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Antifungal activity of hop extracts and compounds against the wheat pathogen *Zymoseptoria tritici*

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ABSTRACT

Searching for alternative methods to conventional pesticides against crop pathogens is a huge challenge. Here, we tested the potential of hop extracts and compounds to be used as biofungicides towards *Zymoseptoria tritici*, the most frequently-occurring and damaging pathogen on wheat crops. Hop (*Humulus lupulus* L.) is known for its benefits on human health conferred by its antifungal, antiviral and antibacterial properties. However, this species has never been examined for its ability to biocontrol phytopathogens.

Hydro-alcoholic crude extracts from different parts of hop (leaves, stems, rhizomes, and female cones also called hops), as well as hops essential oil, were assessed for their activity against *Z. tritici* using spotting bioassays on PDA medium. Moreover, the major phenolic compounds purified from the hydro-alcoholic extract of hops following a bioguided fractionation and the three major terpenes of hops essential oil, previously characterized by GC–MS, were also tested using a microdilution method. Furthermore, checkerboard method was used to assess the potential co-action of hops essential oil and the synthetic fungicide bixafen.

Dose-response-curves revealed that only the crude extract and the essential oil from hops significantly decreased fungal growth. Among the purified prenylated chalcones and acylphloroglucinol derivatives, only desmethylxanthohumol and co-humulone showed an antifungal activity against *Z. tritici* with a half-maximal inhibitory concentration of 0.2 and 0.11 g L^{-1} respectively. The three major terpenes of hops essential oil were not active but an additive to synergistic effect was observed when the essential oil is combined to bixafen. This study provides new insights into the valorization of antimicrobial properties of hop in crop protection.

1. Introduction

Hop (*Humulus lupulus* L., Cannabaceae) is recognized as a medicinal plant. According to the European Medicinal Agency, female inflorescences (hops) are used as traditional herbal medicinal product for relief of mild symptoms of mental stress and to aid sleep (European Medicines Agency, 2014). In addition to this official indication, the female inflorescences are also particularly recommended to treat digestive disorders, but also to reduce symptoms associated with menopause thanks to their estrogenic properties (Zanoli and Zavatti, 2008). From a chemotaxonomic point of view, hop synthesizes original phenolic compounds, including prenylated chalcones and acylphloroglucinol derivatives (α-acids and β-acids). Many biological properties are attributed to these compounds (Gerhäuser, 2005a; Langezaal et al., 1992; Zanoli and Zavatti, 2008). Although hop is in particular recognized for its antimicrobial properties (Bocquet et al., 2018), this species has never been studied for its activity against plant pathogens.

Septoria tritici blotch (STB) is a devastating foliar disease of wheat (*Triticum aestivum L.*, Poaceae), caused by the ascomycete fungus *Zymoseptoria tritici* (syn. *Mycosphaerella graminicola*). Wheat is the second most important food crop in the world, with 720 million tons harvested in 2015 (FAO, 2017). *Z. tritici* induces the disease on wheat leaves by forming yellowish necrotic lesions bearing pycnidia (dark fungal-asexual bodies). *Z. tritici* mainly causes extensive damages in areas with a humid climate such as North-Western Europe (O'Driscoll et al., 2014). Rain and moisture promote the dissemination of the fungus. In 2015, STB was the most destructive foliar disease with 61% of affected wheat crops (Agriculture and Horticulture Development Board, 2016). The economic losses are both related to the reduction of crop yields, but also to the significant cost generated by the use of fungicides. The control of this pathogen relies mainly on the use of conventional fungicides such as DeMethylation Inhibitors (DMIs) and

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Succinate Dehydrogenase Inhibitors (SDHIs) (Fones and Gurr, 2015). The management of crop diseases, including STB, is submitted to two main constraints: (i) occurrence and widespread of fungicide resistance in most plant pathogenic fungi (Fraaije et al., 2005; Torriani et al., 2009) and (ii) societal and politic pressures aiming at reducing the use of conventional fungicides in crop protection because of their potential impacts on both the environment and human health (Damalas and Eleftherohorinos, 2011).

Multiple methods are proposed to reduce the use of conventional pesticides but biocontrol agents, including bio-fungicides, are particularly sought as safe and eco-friendly protection tools. Even if they currently make up only 5% of the worldwide crop protection market, they should be able to reach a larger share of the market by 2025 (Olson, 2015). Bio-fungicides can be insect predators (Lang, 2003), microorganisms, microbial metabolites, minerals, ions, plant extracts, plant metabolites (Siah et al., 2018). The most studied plant extract for induced resistance is an aqueous formulation of a concentrated ethanolic extract of Reynoutria sachalinensis (F. Schmidt) Nakai (Polygonaceae), marketed with the name Milsana®. This product conferred protection of various plants against a wide spectrum of fungal pathogens (Siah et al., 2018). Essential oils are also known to be effective bio-fungicides, acting on fungal development and virulence. Thyme essential oil has already been shown to display antifungal activity against *Z. tritici* and to induce the repression of genes encoding for efflux pumps involved in fungicide fungal resistance (Dayan et al., 2009; Ben Jabeur et al., 2014). Plant extracts and their purified metabolites can also demonstrate a direct antifungal action, such as the phenanthrene derivative effusol isolated from *Juncus maritimus*, which was shown to display an antifungal activity against *Z. tritici* (Sahli et al., 2017).

Eco-friendly fungicides with a good degree of selectivity and with a new mode of action would be a long-term solution to overcome pathogen resistance and to ensure sustainable crop protection (Dayan et al., 2009). In this way, we assessed for the first time the antifungal activity of different parts of hop (leaves, stems, rhizomes and female cones), as well as essential oil of hops, against *Z. tritici*, using *in vitro* bioassays, in order to evaluate their potential be used as bio-fungicides. A bioguided fractionation using chromatographic techniques was performed in order to purify and identify antifungal compounds responsible for the activity. Checkerboard method was also used to assess the potential co-action of the essential oil with the synthetic fungicide bixafen.

