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Evaluation of productive and methanogenic potential of Mediterranean crops

PhD Thesis

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Preface

The aim of the thesis was the evaluation of the feasibility of using three Mediterranean crops for biomethane production via anaerobic digestion.

The thesis is organized in three main chapters. The first Chapter is a general introduction that presents the state of art of bioenergy, biomethane and lignocellulosic crops.

The results of field and laboratory experiments are reported in Chapter 2 which consist of five published papers and one not yet published.

Paper I presents the evaluation of a more than 20-year old plantation of 40 genotypes of giant reed (*Arundo donax* L.) collected around Southern Italy in terms of biomass and biomethane yield under two levels of irrigation (100% ETm and rainfed).

In the context of reducing biomass recalcitrance of giant reed in order to increase biomethane yield, in the Paper II, harvest time and nitrogen fertilization treatments were adopted to assess the suitability of the lignocellulosic herbaceous crop *Arundo donax* L. as a biomass feedstock for advanced biomethane production.

The thesis continues with biomass characterization of *Arundo donax* L. and *Saccharum spontaneum* subsp. *aegyptiacum* (Willd.) Hack and their potential use for biomethane production during anaerobic digestion process after a thermal pretreatment (Paper III).

Paper IV investigates the effect of a biological pretreatment of *Arundo donax* L. obtained from two different harvesting time (autumn and winter) on biomethane production by anaerobic digestion using two white rot fungi (*Pleurotus ostreatus* and *Irpex lacteus*).

Paper V reports the results of field experiment conducted to compare

different genotypes of Castor (*Ricinus communis* L.) breed from native perennial plants in the Mediterranean basin in terms of seed and oil yield.

The seeds of the genotypes with high seed yield were used for a field experiment that investigated the optimization of the cultivation techniques through the evaluation of agronomical inputs reduction in order to increase seed yield. Capsule husks represent agricultural lignocellulosic residues obtained from the oil extraction process. We hypothesized to recover this lignocellulosic residue to produce biomethane through anaerobic digestion after a biological pretreatment using white-rot fungi (Paper VI).

The general conclusions of the results obtained are presented in Chapter 3.

Abstract

Anaerobic digestion process to produce methane is becoming the most used technology worldwide to address concerns about global warming, energy security and the need for sustainable waste management. Anaerobic digestion of lignocellulosic biomass is a highly appreciated and encouraging technology since allows to avoid the conflict over biomass for food/feed or energy.

As a result, non-food lignocellulosic biomass has emerged as promising choice to methane production through anaerobic digestion.

The main disadvantage of using lignocellulosic biomass is its recalcitrance to hydrolysis due to the high lignin content on cell wall.

This thesis deals with evaluation of the feasibility of using three Mediterranean crops for biomethane production via anaerobic digestion.

Different genotypes of biomass crops suitable to grow in semi-arid Mediterranean environment (*Arundo donax* L., *Saccharum spontaneum* ssp. *aegypticum* and *Ricinus communis* L.) were evaluated in terms of input requirement, biomass quality, biomass yield and seed yield.

The lignocellulosic biomass of *Arundo donax* and *Saccharum spontaneum* was evaluated in reference to the biomethane production carrying out different pretreatments, while for *Ricinus communis*, lignocellulosic capsule residue from castor oil extraction was used to produce biomethane via anaerobic digestion processes.

Sintesi

Le energie da fonti rinnovabili offrono un'opportunità eccezionale per limitare le emissioni di gas serra e ridurre il riscaldamento globale sostituendo le fonti energetiche convenzionali (basate su combustibili fossili) e quindi mitigare i cambiamenti climatici.

Tra le diverse filiere energetiche, quella del biogas si integra maggiormente con il comparto agricolo, anche in termini di sotenibilità, in quanto consente l'utilizzo di colture energetiche idonee alla coltivazione in terreni marginali, la gestione in maniera più efficiente di alcuni residui agricoli e la valorizzazione dei sottoprodotti da un punto di vista energetico.

Lo scopo del presente lavoro di tesi è valutare la qualità e la resa della biomassa di diverse colture adatte a crescere in ambiente semiarido mediterraneo (*Arundo donax* L., *Saccharum spontaneum* ssp. *aegypticum* e *Ricinus communis* L.) ed il loro potenziale metanigeno.

In particolare, la biomassa lignocellulosica di *Arundo donax* e *Saccharum spontaneum* ed i residui lignocellulosici ottenuti dalla lavorazione del seme di *Ricinus communis* sono stati sottoposti a pretrattamenti di tipo termico e fungino (utilizzando i funghi white-rot *Pleurotus ostreatus* e *Irpex lacteus*) e valutati in termini di produzione di biometano tramite il processo di digestione anaerobica.

List of abbreviations

AD	Anaerobic digestion
ADF	Acid detergent fiber
ADL	Acid detergent lignin
AFEX	Ammonia fiber explosion
ANOVA	Analysis of variance
BMP	Biochemical methane potential
BMY	Biomethane yield
BOD	Biological oxygen demand
CL	Cellulose
COD	Chemical oxygen demand
DMY	Dry matter yield
ETm	Maximum evapotranspiration
GHG	Greenhouse gas
HL	Hemicellulose
LUC	Land-Use-Change
NDF	Neutral detergent fiber
NDS	Neutral detergent soluble
NIR	Near InfraRed spectroscopy
REDII	Renewable Energy Directive
TS	Total solids
VFAs	Volatile fatty acids
VS	Volatile solids
WRF	White-rot fungi

Keywords: biomass, perennial energy crops, *Arundo donax* L., *Ricinus communis* L., *Saccharum spontaneum* ssp. *aegypticum*, long term plantation, seed yield, oil yield, anaerobic digestion, bioenergy, biofuel, biomethane potential, pretreatment, fungal pretreatment, *Pleurotus ostreatus*, *Irpex lacteus*.

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Chapter 1- General introduction

World population and global demand for food and energy are rapidly increasing resulting in a consequent depletion of fossil fuels and emergence of environmental concerns such as global warming, greenhouse gas emission and land use change [1]. These factors have greatly contributed to looking for alternative energy sources.

Energy consumption is the main cause of climate change and greenhouse gas emissions. Within the energy sector, heat and electricity production are responsible for most emissions, followed by transportation, manufacturing and construction [2]. Research investigates on production of renewable energy sources to reduce use of fossil fuels and mitigate their adverse environmental effects on air quality. The use of biomass for energy (i.e. bioenergy) is considered to be a promising renewable energy alternatives for reducing the environmental impact [3].

Energy generation from biomass sources can be derived directly such as by burning wood for heating, or indirectly from products such as alcohols or biogas. Interest in biogas as a source of bioenergy has progressively been growing since the biogas can ultimately be used to produce electricity and/or thermal energy or biofuel. Biogas is a biofuel obtained through anaerobic digestion from biomass sources, mainly agricultural residues, sewage sludge, animal manure, microalgae and food waste [4].

Among all possible solutions, anaerobic digestion (AD) represents one of the most promising ways to use biomass [5].

Biogas production depends on different types of materials and their physical and chemical properties and on operating conditions [6].

The main organic materials used to produce biogas by anaerobic digestion include municipal solid waste, industrial wastes and wastewaters, food waste, livestock manure, sewage sludge, energy crops, and microalgae [7].

Among various biofuels, biomethane production via anaerobic digestion is one of the most cost-effective and environmentally friendly technology to produce energy from lignocellulosic feedstocks performed worldwide.

Lignocellulosic substrates used for anaerobic digestion should not directly compete with food or feed crops for the exploitation of limited agricultural land resources [8].

An ideal energy crop for biogas production should have high biomass yields and show adaptability to varying environments even under low requirement of energy, water, and nutrients.

Perennial grasses have demonstrated their capacity to grow under adverse conditions minimizing environmental impacts, due to the reduced inputs requirements [9]. However, major challenges to obtain biomethane from lignocellulosic biomass are the highly recalcitrant structure and the complex chemical composition, which confer the resistance to anaerobic degradation [10].

Pretreatment is a necessary step in the processes of anaerobic digestion to overcome lignocellulosic recalcitrance in order to improve methane production from lignocellulosic substrates [11].

The main aim of this thesis was to identifying various Mediterranean crops suitable for cultivation in marginal lands for their usage towards biomethane production via anaerobic digestion.

The Mediterranean crops evaluated during these investigations were *Arundo donax* L., *Saccharum spontaneum* ssp. *aegypticum* and *Ricinus communis* L.

The project included the evaluation of biomass yield obtained by agronomic trials, the qualitative analysis of biomass composition and the optimization of pretreatment processes to enhance biomethane production yields.

1.1 Bioenergy

The continuous consumption of fossil fuels has led to a decrease of the fuel reserve, higher emission of greenhouse gases, climate change and environmental decline. With these scenarios, research investigates on the development of sustainable renewable fuels to meet the present global energy needs without compromising future sustainability [12].

Biofuels are defined as liquid, solid, or gaseous fuels generated from biomass, such as agricultural crops, forest residue, by-products, or waste. Biofuels shown numerous positive aspects because they are a renewable resource and their use is considered environmentally friendly being their carbon balance close to neutral if compared with fossil fuels that are not renewable since they require millions of years to form [13].

Biofuels are differentiated based on different parameters, including sources of feedstock, conversion process used for their production and physical state.

The main classification refers to the origin of biomass used and groups biofuels in first, second and third generation biofuels.

First-generation biofuels are obtained from dedicated cultivation of food or animal feed crops. Major examples of crops used are rapeseed,

soybean, sunflower, corn, sugarcane, wheat and sugar beet. The extracted oils are converted to biodiesel through the transesterification process while ethanol, a form of bioalcohol, is made through fermentation from corn, sugarcane, etc.

Second-generation biofuels are generated from non-food feedstock, such as dedicated energy crops, agricultural and forest residues and other organic waste.

Third-generation biofuels are produced from algal biomass. The algal oil extracted can then be converted into bioethanol or biodiesel through fermentation and transesterification [14].

Biofuels production offers both advantages and disadvantages in terms of environmental, economic and social aspects.

First-generation biofuels normally offer some advantages in terms of energy production potential, but they come from food crops so contribute to increase food and feeds word prices, food vs fuel debate, impact on biodiversity and land use.

Second-generation biofuels entail benefits in terms of C-mitigation, with a lower impact on greenhouse gas emissions than the traditional fuels from fossil sources. Moreover, second-generation biofuels don't compete with food crops and no arable land is required for growing them, with few negative consequences for the environment arising from development of these biofuels.

Biofuels based on the physical state are categorized as solid, liquid and gaseous.

Generally, any solid biomass material can be described as solid biofuel. Solid biomass is principally any solid feedstock that can be converted into biofuel; Biomass as a solid biofuel can be lignocellulosic biomass (agricultural residues, forest residues, energy crops) and solid waste

(the organic fraction of municipal solid waste, wastewater sludge, food waste). These raw materials offer the advantage of being renewable and getting rid of the waste rather than use fossil fuel.

Liquid biofuels refer to any renewable fuel of natural origin in liquid form. They are mainly used as transport fuels. The main examples of liquid biofuels are biodiesel, biomethanol, bioethanol, biobutanol, biopropanol, bio-oil, etc.

Liquid biofuels show interesting features if compared to fossil fuels: are high combustibility, easy and safer to store and to transport, and they are well enough no explosive.

Biogas/biomethane, biohydrogen, and biosyngas are the commonest examples of gaseous biofuels. They have a wide variety of applications, including for thermal, transport, and heat uses and electricity/power generation [12].

Another classification groups biofuels into two classes: conventional biofuels obtained through well-established technologies such as fermentation, distillation and transesterification from edible feedstocks (ethanol from corn, sugar beet, sugar cane, cereals and cassava; biodiesel from vegetable oils, e.g., soybean, rapeseed, sunflower, palm oil) and advanced biofuels produced from different inedible feedstocks listed in part A of Annex IX of the Renewable Energy Directive.

The Renewable Energy Directive, Directive (EU) 2018/2001, (REDII), established a common framework for the promotion of renewable energy sources in the EU and set a binding target of 32% for the overall share of energy from renewable sources in the EU's gross final consumption of energy in 2030. It also established sustainability and greenhouse gas emissions saving criteria for biofuels, bioliquids and

biomass fuels. The REDII is a recast of the Directive 2009/28/EC (REDI). The feedstocks listed in part A of Annex IX include animal manure and sewage sludge, straw, bagasse, nut shells, husks, biomass fraction of wastes and residues from forestry and forest-based industries, energy crops or algae grown for fuel [15,16].

Perennial grasses, such as giant reed, switchgrass, miscanthus, and ryegrass were included as non-food cellulosic material to produce advanced biofuels and to minimize the overall direct and indirect land use change (LUC) impact [17].

1.2 Lignocellulosic biomass

A great interest has been given to lignocellulosic biomass which represents one of the most abundant source of organic matter on earth. Lignocellulosic materials can be collected as forest, agricultural, industrial, and municipal residues. In addition, lignocellulosic biomass could be obtained from energy crops that do not compete with food crops and can grow in areas not suitable for food crops since several ethical concerns have arisen from using food crops for biofuels production [18].

Lignocellulosic biomass is composed mainly of three types of polymers: cellulose, hemicellulose, and lignin that confer rigidity and protection to the plant cell wall. In addition, it also contains minor amounts of extractives and inorganic materials [19]. The carbohydrate components (cellulose and hemicellulose) are fermentable after hydrolysis, which makes lignocellulosic biomass a suitable feedstock for bioenergy production. However, the inherent characteristics of lignocellulosic biomass, such as structural and chemical properties, make it resistant to biodegradation by enzymes and microbes.

Cellulose is the main component of lignocellulose cell walls. It is a linear polysaccharide homopolymer of cellobiose (glucose disaccharide) strongly attached by b-1, 4 glycosidic bonds. A number of hydroxylic groups are present in the cellulose chains, leading to the formation of hydrogen bonds in the same chains or in vicinal chains. Cellulose chains are interlinked by hydrogen bonds and Van der Waals forces to form microfibrils with high tensile strength [20]. Cellulose molecules have different orientations throughout the structure, leading to different levels of crystallinity. Thus, cellulose consists of two regions: crystalline that is both flexible and strong and shows high crystallinity and amorphous characterized by low crystallinity and usually easier to hydrolyze by chemical reagents and enzymes [21]. Meanwhile, cellulose microfibrils are also attached to each other by hemicellulose and/or pectin, and covered by lignin. Such a specialized and complicated structure renders cellulose resistant to biological and chemical attacks. Cellulose corresponds to around 40-50% of the lignocellulosic biomass and it is one of the most abundant polymers on earth [22].

In contrast to cellulose, hemicellulose is an amorphous, random and branched heteropolymer. Hemicellulose has branches with short lateral chains consisting of different types of sugars. These monosaccharides are: pentoses (xylose and arabinose), hexoses (glucose, galactose, mannose, and/or rhamnose), uronic acid groups (glucuronic acid, methylglucuronic acid, and galacturonic acid) and acetyl groups in varying amounts depending on the plant species [23].

Hemicellulose is closely linked with cellulose via hydrogen bonds and with lignin mainly through covalent bonds contributing to improving the mechanical properties of cell walls such as rigidity and flexibility.

Lignin is the third most abundant polymer in nature. It is a large, complex and amorphous aromatic and hydrophobic three-dimensional heteropolymer consisting of three units: guaiacyl, sinapyl, and p-hydroxyphenyl linked by aryl ether or C-C bonds. The role of lignin is to give rigidity, impermeability and resistance to plants (microbial attacks, oxidative stress).

Lignocellulosic material can be used to produce diverse energy products. There are many processes that could be applied to convert lignocellulosic biomass to different energy products. Lignin is the main barrier to utilization of lignocellulosic biomass in energy production due to its structure highly heterogeneous and its resistance to hydrolysis. It is cross-linked to the holocellulose and forms a complex and highly resistant structure that makes carbohydrates less accessible for bioconversion processes, thus it results the most recalcitrant component of the plant cell wall [24]. The structure formed by the interaction of the cellulose, hemicellulose, and lignin makes lignocellulosic materials often insoluble in water at low temperature and not easily digestible by living organisms.

Different feedstocks contain different amount of lignin that must be partially degraded via pretreatment to enhance biomass digestibility.

1.3 Pretreatment of biomass

Structural characteristics of lignocellulosic biomass provide resistance to biological degradation, limiting the conversion of lignocellulosic substrates into methane. The bio-energy conversion process of lignocellulosic biomass into methane via anaerobic digestion may be limited by its hydrolysis phase as the digestible cellulose and hemicelluloses are covered by a layer of insoluble lignin [25]. Thus, to

use lignocellulosic biomass in anaerobic digestion, are necessary one or more pretreatment steps to facilitate microorganisms access to cellulose and hemicellulose.

The main goal of pretreatment is to break down linkages between polysaccharides and lignin to alters the biomass macroscopic and microscopic size and expose cellulose and hemicellulose to enzymatic attack [26]. Therefore, pretreatment is essential to disrupt the complex lignocellulosic structure and reduce cellulose crystallinity in order to improve the production of fermentable sugars and enable the conversion of structural carbohydrates to monomeric sugars during hydrolysis phase of anaerobic digestion to increase their conversion yield into renewable fuels production [27].

Pretreatment of the lignocellulosic material is carried out to overcome recalcitrance through the combination of chemical and structural changes to the lignin and carbohydrates. Lignocellulosic biomass pretreatments include different categories: physical, chemical, thermal, thermo-chemical and biological [10].

Mechanical- physical pretreatments are used to physically reduce the feedstock size or change biomass structure by means of fragmentation, grinding or milling to increase the surface areas and enhance enzyme accessibility. Physical pretreatment also includes other methods such as irradiation (e.g., ultrasound, gamma ray, and microwave), steam explosion, liquid hot water pretreatment and others.

Thermal pretreatments are based on the use of a solvent (water, CO_2, etc.) at high temperature and sometimes in combination with a high pressure.

Chemical pretreatments refer to the use of chemicals (e.g., acids or strong bases, oxidizing agents, organic or inorganic solvents) to change

physical and chemical characteristics of native lignocelluloses [28].

Pretreatment with alkali such as NaOH, KOH, $Ca(OH)_2$, hydrazine and anhydrous ammonia cause swelling of biomass, which increases the internal surface area of the biomass, and decreases both the degree of polymerization and cellulose crystallinity. Alkaline pretreatment disrupts the lignin structure and breaks the linkage between lignin and the other carbohydrate fractions in lignocellulosic biomass, thus making the carbohydrates in the hetero-matrix more accessible. The reactivity of remaining polysaccharides increases as the lignin is removed. Acetyl and other uronic acid substitutions on hemicellulose that lessen the accessibility of enzymes to cellulose surface are also removed by alkali pretreatments [29–32].

However, most of the alkali is consumed. Alkali pretreatment is most effective with low lignin content biomass like agricultural residues but becomes less effective as lignin content of the biomass increases.

Solutions of dilute (< 4 wt.%) sulfuric acid, hydrochloric acid, and phosphoric acid have been also used to hydrolyze biomass [33]. Concentrated acids are not preferred because they are corrosive and must be recovered to make the pretreatment economically feasible. Dilute acid pretreatment in hydrolysis of hemicellulose to its monomeric units, rendering the cellulose more available. Acid pretreatment may require the use of an alkali to neutralize the hydrolysate. Acid pretreatment has also some drawbacks, such as high cost of the materials used for construction of the reactors, gypsum formation during neutralization after treatment with sulfuric acid, and formation of inhibitory by-products [34].

Pretreatments that combine both chemical and physical processes are referred to as physico-chemical processes.

Steam explosion is a hydrothermal technology that treat biomass with high-pressure saturated steam for few seconds (30 s) to several minutes (20 min), and then pressure is suddenly reduced. The mechanical effects are caused because of sudden reduction in pressure and fibers are separated owing to the explosive decompression that disrupts biomass structure. This process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis [35].

Steam explosion pretreatment has low energy requirement compare to physical size reduction pretreatment and no use of toxic chemicals, thus reducing the environmental impact. It is the most commonly used pretreatment method for lignocellulosic biomass [36].

Ammonia fiber explosion (AFEX) is a physicochemical pretreatment process in which lignocellulosic biomass is exposed to liquid ammonia at high temperature (60- 100 °C) and pressure ($20*10^5$ Pa) for 5 min, and then the pressure is reduced. The AFEX process is very similar to steam explosion. The combined effect of ammonia and high pressure leads to swelling of lignin-carbohydrate matrix, disrupting the lignocellulosic structure and leading to hemicellulose hydrolysis and decrystallization of cellulose [37]. Conventional physico-chemical methods for lignin degradation require large inputs of energy and also cause pollution.

Biological pretreatment can be classified into three categories including fungal, microbial consortium and enzymatic pretreatment [38].

Compared with physical and chemical pretreatment, biological methods are more environment friendly, consume less energy, produce no inhibitors and don’t require chemicals input. Despite the advantages, substantial holocellulose losses (required by microbes during

pretreatment) and long pretreatment time are the major issues associated with biological pretreatments [11].

The commonly utilized microorganisms in this pretreatment of lignocellulosic biomass are filamentous fungi, which can be easily found in the environment such as ground, living plants and lignocellulose wastes [39]. Wood-decay fungi are classified into three main groups, which are white-, brown- and soft-rot fungi [40].

Among them, white-rot fungi (WRF) are mainly involved in biological pretreatment due to their capability to degrade lignin from the holocellulose (cellulose and hemicellulose) surface [39,41] whereas brown- and soft-rot fungi degrade only minimal lignin. White-rot fungi can be differentiated by their delignification mode as selective and non-selective delignification. In selective delignification, mostly lignin and hemicellulose are degraded, while a small amount of cellulose is consumed. Whereas in non-selective delignification lignin, hemicellulose and cellulose are degraded almost equally [42,43]. White rots are able to degrade lignin through the action of lignin-degrading enzymes such as peroxidases (lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and laccase) synthetized during an oxidative process. White-rot fungi have also a hydrolytic mechanism producing hydrolytic enzymes consisting of cellulase, cellobiase, and xylanase responsible to degrade glycosidic linkages in cellulose and hemicellulose releasing monomeric sugars [39].

White-rot fungi species commonly used are *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, *Ceriporia lacerata*, *Pycnoporus cinnabarinus*, *Cyathus cinnabarinus*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus, Bjerkandera adusta*, *Ganoderma resinaceum*, *Trametes versicolor*, *Fomesfomentarius*, *Irpex lacteus* [44–46].

To achieve a cellulose-rich but highly delignified biomass for biofuel production, white rot fungi highly selective and efficiency for delignification in lignin degradation are preferred for fungal pretreatment. In addition, cultivation parameters such as moisture content, feedstock particle size, oxygen concentration and incubation time can also affect the pretreatment performance [41].

Moisture content is important to the fungal establishment and for fungal growth. The optimal range of moisture content of substrate using white-rot fungi is between 70 and 80 % and varies with fungus strain and type of biomass [47].

Particle size of the substrate is another important factor affecting biological pretreatment. Large particle size can limit penetration of fungi into cellulosic biomass and also prevents the diffusion of air, water, and metabolite intermediates into the particles. However, small particle size can reduce inter particle gas circulation, thus not necessarily giving an enhanced delignification rate [48].

The time required for the colonization of the substrate varies with biomass composition and fungus strain. Requirements for long incubation time, due to low lignin removal rates, is one of the main obstacles for the application of fungal pretreatment on a large scale [41]. Therefore, optimization of these parameters is necessary to increase the efficiency of the pretreatment by reducing the carbohydrate loss and pretreatment time [42].

WRF were widely studied in biological pretreatment principally for bioethanol production because they can improve enzymatic hydrolysis and its subsequent sugar yield [41]. However only few works have been conducted regarding the improvement of methane during anaerobic digestion of lignocellulosic biomass by using white-rot fungi.

1.4 Biochemical Methane Potential (BMP) tests

Biochemical methane potential (BMP) test is a popular technique that is used to evaluate the biodegradability and potential to produce methane for different organic materials under anaerobic conditions determining both degradation kinetics profile and the methane yield of any substrate [49].

During the test, a predetermined amount of substrate is mixed with an anaerobic bacteria culture, normally obtained from an active anaerobic digester. The reactors are then constantly mixed for a period of time from 30 to 60 days, until the daily methane production during three consecutive days is <1% of the accumulated volume of methane, and kept at a stable temperature of 35- 40 °C [50,51].

The substrate is degraded through a multistep biochemical process obtaining methane and carbon dioxide as the major final products. Since only the amount of methane is of interest, the carbon dioxide is often removed using a scrubber agent (e.g. ethylamines, alkaline solution). The BMP value is expressed as the volume of methane that can be produced from a unit of organic material substrate defined as volatile solids (VS), chemical oxygen demand (COD) or biological oxygen demand (BOD). The BMP assay, therefore, represents a tool for selecting and screening feedstock to produce biogas via anaerobic digestion and to assess the extent and rate of anaerobic transformation of a given substrate to energy rich methane.

1.5 Anaerobic digestion

Anaerobic digestion (AD) is a complex of microbiological processes in which organic substrate is decomposed by microorganisms in the absence of oxygen to produce biogas. Biogas is constituted mainly of

methane (50-75%), carbon dioxide (25-50%), H_2O (1%–5%) and other minor volatile components, such as N_2, H_2S, NH_3 and siloxanes or other organic hydrocarbons.

During AD process specific groups of bacteria work going through four main stages namely hydrolysis, acidogenesis, acetogenesis and methanogenesis.

During the hydrolysis step, complex organic polymers of biomass such as proteins, carbohydrates and lipids are hydrolyzed into simple soluble monomers like aminoacids, sugars, glycerol and long-chain fatty acids by hydrolytic exo-enzymes (example, cellulase, amylase, protease, and lipase) excreted by fermentative microorganisms.

Hydrolysis is immediately followed by the acid-forming step-acidogenesis. In this step, the feedstock from hydrolysis are converted by acidogenic bacteria to a mixture of volatile fatty acids (VFAs) such as propionic and butyric acid and other minor products such as alcohol. Acetogenesis is the stage where acetogenic bacteria further convert the VFAs to acetate, carbon dioxide, and/or hydrogen.

Methanogenesis is the final reaction of anaerobic digestion and in this stage methane is produced by methanogenic bacteria. These bacteria are capable of metabolizing formic acid, acetic acid, methanol, carbon monoxide, carbon dioxide and hydrogen to methane. This stage has two general pathways that involve the utilization of the two major products of the first three stages of anaerobic digestion, acetic acid and carbon-dioxide, to release methane during methanogenesis stage. As general, the 70% of total methane derives from the conversion of acetate (acetoclastic methanogenesis), while the remaining 30% originates from hydrogen and carbon dioxide (hydrogenotrophic methanogenesis) [52,53].

The microorganisms responsible for the production of methane are the Archaea, very similar to bacteria but belonging to another kingdom and probably their ancestors. Archaea kingdom is divided into two categories: the Crenarchaeota and the Euryarchaeota.

Anaerobic digestion is a very complex process involving over 1000 species of bacteria that are sensitive to environmental conditions and have different vital requirements (pH, temperature, nutrition). The interactions between different microbial functional groups are complex. The imbalance between any two microbial groups will affect the overall reaction rate or even cause accumulation of inhibitors, which may result in failure of the AD process [54]. Among the four microbial groups, methanogens are believed to have the lowest growth rate and are the most sensitive to changes of environmental conditions, such as temperature, pH, and the concentrations of inhibitors. Thus, methanogenesis is commonly considered to be a rate-limiting step in the AD process [55].

However, for the degradation of recalcitrant solid materials, such as lignocellulosic biomass, hydrolysis is also believed to be a rate-limiting step since substrate accessibility to enzymes can be difficult due to several factors such as cellulose crystallinity, degree of polymerization, and, especially, lignin content.

