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Arundo donax L. (giant cane) as a feedstock for bioenergy and green chemistry

Ph.D. Thesis

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Abstract

Arundo donax L. or giant cane is a second-generation energy crop that could be used as a feedstock for bioenergy, i.e. biogas, bioethanol, combustion, and in green chemistry as raw material for several industrial sectors. The genetic characterization of *A. donax* plants revealed that, because of its asexual reproduction, low genetic variability could be detected between plants; the studies on the inflorescences also confirmed the agamic reproduction of the plant. Nevertheless, it could be speculated that some genetic characteristics could be hereditable and a selection of clones could be carried out. The clonal selection showed to be a crucial factor also for the choice of the propagation techniques and their final costs.

A. donax biomass conservation by ensilage was possible without using inoculum. The preservation of giant cane could be performed by the two most used silage techniques, trench and silo-bag; the silo-bag technique was more efficient in preserving the biogas potential than trench one. The potential biogas production of *A. donax* silage in comparison to corn silage was tested by simulation of real-scale anaerobic digestion plant with lab-scale trials. The *A. donax* biogas production was lower than the one obtained with corn due to the chemical characteristics of biomass and the absence of starch but, thanks to the higher biomass yields, achievable biomethane and electric energy referred to the cultivated surface area were much higher than corn and other traditional energy crops.

Riassunto

Arundo donax L. o canna comune è una coltura energetica di seconda generazione che può essere impiegata come feedstock per il biogas, il bioetanolo e per la combustione, nonché nel settore della chimica verde come materia prima per diversi settori industriali. La caratterizzazione genetica di *A. donax* ha dimostrato che è rivelabile una limitata variabilità genetica tra i diversi cloni a causa della riproduzione vegetativa. Gli studi effettuati sulle infiorescenze hanno inoltre confermato la riproduzione agamica della specie; grazie ad alcuni caratteri ereditabili, potrebbe comunque essere possibile una selezione clonale della stessa. È stato inoltre dimostrato come la selezione clonale potrà essere determinante anche nella scelta delle tecniche di propagazione e nel costo finale delle stesse.

La conservazione della biomassa può essere effettuata mediante insilamento senza l'utilizzo di inoculi. Due delle tecniche più diffuse sono state impiegate per l'insilamento: la trincea e il silo-bag, Quest'ultima, è stata più efficiente nella conservazione della biomassa e del potenziale di biogas nel tempo. Le produzioni potenziali di biogas da insilato di canna comune sono state verificate in confronto con il mais mediante simulazioni di impianti di scala reale. La produzione di biogas ottenuta da *A. donax* è stata inferiore al mais a causa delle caratteristiche della biomassa e dell'assenza di amido. Nonostante ciò, grazie alle maggiori rese di biomassa, il metano e l'energia elettrica ottenibili per ettaro di superficie coltivata con *A. donax*, sono stati superiori al mais e ad altre tradizionali colture energetiche.

Preface

Due to the increasing energy demand and the rising concentrations of green house gasses, new renewable sources for energy and green chemistry sectors are required. The biomass from dedicated energy crops is a renewable and sustainable solution to obtain energy and chemical compounds with less impact on the environment. *Arundo donax* L. or giant cane is a new energy crop that could have all the qualities to be a sustainable crop for bioenergy and green chemistry.

The aim of this research was to evaluate the possibility to use the *Arundo donax* L. biomass as a feedstock for the biorefonery concept. In particular, all the aspects about cultivation, sustainability, biomass conversion and economic evaluations were discussed with direct comparison with the traditional energy crops normally exploited nowadays.

Chapter 1 is an overview of *Arundo donax* L. utilizations as a feedstock for energy and green chemistry. In this chapter, *A. donax* physiology and agronomy are discussed highlighting the most important advantages linked to its cultivation. These characteristics could promote the use of giant cane as energy crop for biogas and bioethanol production or for its thermo-conversion. Several industrial uses and extraction of chemical compounds could also exploit *A. donax* as principal feedstock. This chapter collects and discusses all these aspects clarifying the state of the art of the first giant cane applications in a biorefinery concept.

The characterization of *Arundo donax* L. starts with a deep investigation of its origin and genetics, developed in *Chapter 2*. From a collection of 87 clones collected in all the Italian territory and in different kind of environments, a genetic characterization of the plant was carried out. The study revealed that between all the clones low genetic diversity was detectable, suggesting that the agamic reproduction of the plant maintained the most important genetic characteristics. A focus on eight clones tended to demonstrate the heritability of some genetic characteristics; culm height and culm diameter were found as useful parameters for the genetic characterizations of plant and opened the possibility of a selection of heritable features.

The characterization of *A. donax* proceeds in *Chapter 3* with the study of inflorescences from the same collection of clones sampled in all Italian territory. The results confirmed the sterility of *A. donax*: no pollen and no seeds were found despite the large size of the inflorescences. 83 clones of the collection were characterized for their inflorescence size and flowering time, finding a significant correlation between the geographical coordinates of sampling and the flowering time. From the genetic point of view, a deeper study of genetic variability of clones demonstrated the existence of three distinct clusters in which the analyzed clones could be divided.

The possibility to select clones was firstly studied in relation to the first step of the *A. donax* cultivation: the propagation of the plant. In *Chapter 4* the principal propagation techniques of giant cane are discussed. In particular, rhizomes transplantation, hydroponic cultivation and in vitro propagation were compared with the aim of improving the efficiency of these agamic propagation techniques, reducing costs and time required for new plants generation. Hydroponic cultivation appeared a valid propagation method, making possible to obtain vigorous plants with lower costs.

The work continues with *Arundo donax* L. biomass characterization in relation to its potential use for bioenergy sector. The analysis of the exploitation of giant cane for biogas production was investigated by studying its preservation by ensilage and its beahavior during anaerobic digestion process. *Chapter 5* discusses about the storage of *Arundo donax* L. as silage. Two normally used silage techniques, i.e. trench and silo-bag ensilage, were compared by a full-scale approach. The detected chemical parameters were useful to identify the dynamics of fermentation and preservation of biomass and biogas potential. Both techniques were efficient in preserving biomass during time but different behavior was noted for biogas potential preservation. Silo-bag was more efficient in preserving

biomethane potential and biomass energy content than trench silage. Economical evaluation of potential electric energy production highlighted that *A. donax* silage is more profitable and valuable than traditional energy crops for biogas and electric energy production.

The *Chapter 6* overcomes the potential estimations of biomethane and electric energy from *A. donax* silage focusing on the evaluation of the real obtainable productions. In this chapter, the biogas and biomethane productions from giant cane silage and corn silage were compared by using continued stirred tank lab-scale-reactors (CSTR) simulating a real-scale biogas plant. The biomethane production was lower for giant cane if compared with corn due to its more recalcitrance and the absence of starch. Although the less gas production, the higher biomass yields let giant cane to be competitive with corn for biomethane and electric energy production per cultivated surface area. The economical evaluations carried out in this chapter on *A. donax* performances showed lower methane and electric energy costs than corn and than other traditional energy crops.

I

Arundo donax L.: a non-food crop for bioenergy and bio-compound production

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Arundo donax L.: a non-food crop for bioenergy and bio-compound production

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Abstract

Arundo donax L., common name giant cane or giant reed, is a plant that grows spontaneously in different kinds of environments and that it is widespread in temperate and hot areas all over the world. Plant adaptability to different kinds of environment, soils and growing conditions, in combination with the high biomass production and the low input required for its cultivation, give to *A. donax* many advantages when compared to other energy crops. *A. donax* can be used in the production of biofuels/bioenergy by biological fermentation, i.e. biogas and bio-ethanol, but also, by direct biomass combustion. Both its industrial uses and the extraction of chemical compounds are largely proved, so that *A. donax* can be proposed as the feedstock to develop a bio-refinery.

Nowadays, the use of this non-food plant in both biofuels/bioenergy and bio-based compounds production is just beginning, with great possibilities for expanding its cultivation in the future. To this end, this review highlights the potential for using *A. donax* for energy and bio-compounds production, by collecting and critically discussing the data available on these first applications for the crop.

Keywords: Arundo donax L., Methane, Ethanol, Combustion, Bio-based Products, Biorefinery.

1. Introduction

Arundo donax L., common name "giant cane" or "giant reed", is a plant that grows spontaneously in different kinds of environments and that it is widespread in temperate and hot zones all over the world. *A. donax* belongs to the Poaceae family, in the tribe of Arundinaceae along with other species such as *A. plinii* Turra, *A. collina* Tenore, *A. mediterranea* Danin (Mariani et al., 2010) and other ornamental species.

The plant originally developed in East Asia, getting successively into the Mediterranean area and then spreading around the world through human activity (Hardion et al., 2012; Mariani et al., 2010; Polunin and Huxley, 1987). Human domestication occurred in the Mediterranean region (Zeven and Wet, 1982). Another hypothesis suggests that *A. donax* and its related species (*A. plinii, A. collina* and *A. mediterranea* or *A. micrantha*) had origins in the Mediterranean area (Zeven and Wet, 1982); in any case by now the presence of this plant is certified in all inhabited continents and in very different environments.

A. donax is a hydrophyte plant able to grow in soil rich in water, especially near channels, rivers, lakes, ponds and marshes, where it shows the maximum biomass yields.

The phylogenetic origin is not very clear: the chromosome number of *A. donax* is not yet certain because of the high number of chromosomes and their small size, so that different authors have reported different chromosome numbers. Firstly Hunter (1934) counted 110 chromosomes as did Bucci et al. (2013) and Pizzolongo (1962). However, Christopher and Abraham (1971) reported 108 chromosomes, while Haddadchi et al. (2013) reported 84 chromosome (2n = 7x = 84). It can be guessed that different ploidy levels of *A. donax* may depend on the different territory in which the plant has grown and of course may depend on its evolutionary history (Table 1).

The variability in chromosome number has been observed in the related species *P. australis*, where both euploids and aneuploids plants have been found (euploid numbers, between 3x and 12x, with x = 12); the more frequently found varieties in Europe are tetraploids while in Asia the more widespread varieties are octoploids with 96 chromosomes (Clevering and Lissner, 1999). Such genetic plasticity in aquatic plants is not surprising, taking into consideration the vegetative reproducing system that increases the probability of accumulating chromosomal mutations (not filtered by the process of meiosis). A simple hypothesis to explain the formation of the 2n = 108 or 110 *A. donax* sterile species, is based on the fusion of reduced (n = 36) and unreduced (n = 72) gametes from fertile progenitors (2n = 72) such as *A. plinii* (Bucci et al., 2013; Hardion et al.,

2012). Regarding the genotypic diversity among clonal populations sampled in different regions, again, data are different depending on the country studied and molecular markers used (Table 2).

However, genetic diversity has been very low, as expected for an agamic-reproducing plant, with the exception of data reported by Haddadchi et al. (2013) in Australia and Khudamrongsawat et al. (2004) in USA, although in this last case more probably the result obtained depended on the type of molecular marker used (RAPD). Despite its low genetic diversity, heritable phenotypic differences among clones of *A. donax* have been reported that could be explored to improve several plant characteristics such as number of culms, culm diameter and height (Cosentino et al., 2006; Pilu et al., 2014). For this reason the genetic improvement of this plant, aiming to obtain better performance as an energy crop, needs to be mainly based on clonal selection. Other techniques useful to modify the genome of this energy crop could be based on physical and chemical mutagenesis (Dhir et al., 2010; Takahashi et al., 2010), although *A. donax* is a polyploid plant and inducing its mutagenesis is likely to be very difficult. In fact genetic improvement of *A. donax* by physical and chemical mutagenesis, could be hampered due to the gene redundancy in the polyploid plants: the most frequent recessive mutations occurring would be masked by the dominant wild-type alleles. The rare events of phenotypic modifications, useful for genetic improvement, would be caused by rare gain of function mutations or genomic rearrangement (Comai, 2005).

Micro-projectile bombardment-mediated transformation of *A. donax* has been performed, obtaining transient expression of green fluorescent protein (GFP) and ß-glucuronidase (GUS) genes, although high frequency plant regeneration from embryogenic callus is still to be optimized (Dhir et al., 2010). Genetic improvement of this species would be aimed to improve the traits biomass production, quality and the capacity of plants to propagate efficiently *in vitro* culture or by propagules.

2. *Arundo donax* L. reproduction

A. donax is a sterile plant (Else, 1996; Witje et al., 2005) because of the absence of division of the megaspore cell mother (Bhanwra et al., 1982). Hence being unable to produce seeds, sowing cannot be used to propagate the plant for agriculture purposes (Khudamrongsawat et al., 2004). Because of its sterility, *A. donax* has developed asexual vegetative reproduction, allowing its rapid spread thoughout the world; new plants can be

generated every year directly from rhizomes, the underground structure of the plant (Figure 1): in a temperate climate, during spring and summer the rhizomes explore the soil laterally and allow the expansion of the plant with the creation and germination of buds. In addition, rhizome fragments can be obtained from live plants; this requires healthy rhizomes and the right environmental conditions for germination. These fragments can be planted from winter to early spring with a plant density of 1 plant m^{-2} and a reciprocal distance of 1 m (Bezzi et al., 2006).

Beside rhizome propagation, another natural plant reproduction method consists of rooting at the nodes, i.e. *A. donax* is able to generate new individuals from stem fragments coming from breakage and/or from lateral canes that bend down, getting in touch with the soil. In the presence of the right conditions (e.g. moisture, etc.) these fragments can produce roots from nodes, allowing the development of new shoots (Boland, 2006). Thanks to this ability, the principal means for canes to disperse is via water, i.e. *A. donax* usually grows along water bodies; during floods stem fragments transported by water, in the presence of the right conditions, can generate new canes (Brinke, 2010; Decruyenaere and Holt, 2005; Else, 1996). Some authors report that fragments can produce new plants only if there is an axillary bud present (Boose and Holt, 1999; Else, 1996; Wijte et al., 2005). The natural reproduction of *A. donax* could be explored for agronomic purposes (Figure 2). The cane fragments can be used to produce propagules characterized by the presence of developed below- and above-ground structures that allow a very high percentage of plant establishment (~100%) and the successive development of vigorous plants, right from the first year of plantation.

Developed plants can be produced, also, by hydroponic and micropropagation techniques, producing plants with both roots and an active photosynthetic apparatus. The hydroponic technique is based on the natural plant ability to develop new plants from nodes. Mature cane fragments can be cut into smaller pieces (about 15 cm) containing at least one node and put into water under particular conditions, so that each node is able to generate one shoot with its own roots (Ceotto et al., 2010; Pilu et al., 2014). Micro-propagation refers to *A. donax in vitro* reproduction technique (Takahashi et al., 2010) starting from axillary buds, and inducing tillering, by using media supplemented with 0-1 mg L^{-1} of BA (6-benzyladenine) and 0-0.1 mg L^{-1} of NAA (1-naphthaleneacetic acid). It is possible to promote root formation and root growth by modifying the BA/NAA ratio, hence the "seedlings" obtained can be transplanted into pots for continued development. It is also

possible to induce callus formation using a medium containing 2,4-D (2,4-dichlorophenoxyacetic acid) alone; the callus produced is able to generate shoots useful for propagation after its transfer into a hormone-free medium (Takahashi et al., 2010).

3. *Arundo donax* L.: lifecycle and agronomy at temperate latitudes

In a temperate climate the *A. donax* lifecycle begins in March with the sprouting of new canes from rhizomes (Figure 1); therefore rhizomes play a key role in this phenological phase (Nassi o di Nasso et al., 2013).

This stage is crucial for plant productivity because it determines future plant yield and growth rapidity. Generally, the first growing stages are the most important: if the plant suffers early water shortages, biomass height and yield are lower than normal (Abichandani, 2007; Perdue, 1958; Watts, 2009). This happens especially when cane is located on natural terraces or along the watershed line.

During spring time plant growth is very fast with an important biomass production that gives to *A. donax* the status of one of the biggest herbaceous plants in the world: its height ranges from 2 to 8 m (Perdue, 1958) but in some areas, such as temperate and subtropical climate zones and according to water availability, the plants can reach over 9 m in height (Giessow et al., 2011).

The fast growth and the ability of *A. donax* to accumulate biomass are the most important characteristics distinguishing this plant from other herbaceous plants. The growth velocity is assisted by the fact that *A. donax*, although it is a C_3 grass, has the typical development of a C_4 plant. Unlike normal C_3, *A. donax* shows high photosynthetic yields thanks to the greater saturation level of the photosystem than other C_3 plants, allowing high biomass accumulation (Papazoglou et al., 2005; Papazoglou et al., 2007; Rossa et al., 1998).

New canes develop during early summer but they are smaller and thinner than the growth during spring. Plant flowering occurs from August to October, according to the climatic conditions. Plant senescence begins at the end of November and continues during winter months; during this period the plant turns yellow but it is not dead inside. The stalk tissues, in fact, are still alive inside allowing a faster vegetative restart in the successive season (February). In February, canes restart vegetative activity, developing nodal buds and generating secondary branches; these structures typically appear earlier than new canes coming from the rhizomes. During this phase the rhizome is very important as it releases

nutrients (Nassi o di Nasso et al., 2013) allowing rapid growth at the beginning of the season.

Both environmental and climatic conditions may cause prolongation or shortening of the different phenological phases: in warmer places, the winter dormancy is shorter than in other areas or completely absent in the case of subtropical climates. High nitrogen concentrations, also, could lead to no dormancy during the year (Decruyenaere and Holt, 2005).

A. donax cultivation consists in soil preparation, transplantation of the propagules, irrigation, fertilization and harvest. The most important steps are related to the first year when the operations for planting and the plant establishment are required. Soil preparation consists in soil ploughing (about 40-45 cm depth for rhizomes and less for propagules) (Fiala, 2009) and fertilization; transplanting of rhizomes can be done between March and April at a depth of 15-20 cm (Fiala, 2009). In warmer climates, such as South Florida, the planting period is between December and February at a depth of 20-25 cm (Gilbert et al., 2010). If "seedlings" are produced from micro-propagation or hydroponic reproduction techniques, the useful time period for planting becomes longer (till June) because the plants have already developed a root apparatus.

On the whole, and referring to a temperate climate, about 5,000-10,000 plants Ha^{-1} are required to ensure good production after 1-2 years from the plantation, with low agronomic inputs such as irrigation, fertilization and phytosanitary interventions. In warmer climates, it is suggested that the plats Ha^{-1} could be more, i.e. 10,000-20,000, in order to cover in just one year the cultivated surface and produce the first yield in 7-12 months (Gilbert et al., 2010). Nowadays, new crop approaches suggest the reduction of the plant density, while keeping the same biomass yields. Angelini et al., (2005) suggested that a lower plant density could encourage production of more tillers and stalks and, consequently, more plant productivity than using high-density planting.

A. donax establishment in the field represents the most delicate moment for its cultivation: during this period the plants can suffer from water shortage stress, requiring additional irrigation; in addition, if weeds are dominant among the younger cane shoots, herbicide treatment may be necessary. During the second year the strong development of *A. donax* suppresses weeds and normally does not need additional water.

After the first year of plantation, *A. donax* does not need organic or inorganic fertilization to complete its lifecycle and to achieve high yields, but the application of fertilizers

enhances biomass production. The addition of nutrients (Angelini et al., 2005; Christou et al., 2003; Gilbert et al., 2010), especially nitrogen (Borin et al., 2013; Quinn et al., 2007) promotes a better development of rhizomes and consequently of new sprouts, allowing yields increases. Nutrient availability defines, also, qualitative improvement of the biomass, above all if it is related to a specific use, e.g. for combustion (Nassi o di Nasso et al., 2010). However, fertilization is only recommended but not necessarily required because *A. donax* transfers nutrients and compounds from the aboveground parts to the rhizome before winter senescence (Bell, 1997; Smith and Slater, 2011).

The high biomass production can represent a problem for agricultural operations such as crop mowing because of the plant height (normally more than 6 m) and plant moisture content: this latter sometimes exceeding 50 % of wet weight (w.w.) (Monti et al., 2006). High moisture content causes an increase in crop weight and consequently of costs for its handling, storing and conservation, above all, in the case of biomass combustion (Martelli and Bentini, 2013). On the other hand, this or higher moisture content is welcome when fresh biomass is to be ensiled (e.g. for biogas production).

Recent studies (Pari et al., 2012) indicated that the harvest of a tall plant like *A. donax* is not difficult when using the ordinary agricultural equipment used for other crops such as sorghum and corn, at least with slight modifications (Pari et al., 2012). Nevertheless specific stalk shredders are suggested for use on canes with a 2-3 cm diameter and moisture contents over 40-50% (w/w) (Fiala, 2009).

An *A. donax* plantation produces biomass for about 10-15 years, or even more if it is harvested every year and adequately fertilized. In any case in the last years of cultivation, biomass yields decline (Angelini et al., 2009).

The long-term cultivation of *A. donax* could present technical problems for soil restoration at the end of the crop cycle, as it requires all plants to be removed and rhizomes to be destroyed by both herbicides and soil ploughing. Unfortunately no published data or relevant observations are available on this theme, although the experience of the Authors of this review indicate that *A. donax* is sensitive to glyphosate: the use of 200-400 L Ha^{-1} of 1.5 % glyphosate solution during early spring is sufficient to create damage on sensitive new canes and then death. The apical buds died in about 10-20 days and the herbicide solution inhibited the germination of new shoots.

4. *Arundo donax* L. as feedstock: advantages and disadvantages

The adaptability of the plants to different kinds of environments, soils and growing conditions, in combination with the high biomass production and the low input required for its cultivation, confer on *A. donax* many advantages when compared to other energy crops (Table 3).