2. Materials and methods

2.1. Phytochemical analysis

2.1.1. Preparation and fractionation of hop extracts

Hop was harvested at maturity stage at the Beck farm (Bailleul, Northern France). The entire plant of the selected cultivar Nugget was first dried for 10 days at room temperature. Then, the different parts (female cones, leaves, rhizomes and stems) were separated, powderized and stored in the dark. Crude extracts of each part were obtained using an EtOH/H_2O (9:1) mixture-based extraction. Macerations were carried out during two hours, three successive times and a full night, stirring in the dark. The solvent extraction was then evaporated to obtain dry hydro-alcoholic extracts. The percentage yields (PY) obtained on a dry weight basis (%) of each crude extract are: female cones (35.5%), leaves (20.3%), rhizomes (21.1%) and stems (17.2%). All solvents used below, including ethanol, come from VWR Prolabo® (France).

The fractionation of the active part was performed using a liquid/liquid extraction with methylene chloride (MC) in proportion CH_2Cl_2/H_2O (5:5). Anhydrous sodium sulfate (Na_2SO_4) was added to the organic phase, enriched in non-polar phenolic compounds, to remove traces of water. After filtration, MC was evaporated, and the aqueous phase was freeze-dried, to obtained two sub-extracts with percentages yields of 50.9% and 12.2% respectively. The essential oil was obtained from the cone powder, using a Clavenger apparatus. Hydro-distillation of 4 h allowed to obtain an optimum amount of the essential oil with a yield of 6.3 mL per kg of dry cones.

2.1.2. HPLC analysis of the active sub-extract

The MC sub-extract of cones was analyzed by reverse phase High Performance Liquid Chromatography (HPLC, Shimadzu®), using a Shimadzu binary LC-10AS pump and a SCL-10A UV–vis detector. Analysis were performed on a VisionHT C18 HL (5 µm, 250 mm x 4.6 mm) column (Grace, Epernon, France), preceded by a C18 HL (7.5 mm x 4.6 mm) pre-column (Grace, Epernon, France). The elution was carried out with acetonitrile and water + 0.1% of acid formic. The gradient of acetonitrile was the following: 10–75% (0–5 min), 75% (5–30 min), 75–100% (30–35 min), and 100% (35–45 min). Injections of 20 µL of a 10 mg/mL plant extract solution in methanol were performed. The main wavelengths used are 254 nm and 370 nm.

2.1.3. Purification and identification of phenolic compounds

Phenolic compounds were purified from the MC sub-extract of hop's cones. It was first fractionated by Centrifugal Partition Chromatography (CPC, Armen instruments®). CPC is a liquid/liquid chromatography widely used for the separation and the purification of natural compounds. It is based on the partition of a sample in a biphasic immiscible liquid system (Kedzierski et al., 2014). The system consists of a rotor of 250 mL, connected to two Shimadzu® LC-20AP pumps, a CBM-20A controller, and a SPD-M20A diode array detector. The Arizona P system (heptane/ethyl acetate/methanol/water, 6:5:6:5) composed the mobile and stationary phases. Injections were carried out with 2 g of the MC sub-extract solubilized in 10 mL of a mixture mobile and stationary phases (5:5). The analysis takes 60 min at 8 mL/min and 1600 rpm. The extrusion, recovery of the stationary phase, takes 10 min at 30 mL/min with the same rotor speed. This method allowed to purify xanthohumol, the major prenylated chalcone of hops.

Several pre-purified fractions were also obtained from the CPC, from which other compounds were isolated using preparative HPLC. The system is composed of two Shimadzu® LC-20AP pumps, a CBM-20A controller, and a SPD-M20A diode array detector. The stationary phase was a VisionHT HL C18 (5 µm, 250 × 22 mm) column (Grace). The mobile phase and the gradient used were the same as HPLC-UV analysis. Injections of 500 µL of a 60 mg/mL fractions solution in methanol were performed. The flow rate was set at 12 mL/min. The main wavelengths used are 254 nm and 370 nm. This process allowed to purify another chalcone, desmethylxanthohumol, and acylphloroglucinol derivatives, or bitter acids: co-humulone and humulone for the α-acids, co-lupulone and lupulone for the β-acids (Fig. 1).

The structural elucidation of compounds was conducted with High Resolution-Mass Spectrometry (HR-MS) and Nuclear Magnetic Resonance (NMR). NMR spectra were recorded on a Bruker DPX-500 spectrometer. Mono- (^1H and ^{13}C) and bi-dimensional (COSY, HSQC, HMBC) spectra were carried out for each compound. HR-MS analysis was carried out in positive mode with a range of *m/z* 100–1000, using a Thermo Fisher Scientific Exactive Orbitrap Mass Spectrometer equipped with an electrospray ion source.

2.1.4. Analysis of the essential oil of cones

Gas-Chromatography coupled to Mass Spectrometry (GC–MS/MS) was used to identify the major compounds of the essential oil. A Varian VF–1 ms capillary column (30 m x 0.25 mm) attached to a Varian CP-3800 gas chromatography and a Varian Saturn 200 spectrometer. The temperature gradient was as following: 40 °C (5 min) and 200 °C at 2 °C/min (5 min), 250 °C at 10 °C/min. The essential oil was previously solubilized at 1% in hexane, and 1 µL was injected at 250 °C by a Varian CP-8400 auto-sampler, with a split of 50:50 using helium as carrier gas. Kovats indexes were determined for each major compound. They were obtained by normalizing the retention time of each compound related to the retention time of n-alkanes adjacent eluent. The Kovats index is