Therefore, to overcome the biomass recalcitrance of lignocellulosic biomass and expose the polymer chains of cellulose and hemicellulose to microbial so as to increase the efficiency of AD and to improve methane yield, a biomass pretreatment process prior to anaerobic digestion is usually required [56]. Lignin is the most recalcitrant component to anaerobic biodegradation and shields cellulose and

hemicelluloses [57], reducing the available surface area for enzymatic attack and hampering the degradation of structural carbohydrates.

1.6 Lignocellulosic species

Arundo donax L.

Giant reed (*Arundo donax* L.) is a lignocellulosic perennial rhizomatous grass belonging to the subfamily Arundinoideae of the Poaceae family. *A. donax* is generally considered as native of Mediterranean basin and it is cultivated in Asia, southern Europe, North Africa and the Middle East, but the origin of this species remains unknown. It spontaneously widespread in tropical and warm-temperate areas of the world and widely used by man for its multiple uses such as fishing, construction, music, erosion control and recently as promising energy crop in southern Europe [58–60]. It has a C3 photosynthetic pathway, but has a photosynthesis rate and productivity that are similar to those of C4 species [61].

As a perennial crop, giant reed can positively affect soil quality, since it contributes to reduce the risk of soil erosion and to increase the soil organic matter content [62]. Giant reed shows many advantages when compared to other energy crops, like (i) the adaptability to different types of environments, soils and growing conditions, (ii) the high biomass production and (iii) the low input required for its cultivation (use of irrigation, fertilizers, pesticides) [58].

Giant reed is considered a drought-tolerant species that can achieve high biomass yields also under high salinity conditions [63]; it can grow in marginal or sub-marginal lands reducing competition with food crops for soil use [64,65]. Thanks to its high production of biomass yield also in marginal land, giant reed has recently been proposed as energy crops

for producing biogas [58,59,66–68].

Saccharum spontaneum spp. aegyptiacum

Saccharum (*Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) Hack.), is a perennial herbaceous rhizomatous grass of the Poaceae family, native of northern Africa and widespread in South Mediterranean regions [69]. It is well adapted to semi-arid regions of the Mediterranean area [70].

Saccharum spontaneum spp. *aegyptiacum* has a range of agronomic, physiologic and qualitative desirable traits of biomass crop, namely C4 plant, high biomass yield, high water efficiently, able to assimilate CO_2 during drought-stress periods, high cellulose and hemicellulose content [71,72] and it is a potential species for biomass production suitable for environment characterized by drought stress and high temperatures [73].

Arundo, Saccharum and Castor are species that could be cultivated for energy purposes and show many characteristics of the energy crops: don't compete with food crops for land use, require low agronomic inputs, exhibit high rusticity, tolerance to biotic and abiotic stress and provide several products for the agroenergy chain such as biodiesel, bioethanol and biogas.

Ricinus communis L.

Castor (*Ricinus communis* L.) is a non- food, drought resistant, energy crop well known since antiquities. Castor is member of the Euphorbiaceae family that is found across all the tropical and semi-tropical regions of the world. Castor plant is very tolerant to different weather conditions and is able to grow in marginal soils [74]. Castor is

an industrial oilseed crop and has been cultivated almost entirely for pharmaceutical and industrial applications. Castor seed contains between 40% and 60% oil, and castor oil is composed by the following fatty acids: 91–95% ricinoleic acid, 4–5% linoleic acid and 1–2% palmitic and stearic acids [75]. Ricinoleic acid can be chemically transformed to obtain various commercial products of interest, such as lubricants, inks, biopolymers, and biodiesel, making castor oil a product of high interest for agriculture, cosmetic and industrial sectors [76]. Moreover, it is considered as a second-generation raw material for the production of bioenergy or industrial purposes [77].

Castor has a high potential for use as a biorefining feedstock [78,79]. Because of the present mainly of ricinoleic acid, castor oil is provided by attractive properties, such as high viscosity, high miscibility, low iodine content, low freezing point which make it an excellent raw material for producing biodiesel.

Castor is used mainly to obtain oil from its seed but in the castor oil production process two main by-products are generated: capsule husks and oil-cake. Capsule husks are obtained from separation of seeds from the fruits, while the cake is produced when the oil is extracted from the seed by pressing.

Castor cakes present a high N content, but due to the presence of toxic ricin, the use of cake as animal feed is not possible. In the husks, ricin residue is usually found in the form of seed pieces. The husks could be utilized as a high-fiber, low-nitrogen animal feed [80].

A process of detoxification is necessary, however there is no efficient and low-cost methods. While a feed option is not feasible, most of the non-edible oil cakes generated worldwide, included castor cake, are used mainly as organic fertilizers, due to their N, P and K contents.

Castor oil is currently used for biodiesel production by transesterification, while the whole biomass including stems and leaves, and oil extraction by- products, such as capsule husks and oil-cake, are potentially applicable as feedstocks for ethanol and biogas production for valorizing residual biomass [81].

However, similar to others lignocellulosic materials, to use these biomass residues in bioenergy production process is necessary a pretreatment to expose their compact structure to enzymatic hydrolisis.

Chapter 2 - Results

2.1 Biomethane potential of an old plantation of giant reed genotypes with two irrigation levels

Based on: Piccitto A, Corinzia SA, Scordia D, Calcagno S, Ciaramella BR, Patanè C, Cosentino SL, Testa G. Biomethane Potential of an Old Plantation of Giant Reed Genotypes with Two Irrigation Levels. In European Biomass Conference and Exhibition Proceedings; 2020; pp 234–237.

During the last years, several researches investigated the potential productivity of giant reed, under different environments and management practices [58,59,81]. However, most of these studies refer to short-term field trials, while the long-term effects of different management practices, such as irrigation or fertilization on productivity, have not been widely evaluated. As first trial of this project, the effects of two levels of irrigation (100% ETm and rainfed) on biomass and biomethane yields of a more than 20-year old plantation of 40 genotypes of giant reed (*Arundo donax* L.) collected around Southern Italy have been evaluated. This giant reed field trial was established in 1997 at the experimental field of the University of Catania.

Cosentino et al. (2006) [65] reported for the 40 genotypes studied a dry matter yield, in the average, of 10.6 Mg ha^{-1} in the first year and 22.1 Mg ha^{-1} in the second year of growth. The genotype 4 and the genotype 20 maintained their high productive aptitude in both years; they yielded, respectively, 13.1 and 14.1 Mg ha^{-1} in the first year and 34.2 and 26.9 Mg ha^{-1} in the second one.

Giant reed is able to keep high productivity even after more than 20 years of cultivation, both under rainfed and irrigated conditions.

The highest dry matter yield was reached by genotype 2 (12.78 Mg ha^{-1}) in rainfed condition and genotype 27 (19.3 Mg ha^{-1}) in the irrigated treatment.

The average yield of the genotypes was 7.4 Mg ha^{-1} in rainfed condition and 12.4 Mg ha^{-1} in the irrigated treatment. As reported by scientific literature, it is known the decrease of the biomass yield in old plantations of perennial grasses.

Angelini et al. (2009) [63] identified three phases in growth of giant reed: an increasing phase from the first to the third year, a stationary phase from the fourth to the eighth year, and a decreasing phase from the ninth to the twelfth year of growth.

Mid- and long-term studies have shown that the yields of the *A. donax* crop varied from year to year and the lifespan of the *A. donax* cycle is not fully known, however some studies have highlighted the capability of the crop to achieve relevant biomass yield in North Italy for at least 16 years [83] and others have obtained dry biomass yield higher than 20 t ha^{-1} with a plantation over 10 years cultivated without irrigation [63].

The biomethane potential yield obtained from giant reed ranged from 347.5 m^3 ha^{-1} (genotype 40) to 1127.3 m^3 ha^{-1} (genotype 2) in rainfed condition and from 653.6 m^3 ha^{-1} (genotype 40) to 1666.4 m^3 ha^{-1} (genotype 27) in the irrigated treatment. Several genotypes maintain high biomass yield and thus, high biomethane potential yield, even after 20 years from plantation.

Differences on biomethane production observed among genotypes were mainly due to the difference in dry biomass yield that was affected by water availability.

The variability of biomass yield and biomethane potential yield among genotypes is high. Almost all the genotypes showed a positive response to the irrigation, which is the main limiting factor in Mediterranean environments. Regarding the chemical composition, cellulose, as expected, is the main fraction of giant reed biomass, followed by hemicellulose and neutral detergent soluble fraction (NDS). Lignin and acid insoluble ash are present in lower amounts.

Irrigated thesis shows higher concentration of cellulose, hemicellulose and lignin than rainfed thesis, while NDS and acid insoluble ash are present in lower amounts. Results of fiber analysis are in agreement with those found by previous studies [84] [85].

The theoretical biomethane yield, calculated on the basis of the biomass chemical composition, using coefficients from literature [86], was higher than experimental one due to the recalcitrance of the lignocellulosic matrix of *A. donax* biomass for the presence of lignin within the lignocellulosic biomass, which covers cellulose and hemicellulose fibers, reducing the degradability of these polymers by hydrolytic bacteria.

Therefore, it is necessary reduce biomass recalcitrance of lignocellulosic crops to enhance the biomethane production. This goal could be achieved by means of agronomic practices on field or undergoing lignocellulosic biomass to a preliminary pretreatment.

2.2 Advanced biomethane production by *Arundo donax* under changing harvest time and nitrogen fertilization

Based on: Scordia D, Calcagno S, Piccitto A, Corinzia SA, Testa G, Ciaramella BR, Cosentino SL. Advanced Biomethane Production by *Arundo Donax* under Changing Harvest Time and Nitrogen Fertilization. In European Biomass Conference and Exhibition Proceedings; 2020; pp. 222–227.

To evaluate the suitability of the lignocellulosic herbaceous crop *Arundo donax* as a biomass feedstock for advanced biomethane production, harvest time and nitrogen fertilization treatments were adopted to reduce biomass recalcitrance thereby increasing biomethane yield. The biomass used was harvested in the growing season 2017-2018, corresponding to the 20th year of plantation, from experimental farm of University of Catania.

In agreement with others studies, as expected, fertilized crops gave a higher biomass than unfertilized [82,87,88]. The fertilization with 80 kg N ha^{-1} with the winter harvest (WN80) showed the highest values of dry matter yield (15.46 ± 1.85 Mg ha^{-1}). The unfertilized winter period instead showed the overall lowest dry matter yield value (9.51 ± 1.02 Mg ha^{-1}). In the absence of nitrogen fertilization, the autumn harvest produced more than the winter, however, the effect of fertilization was less marked for the autumn than for the winter harvest. Generally, previous studies [82] [89] reported an increased yield in winter instead of autumn harvest even if the difference between the two harvest times are evident mainly at the initial 4 years of crop growth [90]. The biomass composition of the dry biomass showed, in general, a greater content in soluble substances (NDS) and ashes in the fertilized treatment as compared with the unfertilized one, and in the autumn than to the winter harvest. By contrast, the content of hemicellulose, cellulose and the acid detergent lignin was higher in the winter than the

autumn harvest and in the unfertilized as compared with the fertilized biomass. In detail, on the average of experimental factors, the content of hemicellulose was significantly influenced by the time of harvest, across the average of nitrogen fertilizers, with higher values in the autumn than winter (31.3 versus 29.6%, respectively), however, nitrogen fertilization did not show differences (30.5% on average). On the other hand, the % of cellulose was significantly higher in the unfertilized as compared with that fertilized treatment across the average of harvest regimes (37.2 vs 32.2%, respectively), while the harvest time, across the average of fertilizations, did not show a significant effect (34.7% on average). The % of acid detergent lignin (ADL) was significantly higher in the winter than autumn harvesting time, in the average of nitrogen fertilizers (9.8 against 8.9%, respectively); nitrogen fertilization, on average of harvest time, did not show a significant effect on the % in ADL (9.3% on average). The ash content (ASH), was significantly influenced both by the harvest, on the average of fertilizations (6.69 and 5.40% in autumn and winter, respectively), and by the fertilizations on the average of harvest time (5.88 and 6.21% in unfertilized and fertilized, respectively). Previous studies have explained the ash content decrease and the lignin increasing trend from autumn to winter, as a consequence of a progressive loss of leaves, which are characterized by a lower lignin content and higher ash and silicon content than stems [61,91–93].

Finally, the effect of nitrogen fertilization, across the average of the harvest time, significantly increased the % of soluble substances - NDS (21.4 and 17.4% in N80 and N0, respectively), while the content in NDS, although higher in the autumn, did not show statistically

significant differences as compared with the winter period across the average of nitrogen fertilization (19.44% on average).

In the different experimental treatments, the final BMP was significantly higher in giant reed WN80 (123.4 Nml g^{-1} VS), followed by the AN80 (118.1 Nml g^{-1} VS), by AN0 (106.2 Nml g^{-1} VS) and finally by the WN0 (100.3 Nml g^{-1} SV). Nitrogen fertilizers increased the production of advanced biomethane compared to unfertilized crops. In absolute values, the fertilized winter harvest (WN80) showed the highest biomethane yield per hectare (1717±203 m^3 CH_4 ha^{-1}), followed by AN80 (1580±96 m^3 CH_4 ha^{-1}). On the other hand, the autumn harvested crops produced higher values than the winter one among the unfertilized (1105±86 and 859±93 m^3 CH_4 ha^{-1} for AN0 and WN0, respectively). On the average of the study treatments, the harvest time did not show significant differences (982 m^3 CH_4 ha^{-1} on average), on the contrary, the production of advanced biomethane responded positively to nitrogen fertilization, that on the average of the harvest time was 1648 m^3 CH_4 ha_{-1} for N80 and 982 m^3 CH_4 ha^{-1} for N0, respectively).

The results highlighted that the effect of nitrogen fertilization has positively influenced the anaerobic digestion and the production of advanced biomethane on the unit land area, due mainly to a combination of biomass yield and composition, such as higher NDS and protein. The harvest time does not seem to have a significant effect, however, in the absence of nitrogen input, the autumn time showed a substrate less recalcitrant by the bacterial flora thanks to a higher content in soluble substances (NDS) and hemicellulose, and a lower content in acid detergent lignin (ADL) compared to the winter harvest time.

2.3 Evaluation of the thermal pretreatment on the methanogenic potential of two lignocellulosic crops: *A. donax* and *S. spontaneum*

Based on: Piccitto A, Corinzia SA, Scordia D, Calcagno S, Ciaramella BR, Cosentino SL, Testa G. Evaluation of the Thermal Pretreatment on the Methanogenic Potential of Two Lignocellulosic Crops: *A. Donax* and *S. Spontaneum*. In European Biomass Conference and Exhibition Proceedings; 2020; pp 494–497.

The main drawback of biomethane production from lignocellulosic perennial rhizomatous grasses is the recalcitrance of biomass to be degraded into monomers for energy purpose due to the elevated presence of lignin.

Several pretreatments have been proposed to enhance the degradability of lignocellulosic biomass (physical, chemical, biological, etc.) and to obtained higher biomethane yields.

The biomass composition and the biochemical methane potential (BMP) yield of two crops suitable for the Mediterranean environment, *Arundo donax* L. and *Saccharum spontaneum* subsp. *aegyptiacum* (Willd.) Hack, have been investigated evaluating the effect of a hydrothermal pretreatment carried out using an autoclave (model 1000 ML Zipperclave Assembly, Parker) with distilled water at 160 °C for 10 minutes.

The amount of solid biomass that has been recovered after the pretreatment is around 90% in both crops.

S. spontaneum showed the highest biomass yield both as untreated (17.68 Mg VS ha^{-1}) and pretreated biomass (15.98 Mg VS ha^{-1}) in comparison with *A. donax* (13.86 and 12.50 Mg VS ha^{-1} for untreated and pretreated biomass, respectively). Pretreated biomass yield was lower than untreated biomass yield for both crops due to the partial solubilization of neutral detergent soluble (NDS) fraction and

hemicellulose that occurred during the hydrothermal pretreatment. As reported by Caparros et al. (2006), after the hydrothermal pretreatment, a progressive decrease in yield and simultaneously an increase in the solubilized fraction is observed, especially from 185 to 200 °C due to more effective hemicellulose degradation [94].

S. spontaneum untreated biomass differed from *A. donax* for lignin content (higher in *A. donax*) and ash content (higher in *S. spontaneum*). Cellulose and hemicellulose were similar in both untreated biomasses.

The biomass quality of both studied crops is comparable in its carbohydrates composition to other lignocellulosic matrices used for second generation biofuel production, such as wood and others herbaceous crops, making these crops adequate substrates for the anaerobic digestion technology [71].

The hydrothermal pretreatment resulted in a decrease of the share of acid insoluble ash, NDS fraction and hemicellulose in comparison with the untreated biomass. As a consequence of the solubilization of these components, the share of cellulose and lignin increased.

Hydrothermal pretreatment, is successful in removing hemicellulose while only mildly degrading lignin and cellulose [95]. During hydrothermal pretreatment is common that fragmented lignin reacts by itself or with carbohydrate oligomers forming compounds that precipitate when cooled and reattach to the pretreated fibers [96,97]. This phenomenon could explain the absence of lignin degradation during the pretreatment.

Nevertheless, the cell wall structure results modified by pretreatment and the biomass recalcitrance reduced so as to improve hydrolysis during anaerobic digestion because of the cellulosic microfibrils are better exposed to enzymes for depolymerization to monomeric sugars.

The change in biomass composition caused by the hydrothermal pretreatment resulted in an increase of the theoretical BMP per gram of volatile solid for both crops: *A. donax* BMP increased from 321.16 to 324.05 Nml g^{-1}VS, *S. spontaneum* BMP increased from 325.42 to 338.14 Nml g^{-1}VS. The higher BMP is ascribable to the increase in the share of cellulose on the total biomass. Even the experimental BMP test showed a higher BMP per gram of volatile solid for pretreated biomass in comparison with untreated biomass for both crops: *A. donax* BMP increased from 100.3 to 109.7 Nml g^{-1}VS, *S. spontaneum* BMP increased from 84.9 to 153.4 Nml g^{-1} VS. Contrarily, the theoretical BMP yield for pretreated biomass is lower than the theoretical BMP yield for untreated biomass in spite of the increase in BMP per gram of volatile solid. This is due to the lower yield of solid biomass as a consequence of the solubilization that occurs during the hydrothermal pretreatment. *A. donax* BMP yield decreased from 4240 to 3841 m^3 ha^{-1}, *S. spontaneum* BMP yield decreased from 5606 to 5259 m^3 ha^{-1}. The highest yield of *S. spontaneum* in comparison with *A. donax* is due to the higher untreated and pretreated biomass yield.

The experimental BMP yield for pretreated biomass is similar to the experimental BMP yield for untreated biomass in *A. donax* (1390 and 1372 m^3 ha^{-1} for untreated and pretreated biomass respectively), while in *S. spontaneum* experimental BMP yield for pretreated biomass is higher than the experimental BMP yield for untreated biomass (1372 and 2452 m^3 ha^{-1} for untreated and pretreated biomass, respectively).

2.4 Advanced biomethane production from biologically pretreated giant reed under different harvest times

Based on: Piccitto A, Scordia D, Corinzia SA, Cosentino SL, Testa G. Advanced biomethane production from biologically pretreated giant reed under different harvest times. Article under review on *Agronomy*.

Compared with physical and chemical pretreatment, biological methods are more environmentally friendly, consume less energy, produce no inhibitors and don't require chemicals input.

The present research investigates the effect of fungal pretreatment of *Arundo donax* L. obtained from two different harvesting time (autumn and winter) on biomethane production by anaerobic digestion using two white rot fungi (*Pleurotus ostreatus* and *Irpex lacteus*).

The analysis of variance (ANOVA) of the harvest time main effect showed significant differences for ADL and ash biomass components, while hemicellulose, cellulsose and NDS did not differ. Total aboveground biomass yield (DMY) and yield components ((i.e., dry biomass composition yield) were significanly affected by harvest time, except the ADL yield.

The ADL content was 10.4% w/w in winter and 9.6% w/w in autumn harvest, while ash content was higher in autumn than winter (1.2 and 0.7% w/w, respectively). Although not significant, hemicellulose and NDS content were higher in autumn (29.1 and 24.2% w/w, respectively) than winter (29.0 and 23.9% w/w, respectively), while the cellulose content was 36.9 and 35.9% w/w in winter and autumn, respectively.

The aboveground dry matter yield (DMY) was higher in autumn than winter, 11.64 Mg ha^{-1} and 10.38 Mg ha^{-1}, respectively. The autumn harvest produced higher yield components: cellulose represented the largest part of giant reed yield, reaching 4.2 Mg ha^{-1} in autumn and 3.8 Mg ha^{-1} in winter harvest, followed by hemicellulose (3.4 and 3.0 Mg

ha^{-1} for autumn and winter, respectively), NDS (2.8 and 2.4 Mg ha^{-1} for autumn and winter, respectively), ADL (1.1 and 1.0 Mg ha^{-1} for autumn and winter, respectively) and ash (0.14 and 0.08 Mg ha^{-1} for autumn and winter, respectively).

Losses of cellulose, hemicellulose, and lignin, with a consequent reduction of organic matter, can be used to evaluate the degradation pattern of different white-rot fungi. The ANOVA showed that biomass chemical composition was significantly modified by fungi growth. The effect of pretreatment was significant on cellulose and lignin content, while harvest time on dry matter, hemicellulose, cellulose and lignin. Significant interactions "pretreatment × harvest time" were observed for dry matter, hemicellulose and cellulose.

The degradation of dry matter, cellulose, hemicellulose and lignin in GRB increased for both *P. ostreatus* and *I. lacteus* treatment. Degradation of dry matter and components during pretreatment showed an increasing trend with time for both fungi. High percentage of degradation of dry matter was observed for the biomass of autumn harvest for both *P. ostreatus* (26.9%) and *I. lacteus* (26.7%) treatment after 30 days of incubation (Figure 3A). For hemicellulose and cellulose, maximum degradation rates were observed for *I. lacteus* in the winter harvest with a loss of 20.5% and 18.1% respectively. The highest value of lignin loss was obtained by *P. ostreatus* in both autumn (27.1%) and winter (31.5%) harvest time.

Hemicellulose and lignin were degraded more than cellulose during fungal pretreatment, mainly with *P. ostreatus*. This is confirmed by selectivity value, defined as lignin degradation over cellulose loss. It is important to evaluate the selective lignin-degrading capability of white rot fungi. The highest selectivity value of 2.7 with lignin degradation of

31.5% was reached from *P. ostreatus,* indicating that *P. ostreatus* selectively degraded hemicellulose and lignin over cellulose. The low degradation of cellulose has a positive impact on the anaerobic digestion process because cellulose is considered the main substrate for anaerobic microorganisms to produce biogas.

The ANOVA showed that daily and cumulative biomethane production were significantly influenced by the incubation time, the pretreatment and by the harvest time. Significant interactions "pretreatment × harvest time" were also observed. Daily production (Nml g^{-1} VS d^{-1}) and cumulative methane production (NmL g^{-1} VS) during anaerobic digestion of untreated and fungal pretreated giant reed are displayed in Figure 4. The daily production curves for the pretreated samples showed the same trend for both harvesting time for each fungal strain used. The daily biomethane peaks (15.6 and 12.6 Nml g^{-1} VS d^{-1}) were highest in the biomass pretreated by *P. ostreatus* after 17 days of digestion for winter and autumn harvest, respectively. Winter giant reed pretreated by *I. lacteus* showed the maximum peak (6.2 Nml g^{-1} VS d^{-1}) after 18 days of incubation, while the autumn one reached the peak of 6.1 Nml g^{-1} VS d^{-1} after 23 days. Cumulative biomethane production was observed for 30 days until biomethane yield reached a plateau at the end of exponential phase. The initial lag phase lasted around three days until the complete adaptation of the bacterial flora to the lignocellulosic substrate. The methane yield obtained for the untreated giant reed biomass of autumn and winter harvest was 97.6 NmL g^{-1} VS and 91.8 NmL g^{-1} VS, respectively. *P. ostreatus* pretreated giant reed biomass achieved the highest BMP values, 130.9 Nml g^{-1} VS and 103.8 Nml g^{-1} VS for the winter and the autumn harvest, respectively, showing an improvement of the anaerobic digestion after fungal

pretreatment. On the contrary, the pretreatment using *I. lacteus* was uneffective and produced lower cumulative methane yield than the untreated giant reed, 77.4 Nml g^{-1} VS and 73.3 Nml g^{-1} VS for winter and autumn harvest, respectively. *I. lacteus* pretreatment resulted in a loss of both holocellulose and lignin, indicating that this strain was less selective than *P. ostreatus*.

The ANOVA revealed that pretreatment, harvest time and interaction were significant on biomethane yield (BMY). The BMY was greater for the autumn harvest than winter in the untreated biomass (1078.4 m^3 CH_4 ha^{-1} and 905.8 m^3 CH_4 ha^{-1}, respectively) as consequence of the higher biochemical methane potential and higher dry biomass yield of autumn biomass.

P. ostreatus pretreatment of winter harvest showed the highest biomethane yield per hectare (1284.5 m^3 CH_4 ha^{-1}), followed by autumn *P. ostreatus* pretreated biomass (1126.5 m^3 CH_4 ha^{-1}). Despite the lowest dry biomass yield of winter, the biomethane production per hectare depended mostly on the higher BMP showed by winter biomass pretreated by *P. ostreatus. I. lacteus* pretreated biomass achieved the lowest values on biomethane yield, 791.9 m^3 CH_4 ha^{-1} and 761.4 m^3 CH_4 ha^{-1} for autumn and winter biomass, respectively.

2.5 Oil production of diverse mediterranean Castor genotypes

Based on: Piccitto A, Calcagno S, Copani V, Testa G, Scordia D, Patanè C, Cosentino SL. Oil Production of Diverse Mediterranean Castor Genotypes. In European Biomass Conference and Exhibition Proceedings; 2021; pp 362–365.

28 genotypes of castor (*Ricinus communis* L.) breed from native perennial plants collected across the semiarid Mediterranean basin were compared to evaluate seed yield and oil yield.

Field experiments were conducted over the period 2019-2020 at the Experimental farm of the University of Catania.

The seed yield was 542 and 1993 kg ha^{-1}, on average, for primary and secondary racemes, respectively.

The total seed yield was mainly affected by yield of secondary racemes with a percentage that ranged between 68 and 85%, according to Severino et al., the contribution of primary racemes to the total seed varies from 14 to 69% [9].

The total seed yield ranged between 3022 (genotype 27) and 1735 (genotype 12) kg ha^{-1}, which were statistically different.

Seed characteristics are not determined by the raceme order, but by an interaction of environmental conditions and genetic factors.

The percentage of oil content in castor seeds ranged between 40.4 (genotype 28) and 46.1% (genotype 12), with an average of 42.7% for the primary raceme and between 43.2 (genotype 12) and 48.1% (genotype 10), with an average of 46.1% for the secondary racemes.

In accordance with study of Souza et al. [12] a lower seed oil content was found in the primary than in the secondary racemes.

The main contribution to the total oil yield is given by the seed yield as reported by Koutroubas et al. [13].

Oil yield varied from 166 (genotype 18) to 270 (genotype 16) kg ha^{-1}

with an average of 231 kg ha^{-1} for the primary raceme. The oil yield was from 518 (genotype 12) to 1206 (genotype 27) kg ha^{-1} with an average of 918 kg ha^{-1} for the secondary racemes.

2.6 Biologically pretreated Castor capsule husks for advanced biomethane production

Capsule husks, a lignocellulosic residues obtained from the oil extraction process, were pretreated by two white-rot fungi (*Pleurotus ostreatus* and *Irpex lacteus*) to evaluate the experimental biomethane potential through anaerobic digestion

The analysis of variance (ANOVA) of nitrogen fertilization effect as main effect did not show significant differences for biomass composition

Although not significant, NDS content was higher in the unfertilized (43.9% w/w) as compared with fertilized treatment (34.1% w/w), while the content of hemicellulose and cellulose were higher in the fertilized residue (20.9 % and 32.2 % w/w, respectively) than unfertilized one (18.5% and 25.9% w/w, respectively). The fertilized residue had a greater content of lignin (11.1% w/w) and ash (1.6% w/w) than the unfertilized (10.4% and 1.3% w/w respectively).