A. donax produces high amount of biomass per surface unit if compared to traditional energy crops; yield depends on several factors such as the age of the plants, pedo-climatic conditions, plant density and agronomics, so that high variability is reported in the literature (Table 4). Production over 150 Mg Ha^{-1} (Giessow et al., 2011; Sharma et al., 1998; Spencer et sl., 2006) is very high and referred to wild fields in warmer climates (California and India) so not comparable with others data referred to cultivated fields, mostly placed in temperate or Mediterranean climates. The temperature can justify the highest yields because due to the warmer climate a no clear dormant period during winter was noted (Decruyenaere and Holt, 2005). Average biomass production (aboveground part of plant) is around 15.5 kg dry matter (DM) m^{-2} (Giessow et al., 2011). The underground part of the plant has been less studied than the aboveground one; it was estimated that it can produce about 22.5% of the aboveground biomass (Sharma et al., 1998), so that a total biomass production of *A. donax* was estimated as ~20 kg DM m^{-2}; as a yardsticks, corn produces in fertile soil ~ 6 kg DM m^{-2}. Angelini et al. (2009), monitoring *A. donax* for 12 years, reported an average biomass yield of 30-40 Mg Ha^{-1} of DM per year.

A. donax can grow in different environmental conditions (Lewandowski et al., 2003): salinity does not seem to affect plant growth (Peck, 1998; Perdue, 1958) and it is possible to achieve high biomass yields under high salinity conditions thanks to its halophyte behaviour; for example, it was reported that in Australia *A. donax* could be a good solution for several saline soils (Williams et al., 2008). In California, for example, *A. donax* easily grows along beaches and in estuaries with brackish water conditions (Else, 1996). Water availability is not a limiting factor for plant growth: it was reported that *A. donax* could resist both soil characterized by water lack and soil, which was water-saturated (Lewandowski et al., 2003).

A. donax cultivation is possible in almost all climatic regions, with limitations only due to the cold; recently, researchers have been developing clones resistant to cold stress, allowing *A. donax* cultivation in colder climates (Pompeiano et al., 2013).

A. donax adaptability makes this plant suitable for marginal and abandoned lands (Lewandowski et al., 2003), i.e. soils that are not suitable for traditional agriculture. For example Nassi o di Nasso et al., (2013) showed competitive yields of *A. donax* (20 Mg Ha^{-1} DM) on low fertility sandy soil.

A. donax showed, also, a strong adaptability to soil containing high macro-elements content, such as N (Ambrose and Rundel, 2007) and P (Williams et al., 2008).

Wildfire, in particular, may promote *A. donax* expansion and it has been observed that after a fire *A. donax* is able to re-sprout, producing additional biomass (Coffman et al., 2010).

Because of high biomass production attention must be paid to the impact of *A. donax* cultivation on soil properties. Literature speculate that prolonged nutrient uptake could reduce the availability of the main elements in the soil, especially N (Angelini et al., 2009; Borin et al., 2013). On the other hand other Authors reported how *A. donax* cultivation improved the organic matter content and the microbial biomass (Riffaldi et al., 2012). In particular, because *A. donax* is a no tillage crop soil accumulates organic matter more than with other cultures such as the cropping sequences (legumes and cereal conventionally cultivated) and natural grassland (with forage removal) (Riffaldi et al., 2012). Christou et al. (2003) highlights also the *A. donax* importance on preserving soil erosion in slope and the nitrate leaching.

From an economic point of view, the excellent biomass yields compensate for both plantation and water supply costs (Giessow et al., 2011) and herbicide treatments during the first crop year (Lewandowski et al., 2003). Soldatos et al., (2004) estimated in Greece a total cost production for *A. donax* per cultivated Ha and Mg dry matter produced, of about 1,200 € and 77 €, respectively. As comparison, the cost for *Miscanthus* cultivation was reported to be the same as that of *A. donax* per surface unit, but it was much higher when it was compared with the biomass unit (91 € Mg^{-1} DM). Currently, in Italy, estimated costs to produce *A. donax* are of about 700-1000 € Ha^{-1} y^{-1} (as the average of 10 years) and 13-20 € Mg^{-1} DM (as the average of 10 years and 10,000 plant Ha^{-1}) in the Po Valley, North Italy (Arundo Italia s.r.l., 2013, personal communication). These investment costs are much lower than those reported for other energy crops cultivated in the same pedo-climatic region such as corn, sorghum, rye and triticale (~ 1,187-2,136 € Ha^{-1} y^{-1}) or crop rotations, i.e. triticale plus corn and triticale plus sorghum (3,346-2,903 € Ha^{-1} y^{-1}) (Schievano et al., 2014). The difference between the estimated cost for Italy and Greece can be explained by

taking into consideration the lower yield and the need for irrigation in the Greek context (Soldatos et al., 2004), in comparison with Northern Italy.

The most expensive aspect of *A. donax* culture is the initial investment to purchase "seedlings" material (rhizome or micro-propagated plants) (e.g. 3,000-5,000 € Ha^{-1}). These costs are those currently applied in an Italian commercial context during the *A. donax* sales campaign on 2013 and 2014 that involved about 40 different farms (Arundo Italia s.r.l., 2014 - personal communication). Actually, no more data are available about the investments because there are few commercial realities assessed on *A. donax*. In a Florida context, estimations suggest that the principal agronomic interventions costs could be similar to the sugarcane cultivation (Gilbert et al., 2010).

The great adaptability of *A. donax* to different pedo-climatic conditions, the high biomass production and the plant tolerance to microelements concentration in both soil and water, suggest the use of this species for phytodepuration purposes.

A. donax has been used for phytoremediation of soil contaminated by Hg (Han et al., 2005, Sagehashi et al., 2011), Cd (Guo and Miao, 2010; Han et al., 2005; Papazoglou et al., 2005; Papazoglou et al., 2007; Sagehashi et al., 2011; Yang et al., 2012;), Cr (Kausar et al., 2012), As (Guo and Miao, 2010; Mirza et al., 2010; Mirza et al., 2011;Yang et al., 2012), Pb (Guo and Miao, 2010; Yang et al., 2012), Ni (Papazoglou et al., 2005; Papazoglou et al., 2007; Van der Merwe et al., 1990), Mn, Zn and Fe (Van der Merwe et al., 1990). Moreover *A. donax* has been used for treating urban wastewater (Mandi and Abissy, 2000), aqueous solutions from industrial processes (Zhang et al., 2008) and in general wastewater containing organic compounds (Sudha and Vasudeva, 2009).

The capacity of *A. donax* to grow in different environments and to resist the competition of other plant species, has promoted the colonization of different areas (Quinn et al., 2007; Virtue et al., 2010), in particular the riparian habitats (Quinn and Holt, 2008). *A. donax* does not produce fertile seeds (Else, 1996; Khudamrongsawat et al., 2004; Witje et al., 2005) and its dispersion is limited to the rhizomes and cane fragments, especially near rivers (Williams et al., 2008). Human activity contributes, also, to the diffusion of *A. donax*: both the maintenance and the cleaning of canals and rivers, including stem cutting, generate a large amount of fragments that follow the water current and originate new plants (Boland, 2008). The combination of all these factors allows a great propagation of *A. donax* in an ecosystem: some alluvial areas are covered by up to 44% of *A. donax* canes (Giessow et al., 2011). In California *A. donax* became a serious threat for the equilibrium

of the seacoast, rivers and marshes (Dudley, 2000; Lawson et al., 2005; Rieger and Kreager, 1989; Tracy and DeLoach, 1998;). As a consequence of that experience, some Authors indicated that *A. donax* cultivation is not recommended in floodplains and riparian areas, because of the risks for the natural equilibrium of the ecosystem (Virtue et al., 2010).

Nevertheless, only rarely and under specific conditions does *A. donax* represent a threat for native plants by altering the ecosystem (Giessow et al., 2011; Herrera and Dudley, 2003; Lowe et al., 2000; Mack, 2008; Spencer et al., 2005; Tracy and DeLoach, 1998), and it does not represent a threat above all when it is used as energy crop in an agricultural area, where its weed potential is limited by the natural sterility of the plant.

A. donax control can be achieved by developing new techniques beside the traditional mechanical control (Bell, 1997; Lawson et al., 2005). Tracy and DeLoach (1998) suggested the use of biotic agents specific for *A. donax*, such as insects or arthropods. The same Authors reported that over 30 species of arthropods and some herbivores are suitable for *A. donax* control. The studies of Goolsby and Moran, (2009) demonstrated that a monophagous arthropod, e.g. *Tramersa romana* Walker, could causes important damage to the cane structure without interacting with the native species. Other insects have been recently studied because they could selectively attack the culms, newly emergent culms and roots (Mack, 2008).

5. *Arundo donax* L. as a feedstock to produce bioenergy and bio-based compounds

The importance of *A. donax* as a non-food crop is justified not only for the high biomass yields, low agronomic input and low production costs, but, also, for its flexibility of usage. *A. donax,* in fact, finds application in the production of bioenergy/biofuels by biological fermentation, i.e. biogas (Ragaglini et al., 2014) and bio-ethanol (Jaradat, 2010) but, also, for direct biomass combustion (Dahl and Obernberger, 2004). Additional industrial uses and the extraction of chemical compounds are largely proved (Tracy and DeLoach, 1998).

5.1. Biogas

A. donax can be efficiently used for biogas production in substitution or partial integration with the traditional energy crops in co-digestion with animal slurries and/or other biomasses.

Unfortunately, very few data are available in the literature about *A. donax* productivity by anaerobic digestion because its use is only beginning to be studied. Available data indicate that *A. donax* has a lower anaerobic biogasification potential (ABP) than traditional energy crops such as corn, sorghum, rye and triticale (Schievano et al., 2014) (Table 5), i.e. *A. donax* produces about 75 %, 94%, 77% and 120 % of ABP of corn, rye, triticale and sorghum, respectively.

Lower productivity with respect to those of cereals is due to the fact *A. donax* is a sterile plant that does not produce seeds containing an energy reserve (starch), unlike cereals (e.g. corn). As a consequence of that, the chemical composition of the aboveground part of *A. donax* consists solely of the presence of structural polymers, i.e. hemicellulose, cellulose and lignin (Table 6), that are less degradable than starch because of natural recalcitrance (Himmel et al., 2007) caused by the 3D cell wall structure (Adani et al., 2011).

Despite the fact that *A. donax* produces less biogas than traditional energy crops per DM unit, when its productivity is compared by referring to the surface area unit (Ha), the high biomass productivity per hectare determines that its bio-methane production is higher than those of other energy crops (Table 7).

Bio-methane production for *A. donax* could vary significantly depending on different environments and agronomics (Table 7). Bio-methane production reported ranges from 9,580 Nm^3 CH_4 Ha^{-1} to 19,440 Nm^3 CH_4 Ha^{-1} (12,618 ± 3,588 Nm^3 CH_4 Ha^{-1} as the average calculated from Table 7), estimates that are in any case higher than those reported for traditional energy crops.

The environmental conditions and agronomic techniques employed for biomass production can affect the bio-methane yields either positively or negatively. For example, in non-irrigated parcel trials of 4 m^2 (Landriano, Pavia, University experimental farm, 45° 18' North, 9° 15' East), potential bio-methane production from *A. donax* (3 years old) cut in early October, resulted in 19,440 Nm^3 CH_4 Ha^{-1}, i.e. a biomass yield of 71.8 ± 8.4 DM Ha^{-1}, ABP of 524 ± 2 Nm^3 Mg DM^{-1} and CH_4 content of 64 % v/v (Gruppo Ricicla – DiSAA, unpublished results). This production was higher than that obtained during the same year by a "two mowing" approach: the first made in early June (biomass yield of 25.7 ± 5.3 Mg DM Ha^{-1}, ABP of 495 ± 7 Nm^3 Mg DM^{-1} and CH_4 content of 64 % v/v.) and the second made in early October (biomass yield of 13.5 ± 1.8 Mg DM Ha^{-1}, ABP of 482 ± 7 m^3 Mg DM^{-1} and CH_4 content of 64 % v/v), for a total production of 9,930 Nm^3 CH_4 Ha^{-1}.

The “one mowing” approach gave methane production much higher than that obtainable, in the same geographic area, by using double energy crops in succession, such as: grass plus corn, triticale plus corn and sorghum plus triticale, i.e. 10,245 ± 1,082 Nm^3 CH_4 Ha^{-1}, 12,179 ± 1,532 Nm^3 CH_4 Ha^{-1} and 9,465 ± 1,521 Nm^3 CH_4 Ha^{-1}, respectively (Schievano et al., 2014).

Pedo-climatic conditions influence *A. donax* production; for example Ragaglini et al., (2014) reported for central Italy (San Piero a Grado, Pisa, 43° 40’North, 10° 20’ East), that double mowing was better than only one, producing 11,585 – 12,981 Nm^3 CH_4 Ha^{-1} and 9,580 Nm^3 CH_4 Ha^{-1}, respectively. In this case the different pedo-climatic conditions also affected biomass DM content that ranged from 39.6 % to 46.9 % w.w. for the double harvest system and of 51 % w.w. for the single one (Ragaglini et al., 2014). These results contrast with those reported in this work that indicate a DM content of 19.7 – 28.5 % w.w. and 34.5 % w.w. for the double and single mowing, respectively. These differences were due, probably, to diverse water availability for the plants, i.e. high availability for the Padania basin which is characterized by a permanent high aquifer, and low availability for Central Italy trials (Ragaglini et al., 2014). Again, water availability may probably have played an important role in determining the advantage in one or two mowings, i.e. water scarcity in the advanced season limited plant development in Central Italy (Ragaglini et al., 2014) accelerating plant maturation and lignification.

A. donax pre-treatment could be an excellent solution to increase biomass biodegradability during anaerobic digestion by reducing natural recalcitrance. Di Girolamo et al., (2013) treated *A. donax* biomass with steam cooking (temp. = 180°C, time = 10 min) increasing bio-methane production by 12 %. Dried samples of *A. donax* (DM of 87%) treated by steam explosion (temp. = 220 °C, time = 7 min) (details in Adani et al., 2011), when tested for biogas production under batch conditions, increased bio-methane production by more than 60 %, i.e. from 310 Nm^3 Mg^{-1} to 450 Nm^3 Mg^{-1} (Gruppo Ricicla – DiSAA, unpublished results). The increase of bio-methane yield was due to the breakdown of the 3D structure of cell walls, which was well documented by the increase of glucose yield in the treated sample after enzymatic tests performed on both untreated and treated *A. donax* samples (Adani et al., 2011).

A problem resulting from the production of biogas from *A. donax* is the presence of siloxanes in the biogas. Siloxanes are volatile compounds generated from silicon present in

A. donax (Chauhan et al., 2011; Monti et al., 2008; Nassi o di Nasso et al., 2010) during anaerobic digestion.

Siloxanes are particularly dangerous to the mechanical structure of reactors and motors because of their abrasive characteristics (Accettola and Guebitz, 2008; Dewil et al., 2006; Schweigkofler and Niessner, 2001;). Soil type, the use of fertilizers and farm machinery (Rasi et al., 2013) can affect the presence of siloxanes in biogas. Anaerobic digestion of biomasses gives cyclosiloxanes as the most abundant siloxanes, that are, in any case, less concentrated than those reported to be produced during anaerobic digestion of wastewater and of MSW in landfills (Rasi et al., 2010). Although literature indicates that silicon content for *A. donax* is higher than those of other energy crops, ranging from 6.2 g kg^{-1} DM in stems to 17.2 g kg^{-1} DM in leaves (Monti et al., 2008), no data on biogas siloxanes from *A. donax* are available. A lab-scale test performed by the Authors of this review, indicated a concentration of siloxanes in the headspace of vials of 20 mL during anaerobic batch test with *A. donax* (60 days length), of 0.203 g m^{-3} headspace, that was much less than that detected for an identical test performed with corn (0.585 g m^{-3} headspace). The type of silicon compounds detected were the same as those found by Rasi et al. (2013), studying grass and corn biogases, i.e. cyclosiloxanes (D3, D4 and D5 types).

5.2. Bio-ethanol

Second generation bio-ethanol is obtained from lignocellulosic materials (Alvira et al., 2010, Menon and Rao, 2012) containing C_6 and C_5 sugars polymers (Chandel et al., 2007). The principal advantages of the second-generation bio-ethanol are connected to the high biofuels yield per Ha, positive energetic balance and negative greenhouse gas emissions (Kheshgi et al., 2000).

Thanks to the high biomass yield per Ha (Table 4) and fiber composition (Table 6), *A. donax* produces a large amount of bio-ethanol per Ha, generally higher than those obtained by using other energy crops. It was been reported that *A. donax* produces around 11,000 L Ha^{-1} that is 50 % more than ethanol coming from sugar cane and sugar beet and 20% more than *Miscanthus* production (Qin et al., 2011) (Table 8). New promising energy crops like cassava, produce, on average, 45 % less ethanol in comparison with *A. donax.*

The natural resistance of lignocellulosic material to degradation may oblige operators to perform biomass pretreatment prior to the saccharification process, to remove recalcitrance (Adani et al., 2011), i.e. removing hemicellulose, rearranging lignin structure, preparing

cellulose for subsequent hydrolysis into monomers (Scordia et al., 2011). Very few data are reported for *A. donax* and generally they represent lab-scale approaches.

Scordia et al., (2011) used dilute oxalic acid to remove hemicellulose selectively from *A. donax*, since this acid is more selective in breaking the *β*-(1-4) linkage than others, e.g. sulphuric acid. Severity factors of 2.76 (Process parameters: Temp. = 190°C, Time = 25 min and oxalic acid concentration = 5 % w/w, solid load of 2 % w/v) allowed a glucose recovery after treatments of 100 %. Successive simultaneous saccharification and fermentation processes gave ethanol production of 18 g L^{-1} of ethanol (solid loading of 10 % w/v, incubation time of 72 h at pH 6 with *Schefferomyces (Pichia) stipitis*). Ethanol yield was 57 % of the theoretical value, which was relatively low for an industrial process.

Two stages microwave/chemical pre-treatment (microwave/NaOH performed at temp. = 120°C, time = 5 min and NaOH concentration = 0.5 % w/v plus microwave/H_2SO_4 performed at temp. = 180°C, time = 30 min and H_2SO_4 concentration = 0.5 % w/v) reduces hemicellulose content, i.e. xylan and arabinan were reduced by 65 % and 100 %, respectively (Komolwanich et al., 2014). As a consequence of that, monomeric sugar and glucose yields, compared to the starting dry biomass, were of 31.9 % and 26.4 % respectively.

More interesting are results presented by De Bari et al., (2012), in which they studied the effect of one step stem explosion treatment (SE) with and without acid catalysis, in comparison with *A. donax* samples coming from a Chemtex pilot scale plant (Mossi Ghisolfi Group, Italy), from which the full scale plant of Crescentino (Beta Renewables, 2014) was then developed. Since the process is commercially protected, no process details are available; however the pilot process considers a two-step pre-treatment in which hemicellulose is extracted before treating the biomass with an innovative SE process (De Bari et al., 2012). The best results obtained, expressed as hydrolysis yield compounds (i.e. glucose) indicated a glucose yield of 67.4 %, i.e. ~ 74 g glucose L^{-1} in a bioreactor equipped with a helical impeller (i.e. pH 5.5., solid loading of 20% w/v, Novoymes enzymatic preparation: Cellic^{TM} Ctec2 0.28 g g^{-1} $glucan^{-1}$, temp. = 50°C, incubation time of 48h). One step acid-catalysed SE gave similar results (De Bari et al., 2012). Melis (2010) reported for the full scale plant a bio-ethanol production of 12,690 – 15,228 L Ha^{-1} (*A. donax* production of 45 Mg DM Ha^{-1}), which is a much higher bio-ethanol production than that reported from other energy crops (Table 8).

5.3. Combustion

Thanks to both the high biomass production (Table 4) and the heating value, which is comparable to those of other energy crops (Table 9), *A. donax* is promising for the combustion process.

A. donax high heating value (HHV) was calculated, as average, to be of 18.7 ± 1.2 MJ kg^{-1} that is not statistically different (ANOVA, Tukey test, $P < 0.05$) from those of both herbaceous i.e., miscanthus and switchgrass (HHV of 18.7 ± 0.9 MJ kg^{-1} and 19.1 ± 1.5 MJ kg^{-1}, respectively) and woody biomasses such as poplar (HHV of 19.5 ± 0.2 MJ kg^{-1}). Considering in a temperate climate an *A. donax* biomass production of 37.7 Mg DM Ha^{-1} (Angelini et al., 2009) (Table 4) and those of miscanthus, switchgrass and poplar, i.e. 15-30, 10-25 and 9-20 Mg DM Ha^{-1}, respectively (Bezzi et al., 2006), it can be deduced that *A. donax*, potentially, can produce about respectively 40.3 %, 52.6 % and 59.9 % more energy per Ha, although a direct comparison has not yet been made.

The use of *A. donax* for combustion processes could show some problems connected to the chemical composition of tissues. The amount of ash produced is remarkable when compared with other energy crops (Coulson et al., 2004; Monti et al., 2008; Nassi o di Nasso et al., 2010). Monti et al. (2008) (Bologna, University experimental farm, 44° 33' North, 33 m a.s.l.) highlighted that the amount of ash comes principally from leaf tissues, i.e. ash content of 11.3 % DM and 3.2 % DM for leaves and stems respectively, to be compared with those of other energy crops such as *Miscanthus* (6.2 % DM and 2.9 % DM for leaves and stems, respectively) and switchgrass (7-7.6 % DM and 2.3-2.6 % DM for leaves and stems, respectively). Plant age seems to influence ash content: data of Authors of this review indicate for *A. donax* (Landriano, Pavia, University of Milan experimental farm, 45° 18' North, 9° 15' East) a decrease of the total ash content with plant age (whole plants), i.e. from 9.9 % DM for May-samples to 3 % DM for March-samples. A correct agronomic technique can reduce ash content, improving heating values; Nassi o di Nasso et al., (2010) demonstrated that both fertilization and harvest time influenced tissues' mineral content (Si). In particular, silicon content seems to decrease with high fertilization and autumn harvest. Anyway, *A. donax* has ash content similar to other crops, such as switchgrass (Coulson et al., 2004; Monti et al., 2008). It was reported that elements such as K and Cl and corrosive compounds (Si), released during thermal processing, give rise to technical problems on machineries (Coulson et al., 2004). Moreover attention must be paid to the ash content because it reduces thermal conversion efficiency (Coulson et al., 2004).

