Crop Protection 55 (2014) 109–118

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Antifungal activity of liquid waste obtained from the detoxification of steam-exploded plant biomass against plant pathogenic fungi

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ARTICLE INFO

Article history:
Received 11 March 2013
Received in revised form
23 September 2013
Accepted 25 September 2013

Keywords:
Antifungal activity
Plant pathogenic fungi
Renewable plant resources
Steam explosion liquid waste
Sustainable horticulture

ABSTRACT

The antifungal activity of steam-exploded liquid waste (SELW) produced by the detoxification of steam-exploded biomass of *Miscanthus sinensis*, *Arundo donax* and wheat straw for 2nd generation industrial bioethanol production was studied against plant pathogenic fungi for the first time. Quantification of fermentation inhibitors (2-furaldehyde, 5-hydroxymethylfurfural, acetic and formic acid) was carried out by standard methods. Mycelial growth inhibition and conidial germination of eight fungal strains [*Alternaria alternata*, *Botrytis cinerea*, *Colletotrichum acutatum*, *Cladosporium fulvum*, *Fusarium oxysporum* f. sp. *lycopersici* (FOL), *F. oxysporum* f. sp. *melonis* (FOM), *F. solani* f. sp. *pisi* and *Verticillium dahliae*] were evaluated *in vitro* at different dilution rates (1:2, 1:4 and 1:8) for each SELW. Disease suppressiveness was assessed *in vivo* in eight horticultural pathosystems (*A. alternata*/tomato, *B. cinerea*/tomato, *C. acutatum*/strawberry, *C. fulvum*/tomato, FOL/tomato, FOM/melon, *F. solani* f. sp. *pisi*/pea and *V. dahliae*/eggplant) under greenhouse conditions. Pathogen suppression by each SELW, applied both in spray form on to tomato leaves and skins of tomato and strawberry, and by means of dipping method in watering suspensions on to seedling root systems of tomato, melon, pea and eggplant, was evaluated at dilution rates of 1:2, 1:4 and 1:8 using irrigation water during curative and preventive treatments. Investigations carried out *in vitro* showed that *M. sinensis* SELW was more suppressive than wheat straw SELW, and *A. donax* SELW was less suppressive than wheat straw SELW at the lowest dilution rates. Relationships between the concentration of 2-furaldehyde, acetic and formic acid present in SELWs and their antifungal effect were found. Moreover, *B. cinerea*, *C. fulvum*, *V. dahliae*, *A. alternata*, *C. acutatum* and *F. solani* f. sp. *pisi* were more effectively inhibited than FOL and FOM. Investigations performed *in vivo* showed that SELWs of *M. sinensis* and wheat straw can be diluted at a 1:2 rate and used for controlling six fungal diseases during preventive treatments. In particular, they can be used by dipping of the root systems during transplanting in the case of *F. solani* f. sp. *pisi*/pea and *V. dahliae*/eggplant; on the other hand, they can be nebulized on to the leaves and fruits before symptoms appearance in the case of *A. alternata*/tomato, *B. cinerea*/tomato, *C. acutatum*/strawberry and *C. fulvum*/tomato. This study is of particular interest because it points out how these SELWs could be employed in horticultural crop protection in Southern Italy, thereby making it possible to effectively combine industrial production of 2nd generation biofuels with sustainable horticulture under greenhouse conditions.

1. Introduction

Since the 1990s, the steam-explosion (SE) process has been a subject of great interest as regards 2nd generation industrial bioethanol production from several renewable plant resources, such as pruned wood, energy crops and plant wastes (Focher et al., 1991). Second generation bioethanol from different lignocellulose sources, such as annual and perennial energy crops (*Miscanthus sinensis* × *giganteus*, *Arundo donax* L., *Panicum maximum* Jacq., *Sorghum bicolor* L. Moench and *Cynara cardunculus* L.), short rotation forestry species (*Populus tremula* L., *Eucalyptus* spp., *Salix purpurea* L. and *Robinia pseudoacacia* L.) and green wastes (corn stalks,

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http://dx.doi.org/10.1016/j.cropro.2013.09.005

rice husks, straws and woody shaving), is considered to be important for its potential uses as a biofuel in Italy (Ballerini et al., 1994; Nunes and Pourquie, 1996; Pari, 2011).

Pre-treatment of biomass by SE involves a thermo-mechanic-chemical process performed in different steps: saturated steam penetrates the lignocellulose structures by diffusion in a reactor; it partially or completely condenses on contact with cold biomass, wetting the material; thereby the acetyl groups of the hemicellulose fraction are hydrolysed, forming furfural, acetic, formic and uronic acid. The pre-treatment ends with a sudden decompression to atmospheric conditions which mechanically defiberize the biomass by means of the instantaneous vaporization of liquid water. Treatment severity in terms of temperature and time typically ranges from 180 to 220 °C and lasts between 1 and 10 min, respectively.

The chemical composition of steam-exploded biomass (SEB) varies widely, depending on raw materials and treatment severity (Zimbardi et al., 2007). This process also produces volatile compounds that inhibit microbial fermentation; these compounds include products of sugar dehydration and lignin depolymerization, such as organic acids, furfural, aldehydes and phenolic acid (Zimbardi et al., 2007). In particular, highest severity promotes the degradation of glucose and xylose to furfural, acetic and formic acid, which have very good inhibitory effect on ethanologenic yeasts (*Saccharomyces cerevisiae* and *Pichia stipis*) employed in 2nd generation bioethanol production carried out in pilot plants (De Bari et al., 2004; Navarro, 1994).

