manner and with the extract obtained using water as solvent as the most active. The germination of *A. alternata* conidia was also inhibited by these *C. annuum* extracts, in the range 40–53% in comparison to the untreated control and with the extracts obtained using ethanol or ethyl acetate as solvents having the highest bioactivity. Similarly, soft rot caused by *A. alternata* cherry tomato fruits was reduced by the treatment (10 and 25 mg/ml) with these *C. annuum* extracts and with ethanol and ethyl acetate extracts as the most active.

Similarly, water and alcoholic extracts from pomegranate peel have been proven effective in controlling post-harvest rot and decay of fruits and crops, caused by several fungal phytopathogens such as *Penicillium* species, mycotoxigenic *Aspergillus*, *Botrytis cinerea*, and *Colletotrichum gloeosporioides*. Their bioactivity is also conserved when used in bioformulations with edible natural coatings, such as chitosan and alginate [28–30]. The antifungal properties of pomegranate peel are attributable to ellagitannins, such as punicalin, punicalagin, and ellagic acid, as

well as gallotannins [31]. By comparing different extraction solvents, the recovery of bioactive tannins as well as anthocyanins was higher when using hydroalcoholic solutions at high concentrations, instead of just hot water. The 80% ethanol/water extract from pomegranate peel, concentrated by evaporation, was demonstrated highly effective to reduce the development of *Botrytis cinerea* rots on table grape and to control olive anthracnose caused by *Colletotrichum* spp. [32, 33]. An inhibitory effect was demonstrated for an ethanol extract of pomegranate peel for *A. alternata*, *Fusarium oxysporum*, *Phoma destructiva*, *Rhizoctonia solani*, and *Sclerotium rolfsii* [34]. Conflicting results have been obtained with a pomegranate peel water extract, amended to PDA medium. While at 8.60 and 17.20 mg/ml, the ability to inhibit *A. alternata* and *Fusarium* spp. was confirmed, and the antifungal activity against *Stemphylium botryosum* demonstrated for the first time, no decrease of the growth rates of *P. expansum* and *B. cinerea* was induced for this pomegranate peel water extract, as well as no effect found on *P. digitatum* [35]. The inhibitory activity was shown to be correlated with the concentration of total polyphenolics and in particular with punicalagins. However, according to the extraction procedure adopted, organic acids and other primary and secondary metabolites were known to be present in this water extract [36], which can account for the unexpected and conflicting results on its antifungal activity.

Tannin extracts from waste biomass of chestnut (*Castanea sativa*) have been shown to be highly promising for their potential use as natural fungicides in plant protection. They are obtained by solvents such as water and ethanol classified as generally recognized as safe (GRAS) to powdered dried chestnut burs. The inhibitory activity found on the growth of *A. alternata*, *F. solani*, and *B. cinerea* was dependent from the extraction procedure adopted as well as from the different sensitivity of the fungal species examined. However, it was found to be mainly attributable to ellagic acid, with EC50 values ranging from 13.33 to 112.64 μg/mL [37].

Condensed tannins, such as catechins, have been found in water extracts from chili (*Capsicum frutescens*) (8.50 mg/ml) and garlic (*Allium sativum*) (6.93 mg/ml) extracts, having strong antifungal activity *in vitro* against *C. gloeosporioides*, *Fusarium*, and *Phomopsis* spp. [38].

3.2 Tannins against plant pathogenic bacteria

The control of bacterial diseases of plants is extremely challenging because of the complex biological cycle of these phytopathogens, spanning from epiphytic and/or endophytic asymptomatic stages to survival into soil, water, or other wild host or nonhost plants, although their impact causes serious economic losses concerning yield and quality of a huge number of crops. Moreover, most of the management of bacterial diseases of plants still relies on the preventive use of copper compounds as bactericide, with a wide spectrum of well-known negative ecotoxicological consequences. In spite of that, studies concerning the search and the development of botanical extracts alternative to copper treatment against phytopathogenic bacteria are surprisingly less common than those for fungi and for bacteria pathogens on humans.