With reference to environmental aspects, some studies indicated that *A. donax* combustion may produces some harmful compounds (NO_x, HCl, SO_2, CO) and fine dusts at higher levels than other crops like switchgrass, cardoon, *Miscanthus* and pine (Dahl and Obernberger, 2004). In particular, for *A. donax,* as for *Miscanthus*, combustion tests revealed that these emissions are principally due to the entrainment of particles that increase the values of CO and dusts. It was suggested that the total amounts of gas and dusts emitted are comparable to those from other crops (e.g. *Miscanthus*) (Jeguirim et al., 2010).

5.4. Industrial use and bio-based compounds

A. donax can find other applications in addition to energy/biofuel production, consisting in the direct use of canes and/or in the extraction of chemical compounds for industrial use (Figure 2) (Table 10).

As well as for the other uses, industrial applications are economically justified because of the low cost of biomass production and the high plant productivity (Tracy and DeLoach, 1998).

In the past, *A. donax* was used for the production of musical instruments such as flutes and related instruments (Obataya and Norimoto, 1999; Perdue, 1958) and it still provides reeds for the mouthpieces of oboes, clarinets, bassoons and saxophones. The *A. donax* chemical composition, i.e. water-soluble extractive (e.g. glucose, fructose and sucrose) can modify the reed properties changing acoustic properties (Obataya and Norimoto, 1999).

Thanks to the cane strength, *A. donax* was used in the past to reinforce rural buildings (Barreca, 2012). Nowadays, *A. donax* is still employed for prefabricated wall panel and chipboards (Ferràndez-Garcìa et al., 2012; Flores et al., 2011; Flores-Yepes et al., 2012). The production of particleboards starting from *A. donax* represents another interesting application (Garcìa-Ortuño et al., 2011). Thanks to the strength of its structure and to the huge amount of cellulose contained, *A. donax* is also used in the paper industry. The plant internodes were found to be the more suitable parts for pulping and papermaking thanks to the lower lignin content after treatment and to the viscosity, strength and brightness properties of the pulp (Shatalov and Pereira, 2002). In order to obtain optimal characteristics regarding tensile index, burst index and tear index, a method was assessed for pre-treating biomass by auto-hydrolysis, isolating the cellulose useful for papermaking (Caparròs et al., 2007b). The *A. donax* acetone and chloroform soluble extractives play a

role in defining the paper properties and quality, because these lipophilic extractives have different behaviour during conversion processes. Coelho et al. (2007) reported that the lipophilic fraction is about the 1.56 % of fiber content; the compounds are preferentially characterized by n-fatty acids (41 %), sterols (19 %), monoglycerides (13 %), fatty alcohols (7 %), steryl glucosides (6 %). The high amount of these compounds can give rise to sticky deposits on machineries and requires higher chemical consumption during conversion processes (Coelho et al., 2007).

New studies are providing new and more efficient techniques for the extraction, isolation and characterization of the mixture of cellulose and lignin (Neto et al., 1997; Seca et al., 2000) that are eligible raw materials for several industrial products. The chemical structure of lignin suggests that it is a possible source of chemical compounds, such as *p*-hydroxyphenylpropane (Seca et al., 2000) for biopolymer and plastic production. Moreover, the production of bio-oil by the simultaneous de-polymerization of fibers (hemicellulose, cellulose and lignin) at high temperature has been reported. The bio-oil is a mixture of complex organic compounds that has the properties of a wood protector: in particular, the bio-oil shows hydrophobic properties and confers on wood resistance against biotic agents, fungi and termite attack (Aysu and Küçük, 2013; Temiz et al., 2013).

Chemical and thermal treatments of *A. donax* allow the extraction of compounds usable in the pharmaceutical, cosmetic and food sectors.

A. donax can be a good source of levulinic acid and γ-valerolactone (Raspolli Galletti et al., 2013). From these acids it is possible to obtain solvents, antifreeze, food flavourings and intermediates for plastic and pharmaceutical products; the γ-valerolactone is also used as additive for ethanol and diesel fuel (Raspolli Galletti et al., 2013). From the vegetal structures is possible to obtain great amounts of xylose that, after appropriate processing, produce xylitol, a sugar utilized in the confectionery and food for diabetics (Shatalov and Pereira, 2012).

The extractable compounds, such as alkaloids, are also employed as active compounds for medicines (Tracy and DeLoach, 1998) and for the formulation of insecticides (Miles et al., 1993). Great interest is taken in plant alkaloids because they can act as active principles in pharmaceutical preparation. Ghosal and Dutta (1969) first suggested that the indole-based compounds extracted from *A. donax* can be used for different applications. There are numerous studies on the isolation and identification of alkaloids from *A. donax* and their distribution between rhizome, stem, leaves and flowers (Khuzhaev et al., 1994). New

alkaloids have been recently identified, especially indole alkaloids (Khuzhaev, 2004; Khuzhaev et al., 2004a; Khuzhaev et al., 2004b; Zhalolov et al., 2000; Zhalolov et al., 2002a; Zhalolov et al., 2002b) in addition to the first nine isolated by Khuzhaev and Aripova, (1998). The pharmacological properties of these alkaloids have been studied, finding that some of them, in particularly arundamine (Li et al., 2007) (Figure 2) and donasine (Jia et al., 2008), are effective in reducing fever. The isolation of alkaloids could be also explored for the chemical preparation of formulations against insects or aphids (Salem, 1991).

The huge yields and the fast-growing habit of *A. donax* justify the search for useful products derived from the lignocellulosic material. This is the case when using *A. donax* as raw material for the production of activated carbons for the treatment and the purification of wastewater from toxic heavy metals (Basso et al, 2002a; Vernersson et al., 2002). The active carbons produced showed important and efficient properties in removing heavy metals, such as cadmium, nickel (Basso et al., 2002[b]), hexavalent chromium (Sun et al., 2013) and organic compounds, such as benzene and toluene (Basso and Cukierman, 2005) from aqueous solution.

Besides the activated carbons, resins can be obtained from *A. donax* fiber. The advantage of the resins is especially based on their great adsorption capacity for ionic pollutants. Xu et al., (2012) demonstrated that this material could remove water pollutants from wastewater, such as NO_3^-, SO_4^{2-}, PO_4^{3-}, $NO_2^-$and AsO_2^-.

6. *Arundo donax*: from bioenergy/biofuel to bio-refinery concept

The use of *A. donax* for different energetic purposes can be evaluated in terms of total energy production per surface unit (Ha). Taking into consideration an *A. donax* production of 37.7 Mg Ha^{-1} DM (Angelini et al., 2009) (Table 4), a total energy production per surface unit (Ha) was calculated of 338 GJ Ha^{-1}, 238 GJ Ha^{-1} and 268 GJ Ha^{-1}, for bio-methane, bio-ethanol and combustion, respectively (Table 11). These results, although they can vary a lot because of the arbitrary assumption made regarding DM production per Ha, indicate that the transformation of *A. donax* in bio-methane is better in terms of total energy produced per Ha than the other two solutions. On the other hand both bio-methane and bio-ethanol production have the advantage, in contrast to the biomass combustion, of producing secondary products that can be employed to produce more energy and/or bio-products, developing a bio-refinery approach.

Anaerobic digestion leads to the co-production of energy/bio-methane but, also, of digestate that can be used to produce both organic and mineral fertilizers (Ledda et al., 2013). Recently a bio-refinery concept focused on anaerobic digestion has been developed (Manenti and Adani, 2014), and is re-proposed in this review (Figure 3a).

Bio-ethanol production, as well, resulted in the recovering of residual biomass to be used to produce bio-methane and or bio-based compounds and fertilizers (Figure 3b). In this way, the total energy produced per surface unit increases a lot. For example, for corn stalks in a Romanian context, it has been reported that the combined production of bio-ethanol and bio-methane could potentially increase the total energy production per DM unit by about 70% (Vintilă et al., 2013). Unfortunately no detailed data exist for both bio-methane and bio-ethanol bio-refineries for *A. donax*. Bio-ethanol bio-refinery from a lignocellulosic feedstock has been partially suggested by the literature (Barta et al, 2010; Ekman et al., 2013). Recently, an Italian private firm (Beta Renewables, 2014) is developing with *A. donax* a full scale bio-refinery concept, but unfortunately due to commercial knowledge protection, no detailed data were available for this review (Figure 3b).

7. Conclusions and outlook

A. donax is a perennial plant widespread all over the world and characterized by great adaptability. Data collected in this work indicate that, despite the variability in biomass production, *A. donax* produces much more biomass than other energy crops, leading to much higher fuel/energy production per unit surface area. All these data suggest using this crop in substitution for traditional energy crops, reducing, also, the biomass production costs. Unfortunately very few data are available regarding the use of *A. donax* for fuel/energy, and so, much remains to be done in terms of full field investigation.

Plant versatility and the wide use of its products indicate that *A. donax* is suitable for the development of a bio-refinery concept. Again, this topic needs to be completely investigated and fully developed.

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di biogas, multifunzionalita'". The Authors wish to thanks Arundo Italia s.r.l. for the personal information given.

Table 1 – Number of chromosomes and seed production reported for *A. donax* by different authors.

Country	Chromosome number	Percentage of seed production	References
Italy	110	0	Pizzolongo (1962)
Italy	110	0	Bucci et al. (2013)
India	108	0	Christopher and Abraham (1971)
Australia	84	0	Haddadchi et al. (2013)

Table 2 – Genotypic diversity of *A. donax* among different countries and authors. Modified from Haddadchi et al. (2013).

Country	Molecular markers	Number of loci tested	G/N[a]	References
Italy	ISSR	10	0.083	Mariani et al. (2010)
Italy	SSR/STS	10	0.093	Pilu et al. (2014)
France	SRAP	12	0.050	Ahamad et al. (2008)
USA	Isozyme	2	0.092	Khudamrongsawat et al. 2004
USA	RAPD	14	0.460	Khudamrongsawat et al. 2004
USA	SRAP	10	0.011	Ahamad et al. (2008)
Australia	ISSR	10	0.815	Haddadchi et al. (2013)

[a]G is unique genotype; N is number of samples; G/N is the proportion of distinguishable genotypes.

Table 3 – Summary of the most important advantages and disadvantages of *A. donax* cultivation.

	Advantages	Disadvantages
Agronomical aspects	High biomass yields per Ha.	Not recommended in floodplain.
	Adaptation to different kind of environments.	High initial investment costs.
	Adaptation to different kinds of soils.	Potential weed in no-agricultural area.
	Use of marginal lands.	
	Lower tillage and cost than traditional crops.	
	No seeds reproduction.	
	Vegetative reproduction by micro-propagation or by rhizome.	
Environmental aspects	Phytodepuration properties.	
	Strong reduction in using chemicals.	
	Promoting biodiversity.	
	Promoting soil organic matter accumulation.	
Utilization aspects	More biogas production per cultivated area.	Lower biogas production per dry matter ton than traditional crops.
	More bio-ethanol production per cultivated area.	Pretreatment need.
	Green Chemistry.	
	Different industrial uses.	
	Developing bio-refinery.	

Table 4 – *A. donax* yields in different countries.

Country	Description	Dry matter (Mg Ha^{-1})	References
U.S.A.	Wild field	171	Spencer et al., 2006
U.S.A.	Wild field	155	Giessow et al., 2011
U.S.A.	Annual yield from wild field (post fire)	49	Ambrose and Rundel., 2007
India	Wild field	36-167	Sharma et al., 1998
Australia	Annual yield from crop (saline soil)	29-45.2	Williams et al., 2008
Spain	Annual yield from crop (year I)	1.3	Hidalgo and Fernandez, 2000
Spain	Annual yield from crop (year II)	20	Hidalgo and Fernandez, 2000
Spain	Annual yield from crop (year III)	45.9	Hidalgo and Fernandez, 2000
Greece	Annual yield from crop	30-40	Papazoglou et al., 2007
Greece	Annual yield from crop	15.6	Soldatos et al., 2004
Spain	Annual yield from crop (year I)	1.37	Christou et al., 2005
Spain	Annual yield from crop (year II)	2.7-8.7	Christou et al., 2005
Spain	Annual yield from crop (year III)	6.3-18.6	Christou et al., 2005
Northern Italy	Annual yield from crop (year I)[a]	5.4-6	Gruppo Ricicla - DiSAA, unpublished results
Northern Italy	Annual yield from crop (year II)[a]	26-52	Gruppo Ricicla - DiSAA, unpublished results
Northern Italy	Annual yield from crop (year III)[a]	74	Gruppo Ricicla - DiSAA, unpublished results
Central Italy	Annual yield from crop (average of 12 years)	37.7	Angelini et al., 2009
Central Italy	Annual yield from crop (year III) (marginal land)	20	Nassi o Di Nasso et al., 2013
Southern Italy	Annual yield from crop (year I)	10.6	Cosentino et al., 2006
Southern Italy	Annual yield from crop (year II)	22.1	Cosentino et al., 2006
Southern Italy	Annual yield from crop	26-37	Cosentino et al., 2008
Southern Italy	Annual yield from crop (year II)	31.1	Mantineo et al., 2009
Southern Italy	Annual yield from crop (year III)	38.8	Mantineo et al., 2009
Southern Italy	Annual yield from crop (year IV)	34.9	Mantineo et al., 2009

[a] data evaluated during 2012.

Table 5 – Comparison between batch biogas production (ABP) (Nm^3 Mg^{-1} DM^{-1}) between *A. donax* and other energy crops. Modified from Schievano et al. (2014).

	Fresh matter	**Dry matter content**	**Dry matter**	**ABP**	**References**
	Mg Ha^{-1}	%	Mg Ha^{-1}	Nm^3 Mg^{-1} DM^{-1}	
A. donax	129	35	45	524 ± 2	Gruppo Ricicla – DiSAA, unpublished results
Corn	71	30.4	21.5	694 ± 43	Schievano et al. (2014)
Triticale	90	18.3	16.5	677 ± 79	Schievano et al. (2014)
Corn + Triticale	136	25	34.1	631 ± 79	Schievano et al. (2014)
Sorghum	122	15.1	19.4	423 ± 87	Schievano et al. (2014)
Grass	50	21.8	10.9	576 ± 28	Schievano et al. (2014)
Rye	31	33.4	8.7	556 ± 15	Schievano et al. (2014)

Table 6 – *A. donax* fiber composition.

Extracts % DM	**Hemicellulose** % DM	**Cellulose** % DM	**Lignin** % DM	**Ashes** % DM	**References**
n.d.	24.4	39.1	19.2	4.2	Komolwanich et al., 2014
12.2[a]	20.3	33.8	24.0	n.d.	Shatalov and Pereira, 2013
n.d.	19.2	29.7	22.1	n.d.	Ruan et al., 2013
13	32.0	29.2[b]	20.9	4.8	Garcìa-Ortuño et al., 2011
n.d.	20.9	34.8	23	n.d.	Franscisco et al., 2010
n.d.	29.8	34.8	23	n.d.	Caparròs et al., 2007b
11.2-13[a]	28.5-32	29.2-32.9[b]	21	4.8-6.1	Shatalov and Pereira, 2002
21.6[c]	14.5[d]	39.6[d]	24.3[d]	5.3[d]	Gruppo Ricicla - DiSAA, unpublished results

[a] Extraction in dichloromethane, ethanol and water.
[b] Reported as α-Cellulose.
[c] Extraction in sodium lauryl sulfate (Van Soest et al., 1991) of plants collected on October 2012.
[d] Plants collected on October 2012.

Table 7 – Potential bio-methane production of *A. donax* and different energy crops.

Crop	Further description	Bio-methane production $Nm^3\ CH_4\ Ha^{-1}$	References
Corn		5,453	Thyø and Wenzel, 2007
		7,050 – 7,768	Oslaj et al., 2010
	Early/medium ripening	5,300 – 8,500	Amon et al., 2004
	late ripening	7,100 – 9,000	
	1st harvest	8,430	Schievano et al., 2014
	2nd harvest	6,705	
	3rd harvest	5,862	
		8,000 – 10,000	Amon et al., 2007
		1,660 – 12,150	Murphy et al., 2011
Sorghum		3,917	Schievano et al., 2014
		2,500	Seppälä et al., 2013
		2,124 – 8,370	Murphy et al., 2011
Barley		4,493 – 4,747	Gatta et al., 2013
		1,144 – 2,428	Murphy et al., 2011
Wheat		1,244 – 4,505	Murphy et al., 2011
Triticale		5,221	Gatta et al., 2013
		5,345	Schievano et al., 2014
		1,000 – 5,944	Murphy et al., 2011
Rye		535 – 930	Murphy et al., 2011
		2,729	Schievano et al., 2012
Cereals	(average of wheat, triticale, rye)	3,200 – 4,500	Amon et al., 2007
Sunflower		2,771 – 4,695	Amon et al., 2007
		832-2,880	Murphy et al., 2011
Grass		2,682 – 6,305	Murphy et al., 2011
		3,541	Schievano et al., 2014
Sugar beet		1,954 – 6,309	Murphy et al., 2011
A. donax	one mowing	9,580	Ragaglini et al., 2014
	two mowing	11,585 – 12,981	Ragaglini et al., 2014
	one mowing, 3 years old plant (2012)	19,440	Gruppo Ricicla - DiSAA, unpublished results
	two mowing, 3 years old plant (2012)	9,930	Gruppo Ricicla - DiSAA, unpublished results

Table 8 – Bio-ethanol production of *A. donax* and different energy crops.

Crop	**Biomass Yield** Mg Ha^{-1}	**Bio-ethanol production** L Ha^{-1}	**References**
Cassava	12	1,750 – 5,400	Duku et al., 2011
	12.2	2,746 – 6,717	Ziska et al., 2009
	40	6,000	Jansson et al., 2009
	n.d.	6,313	Dai et al., 2006
Sweet sorghum	35	2,800	Jansson et al., 2009
	n.d.	3,000	Ravindranath et al., 2011
	54-69	3000	Almodares and Hadi, 2009
	1.03	3,000 – 6,000	Duku et al., 2011
Sugar beet	30-40	5,000 – 6,000	Almodares and Hadi, 2009
	47.1	5,150	Adams et al., 2009
	n.d.	5,891	Ziska et al., 2009
Sugarcane	70-80	3,000 – 5,000	Almodares and Hadi, 2009
	254	4,000 – 8,000	Duku et al., 2011
	70	4,900	Jansson et al., 2009
	n.d.	5,000	Ravindranath et al., 2011
	n.d.	6,195	Ziska et al., 2009
	68.3	6,756	Adams et al., 2009
Winter rye	5.4	2,275	Rosenberger et al., 2002
Winter triticale	5.3	2,374	Rosenberger et al., 2002
	5.8	2,651	Rosenberger et al., 2002
	7.6	3,506	Rosenberger et al., 2002
Wheat	2.8	1,010	Adams et al., 2009
	4	1,560	Jansson et al., 2009
	6.1	2,750	Rosenberger et al., 2002
	4.1	1,801	Rosenberger et al., 2002
Pea	7.6	2,750 – 3,505	Rosenberger et al., 2002
Corn	10.4	700 – 3,000	Duku et al., 2011
	5	2,050	Jansson et al., 2009
	n.d.	2,370	Ravindranath et al., 2011
	7.9	3,232	Qin et al., 2011
Corn starch	3.7	1,588	Qin et al., 2011
	4.8	2,010	Adams et al., 2009
	n.d.	4,000	Keshwani and Cheng, 2009
Corn stover	n.d.	700 – 800	Keshwani and Cheng, 2009
	4.1	1,644	Qin et al., 2011
	n.d.	2,000	Keshwani and Cheng, 2009
Rice	5	2,250	Jansson t al., 2009
Switchgrass	7.3	2,895	Qin et al., 2011
	n.d.	2,000 – 4,000	Keshwani and Cheng, 2009
	15	5,000 – 6,000	Keshwani and Cheng, 2009
Miscanthus	22.1	8,812	Qin et al., 2011
A. donax	45	11,000	Williams et al., 2008
	45	11,000	Jaradat, 2010
	45	12,690 – 15,228	Elaborated from Melis, 2010

Table 9 - High Heating Value (HHV) of *A. donax* and different lignocellulosic and woody species.

Crop	Further description	HHV MJ kg^{-1}	References
Corn stover		18.4	Kumar et al., 2008
Thistle		17.6	Coulson and Bridgwater, 2004
Cotton stalk		17.4	Munir et al., 2009
		13.5	Sun et al., 2010
Switchgrass		17.8	Dahl and Obernberger, 2004
		18.8	Hu et al., 2010
		20.7	Pilon and Lavoie, 2013
Miscanthus		19.6	Bonanno et al., 2013
		17.8	Jeguirim et al., 2010
		18.5	Melligan et al., 2011
		17.7	Michel et al., 2006
		19.6	Dahl and Obernberger, 2004
Sugarcane bagasse	After sugar extraction in sugar mill	16.8	Munir et al., 2009
Pine	Wood pine	18.0	Mante and Agblevor, 2010
		20.0	Kim et al., 2010
	Average of *P. elliotti* and *P. taeda*	18.6	Baker et al., 2010
	Austrian wood (*P. nigra*)	20.3	Dahl and Obernberger, 2004
Poplar	Average of four poplar clones	19.7	Pannacci et al., 2009
		19.3	Kieseler et al., 2013
	White poplar	19.5	Kyriakopoulos et al., 2010
A. donax		18.4	Bonanno et al., 2013
		20.1	Rabemanolontsoa and Saka, 2010
		17.2	Jeguirim et al., 2010
		19.8	Dahl and Obernberger, 2004
		18.0	Coulson and Bridgwater, 2004

Table 10 – Industrial uses and extractable compounds of *A. donax*.