Volatile compounds, which have a severe negative impact on the ethanologenic microorganisms involved in the fermentation, must be removed from the SEB before the process. This detoxification procedure was usually carried out inside special reactors by means of three-four consecutive washings in water; however, this led to the removal of not only the inhibitors but also of some soluble hydrocarbons useful in the bioethanol conversion process. A technological system which is able to combine biomass detoxification with a steam and air flow technology, without the addition of any chemical substances or loss of hydrocarbons, was studied in Italy. In fact, for one such industrial system for removing furfural, acetic and formic acid and other volatile compounds, the Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA) has already obtained a patent N° 753/2012/ENEA (Arcieri et al., 2012). These volatile molecules were removed by evaporation, condensed by hydro-distillation, recovered in the aqueous phase (commonly known as “steam-exploded liquid waste” or SELW), purified and employed as co-products in the pharmaceutical and chemical industries.

Recent research performed in Italy also demonstrated that SEBs of *M. sinensis*, *A. donax* and wheat straw can be employed in crop protection as alternatives to commercial compost used in horticulture. In fact, they have been used effectively as substitutes for compost since they have properties capable of suppressing some particularly diffuse soil-borne plant pathogens. In particular, SEBs of *M. sinensis* and *A. donax*, tested in seven soil-borne crop-pathogen systems under greenhouse conditions with applications at three increasing rates in pots [10, 20 and 30% (w/w) of mixed soil], proved to be more suppressive than commercial compost and SEB of wheat straw (De Corato et al., 2011a, 2011b, 2011c). The suppressiveness of SEB could be attributed to the inhibitory effect of furan molecules, such as 2-furaldehyde and 5-hydroxymethylfurfural (De Corato et al., 2010) produced during the chemical conversion of glucose and xylose (Tanahashi, 1990). Moreover, the nature of the mechanisms by which carbohydrates are converted into 5-hydroxymethylfurfural has now been revealed by specific molecular studies (Khokhlova et al., 2012). Some hypotheses regarding the most likely or most hypothesized action mechanisms of these substances have also been reported in the literature. In particular, damage to cell membrane integrity, inhibition of essential enzymes and negative interaction with DNA/RNA are likely to be involved (Ibraheem and Ndimba, 2013).

In this research work, the antifungal activity of SELWs obtained from two energy crops (*M. sinensis* and *A. donax*) and one type of green waste (wheat straw) were assessed *in vitro* and *in vivo* against eight plant pathogenic fungi that are harmful to tomato (*Lycopersicon esculentum* Mill.), melon (*Cucumis melo* L.), strawberry (*Fragaria* × *ananassa*), pea (*Pisum sativum* Asch. *et* Gr.) and eggplant (*Solanum melongena* L.). The main purpose of this work was to evaluate the potential employment of these SELWs under greenhouse conditions for horticultural crop protection in Southern Italy.

2. Material and methods

2.1. Steam-explosion treatment and detoxification of steam-exploded biomass

The biomasses considered in this work were collected in different Regions of Southern Italy (Puglia, Basilicata and Calabria). SE pre-treatment with *M. sinensis*, *A. donax* and durum wheat straw biomass was carried out in a pilot plant ST.E.LE. (STeam Explosion LEgno – Mod. Stake Tech System Digester) located at ENEA TRISAIA (Rotondella, Matera, Italy) by processing 300 kg/h of fresh or air-dried biomass at 207 °C × 6 min.

The detoxification treatment of *M. sinensis*, *A. donax* and wheat straw SEBs was carried out in a special reactor located at ENEA TRISAIA, inside of which SEB was subjected to 130 °C × 5 h. The evaporated molecules were then condensed in the aqueous phase, collected in bottles or tanks and stored at room temperature for further use. All samples were sterilized by filtration before *in vitro* bioassays.

2.2. Quantification of volatile microbial inhibitors

Three replicated samples of each SELW were analysed for the quantitative determination of the many volatile microbial inhibitors. 2-furaldehyde (furfural), 5-hydroxymethylfurfural (HMF) and formic acid (expressed as g/L) were extracted from crude SELWs by water washing and determined by the High Pressure Liquid Chromatography (HPLC) technique. Acetic acid (expressed as g/L) was extracted from crude SELWs with the same method and quantified by the High Performance Ionic Chromatography (HPIC) technique.

The concentrations of furfural, HMF and formic acid were determined by HPLC system (Mod. HP 1100, Series with DAD detector). Three replicated aliquots of each sample (20 μL) were filtered by 0.2 mm filters (Millipore, Milan, Italy), manually injected and eluted through a Phenomenex Synergi Fusion-RP 80 column. Acetonitrile/water gradient (eluent) was used as follows: from 0 to 15 min with CH_3CN 3%, from 15 to 27 min with CH_3CN 10%, from 27 to 35 min with CH_3CN 17%, from 35 to 50 min with CH_3CN 30% and from 50 to 60 min with CH_3CN 50%.

The concentrations of acetic acid were determined using a HPIC system (Mod. Dionex LC30) equipped with automatic injector (Mod. AS50). Three replicated aliquots of each sample (20 μL) were filtered by 0.2 mm filters (Millipore, Milan, Italy), automatically injected and eluted through a Nucleogel Ion 300 OA column. Sulphuric acid (H_2SO_4 0.01 N) was used as an eluent at a temperature of 40 °C and a flow rate of 0.4 mL/min. Detector refractive index ED50 was used.