Condensed tannins based on flavan-3-olic units, such as catechins and epicatechins, have been shown to be the most abundant polyphenolic metabolites found in the water extracts of grape seeds and green tea. Grape seed polyphenolic extracts entirelies consist of several catechins and epicatechins with molecular weights ranging from 290 to 1170 Da and of free gallic acid, while in green tea extracts epigallocatechin gallate and epicatechin gallate represent 96% and 4% of the total tannins, respectively. By using an *in vitro* plant model system, both these tannin

extracts were demonstrated to be able to give a statistical reduction of the hyperplastic symptoms produced by the inoculation of *Pseudomonas savastanoi* pv. *nerii* strain Psn23 on cuttings from 2-year-old twigs of *Nerium oleander*, in comparison to those untreated. In addition, a significant decrease of bacterial multiplication was observed on tannin-treated plants, which was comparable to the in planta growth of the Δ*hrpA* nonpathogenic mutant of Psn23 [39].

A strong antibacterial activity against the destructive causal agent of tomato bacterial wilt *Ralstonia solanacearum*, both *in vitro* and *in vivo*, was found for tannins extracted from *Sedum takesimense* and *Sapium baccatum* [40].

The profiling of the phenolic compounds present in young leaves of the two apple cultivars "Enterprise" and "Idared," highly resistant and highly susceptible to fire blight, respectively, was estimated and evaluated both before and after *E. amylovora* infection. According to this data, the activity of 13 selected phenolics was tested *in vitro* against *E. amylovora*, at 10, 50, and 100 mM in aqueous solution. Gallic acid was among the most effective to suppress the bacterial growth. Moreover, its efficacy was confirmed *in vivo*, by significantly limiting the development of disease and *E. amylovora* infection on pear fruitlet slices when applied as 100 mM aqueous solution.

3.3 Tannins and their mechanisms for plant disease control

The biological activity of tannins strongly depends from their highly variable chemical structure, and tannins basically can act as metal ion chelating and protein complexing agents and antioxidants, in addition to their well-known antimicrobial properties. However, in view of their potential application in plant protection, a deeper knowledge about their mode of action would be desirable.

Experiments carried on bacterial phytopathogen *Clavibacter michiganensis* with the ellagitannin HeT extracted from strawberry leaves demonstrated that its bactericide activity is related to a dose-dependent inhibition of the oxygen consumption rate and respiration, as a consequence of its interaction with cell membranes [41]. The absence of any toxicity was assessed for several tannins, such as epigallocatechin gallate and catechin, up to 1 μM by using as a target the membrane protein Ca^{2+}-ATPase from the sarcoplasmic reticulum (SR). SR belongs to the ubiquitous and highly conserved proteins of the so-called P-type ATPase family, whose members are present in the cellular membrane of any living organism and involved in numerous transport processes. Conversely, copper suppresses almost completely Ca^{2+}-ATPase activity at just 0.1 μM concentration Cu^{2+} ions [39].

An alcoholic extract obtained from the peel of pomegranate, and mainly containing tannins, was found active as resistance inducer. This extract elicits defense responses when applied to harvested citrus fruits, expressed as an increase in reactive oxygen species and in the expression of five genes which are pivotal during the activation of plant post-infectional defense [33].

Tannins have been also shown to possess noticeable inhibition abilities on some enzymatic activities strictly related to the virulence of phytopathogenic bacteria. The virulence of the *Dickeya chrysanthemi* is mainly dependent from its production and secretion of several cell wall-degrading enzymes, such as pectate lyases and proteases. Tannic and gallic acid are efficient to give a total inhibition of *D. chrysanthemi* pectate lyase at concentrations of 400 and 800 μg/ml, respectively [42]. At last, tannins can also negatively interfere with other bacterial systems which are essential for their pathogenicity and virulence on plants, such as the Type Three Secretion System and the Quorum Sensing, respectively [39, 43].