Material - compound	Use	Further description	Yield g kg^{-1} DM^{-1}	References
Raw material	Musical instruments		-	Perdue, 1958
			-	Obataya and Norimoto, 1999
Raw material	Buildings		-	Barreca, 2012
Raw material	Chipboards and particleboards		-	Garcìa-Ortuno et al., 2011
			-	Flores et al., 2011
			-	Flores et al., 2012
			-	Ferrandez-Garcìa et al., 2012
Raw material	Paper	Alkali pretreatment (Na2O, 175°C)	-	Shatalov and Pereira, 2002
		Hydrothermal pretreatment (150°C to 200°C)	-	Caparros et al., 2007b
Raw material	Resins		-	Xu et al., 2012
Raw material	Bio-oil	Pyrolysis (450-525°C)	297	Temiz et al., 2013
		2-butanol solvent, 10% NaOH, 285°C	674	Aysu and Küçük, 2013
		Acetone, 10% NaOH, 285°C	603	
Raw material	Activated carbon	H_3PO_4 solution, 500°C	-	Basso et al., 2002b
		H_3PO_4 solution, 400-450°C and 500°C	-	Vernersson et al., 2002
		H_3PO_4 solution, 500°C	-	Basso and Cukierman, 2005
		H_3PO_4 solution, 400°C	-	Sun et al., 2013
Xylose	Production of xylitol	H_2SO_4 1.27 %, 142°C	218	Shatalov and Pereira, 2012
Xylo-oligosaccharides	Production of functional food	Hydrothermal pretreatment, 150°C to 195°C	145	Caparros et al., 2007a
Levulinic acid	Solvent, antifreeze, food flavoring, intermediate for medicine and plastic	HCl 0.4 M, 180°C	233	Raspolli Galletti et al., 2013
γ−valerolactone	Fuel substitution	NbO 0.25 meq, 70°C, 3 MPa	166	Raspolli Galletti et al., 2013
Indole compounds	Pharmaceutical preparation		-	Ghosal and Dutta, 1967
Arundamine	Pharmacological preparation		-	Li et al., 2007
Donasine	Pharmacological preparation		-	Jia et al., 2008
Indole compounds	Insecticide formulation		-	Miles et al., 1993
p-hydroxyphenil-propane	Production of biopolymer and plastic		-	Seca et al., 2000

Table 11 – Potential energy production per surface unit obtainable from different uses of *A. donax* as energy feedstock.

Bio-methane		Bio-ethanol		Combustion	
Biomass production[a]	37.7 Mg Ha^{-1} DM	Biomass production[a]	37.7 t Ha^{-1} DM	Biomass production[a]	37.7 t Ha^{-1} DM
Bio-methane production[b]	351 Nm^3 CH_4 Mg^{-1} DM	Bio-ethanol production[e]	299 L Mg^{-1} DM	Moisture content[h]	50%
Plant efficiency[c]	80.8%	CH_3CH_2OH density[f]	788 kg $m^{3\ -1}$	Water Specific Heat	4.18 kJ kg^{-1} $°K^{-1}$
				Water Latent Heat Vaporization	2.27 MJ kg^{-1}
LHV[d]	31.6 MJ $m^{3\ -1}$	LHV[g]	26.8 MJ kg^{-1}	LHV[i]	16.8 MJ kg^{-1}
Energy yield	338 GJ Ha^{-1}	Energy yield	238 GJ Ha^{-1}	Energy yield	268 GJ Ha^{-1}

[a] average of dry biomass production in a temperate climate (43°40'N, 10°19'E, Pisa, Italy) (Angelini et al., 2009) (Table 4).
[b] bio-methane production calculated from *A. donax* ABP (Table 6) with a CH_4 content of 67.0±3 % v/v (Gruppo Ricicla - DiSAA,unpublished results).
[c] biogas plant efficiency evaluated in CSTR trials (Gruppo Ricicla - DiSAA, unpublished results).
[d] methane low heating value reported by Schievano et al., 2014.
[e] bio-ethanol production reported by Williams et al., 2008.
[f, g] ethanol density and low heating value reported by Rakopoulos et al., 2007, Qi et al., 2010, Rakopoulos et al., 2011.
[h] moisture content as reported by Monti, 2006, Fiala, 2009.
[i] low heating value calculated as average from Bezzi et al., 2006, Fiala, 2009.

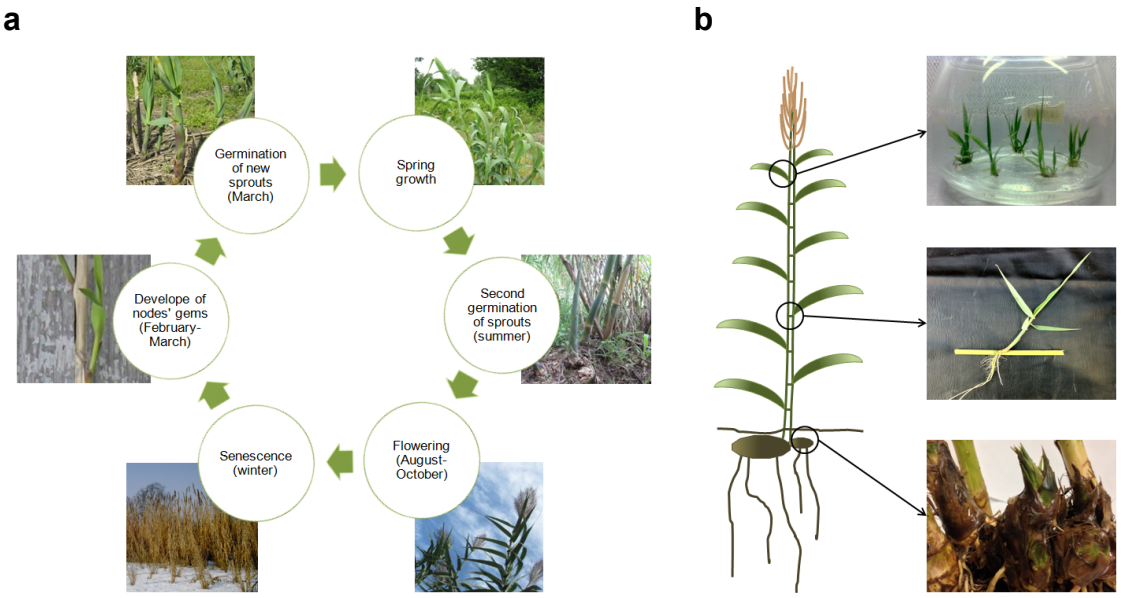

Figure 1 – a) *A. donax* lifecycle in a temperate climate and b) *A. donax* reproduction techniques: micro-propagation (from axillary bud) (top), hydroponic technique (from nodes) (middle) and rhizome propagation (from rhizomes buds) (bottom).

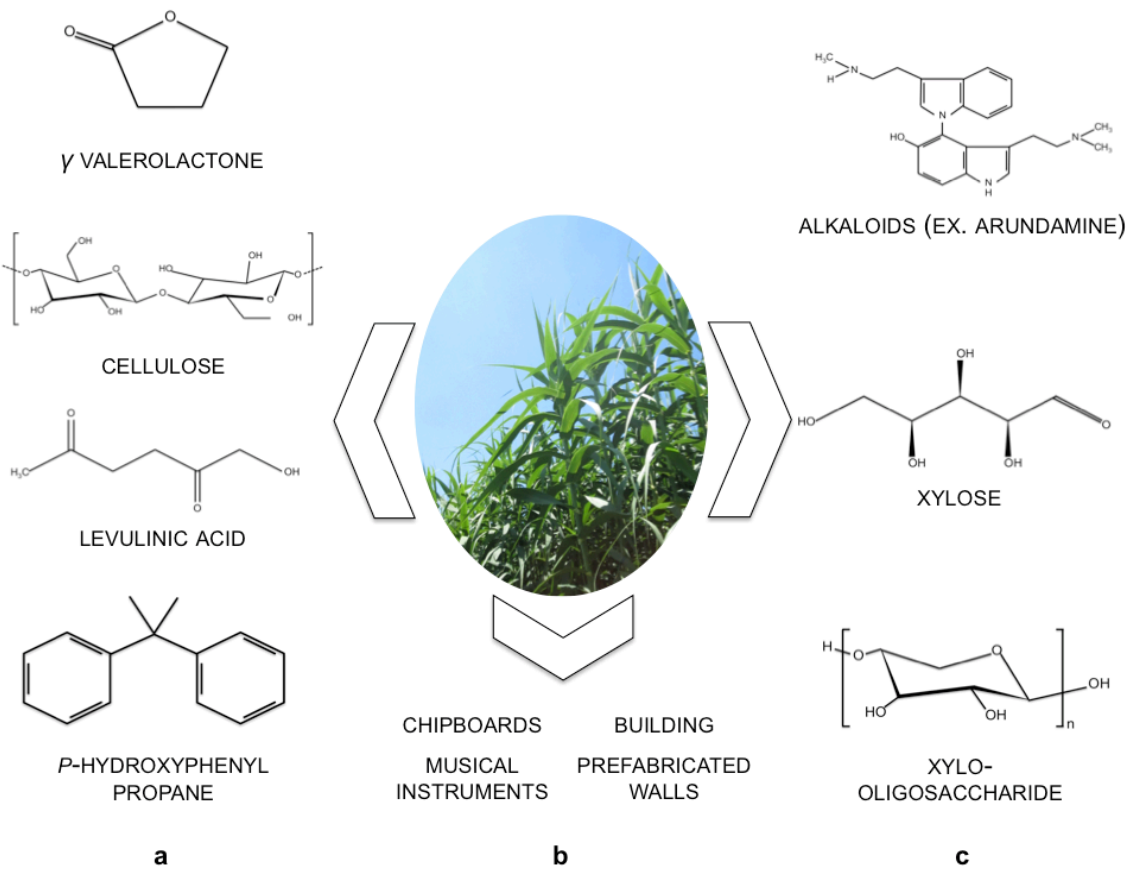

Figure 2 – Examples of possible *A. donax* derived compounds or possible utilization of biomass in several industrial sectors: a) derived compounds after various types of pretreatments; b) *A. donax* biomass employed as raw material; c) *A. donax* extractable compounds.

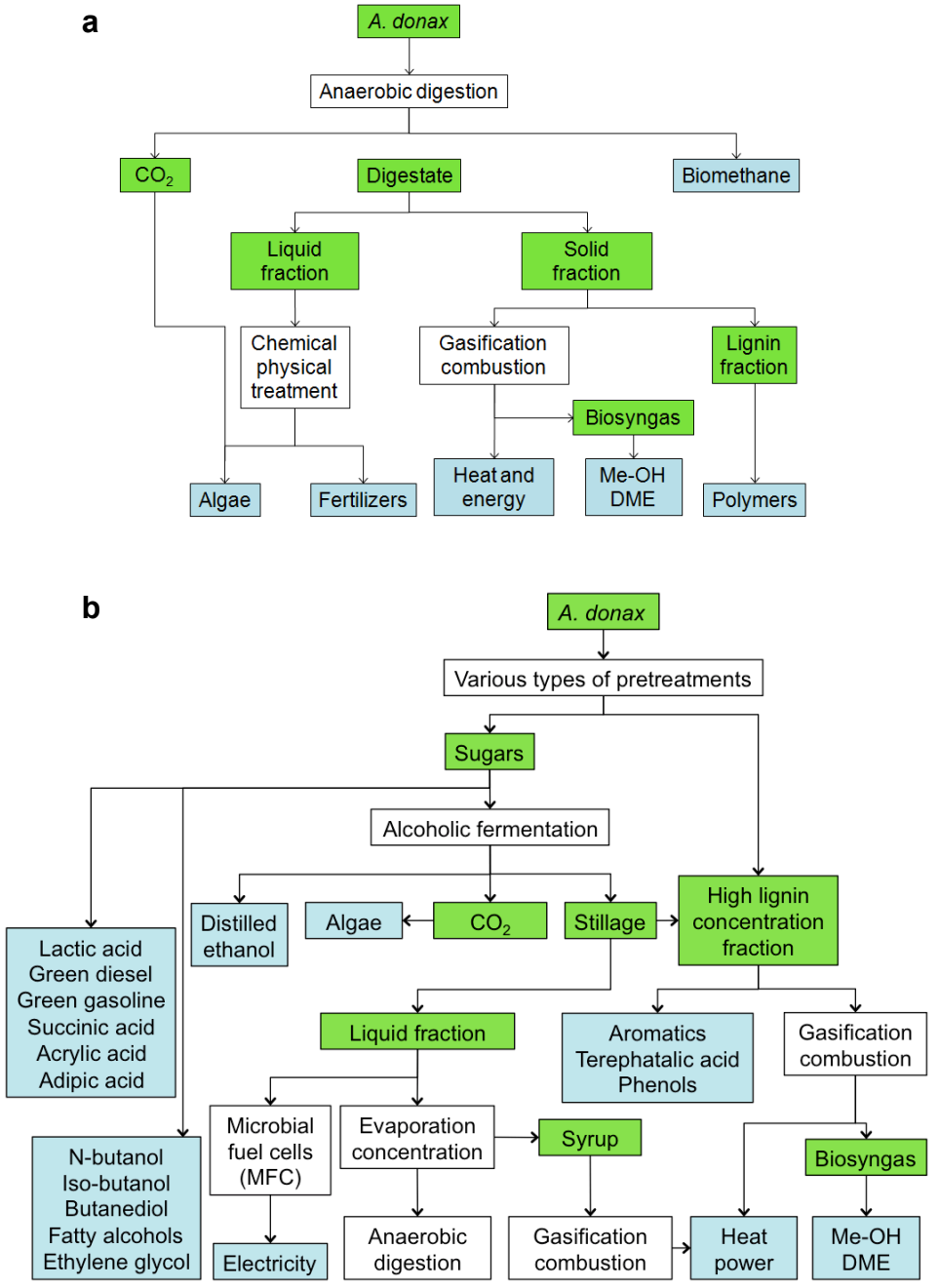

Figure 3 – Examples of *A. donax* bio-refineries: a) bio-refinery from anaerobic digestion process (readapted from Manenti and Adani, 2014); b) bio-refinery from pretreated biomass for sugar release (readapted from Barta et al., 2010; Beta Renewables, 2014; Ekman et al., 2013). The green boxes indicates the feedstock, the white boxes the biological and/or chemical-physical processes and the light blue boxes the products.

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II

Genetic characterization of an Italian giant reed (*Arundo donax* L.) clones collection: exploiting clonal selection

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Genetic characterization of an Italian giant reed (*Arundo donax* L.) clones collection: exploiting clonal selection

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Abstract

Arundo donax is a perennial rhizomatous plant growing spontaneously all over the world as an invasive plant reaching more than 8 m in height. It is a sterile plant which reproduces itself only agamically, through rhizomes and cane fragments which are transported by water or through human action. Although *A. donax* is an invasive plant it is considered one of the most promising energy crops, as it is characterized by a high energy balance. In this work we collected 87 *A. donax* clones from around Italy and with the aim of characterizing the genetic structure of the population, we studied the genetic diversity by using molecular markers (a survey of SSRs and genes from maize) and, for the first time, by sequence comparison using the *purple plantl1* maize homologous orthologous gene. The results obtained showed a low genetic diversity as expected for an agamic plant. However, although MANTEL analysis did not show any statistical difference regarding the geographical distribution of these clones, we noticed by PCA/AMOVA analysis the presence of three different genotypes. Among a survey of eight clones studied in detail we found a high correlation between parent–progeny for the traits culm height and culm diameter and a heritability (h2) of the same traits respectively of 0.21 and 0.34 which appeared promising for clonal selection. Finally based on data collected regarding molecular analysis, chromosome number, epidermal cell size and chlorophyll content, we advance the hypothesis that *A. donax* may be a polyploid derived from *Arundo plinii.*

Keywords: Energy crop; Arundo donax; Clones collection; Genetic diversity; Heritability; Polyploid.

1. Introduction

Arundo donax is a perennial grass that grows spontaneously almost all over the world (Perdue 1958; Faix et al. 1989). It is found in the riparian zones and in highly anthropic disturbed ecosystems (Coffman et al. 2010) where it often becomes an invasive weed compromising biodiversity (Herrera and Dudley 2003) and increasing wildfire and flooding risks (Quinn and Holt 2008). The most common method of propagation is by fragments of the rhizomes, which are irregularly transformed underground stems containing reserve compounds (Perdue 1958; Spencer et al. 2008). The roots can reach 5 m in depth (Perdue 1958; Frandsen 1997) and canes reach 8–10 m in height and 3–4 cm in diameter. The flat leaves, 5–8 cm wide and 30–100 cm long, are inserted alternately in two ranks (Perdue 1958; Tucker 1990; Pilu et al. 2013). In our latitude the sprouting of the new canes from rhizomes begins in early March and it continues during the whole vegetative season until flowering. This takes place between August and November, and the inflorescences, large plume-like panicles 30–100 cm long (Tucker 1990), do not produce seeds (Bell 1997; DiTomaso and Healey 2003; Bucci et al. 2013; Haddadchi et al. 2013). In winter the canes become yellow and generally lose leaves and inflorescences. *Arundo donax* is supposed to have spread from Asia, its native center, to America, passing through the Mediterranean area (Polunin and Huxley 1987; Lewandowski et al. 2003; Ahmad et al. 2008; Mariani et al. 2010). Other authors argue that this plant originated in Mediterranean regions from native species (Zeven and Wet 1982). The reproduction is exclusively asexual through vegetative propagation (Decruyenaere and Holt 2001; Ceotto and Di Candilo 2010) taking place when rhizomes and cane fragments are broken up and transported by flooding or by human agents (Perdue 1958; Boland 2006). In particular, recently, land movement by bulldozers has significantly contributed to *A. donax* spread by moving the propagules from one site to another (Boose and Holt 1999; Boland 2008). However the ancient spread of *A. donax* was mainly because of its utilization for several domestic purposes such as the making of walking-sticks, baskets, mats, fishing rods, fences, plant stakes and in musical instruments such as the reeds for clarinets and saxophones (Perdue 1958; Zohary 1962; Pilu et al. 2013). At the moment there is no large scale cultivation and only a few experimental fields have been established. However between the 1930s and the 1960s *A. donax* was cultivated in Northern Italy to produce the

raw material for a textile fiber called Rayon, but after the second world war, the increased production costs and the competition with chemical products derived from petroleum made this industrial production unsustainable (Facchini 1941; Perdue 1958). The economic interest for this species has recently returned with the development of biomass energy. In fact the vegetative vigor, the great production of dry matter per hectare and the low input required in cultivation make *A. donax* an important and promising energy crop (Angelini et al. 2005, 2009). The *A. donax* biomass could be used to produce solid biofuel such as chips and pellets for direct combustion (Dahl and Obernberger 2004) and gasification (Jeguirim and Trouvé 2009), bioethanol to generate the power for transportation (Jeon et al. 2010; Adani et al. 2011) and biogas for cogeneration.

In Mediterranean regions three other *Arundo* species are present: *A. plinii*, *A. collina* and *A. mediterranea* also named *A. micrantha* (Danin 2004; Hardion et al. 2012). Another closely related species is *Phragmites australis,* which shows several morphological traits reminiscent of *A. donax* and also grows in similar environmental conditions (Clevering and Lissner 1999). The phylogenetic origin of *A. donax* is not clear, even though some genetic evidence suggests that the great vegetative vigor and the absence of fertile seeds is due to the fact that this plant is probably a polyploid species (Pizzolongo 1962; Bucci et al. 2013; Haddadchi et al. 2013). The chromosome number of *A. donax* is not yet certain because of the difficulty in obtaining good preparations of metaphase plates due to the high number of chromosomes and their small size: Hunter in 1934 reported 110 chromosomes as did Pizzolongo in 1962 and Bucci et al. 2013, while Christopher and Abraham in 1971 reported 108, Gorenflot et al. in 1972 reported 112 and Haddadchi et al. in 2013 reported 84 chromosomes ($2n = 7x = 84$). The genetic diversity found among different clones and regions appears quite low as expected for an agamic plant. In fact the G/N proportion (G unique genotype; N, number of samples) of distinguishable genotypes resulted in 0.083 in Italy using ISSR markers (Mariani et al. 2010), 0.050 in France (Ahmad et al. 2008) and 0.011 in USA using SRAP markers (Ahmad et al. 2008). Nevertheless Haddadchi et al. in 2013 obtained a G/N proportion of 0.83 using ISSR markers and thus showed that in Australia the genetic diversity is higher than that previously reported in other countries. However despite its low genetic diversity, some phenotypic differences have been reported among the clones collected in southern Italy (Cosentino et al. 2005): they could be exploited to improve some useful characteristics with the aim to enhance by clonal selection the performance of *A. donax* as an energy crop.

In this work we studied the genetic diversity in a collection of *A. donax* sampled all over Italy, we calculated the heritability of height and culm diameter and finally, taking into account the data on epidermal cell size and chlorophyll content, we propose some hypotheses regarding the origin of the species.

2. Materials and methods

2.1. Plant materials

Arundo donax clones collection, one clone of *Arundo plinii* and one clone of *Phragmites australis* were obtained, sampling the plant rhizomes from all around Italy. The rhizomes were planted in the experimental field of the University of Milan located in Landriano (N 45°18′, E 9°15′) (Italy) to constitute the collection of 86 *A. donax* putative clones (GPS, Global Positioning System coordinates of clones locations are shown in Fig. S1).

2.2. Hydroponic cultivation and transplantation

We propagated the clones using hydroponic cultivation starting from cane nodes. Samples of mature canes were cut into smaller fragments containing one node (about 10 cm long) and put into a box filled with tap water, at a density of about one node per 300 cm3. After 2–3 weeks, each node generated one shoot which started to root after 2–3 weeks at 25 °C. The young plantlets were then transferred into pots for about 3 weeks before being transplanted into the experimental field.