The ratio of structural carbohydrates (hemicellulose and cellulose) over lignin was determined in addition to the analysis of the various fractions of lignocellulose residue. This measurement may be used to estimate the digestibility of the substrate being tested. For fertilized residue, the highest ratio was recorded (4.8).

Biomass composition was modified by fungi growth with a consequent reduction of organic matter. Losses of cellulose, hemicellulose, and lignin can be used to evaluate the degradation pattern of different white-rot fungi.

The ANOVA showed that biomass chemical composition was significantly modified by fungi growth.

The effect of pretreatment and of nitrogen fertilization was significant on dry matter loss. Significant interactions ″pretreatment×fertilization” were observed for hemicellulose and lignin.

The degradation of dry matter, cellulose, hemicellulose and lignin of capsules residue showed an increasing trend with time for both fungi. High percentage of degradation of dry matter was observed for *I. lacteus* for both unfertilized (20%) and fertilized (19%) substrates after 30 days of incubation. The percentages of degradation of dry matter for *P. ostreatus* were of 14.5 % and 13.8 % for N0 and N120 treatments.

For hemicellulose degradation, the highest loss was observed for *P. ostreatus* in both unfertilized and fertilized treatment (14.7% and 14.6%, respectively) (Fig. 2B). By contrast, for cellulose, highest degradation was observed for *I. lacteus* with a loss of 15.2% and 14.6% in unfertilized and fertilized treatment, respectively.

The highest value of lignin loss was obtained by *P. ostreatus* in both unfertilized (21.4%) and fertilized (20.8%) samples.

The ANOVA showed that daily biomethane production was significantly influenced by incubation time and pretreatment. Cumulative biomethane production was significantly influenced by incubation time, pretreatment and fertilization. Significant interactions ″pretreatment × fertilization” were also observed on cumulative biomethane production.

The daily biomethane production showed the highest peaks for untreated capsules N0 and N120 (6.1 and 6.7 Nml g^{-1} VS d^{-1}, respectively) after 19 days of digestion.

Capsules pretreated by *I. lacteus* showed the maximum peak (5.6 Nml g^{-1} VS d^{-1}) on the 17^{th} day for both fertilization levels (N0 and N120),

while *P. ostreatus* pretreated biomass showed the peaks of daily methane production lower than the others thesis (4.5 and 4.1 Nml g^{-1} VS d^{-1} for N0 and N120, respectively) reached after 17^{th} and 13^{th} days respectively.

Cumulative bioethane production was observed for 30 days until biomethane yield reached a plateau at the end of exponential phase. The initial lag phase lasted around three days until the complete adaptation of the bacterial flora to the lignocellulosic substrate.

The ANOVA revealed that pretreatment were significant on biomethane yield (BMY).

The methane production obtained for the untreated capsules husks N0 and N120 was 62.4 NmL g^{-1} VS and 75.8 NmL g^{-1} VS, respectively.

P. ostreatus pretreated capsules achieved values of 52.6 Nml g^{-1} VS and 54.4 Nml g^{-1} VS for N0 and N120 fertilization respectively.

The methane yield reached by *I. lacteus* pretreated capsule husks was 56.3 Nml g^{-1} VS and 58.7 Nml g^{-1} VS for for N0 and N120 fertilization levels, respectively.

The capsule yield, as biomass residue after the shelling of the seeds, obtained during the first year of cultivation was used to estimate the potential residue of a castor cultivation in Mediterranean environment. This value was equal to 3.0 Mg ha^{-1} and 2.5 Mg ha^{-1} for N0 and N120, respectively.

The biomethane yield was greater for both untreated N0 and N120 biomass than fungal pretreated (162.9 m^3 CH_4 ha^{-1} and 163.4 m^3 CH_4 ha^{-1}, respectively) as consequence of the highest biochemical methane potential.

Among the fungal pretreated thesis, *I. lacteus* showed the highest biomethane yield per hectare (150.3 m^3 CH_4 ha^{-1} and 123.3 m^3 CH_4 ha^{-1} for N0 and N120, respectively) while *P. ostreatus* achieved values of 130.5 m^3 CH_4 ha^{-1} for N0 and 110 m^3 CH_4 ha^{-1} for N120.

Chapter 3- Concluding Remarks

This PhD project investigated the feasibility of utilizing lignocellulosic biomass to produce biomethane in the anaerobic digestion.

Biomethane production can be considered a solid and mature technology for the sustainable use of agricultural biomass as a source of biofuel, especially when the substrate for anaerobic digestion is a non-food crop that does not compete with food crops for the land use and other resources.

A. donax and *S. spontaneum* are two promising biomass crops for rainfed agriculture in the south Mediterranean basin, where soil water supply is limited throughout the summer.

The high cellulose and hemicellulose content of both crops suggests that they are suitable for biomethane generation.

Arundo donax grown in low or no input in rainfed condition, is able to support satisfactory dry biomass production, even after 20 years from plantation, and in environments where water is the limiting factor for the production of crops owing spring-summer cycle.

The harvest time does not seem to have a significant effect on biomethane production, however, in the absence of nitrogen input, the autumn time showed a substrate less recalcitrant by the bacterial flora thanks to a higher content in soluble substances (NDS) and hemicellulose, and a lower content in acid detergent lignin (ADL) compared to the winter harvest time.

The influence of lignin inside the lignocellulosic matrix, which surrounds the cellulose and hemicellulose fibers and reduces the degradability of these polymers by bacterial activity, is the principal obstacle to this process.

Several pretreatments have been proposed to improve the degradability of lignocellulosic biomass: most physical and chemical pretreatments using acid, alkali, microwave, steam explosion, ionizing radiation, or combined processes require specialized equipment, consume a lot of energy, and can produce inhibitors that can interfere with the subsequent hydrolysis phase of anaerobic digestion. A hydrothermal pretreatment lowers the requirement for specialized apparatus and avoids the formation of chemicals that could hinder methane conversion.

The hydrothermal pretreatment allows to alter the structure and composition of the lignocellulosic matrix, by interrupting the continuity between the lignin and the cellulose and hemicellulose fibers and partially solubilizing hemicellulose and NDS fraction. The hydrothermal pretreatment returns a liquid fraction, rich in NDS and monomers of hemicellulose, which is suitable for further energetic purposes, such as fermentation for bioethanol production or anaerobic digestion as a co-digestion with the solid fraction or separated digestion. The composition and the energetic potential of the liquid fraction should be investigated furthermore.

The alteration of the lignocellulosic biomass by the hydrothermal pretreatment resulted in the increase of the experimental yield, despite the higher content of lignin and the lower total content of digestible fractions (hemicellulose, cellulose and NDS).

Biological pretreatment is more environmentally friendly than physical or chemical pretreatment since it uses less energy, produces no inhibitors, and does not require the use of chemicals. However, biological pretreatments have some drawbacks, including significant

holocellulose losses (needed for microorganisms growth during pretreatment) and a long pretreatment time.

Fungal pretreatment with *P. ostreatus* and *I. lacteus* carried out on *Arundo donax* changed the structure and the composition of giant reed biomass; especially, *P. ostreatus* showed a better efficiency on selectively degrading lignin compared to *I. lacteus*.

In our experiments *I. lacteus* showed a lower lignin degradation and a greater cellulose loss than *P. ostreatus*, resulting in a major consumption of holocellulose. The holocellulose losses during pretreatment, in particular of cellulose that mostly influences methane production, led to a reduced biomethane yield through anaerobic digestion.

The *P. ostreatus* pretreatment showed promising results for anaerobic digestion of giant reed achieved a cumulative yield of 130.9 Nml g^{-1} VS for the winter harvest, whereas *I. lacteus* reported a decrease in methane yield compared to untreated.

The results obtained in terms of seed and oil yield from the field experiments conducted on Castor genotypes confirmed the plant adaptability in the Mediterranean area.

Capsule residues obtained from the oil extraction process were evaluated to produce biomethane throug anaerobic digestion process after a biological pretreatment using *P. ostreatus* and *I. lacteus*.

The composition analysis of capsule husks showed the differences on lignocellulosic substrate resulted by the different fertilization levels, with a greater content of hemicellulose and cellulose on fertilized biomass as compared with the unfertilized.

The significant amount of carbohydrates presents on capsules residue confirm the potential of this substrate to be used on biochemical

conversion and anaerobic digestion for advanced biomethane production.

Fungal pretreatment carried out on capsule husks modified the structure and the composition of biomass degrading cellulose, hemicellulose and lignin. However, the fungal pretreatment using *P. ostreatus* and *I. lacteus* had a negative effect on biomass tested and on biomethane production producing lower cumulative methane yield than the untreated biomass.

In general, this PhD thesis, through the field and laboratory experiments, proved that these investigated crops are suitable to grow in Mediterranean region and to support satisfactory dry biomass production, even after many years of cultivation under low or no input. The high potential of these crops to be used as feedstock for biomethane production is confirmed by the composition analisys of lignocellulosic biomass and by the results of BMP tests.

A pretreatment is necessary to reduce recalcitrance of these substrates and enhance the biomethane yield.

The fungal pretreatment carried out on giant reed biomass using *P. ostreatus* allowed to obtain a biomethane yield greater than untreated biomass, while *I. lacteus* reported a decrease in methane yield compared to untreated.

The fungal pretreatment using *P. ostreatus* and *I. lacteus* had a negative effect on biomethane production from capsule husks.

Further investigations are needed to identify white rot fungi more suitable to pretreat these lignocellulosic biomass and optimize biological pretreatment efficiency.

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Publications of the project

Biomethane potential of an old plantation of giant reed genotypes with two irrigation levels

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ABSTRACT: Giant reed (*Arundo Donax* L.) is a perennial, non-food and low-input energy crop representing a promising solution to produce renewable energy at low cost, especially in marginal areas - i.e. low profitable areas which are prone to land abandonment. This research investigates the effect of two levels of irrigation (100% ETm and rainfed) on a 20-year old plantation of 40 genotypes of giant reed (*Arundo donax* L.) collected around Southern Italy. The experimental methanogenic potential of the biomass was defined trough the BMP test (Biochemical Methane Potential). The trial shows that several genotypes maintain high biomass yield and thus high biomethane potential yield even from old plantations. The variability of biomass yield and biomethane potential yield among genotypes is high. Giant reed genotypes show a positive response to the irrigation, which represent the main limiting factor in Mediterranean environments.

KEYWORDS: biomass, bioenergy, perennial energy crops, biomethane potential.

1. INTRODUCTION

Among renewable energy sources, biomasses derived from non-food or feed sources as energy dedicated crops, agricultural and agro-industrial waste are one of the most interesting solutions in the short and medium term for several reasons: the ability to produce energy in situ or on a short range, the relatively low investments, the opportunity to give an alternative to traditional crops that are unable to withstand the competition of a globalized market, the possibility to storage significant amounts of carbon in the soil, the opportunity to recover marginal and abandoned land by offering new market opportunities to farms avoiding competition with food production [1]. Rhizomatous perennial grasses are suitable for biomass production even in marginal context because of their resistance to pests and diseases and the ability to grow in variable environments, such as drought affected, or under salinity [2]. *Arundo donax* L. can be considered the most suitable bioenergy perennial grass for marginal environments affected by summer drought: it is naturalized in Mediterranean basin and is adapted to low soil water availability and other limiting growing conditions [3, 4, 5]. Biochemical methane conversion of lignocellulosic biomass is a suitable process to obtain removable energy from energy crops. The drawback of biomethane production from perennial rhizomatous grasses are, apart from yield limitation due to low water availability, high cost of crop propagation, which can be reduced by extending the plantation life, and the recalcitrance of lignocellulosic biomass to be degraded into monomers for energy purpose.

2. MATERIAL AND METHODS

The field trial was carried out at the Experimental Farm of the University of Catania (10 m a.s.l., 37°24′ N, 15°03′ E) in a typical Xerofluvents soil [6] evaluating two experimental factors, levels of irrigation (100% of maximum crop evapotranspiration, ETm, and rainfed) as the main factor and the genotype as subfactor. The daily ETm was calculated according to:

$$ETm = E0 \times Kp \times Kc$$

where ETm is the maximum daily evapotranspiration (mm); E0 is the evaporation of class-A pan (mm); Kp is the pan coefficient, equal to 0.80 in semi-arid environment. Crop coefficient (Kc) has been estimated by Cosentino et al. [3] in the same environment. The giant reed plantation has been established in 1995 placing rhizomes with 3 buds at 20 cm of depth with a density of 1 rhizome m^{-2}. The total above ground biomass has been harvested every year before the start of the growing season (early February). The results reported in this study refer to the 2017 harvest. The biomass composition was determined by a Near InfraRed (NIR) spectroscopy (SpectraStar 2500XL-R, Unity Scientific). The spectroscopy analysed the diffuse reflectance between 680 and 2500 nm at 1 nm intervals. The absorption spectra have been used to predict the concentration of hemicellulose, cellulose, acid detergent lignin (ADL), acid insoluble ash and neutral detergent soluble (NDS) using a calibration obtained from spectra and correspondent analytic values measured on lignocellulosic biomass of herbaceous plants adopting the developed calibration for lignocellulosic perennial grasses, as reported in Scordia et al. [7]. The experimental

methanogenic potential of the giant reed genotypes biomass was defined trough the BMP test (Biochemical Methane Potential), using the AMPTS (AMPTS, Bioprocess Control AB, Sweden). The BMP test was run as a batch process lasting 30 days. The ratio between the biomass and the bacterial inoculum has been set at 1:3 between volatile solids of respectively inoculum and substrate. For the purpose of quantification of the produced biomethane, a CO_2 filtering system has been adopted by letting the impurified biogas flow over a sodium hydroxide solution (6N) which allows the removal of CO_2. The experimental BMP has been calculated as the volume of methane detected by the flow meter at standard conditions for temperature and pressure per gram of volatile solid of substrate (Nml gVS^{-1}). The experimental biochemical methane potential yield has been calculated as the product of BMP and biomass yield expressed in volatile solid (gVS ha^{-1}). The theoretical BMP was calculated on the basis of the biomass chemical composition, using coefficients from literature [8], as the sum of the theoretical BMP achievable from each biomass fraction, obtained by multiplying genotypes biomass yield expressed as volatile solid per hectare, the percentage of each fraction in the whole biomass and the respective coefficient reported in Table 1 [8].

Table I: Coefficients for BMP calculation on the basis of biomass composition.

Biomass fraction	Bmp (Nml g^{-1})
Cellulose	378
Hemicellulose	354
Lignin	-194
NDS	313

3. RESULTS AND DISCUSSIONS

During the trial, meteorological trend was typical of southern Mediterranean environment. Minimum temperature was recorded in winter time, falling below 0°C only in a few days. Rainfall, as usual in Mediterranean environment, was concentrated in autumn-winter, when several events were recorded. However, rainfalls were scarce during summer time. During the vegetative growth of giant reed, 449 mm were recorded. Biomass yield ranged from 3.78 Mg ha^{-1} (genotype 40) to 12,78 Mg ha^{-1} (genotype 2) in rainfed condition and from 7,19 Mg ha^{-1} (genotype 40) to 19.3 Mg ha^{-1} (genotype 27) in the irrigated treatment (Figure 1).

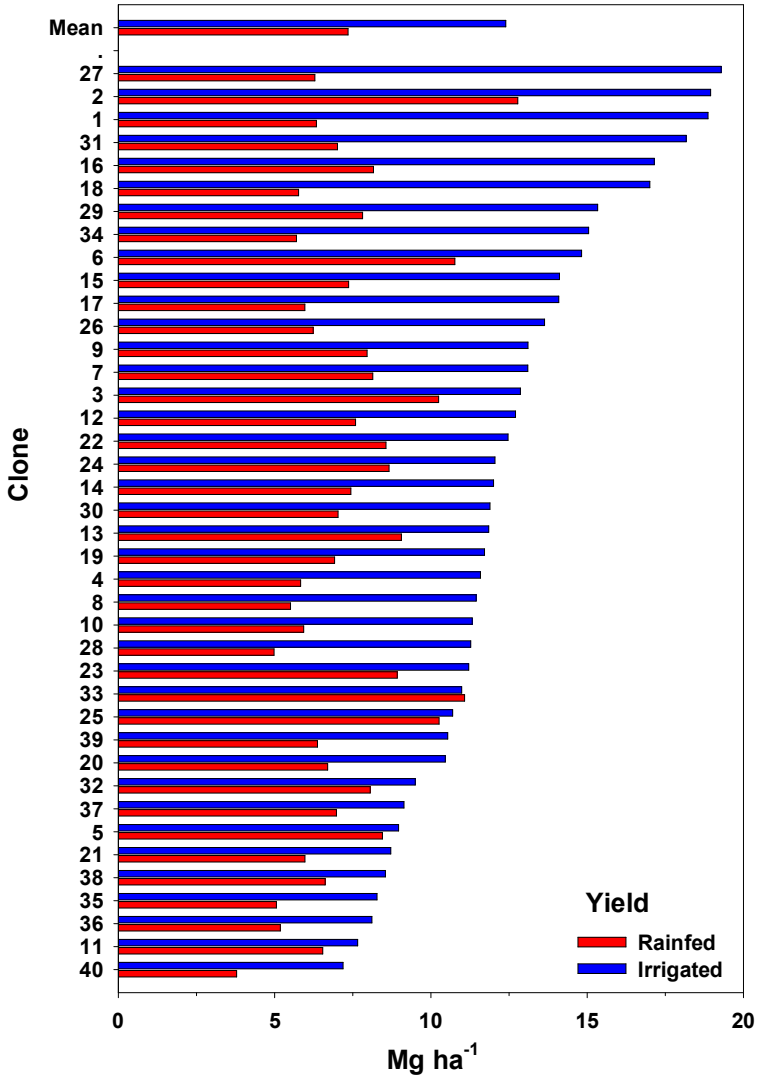

Figure 1: Biomass yield (Mg ha^{-1}) of 40 giant reed genotypes in irrigated and rainfed treatments.

The average yield of the genotypes was 7.4 Mg ha^{-1} in rainfed condition and 12.4 Mg ha^{-1} in the irrigated treatment. Biomethane potential yield ranged from 347.5 m^3 ha^{-1} (genotype 40) to 1127.3 m^3 ha^{-1} (genotype 2) in rainfed condition and from 653.6 m^3 ha^{-1} (genotype 40) to 1666.4 m^3 ha^{-1} (genotype 27) in the irrigated treatment (Figure 2).

As expected for lignocellusic substrate used in anaerobic digestion, the theoretical biomethane potential was higher than experimental one.

This was due to the presence of lignin that limit the cellulose conversion.

As reported above, among genotypes differences were observed in relation to the biomethane production, these was mainly due to the difference in dry biomass yield that was affected by water availability. Giant reed requires abundant water amount to sustain high yield levels but some clones were able to produce high biomass yield even in rainfed conditions, leading to the conclusion that differences amongst clones exist and need to be further studied at physiological and molecular levels for a successful introduction in marginal land

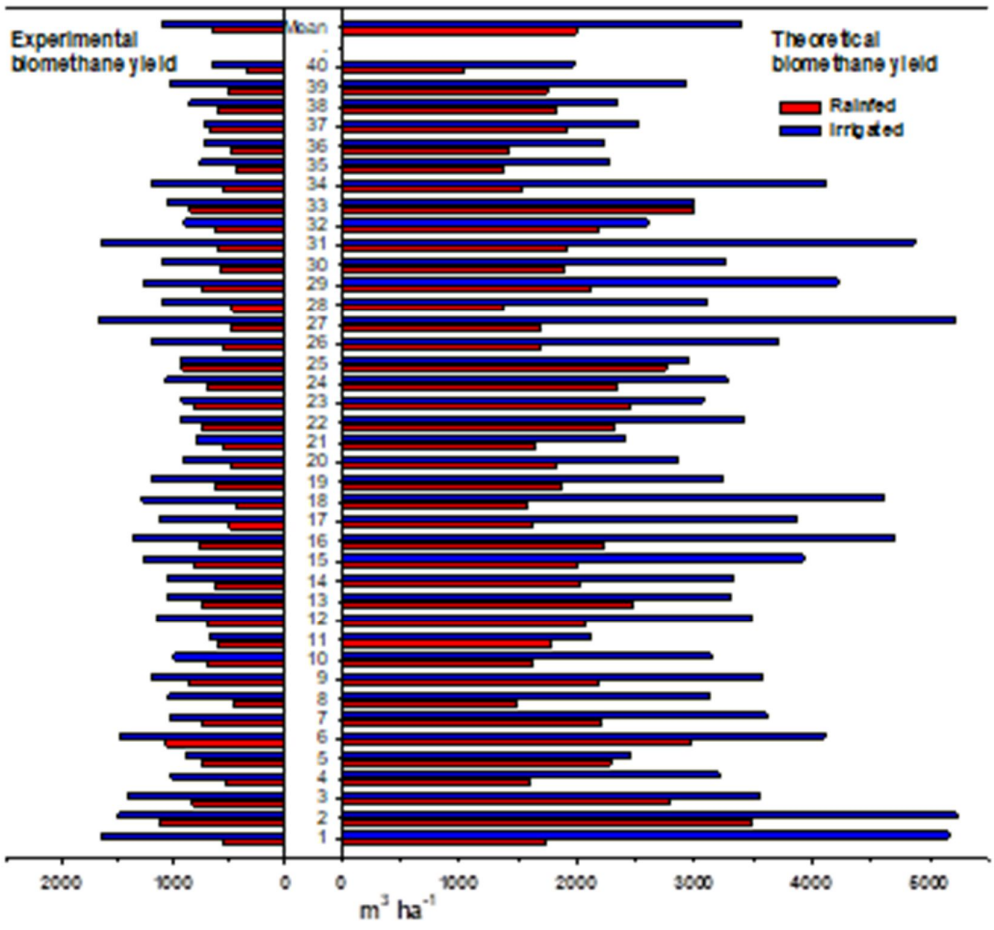

Figure 2: Experimental and theoretical BMP yield of 40 giant reed genotypes

All the genotype benefited from irrigation except for genotype 33, which yielded equally in both theses. Regarding the chemical composition, cellulose is the main fraction of giant reed biomass, followed by hemicellulose and NDS (Figure 3).

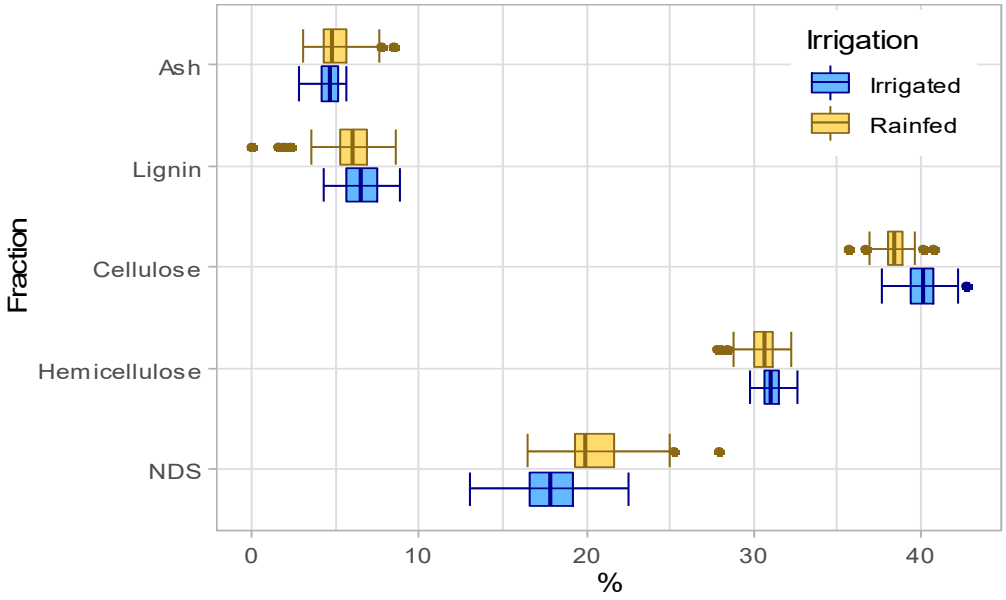

Figure 3: Boxplot showing the proportion of the biomass fractions. The vertical line within the boxes stands for the median; the left and the right sides of the boxes stand for the value of the first and the third quartile respectively. The whiskers represent the range of the values excluding the outliers.

Lignin and acid insoluble ash are present in lower amounts. Irrigated thesis shows higher concentration of cellulose, hemicellulose and lignin than rainfed thesis, while NDS and acid insoluble ash are present in lower amounts. The average biomethane potential yield of the genotypes defined experimentally was 668.6 m^3 ha^{-1} in rainfed condition and 1118.6 m^3 ha^{-1} in the irrigated treatment (Figure 4).

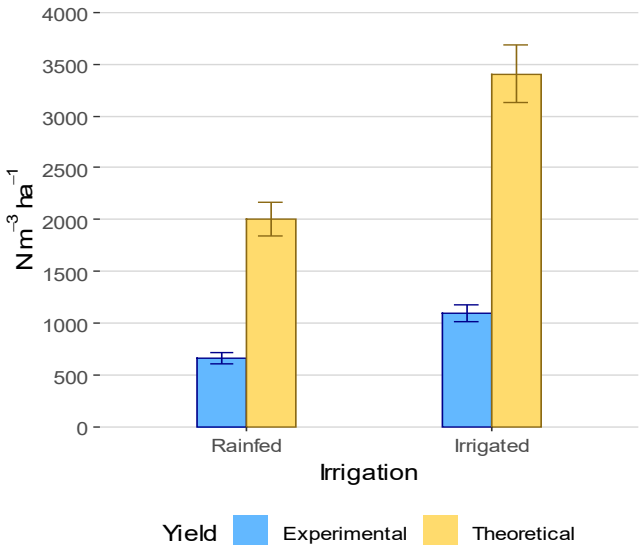

Figure 4: Experimental and theoretical BMP for rainfed and irrigated treatments. Values represent the average of 40 giant reed genotypes. The confidence interval is defined as $\pm 1.96 \frac{s}{\sqrt{N}}$, where s is standard deviation and N is the sample size.

The average biomethane potential yield of the genotypes calculated theoretically was 2002.6 m^3 ha^{-1} in rainfed condition and 3403 m^3 ha^{-1} in the irrigated treatment. These differences are mainly due to the difference between genotypes yield.

4. CONCLUSIONS

Scientific literature reports the decrease of the biomass yield in old plantations of perennial grasses. The trial shows that several genotypes maintain high biomass yield and thus high biomethane potential yield even after 20 years from plantation. The trial shown that several genotypes maintain high biomass yield and thus high biomethane potential yield even from old plantations. The variability of biomass yield and biomethane potential yield among genotypes is high. Almost all the genotypes shown a positive response to the irrigation, which is the main limiting factor in Mediterranean environments.

As expected for lignocellusic substrate used in anaerobic digestion, the theoretical biomethane potential was higher than experimental one due to the presence of lignin within the lignocellulosic matrix, which envelopes the cellulose and hemicellulose fibers, reducing the degradability of these polymers by the bacterial activity.

Among genotypes differences were observed in relation to the biomethane production, these was mainly due to the difference in dry biomass yield that was affected by water availability. Giant reed requires abundant water amount to sustain high yield levels but some clones were able to produce high biomass yield even in rainfed conditions, leading to the conclusion that differences amongst clones exist and need to be further studied at physiological and molecular levels for a successful introduction in marginal land.