4. Polyphenol extracts to combat some bacterial diseases on kiwi crop: effect on soil biochemical functions

4.1 About soil quality

Soil is a natural resource that we must conserve and protect. In this sense, we must ensure that various soil properties (physical, chemical, biological, microbiological, and biochemical), capable of maintaining the quality, sustainability, and functionality of soils, respond to the soil protection criteria. Soil properties effected by the size, activity, and the composition of the microbial biomass included water holding capacity, infiltration rate, erodibility, aggregate stability, nutrient cycling, nutrient capacity, and soil organic matter content (soil function). Soil quality cannot yet be defined in quantitative terms; however, it should be possible eventually to define soil quality and sustainability quantitatively by the appropriate integration of specific quantitative terms, so that the effects of management on soil quality can be determined. Soil quality is a dynamic character, and many significant indicators must be sensitive to small changes in key soil properties. However, tools to detect the impact of changes in soil functionality are needed. Soil enzymes are extremely important in assessing the status or the conditions of the soil environment. This is because enzymes' and microorganisms' activity and biodiversity are related with the most important elements for soil sustainability and functionality (C, N, P, and S). Many extracellular enzymes are absorbed to, complexed with, or entrapped within soil clays and humics, and they may have a long-term stability.

The demand for biofertilizers is increasing since the last decade owing to its eco-friendly characteristics and a worldwide trend to reduce the reliance on chemically derived fertilizers. The Asia-Pacific shared approximately 34% of the total demand in 2011. European and Latin American countries are the leading consumers of biofertilizers, owing to stringent regulations imposed to chemical fertilizers which would eventually be replaced by biofertilizers.

4.2 About biopesticides

In the Evergreen Project, different experiments using polyphenol extracts as biopesticides were carried out. We show (only as an example) some results obtained on soils from a kiwi (*Actinida chinensis*) crop, where some polyphenol extracts were used as biopesticides. The bacteria (*Pseudomonas syringae actinidiae*) were used as pathogen agent, a vascular pathogen, whose most conspicuous symptom is the red-rusty exudation which covers bark tissues on trunks and twigs.

The polyphenol extracts used in this experiment were the following:

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).

In addition, $CuSO_4$ has been used to compare a possible conventional treatment and another way to combat some pathogen microorganisms (biopesticides as polyphenol extracts). The total treatments in this assay were (1) control− (without bacteria), (2) control+ (with bacteria), (3) $CuSO_4$− ($CuSO_4$ without bacteria), (4) $CuSO_4$+ ($CuSO_4$ with bacteria), (5) form 1, and (6) form 2.

In this study, some biological and biochemical parameters measured on soil treated with polyphenol extracts have been shown. The use of biochemical parameters (soil enzyme activities) can be important to know the possible effect of polyphenols on the cycle of the important elements such as C, N, and P.

Application methods for polyphenols and pathogen:

a. Supply polyphenol (form 1/form 2) or $CuSO_4$ (100 c.c.) on soil next to the roots. Let it be absorbed during a week.

b. Spraying polyphenol or $CuSO_4$ solution on aerial part of the plant. Let it be absorbed (24 h).

c. Bacterial inoculation: remove leaves from each plant, petiole included (100% wounds done) along the stem, exposing the wound produced. Inoculate helping with a micropipette 10 μl of bacterial solution on the wound directly. Protect the wound with a film at least during 24 h, and then remove it.

d. Watering polyphenol treatments (100 c.c.) 7 and 15 days later from the bacterial inoculation with the corresponding liquid polyphenol (form 1 or form 2).

Results were the following.

4.2.1 Total organic carbon and total N

One of the most interesting parameters for soil quality is the organic carbon content, indicative of the organic matter content of the soil. The organic C induces fundamentally the productivity and fertility of the soil. Its presence in the soil is of great interest from two points of view: environmental (fixation of C in the soil) and agronomical (soil fertility).

In our experiment (**Table 1**), no significant differences were observed for organic C between the control soil and the soils treated with polyphenols. We know that polyphenols are organic products, and they should be implied in mineralization and humification processes; they could alter soil organic C. However, the addition of polyphenols to the soil did not alter organic C content in the soils studied. This confirms that the doses used for polyphenols in this study are not high enough to affect this type of parameter.

Nitrogen enhances plant growth, improves the quality of crops, and increases seed and fruit production. Nitrogen in the soil is usually supplied by decomposition of organic material, commercial fertilizers, and nitrogen-fixing bacteria. The desirable amount varies between crops; however, too much nitrogen can have adverse effects especially on the environment.