2.3. Heritability estimation

8 clones (Ad10-Ad12, Ad14, Ad19, Ad20, Ad34 and Ad36) were propagated by hydroponic cultivation and transplanted in the experimental field of the University of Milan (Italy). The plants were transplanted in the field on March the 28th in a randomized way, rows and plants within rows were spaced 2 m apart and were then mulched. At the end of the growing season the plants were measured (at least three plants for each clone) for the two parameters diameter and height of the culm. The diameters of the culms were taken at a height of about 1.60 cm from the soil level and the measurements of the culm heights were taken at the tip of the inflorescence. Broad-sense heritability (h2) of the same traits was estimated using the equation $H^2=\sigma^2_G/\sigma^2_P$ where σ^2_G = genetic variance among clones and $\sigma^2_P=\sigma^2_G/\sigma^2_E$ where σ^2_E= environmental variance (Mather and Jinks 1982). Data were analysed using Microsoft Excel data analysis tools.

2.4. PCR amplification

Leaves from all the 86 *A. donax* samples and from the two single clones of *A. plinii* and *P. australis* were crushed and then used for DNA extraction using a protocol developed for maize (Dellaporta et al. 1983). DNA samples were amplified using 7 out of 48 maize simple-sequence repeats (SSR) randomly chosen from MaizeGDB (http:// www. maizegdb. org/ ssr. php). The SSR chosen produced a single band similar to that obtained from corn DNA in term of size of amplicon and repeatability using the condition described below (for each amplifications, we used the Tm recommended by MaizeGDB for maize). The primer sequences of the 7 SSR markers were: umc1082, umc1523, ZAG105; umc1221, bnlg1246, umc1666 and bnlg1250. We also amplified 3 gene sequences out of 12 randomly selected from our primers collection (we used these 3 genes because one single band was obtained by amplification). The genes used were: *ramosa1*, *purple plant1* and *low phytic acid 1/ZmMrp4*. All the primers sequences are shown in Fig. S2.

PCRs were performed in a final volume of 10 µl, containing 10 ng DNA, 0.1 µl M of each primer (forward and reverse), 200 mM of each dNTP, 2.5 mM MgCl2 and one unit of Taq DNA polymerase (Promega, Madison, WI). The reactions were carried out in a thermocycler, programmed for one pre-cycle at 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, specific annealing temperature of the primers for 1 min, 72 °C for 1 min, and a final step at 72 °C for 5 min. The amplified fragments were fractionated by electrophoresis using 3 % (w/v) agarose gels and stained with ethidium bromide (0.1 µg/µl).

2.5. Genetic analysis

Each band obtained by PCR was considered as an independent locus with two alleles: presence (1), or absence (0). A binary matrix was generated and used for statistical analyses. The dendogram was obtained by the algorithm UPGMA (Unweighted Pair Group Method Using Arithmetic Averages), using the Jaccard coefficient of similarity (http:// genomes. urv. es/ UPGMA, Garcia-Vallve, 1999). The output dendrogram was in Phylip Format. For Principal Coordinate Analysis (PCA), Analysis of Molecular Variance (AMOVA) and Mantel analysis we used the program GenALEx v. 6.1 (Peakall and Smouse 2006).

2.6. Pl1 sequence analysis

Genomic DNA extracted from *P. australis*, *A. plinii* and from 37 clones of *A. donax* were amplified by high fidelity PCR, Pfu (polymerase; Stratagene, La Jolla, CA, USA) using primers (PL1F 5′-CATGGCGAAGGCAAATGGAGG-3′, Tm 66 °C, position +905 and PL1R 5′-GTTGCCGAGGAGCCTGTGGA-3′, Tm 66 °C, position +1,183) specific for the 5′ maize *purple plant1* gene (GENBANK/L19494) obtaining an amplified of 279 pb. The *A. donax* clones amplified were: Ad2, Ad3, Ad5-Ad17, Ad19-Ad36, Ad38, Ad68, Ad74 and Ad77. The bands obtained were sequenced (consensus sequences were obtained at least from three independent samples for each clone/species) and aligned (from +963 to +1,178, 216 bp) using the algorithm ClustalW (http:// clustalw. genome. ad. jp). The dendrogram was built using ClustalW and the freely available web service Phylogeny.fr (http:// www. phylogeny. fr/ version2_ cgi/ index. cgi, Dereeper et al. 2008).

2.7. Microscopy

To determine cell sizes, we collected from *A. donax* and *A. plinii* adult plants, grown in the open field or in pots as specified, a central piece of the first leaf (about 15 cm from the tip). The tissues were treated with a clearing solution (160 g chloral hydrate and 20 mg glycerol in 60 ml water), mounted on slides and interference contrast images were taken using an IMAGE R.D1 microscope equipped with an AxioCamRc1 digital camera (Carl Zeiss). Cell measurements were taken on digital photographs using the AxioVision AC imaging software (Carl Zeiss). For each measurement at least three plants were analysed and from each plant two samples were collected from the central part of the first leaf.

2.8. Chlorophyll extraction and quantification

To quantify chlorophylls and carotenoids content we collected from *A. donax* and *A. plinii* adult plants, grown in pots, a piece of tissue from the central part of the first leaf (about 15 cm from the tip). The tissue was ground using liquid nitrogen and the pigments were extracted using a solution of 80 % acetone. The absorbance of the extracts was measured at 645, 663 and 470 nm with a spectrophotometer and the levels of chlorophyll a/b and carotenoids were expressed as mg/g from the Arnon's equations (Arnon 1949). For each measurement at least 3 plants were analysed and from each plant two samples were collected from the central part of the first leaf.

3. Results

3.1. Genetic characterization of the collection using molecular markers

With the aim of characterizing the population of *A. donax* on Italian territory, we collected more than 100 clones which were transplanted and cultivated in the experimental field of the University of Milan (N 45°18′, E 9°15′) together with *A. plinii* and *Phragmites australis* as related species (Fig. 1). 86 out of 100 Arundo clones, (see Fig. 2a and Fig. S1) were characterized with *A. plinii* and *P. australis* using 10 molecular markers obtained from maize as described in Materials and Methods chapter. The dendogram obtained by UPGMA (Unweighted Pair Group Method Using Arithmetic Averages) method showed, as expected, that *A. plinii* was closer to *A. donax* than was *P. australis* (Fig. 2b). Within the *A. donax* clones we observed quite a low genetic diversity among the clones (Fig. 2b) although PCA (Principal Coordinate Analysis) analysis suggested the presence of three distinct populations (Fig. 3c), in fact the first three coordinates explained 71.7 % of the total variation (axis1, 32.36 % and axis 2, 22.16 %). The genetic variation between these populations was 63 % while within populations it was 17 % (AMOVA, PhiPT value: 0.629, $P < 0.001$). However these populations did not reflect the geographic positions of the clones sampled as shown by the coloured map (white for pop. 1, red for pop. 2 and yellow for pop. 3) in Fig. 2a. To confirm these data we performed the Mantel test using the genetic and geographical distance matrix: the result obtained showed no statistical relationship between the two matrices (R2 = 0.008). Furthermore these populations did not show any difference for the phenotypic traits analysed (height, diameter and number of culms) even though differences between the single clones were noticed (data not shown).

3.2. Genetic characterization of the collection by pl1maize homologous orthologous sequencing

We used another strategy to characterize the clones collected: we looked for maize homologous orthologous genes in *A. donax* by amplifying a survey of genes randomly chosen. Three gene sequences (out of 12 randomly selected), *ramosa 1* (accession number AY957396), *purple plant1* (accession number L19494) and *low phytic acid 1/ZmMrp4* (accession number EF586878) were amplified using primers from our collection. We sequenced the *pl1* amplified sequences obtained from 37 clones representing a subset of our *A. donax* clones collection, *A. plinii* and *P. australis* (see "Materials and methods" section). No differences in the 210 bp nucleotide sequences were noticed among the *A.*

donax clones analysed (data not shown), confirming the low genetic diversity in the population in Italy. However, alignment (Fig. 3a) with *A. plinii* (210 bp), *P. australis* (209 bp) and maize sequences (216 bp, from +963 to +1,178 with respect to GENBANK/L19494 sequence) allowed us to draw a dendrogram by the CLUSTALW program (Fig. 3b). The data obtained showed that *A. donax* is evolutionarily closely related to *A. plinii* (8 nucleotides change) whilst *P.australis* and *Z. mays* are, as expected, more distant (Fig. 3c).

3.3. Heritability of phenotypic characters

With the aim to establish if the phenotypic differences observed among the clones collected, in particular height and diameter of the culms (data not shown), were inheritable we selected a survey of 8 clones (Ad10, Ad11, Ad12, Ad14, Ad19, Ad20, Ad34 and Ad36) and using hydroponic cultivation we propagated the clones starting from the nodes present on the culms (Fig. 4a, b). By so doing we obtained 5 Ad10, 6 Ad11, 4 Ad12, 7 Ad14, 13 Ad19, 18 Ad20, 6 Ad34 and 3 Ad36 plants transplanted and growth (as described in Materials and Methods chapter), measurements were taken at the end of the growing season (Fig. 4c). The data obtained demonstrated a statistical difference among clones regarding the height and the diameter of the culms (Table 1).

The clone with the highest diameter (Ad19, 32.65 mm) exceeded the clone with lowest value by 47.73 % (Ad20, 22.10 mm) and the difference between the progeny in the first year respectively was of 53.70 %. In the case of the culm height the tallest clone was Ad36 (713.33 cm) and the lowest Ad20 (602.66 cm) with a difference of 18.36 % that became 30.17 % between the progeny. Simple parent-progeny Spearman correlation coefficients (r) were calculated from the mean values of these traits and tested for significance. Statistical analysis showed a significant correlation for both traits analyzed (Table 1). Furthermore we estimated the broad-sense heritability (h2) of these two traits (height and diameter of the culms) and we found respectively the value of 0.21 and 0.34 (Table 2).

3.4. Determination of leaf epidermal cellular dimensions and chlorophyll content in A. donax and A. plinii

So far the origin and the cause of *A. donax* sterility are still an open question. We can conjecture that *A. donax* may be a polyploidy species derived in some way from *A. plinii*. This hypothesis is supported by the similarity between these two species and chromosome

size and number, which is higher in *A. donax* (in Italy 110 vs 72, Pizzolongo 1962). Usually polyploid plants undergo an increase in cell size (in particular stomata cells) and sometimes also an increase in chlorophyll content (Mishra et al. 1991; Joseph and Randall 1981). To support our hypothesis we determined these two parameters in *A. donax* and in *A. plinii*. We observed in *A. donax* plants growth either in pots or in the field, a consistently larger size of leaf epidermal cells (length and width) and stomata length/dimensions with respect to *A. plinii* (Fig. 5; Table 3). For chlorophyll content, we also observed a higher value in *A. donax* with respect to *A. plinii* (Table 4).

4. Discussion

In recent years, interest in *A. donax* as an energy crop has grown due to its high production of biomass per hectare and a good value of energy returned on energy invested in low input cultivation conditions (Cleveland et al. 1984). In particular in Mediterranean regions it appears to be the best perennial energy crop in comparative studies on biomass yield (Lewandowski et al. 2003). In Italy, a mean yield of about 40 Mg/dry matter was obtained in trials conducted for 7 years in Northern Italy (Di Candilo et al. 2005), a value of about 38 Mg/dry matter in Central Italy in a study during 12 years (Angelini et al. 2009) and up to 70 Mg/dry matter in conditions of high water table (unpublished results of our group). However these results have been obtained without any systematic exploration of the potential that could be expressed by the genetic variation present among the different clones found in natural populations. For this reason we sampled all over Italy for the clones that were transplanted in our experimental field located in Landriano (Fig. S1). These plants were characterized, from a genetic point of view, using molecular markers obtained from maize. Seven out of 48 maize SSRs and 3 out of 12 genes randomly chosen from MaizeGDB gave rise to a robust amplification and were used to build a binary matrix used for in silico investigations. The results obtained allowed us to establish the number of distinguishable genotypes, G = 8 and the G/N proportion = 8/86 = 0.093 (where N = number of samples) of distinguishable genotypes (data not shown). The data obtained were in agreement with those previously reported by Mariani and colleagues in Italy (Mariani et al. 2010) and Ahmad and colleagues in France and the USA (Ahmad et al. 2008) where the G/N value were respectively of 0.083, 0.050 and 0.011. The lower value found in the USA could be explained by assuming that *A. donax* probably spread from the Mediterranean area to America, undergoing a bottleneck which further reduced the genetic

diversity compared with that observed in Europe (Polunin and Huxley 1987; Lewandowski et al. 2003; Ahmad et al. 2008; Mariani et al. 2010). Furthermore a recent paper reported a higher G/N value of 0.83 in Australia (Haddadchi et al. 2013) due probably to the proximity to Asia, the putative center of *A. donax* origin (Polunin and Huxley 1987; Mariani et al. 2010, Hardion et al. 2012). However, despite the low G/N value the utilization of the UPGMA algorithm and PCA analysis (Fig. 2) showed the presence of three distinct populations on Italian territory (AMOVA, PhiPT value: 0.629, $P < 0.001$) with no relationship with the geographical position of sampling (MANTEL analysis, R = 0.008). Another approach used, for the first time, to assess the genetic diversity among the clones collected and the related species *A. plinii*, *P. australis* and *Z. mays* was the sequence comparison of the maize homologous orthologous *purple plant1* (accession number L19494) gene in *A. donax.* Sequencing 37 clones representative of all the Italian territory we did not find any difference in the 210 bp nucleotide sequences, confirming the low genetic diversity observed using the previous molecular markers. The alignment among *A. donax*, *A. plinii*, *P. australis* and *Z. mays* showed different polymorphisms (Fig. 3a) allowing us to outline a phylogenetic tree where the closer relationship between *A. donax* and *A. plinii* was confirmed. Hence having confirmed the high genetic uniformity among *A. donax* populations, we planned to check for the presence of phenotypic diversity among the clones. For this we propagated, using hydroponic cultivation, a group of 8 clones randomly selected from the collection and we measured the transplanted progenies in the field for the two traits height and diameter of the culm (Fig. 4). We utilized hydroponic cultivation for initial propagation to minimize the effect of the propagules' dimensions on the plant adult size because of the difficulties in selecting rhizomes homogeneous for size and number of buds in each propagule. The results obtained at the end of the season showed a statistically significant difference between the clones and a high correlation between parent-progeny phenotypes for the parameters diameter and culm height (Table 1). Furthermore we estimated the heritability (h2) of the same trait obtaining the values of 0.21 and 0.34 (Table 2). In literature only the work of Cosentino and colleagues reported phenotypic differences among clones and estimated the heritability (comprised between 23 and 48 %) regarding yield, stem weight, stem density and stem height utilizing about 40 clones collected in Sicily and Calabria (Cosentino et al. 2005). We can conjecture that other differences could be present for important traits involved in the performance of *A. donax* as an energy crop such as silica and lignin content. Further

work will be required to characterize the whole collection for all the useful traits for an energy crop. Hence, despite the low genetic diversity measured by molecular markers, our data confirmed that among *A. donax* clones there are some inheritable and promising phenotypic differences that could be exploited for clonal selection. However we cannot exclude the idea that the differences observed might be due to epigenetic modifications (change in gene methylation level) caused by the peculiar environment where the plants were sampled as reported for example by Cubas et al. in the case of the perennial plant *Linaria vulgaris*, in which was found that a change in *Lcyc* gene methylation (homologue of the *cycloidea* gene in Arabidopsis) was associated to an epigenetic mutation causing a heritable change in floral symmetry (Cubas et al. 1999). Of course in this case these differences would tend to get lost during the succeeding years, not being caused by nucleotide changing, making frustrating the work of clonal selection: more experiments will be necessary to assess this point.

In this work we also point out that the origin of *A. donax* still remains an open issue: the similar morphology (Fig. 1) and the sequences data obtained aligning the homologous orthologous of *pl1* maize gene and the derived phylogenetic tree (Fig. 3) pushed us to further investigate this aspect starting from the hypothesis that *A. donax* could be a sterile triploid species derived from a progenitor such as *A. plinii* ($2n = 72$). To support this hypothesis we measured the leaf epidermal cell size (Table 3) and the chlorophyll content (Table 4) since it is well known that there is a correlation between polyploid plant/genome copy number and the size/volume of cells/organs and often also with the chlorophyll content (Mishra et al. 1997). In Table 1 and Table 2, *A. donax* shows statistically higher values for the parameters of stomatal cell length (abaxial side of mature leaves), leaf epidermal cell size (length and width) and chlorophyll content compared to *A. plinii*. These data are in accordance with the hypothesis made previously, though we cannot exclude a polyphyletic origin of this species. In fact it seems that *A. donax* has different chromosome numbers depending on the country where is studied: 110 chromosomes in Italy (Pizzolongo 1962; Bucci et al. 2013), 108 chromosomes in India (Christopher and Abraham 1971) and 84 chromosomes ($2n = 7x = 84$) in Australia (Haddadchi et al. 2013). This is not surprising since similar results have been observed for *P. australis*, where different euploid numbers, between 3× and 12× have been found: in Europe these plants are tetraploids while in Asia, the octoploids with 96 chromosomes are the plants more commonly found (Clevering and Lissner 1999). However, of course, more work will be

necessary to address this issue, in particular it will be interesting to look for the *A. donax* progenitor in Asia, the putative centre of origin.

For the genetic improvement of this species, in the near future we can imagine that different techniques may be used to modify this crop, based on chemical and physical mutagenesis and genetic transformation (Takahashi et al. 2010; Dhir et al. 2010). First indications about the feasibility of this latter technique came from experiments where transient expression was obtained, transforming somatic cells with green fluorescent protein (GFP) and ß-glucuronidase (GUS) genes, using microprojectile bombardment (Dhir et al. 2010). In conclusion, further work will be necessary to better explore the genetic diversity in this species and to assess if clonal selection will be effective in the long term: *A. donax* has becoming a promising reality in the field of renewable energy based on biomass production, and efforts should be made to better characterize this plant from every point of view to fully explore its potential.

Acknowledgments

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Table 1 - Parent-clonal progeny correlation of the 8 Ad clones selected.

	Culm diameter (mm)		Culm height (cm)	
	Parent	Progeny	Parent	Progeny
Ad10	24.33±0.58a	11.98±0.70abc	634.45±15.34ab	247.86±16.20abc
Ad11	25.50±0.94a	13.92±1.19bcd	686.66±10.93bc	295.15±31.02bcd
Ad12	25.45±0.68a	11.93±0.72abc	648.75±7.59b	256.66±16.90abc
Ad14	23.12±0.44a	10.30±0.44a	631.33±12.35ab	207.32±10.52a
Ad19	32.65±0.45b	17.20±0.54d	653.36±11.83bc	328.44±11.64d
Ad20	22.10±0.92a	11.19±0.24ab	602.66±15.28a	236.57±7.35ab
Ad34	25.02±0.75a	11.90±0.39ab	631.68±16.17ab	226.86±8.68ab
Ad36	30.34±1.02b	14.09±0.59c	713.33±18.39c	307.95±15.35cd
R	0.904*		0.857*	

R Spearman Correlation, * indicate significance at the 0.05 level of probability. Mean values with different letters are significantly different in the column at the 0.05 level. The parent measurement represent the average values (culm diameter and culm eight) of the clones in the second year of cultivation whilst the progeny represent the average values of the plants in the first year. SE is shown. Means calculated from n = 18 to n = 129 culms measured.

Table 2 - Calculation of broad-sense heritability (h2) for height (expressed as cm) and diameter (expressed as mm) of the culm in *A. donax.*

Variation sources	Deviance		df	Variance (σ^2)		$h^2\ \sigma^2_G/(\sigma^2_G+\sigma^2_E)$	
	Height	Diam.		Height	Diam.	Height	Diam.
Among culms in different clones	717,82	2,248	m−1=7	102,545 $\sigma^2_E+n\sigma^2_G$	321 $\sigma^2_E+n\sigma^2_G$	0.21	0.34
Among culms within each clone	2,745,540	4,625	m(n−1)=376	$7{,}302\sigma^2_E$	$12.3\sigma^2_E$		
Total	3,463,360	6,873	N−1=383				

m = nr different clones (8); n = nr culms measured per each clones (48); N = total culms measured (384)
df degrees of freedom

Table 3 - Measurements of stomata cells length (abaxial side of mature leaves) and leaf epidermal cells size (length and width) in *A. donax* vs *A. plinii* (expressed as μm) cultivated in pots and in field.

	A. donax	*A. plinii*
Leaves from field		
Stomata cell length	46.15±0.44	37.55±0.27
Epidermal cell length	122.78±4.10	100.61±2.44
Epidermal cell width	22.82±0.59	16.41±0.51
Leaves from pots		
Stomata cell lengtha	48.97±0.28	29.44±0.27
Epidermal cell length	129.54±2.80	71.11±2.37
Epidermal cell width	23.94±0.47	13.73±0.29

Standard error is shown. Mean calculated from >45 measurements. Significant differences were noticed between the two species in all the mean values obtained based on *t* test ($n > 45$, $P < 0.05$).

Table 4 – Determination of chlorophylls a, b, a+b and carotenoids (expressed as mg/g of fresh tissue) in the leaf of *A. donax* vs *A. plinii* cultivated in field. Significant differences were noticed between the two species in all the mean values obtained based on *t* test ($n = 10$, $P < 0.05$).

	A. donax	*A. plinii*
Chlorophyll a	2.34±0.11	1.77±0.11
Chlorophyll b	0.77±0.04	0.61±0.04
Chlorophyll a+b	3.12±0.15	2.38±0.16
Carotenoids	0.71±0.03	0.56±0.04

Standard error is shown. Mean calculated from 10 measurements.