The difference between experimental and theoretical biomethane potential yield is ascribable to the recalcitrance of the lignocellulosic matrix of *A. donax* biomass, in which cellulose fibers are enveloped into the lignin polymer. Therefore, a thermal pretratment of the

lignocellulosic biomass could enhance the biomethane potential yield by reducing the recalcitrance toward the biological hydrolysis of the biomass components.

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Lignocellulosic biomass production of Mediterranean wild accessions (*Oryzopsis miliacea*, *Cymbopogon hirtus*, *Sorghum halepense* and *Saccharum spontaneum*) in a semi-arid environment. Field Crops Research, 214, 56-65.

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Advanced biomethane production by *Arundo donax* under changing harvest time and nitrogen fertilization

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ABSTRACT: According to the RED II (2018/2001/EU), Member States must supply a minimum of 14% of the energy consumed in road and rail transport by 2030, of which the contribution of advanced biofuels and biogas must reach at least 3.5%. Reduce biomass recalcitrance of high-yielding lignocellulosic crops by means of agronomic strategies would significantly contribute to the advanced biofuel production goal. Lignocellulose is the lowest cost raw material on earth, it is a no-food biomass and its use alone or in mix with other biomasses can strongly increase the biomass availability for advanced biomethane production. The present study evaluated the suitability of the lignocellulosic, herbaceous *Arundo donax* as a biomass feedstock for advanced biomethane production. Harvest time and nitrogen fertilization treatments were adopted to reduce biomass recalcitrance thereby increasing biomethane yield. Biochemical Methane Potential (BMP) was evaluated on batch anaerobic fermenters in mesophilic conditions. The BMP at 30 days of incubation was influenced by the investigated treatments, the incubation time and the interaction of these two factors. The trend showed a lag phase for the first 5 days of testing, due probably to the adaptation of the bacterial flora to the lignocellulosic matrix, followed by an exponential increase up to approximately 18 days; after that a slight increase tending to an

asymptotic trend in the final phase (up to day 30) was observed. The highest BMP was reached by the combination of winter harvest (W) and 80 kg N ha^{-1} (123.4 Nml CH_4 g^{-1} SV), followed by the autumn harvest (A) and 80 kg N ha^{-1} (118.1 Nml CH_4 g^{-1} SV). The unfertilized treatments showed an opposite BMP, with the autumn higher than winter harvest (106.2 and 100.3 Nml CH_4 g^{-1} SV, respectively). In terms of biomethane yield per unit land area, WN80 showed the highest (1717 ± 203 m^3 CH_4 ha^{-1}) and WN0 the lowest (859 ± 93 m^3 CH_4 ha^{-1}). Nitrogen fertilization looks a promising strategy to reduce biomass recalcitrance for bioconversion by anaerobic digestion mainly due to the higher content of neutral detergent soluble and protein; however, it should be proved by an energy, economic and environmental analysis to ascertain an overall sustainability.

KEYWORDS: biofuel, giant reed, perennial grass, anaerobic digestion; biomethane; lignocellulose; mediterranean.

1. INTRODUCTION

Anaerobic digestion (AD) for biogas production is a key technology to transform crops and residues to a gaseous biofuel. Leading countries are Germany and Italy, however, the main feedstock used so far is maize [1, 2]. As known, using food crops for biofuel production can increase the competition between food and fuel, increase market price of raw materials along with competition for natural resources and land use changes (LUC). Furthermore, food annual crops are high input requiring, thus their contribution toward climate change mitigation is negative if the biomass energy content is used for fuel rather than for food.

Using lignocellulosic non-food crops rather than maize and other food/feed crops, can prevent etic, competing and many other environmental issues as raised by the latest European directive on renewable energy production, the so-called RED II [3]. In the RED II, perennial grasses, such as switchgrass, miscanthus, giant reed and ryegrass were included as non-food cellulosic material to produce advanced biofuels and to minimize the overall direct and indirect LUC impacts [4].

Many researches and projects addressed the environmental and energetic sustainability of perennial grasses, the ability to grow in variable environments, including marginal lands, leading to a low competition for land and other resources with food crops [5]. These species are established only once and harvested yearly, in a plantation life-time spanning from 10 to 25 years [6], resulting in highly positive energy and greenhouse gas (GHG) balances [7]. One of the most important sustainability characteristics of perennial grasses is the lignocellulosic structure of their cell walls that contributes to the natural resistance to pest and diseases [8]. Taken together, perennial grasses are a low-cost biomass feedstock in contrast with oil crops, sugars, cereals and other starch-rich crops used for biofuel production.

However, in the AD of lignocellulosic materials, hydrolysis may be constrained by high lignin content and crystalline cellulose, resulting in low methane (CH_4) output. Hence, pretreatments, aiming at removing or rearrange lignin structure and make more accessible both hemicellulose and cellulose to hydrolytic microorganisms might be envisaged [9]. In this regard, Di Girolamo et al. (2013) [10], showed that untreated giant reed (*Arundo donax* L.) biomass exhibited a potential CH_4 yield of 273 ml g^{-1} volatile solid (VS); four pre-treatments

without acid catalyst achieved a 4-23% CH_4 yield gain, while pretreatments with H_2SO_4 as catalyst incurred in a methanogenic inhibition.

Biomass recalcitrance can be modified directly on field by modulating agronomic practices, such as harvest time. Ragaglini et al. (2014) [11] found out that harvest time significantly influenced biomass AD of giant reed. Although biomass yield was higher in a single harvest per year, harvesting twice per year increased the CH_4 yield per hectare by 20–35% (9.452 and 11.585-12.981 Nm^3 CH_4 ha^{-1}, respectively), as consequence of the highest biochemical methane potential (BMP) achieved by juvenile stages of the crop, and a better digestion kinetics due to a lower biomass recalcitrance. Similar results were reported by Kiesel et al. (2017) [12] with five miscanthus genotypes, grown at three locations in six harvest dates. Generally, green harvest (as early sampling in August) improved the net energy yield of AD due to a combination of biomass yield per hectare, and substrate specific methane yield (e.g., organic and inorganic compounds in the biomass). In the present study, a long-term plantation of giant reed (*Arundo donax* L.), was used for AD without any biomass pretreatment. Giant reed is a C3 perennial grass which had demonstrated a high tolerance to several abiotic stresses, such as drought and salinity and the ability to grow on steep slopes mitigating soil erosion [13, 14, 15]. The plantation is managed in rainfed conditions in a semi-arid Mediterranean environment, dominated by cool and wet winters and high summer temperatures and prolonged drought. Harvest time and nitrogen fertilization treatments were adopted to reduce biomass recalcitrance thereby increasing biomethane yield.

2. MATERIAL AND METHODS

2.1 Field trial description

Giant reed (*Arundo donax* L.) is grown at the Experimental farm of the University of Catania (37°24'N, 15°03'E, 10 m s.l.m.). Briefly, plantlets from nodal cutting were established in 1997 at a plant density of 2.5 plants m^{-2}. Soil was prepared by a ploughing at 40 cm soil depth, followed by a lighter harrowing at 25 cm. At transplant and successive three years, nitrogen fertilization was differentiated (0 kg N ha^{-1}, 60 kg N ha^{-1} and 120 kg N ha^{-1}). Irrigation water was optimally kept for first year to ensure plant survival and the successive three years was differentiated as 100% of maximum evapotranspiration (ETm) restoration, 50% of ETm restoration, and rainfed conditions. Further details are reported in Cosentino et al. 2014 [13].

From 2001 and up to 2011, the giant reed plantation was managed in rainfed conditions, without neither irrigation nor nitrogen fertilization differentiation, weeding or crop protection, as well as other agronomic practices. Only the aboveground biomass was harvested yearly on winter time.

From 2011, corresponding to the 14^{th} growing season, nitrogen fertilization at two levels (N0 - 0 kg N ha^{-1}, and N80 - 80 kg N ha^{-1}) and harvest time (autumn – A, and winter - W) on three replicated plots of 134 m^2 (8 x 17 m) for each treatment was carried out up to present.

Nitrogen fertilization was applied as ammonium sulfate (a.i. 21%) after the autumn cut since crops have to approach crop senescence in winter, while after the winter cut the ammonium nitrate (a.i. 27%) was used since crops are will approach the spring regrowth.

The biomass used in the present experiment was harvested in the growing season 2017/2018, corresponding to the 20th year of plantation. During the study growing seasons, meteorological conditions and potential evapotranspiration (ET0) were continuously measured through a weather station connected to a data logger (Delta-T, WS-GP1 Compact) and a Class A evaporation pan (mm d^{-1}). The dryness index was calculated as precipitation to the potential evapotranspiration (P/PET), according to the Joint Research Center study for delineation of agricultural area affected by biophysical constraints [16], both on autumn and on winter growing season. Aboveground biomass was determined by removing edge plants in all sides of the plots to obtain a sampling area for biomass weight of 6 m^2 (3 x 2 m). Biomass was cut 5 cm above ground level and fresh sub-samples were randomly collected, immediately weighted and then dried to a constant weight at 65°C. The percentage dry weight was used to calculate the dry biomass yield, which was referred to the unit land area (DMY, Mg ha^{-1}).

2.2 Determinations and bioconversion

Dry sub-samples were ground through a 1-mm sieve in an IKA mill (IKA-WERFE, Gmbh & Co., KG, Staufenim Breisgau, Germany) for biomass composition determinations. Hemicellulose (HL), cellulose (CL), acid detergent lignin (ADL), ash (ASH) and the neutral detergent soluble (NDS) were determined by a near-infrared spectrometer (NIR, SpectraStar™ 2500XL-R, Unity Scientific, USA) in a previously developed calibration for lignocellulosic perennial grasses, as reported in Scordia et al. (2017) [17]. The BMP test (Biochemical Methane Potential) was performed by an automatic methanogenic potential detection system (AMPTS, Automatic Methane Potential Test System,

Bioprocess Control AB, Sweden). Total and volatile solids were determined both for the organic substrate and the inoculum in order to obtain an inoculum to substrate ratio of 3 inside each batch. The total solids were obtained drying the biomass in a ventilated oven at 105 °C until constant weight. Samples were then burnt in a muffle furnace at 550 °C for 5 h for the volatile solids. The BMP tests was run for 30 days of incubation at mesophilic conditions (37±1°C) and biomethane evolution was measured by μFlow biogas detectors connected to the Universal DataLogger for realtime measurement (Bioprocess Control AB, Sweden).

2.3 Statistical analysis

Biomass yield data and biomass composition data were subjected to the two-way analysis of variance (ANOVA) according to the randomized block design, with fertilization and harvest time as fixed factors (CoStat, version 6.0). Biomethane production through the incubation time was analyzed by a two-way ANOVA using repeated measurements in time, where the incubation time represents the within-factor, and the fertilization and harvest time the between-factor (SPSS, PASW Statistics 18). When data failed Mauchly's sphericity test, the univariate results were adjusted by using the Greenhouse-Geisser Epsilon and the Huynh-Feldt Epsilon correction factors. When univariate results satisfied sphericity tests for within-subjects effects, the F-values and associated P-values for between-subjects effects were tested. Differences between means were evaluated for significance using the Student-Newman-Keuls (S.N.K.) test at 95% confidence level.

3. RESULTS AND DISCUSSIONS

3.1 Meteorological conditions

In the autumn season (September to September), annual average temperatures of 24.2 °C for the maximum, 13.0 °C for the minimums and 18.6 °C for the mean were recorded (Figure 1). The winter season (February-February) was cooler, 22.8°C and 18.1°C for maximum and mean air temperature, respectively, while the minimum temperatures were warmer, 13.4 °C. Rainfall were more abundant in the winter than in the autumn season, 518.5 and 354.1 mm, respectively. Overall, the reference evapotranspiration (ET0) was higher in the autumn season than in the winter (1101.2 and 985.5 mm, respectively), with an average of 2.89 and 2.59 mm day^{-1} respectively. Obviously, the period with the highest ET0 was from late spring to late summer (4.21 mm day^{-1}). The drought index, expressed by the ratio between annual rainfall and potential evapotranspiration in the same period (P/PET) was much lower in autumn (0.32) than to the winter season (0.53), and both were below the 0.6 threshold suggested by the JRC study [13]. Therefore, the environment in which the test was conducted can be considered affected by dryness.

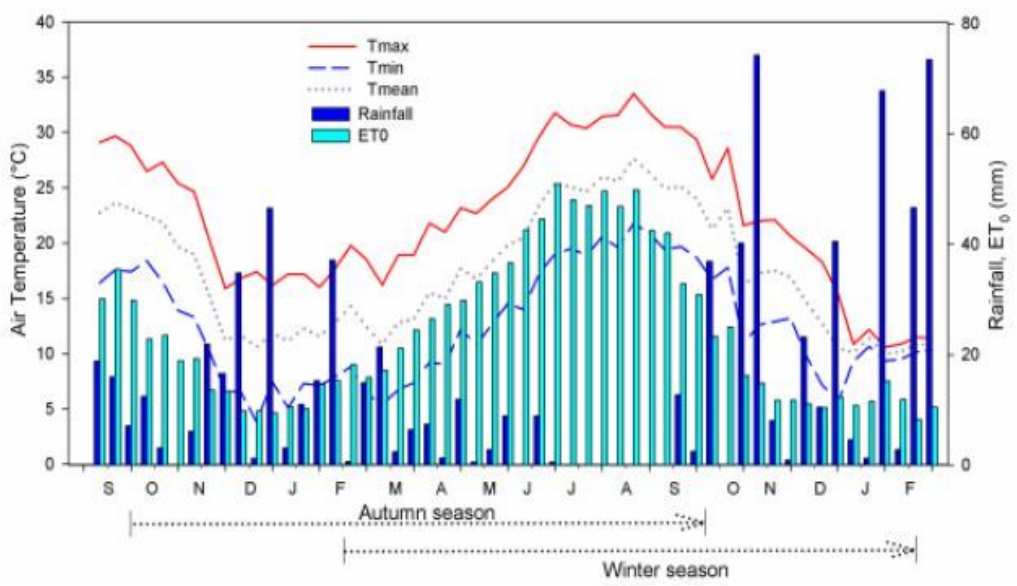

Figure 1. Meteorological trend (air temperature and rainfall) and reference evapotranspiration (ET0) at the autumn and winter growing season of giant reed (*Arundo donax*) grown at the Experimental farm of the University of Catania, Italy (10 m a.s.l., 37°25' N lat., 15°03' E long.).

3.2 Yield and composition

The analysis of variance (ANOVA) of main effects (harvest time - H, and nitrogen fertilization - F) and the interactions (H×F) on the dry matter yield (DMY), and on the content of hemicellulose (HC), cellulose (CL), acid detergent lignin (ADL), ash (ASH) and neutral detergent soluble (NDS) is shown in Table 1. The effect of the harvest time was significant on the HC, ADL and ASH content, while nitrogen fertilization effect on DMY, CL, ASH and NDS. Significant interactions H×F were observed for CL and ASH.

Table I: ANOVA for harvest (H) and fertilization (F) main effect, and interaction on aboveground biomass dry matter yield (DMY), hemicellulose (HL), cellulose (CL), acid detergent lignin (ADL), ash (ASH) and neutral detergent soluble (NDS). Degree of freedom (df), adjusted mean square (Adj MS) and statistical significance indicated by *P≤0.05, **P≤0.01. Not significant (ns).

Source	df	DMY	HC	CL	ADL	ASH	NDS
		Adj MS					
H	1	1.61^{ns}	8.86^{*}	2.61^{ns}	8.59^{*}	5.04^{**}	4.08^{ns}
F	1	64.21^{**}	4.92^{ns}	74.36^{**}	0.99^{ns}	0.33^{*}	47.20^{**}
H×F	1	5.20^{ns}	1.51^{ns}	7.23^{*}	1.65^{ns}	1.65^{*}	2.11^{ns}
Error	6	2.60	0.99	6.07	0.28	0.26	1.36

Figure 2 shows the DMY of the interactions and the separation of the means of the main effects. On the average of fertilizations, there were no significant differences in terms of DMY between autumn and winter harvesting (12.25 Mg ha^{-1} on the average). By contrast, the N80 treatment, on average of the harvest periods, showed significantly higher DMY values compared to N0 (15.16 and 10.54 Mg ha^{-1}, respectively). Overall, the fertilization with 80 kg N ha^{-1} with the winter harvest (WN80) showed the highest values (15.46 ± 1.85 Mg ha^{-1}). The unfertilized winter (WN0) period instead showed the overall lowest DMY value (9.51 ± 1.02 Mg ha^{-1}). In the absence of nitrogen fertilization, the autumn harvest produced more than the winter, however, the effect of fertilization was less marked for the autumn than for the winter harvest.

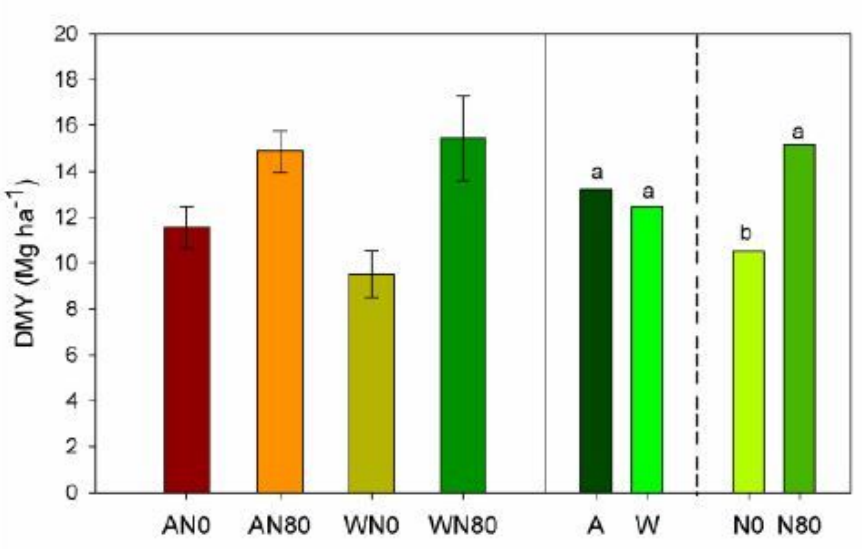

Figure 2. Biomass dry matter yield (DMY, Mg ha^{-1}) of gaint reed (*Arundo donax*) under harvest regimes (autumn – A, and winter – W), and nitrogen fertilization levels (N0 - 0 kg N ha^{-1}, and N80 - 80 kg N ha^{-1}). On the left, mean separation, with different letters representing statistically significant means according to the SNK test ($P \leq 0.05$).

The biomass composition of the dry biomass showed, in general, a greater content in soluble substances (NDS) and ashes (ASH) in the fertilized treatment as compared with the unfertilized one, and in the autumn than to the winter harvest. By contrast, the content of structural polysaccharides, such as hemicellulose and cellulose, and the acid detergent lignin (HC, CL and ADL, respectively) was higher in the winter than the autumn harvest and in the unfertilized as compared with the fertilized biomass (Figure 3). Similar results were observed in Monti et al. 2015 [18], and Zanetti et al. 2019 [19].

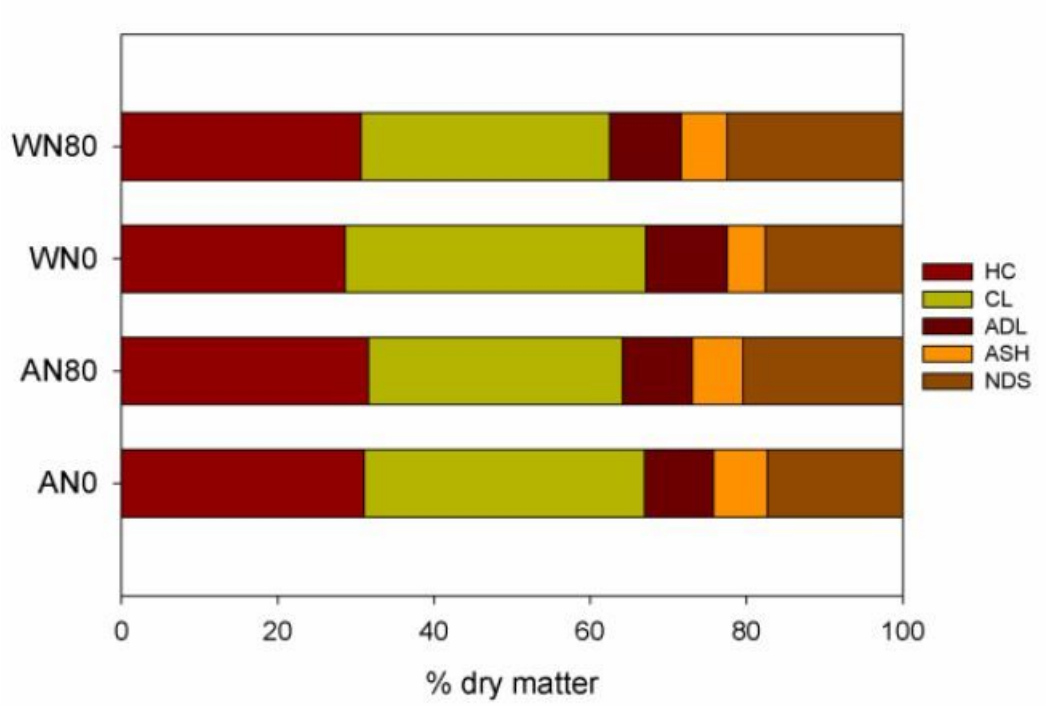

Figure 3. Biomass composition (% w/w), such as hemicellulose (HL), cellulose (CL), acid detergent lignin (ADL), ash (ASH) and neutral detergent soluble (NDS) of giant reed (*Arundo donax*) under harvest regimes (autumn – A, and winter – W), and nitrogen fertilization levels (N0 - 0 kg N ha^{-1}, and N80 - 80 kg N ha^{-1}).

In detail, on the average of experimental factors, the content of HC was significantly influenced by the time of harvest, across the average of nitrogen fertilizers, with higher values in the autumn than winter (31.3 versus 29.6%, respectively), however, nitrogen fertilization did not show differences (30.5% on average). On the other hand, the % of CL was significantly higher in the unfertilized as compared with that fertilized treatment across the average of harvest regimes (37.2 vs 32.2%, respectively), while the harvest time, across the average of fertilizations, did not show a significant effect (34.7% on average). The % of ADL was significantly higher in the winter than autumn harvesting time, in the average of nitrogen fertilizers (9.8 against 8.9%, respectively); nitrogen fertilization, on average of harvest time, did not show a significant effect on the % in ADL (9.3% on average). The ash

content (ASH), was significantly influenced both by the harvest, on the average of fertilizations (6.69 and 5.40% in autumn and winter, respectively), and by the fertilizations on the average of harvest time (5.88 and 6.21% in unfertilized and fertilized, respectively). Finally, the effect of nitrogen fertilization, across the average of the harvest time, significantly increased the % of soluble substances - NDS (21.4 and 17.4% in N80 and N0, respectively), while the content in NDS, although higher in the autumn, did not show statistically significant differences as compared with the winter period across the average of nitrogen fertilization (19.44% on average).

3.3 Advanced biomethane production

The Biochemical Methane Potential test (BMP) which represents the net amount of methane produced by the anaerobic fermentation of a mass unit in a given time and certain test conditions, was used to compare the advanced biomethane production by giant reed under harvest regimes (autumn – A, and winter – W), and nitrogen fertilization levels (N0 - 0 kg N ha^{-1}, and N80 - 80 kg N ha^{-1}). The BMP tests the normal milliliters of biogas (biomethane in this case) per grams of volatile solids (NmL g^{-1} VS) evolved in the 30 days of incubation of the ground biomass and the inoculum. The conditions to run the BMP were mesophilic (37±1°C), with an inoculum to substrate ratio of 3:1. Figure 4 showed that the evolution of advanced biomethane production in the incubation time was significantly influenced by the main factors, the incubation time and the interaction of these.

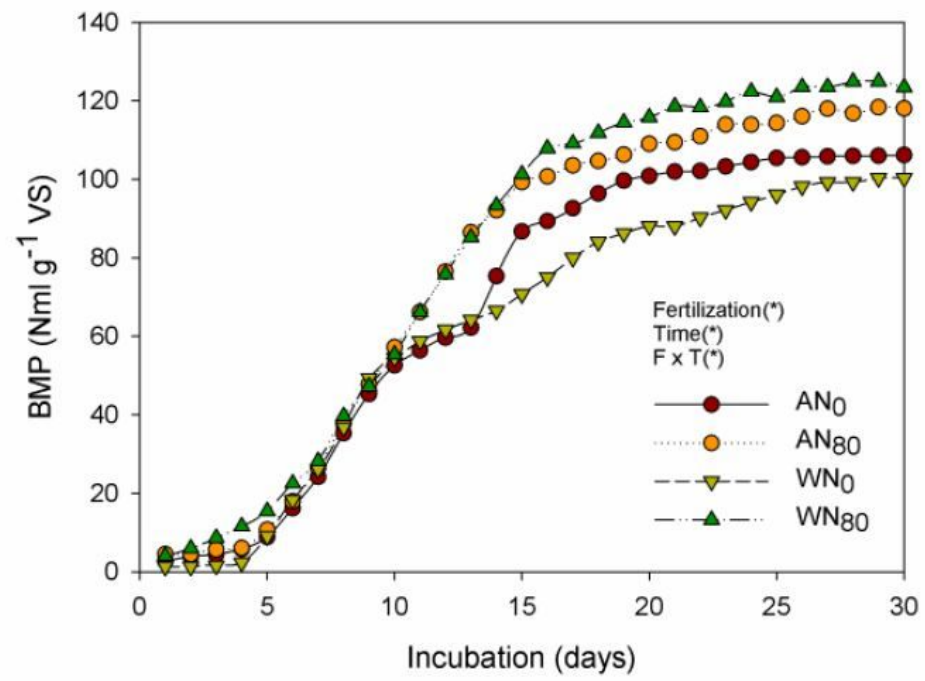

Figure 4. Evolution of advanced biomethane production of giant reed (*Arundo donax*) under harvest regimes (autumn – A, and winter – W), and nitrogen fertilization levels (N0 - 0 kg N ha^{-1}, and N80 - 80 kg N ha^{-1}). Treatments and time effects statistically significant per P≤0.05(*).

All substrates had an initial lag phase lasting around five days of testing, probably due to adaptation of the bacterial flora to the lignocellulosic matrix, followed by an exponential increase in BMP up to approximately 18 days after incubation; subsequently, the increase was less than proportional, approaching an asymptotic trend in the final phase (up to day 30). In the different experimental treatments, the final BMP was significantly higher in giant reed WN80 (123.4 Nml g^{-1} VS), followed by the AN80 (118.1 Nml g^{-1} VS), by AN0 (106.2 Nml g^{-1} VS) and finally by the WN0 (100.3 Nml g^{-1} VS).

Nitrogen fertilizers increased the production of advanced biomethane compared to unfertilized crops. In absolute values, the fertilized winter harvest (WN80) showed the highest biomethane yield per hectare (1717±203 m^3 CH_4 ha^{-1}), followed by AN80 (1580±96 m^3 CH_4 ha^{-1}).

On the other hand, the autumn harvested crops produced higher values than the winter one among the unfertilized (1105±86 and 859±93 m^3 CH_4 ha^{-1} for AN0 and WN0, respectively). On the average of the study treatments, the harvest time did not show significant differences (982 m^3 CH_4 ha^{-1} on average), on the contrary, the production of advanced biomethane responded positively to nitrogen fertilization, that on the average of the harvest time was 1648 m^3 CH_4 ha^{-1} for N80 and 982 m^3 CH_4 ha^{-1} for N0, respectively).

