**Chapter N**

**Sweet chestnut fractions from sustainable circular process for the control of phytopathogenic oomycetes**

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**Abstract.** One natural, sustainable extract of *Castanea sativa* Mill. was tested for *in vitro* antimicrobial activity against three phytopathogenic oomycetes of agronomic interest: *Pythium dissotocum*, *P. sylvaticum* and *P. ultimum*. The extract is obtained from chestnut culture waste by hot water extraction and membrane fractionation/concentration. The HPLC-DAD-MS chemical characterization of bioactive secondary metabolites highlighted the presence of gallic and ellagic hydrolysable tannins (26.0% *vs* weight of powder), with a prevalence of vescalagin and castalagin (4.8% and 9.8%). The extract showed a good inhibition of the mycelial growth, in particular for *P. sylvaticum*, for which an inhibition of 79.4% with 0.1% w/V extract was observed. For *P. dissotocum* and *P. ultimum* the inhibition was 57.1% and 56.5% respectively, at 0.1% extract. Furthermore, in all tests a different phenotype (more lax mycelium) was observed. These evidences could open perspectives for using sweet chestnut tannins for the contrast of phytopathogenic *Pythium*, e.g. in sowing substrates or for crops bio-defence, or even in substrates for hydroponic crops, given the dependence of *Pythium* spp. on aquatic environments. This study is in line with the growing interest towards eco-sustainable solutions for reducing chemical inputs in agriculture.

**Keywords.** *Castanea sativa* Mill., HPLC-DAD-MS, hydrolysable tannins, antimicrobial activity, *Pythium* spp., oomycete

**1 Introduction**

Sweet chestnut growing in Italy has ancient origins and in many areas it played, and continues to play, an important role in defining the characteristics of landscape and territory. To date, the land occupied by chestnut trees in Italy is about 800,000 ha across all regions, but in recent years socio-economic changes and the spread of pathologies caused a decrease of the activities with a corresponding increase in imports from abroad, in particular of fruits for food purposes (Castellini *et al*., 2009). The enhancement of chestnut growing in the current context requires the application of modern and innovative strategies that involve the supply chains traditionally associated with that of chestnut and their integration with new supply chains, in order to promote the recovery at a productive, economic and social level. The recovery of waste and by-products of both food and wood production activities, and their use to obtain new products applicable in multiple sectors according to circular economy models, can guarantee the evolution of the sector towards greater environmental and economic sustainability and allow the connection between companies and realities operating in different sectors (Romani et al., 2020). The presence of considerable amounts of hydrolysable tannins with antioxidant and antimicrobial properties in the woody tissues of sweet chestnut, suggests the possibility of using the waste to produce natural extracts rich in bioactive compounds, to be used as semi-finished or finished products with high added value for applications in sectors such as textiles/tanning, agronomics, cosmetics, feed, food/nutraceuticals and wine (Pagliaro et al., 2021; Pizzi, 2021; Romani et al., 2021). The sweet chestnut dry extract investigated in this work is an industrial fraction obtained by green methodologies in the plant of Gruppo Mauro Saviola in Radicofani (Si), allowing the sustainable re-use of wood chips yielded as a by-product during the processing of wood. As previously described (Campo et al., 2016; Lucarini et al., 2018), after hot water extraction the raw extract is purified and concentrated by a series of filtration steps on membranes with different molecular cut-offs, to obtain fractions enriched in different bioactive hydrolysable tannins. The two commercial standardized products are a liquid purified and concentrated fraction and the spray-dried powder obtained from this latter, that is the dry extract object of the present study in which some of the possible agronomic applications were evaluated. More in detail, quali-quantitative HPLC-DAD-MS analysis was performed for the chemical characterization of the hydrolysable tannins in the dry extract, before testing it for *in vitro* antimicrobial activity against three phytopathogenic oomycetes of agronomic interest: *Pythium dissotocum*, *P. sylvaticum* and *P. ultimum*. A good inhibition of the mycelial growth was observed and a different phenotype (more lax mycelium) with doses of 0.1%-0.5% w/v, confirming the antimicrobial effect of sweet chestnut tannins towards the tested *Pythium*. This preliminary study will be followed by further *in vitro* and *in vivo* tests to assess possible new perspectives for sweet chestnut tannins based products in the contrast of phytopathogenic *Pythium*. The innovative applications could be particularly useful e.g. for sowing substrates for horticultural and ornamental plants or for crops bio-defence, in line with the growing interest in eco-sustainable solutions for the reduction of chemical inputs in agriculture. A further area of study will concern the possible application of the extract in substrates for hydroponic crops, a sector in progressive diffusion, given the dependence of *Pythium* spp. on aquatic environments.