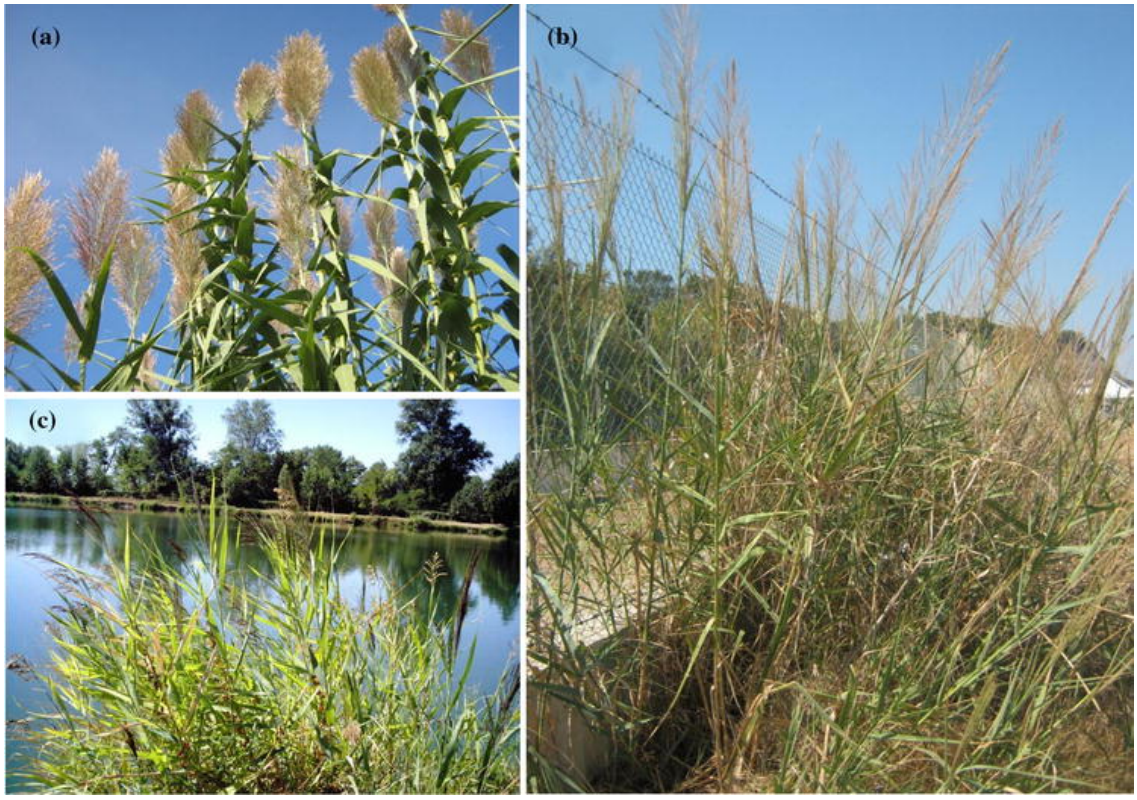

Figure 1 - *Arundo donax* (**a**), *Arundo plinii* (**b**) and *Phragmites australis* (**c**) plants.

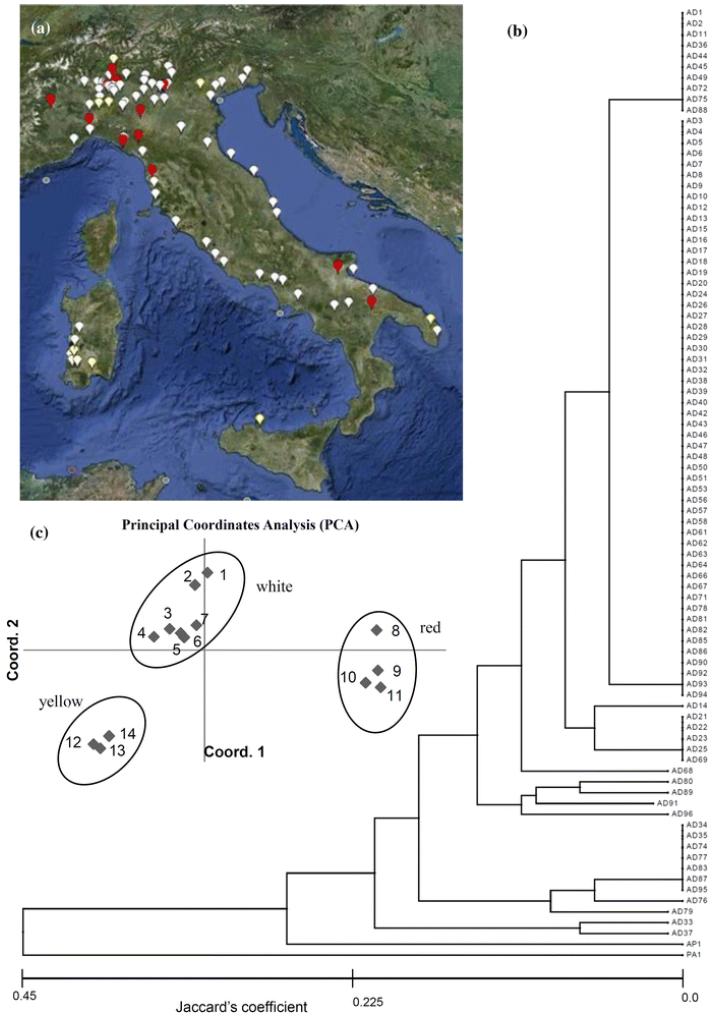

Figure 2 - Map of Italy showing the geographical positions of the 86 *A. donax* putative clones sampled in this study, every spot (*white*, *red* and *yellow*) represents the location of one clone sampled (a). Dendogram of *A. donax* clones collection (AD), obtained by 10 molecular markers using Unweighted Pair-Group Mean Arithmetic method (UPGMA) grouping (b). We used *A. plinii* (AP1) and *P. australis* (PA1) to root the tree. Principal coordinate analysis (PCA) of the clones collected based on 10 molecular markers. *1*: Ad89; *2*: Ad21-Ad23, Ad25, and Ad69; *3*: Ad33; *4*: Ad37; *5*: Ad3; *6*: Ad4-Ad10, Ad12, Ad13, Ad15-Ad20, Ad24, Ad26-Ad32, Ad38, Ad39, Ad40, Ad42, Ad 44, Ad46-Ad48, Ad50, Ad51, Ad53, Ad56-Ad58, Ad61-Ad64, Ad66, Ad67, Ad71, Ad78, Ad81, Ad82, Ad85, Ad86 and Ad90-Ad94; *7*: Ad80; *8*: Ad14; *9*: Ad96; *10*: Ad1, Ad2, Ad11, Ad36, Ad43, Ad45, Ad49, Ad72, Ad75 and Ad88; *11*: Ad68; *12*: Ad76; *13*: Ad79; *14*: Ad34, Ad35, Ad74, Ad77, Ad83, Ad87 and Ad95 (c). The *white* spots in (a) represent the sampling location of clones from 1 to 7, in *red* are represented the clones from 8 to 11 and in *yellow* the clones from 12 to 14 with respect to the result of PCA analysis.

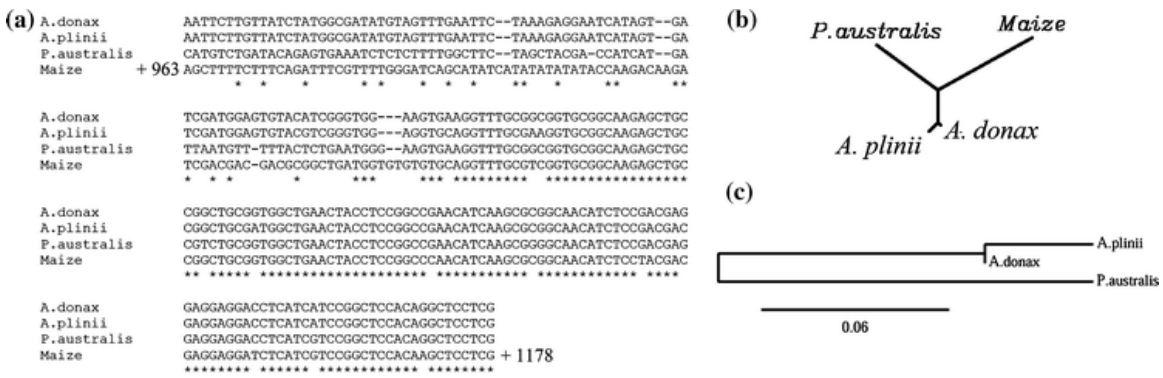

Figure 3 - Multiple sequence alignment among *A. donax*, *A. plinii*, *P. australis* and *Z. mays* sequences (a). *A. donax*, *A. plinii* and *P. australis* sequences were obtained amplifying genomic DNA using Pl1F and Pl1R primers specific for 5′ Pl1 gene, for the maize sequence we used the L19494 Pl accession. (b) Dendrogram obtained from the above alignment using ClustalW program and (c) dendrogram obtained using only the sequences of *A. donax*, *A. plinii* and *P. australis* carried out using Phylogeny.fr program.

Figure 4 - Phenotypic differences between the A18 clone with small diameter culms (**a**) and the A19 clone with large diameter culms (**b**). (**c**) Progenies obtained from clones selected and propagated by cane cuttings propagation (using mulch). The photos were taken in the experimental field of the University of Milano located in Landriano (N 45°18′, E 9°15′) in late July.

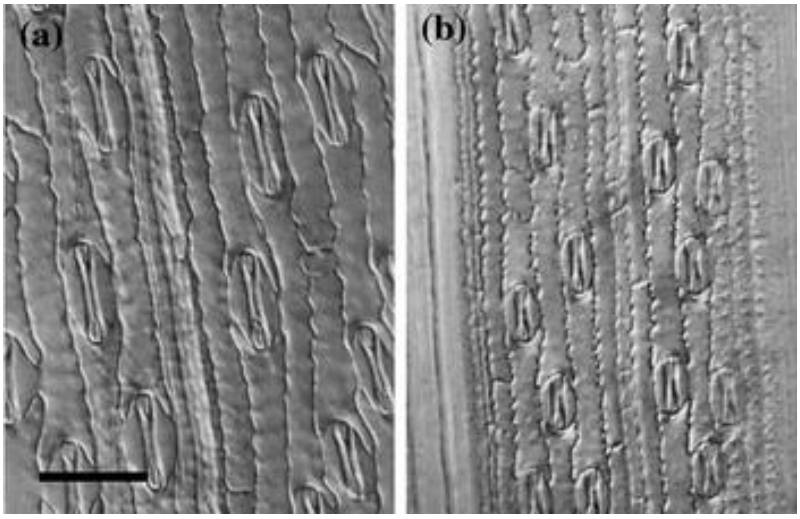

Figure 5 - Histological analysis of leaf epidermal cell (adaxial side of mature leaves) from *A. donax* (a) and *A. plinii* (b) plants cultivated in pots. *Bar* = 50 μm.

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III

Study on the inflorescence of *Arundo donax* L. clone sampled in Italy

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Study on the inflorescences of *Arundo donax* L. clone sampled in Italy

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Abstract

The giant reed, *Arundo donax* L. (Poaceae), is a perennial plant species widespread almost all over the world. It is not only considered as an invasive plant, but it also represents one of the most promising energy crops because of its vegetative vigor, high yields, and the low input required for its cultivation. Giant reed reproduces itself only agamically through rhizomes and cane fragments, usually transported by water or through human action. In this work, inflorescences from a collection of clones sampled all over Italy were studied. The resultsc onfirmed the sterility of *A. donax*: no pollen was observed and no seeds were found despite the large size of the inflorescences. Furthermore, 83 clones of the collection were characterized for their inflorescence size and flowering time. We found, for the first time, a significant correlation between the geographical coordinates (latitude) of sampling and the flowering time (r=-0.902 and r=-0.836, respectively at the 4th and at the 5th year after clones transplantation in our experimental farm). A survey of 15 clones, chosen to represent all the Italian territory, was also studied from the genetic point of view using 10 simple sequence repeats molecular markers, to further explore the genetic variability of this species in Italy, and to verify the presence of correlations with the phenotypic differences observed. Although PCA (Principal Component Analysis), AMOVA (Analysis of Molecular Variance), and the calculation of the average pairwise relatedness of populations analysis suggested the presence of three distinct clusters, no correlation was

observed between genetic data and inflorescences' phenotypic data.

Keywords: Arundo donax; Clones collection; Energy crop; Genetic diversity; Inflorescence.

1. Introduction

Arundo donax, commonly named giant cane or giant reed, is a rhizomatous perennial plant belonging to the Poaceae family (Arundinae tribe), growing almost all over the world in subtropical and temperate regions (Perdue, 1958; Faix et al., 1989). It is widespread in riparian zones and in highly disturbed ecosystems (Coffman et al., 2010) where it can compromise biodiversity (Herrera and Dudley, 2003) and increase wildfire and flooding risks (Quinn and Holt, 2008; Coffman et al., 2010; Giessow et al., 2011). Even though *Arundo donax* invasiveness represents a problem in several geographical regions, especially in California and Southern Europe (Perdue, 1958; Dudley, 2000), economic interest in this species has recently grown and it is now considered one of the most promising energy crops (Angelini et al., 2005, 2009). *A. donax* biomass can be used to produce biogas, second generation biofuels, such as bioethanol (Jeon et al., 2010; Adani et al., 2011), and solid biofuels such as chips and pellets for direct combustion (Dahl and Obernberger, 2004) and gasification (Jeguirim and Trove, 2009). The biomass can also be easily conserved for a longtime as silage, and used according to the industrial plant's needs. *A. donax* is a sterile plant (Bell, 1997; DiTomaso and Healey, 2003; Balogh et al., 2012; Bucci et al., 2013; Haddadchi et al., 2013), and it does not produce seeds even though the inflorescences, 30–100 cm long (Tucker, 1990), carry thousands of hermaphrodite flowers. So it reproduces itself only agamically (Decruyenaere and Holt, 2001; Ceotto and Di Candilo, 2010), through rhizome and stem fragments with at least one bud, which are transported by water or through human action that has largely contributed to *A. donax* dispersion, because of the mechanical characteristics of the culms (Perdue, 1958) that made them suitable for many different purposes (Perdue, 1958; Zohary, 1962; Pilu et al., 2012). Furthermore, recently land movement by bulldozers significantly contributes to *A. donax* diffusion (Boose and Holt, 1999; Boland, 2008). Because of its wide distribution, the center of origin of this species is still not certain: the Arundo genus is supposed to have originated in East Asia, reached the Mediterranean area and then spread all over the world (Polunin and Huxley, 1987; Ahmad et al., 2008; Mariani et al., 2010;

Hardion et al., 2012). However, the recent paper of Hardion et al. (2014[a,b]) would clearly indicate that *A. donax*'s center of origin is located in Middle East, confirming in parthypotheses on this question (Hardion et al., 2014[a]). The great vegetative vigor and the absence of fertile seeds suggest that this plant is probably a young polyploid species (Pizzolongo, 1962; Bucci et al., 2013; Haddadchi et al., 2013). In studies on genetic variability performed using molecular markers, Haddadchi et al. (2013) found a higher genetic diversity in Australia than that previously reported in other countries (Ahmad et al., 2008; Khudamrongsawat et al., 2004; Mariani et al., 2010), obtaining a G (distinguished genotypes)/N (number of samples) proportion of 0.83 using ISSR markers.

Because of its sterility, as previously reported, *A. donax* can be propagated to establish new fields, only by agamic way using rhizome, stem fragments (Ceotto and Di Candilo, 2010; Pilu et al., 2013; Corno et al., 2014), and in vitro culture techniques (Takahashi et al., 2010). All these methods are expensive, considering that a field establishment rates, for biomass production, may vary from about 2500 to 10,000–20,000 propagules per hectare (Ceotto and Di Candilo, 2010; Pilu et al., 2013). On the other hand, giant reed sterility could be also considered a favorable trait for biomass production, as it prevents the propagation of this plant through seeds in places other than the ones where it is cultivated (Balogh et al., 2012).

Despite their low genetic variability, the different clones show some phenotypic differences, as reported by Cosentino et al. (2005) in Southern Italy and by Pilu et al. (2014) in Northern Italy: differences among clones could be exploited to improve *A. donax* performances as an energy crop, in fact some of the monitored characters seem to be heritable (e.g., culm height and culm diameter) (Pilu et al., 2014). The genetic improvement of this crop can be addressed by finding traits useful to obtain a better energy crop, modifying its chemical composition, increasing biomass production and the capacity to be propagated efficiently by in vitro culture or by propagules. Such genetic improvement, considering its sterility, will be mainly based on clonal selection (Pilu et al., 2013). For this reason, various groups are exploring the genetic variability of this species as well as other genetic improvement techniques based on physical and chemical mutagenesis and on genetic transformation (Takahashi et al., 2010; Dhir et al., 2010; Pilu et al., 2013). Another important phenotypic character to take in consideration in genetic improvement programs is the flowering time because the development of the inflorescence corresponds with the end of the vegetative growing season, hence representing an

important factor which affects biomass yield. The *Arundo donax* inflorescence is a big single terminal plume-like panicle, highly branched, carrying many flower spikelets: *A. donax* develops one inflorescence for each cane at the end of the growing season, usually in September–October in our latitudes.

So far, no paper regarding the study of development, structure, and inflorescence time has been published on *A. donax*. In this work, we studied a collection of *A. donax* clones, in particular looking at the inflorescence structure in terms of size, number of spikelets, sterility, flowering time, and the genetic structure of a subset of clones representing the Italian territory.

2. Materials and methods

2.1. Arundo donax clones collection

The plant material was represented by 84 putative clones (Table S1), whose rhizomes fragments were sampled on the Italian territory at different latitudes (Northern, Central and Southern Italy) in the period 2010–2011, and grown on in the experimental field of the University of Milan situated in Landriano (N 45°180', E 9°150'). The clones of the collection were propagated using rhizome fragments whose weight was 300 to 500 g. Rhizomes were transplanted about 20 cm below the soil surface. The ground was kept free of weeds during the first year after transplantation and plants were irrigated as needed.

2.2. Inflorescence measurements

Forty nine (49) inflorescences were randomly sampled at maturity from different clones of the collection and air dried at room temperature. The primary branches of nine randomly sampled inflorescences were taken off the main rachis and weighed. The primary branches (directly attached to the main rachis) of a single inflorescence were measured considering length (cm), weight (g) and number. All the spikelets which they carried were manually removed and counted.

2.3. Check for the presence of pollen and seeds

Eighty four (84) clones of the collection were shaken during flowering to look for the presence of pollen. To check the presence of seeds, 49 inflorescences, randomly sampled at maturity from different clones, were shaken over a white sheet and the residues fallen were observed using a magnifying lens.

2.4. Study of clones flowering

Eighty four (84) clones of the collection were observed at the end of the growing season in 2013 (1 October) and in 2014 (21 September), respectively at the 4th and at the 5th year after sampling around Italy. They were classified for the developmental stage of their inflorescences according to the following scheme:

(1) Totally absent,

(2) Barely visible,

(3) Partially opened,

(4) Completely opened,

(5) Mature (dry),

Inflorescences were also classified on the basis of their size into two classes: class 1,\45 cm in length, approximately;

class 2,[45 cm in length, approximately.

2.5. PCR amplification

Leaves from 15 *A. donax* clones chosen to represent all the Italian territory were sampled. Leaves were crushed and the DNA extraction was performed adopting a protocol developed for maize (Dellaporta et al., 1983). DNA samples were amplified using 10 simple-sequence repeats (SSRs) molecular markers previously used by Tarin et al. (2013). The 10 SSR markers were as follows: Ad_B7, Ad_G8, Ad_A3, Ad_B1, Ad_F8, Ad4_13, Ad_E10, Ad5_4, Ad4_6, Ad_H1. All the primers sequences and the corresponding Tm are shown in Table S5.

PCRs were performed in a final volume of 10 ll, containing 10 ng DNA, 0.1 llM of each primer (forward and reverse), 200 mM of each dNTP, 2.5 mM MgCl2, and one unit of Taq DNA polymerase (Promega, Madison, WI). Reactions were carried out in a thermocycler, programmed for one pre-cycle at 94 _C for 2 min, followed by 35 cycles at 94 _C for 1 min, specific annealing temperature of the primers for 1 min, 72 _C for 1 min, and a final step at 72 _C for 5 min. The amplified fragments were fractionated by electrophoresis using 3 % (w/v) agarose gels and stained with ethidium bromide (0.1 lg/ll).

2.6. Genetic analysis

Each band obtained by PCR was considered as an independent locus; a different number was attributed to each allele. The generated matrix was used for statistical analyses.

Principal Coordinate Analysis (PCA), Analysis of Molecular Variance (AMOVA), and Mantel analysis were performed using the program GenALEx v. 6.2 (Peakall and Smouse, 2006).

3. Results

3.1. Inflorescence structure

A. donax is considered a sterile plant, nevertheless it is able to produce a big inflorescences that can reach the length of 1 m (Fig. 1). Flowering takes place at the end of the growing season, between August and November at our latitudes: the inflorescence matures progressively and the glumes' color progressively changes from pale green to purple-reddish. After drying they turn chestnut-brown/ yellow at the end of the growing season. The inflorescences, that progressively rise from the culms' apices, are constituted by a main rachis to which are attached the primary branches and the secondary ones that carry the spikelet pairs (Fig. 2). When the plume-like inflorescences are completely opened, the thousands of hermaphrodite flowers emit anthers (Fig. 3). With the aim to better characterize the inflorescence architecture, a dry inflorescence of about 80 cm was measured and all its spikelets were counted (Table S2). The inflorescence was composed of 127 primary branches carrying 15,270 spikelets, and on average, every single branch carried 120 spikelets. The total weight of the branches was 38.13 g with an average value of 0.3 g for single branch (Table 1). The net weight of all the spikelets was 27.2 g, so the average weight of a single dry spikelet was 1.78 mg. Hence the spikelets were found to represent the greater part of the primary branches' total weight (71.15 % on average); the remainder (28.85 %) was represented by the branches to which the spikelets were attached. The primary branch length was found to be very variable, with an average value of 20.6 cm on the measured inflorescence (Table 1): in the middle portion of the inflorescence it could be over 30 cm in length, while the ones near the inflorescence apex were found to be shorter (3–4 cm).