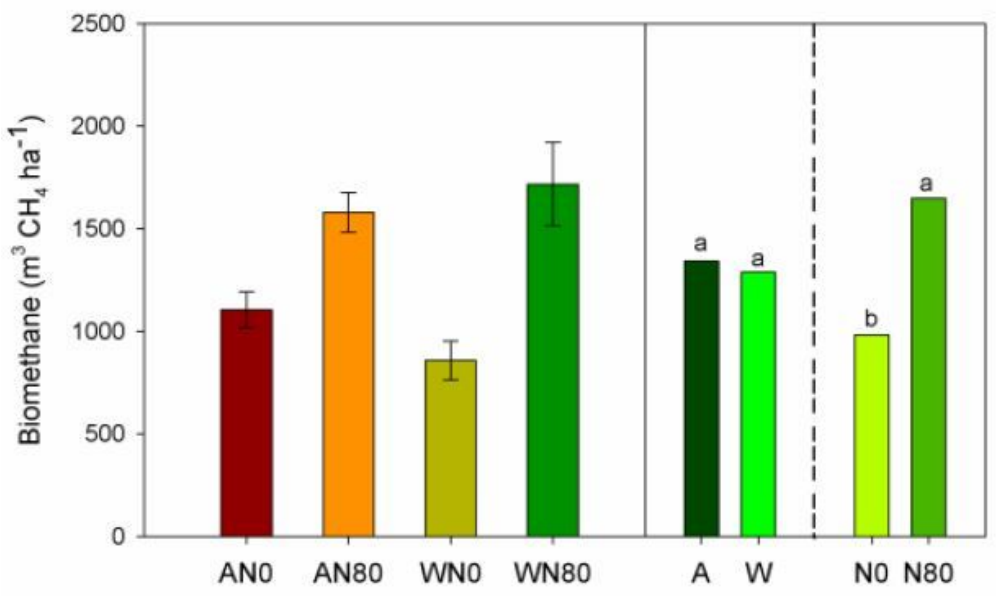

Figure 5. Biomethane production (m^3 CH_4 ha^{-1}) of gaint reed (*Arundo donax*) under harvest regimes (autumn – A, and winter – W), and nitrogen fertilization levels (N0 - 0 kg N ha^{-1}, and N80 - 80 kg N ha^{-1}). On the left, separation of means, with different letters representing statistically significant means according to the SNK test (P≤0.05).

4. CONCLUSIONS

Biomethane production can be considered a solid and mature technology for the sustainable use of agricultural biomass as a source of biofuel, especially when the substrate for anaerobic digestion is a non-food crop that does not compete with food crops for the land use and other resources. Giant reed (*Arundo donax* L.) investigated in this study is a non-food, lignocellulosic crop, tolerant to drought and with good yield persistence over its lifespan, which has gained considerable interest from researchers of the Mediterranean environment. This crop has been included in the list of substrates to be used for the production of renewable energy and advanced biofuels, as for the Annex IX part A of RED II (EU 2018/2001), with which its contribution to the achievement of the objective proposed in Article 25 (14% the contribution of biofuels in the transport sector of which 3.5% from advanced biofuels) can be double counted.

Main findings have shown that *Arundo donax* grown in low (80 kg N ha^{-1}) or no input in rainfed condition, is able to support satisfactory dry biomass production (from 9.5 Mg ha^{-1} in the worst conditions - WN0 - to 15.4 Mg ha^{-1} in the best - WN80), even in the 20^{th} year of cultivation, and in environments where water is the limiting factor for the production of crops owing spring-summer cycle. In fact, in the season analyzed in this work, the drought index, expressed by the ratio between annual precipitation and potential evapotranspiration in the same period (P/PET), was always lower than the threshold value of 0.6 set by the JRC study, both in the autumn (0.32) and in the winter (0.53) season.

The production of advanced biomethane was about three to four times lower than that obtainable from crops most used for this purpose, such

as maize in the best growing conditions. However, the energy, economic and environmental costs necessary to support these productions should be carefully considered with respect to the cultivation of a multi-year species with low or zero requirements in external agronomic inputs (water, fertilizers, plant protection products, etc.). Furthermore, if the aforementioned directive (RED II) is taken into account, the results obtained should be multiplied by a factor of two, as a result of the double counting, thus becoming well comparable to those of the crops currently used in anaerobic digestion.

In conclusion, the results highlighted that the effect of nitrogen fertilization has positively influenced the anaerobic digestion and the production of advanced biomethane on the unit land area, due mainly to a combination of biomass yield and composition, such as higher NDS and protein. The harvest time does not seem to have a significant effect, however, in the absence of nitrogen input, the autumn time showed a substrate less recalcitrant by the bacterial flora thanks to a higher content in soluble substances (NDS) and hemicellulose, and a lower content in acid detergent lignin (ADL) compared to the winter harvest time.

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Evaluation of the thermal pretreatment on the methanogenic potential of two lignocellulosic crops: *A. donax* and *S. spontaneum*

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ABSTRACT: Giant reed (*Arundo Donax* L.) and African fodder cane (*Saccharum spontaneum* ssp. *aegypticum*) are perennial, non-food and low-input energy crop representing a promising solution to produce renewable energy at low cost, especially in marginal areas - i.e. low profitable areas which are prone to land abandonment. This research investigates the biomass composition and the biochemical methane potential (BMP) yield of these crops and the effect of a hydrothermal pretreatment on the biomass composition and on BMP yield.

KEYWORDS: biomass, bioenergy, perennial energy crops, pretreatment, biomethane potential.

1. INTRODUCTION

Among renewable energy sources, biomasses derived from non-food or feed sources as energy dedicated crops, agricultural and agro-industrial waste are one of the most interesting solutions in the short and medium term for several reasons: the ability to produce energy in situ or on a short range, the relatively low investments, the opportunity to give an alternative to traditional crops that are unable to withstand the competition of a globalized market, the possibility to storage significant amounts of carbon in the soil, the opportunity to recover marginal and abandoned land by offering new market opportunities to

farms avoiding competition with food production [1]. Rhizomatous perennial grasses are suitable for biomass production even in marginal context because of their resistance to pests and diseases and the ability to grow in variable environments, such as drought affected, or under salinity (Scordia et al., 2019). Up to now, the most suitable perennial grasses for biomass production in marginal environments affected by low soil water availability during summer are two undomesticated grasses naturalized in the Mediterranean basin: giant reed (*Arundo donax* L.) and African fodder cane (*Saccharum spontaneum* ssp. *aegypticum*) [3, 4, 5, 6, 7, 8]. African fodder cane is a C4 grass with high biomass yield across several levels of soil water availability, from rainfed condition to 100% of maximum crop evapotranspiration restoration, with a high radiation use efficiency and water use efficiency and produces a biomass with satisfactory quality [4, 5]. Giant reed is a perennial grass with a C3-photosyntehic pathway that shows CO_2 assimilation rate, light interception and biomass yield similar to that of C4 plants [6], while having a higher transpiration rates typical of the C3 [2].

The high tolerance of giant reed to extended drought spells is due to the long and deep root system, which has the capacity to extract deep water even at 1.80 m [3]. Its biomass quality and bioconversion suitability has been also investigated, both for biochemical and thermochemical conversion pathways [9, 10, 11, 12, 13]. Biochemical methane conversion of lignocellulosic biomass is a suitable process to obtain removable energy from energy crops. The main drawback of biomethane production from perennial rhizomatous grasses is, apart from yield limitation due to low water availability, the recalcitrance of lignocellulosic biomass to be degraded into monomers for energy

purpose. Several pretreatment have been proposed to enhance the degradability of lignocellulosic biomass: most physical and chemical pretreatment using acid, alkali, microwave, steam explosion, ionizing radiation or combined processes require special instrument, are higly energetic consuming and can generate inhibitors which can adversely affect the following enzymatic hydrolysis and fermentation if the aim is the bioethanol production, or the anaerobic digestion if the aim is the production of biomethane. A hydrothermal pretreatment reduces the need of specialistic machinery and avoid the generation of compounds that could inhibit the anaerobic digestion by methagenic bacterial consortium. The aim of the study is to evaluate the biochemical methane potential (BMP) and BMP yield from two lignocellulosic crops suitable for the Mediterranean environment, *Arundo donax* L. and *Saccharum spontaneum* subsp. *aegyptiacum* (Willd.) Hack, and the effect of a hydrothermal pretreatment on the biomass composition and on BMP yield.

2. MATERIAL AND METHODS

The field trial was carried out at the Experimental Farm of the University of Catania (10 m a.s.l., 37°24′ N, 15°03′ E) in a typical Xerofluvents soil (USDA, 1999). Plants were grown in rainfed condition.

The qualitative analysis of the untreated biomass was performed using the Van Soest method on milled biomass. A hydrothermal pretreatment was carried out using an autoclave (model 1000 ML Zipperclave Assembly, Parker) with distilled water at 160 °C for 10 minutes. The autoclave is composed of a 1 liter sealed stainless steel reactor, provided with an internal stirrer. The core temperature is maintained by heating jacket. Stirrer angular frequency, core and jacket temperature, core pressure are controlled by a control unit (Unit Record Controller, URC). The biomass to be treated has been milled with a IKA miller with a sieve dimension of 2.5 mm. The hydrothermal pretreatment has been performed with 50 g of milled biomass and 500 ml of distilled water. Pretreatment duration has been counted since the reaching of the set core temperature. After 10 minutes, the heater has been switched off and the biomass sample has been taken after the core temperature reached the room temperature. The sample has been filtered with a 0.5 mm sieve in order to separate the solid from the aqueous fraction. The subsequent analyzes (Van Soest fiber composition analysis and BMP test) have been performed on the solid fraction of the pretreated biomass. The Biochemical Methane Potential (BMP) test, performed by the AMPTS (Bioprocess Control), was used to define the experimental methanogenic potential of the biomass. The BMP test was run as a batch process for 30 days of incubation at mesophilic conditions (37±1°C)

and the biomethane outflow was measured by μFlow biogas detectors connected to the Universal Data Logger for realtime measurement (Bioprocess Control AB, Sweden). The ratio between the biomass and the bacterial inoculum has been set at 1:3 between volatile solids of respectively inoculum and substrate. The total solids were obtained drying the biomass in a ventilated oven at 105 °C until constant weight. Samples were then burnt in a muffle furnace at 550°C for 5 h for the volatile solids determination. For the purpose of quantification of the produced biomethane, a CO_2 filtering system has been adopted by letting the impurified biogas flow over a sodium hydroxide solution (6N) which allows the removal of CO_2. The experimental BMP has been calculated as the volume of methane detected by the flowmeter at standard conditions for temperature and pressure per gram of volatile solid of substrate (Nml gVS^{-1}). The experimental methane yield has been calculated by multiplying the experimental BMP and the biomass yield expressed in gVS ha^{-1}. The experimental methanogenic potential was compared with the theoretical potential, calculated on the basis of the biomass chemical composition, using coefficients from literature, as the sum of the theoretical BMP achievable from each biomass fraction, obtained by multiplying genotypes biomass yield expressed as volatile solid per hectare, the percentage of each fraction in the whole biomass and the respective coefficient reported in Table 1 [14].

Table 1 Coefficients for BMP calculation on the basis of biomass composition. NDS: Neutral detergent soluble fraction

Biomass fraction	Bmp (Nml g^{-1})
Cellulose	378
Hemicellulose	354
Lignin	-194
NDS	313

3. RESULTS AND DISCUSSIONS

S. spontaneum showed the highest yield both as untreated and pretreated biomass in comparison with *A. donax* (

Table **2**). Pretreated biomass yield is lower than untreated biomass yield for both crops due to the partial solubilization of acid insoluble ash, neutral detergent soluble (NDS) fraction and hemicellulose that occurred during the hydrothermal pretreatment (Error! Reference source not found.). The amount of solid biomass that has been recovered after the pretreatment is around 90% in both crops.

Table 2 Biomass yield for *A. donax* and *S. spontaneum*

Thesis	Yield (Mg VS ha^{-1})
A.donax	13.86
A.donax pretreated	12.50
S. spontaneum	17.68
S. spontaneum pretreated	15.98

S. spontaneum untreated biomass differed from *A. donax* for lignin content (higher in *A. donax*) and ash content (higher in *S. spontaneum*). Cellulose and hemicellulose were similar in both untreated biomasses. The hydrothermal pretreatment resulted in a decrease of the share of acid insoluble ash, NDS fraction and hemicellulose in comparison with the untreated biomass. As a consequence of the solubilization of these components, the share of cellulose and lignin increased (Figure 2).

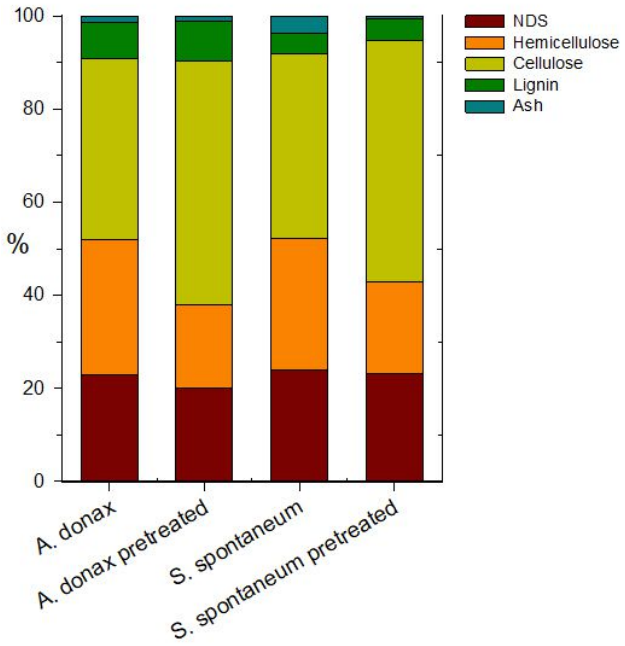

Figure 2 Biomass composition according to the Van Soest method of untreated and pretreated biomass of *A. donax* and *S. spontaneum*.

The change in biomass composition caused by the hydrothermal pretreatment resulted in an increase of the theoretical BMP per gram of volatile solid for both crops: *A. donax* BMP increased from 321.16 to 324.05 Nml gVS^{-1}, *S. spontaneum* BMP increased from 325.42 to 338.14 Nml gVS^{-1}. The higher BMP is ascribable to the increase in the share of cellulose on the total biomass (Figure 3). Even the experimental BMP test showed a higher BMP per gram of volatile solid for pretreated biomass in comparison with untreated biomass for both crops: *A. donax* BMP increased from 100.3 to 109.7 Nml gVS^{-1}, *S. spontaneum* BMP increased from 84.9 to 153.4 Nml gVS^{-1} (Figure 4). Contrarily, the theoretical BMP yield for pretreated biomass is lower than the theoretical BMP yield for untreated biomass in spite of the increase in BMP per gram of volatile solid. This is due to the lower yield of solid biomass as a consequence of the solubilization that occurs

during the hydrothermal pretreatment (Figure 4). *A. donax* BMP yield decreased from 4240 to 3841 Nm^3 ha^{-1}, *S. spontaneum* BMP yield decreased from 5606 to 5259 Nm^3 ha^{-1}. The highest yield of *S. spontaneum* in comparison with *A. donax* is due to the higher untreated and pretreated biomass yield.

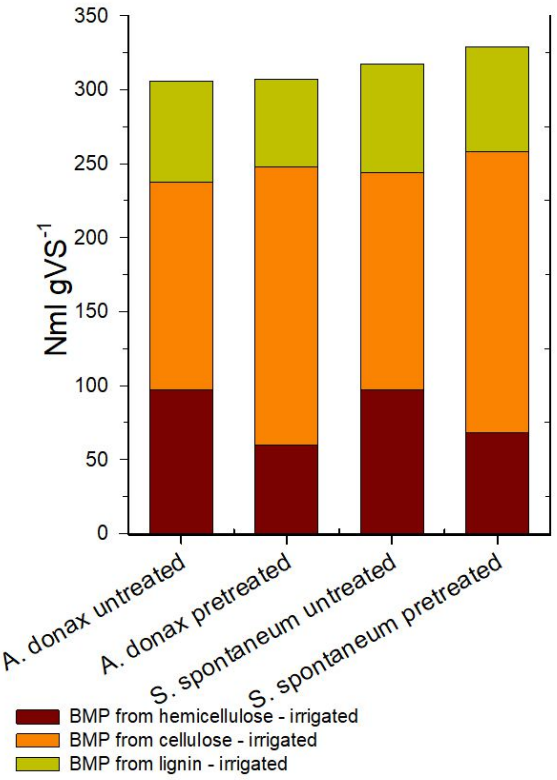

Figure 3 BMP from biomass fraction of untreated and pretreated biomass of *A. donax* and *S. spontaneum*.

The experimental BMP yield for pretreated biomass is similar to the experimental BMP yield for untreated biomass in *A. donax* (1390 and 1372 Nm^3 ha^{-1} for untreated and pretreated biomass respectively), while in *S. spontaneum* experimental BMP yield for pretreated biomass is higher than the experimental BMP yield for untreated biomass (1372 and 2452 Nm^3 ha^{-1} for untreated and pretreated biomass, respectively) (Figure 5).

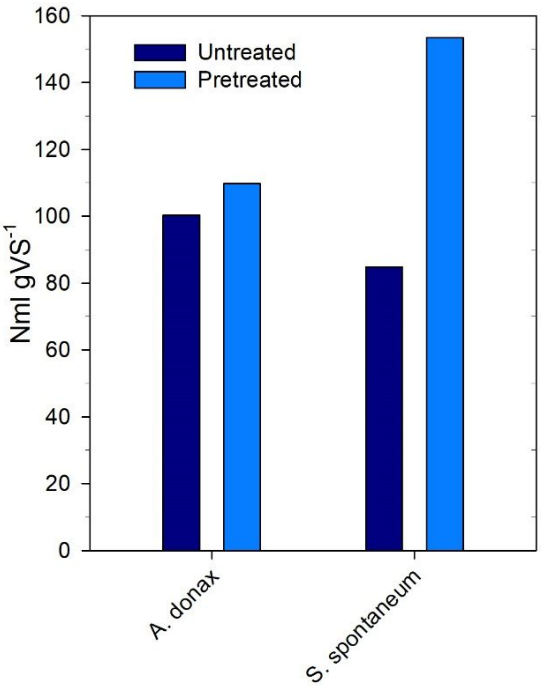

Figure 4 Experimental BMP of untreated and pretreated biomass of *A. donax* and *S. spontaneum***.**

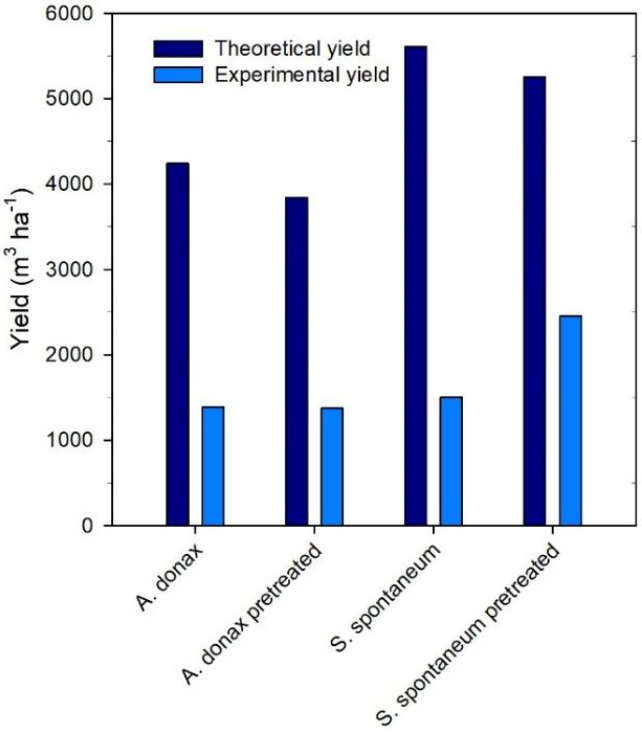

Figure 5 Experimental and theoretical BMP yield of untreated and pretreated biomass of *A. donax* and *S. spontaneum*.

4. CONCLUSIONS

A. donax and *S. spontaneum* are two promising biomass crops for the rainfed cultivation in the south Mediterranean basin affected by low soil water availability during summer. For both crops, the high cellulose and hemicellulose content suggests the suitability for biomethane production. The main hindrance to this process is the effect of the lignin within the lignocellulosic matrix, which envelopes the cellulose and hemicellulose fibers, reducing the degradability of these polymers by the bacterial activity [9, 10, 11, 12, 13]. The hydrothermal pretreatment allows to alter the structure and composition of the lignocellulosic matrix, by interrupting the continuity between the lignin and the cellulose and hemicellulose fibers and partially solubilizing hemicellulose and NDS fraction. The hydrothermal pretreatment returns a liquid fraction, rich in NDS and monomers of hemicellulose, which is suitable for further energetic purposes, such as fermentation for bioethanol production [9, 10, 11, 12, 13] or anaerobic digestion as a co-digestion with the solid fraction or separated digestion. The composition and the energetic potential of the liquid fraction should be investigated furthermore. The alteration of the lignocellulosic biomass by the hydrothermal pretreatment resulted in the increase of the experimental yield, despite the higher content of lignin and the lower total content of digestible fractions (hemicellulose, cellulose and NDS).

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Advanced biomethane production from biologically pretreated giant reed under different harvest times

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ABSTRACT: Increasing energy demand and fossil fuel consumption causing global warming has motivated research to find alternative energy sources such as biofuels. Giant reed (*Arundo donax* L.), a lignocellulosic, perennial, rhizomatous grass has been proposed as an important bioenergy crop for advanced biofuel in the Mediterranean area. Anaerobic digestion for advanced biomethane seems a promising approach, however, the presence of lignin in lignocellulosic biomass represents the main obstacle to such production due to its recalcitrance. Thus, to use effectively lignocellulosic biomass in anaerobic digestion one or more pretreatment steps are needed to aid microorganisms access to the plant cell wall. To this end, the present study investigated the effect of fungal pretreatment of giant reed obtained from two different harvesting time (autumn and winter) on biomethane production by anaerobic digestion using two white rot fungi (*Pleurotus ostreatus* and *Irpex lacteus*, respectively).

The highest lignin degradation was at 30 days for *P. ostreatus* in both autumn (27.1 %) and winter (31.5 %) harvest time. *P. ostreatus* pretreatment showed promising results for anaerobic digestion of giant reed achieving a cumulative yield of 130.9 Nml g^{-1} VS for the winter

harvest, whereas *I. lacteus* reported a decrease in methane yield as compared with the untreated (77.4 Nml g^{-1} VS and 73.3 Nml g^{-1} VS for winter and autumn harvest, respectively). *I. lacteus* pretreatment resulted in a loss of both holocellulose and lignin, indicating that this strain was less selective than *P. ostreatus*.

Further studies are necessary to identify white rot fungi more suitable to lignocellulosic biomass and optimize biological pretreatment conditions to reduce its duration.

KEYWORDS**:** *Arundo donax* L., long term plantation, anaerobic digestion, biofuel, white rot fungi, fungal pretreatment.

1. INTRODUCTION

World population and global demand for food and energy are rapidly increasing resulting in a consequent depletion of fossil fuels and emergence of environmental concerns such as global warming, greenhouse gas emission and land use changes [1]. These factors have greatly contributed to looking for alternative energy sources.

Fossil energy consumption is the main cause of climate change and greenhouse gas emissions. Within the energy sector, heat and electricity production are responsible for most emissions, followed by transportation, manufacturing and construction [2]. Research investigates on production of renewable energy sources to reduce use of fossil fuels and mitigate their adverse environmental effects on air quality. The use of biomass for energy (i.e. bioenergy) is considered to be a promising renewable energy alternative for reducing the environmental impact [3].

Energy generation from biomass sources can be derived directly such as by burning wood for heating, or indirectly from products such as alcohols or biogas. Interest in biogas as a source of bioenergy has progressively been growing since the biogas can ultimately be used to produce electricity and/or thermal energy or biofuel. Biogas is a biofuel obtained through anaerobic digestion from biomass sources, mainly agricultural residues, sewage sludge, animal manure, microalgae and food waste [4].

Among all possible solutions, anaerobic digestion represents one of the most promising ways to use biomass [5].

However, biogas production from different types of materials is still difficult due to the physical and chemical properties of the biomass and operating conditions [6].

Lignocellulosic feedstock is one of the most abundant organic resource derived from agricultural residuals, forestry, urban wastes and dedicated energy crops. It represents a renewable resource, widely available and rich in complex carbohydrates; these characteristics make it a promising candidate for second generation bioenergy production, such as bioethanol, biomethane and bio-oil in order to reduce dependency on limited fossil fuels sources, greenhouse gas emissions and environmental pollutions [7].

Among various biofuels, biomethane production via anaerobic digestion is one of the most cost-effective and environmentally friendly technology to produce energy from lignocellulosic feedstocks performed worldwide.

Lignocellulosic substrates used for anaerobic digestion should not directly compete with food or feed crops for the exploitation of limited agricultural land resources [8].

An ideal energy crop for biogas production should have high biomass yields and show adaptability to varying environments even under low requirement of energy, water, and nutrients.

Perennial rhizomatous grasses have demonstrated their capacity to grow under adverse conditions minimizing environmental impacts, due to the reduced inputs requirements [9].

Giant reed (*Arundo donax* L.) is a lignocellulosic, perennial, rhizomatous grass diffused in the Mediterranean area, which is considered a promising energy crop in southern Europe [10–12]. As a perennial crop, giant reed can positively affect soil quality, since it contributes to reduce the risk of soil erosion and to increase the soil organic matter content [13]. Giant reed shows many advantages when compared to other energy crops, like (i) the adaptability to different types of environments, soils and growing conditions, (ii) the high biomass production and (iii) the low input required for its cultivation (use of irrigation, fertilizers, pesticides) [10].

Giant reed is considered a drought-tolerant species that can achieve high biomass yields also under high salinity conditions [14]; it can be grown in marginal or sub-marginal lands reducing competition with food crops for soil use [15,16]. Thanks to its high biomass yield also in marginal land, giant reed has recently been proposed as energy crops for producing biogas [10,11,17–19].

However, major challenges to obtain biomethane from lignocellulosic biomass are the highly recalcitrant structure and the complex chemical composition, which confer the resistance to anaerobic degradation [20]. Pretreatment is a necessary step in the processes of anaerobic digestion to overcome lignocellulosic recalcitrance in order to improve methane production from lignocellulosic substrates [21]. Compared with

physical and chemical pretreatment, biological methods are more environment friendly, consume less energy, produce no inhibitors and do not require chemicals input. The commonly microorganisms utilized in this pretreatment are filamentous fungi, mainly white-rot fungi for their ability to degrade lignin selectively.

The objectives of this study were to (1) evaluate the effects of two different harvesting time (autumn and winter) on giant reed biomass production; (2) compare fungal pretreatment of giant reed using two white rot fungi (*Pleurotus ostreatus* and *Irpex lacteus*); and (3) evaluate the effect of fungal pretreatment of giant reed biomass on biomethane production by anaerobic digestion.

2. MATERIALS AND METHODS

2.1 Agronomic data

The field experiment was carried out at the Experimental farm of the University of Catania, Italy (10 m a.s.l., 37°25' N lat., 15° 03' E long.) in a typical xerofluvent soil. The Giant reed (*Arundo donax* L.) field was established using plantlets obtained from nodal cuttings in 1997 at a plant density of 2.5 plants m^{-2}. Further details are reported in Cosentino et al. (2014) [11]. From 2002 the field was left without any fertilisation and irrigation.

From 2011 two harvest time (autumn and winter) on three replicated plots of 134 m^2 (8 x 17 m) was carried out up to present. The biomass used in this study was harvested during the 2020/2021 growing season, which represents the 23rd plantation's year. The dates of harvest were 12th October and 11th February. During the study growing seasons, meteorological conditions and potential evapotranspiration (ET0) were

continuously measured using a weather station connected to a data logger (Delta-T, WS-GP1 Compact) and a Class A evaporation pan (mm d^{-1}).