**2 Materials and methods**

**2.1 Chemicals**

All solvents (HPLC grade) and formic acid (ACS reagent) were purchased from Sigma Aldrich Chemical Company Inc. (Milwaukee, Wisconsin, USA). Gallic and ellagic acids, of analytical grade, were purchased from Extrasynthèse S.A. (Lyon, Nord-Genay, France). HPLC-grade water was obtained via double-distillation and purification with a Labconco Water Pro PS polishing station (Labconco Corporation, Kansas City, USA). The PDA (Potato Dextrose Agar) was purchased from VWR International (Radnor, Pennsylvania, USA).

**2.2 Extract**

The sweet chestnut dry extract was a commercial fraction furnished by GRUPPO MAURO SAVIOLA Srl (Viadana, MN, Italy). This fraction is obtained after ten circular and solvent free process streams by the industrial tannin extraction and concentration/purification plant operating in Radicofani (SI, Italy), previously described (Campo et al., 2016; Lucarini et al., 2018).

**2.3 HPLC-DAD-MS analysis**

The extract was analyzed with a HP-1260 liquid chromatograph equipped with a DAD detector and a MSD API-electrospray (Agilent Technologies, Santa Clara, CA) in negative ionization mode. A Luna, C18 250×4.60 mm, 5 μm column (Phenomenex, Torrance, CA), at 26 °C was used. Eluents: 5% HCOOH in water and CH3CN. A four-step linear solvent gradient from 100% H2O up to 100% CH3CN was performed over a 55 minutes period, as previously described (Campo et al., 2016; Lucarini et al., 2018). Mass spectrometer operating conditions: gas temperature 350 °C, flow rate 10.0 L/min, nebulizer pressure 30 psi, quadrupole temperature 30 °C and capillary voltage 3500 V. Fragmentor 120 eV. Tannins were identified by comparing their retention times, UV-Vis and mass spectra with those of the commercial standards. Compounds were quantified using five-point regression curves (r2 ≥0.9998) in gallic and ellagic acids. Analyses were carried out in triplicate and the results were recorded as mean values with standard deviations ≤ 5%.

**2.4 *In-vitro* test**

The species used for the *in-vitro* test were *Pythium dissotocum* Py\_diss01, *P. sylvaticum* Py\_sylv02 e *P. ultimum* Py\_ult36 (CREA-AA Bologna), maintained on PDA at 25°C in darkness. The substrate was prepared by adding the sweet chestnut extract to the PDA in the concentration of 0% (control), 0.01%, 0.05%, 0.1%, 0.5% and 1.0% w/v. The pH was corrected to 5.6 with a KOH solution. The plates (85mm) were inoculated in the center with a 5mm disc of mycelium obtained from an active growing colony and incubated at 25°C in the darkness. The two perpendicular colony diameters were registered every 4-6 hours until the plate edge was reached. The growth inhibition (%) was calculated as follows:

[(diam.ctrl - diam.treated)/diam.ctrl]\*100

The final result is obtained with the mean of 5 replicates and two repetitions of the same experiment. The statistical analysis was made with DSAASTAT through the variance analysis (ANOVA). The mean separation was made using Fisher's LSD prior the angular transformations of the percentage values and the statistical significance was evaluated with a p<0,01.

**3 Results and discussion**

**3.1 Chemical characterization of the tannin content**

The HPLC-DAD-MS quali-quantitative chemical characterization of hydrolysable tannins in the sweet chestnut dry fraction is shown in Table 1.