3.2. Study of Arundo donax sterility

To definitely assess if the *A. donax* is sterile, we characterized a clones collection representing all the Italian territory: 84 clones were sampled at different latitudes and their rhizome fragments were transplanted and cultivated in the experimental field of the University of Milan (N 45°180', E 9°150') (Table S1). The clones were observed during

flowering, by observing anthers' emission from the spikelets (Fig. 3), but despite this, no pollen has ever been observed. Furthermore, to check on the eventual presence of seeds, 49 of the biggest inflorescences were randomly sampled from *A. donax* plants from the clones collection. The residues obtained by shaking the inflorescences over a white sheet were observed, looking for seeds, also using a magnifying lens. No seed was found. With the purpose of expressing the data on sterility according to the number of spikelets present on the inflorescences analyzed, we used the regression equations obtained by the correlations between the number of spikelets carried by the inflorescences, and two parameters which were easier to measure, the branch length and branch weight (Fig. 5). Hence to avoid a long process of counting of the spikelets present on the inflorescence, we used the following regression equation: considering (X) the branch weight (g) and (Y) the number of spikelets, we obtained the following: Y=377.1X+6.99 and considering (X) the branch length (cm), and (Y) the number of spikelets; the following equation was obtained Y=10.65X-99.2. The correlation between the primary branches weight and the number of spikelets was found to be higher than the one between the primary branches length and the number of spikelets, as attested by the Spearman correlation coefficients, respectively, r=0.976 and r=0.845. Hence we decide to verify the reliability of the best regression equation obtained using the primary branches' weight: 20 primary branches, randomly sampled from different inflorescences, were weighed and the spikelets which they carried were counted (Table 2). The total number of spikelets was 4995, and the expected figure, calculated by the addition of the values referring to the single primary branches, was 5672.74. The average ratio between the expected and observed numbers of spikelets was 1.15. On the basis of these data, the equation was then modified to better estimate the number of spikelets obtaining, the new equation Y=(377.16X+6.99)*0.869 where (X) was the branch length (cm) and (Y) the number of spikelets, the 0.869 factor was calculated as the ratio 1/1.15 (Table 2). Hence we determined the average weight (49.80 g) of dry primary branches (X) weighing all the primary branches from 9 biggest inflorescences randomly sampled from the collection (data not shown). The average number of spikelets (Y) carried by a single inflorescence was then estimated, using the equation Y=(377.16X+6.99)*0.869, and amounted to about 16,331, a value very similar to that counted in Table 1 (15,270). Finally using these data, we estimated the number of spikelets carried by the 49 inflorescences (obtained by multiplication 49*16,331) shaken without finding any seed: 800,219.

3.3. Inflorescence characterization: size and flowering time

Arundo donax inflorescences are very big as they can reach or be longer than 80 cm in length. Two classes were established to classify inflorescences by their length: class 1\45 cm; class 2[45 cm (Fig. 4).

Eighty three clones of the collection were observed at the end of the growing season at the fifth year after rhizome transplantation (spring 2010) and classified considering their inflorescence size (Table S3). No correlation was found between the size of the inflorescences and the geographical coordinates of the clones' sampling locations (data not shown).

The collected clones were also classified in five classes considering the developmental stage of their inflorescences to evaluate flowering time (Table S3). Clones were grouped considering the latitude coordinates of their sampling locations, and the average value of the inflorescences' developmental stage was calculated for each group (Table 3). A statistically significant negative correlation was found between the average inflorescences' developmental stage and the latitude in degrees, both at the first and at the second year after transplantation (Fig. 6), showing r values of -0.902 and -0.836, respectively.

3.4. Genetic characterization using SSRs molecular markers

With the aim to find a correlation among the inflorescence data and the genetic structure of the clones collected, 15 *Arundo donax* clones, chosen to represent all the Italian territory, (Fig. 7a and Table S4) were characterized using 10 simple sequence repeats (SSRs) molecular markers previously used by Tarin et al. (2013) (Table S5 and S6 and Figure S1). We found among the analyzed clones seven different alleles using Ad_B7, three alleles using Ad_G8, Ad_A3, Ad_B1, Ad_F8, Ad4_13, Ad5_4, Ad4_6 and two alleles using Ad_E10, Ad_H1 SSR molecular markers. The analysis showed that the AD68 and Ad71 clones are undistinguishable while the other carried a unique molecular pattern (Table S6). Hence the ratio between the number of distinguishable genotypes (G=14) and the number of samples (N=15) was 0.933 (G/N).

Genetic diversity within the clones was observed, as attested by the principal coordinate analysis (PCA), suggesting the presence of three distinct clusters (Fig. 7b). The molecular variance among these clusters was 53 %, while within clusters it was 47 % (AMOVA, PhiPT value: 0.526 P\ 0.010). The calculation of the average pairwise relatedness of populations, and statistical testing by random permutation (permutation=999,

bootstraps=999) showed also an indication of the presence of these clusters (Fig. 7 c). However, the three clusters did not reflect the sampling geographical location of the clone as shown by the Mantel test performed using the genetic and geographical distance matrix: no statistical relationship was found between the two matrices (R2=0.0087). Furthermore, no significant differences were observed among the three clusters neither considering the flowering time nor considering the inflorescence size (data not shown).

4. Discussion

Arundo donax was studied, at first, because of its invasiveness that makes its presence a problem in several geographical regions, especially in California and Southern Europe (Perdue, 1958; Dudley, 2000; Giessow et al., 2011). This characteristic can also be considered an advantage when using *A. donax* as an energy crop. In particular in Mediterranean regions, it appears to be the best perennial energy crop in comparative studies on biomass yield (Lewandowski et al., 2003), so the economic interest for this species has recently increased. Despite its invasiveness, *A. donax* is considered a sterile plant even if it is able to produce inflorescences carrying thousands of hermaphrodite flowers (Perdue, 1958; DiTomaso and Healey, 2003; Dudley, 2000). The inflorescence structure is typical of the Poaceae family, with a main lignified rachis to which are attached several primary branches (Figures 1–4). Usually 2 to 4 primary branches are grouped with a single point of attachment. Primary branches are often further ramified, and their length may be variable from a few to more than 30 cm. The longer ones are usually attached to the middle portion of the inflorescence. Primary branches carry thousand of spikelets, whose color turns from pale green to purple-reddish during maturation.

At maturity small yellow anthers, similar in shape and size to those of Zea mays, are emitted from the spikelets (Fig. 3). Despite the big inflorescence, *A. donax* seems to be sterile as reported previously by several authors (Perdue, 1958; Pizzolongo, 1962; Dudley, 2000; DiTomaso and Healey, 2003; Balogh et al., 2012 ; Hardion et al., 2012; Bucci et al., 2013; Haddadchi et al., 2013). In this work, to definitively assess this issue on Italian territory, in fact to ascertain *A. donax* sterility, 83 clones sampled in Italy territory at different latitudes were observed in our collection during flowering, looking for pollen. Even though the anthers are emitted, pollen was not observed on *Arundo donax* inflorescences, but only on the inflorescences of the related species *Arundo plinii* which was cultivated in the same experimental field (data not shown). Furthermore, with the aim

to find seeds, 49 inflorescences randomly sampled from the clones of the collection were shaken over a white sheet and the residues fallen were observed using a magnifying lens but nevertheless no seed was found.

Estimating the total number of observed spikelets by a regression equation (weight of the primary branches and number of carried spikelets) allows us to assert that no seeds were observed on about 800,200 spikelets (Table 2).

However in Iran, Afghanistan, China, and Pakistan caryopses have been found in this species probably due to a low level of ploidy (Hardion et al., 2014[a]).

In fact this sterility could be linked to the ploidy level, this species seems to have 110 chromosomes in Italy (Pizzolongo, 1962; Bucci et al., 2013), 108 chromosomes in India (Christopher and Abraham, 1971), and 84 chromosomes in Australia (Haddadchi et al., 2013). Differences in ploidy levels, found by different authors, could be linked to the evolutionary history of this species which is probably very recently evolved from the phylogenetic point of view (Hardion et al., 2014[a,b]).

Similar results have also been observed when considering the related species Phragmites australis (Cav.), showing different euploid numbers, between 3x and 12x, depending on the sampling location: in Europe these plants are tetraploids with 48 chromosomes, while in Asia they are usually octoploids with 96 chromosomes (Clevering and Lissner, 1999). Recently, another explanation of the sterility of Arundo taxa was suggested by Hardion et al. (2015) based on the fact that high ploidy levels not always induce sterility. In fact growing *A. plini* having ploidy level of 129 and 189 and A. donaciformis (189), the authors found seeds only in ex situ admixed condition while in ex situ isolation no seeds were recovered (Hardion et al., 2015). This result strongly suggests the presence of autoincompatibility processes in the genus as reported previously in Phragmites (Ishii and Kadono, 2002). However, as showed also in this work, being *Arundo donax* enable to produce pollen we can assert that the sterility in this species is due to high ploidy levels or other causes so far not yet identified.

Another aim of this work was to understand if there was difference among the clones, transplanted in the same field, concerning the date of flowering. Comparing 83 clones and considering their inflorescences' developmental stages (Table S3), a high negative correlation between this parameter and the latitude of their sampling location was observed: r values of - 0.902 and - 0.836, respectively, at the fourth and at the fifth year after rhizomes transplantation (Fig. 6 and Table 3). To the best of our knowledge, this

result represents the first evidence of a correlation between a phenotypic character and the geographical position of sampling in *A. donax*. These results agree with those obtained by Lim et al. (2014), who found a negative correlation between the sampling latitude and different phenotypic characteristics: stem diameter (r=-0.495), stem dry weight (r=-0.393), heading date (r=-0.914) and estimated yield (r=-0.425), in 66 clones of the related genus Miscanthus sampled in East Asia.

Flowering time represents the end of the growing season for the vegetative canes, so we supposed there might exist a negative correlation between the sampling latitude and the yield, as found in Miscanthus by Lim et al. (2014). Nevertheless this correlation was not observed for the Italian clones studied (data not shown). The observed gap in flowering time among clones sampled at different latitudes is only of a few days, so the difference is probably too limited to have a visible effect on yield, while this latter parameter could also be influenced by a number of other genetic and environmental factors. However, further studies will be necessary to assess this issue, in particular clones sampled both in subtropical and in warm-temperature regions need to be compared, as a wider difference in the sampling latitude will probably amplify the effect on flowering time.

The strong correlation between latitude of sampling and flowering time seems to indicate a phenomenon of adaptation of the clones to the environmental conditions, maybe related to the photoperiod or to the temperatures. In this case, the ability of the species to adapt the length of the life cycle to the environmental conditions may be rather surprising, especially if we consider that propagation is only agamic in this species, so genetic variability among clones is expected to be limited. Moreover, a correlation characterized by such high r values is surprising considering that in the last centuries human action has largely contributed to *Arundo donax* diffusion on the Italian territory and elsewhere, mixing the clones taken from different environments. So we cannot exclude the idea that the differences observed and the high correlation found may be due to relatively fast epigenetic modifications caused by the particular environments where the plants were sampled (Baulcombe and Dean, 2014).

In fact it is well known that the inflorescence architecture, on which the reproductive success of a species normally depends, is determined by the inflorescence meristem that is heavily influenced by environmental and developmental signals (Teo et al., 2014). The promotion of flowering as a consequence of vernalization (the exposure of the developing plant to a period of cold temperature) is a process which is epigenetically controlled and

the memory of the vernalized state of the key flowering genes is reset in the next sexual generation (Dennis and Peacock, 2007). The epigenetic modifications of the key regulatory genes are also known to be responsible for the variations of flowering time observed in polyploids (Mayfield et al., 2011).

Also the induction of flowering by vernalization, effective in many species in temperate regions, is the consequence of the epigenetic modification of the key flowering genes. This modification is inherited through mitotic division during the development of the plant until the transformation of the vegetative meristem to reproductive meristem. With the production of the inflorescence the flowering genes return to their original activity state (Dennis and Peacock, 2007). Hence we can thus suppose that in *A. donax*, the absence of sexual reproduction may prevent the resetting of the epigenetic modification of the flowering genes that can maintain the epigenetic effects on their activity, and thus the consequently altered flowering time, for longer.

Nevertheless, if these differences were due to epigenetic modifications, they could tend to get lost during the following years, making genetic improvement by clonal selection in vain. However, as shown in this study, the observed differences in the flowering time among clones persisted for at least 5 years.

As previously reported, the genetic variability in this species is very low: the highest genetic variability was found in Australia by Haddadchi et al. (2013), obtaining a G/N proportion of 0.83 using ISSR markers. In other countries, a lower diversity has been reported (Khudamrongsawat et al., 2004; Ahmad et al., 2008; Mariani et al., 2010). The lowest genetic variability has been observed in the United States of America (Ahmad et al., 2008), where this species was probably introduced only recently, undergoing a bottleneck that further reduced the genetic variability, compared with the giant reed observed in Europe (Polunin and Huxley, 1987; Lewandowski et al., 2003; Ahmad et al., 2008; Mariani et al., 2010). Recently Hardion et al. (2014[a,b]) studied the origin of *A. donax* using chloroplast DNA sequencing and morphometric analysis on 127 herbarium specimens collected across subtropical Eurasia. The analysis showed that out of 13 haplotypes identified, only one (the more robust morphotype distinguishable from all other Asian samples) is shared with invasive samples worldwide, and its nearest phylogenetic relatives are located in the Middle East (Hardion et al., 2014[a]).

In our study, we also used molecular markers to try to find a correlation between genetic structure and inflorescence phenotype. However, using a subset of 15 clones from our

collection, chosen to represent all the Italian territory (Fig. 7a and Table S4) and 10 simple sequence repeats molecular markers (Table S5), we did not observe any correlation (data not shown), although PCA (Fig. 7 b), AMOVA analysis and the following calculation of the average pairwise relatedness of populations (Fig. 7c), suggested that the 15 clones considered could be divided into three distinct clusters. Hence the three clusters neither correlated with the sampling geographical location of the clones nor inflorescences size and the flowering stage of clones (data not shown). Of course this result will be strengthened by analyzing more samples from our collection and from other countries and using a sequencer to size the alleles found, in fact using agarose gels we could lose some polymorphisms/alleles (the capacity to discriminate of agarose gels is limited, in the best conditions, to 4-6 bp).

Further work is necessary to assess whether the phenotypic differences among clones will be maintained in the following years. In particular: are we observing epigenetic variation or stable genetic variation? The answer to this question will be very useful in future clonal selection programs.

To conclude, *A. donax* is assuming an important role in renewable energy production based on biomass, as its cultivation represents a cheap and efficient way to produce bioenergy. However, some important issues related to its sterility emerge. First of all, it will be necessary to define how to propagate this culture in the fastest and cheapest way, since it reproduces itself only agamically (Decruyenaere and Holt, 2001; Ceotto and Di Candilo, 2010). Sterility will also represent a problem for future genetic improvement, forcing us to base it mainly on clonal selection. For this reason, it will be necessary to better explore the genetic variability among clones and to assess whether clonal selection can be effective in the long term.

Acknowledgment

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Table 1 – Measurements of an *Arundo donax* inflorescence

	Average values	Entire inflorescence
Number of primary branches		127
Primary branch length (cm)	20.6±9.21	2616.40
Primary branch weight (g)	0.3±0.3	38.131
Number of spikelets	120±116	15,27

Data referred to a single inflorescence air dried at room temperature of 80 cm length. Standard deviation is shown.

Table 2 – Estimation of number of spikelets present on primary branches by regression equation.

Primary branch	Weight (g)	Number of spikelets		Expected/Observed ratio	Number of spikelets Expected using 0.869 correction factor[b]
		Observed	Expected[a]		
1	1.73	486	659.48	1.36	573.09
2	1.64	510	625.53	1.23	543.59
3	1.40	387	535.01	1.39	464.92
4	1.29	409	493.53	1.21	428.88
5	1.26	411	482.21	1.17	419.04
6	0.82	266	316.26	1.19	274.83
7	0.72	289	278.55	0.96	242.06
8	0.72	242	278.55	1.16	242.06
9	0.69	219	267.23	1.23	232.22
10	0.69	362	267.23	0.74	232.22
11	0.54	235	210.66	0.90	183.06
12	0.52	162	203.11	1.26	176.50
13	0.52	182	203.11	1.13	176.50
14	0.44	243	172.94	0.72	150.28
15	0.43	107	169.17	1.58	147.01
16	0.42	185	165.40	0.91	143.73
17	0.33	101	131.45	1.31	114.23
18	0.19	69	78.65	1.15	68.35
19	0.19	91	78.65	0.88	68.35
20	0.13	39	56.02	1.52	48.68
Average	0.73±0.48[c]	249.75±139.67	283.63±181.09	1.15	246.48±157.37
Total	14.67	4995	5672.74		4929.61
R^d	0.947				

Comparison between the observed and the expected number of spikelets carried by single primary branches randomly taken from different inflorescences. Data refer to air dried primary branches

[a] The number of spikelets is estimated using the following equation: Y = (377.16X ? 6.99); (X = weight of the primary branch, Y = number of spikelets)

[b] Estimation obtained using the equation corrected introducing a 0.869 factor (obtained as 1/1.15, representing the average ratio between the expected y values using the first equation, and the observed ones), as follows: Y = (377.16X ? 6.99) 9 0.869

[c] Average values and standard deviations are shown

R^d Spearman Correlation

Table 3 – Latitude and flowering stage.

Latitude (N)	Flowering stage	
	Fourth year	Fifth year
45°	1.33	1.69
44°	1.08	2.33
43°	1.29	1.57
42°	1.67	2.33
41°	1.43	2.86
40°	2.19	2.25
39°	2.08	3.17
38°	2.50	4.00

Comparison among the clones' sampling latitude and the average developmental stage of their inflorescences (data taken as shown in Table S3). Data were collected on 01/10/13 (fourth year after transplantation) and on 21/09/14 (fifth year).

Figures 1–4 – Size and structure of the *Arundo donax* inflorescence. Mature inflorescence (1), from left to right, primary branch, secondary branches and spikelet (2). Anthers, Bar = 1 cm (3). Classes of inflorescence size used in this work (4): less than 45 cm in length, corresponding to the class 1 (on the right), and over 45 cm in length, corresponding to the class 2 (two inflorescences on the left).

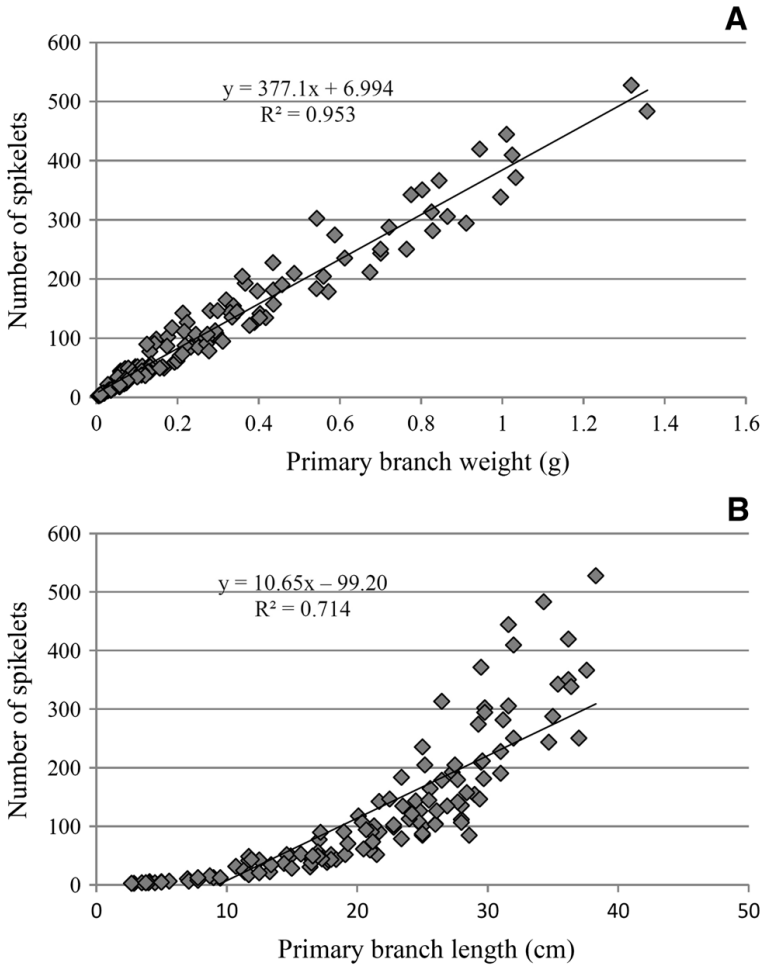

Figure 5 – Fig. 5 Correlation between the primary branch weight (g) and the number of spikelets carried (a). Correlation between the primary branch length (cm) and the number of spikelets carried (b). Data refer to primary branches carried by a single inflorescence.