At harvest, edge plants were removed in each plot and the aboveground biomass from a sampling area of 6 m^2 (3 x 2 m) was weighted. A sample of plants was collected in order to subdivide the plants in stems and leaves.

Fresh sub-samples were randomly collected, weighted, and dried to a constant weight at 65°C to determine the dry biomass yield which was referred to as the unit land area (DMY, Mg ha^{-1}).

2.2 Characterization of feedstock

After being oven-dried at 65 °C, samples of giant reed biomass were milled and stored for chemical analysis and pretreatment.

The total solids (TS), volatile solids (VS), and chemical composition of feedstock were determined before pretreatment. TS correspond to the residue after 24 hours drying period at 105°C. It is expressed in percent of the sample initial weight. Dry residue is, then, burnt 5 h at 550°C. VS are the combusted organic matter (expressed in percent of TS) whereas residue after ignition is the mineral matter (ash). TS and VS measurements were realized in duplicates.

The total fiber composition was determined as neutral detergent soluble (NDS), neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) according to Van Soest method [22] through a Fiber Analyzers (Fibertec Velp Scientifica, model FIWE). The hemicellulose and cellulose contents were calculated as the difference between the NDF and ADF, and the ADF and ADL, respectively. To determine the ash, ADL residue was ignited in muffle

furnace at 550°C for 5 hours and lignin content was calculated as the difference between ADL and ash.

2.3 Inoculum preparation

Two fungal strains used in this study were *Pleurotus ostreatus* (MUT00002977) and *Irpex lacteus* (MUT00005918), purchased from Mycotheca Universitatis Taurinensis (MUT) of the Department of Life Sciences and Systems Biology, University of Turin (Italy).

Fungi were activated on Malt Extract Agar plates and incubated at 26°C for 7 days. Sterile giant reed biomass colonized with *P. ostreatus* and *I. lacteus* was used as inoculum for the fungal pretreatment experiments. To prepare the inoculum, 30 g (dry basis) of giant reed biomass (GRB) were placed in 0.5 L reactors, in which deionized water was added to obtain a moisture content of 70%. Reactors were autoclaved at 121°C for 20 minutes followed by cooling down to room temperature. Subsequently, four agar disc of 7-day-old mycelia (approximately 1 cm in diameter) were aseptically added to sterilized giant reed biomass (GRB) and incubated at 26 °C until full colonization. At the end of colonization, which occurred 4 weeks after the start of incubation, fungal-colonized GRB was thoroughly mixed and used as inoculums for the successive fungal pretreatment of GRB.

2.4 Fungal Pretreatments

Sterile GRB and inoculum (fungal-colonized GRB) were mixed and added to 0.5 L reactors. Fungal pretreatments were performed at 30% (dry weight basis) inoculum ratio. Deionized water was added to reach 70% moisture content. Reactors were covered with cotton plugs and

incubated at 26°C for 30 days. Sterile GRB was considered as a negative control.

Samples were collected at day 10, 20 and 30. Fungal-treated samples from each sampling time were subjected to composition analysis.

For each sampling and at the end of the pretreatment, samples were taken out of the reactors, thoroughly mixed and dried at 65°C in a ventilated oven for 24 h before cellulose, hemicellulose and lignin content determination.

The dry matter loss and degradation of cellulose, hemicellulose and lignin during the pretreatment were expressed as percentage of the initial dry weight and fiber fractions before fungal pretreatment.

2.5 Biochemical Methane Potential (BMP) tests

The BMP test was performed by an automatic methanogenic potential detection system (AMPTS II, Automatic Methane Potential Test System, Bioprocess Control AB, Sweden). The AMPTS II is a standardized laboratory set-up specially designed for automatic BMP determination of any biodegradable material. It consists of 15 parallel reactors and the same number of gas flow meters (flow cells) attached to a detection unit for online, automatic data acquisition.

The experiment was conducted in reactors of 500 mL each, in which substrates and inoculum were mixed at a ratio of 1:3 in terms of grams of VS at mesophilic conditions (38±1°C) with continuously mixing. All tests were performed in triplicate.

TS and VS were determined both for the organic substrate and the inoculum as reported above.

Each reactor was connected to a 80 mL trap bottle of 3 M sodium hydroxide solution used for absorbing CO_2 from the raw gas. The

remaining gas after scrubbing passed to ultra-low gas flow meters which were connected to the data analytical and acquisition system. The BMP test was run for 30 days.

Additionally, blank samples, only containing inoculum, were incubated as well. The resulting methane production of the substrate was determined by subtracting methane production of the blank (inoculum) from the substrate sample (substrate + inoculum). The final value of cumulative methane production at the end of the test was defined as the experimental BMP of the substrate.

2.6 Biomethane Potential per hectare

The biomethane yields per hectare of giant reed cultivation (m^3 CH_4 ha^{-1}) was calculated as the product of biomethane potential and dry biomass yield expressed in volatile solid (gVS ha^{-1}).

2.7 Statistical Analysis

Data were analyzed according to the randomized block design. Before conducting the ANOVA, the Bartlett's test was run to verify the assumption of homogeneity of variances. Biomass dry matter yield, biomass composition, and yield content of the hemicellulose, cellulose, ADL, ash and NDS, were analyzed by one-way ANOVA with harvest time as fixed effect. The biomethane yield was analyzed by a two-way ANOVA with fungal pretreatment and harvest time as fixed effect. Degradation of giant reed dry matter, hemicellulose, cellulose and acid detergent lignin after 10, 20 and 30-day fungal pretreatment, the daily and cumulated biomethane of untreated and fungal pretreated giant reed after 30-day incubation were analyzed by the repeated measure ANOVA. Time represented the within-factor, while the fungal

pretreatment and harvest time the between-factor effect (SPSS, PASW Statistics 18). When data failed Mauchly's sphericity test for sphericity, the univariate results were adjusted by using the Greenhouse-Geisser Epsilon and the Huynh-Feldt Epsilon correction factors. When univariate results satisfied sphericity tests for within-subjects effects, the F-values and associated P-values for between-subjects effects were tested. Differences between means were evaluated for significance using the Tukey test at 95% confidence level.

3. RESULTS

3.1 Meteorological conditions

During the autumn season (September to September) annual average temperatures were 23.9°C for the maximum, 12.8°C for the minimum and 18.1 °C for the mean temperature (Figure 1). The winter season (February-February) was cooler, 23.4°C, 12.3°C and 17.6°C for the maximum, the minimum and the mean air temperature, respectively.

Rainfall were more abundant in the winter than in the autumn season, 776.4 and 674.6 mm, respectively. The reference evapotranspiration (ET0) was higher in the autumn season than in the winter (1505.2 and 1465.8 mm respectively), with an average of 4.12 and 4.02 mm day^{-1} respectively.

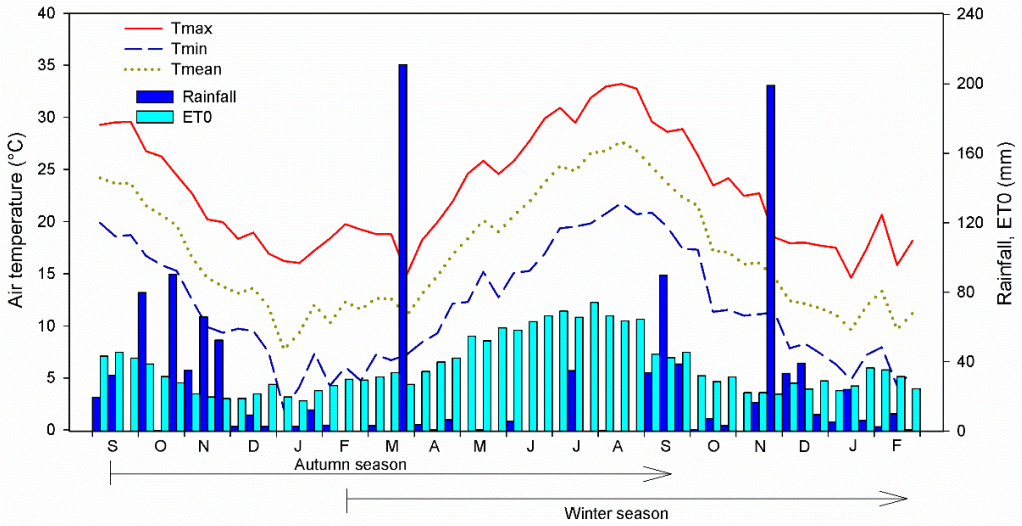

Figure 1. Meteorological trend (air temperature and rainfall) and reference evapotranspiration (ET0) at the autumn and winter growing season of giant reed (*Arundo donax*) grown at the Experimental farm of the University of Catania, Italy (10 m a.s.l., 37°25' N lat., 15°03' E long.).

3.2 Biomass composition and yield components

The analysis of variance (ANOVA) of the harvest time main effect showed significant differences for ADL and ash biomass components, while hemicellulose, cellulsose and NDS did not differ (Table 1). Total aboveground biomass yield (DMY) and yield components ((i.e., dry biomass composition yield) were significanly affected by harvest time, except the ADL yield.

The ADL content was 10.4% w/w in winter and 9.6% w/w in autumn harvest, while ash content was higher in autumn than winter (1.2 and 0.7% w/w, respectively). Although not significant, hemicellulose and NDS content were higher in autumn (29.1 and 24.2% w/w, respectively) than winter (29.0 and 23.9% w/w, respectively), while the cellulose content was 36.9 and 35.9% w/w in winter and autumn, respectively (Figure 2A).

The aboveground dry matter yield (DMY) was higher in autumn than winter, 11.64 Mg ha^{-1} and 10.38 Mg ha^{-1}, respectively (Figure 2B). The

autumn harvest produced higher yield components: cellulose represented the largest part of giant reed yield, reaching 4.2 Mg ha^{-1} in autumn and 3.8 Mg ha^{-1} in winter harvest, followed by hemicellulose (3.4 and 3.0 Mg ha^{-1} for autumn and winter, respectively), NDS (2.8 and 2.4 Mg ha^{-1} for autumn and winter, respectively), ADL (1.1 and 1.0 Mg ha^{-1} for autumn and winter, respectively) and ash (0.14 and 0.08 Mg ha^{-1} for autumn and winter, respectively).

Table 1. One-way ANOVA for main effect (harvest) on biomass dry matter yield (DMY), hemicellulose content and yield (H, % and Y) cellulose content and yield (C, % and Y), lignin content and yield (ADL, % and Y), neutral detergent soluble content and yield (NDS, % and Y), ash content and yield (ASH, % and Y). Degree of freedom (df) and adjusted mean square significance: P≤0.001 (***), P≤0.01 (**), P≤0.05 (*), Not significant (ns).

Source	df	DMY	H		C		ADL		NDS		ASH	
		-	%	Y	%	Y	%	Y	%	Y	%	Y
Reps	2	0.002^{ns}	0.285^{ns}	0.003^{ns}	0.174^{ns}	0.001^{ns}	0.002^{ns}	0.001^{ns}	0.936^{ns}	0.102^{ns}	0.001^{ns}	0.001^{ns}
Harvest	1	2.53***	0.108^{ns}	0.225***	1.653^{ns}	0.190*	0.777*	0.004^{ns}	2.151^{ns}	0.288*	0.360*	0.007**
Error	2	0.001	0.003	0.001	0.684	0.008	0.022	0.002	0.729	0.008	0.005	0.001

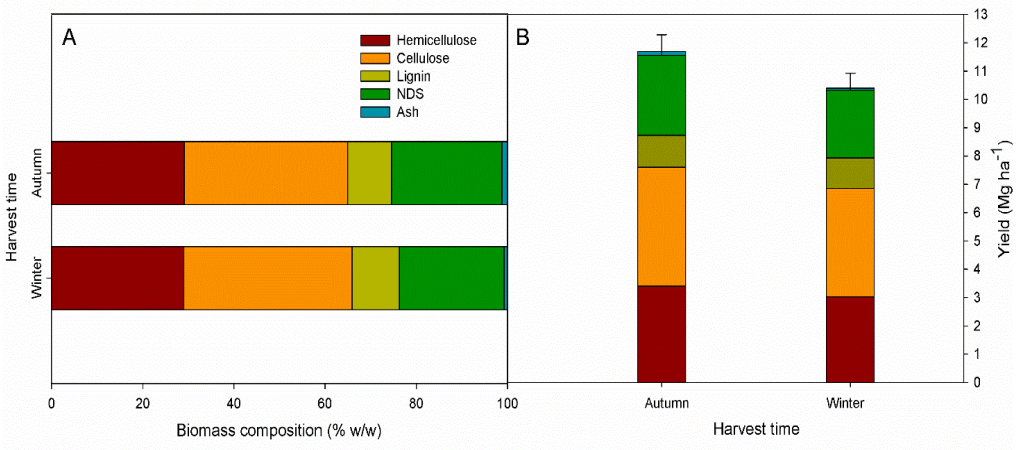

Figure 2. A) Biomass composition (% w/w) and B) aboveground dry matter yield and yield components (Mg ha^{-1}) of giant reed (*Arundo donax*) at the autumn and winter harvest regimes.

3.3 Pretreatment effects on lignocellulosic biomass

Losses of cellulose, hemicellulose, and lignin, with a consequent reduction of organic matter, can be used to evaluate the degradation pattern of different white-rot fungi. The ANOVA showed that biomass chemical composition was significantly modified by fungi growth (Table 2).

Table 2. Repeated measure ANOVA on degradation of giant reed dry matter (DM), hemicellulose (H), cellulose (C) and acid detergent lignin (ADL) during 10, 20 and 30-day fungal pretreatment (*I. lacteus* and *P. ostreatus*) in winter and autumn harvest. Degree of freedom (df) and adjusted mean square significance: P≤0.001 (***), P≤0.01 (**), P≤0.05 (*), Not significant (ns).

Source	df	DM	H	C	ADL
Reps	2	0.320^{ns}	0.154^{ns}	2.39^{ns}	0.52^{ns}
Time	2	420.59***	582.30***	354.51***	1298.45***
Pretreatment (P)	3	5.73^{ns}	0.646^{ns}	20.33***	43.98***
Harvest (H)	1	20.59***	29.53**	2.58*	54.05***
P x H	3	46.95***	9.25*	12.96***	0.40^{ns}
Error	24	2.86	2.87	0.62	3.23

The effect of pretreatment was significant on cellulose and lignin content, while harvest time on dry matter, hemicellulose, cellulose and lignin. Significant interactions “pretreatment × harvest time” were observed for dry matter, hemicellulose and cellulose. The degradation of dry matter, cellulose, hemicellulose and lignin in GRB increased for both *P. ostreatus* and *I. lacteus* treatment (Figure 3).

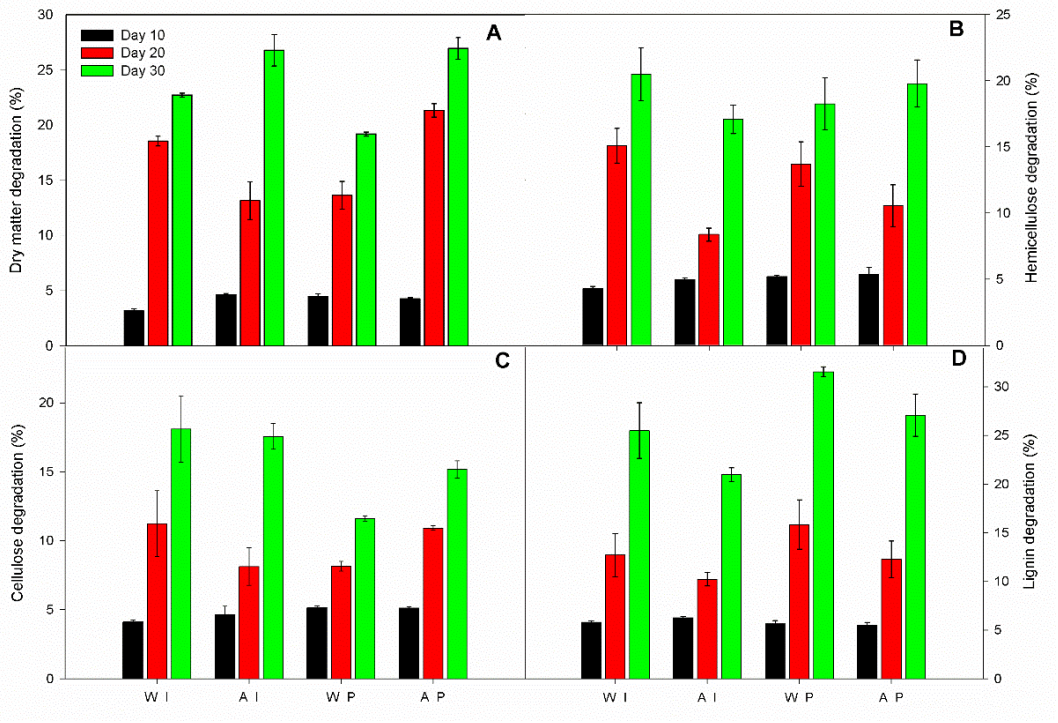

Figure 3. Degradation (%) of giant reed components: (A) dry matter, (B) hemicellulose, (C) cellulose and (D) lignin during 10, 20 and 30-day fungal pretreatment in winter and autumn *I. lacteus* pretreatment (WI and AI, respectively), and winter and autumn *P. ostreatus* pretreatment ((WP and AP, respectively). Significant interaction (LSDint P≤0.05) for: i) dry matter (2.76), hemicellulose (2.85), cellulose (1.33), ADL (3.03).

Degradation of dry matter and components during pretreatment showed an increasing trend with time for both fungi. High percentage of degradation of dry matter was observed for the biomass of autumn harvest for both *P. ostreatus* (26.9%) and *I. lacteus* (26.7%) treatment after 30 days of incubation (Figure 3A). For hemicellulose and cellulose, maximum degradation rates were observed for *I. lacteus* in the winter harvest with a loss of 20.5% and 18.1% respectively (Figure 3B-C). The highest value of lignin loss was obtained by *P. ostreatus* in both autumn (27.1%) and winter (31.5%) harvest time (Figure 3D).

Hemicellulose and lignin were degraded more than cellulose during fungal pretreatment, mainly with *P. ostreatus*. This is confirmed by selectivity value, defined as lignin degradation over cellulose loss. It is

important to evaluate the selective lignin-degrading capability of white rot fungi. The highest selectivity value of 2.7 with lignin degradation of 31.5% was reached from *P. ostreatus,* indicating that *P. ostreatus* selectively degraded hemicellulose and lignin over cellulose. The low degradation of cellulose has a positive impact on the anaerobic digestion process because cellulose is considered the main substrate for anaerobic microorganisms to produce biogas.

3.4 Methane production

The ANOVA showed that daily and cumulative biomethane production were significantly influenced by the incubation time, the pretreatment and by the harvest time. Significant interactions "pretreatment × harvest time" were also observed (Table 3).

Table 3. Repeated measure ANOVA on daily and cumulated biomethane (DCH4 and ∑CH4, respectively) of untreated and fungal pretreated giant reed in winter and autumn harvest after 30-day incubation. Degree of freedom (df) and adjusted mean square significance: P≤0.001 (***), P≤0.01 (**), P≤0.05 (*), Not significant (ns).

Source	**df**	**DCH_4**	**$\sum CH_4$**
Reps	2	4.44^{ns}	116.84^{ns}
Time	90	36.21***	7843.8***
Pretreatment (P)	2	85.45**	27093.3**
Harvest (H)	1	10.50*	5141.1**
P x H	3	13.80***	1444.9***
Error	460	0.68	19.9

Daily production (Nml g^{-1} VS d^{-1}) and cumulative methane production (NmL g^{-1} VS) during anaerobic digestion of untreated and fungal pretreated giant reed are displayed in Figure 4. The daily production curves for the pretreated samples showed the same trend for both

harvesting time for each fungal strain used (Figure 4A). The daily biomethane peaks (15.6 and 12.6 Nml g^{-1} VS d^{-1}) were highest in the biomass pretreated by *P. ostreatus* after 17 days of digestion for winter and autumn harvest, respectively. Winter giant reed pretreated by *I. lacteus* showed the maximum peak (6.2 Nml g^{-1} VS d^{-1}) after 18 days incubation, while the autumn one reached the peak of 6.1 Nml g^{-1} VS d^{-1} after 23 days.

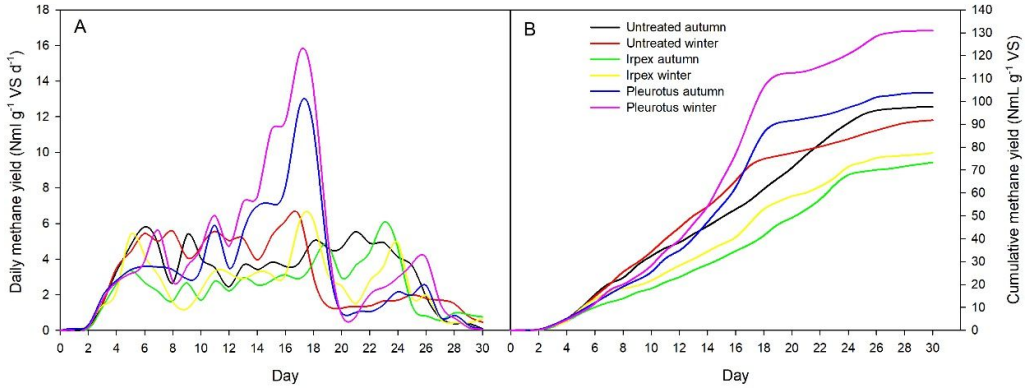

Figure 4. (A) Daily methane yield (Nml g^{-1} VS d^{-1}) and (B) cumulative methane yield (NmL g^{-1} VS) for giant reed biomass under harvest regimes (autumn and winter) and fungal pretreatments (*I. lacteus* and *P. ostreatus*). Significant interaction (LSDint P≤0.05) for: i) DCH_4 (1.34) and $\sum CH_4$ (7.22).

Cumulative biomethane production was observed for 30 days until biomethane yield reached a plateau at the end of exponential phase (Figure 4B). The initial lag phase lasted around three days until the complete adaptation of the bacterial flora to the lignocellulosic substrate. The methane yield obtained for the untreated giant reed biomass of autumn and winter harvest was 97.6 NmL g^{-1} VS and 91.8 NmL g^{-1} VS, respectively. *P. ostreatus* pretreated giant reed biomass achieved the highest BMP values, 130.9 Nml g^{-1} VS and 103.8 Nml g^{-1} VS for the winter and the autumn harvest, respectively, showing an improvement of the anaerobic digestion after fungal pretreatment. On

the contrary, the pretreatment using *I. lacteus* was uneffective and produced lower cumulative methane yield than the untreated giant reed, 77.4 Nml g^{-1} VS and 73.3 Nml g^{-1} VS for winter and autumn harvest, respectively. *I. lacteus* pretreatment resulted in a loss of both holocellulose and lignin, indicating that this strain was less selective than *P. ostreatus*.

3.5 Methane yields per hectare

The ANOVA revealed that pretreatment, harvest time and interaction were significant on biomethane yield (BMY) (Table 4).

Table 4. Two-way ANOVA on biomethane yield (BMY) of untreated and fungal pretreated giant reed in winter and autumn harvests. Degree of freedom (df) and adjusted mean square significance: P≤0.001 (***), P≤0.01 (**), P≤0.05 (*), Not significant (ns).

Source	df	BMY
Reps	2	147.34^{ns}
Pretreatment (P)	2	275782***
Harvest (H)	1	920**
P x H	2	40748***
Error	10	106

The BMY was greater for the autumn harvest than winter in the untreated biomass (1078.4 m^3 CH_4 ha^{-1} and 905.8 m^3 CH_4 ha^{-1}, respectively) as consequence of the higher biochemical methane potential and higher dry biomass yield of autumn biomass (Figure 5).
P. ostreatus pretreatment of winter harvest showed the highest biomethane yield per hectare (1284.5 m^3 CH_4 ha^{-1}), followed by autumn *P. ostreatus* pretreated biomass (1126.5 m^3 CH_4 ha^{-1}). Despite the lowest dry biomass yield of winter, the biomethane production per

hectare depended mostly on the higher BMP showed by winter biomass pretreated by *P. ostreatus*. *I. lacteus* pretreated biomass achieved the lowest values on biomethane yield, 791.9 m^3 CH_4 ha^{-1} and 761.4 m^3 CH_4 ha^{-1} for autumn and winter biomass, respectively.

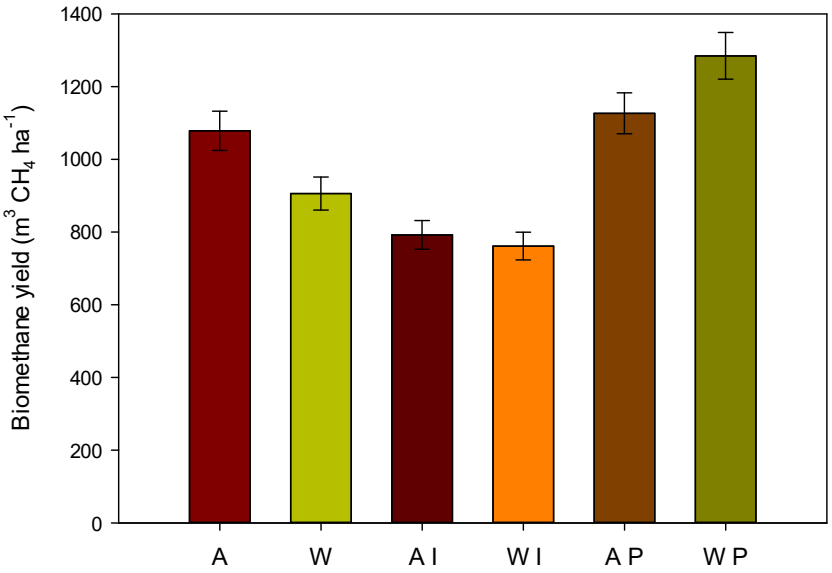

Figure 5. Biomethane production (m^3 CH_4 ha^{-1}) of untreated giant reed biomass (autumn - A and winter - W), autumn and winter pretreated with *I. lacteus* (AI and WI, respectively), autumn and winter pretreated with *P. ostreatus* (AP and WP, respectively). Significant interaction (LSDint $P \leq 0.05$) for BMY (18.73).

4. DISCUSSION

Giant reed showed higher dry biomass yield on autumn than winter (11.7 Mg DM ha^{-1} and 10.4 Mg DM ha^{-1}, respectively). This production was in line with data reported by previous long-term productivity trials on giant reed [14,17,23,24]. The higher yield in autumn harvest was linked to the higher leaves yield, which decreased during the winter period due to senescence and losses, while stems yield slightly increased (data not shown).

The composition analysis of biomass showed the differences on lignocellulosic matrix resulted by harvest time, with a greater content

of hemicellulose, NDS and ash, accompanied by a lower amount of lignin on autumn harvested biomass as compared with the winter one. Biomass yield and composition agree with values reported for this species by Scordia et al. 2020 and Zanetti et al. 2019 [25,26]. High concentrations of cellulose and hemicellulose confirm the wide interest for biochemical conversion and anaerobic digestion of giant reed for advanced biomethane production.

This study proved that untreated lignocellulosic biomass is highly recalcitrant to the anaerobic digestion, thus, a pretreatment step to enhance the bioconversion process is necessary. Several pretreatments carried out on giant reed biomass were performed using physical methods, chemicals (acids, bases or solvents) or severe conditions (high temperature and /or pressure). Di Girolamo et al. (2013) reported the effects of hydrothermal pretreatments performed at varying time, temperature and catalyst on giant reed resulting that hydrothermal pretreatments without acid catalyst contributed to increase methane yield of giant reed (average, +12%), whereas pretreatments with H_2SO_4 incurred a methanogenic inhibition [27]. A recent work studied the effects of a milling pretreatment on giant reed stems, reporting a methane yield of 89.5 $Nm^3\ t^{-1}$ VS for untreated giant reed stems, whereas the processed material reached a methane production of 212.8 $Nm^3\ t^{-1}$ VS [28].