The differences found in soil total N in our experiment (**Table 1**) can be attributable to the variability of soil and our technical analyses; for this reason, it can be

	N total (g/100 g)	**C total (g/100 g)**	**Corg (g/100 g)**
Control−	0.210 a	8.153 a	3.516 a
Control+	0.606 b	9.716 ab	5.153 a
$CuSO_4$−	0.526 b	10.363 b	5.116 a
$CuSO_4$+	0.533 b	10.313 b	4.113 a
Form 1	0.533 b	11.066 b	4.546 a
Form 2	0.556 b	10.600 b	5.673 a

The same letter for each parameter indicates no significant differences between treatment (Tuckey's method, $p < 0.05$).

Table 1.
Total N, total C, and total organic carbon, measured in kiwi soils at the end of the experiment.

indicated that the variations observed in this parameter cannot probably be due to polyphenols' addition.

4.2.2 Enzymatic activities

Enzyme activities related to the cycle of elements (carbon, nitrogen, phosphorus, or sulfur) are of paramount importance in soil quality. Among these enzymes we propose the study of phosphatases, ureases, proteases, and different enzymes related to C cycle such as β-glucosidases. Indicators of the microbial population activity (dehydrogenase activity) will give an accurate notion of the impact of the addition of these products on microbial activity. For a general assessment of the functional and structural changes in microbial community, we have carried out several measurements based on soil enzymes.

Most enzymes found in the soil, in particular the hydrolases, are extracellular and have a great environmental interest. In addition, these extracellular enzymes may be free and exposed to rapid denaturation or immobilized together with mineral or organic colloids. Generally, those immobilized enzymes in mineral and/or organic colloid change in their status, nature, and properties (such as kinetics, stability and mobility of enzymes) and are less prone to proteolytic denaturalization, since they are physically and chemically associated with other surrounding chemical compounds.

4.2.3 Phosphatase

The agronomic and biotechnological importance of phosphatase is that it activates the transformation of organic P into inorganic forms of P available to plants. We have determined alkaline phosphatase since we have worked with basic soils (**Table 2**). Phosphatases are inhibited by inorganic P, the final product of their enzymatic reaction. This is due to a feedback inhibition, so phosphatases are synthesized only when available P is deficient.

In our study, no statistical differences between treatments were appreciated in this enzyme activity. This indicates that the addition to the soils of polyphenol products does not change the phosphorous cycle in the soil. Some differences in this enzyme activity were noted throughout the experimental period, greater phosphatase activity being detected at the start than at the end of the experiment. This fact could be due to P mobilization from organic to inorganic forms, in order to make it available to plants. The P cycle, studied by phosphomonoesterase activity, seems not to be affected by the polyphenol addition to the soil since little differences were observed as regards phosphatase activity between the soils treated with polyphenols

Phosphatase activity (μmol PNF h^{-1} g^{-1} soil)	**T0**	**Tf**
Control−	6.779 b	4.028 a
Control+	7.118 b	5.197 a
$CuSO_4$−	6.072 ab	5.373 a
$CuSO_4$+	6.206 ab	5.560 a
Form 1	6.391 ab	5.886 a
Form 2	5.349 a	5.324 a

The same letter for each parameter indicates no significant differences between treatments (Tuckey's method, $p < 0.05$).

Table 2.
Evolution of soil alkaline phosphatase activity in kiwi soils (initial, To, and final Tf).

and the control. Anyway, a slight increase in phosphatase activity was observed when polyphenols were introduced into the soil.

A negative effect on soil phosphatase was observed when $CuSO_4$ is used as pesticide. It indicates that this conventional treatment can affect to P cycle in the soil.

4.2.4 β-Glucosidase

β-Glucosidase is a hydrolase which intervenes in the C cycle, acting especially in the hydrolysis of the β-glucoside bonds of long carbohydrate chains. The hydrolysis of these substrates plays an important role in the attainment of energy from the soil by microorganisms.

C cycle linked to β-glucosidase activity was not affected by the utilization of polyphenols, as shown in **Table 3**. The activity of this enzyme did not change with time in a significant way, similar activity values being observed at the start and end of the experimental period. Both polyphenols directly (form A and B and also the Cu salt introduction in soil) and their impact on the soil biota do not affect the carbon cycle.