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|  Table 1. Quali-quantitative HPLC-DAD-MS characterization of the hydrolyzable tannins in sweet chestnut dry extract. Data expressed in mg compounds per g of extract. |
|  | mg/g |
| Vescalin | 9.260 |
| Castalin | 8.126 |
| Pedunculagin I | 9.974 |
| Monogalloyl glucose | 3.811 |
| Gallic acid | 16.157 |
| Vescalagin | 47.633 |
| Dehydrated tergallic-C-glucoside | 9.262 |
| Castalagin | 97.742 |
| Digalloyl glucose | 19.618 |
| Trigalloyl glucose | 20.566 |
| Tetragalloyl glucose | 7.703 |
| Ellagic acid | 6.072 |
| Pentagalloyl glucose | 4.258 |
| **Tot** | **260.180** |

Both gallic and ellagic tannins were found, and the composition confirms the standardization and stability of the commercial natural extract, for which in-depth analyses were carried out on previous batches (Campo et al., 2016). The total tannin content in the powder is 26% weight, with a prevalence of gallic acid, vescalagin and castalagin, representative compounds of the vegetal species under study (1.6%, 4.8% and 9.8% respectively). It is also interesting to note the small relative amounts of partial hydrolysis-derived compounds like vescalin, castalin and pedunculagin: despite the extraction conditions in high temperature water, high molecular weight tannins are mostly preserved during extraction. The extract was chemically stable as demonstrated by control HPLC-DAD-MS analysis after 6 and 12 months.

**3.2 *In-vitro* test**

The sweet chestnut extract demonstrated a good inhibitory activity against the mycelial growth of the tested *Pythium* (Table 2). The best results were obtained against *P. sylvaticum*, with scarce effect at the lowest dose (19.4% inhibition at 0.01%) but higher effects with the next two doses (73.0% and 79.4% of inhibition at 0.05% and 0.1%). Higher concentrations showed a 100% inhibition value. The same effect was observed with *P. dissotocum*, with an inhibition of 13.9% at 0.01% dose; reaching 44.3, 57.1% and 99.4% with extract concentrations of respectively 0.05%, 0.1% and 0.5%. Also in this case a 100% inhibition percentage was observed at 1.0% extract. Similarly, against *P. ultimum* the inhibition percentage was 3.0% at 0.01% sweet chestnut extract, going up to 48.2%, 56.5% and 96,6% with doses of respectively 0.05%, 0.1% and 0.5% w/v. Again, at 1.0% w/v the inhibition was 100%. In all the tests, aside from the reduced colony growth speed, it was also observed a minor mycelial density.

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|  Table 2. Inhibitory value (%) for each pathogen and extract concentration. |
|  | 0.01% | 0.05% | 0.1% | 0.5% | 1.0% |
| *Pythium dissotocum* | 13,29a | 44,27b | 57,14c | 99.38d | 100.0d |
| *Pythium sylvaticum* | 19.44a | 73.05b | 79.39c | 100.0d | 100.0d |
| *Pythium ultimum* | 3.13a | 48.18b | 56.51c | 96.56d | 100.0e |

In the literature there is some evidence about the vulnerability of *Pythium* against tannins. In a paper by Khan *et al.* (1996) a 100% inhibition was observed of *P. aphanidermatum* using 0.1% gallic acid. In other studies on lines of cotton (Kenneth, 2009), broad bean (Kantar *et al.*, 1996) and peas (Kraft, 1974) a correlation was observed between tannins and other polyphenols in the seeds integuments or in the seedlings exudates, and the resistance against some *Pythium*. The present work confirms this hypothesis, demonstrating that sweet chestnut hydrolysable tannins have a repressive activity against the mycelial growth of the tested pathogens. Concentration of 0.5% determinate and inhibition just under 100%, while a 1.0% dose allowed the complete inhibition of all the *Pythium* tested.

**4 Conclusions and future perspectives**

The results shown are quite promising and more tests should be necessary to confirm *in-vivo* these evidences. If confirmed, innovative applications could be hypothesized in the development of useful products for the defence against pythopatogens *Pythium*, also in line with the growing interest toward eco sustainable solutions for the reduction of chemical inputs in agriculture.

The possible applications range from the use into the sowing substrates for ornamental and vegetables seedlings to prevent *Pythium* infestations, up to the development of products aimed at the bio-defence of cultures. Moreover, due to the dependence of *Pythium* spp. from the aquatic environment and the damages that can cause against hydroponic cultures (Kanjanamaneesathian *et al*., 2014), it could be interesting to investigate the application of tannins in this sector, especially for what concerns the prevention.

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