Fungal pretreatment with *P. ostreatus* and *I. lacteus* employed here changed the structure and the composition of giant reed biomass; especially, *P. ostreatus* showed a better efficiency on selectively degrading lignin as compared with *I. lacteus*. Previous studies reported similar results for *P. ostreatus* pretreatment applied to corn stover and rice straw [29,30] and the effect of other white rot fungi used on

lignocellulosic biomass, such as rice straw, wheat straw and corn stover [31–33], but very few studies described the effect of fungal pretreatment on giant reed biomass [34,35].

P. ostreatus is the most studied white rot fungus for its ability to produce hydrolytic ligninolytic enzymes in different lignocellulosic biomass. Mustafa et al. [32] found out that *P. ostreatus* treatment of rice straw at 75% moisture content and 20 day incubation time led to a lignin degradation of 33.4%. Taniguchi et al. [36] reported P. ostreatus as the most efficient fungus to selectively degrade the lignin but not the holocellulose component comparing four different strains of white-rot fungi (*Trametes versicolor*, *Phanerochaete chrysosporium*, *P. ostreatus* and *Ceriporiopsis subvermispora*) to pretreat rice straw.

Previous studies reported good results in terms of selectivity also for *I. lacteus*. For example, Yu et al. [37] investigated the effect of fungal pretreatment with *I. lacteus* on sodium hydroxide pretreatment of corn stalks under mild reaction condition, reporting that *I. lacteus* showed selective lignin-degrading ability and significantly facilitated lignin degradation. However, as reported by Wan and Li (2012), fungal degradation rate varies with different feedstocks and fungal selectivity depends on the species and on the pretreatment time [38].

In our experiments *I. lacteus* showed a lower lignin degradation (21% and 25.5% for autumn and winter biomass, respectively) and a greater cellulose loss (17.6% and 18.1% for autumn and winter biomass, respectively) than *P. ostreatus*, resulting in a major consumption of holocellulose. The holocellulose losses during pretreatment, in particular of cellulose that mostly influences methane production, led to a reduced biomethane yield through anaerobic digestion. The *P. ostreatus* pretreatment showed promising results for anaerobic

digestion of giant reed, reaching a cumulative yield of 130.9 Nml g^{-1} VS for the winter harvest, whereas *I. lacteus* showed a decrease in methane yield as compared with the untreated control. The low methane yields obtained by *I. lacteus* were due to the negative effect of pretreatment on biochemical methane potential (and high component degradation).

Regarding the daily production trend, the main differences observed were due to fibrous composition of the biomass after pretreatment. The pretreatment step allowed to alter lignocellulosic structure removing lignin, ultimately to increase the accessible surface area. Thus, after the conversion of readily-available soluble fraction (i.e., NDS content), pretreated samples were more susceptible to an enzymatic attack resulting in a better digestibility of cellulose. This was observed on *P. ostreatus* that reached the highest biomethane yield thanks to the selective lignin consumption that led to a higher cellulose proportion in the digested biomass. On the contrary, *I. lacteus* showed a reduction of methane production due to the elevate content of cellulose degraded during pretreatment.

Regarding the harvest time, the untreated autumn biomass produced higher biomethane due to the higher proportion of leaves which contain a higher amount of soluble substances and a lower content of lignin compared to the winter harvest time. The winter harvested pretreated samples, on the contrary, showed an increased production compared to the autumn one due to the greater content of lignin degraded during pretreatment that led to a rise of soluble and cellulose fraction suitable for anaerobic digestion.

In accordance with other studies, *P. ostreatus* confirmed its capability to improve anaerobic digestion showing an outstanding degrading

lignin rate [30–32,36,39]. Regarding the performance of *I. lacteus*, results suggested that this strain did not improve the methane production rather caused a decrease of cumulative yield compared to the control. Similarly, other works reported *I. lacteus* as non-selective due to an extended consumption of polysaccharides over lignin [39].

The methane yield per hectare obtained by an old plantation (23 years) of giant reed was quite low if compared to that produced by the most used biomasses, like maize and sorghum. However, the present plantation has not received any agronomic input (irrigation, fertilization, weed or pest control) and only the harvest could account for energy expenses. Hence, cultivating perennial grasses, as giant reed, for anaerobic digestion can contribute to environmental benefits, economic returns and low risk of land use change (if grown in marginal lands) as compared with the most productive annual feedstock.

5. CONCLUSIONS

This study highlighted the potential of giant reed to produce satisfactory dry biomass yield and biomethane production even in the 23-year of cultivation in the semiarid Mediterranean environment.

The white-rot fungus *P. ostreatus* showed high values of lignin degradation in both autumn and winter harvest time and enhanced the methane yield of recalcitrant giant reed biomass during anaerobic digestion. By the contrast, the pretreatment using *I. lacteus* produced lower cumulative methane yield than the untreated giant reed due to the high percentage of holocellulose lost during the pretreatment.

The application of a biological pretreatment, using white-rot fungi, allows to improve the methane yield degrading lignin from lignocellulosic biomass through a safe and environmentally friendly

pretreatment without the requirement of high energy, chemicals or expensive instruments needed by the widely used pretreatment methods, such as physical and thermochemical processes.

However, further studies are necessary to ascertain the best harvest time window to accumulate the highest biomass dry matter yield and components, while preserving the plantation in the long-term. In addition, more work should still be done on the appropriateness of white-rot to identify those more suitable to a wide spectrum of lignocellulosic feedstock and optimize biological pretreatment conditions to reduce its duration.

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Oil production of diverse mediterranean Castor genotypes

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ABSTRACT: Castor (*Ricinus communis* L.) is a member of the Euphorbiaceae family that is found across all the tropical and semi-tropical regions of the world. Castor is considered to be one of the most promising nonedible oil crop, due to its high annual seed production and yield, and adaptability to semiarid climate and adverse growing conditions. Castor plant is an important renewable resource that has a high potential for use as a biorefining feedstock. Castor oil can be used for biodiesel production, while the main by-products generated in the castor oil production (capsule husks and meal) and the residual biomass, are potentially applicable as feedstocks for advanced ethanol and biogas.

Field experiments were conducted over the period 2019-2020 at the Experimental farm of the University of Catania to compare 28 genotypes of Castor (*Ricinus communis* L.) breed from native perennial plants in the Mediterranean basin in terms of seed and oil yield. The total seed yield ranged between 3022 and 1735 kg ha^{-1}. Oil content in castor seeds was on average 42.7 % and 46.1 % for the primary and secondary racemes, respectively. Oil yield reflected the changes in seed yield obtaining on average 1149 kg ha^{-1}.

KEYWORDS: *Ricinus communis* L., seed yield, oil yield, Mediterranean environment.

1. INTRODUCTION

Castor bean (*Ricinus communis* L.) is a non-edible multipurpose oilseed species cultivated worldwide from tropic to arid areas. it belongs to the Euphorbiaceae family, originated from Eastern Africa and most probably from Ethiopia, as in this country are found the higher number of wild and semi-cultivated types worldwide [1,2].

All parts of castor plant are toxic to humans and animals due to the presence of toxins as ricinine, a toxic alkaloid, and Ricinus communis agglutinin, while the seeds are toxic because of the presence of ricin, a highly poison ribosome-inactivating lectin [3]. Castor oil does not contain ricin because this protein is insoluble in oil, and any residual ricin is eliminated in the refining process but is retained in the meal [4].

Castor is an industrial crop cultivated for the oil found in its seed that ranges between 28% and 59% according to germplasms or accessions [5]. This inedible oil contains ricinoleic acid, which constitutes 79% to 92% of the fatty acid content in the seed-oil. Castor oil is considered a high-value oil for the agriculture, medicine, and cosmetic sectors because ricinoleic acid can be chemically transformed to obtain various commercial products of interest, such as lubricants, inks, paints, coatings, biopolymers, and biodiesel [2,6].

Moreover, it is considered as a second-generation raw material for the production of bioenergy or industrial purposes [7].

Seed yield and oil content of castor plants are dependent on many factors including genotype, environmental conditions, agronomic practices and harvesting practices [8].

Seed yield depends on the number of racemes per plant, the number of capsules per raceme and the thousand seed weight. Under natural conditions, the castor plant has many racemes, depending on the number of branches, that develop progressively over the life of the plant [9].

The number of capsules per raceme depends on the number of female flowers on the raceme. Castor plants are normally monoecius, with male flowers on the upper portion of the raceme and female on the lower. Flowers of both types can also be interspersed along the length of the raceme.

The proportion of male and female flowers on each raceme varies and can be influenced by the environment.

High temperatures, above 35 °C, and water stress during the flowering and oil formation can reduce the seed oil content [10].

Racemes appear in the apex of stems. The primary stem ends in a raceme after producing a given number of leaves. Lateral branches can potentially grow from any leaf axil. Secondary branches are those originated from the primary stem, and tertiary are those developed from a secondary branch.

A primary raceme is that developing in the primary stem, secondary racemes are in the apex of secondary branches, and so on. Developing new branches and racemes can continue indefinitely due to the indeterminate nature of castor. This classification is an indirect way to track the time that racemes developed. This approach assumes that primary racemes initiate and mature before secondary racemes, and tertiary racemes appear and mature later than the secondary ones. Differences found among racemes of increasing orders are likely to be associated with environmental and physiological conditions that changed along the growing season [11].

The present study compared 28 genotypes of castor (*Ricinus communis* L.) breed from native perennial plants collected across the semiarid Mediterranean basin.

2. MATERIAL AND METHODS

2.1 Field trial description

Field experiments were conducted over the period 2019-2020 at the Experimental farm of the University of Catania, Italy (10 m a.s.l., 37°25' N lat., 15° 03' E long.). in a typical xerofluvent soil. The seeds of 28 genotypes were collected from plants in a site of Gafsa, in southwest Tunisia.

The soil of the experimental area was ploughed before sowing and fertilized with 70 kg/ha N as ammonium nitrate and 60 kg/ha P_2O_5 as mineral perphosphate. Sowing was carried out in July 2019.

Castor seed were sown at 4 to 5 cm depth to 1 m intervals within row and 1.5 m apart (sowing density 0.66 m^2 plants). The plants were irrigated periodically until maturity according to the maximum available soil water content in a 0.6 m soil depth where root system is predominantly developed. Irrigation was scheduled when the sum of daily maximum crop evapotranspiration (ETm) corresponded to the volume, subtracting rainfall events from the calculation.

The experiment was arranged in a randomized block design with four replicates and genotypes were randomly distributed.

2.2 Measurements and calculations

During the growing season, for each genotype, the main phenological phases (seedling emergence, flowering, brown full capsule, seed physiological maturity) were monitored. The complete browning of

capsules was considered as stage of physiological maturity.

The first harvest was carried out in December 2019 on primary racemes, while fruits of racemes of higher orders were collected in the next harvests according to the different flowering time.

At the harvest the insertion height and the length of the first raceme were measured. Thereafter, the number of capsules per raceme was measured. The first raceme and the other racemes were separately collected for seed yield.

The seeds were separated from capsule residue to obtain clean seeds. A knife mill (GM200, Retsch) was used to crush seed into paste (cake) in preparation for oil extraction.

The oil content was determined according to Randall method by the use of a solvent extractor SER 148 Velp Scientific.

2.4 Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) with genotypes as main effect and according to the experimental layout. The mean separation was tested by the SNK test per $P \leq 0.05$.

In order to group the studied genotypes a hierarchical cluster analysis was performed using complete linkage method adopting a measure of dissimilarity based on the Euclidean distance metric and considering the length of sowing maturity period, the oil content (%) of both primary and secondary racemes, the seed yield of both the first and secondary racemes, the height of first raceme, the length of the first raceme (R software).

3. RESULTS AND DISCUSSIONS

Physiological maturity is reached on average after 147 days from sowing (Figure 1). The latest maturity genotype was #28 (160 days), while the earliest was the #13 (132 days).

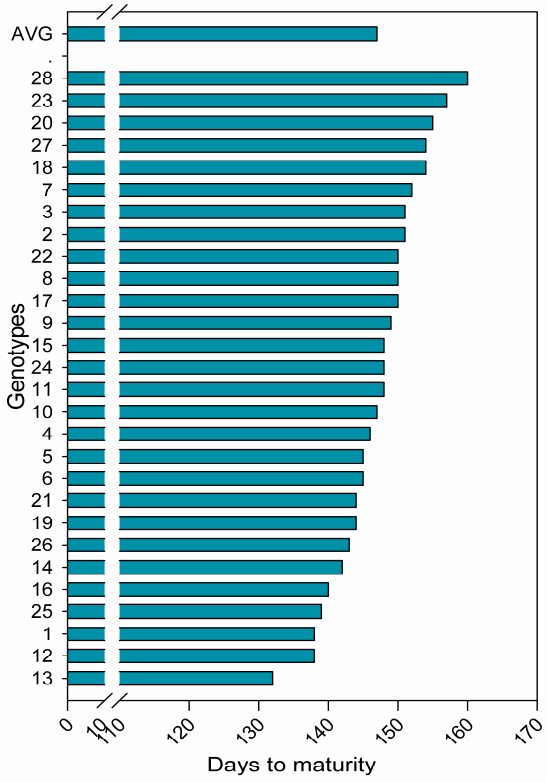

Figure 1. Phenological stage "sowing to physiological seed maturity" of 28 castor genotypes.

The cluster analysis based on complete linkage method divided genotypes into 6 groups (Figure 2). Group 1 had lower mean values for insertion height of primary raceme (cm), seed yield of primary and secondary racemes (kg ha^{-1}) and maturity date but had the largest value for seed oil content of primary raceme. Group 3 had the largest mean values for insertion height and length (cm) of primary raceme and seed yield of primary raceme (kg ha^{-1}). Group 4 had the largest mean values for seed yield of secondary racemes (kg ha^{-1}) but the lower for seed yield

of primary raceme (kg ha^{-1}). Group 6 had the largest mean values for seed yield of secondary racemes (kg ha^{-1}) (Table 1). This analysis, performed on yield components and the cycle length, allowed to define the genotypes to use for the next breeding program.

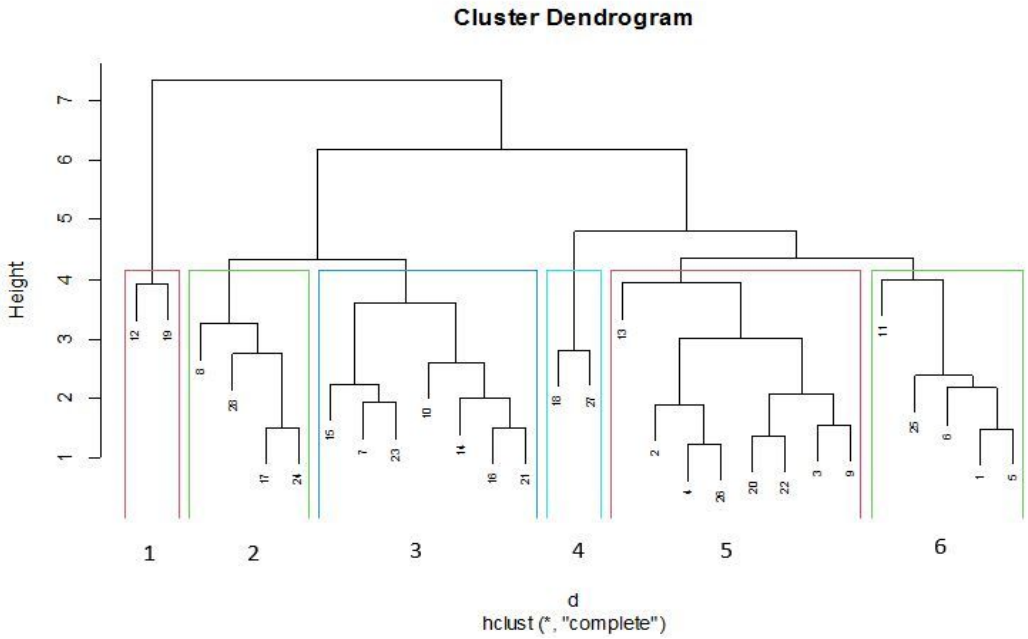

Figure 2: Graphical representation of the genetic divergence among the 28 genotypes of castor generated using complete linkage method adopting a measure of dissimilarity based on the Euclidean distance metric.

Table I: Mean of the descriptors for each of the 6 groups formed by analyzing the 28 of castor beans.

Group	Insertion height primary raceme (cm)	Length primary raceme (cm)	Maturity (days)	Seed yield primary raceme (kg ha^{-1})	Seed yield secondary racemes (kg ha^{-1})	Seed oil content primary raceme (%)	Seed oil content secondary racemes (%)
1	59.66	29.99	141	480.31	1551.03	45	43
2	70.54	31.69	152	550.49	1731.25	41	45
3	73.87	32.93	147	597.04	2087.18	43	47
4	65.97	27.97	154	419.62	2350.72	43	47
5	69.30	28.50	147	517.40	1915.60	42	47
6	70.78	31.99	143	554.93	2117.33	43	44

The seed yield was 542 and 1993 kg ha^{-1}, on average, for primary and secondary racemes, respectively (Figure 3). The total seed yield was mainly affected by yield of secondary racemes with a percentage that ranged between 68 and 85%, according to several studies the contribution of primary racemes to the total seed varies from 14 to 69% [9]. The total seed yield ranged between 3022 (genotype 27) and 1735 (genotype 12) kg ha^{-1}, which were statistically different.

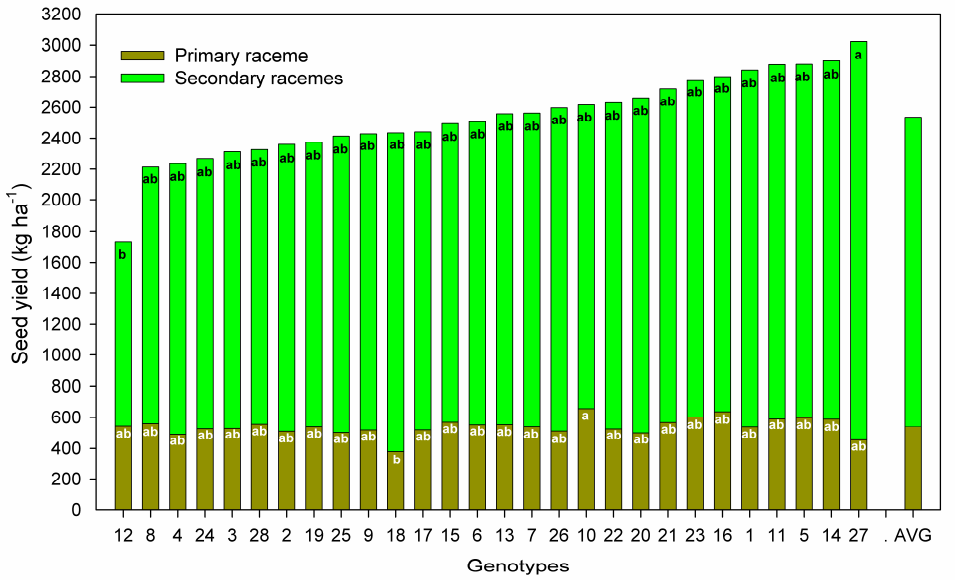

Figure 3. Seed yield of 28 castor genotypes.

Seed characteristics are not determined by the raceme order, but by an interaction of environmental conditions and genetic factors.

The percentage of oil content in castor seeds ranged between 40.4 (genotype 28) and 46.1% (genotype 12), with an average of 42.7% for the primary raceme and between 43.2 (genotype 12) and 48.1% (genotype 10), with an average of 46.1% for the secondary racemes (Figure 4).

In accordance with study of Souza et al. [12] a lower seed oil content was found in the primary than in the secondary racemes.

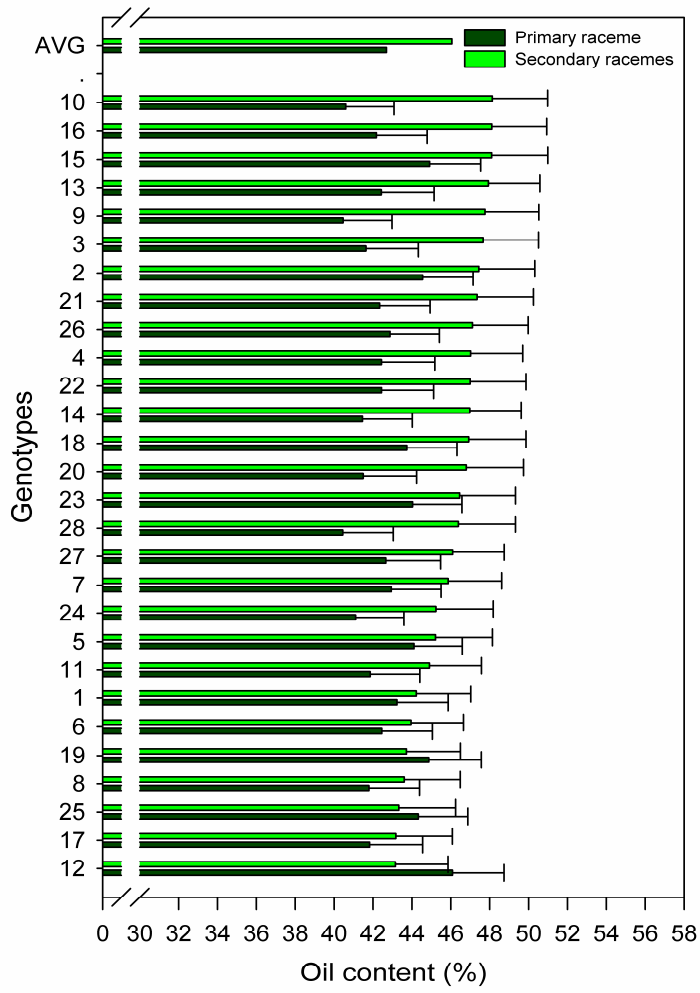

Figure 4: Seed oil content (%) of 28 castor genotypes

The main contribution to the total oil yield is given by the seed yield as reported by Koutroubas et al. [13].

Oil yield varied from 166 (genotype 18) to 270 (genotype 16) kg ha $^{-1}$ with an average of 231 kg ha $^{-1}$ for the primary raceme. The oil yield was from 518 (genotype 12) to 1206 (genotype 27) kg ha $^{-1}$ with an average of 918 kg ha $^{-1}$ for the secondary racemes (Figure 5).

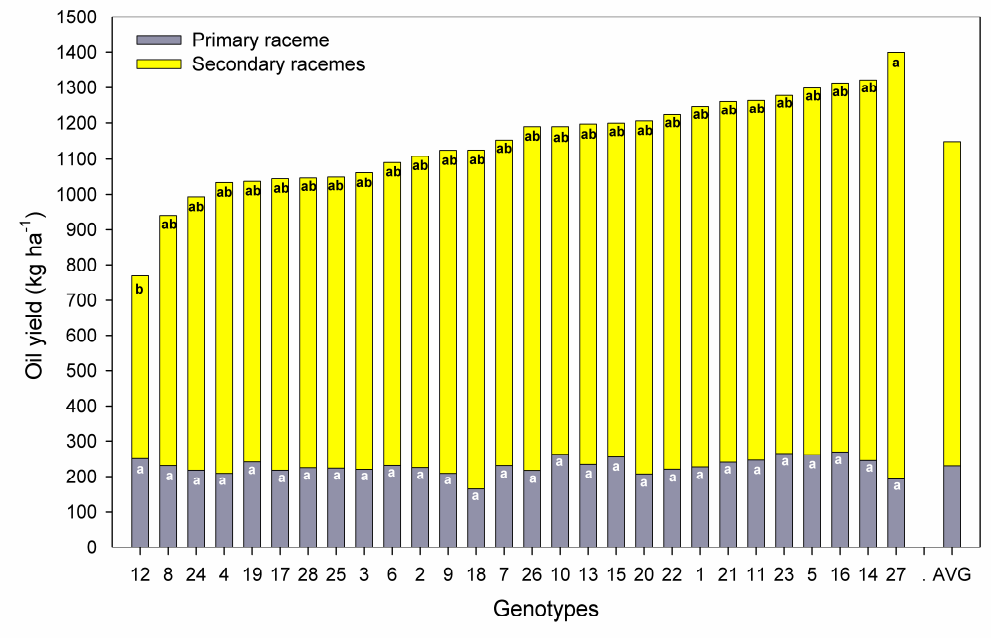

Figure 5: Oil yield (kg ha^{-1}) of 28 castor genotypes.

4. CONCLUSIONS

These results suggest that investigated genotypes are suitable to the Mediterranean climate as reported by preliminary field experiments carried out in southern Sicily and in Tunisia that shown the possibility of exploiting the perennial habit of this species [14,15,16]. A great variability exists among tested genotypes, which were collected from wild semiarid environment of the Mediterranean basis. Besides seed yield and oil content, this preliminary study allows to select other traits, such as physiological, morphological and phenological, which are currently under study, to be selected for breeding improved castor lines suitable to drought prone environment of southern Europe.

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Biologically pretreated Castor capsule husks for advanced biomethane production

ABSTRACT: Castor (*Ricinus communis* L.) is a member of the Euphorbiaceae family that is found across all the tropical and semi-tropical regions of the world. It is an important oilseed crop and its inedible oil is widely used in industrial, pharmaceutical and agricultural sectors.

Castor plants show a high potential to be converted and used for biofuels production. Castor oil can be used for biodiesel production, while the main by-products generated in the castor oil production (capsule husks and castor oil cake) and the residual biomass, are potentially applicable as feedstocks for advanced ethanol and biomethane production. The production of biomethane through anaerobic digestion using agricultural waste has been widely adopted as innovative, sustainable and cost-effective technology.

The aim of this work was to evaluating the experimental biomethane potential obtained from capsule husks. Similarly, to others lignocellulosic materials, to use these biomass residues in bioenergy production process is necessary a pretreatment to expose their compact structure to enzymatic hydrolysis, thus a biological pretreatment using white-rot fungi (*Pleurotus ostreatus* and *Irpex lacteus*) was performed.

KEYWORDS: *Ricinus communis* L., anaerobic digestion, biofuel, white rot fungi, fungal pretreatment.

1. INTRODUCTION

Castor plant is very tolerant to different weather conditions and is able to grow in marginal soils. Castor is primarily used to extract oil from its seed, which is mainly used for pharmaceutical and industrial

applications. It is composed by the following fatty acids: 91–95% ricinoleic acid, 4–5% linoleic acid and 1–2% palmitic and stearic acids [1].

Because of the present mainly of ricinoleic acid, castor oil is provided by attractive properties, such as high miscibility, low iodine content, low freezing point, which make it an excellent raw material for producing biodiesel.

This plant, containing seeds and lignocellulosic residues, has a promising potential for biofuel production. The oil extracted from the seeds can be efficiently converted to biodiesel, while the lignocellulosic parts are suitable for ethanol and biomethane production [2], [3].

Two main by-products from castor oil production process are generated: capsule husks and oil-cake. Capsule husks are obtained from separation of seeds from the fruits, while the cake is produced when the oil is extracted from the seed by pressing.

Castor cake presents a high N content, but due to the presence of toxic ricin, the use of cake as animal feed is not possible. In the husks, ricin residue is usually found in the form of seed pieces. The husks could be utilized as a high-fiber, low-nitrogen animal feed [4].

A process of detoxification is necessary, however there is no efficient and low-cost methods. While a feed option is not feasible, most of the non-edible oil cakes generated worldwide, included castor cake, are used mainly as organic fertilizers, due to their N, P and K contents.