Urease activity. Urease catalyzes the hydrolysis of urea or ureic-type substrate to give carbon dioxide and ammonia as reaction products. This term includes all those hydrolases capable of acting on the C-N (non-peptide) bonds of linear amides. They are extracellular enzymes.

At the start of the experiment, some changes in urease activity were observed in the soils treated with polyphenols with respect to the control (**Table 4**). This is indicative that the N cycle is influenced by polyphenols. The soils treated with $CuSO_4$ showed the lowest values of urease activity; it could be due to the heavy metal incidence (Cu) or to the increase of soil salinity. At the end of the experiment, urease activity values were also lower when $CuSO_4$ was used, but the differences were not statistically significant. For this reason, we can say that the microbial populations that synthesize urease do not undergo to experiment changes, and consequently, the N cycle does not show difference between soils.

D-hydrogenase. The biological oxidation of organic compounds occurs by means of dehydrogenation processes, in which intracellular enzymes called dehydrogenases take part. The dehydrogenation activity in soils is determined by different dehydrogenase systems, which are characterized by their high substrate specificity. All these systems are an integral part of the microorganisms; indeed, dehydrogenase activity has been proposed as an indicator of soil microbiological activity and biomass.

Dehydrogenase activity is intracellular and detects the set of cells capable of being activated against various situations; soil samples toward the initial of the

β-glucosidase activity (μmol PNF h^{-1} g^{-1} soil)	**T0**	**Tf**
Control−	0.480 a	0.524 a
Control +	0.527 a	0.531 a
$CuSO_4$−	0.527 a	0.475 a
$CuSO_4$+	0.563 a	0.472 a
Form 1	0.544 a	0.620 a
Form 2	0.549 a	0.573 a

The same letter for each parameter indicates no significant differences between treatments (Tuckey's method, $p < 0.05$).

Table 3.
Evolution of soil β-glucosidase activity in kiwi soils (initial, To, and final Tf).

Urease (μg INTF h^{-1} g^{-1} soil)	**T0**	**Tf**
Control−	0.200 b	0.931 a
Control+	0.246 c	0.945 a
$CuSO_4$−	0.141 a	0.758 a
$CuSO_4$+	0.180 ab	0.726a
Form 1	0.364 d	1.008 a
Form 2	0.329 d	0.806 a

The same letter for each parameter indicates no significant differences between treatments (Tuckey's method, $p < 0.05$).

Table 4.
Evolution of soil urease activity in kiwi soils (initial, To, and final Tf).

D-hydrogenase activity (μg INTF h^{-1} g^{-1} soil)	**T0**	**Tf**
Control−	1.619 a	5.355 b
Control+	1.595 a	5.147 b
$CuSO_4$−	1.391 a	2.972 a
$CuSO_4$+	1.559 a	4.719 b
Form 1	1.174 a	5.274 b
Form 2	1.487 a	5.380 b

The same letter for each parameter indicates no significant differences between treatments (Tuckey's method, $p < 0.05$).

Table 5.
Evolution of soil d-hydrogenase activity in kiwi soils (initial, To, and final Tf).

experiment showed no changes for this oxidoreductase enzyme. Only at the end of the experiment, the soils treated with $CuSO_4$ showed a decrease in the activity of this enzyme. This different behavior between the start and the end of the experiment could be due to the fact that at the start of the experiment, the Cu salt has no time to act, and some more time needed to the effect of the salt on the enzyme activity is noted (**Table 5**).

The main conclusion obtained from the results is that the use of polyphenols such as those prepared in the Evergreen project can be regarded as positive, since it is able to prevent bacterial diseases on crops such as kiwi. Our results indicate that the polyphenols used can be considered as biopesticides. For example, we can indicate that in agriculture, Cu is a metal widely used as a pesticide; however, the accumulation of this metal in soils can become harmful to the quality of that soil. Therefore, the possibility of having alternatives such as polyphenols, capable of acting against certain pathogenic microorganisms, has paramount agronomic and environmental interests. We think that the use of polyphenols should be studied at the level of management. The application of these compounds (the time of application if application should be repeated, if they can be used as preventive treatment, etc.) should be studied, particularly for soil biological properties.

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