Castor oil is currently used for biodiesel production by transesterification, while the whole biomass including stems and leaves, and agro-waste of castor oil extraction, such as capsule husks and oil-cake, are potentially applicable as feedstock for ethanol and biogas production for valorizing residual biomass [5].

This work evaluated the experimental biomethane potential obtained from capsule husks. Similarly, to others lignocellulosic materials, to use these biomass residues in bioenergy production process is necessary a pretreatment to expose their compact structure to enzymatic hydrolysis, thus a biological pretreatment using white-rot fungi was performed.

2. MATERIALS AND METHODS

2.1 Field experiment

Capsule husks were obtained from a field experiment conducted over the period 2020-2021 at the Experimental farm of the University of Catania, Italy (10 m a.s.l., 37°25' N lat., 15° 03' E long) in a typical xerofluvent soil.

Briefly, sowing was carried out in June 2020 at a sowing density of 0.58 plants m^{-2}. Soil was ploughed before sowing and fertilized with 70 kg/ha N.

Two experimental factors were be studied: irrigation and fertilization. Irrigation was applied as main plot and fertilization as sub plot: were be studied four irrigation levels: irrigation only at sowing and restitution of the 30, 60, 100% of the evapotranspiration and three fertilization levels: 0, 60 and 120 kg ha^{-1} of N (as ammonium sulphate). Further details are reported in Calcagno et al. 2021[6].

The mature racemes of different orders were harvested according to the different flowering period. After the harvest the seeds were separated from capsule residue to calculate seed yield and capsule husks yield.

In order to evaluate the effect of nitrogen fertilization on the chemical composition and biomethane potential of the capsule husks, in this laboratory experiment we analyse the optimal irrigation level (I100) and the low and high nitrogen input (NO and N120).

2.2 Characterization of feedstock

Capsule husks were oven-dried at 65 °C, milled and used for chemical analysis and pretreatment.

The total solids (TS), volatile solids (VS), and chemical composition of capsule were determined before pretreatment. TS correspond to the residue after 24 hours drying period at 105 °C. It is expressed in percent of the sample initial weight. Dry residue is, then, burnt 5 h at 550 °C. VS are the combusted organic matter (expressed in percent of TS) whereas residue after ignition is the mineral matter (ash). TS and VS measurements were realized in triplicate.

The total fiber composition was determined as neutral detergent soluble (NDS), neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) according to Van Soest method [7] through a Fiber Analyzers (Fibertec Velp Scientifica, model FIWE). The hemicellulose and cellulose contents were calculated as the difference between the NDF and ADF, and the ADF and ADL, respectively. To determine the ashes, ADL residue was ignited in muffle furnace at 550 °C for 5 hours and lignin content was calculated as the difference between ADL and ash.

2.3 Inoculum preparation

The fungal strains used in this study (*Pleurotus ostreatus* (MUT00002977) and *Irpex lacteus* (MUT00005918)) were purchased from Mycotheca Universitatis Taurinensis (MUT) of the Department of Life Sciences and Systems Biology, University of Turin (Italy).

Fungi were activated on Malt Extract Agar plates and incubated at 26 °C for 7 days. Sterile capsule husks colonized with *Pleurotus ostreatus*

and *Irpex lacteus* were used as inoculum for the fungal pretreatment experiments.

To prepare the inoculum, 30 g (dry basis) of capsule husks were placed in 0.5 L reactors, and then deionized water was added to reach 70% moisture content.

Reactors were autoclaved at 121 °C for 20 minutes, after that cooled down to room temperature. Four agar disc of 7-day-old mycelia (approximately 1 cm in diameter) were aseptically added to sterilized capsule husks and incubated at 26 °C until full colonization.

At the end of colonization, which occurred 4 weeks after the start of incubation, fungal- colonized capsules were thoroughly mixed and used as inoculums for the successive fungal pretreatment of husks.

2.4 Fungal Pretreatments

Sterile capsule husks and inoculum (fungal-colonized capsules) were mixed and added to 0.5 L reactors. Fungal pretreatments were performed at 30% (dry weight basis) inoculum ratio. Deionized water was added to reach 70% moisture content. Reactors were covered with cotton plugs and incubated at 26 °C for 30 days. Sterile capsules husks were considered as a negative control.

Sampling were conducted at days 10, 20 and 30. Fungal-treated samples from each sampling time were subjected to composition analysis.

For each sampling and at the end of the pretreatment, samples were taken out of the reactors, thoroughly mixed and dried at 65°C in a ventilated oven for 24 h before cellulose, hemicellulose and lignin content determination.

The dry matter loss and degradation of cellulose, hemicellulose, and lignin during the pretreatment were expressed as percentage of the initial dry weight and fiber fractions before fungal pretreatment.

2.5 Biochemical Methane Potential (BMP) tests

The BMP test was performed by an automatic methanogenic potential detection system (AMPTS II, Automatic Methane Potential Test System, Bioprocess Control AB, Sweden). The AMPTS II is a standardized laboratory set-up specially designed for automatic BMP determination of any biodegradable material. It consists of 15 parallel reactors and the same number of gas flow meters (flow cells) attached to a detection unit for online, automatic data acquisition.

The experiment was conducted in reactors of 500 mL each, in which substrates and inoculum were mixed at a ratio of 1:3 in terms of grams of VS at mesophilic conditions (38±1°C) with continuously mixing. All tests were performed in triplicate.

TS and VS were determined both for the organic substrate and the inoculum as reported above.

Each reactor was connected to a 80 mL trap bottle of 3 M sodium hydroxide solution used for absorbing CO_2 from the raw gas. The remaining gas after scrubbing passed to ultra-low gas flow meters which were connected to the data analytical and acquisition system. The BMP test was run for 30 days.

Additionally, blank samples, only containing inoculum, were incubated as well. The resulting methane production of the substrate was determined by subtracting methane production of the blank (inoculum) from the substrate sample (substrate + inoculum). The final value of

cumulative methane production at the end of the test was defined as the experimental BMP of the substrate.

2.6 Biomethane Potential per hectare

The biomethane yields of capsule husks per hectare of castor cultivation (m^3 CH_4 ha^{-1}) was calculated as the product of biomethane potential and dry biomass yield expressed in volatile solid (gVS ha^{-1}).

2.7 Statistical Analysis

Data were analyzed according to the randomized block design. Before conducting the ANOVA, the Bartlett's test was run to verify the assumption of homogeneity of variances. Biomass content of the hemicellulose, cellulose, ADL, ash and NDS, were analyzed by one-way ANOVA with fertilization as fixed effect. The biomethane yield was analyzed by a two-way ANOVA with fungal pretreatment and fertilization as fixed effect. Degradation of capsule husks dry matter, hemicellulose, cellulose and acid detergent lignin after 10, 20 and 30-day fungal pretreatment, the daily and cumulated biomethane of untreated and fungal pretreated capsule husks after 30-day incubation were analyzed by the repeated measure ANOVA. Time represented the within-factor, while the fungal pretreatment and fertilization the between-factor effect (Software RStudio, Boston, USA).

3. RESULTS

3.1 Biomass composition

The analysis of variance (ANOVA) of nitrogen fertilization effect as main effect did not show significant differences for biomass composition (Table 1).

Table 1. One-way ANOVA for main effect (fertilization) on hemicellulose content (H), cellulose content (C), lignin content (ADL), neutral detergent soluble content (NDS), ash content (ASH). Degree of freedom (df) and adjusted mean square significance: P≤0.001 (***), P≤0.01 (**), P≤0.05 (*), Not significant (ns).

Source	df	H	C	ADL	NDS	ASH
		%	%	%	%	%
Reps	1	$1.0*10^{-7}$ ns	$1.2*10^{-4}$ ns	$4.4*10^{-5}$ ns	$1*10^{-5}$ ns	$9.6*10^{-7}$ ns
Fertilization	1	$6.0*10^{-4}$ ns	$3.8*10^{-3}$ ns	$6.2*10^{-5}$ ns	$9.5*10^{-3}$ ns	$8.0*10^{-6}$ ns
Error	1	$3.2*10^{-4}$ ns	$5.5*10^{-5}$ ns	$1.4*10^{-5}$ ns	$8.2*10^{-4}$ ns	$2.0*10^{-7}$ ns

Although not significant, NDS content was higher in the unfertilized (43.9% w/w) as compared with fertilized treatment (34.1% w/w), while the content of hemicellulose and cellulose were higher in the fertilized residue (20.9 % and 32.2 % w/w, respectively) than unfertilized one (18.5% and 25.9% w/w, respectively). The fertilized residue had a greater content of lignin (11.1% w/w) and ash (1.6% w/w) than the unfertilized (10.4% and 1.3% w/w respectively) (Figure 1).

The ratio of structural carbohydrates (hemicellulose and cellulose) over lignin was determined in addition to the analysis of the various fractions of lignocellulose residue. This measurement may be used to estimate the digestibility of the substrate being tested. For fertilized residue, the highest ratio was recorded (4.8).

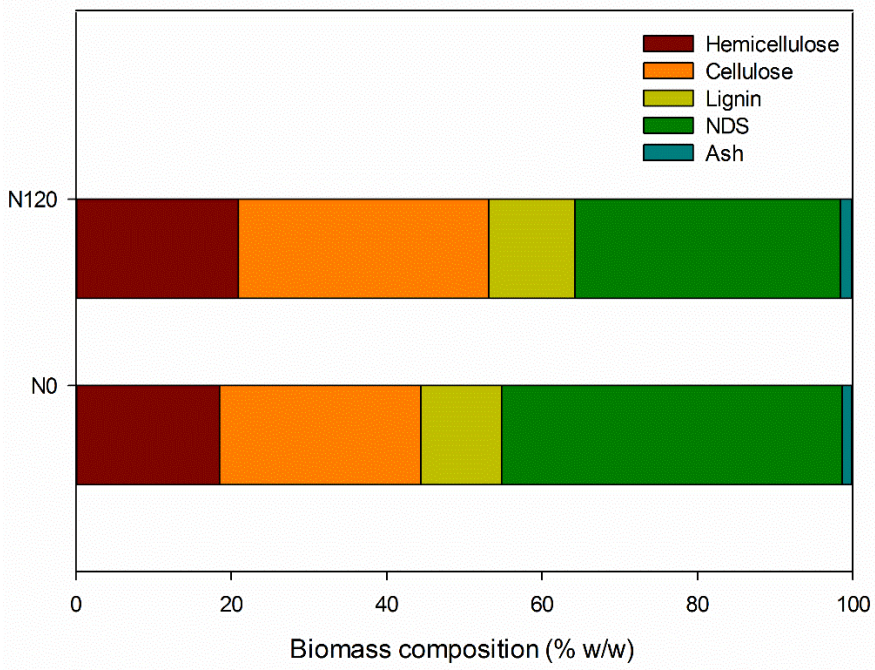

Figure 1: Biomass composition (% w/w) of capsule husks under nitrogen fertilization levels (N0 - 0 kg N ha^{-1} and N120 - 120 kg N ha^{-1}).

3.2 Pretreatment effects on lignocellulosic biomass

Biomass composition was modified by fungi growth with a consequent reduction of organic matter. Losses of cellulose, hemicellulose, and lignin can be used to evaluate the degradation pattern of different white-rot fungi.

The ANOVA showed that biomass chemical composition was significantly modified by fungi growth (Table 2).

Table 2. Repeated measure ANOVA on degradation of capsule husks dry matter (DM), hemicellulose (H), cellulose (C) and acid detergent lignin (ADL) during 10, 20 and 30-day fungal pretreatment (*I. lacteus* and *P. ostreatus*) under fertilization levels. Degree of freedom (df) and adjusted mean square significance: P≤0.001 (***), P≤0.01 (**), P≤0.05 (*), Not significant (ns).

Source	df	DM	H	C	ADL
Reps	1	0.000041^{ns}	0.000056^{ns}	0.000001^{ns}	0.000007^{ns}
Time	2	0.003218***	0.007606***	0.005114***	0.017527***
Pretreatment (P)	1	0.001216***	0.000415^{ns}	0.000158^{ns}	0.000095^{ns}
Fertilization (F)	1	0.000253*	0.000006^{ns}	0.000189^{ns}	0.000021^{ns}
P x F	1	0.000003 ^{ns}	0.000937**	0.000371^{ns}	0.000507**
Error	17	0.000044	0.000109	0.000093	0.000059

The effect of pretreatment and of nitrogen fertilization was significant on dry matter loss. Significant interactions ″pretreatment×fertilization” were observed for hemicellulose and lignin.

The degradation of dry matter, cellulose, hemicellulose and lignin of capsules residue showed an increasing trend with time for both fungi (Fig. 2). High percentage of degradation of dry matter was observed for *I. lacteus* for both unfertilized (20%) and fertilized (19%) substrates after 30 days of incubation. The percentages of degradation of dry matter for *P. ostreatus* were of 14.5 % and 13.8 % for N0 and N120 treatments (Fig. 2A).

For hemicellulose degradation, the highest loss was observed for *P. ostreatus* in both unfertilized and fertilized treatment (14.7% and 14.6%, respectively) (Fig. 2B). By contrast, for cellulose, highest degradation was observed for *I. lacteus* with a loss of 15.2% and 14.6% in unfertilized and fertilized treatment, respectively (Fig. 2C).

The highest value of lignin loss was obtained by *P. ostreatus* in both unfertilized (21.4%) and fertilized (20.8%) samples (Fig. 2D).

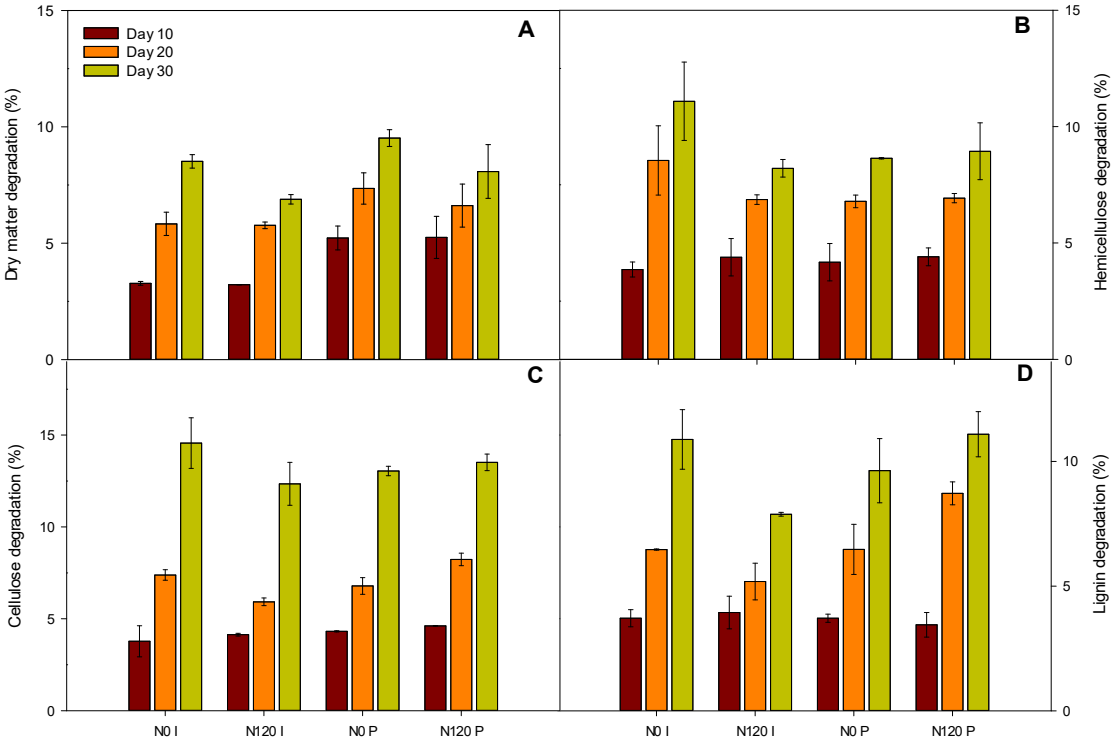

Figure 2. Degradation (%) of capsule husks components: (A) dry matter, (B) hemicellulose, (C) cellulose and (D) lignin during 10, 20 and 30-day fungal pretreatment in unfertilized and fertilized biomass (N0 - 0 kg N ha^{-1} and N120 - 120 kg N ha^{-1}) pretreated by *I. lacteus* (N0I and N120I, respectively) and *P. ostreatus* (N0P and N120P, respectively).

3.3 Methane production

The ANOVA showed that daily biomethane production was significantly influenced by incubation time and pretreatment. Cumulative biomethane production was significantly influenced by incubation time, pretreatment and fertilization. Significant interactions "pretreatment × fertilization" were also observed on cumulative biomethane production (Table 3).

Table 3. Repeated measure ANOVA on daily and cumulated biomethane (DCH_4 and $\sum CH_4$, respectively) of untreated and fungal pretreated capsule husks after 30-day incubation under two levels of nitrogen fertilization. Degree of freedom (df) and adjusted mean square significance: P≤0.001 (***), P≤0.01 (**), P≤0.05 (*), Not significant (ns).

Source	df	DCH_4	$\sum CH_4$
Reps	1	0.0004454*	0.0871***
Time	27	0.0014885***	0.4962***
Pretreatment (P)	2	0.0009394***	0.1501***
Fertilization (F)	1	0.0003646^{ns}	0.1395***
P x F	3	0.0001529^{ns}	0.0587***
Error	320	0.0001131	0.0025

Daily production (Nml g^{-1} VS d^{-1}) and cumulative methane production (NmL g^{-1} VS) during anaerobic digestion of untreated and fungal pretreated capsule husks are displayed in Figure 3.

The daily biomethane production showed the highest peaks for untreated capsules N0 and N120 (6.1 and 6.7 Nml g^{-1} VS d^{-1}, respectively) after 19 days of digestion (Figure 3A).

Capsules pretreated by *I. lacteus* showed the maximum peak (5.6 Nml g^{-1} VS d^{-1}) on the 17th day for both fertilization levels (N0 and N120), while *P. ostreatus* pretreated biomass showed the peaks of daily methane production lower than the others thesis (4.5 and 4.1 Nml g^{-1} VS d^{-1} for N0 and N120, respectively) reached after 17th and 13th days respectively.

Cumulative bioethane production was observed for 30 days until biomethane yield reached a plateau at the end of exponential phase (Figure 3B). The initial lag phase lasted around three days until the

complete adaptation of the bacterial flora to the lignocellulosic substrate.

The methane production obtained for the untreated capsules husks N0 and N120 was 62.4 NmL g^{-1} VS and 75.8 NmL g^{-1} VS, respectively.

P. ostreatus pretreated capsules achieved values of 52.6 Nml g^{-1} VS and 54.4 Nml g^{-1} VS for N0 and N120 fertilization respectively.

The methane yield reached by *I. lacteus* pretreated capsule husks was 56.3 Nml g^{-1} VS and 58.7 Nml g^{-1} VS for for N0 and N120 fertilization levels, respectively.

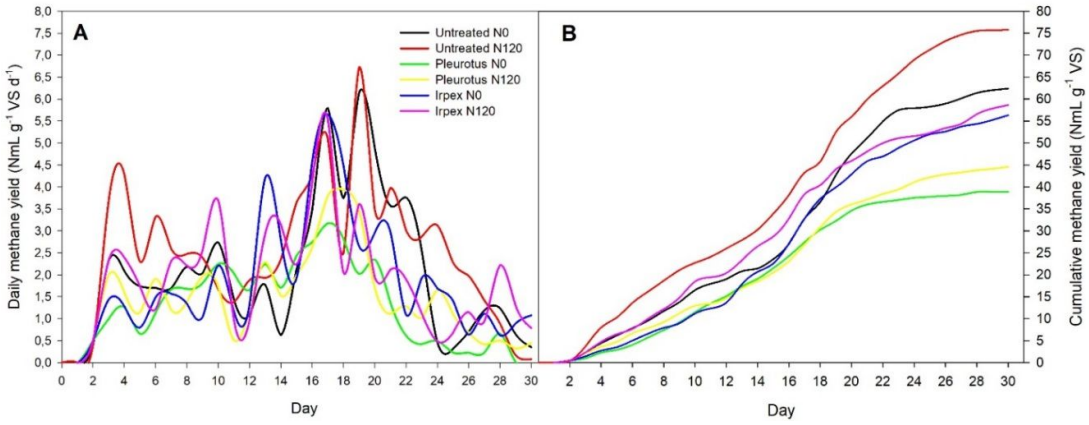

Figure 3: (A) Daily methane yield (Nml g^{-1} VS d^{-1}) and (B) cumulative methane yield (NmL g^{-1} VS) for capsule husks under fertilized regimes (N0 and N120) and fungal pretreatments (*I. lacteus* and *P. ostreatus*).

3.4 Methane yields per hectare

The ANOVA revealed that pretreatment were significant on biomethane yield (BMY) (Table 4).

Table 4. Two-way ANOVA on biomethane yield (BMY) of untreated and fungal pretreated capsule husks under two levels of nitrogen fertilization. Degree of freedom (df) and adjusted mean square significance: P≤0.001 (***), P≤0.01 (**), P≤0.05 (*), Not significant (ns).

Source	df	BMY
Reps	1	0.08033^{ns}
Pretreatment (P)	2	0.18601*
Fertilization (F)	1	0.06742^{ns}
P x F	2	0.01839^{ns}
Error	5	0.03048

The capsule yield, as biomass residue after the shelling of the seeds, obtained during the first year of cultivation was used to estimate the potential residue of a castor cultivation in Mediterranean environment. This value was equal to 3.0 Mg ha^{-1} and 2.5 Mg ha^{-1} for N0 and N120, respectively.

The biomethane yield was greater for both untreated N0 and N120 biomass than fungal pretreated (162.9 m^3 CH_4 ha^{-1} and 163.4 m^3 CH_4 ha^{-1}, respectively) as consequence of the highest biochemical methane potential (Figure 4).

Among the fungal pretreated thesis, *I. lacteus* showed the highest biomethane yield per hectare (150.3 m^3 CH_4 ha^{-1} and 123.3 m^3 CH_4 ha^{-1} for N0 and N120, respectively) while *P. ostreatus* achieved values of 130.5 m^3 CH_4 ha^{-1} for N0 and 110 m^3 CH_4 ha^{-1} for N120.

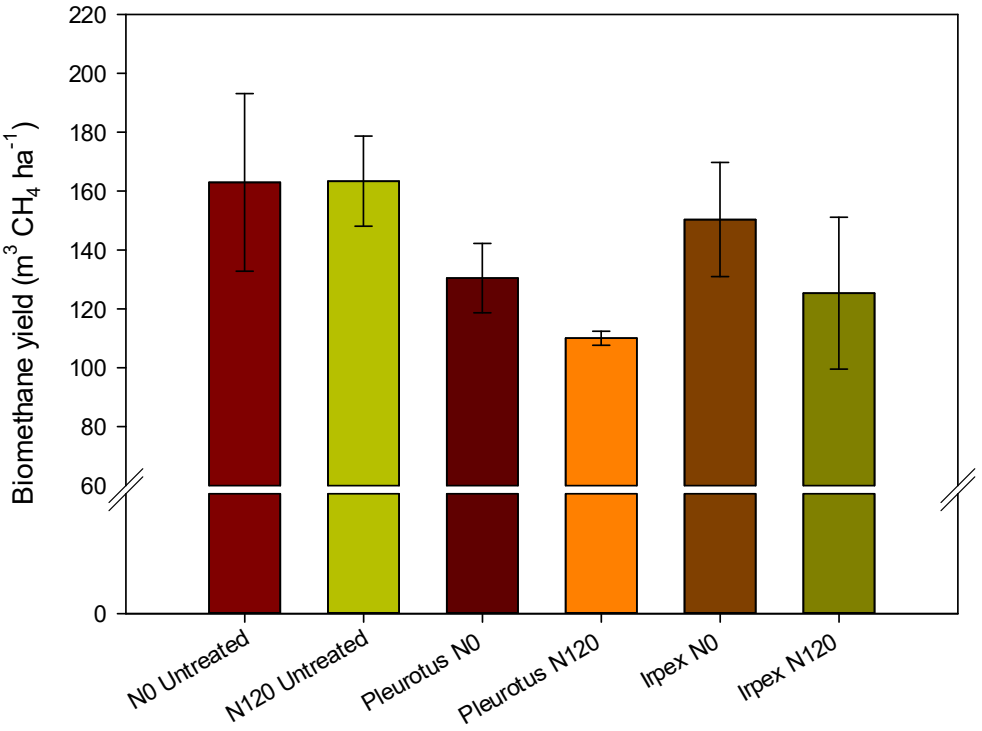

Figure 4: Biomethane production (m^3 CH_4 ha^{-1}) of untreated capsule husks under fertilized regimes (N0 and N120) and fungal pretreatments (*I. lacteus* and *P. ostreatus*).

4. DISCUSSION

The composition analysis of capsule husks showed the differences on lignocellulosic substrate resulted by the different fertilization levels, with a greater content of hemicellulose and cellulose on fertilized biomass as compared with the unfertilized.

The ratio of structural carbohydrates (hemicellulose and cellulose) over lignin was used as indicator to estimate the digestibility of the substrate on anaerobic digestion process.

The highest ratio was reported by the fertilized residue suggested that nitrogen fertilization had a positive effect on lignocellulose susceptibility to anaerobic digestion [8].

The significant amount of carbohydrates presents on capsules residue confirm the potential of this substrate to be used on biochemical conversion and anaerobic digestion for advanced biomethane production. However, their recalcitrant nature due to the present of lignin, is an obstacle in their direct conversion. Thus, a pretreatment is a necessary process to reduce the recalcitrance of the materials and remove lignin, reducing cellulose crystallinity and increasing accessible surface area. Among pretreatments, the biological pretreatment could play key role in order to reduce the use of chemicals and energy inputs. As reported by Zheng et al. (2014), Noonari et al. (2020), van Kuijk et al. (2016) and Wan et al. (2010), the fungal pretreatment have as positive effects on degradation of lignocellulosic biomass [9]–[12].

P. ostreatus showed the highest hemicellulose degradation (14.7% and 14.6% for N0 and N120, respectively) and lignin loss (21.4% and 20.8% for N0 and N120, respectively) in both fertilization levels.

By contrast, *I. lacteus* reported the highest cellulose degradation, 15.2% and 14.6% in unfertilized and fertilized treatment, respectively.

Regarding the fungal strain to use on pretreatment process, previous studies investigated the selective lignin-degrading capability of *P. ostreatus* and *I. lacteus* and reported positive results [13]–[15].

However, in our experiment, the pretreatment using *P. ostreatus* and *I. lacteus* had a negative effect on biomass tested and on biomethane production producing lower cumulative methane yield than the untreated biomass. The efficiency of fungal pretreatment depends on biomass and on adopted process conditions (fungal strain, time, temperatures) [16], [17].

Limited studies have been conducted on potential biomethane production from castor plant and castor seed cake but there is no research on biomethane production from capsule husks.

Bateni et al. (2014) evaluated the effect of alkaline pretreatment at different temperatures and time to improve biomethane yield from castor stem, leaves and castor seed cake. Pretreatment increased the biomethane production of untreated castor stem from 80.8 to 145.5 mL/g VS. In contrast, alkaline pretreatment had a negative effect on the biomethane production from both leaves and castor seed cake [5].

The pretreated capsule husks showed a decrease of biomethane production due to the higher consume of structural polysaccharides, such as hemicellulose and cellulose during the pretreatment. The biomethane yield per hectare of *P. ostreatus* and *I. lacteus* pretreated biomass depended mostly on the higher dry biomass yield of unfertilized biomass, reflecting the difference on capsule yields between the two levels of fertilization (N0 and N120), despite the highest BMP showed by N120 fertilized.

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