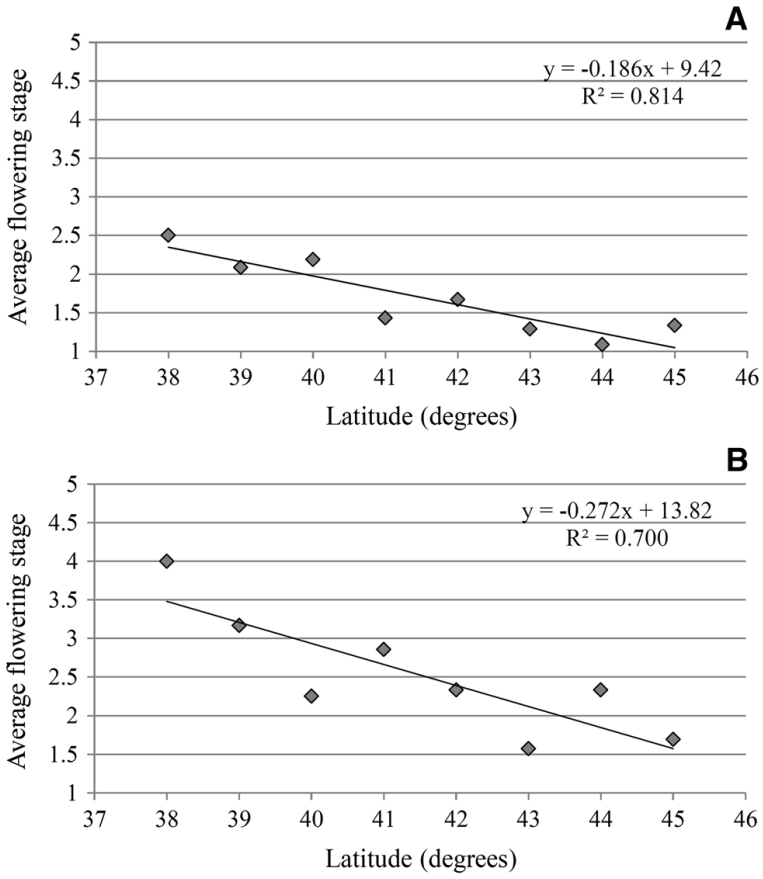

Figure 6 – Correlation among the clones' sampling latitude and the average flowering stage, at the fourth (a) and at the fifth (b) year after rhizomes' transplantation. The inflorescence developmental stage is indicated as follows: (1) Totally absent, (2) Barely visible, (3) Partially opened, (4) Completely opened, (5) Mature (dry). Data were collected on 10/10/13 (fourth year) and on 21/09/14 (fifth year).

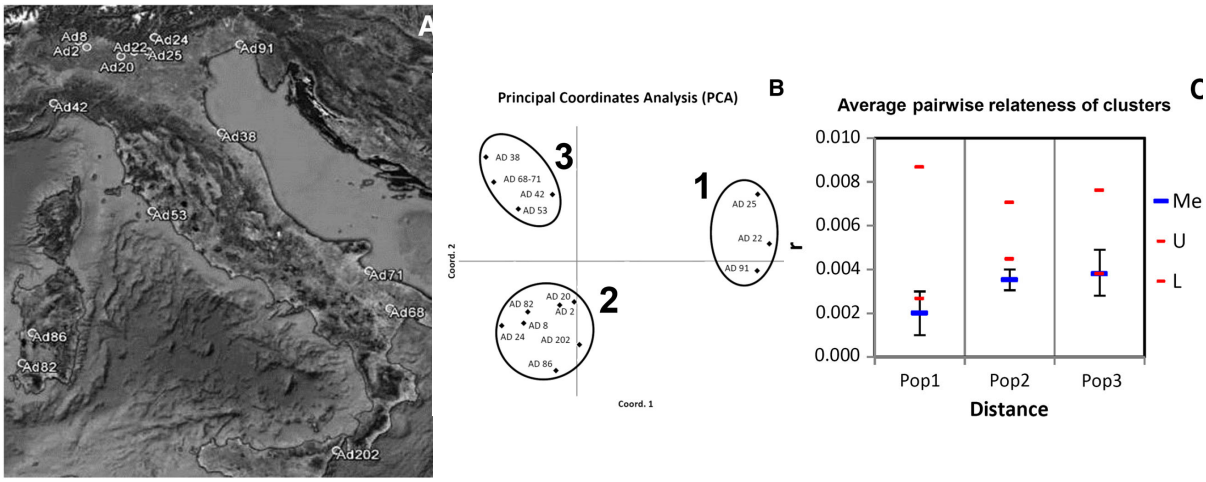

Figure 7 – Map showing the sampling locations of the clones studied through molecular analysis using 10 SSRs molecular markers(a). Principal Coordinates Analysis (PCA) of the clones compared in this study based on Eigen value showing the presence of three clusters (b). Calculation of the average pairwise relatedness of populations, and statistical testing by random permutation (c). Upper (U) and lower (L) confidence limits bound the 95 % confidence interval about the null hypothesis of 'No Difference' across the clusters/populations as determined by permutation (permutation = 999, bootstraps = 999).

Table S1 – Plant material and sampling location. The clones collection is situated in Landriano (PV), Italy (N 45°18', E 9°15'). The 84 clones were sampled in Northern, Central and Southern Italy, years 2010-2011.

Clone	Place	Latitude	Longitude
Ad1	Buccinasco (MI)	45°24'	09°06'
Ad2	Cassina de Pecchi (MI)	45°31'	09°22'
Ad3	Calderara di Reno (BO)	44°32'	11°16'
Ad4	Sforzesca (PV)	45°17'	08°52'
Ad5	Cisliano (MI)	45°26'	08°58'
Ad6	Marudo (LO)	45°16'	09°18'
Ad7	Rozzano (MI)	45°22'	09°08'
Ad8	Cesano Maderno (MB)	45°37'	09°09'
Ad9	Boffalora Sopra Ticino (MI)	45°27'	08°49'
Ad10	Vittuone (MI)	45°28'	08°57'
Ad12	Piacenza	45°01'	09°38'
Ad13	Gazzola (PC)	44°58'	09°34'
Ad15	Civesino (MI)	45°23'	09°15'
Ad16	Alessandria	44°56'	08°37'
Ad17	Bergamo	45°40'	09°38'
Ad18	Scaldasole (PV)	45°07'	08°54'
Ad19	Brescia	45°32'	10°10'
Ad20	Leno (BS)	45°20'	10°16'
Ad21	Calcinato (BS)	45°28'	10°24'
Ad22	Desenzano del Garda (BS)	45°26'	10°37'
Ad23	Nago Torbole (TN)	45°52'	10°52'
Ad24	Ala (TN)	45°44'	10°58'
Ad25	Pescantina (VR)	45°29'	10°51'
Ad26	Chiaravalle (AN)	43°35'	13°21'
Ad27	Mantova	45°09'	10°47'
Ad28	Colombarolo (CR)	45°07'	10°29'
Ad29	Cremona	45°10'	09°57'
Ad30	Spino D'Adda (CR)	45°24'	09°28'
Ad31	Sozzano (BS)	45°39'	10° 12'
Ad32	Civitanova Marche (MC)	43°57'	13°43'
Ad33	Trezzano sul Naviglio (MI)	45°25'	09°03'
Ad34	Castelnuovo Scrivia (AL)	44°59'	08°53'
Ad35	Torricella Verzate (PV)	45°01'	09°10'
Ad36	Fidenza (PR)	44°52'	10°05'
Ad37	San Benedetto Tronto (AP)	42°53'	13°53'
Ad38	Cattolica (RN)	43°57'	12°42'
Ad39	Roseto Abruzzi (TE)	42°39'	13°59'
Ad40	Landriano (PV)	45°19'	09°15'
Ad42	Arenzano (GE)	44°23'	08°40'
Ad43	Montale (SP)	44°11'	09°37'

Table S1 – (...continue)

Clone	Place	Latitude	Longitude
Ad44	Corrodano (SP)	44°14'	09°38'
Ad45	Ovada (AL)	44°37'	08°38'
Ad46	Verzi - Loano (SV)	44°09'	08°14'
Ad47	Forlì	44°20'	12°09'
Ad48	Massa	43°57'	10°11'
Ad49	Fauglia (PI)	43°32'	10°28'
Ad50	Cecina (LI)	43°17'	10°32'
Ad51	Venturina (LI)	43°01'	10°35'
Ad53	Orbetello (GR)	42°26'	11°10'
Ad57	Fiumicino (RM)	41°44'	12°16'
Ad58	Ardea (RM)	41°34'	12°30'
Ad61	Cellole (CE)	41°18'	13°29'
Ad62	Sessa Aurunca (CE)	41°13'	13°54'
Ad63	Sparanise (CE)	41°10'	14°07'
Ad64	Palma Campania (NA)	40°51'	14°32'
Ad66	Balvano (PZ)	40°36'	15°32'
Ad67	Vaglio Basilicata (PZ)	40°39'	15°56'
Ad68	Matera	40°39'	16°35'
Ad69	Bari	40°56'	16°38'
Ad71	Margherita di Savoia (BT)	41°23'	16°06'
Ad72	Foggia	41°28'	15°40'
Ad73	Begliano (TS)	45°49'	13°27'
Ad74	Bellano (LC)	45°59'	09°15'
Ad75	Spagnoi (VR)	45°27'	10°45'
Ad76	Laterza (TA)	40°37'	16°48'
Ad77	Sternata (LE)	40°13'	18°14'
Ad78	Tricase (LE)	39°57'	18°24'
Ad79	Curtarolo (PD)	45°31'	11°49'
Ad80	Musei (CI)	39°18'	08°41'
Ad81	Iglesias	39°18'	08°33'
Ad82	Fluminimaggiore (CI)	39°25'	08°30'
Ad85	Tanca Marchese (OR)	39°44'	08°35'
Ad86	Bauladu (OR)	40°02'	08°41'
Ad87	Elmas (CA)	39°16'	09°04'
Ad88	Lurago d'Erba (CO)	45°45'	09°13'
Ad89	Campalto (VE)	45°29'	12°16'
Ad90	Meolo (VE)	45°34'	12°27'
Ad91	Lignano Sabbiadoro (UD)	45°39'	13°04'
Ad92	Chiarisacco (UD)	45°50'	13°13'
Ad93	Codevigo (PD)	45°15'	12°07'
Ad94	Recetto (NO)	45°27'	08°26'
Ad95	Aspra (PA)	38°06'	13°30'
Ad96	Orbassano (TO)	44°59'	07°30'
Ad202	Nizza di Sicilia (ME)	37°59'	15°24'

Table S2 – Measurement of an *Arundo donax* inflorescence. Data refer to the primary branches of a single inflorescence, of about 80 cm in length, air dried at room temperature. The primary branches are considered from their attachment to the main rachis. Average values and standard deviations are shown.

Primary branch	Length (cm)	Weight (g)	Number of spikelets
1	38.3	1.318	527
2	37.6	0.844	366
3	37	0.700	250
4	36.4	0.996	338
5	36.2	0.803	350
6	36.2	0.944	419
7	35.4	0.775	342
8	35	0.721	287
9	34.7	0.701	243
10	34.3	1.357	483
11	32	1.024	409
12	32	0.764	250
13	31.6	1.010	444
14	31.6	0.864	305
15	31.2	0.828	281
16	31	0.435	227
17	31	0.457	190
18	29.8	0.543	302
19	29.8	0.911	294
20	29.7	0.436	181
21	29.6	0.674	211
22	29.5	0.487	209
23	29.5	1.033	371
24	29.4	0.298	146
25	29.3	0.587	274
26	29	0.337	154
27	28.6	0.251	84
28	28.4	0.436	157
29	28	0.217	111
30	28	0.334	135
31	28	0.245	106
32	27.7	0.403	141
33	27.7	0.396	179
34	27.5	0.559	204
35	27.3	0.367	192
36	26.9	0.417	134
37	26.5	0.826	313

Table S2 – (…continue)

Primary branch	Length (cm)	Weight (g)	Number of spikelets
38	26.5	0.571	178
39	26.1	0.389	126
40	26	0.294	104
41	26	0.279	103
42	25.6	0.320	164
43	25.5	0.346	144
44	25.2	0.360	204
45	25	0.174	86
46	25	0.272	100
47	25	0.232	84
48	25	0.218	87
49	25	0.612	235
50	24.8	0.224	126
51	24.7	0.294	108
52	24.5	0.329	143
53	24.2	0.377	121
54	24	0.293	112
55	23.5	0.402	134
56	23.4	0.542	183
57	23.4	0.277	78
58	22.8	0.148	98
59	22.8	0.176	102
60	22.5	0.280	146
61	21.7	0.213	142
62	21.7	0.143	90
63	21.5	0.096	51
64	21.3	0.269	100
65	21.2	0.212	73
66	21.1	0.272	90
67	21	0.193	59
68	20.7	0.311	94
69	20.5	0.199	61
70	20.4	0.273	106
71	20.1	0.187	117
72	19.3	0.206	70
73	19.1	0.163	51
74	19	0.143	90
75	18.4	0.132	43
76	18	0.132	51
77	18	0.127	43
78	17.8	0.118	42
79	17.7	0.095	38

Table S2 – (…continue)

Primary branch	Length (cm)	Weight (g)	Number of spikelets
80	17.4	0.125	46
81	17.3	0.112	44
82	17.2	0.125	89
83	17.1	0.132	77
84	17	0.135	56
85	17	0.166	49
86	16.6	0.089	44
87	16.6	0.156	49
88	16.5	0.060	44
89	16.5	0.111	37
90	16.4	0.072	30
91	15.7	0.113	52
92	15	0.075	28
93	14.9	0.079	49
94	14.6	0.101	51
95	14.4	0.120	36
96	13.4	0.053	34
97	13.4	0.100	34
98	13.3	0.068	22
99	12.5	0.061	42
100	12.5	0.057	20
101	11.9	0.077	43
102	11.7	0.060	41
103	11.7	0.071	48
104	11.7	0.057	17
105	11.4	0.029	21
106	11.3	0.073	24
107	10.7	0.052	31
108	9.5	0.036	11
109	9.5	0.033	12
110	9	0.031	12
111	8.7	0.044	15
112	7.8	0.018	7
113	7.8	0.036	12
114	7.1	0.019	6
115	7	0.028	10
116	5.6	0.015	6
117	5	0.011	5
118	5	0.011	4
119	4.5	0.007	3
120	4.1	0.014	5

Table S2 – (…continue)

Primary branch	Length (cm)	Weight (g)	Number of spikelets
121	4	0.010	3
122	3.8	0.008	3
123	3.8	0.008	3
124	3.5	0.008	3
125	2.9	0.006	2
126	2.7	0.006	2
127	2.7	0.005	2
Average values	20.6±9.21	0.3±0.3	120±116
Total values	2616.4	38.131	15270

Table S3 – Flowering stage and size of the inflorescences of clones collected. The inflorescence developmental stage is indicated as follows: 1) Totally absent, 2) Barely visible, 3) Partially opened, 4) Completely opened, 5) Mature (dry). The size of the inflorescence, observed at the second year after transplantation, is indicated as follows: class 1 (< 45cm in length), class 2 (> 45 cm in length). Data were collected on 01/10/13 (fourth year) and on 21/09/14 (fifth year).

Clone	Place	Coordinates		Years after transplantation		Inflorescence size class
		Latitude (N)	Longitude (E)	Fourth	Fifth	
Ad1	Buccinasco (MI)	45°24'	09°06'	1	1	1
Ad2	Cassina de Pecchi (MI)	45°31'	09°22'	1	1	1
Ad3	Calderara di Reno (BO)	44°32'	11°16'	1	4	1
Ad4	Sforzesca (PV)	45°17'	08°52'	1	1	1
Ad5	Cisliano (MI)	45°26'	08°58'	3.5	1	1
Ad6	Marudo (LO)	45°16'	09°18'	1	2	1
Ad7	Rozzano (MI)	45°22'	09°08'	1	1	1
Ad8	Cesano Maderno (MB)	45°37'	09°09'	1	2	1
Ad9	Boffalora Sopra Ticino (MI)	45°27'	08°49'	1	1	1
Ad10	Vittuone (MI)	45°28'	08°57'	1	1	1
Ad12	Piacenza	45°01'	09°38'	1	2	1
Ad13	Gazzola (PC)	44°58'	09°34'	1	2	1
Ad15	Civesino (MI)	45°23'	09°15'	1	1	1
Ad16	Alessandria	44°56'	08°37'	1	1	1
Ad17	Bergamo	45°40'	09°38'	1	3	1
Ad18	Scaldasole (PV)	45°07'	08°54'	1	1	1
Ad19	Brescia	45°32'	10°10'	1	3	1
Ad20	Leno (BS)	45°20'	10°16'	1	1	1
Ad21	Calcinato (BS)	45°28'	10°24'	1	2	1
Ad22	Desenzano del Garda (BS)	45°26'	10°37'	1	1	1
Ad23	Nago Torbole (TN)	45°52'	10°52'	1	1	1
Ad24	Ala (TN)	45°44'	10°58'	1	1	1
Ad25	Pescantina (VR)	45°29'	10°51'	1	2	1
Ad26	Chiaravalle (AN)	43°35'	13°21'	1	1	1
Ad27	Mantova	45°09'	10°47'	1	1	1
Ad28	Colombarolo (CR)	45°07'	10°29'	1	2	1
Ad29	Cremona	45°10'	09°57'	1	1	1
Ad30	Spino D'Adda (CR)	45°24'	09°28'	1	2	1
Ad31	Sozzano (BS)	45°39'	10° 12'	1	1	1
Ad32	Civitanova Marche (MC)	43°57'	13°43'	1	1	1
Ad33	Trezzano sul Naviglio (MI)	45°25'	09°03'	1	2	1
Ad34	Castelnuovo Scrivia (AL)	44°59'	08°53'	1	2	1
Ad35	Torricella Verzate (PV)	45°01'	09°10'	1	3	1
Ad36	Fidenza (PR)	44°52'	10°05'	1	3	1
Ad37	San Benedetto Tronto (AP)	42°53'	13°53'	1	3	1

Table S3 – (…continue)

Clone	Place	Coordinates		Years after transplantation		Inflorescence size class
		Latitude (N)	Longitude (E)	Fourth	Fifth	
Ad38	Cattolica (RN)	43°57'	12°42'	1	2	1
Ad39	Roseto Abruzzi (TE)	42°39'	13°59'	1	2	1
Ad40	Landriano (PV)	45°19'	09°15'	3.5	4	1
Ad42	Arenzano (GE)	44°23'	08°40'	1	1	1
Ad43	Montale (SP)	44°11'	09°37'	1	2	1
Ad44	Corrodano (SP)	44°14'	09°38'	1	2	1
Ad45	Ovada (AL)	44°37'	08°38'	1	1	1
Ad46	Verzi - Loano (SV)	44°09'	08°14'	2	2	1
Ad47	Forlì	44°20'	12°09'	1	4	1
Ad48	Massa	43°57'	10°11'	2	2	1
Ad49	Fauglia (PI)	43°32'	10°28'	1	2	1
Ad50	Cecina (LI)	43°17'	10°32'	2	2	1
Ad51	Venturina (LI)	43°01'	10°35'	1	1	1
Ad53	Orbetello (GR)	42°26'	11°10'	3	2	1
Ad57	Fiumicino (RM)	41°44'	12°16'	1	4	1
Ad58	Ardea (RM)	41°34'	12°30'	1	2	1
Ad61	Cellole (CE)	41°18'	13°29'	1	1	1
Ad62	Sessa Aurunca (CE)	41°13'	13°54'	1	2	1
Ad63	Sparanise (CE)	41°10'	14°07'	1	3	1
Ad64	Palma Campania (NA)	40°51'	14°32'	3	3	1
Ad66	Balvano (PZ)	40°36'	15°32'	2	2	1
Ad67	Vaglio Basilicata (PZ)	40°39'	15°56'	2	2	1
Ad68	Matera	40°39'	16°35'	3	3	1
Ad69	Bari	40°56'	16°38'	1	2	1
Ad71	Margherita di Savoia (BT)	41°23'	16°06'	2	4	1
Ad72	Foggia	41°28'	15°40'	3	4	1
Ad73	Begliano (TS)	45°49'	13°27'	2	2	1
Ad74	Bellano (LC)	45°59'	09°15'	1	1	1
Ad75	Spagnoi (VR)	45°27'	10°45'	1	3	1
Ad76	Laterza (TA)	40°37'	16°48'	1	2	1
Ad77	Sternata (LE)	40°13'	18°14'	2	2	1
Ad78	Tricase (LE)	39°57'	18°24'	1	1	1
Ad79	Curtarolo (PD)	45°31'	11°49'	3	2	1
Ad80	Musei (CI)	39°18'	08°41'	1	4	1
Ad81	Iglesias	39°18'	08°33'	4.5	4	2
Ad82	Fluminimaggiore (CI)	39°25'	08°30'	1	4	2
Ad85	Tanca Marchese (OR)	39°44'	08°35'	1	4	1
Ad86	Bauladu (OR)	40°02'	08°41'	3.5	2	1
Ad87	Elmas (CA)	39°16'	09°04'	4	2	1
Ad88	Lurago d'Erba (CO)	45°45'	09°13'	1	2	1

Table S3 – (…continue)

Clone	Place	Coordinates		Years after transplantation		Inflorescence size class
		Latitude (N)	Longitude (E)	Fourth	Fifth	
Ad89	Campalto (VE)	45°29'	12°16'	1	1	1
Ad90	Meolo (VE)	45°34'	12°27'	1	1	1
Ad91	Lignano Sabbiadoro (UD)	45°39'	13°04'	3	2	1
Ad92	Chiarisacco (UD)	45°50'	13°13'	2.5	3	1
Ad93	Codevigo (PD)	45°15'	12°07'	2.5	2	1
Ad94	Recetto (NO)	45°27'	08°26'	1	2	1
Ad95	Aspra (PA)	38°06'	13°30'	2.5	4	2
Ad96	Orbassano (TO)	44°59'	07°30'	1	4	2

Table S4 – Comparison among the clones studied using SSRs molecular markers: sampling location, flowering stage and size of the inflorescences.
The inflorescence developmental stage is indicated as follows: 1) Totally absent, 2) Barely visible, 3) Partially opened, 4) Completely opened, 5) Mature (dry). The size of the inflorescence, observed at the second year after transplantation, is indicated as follows: class 1 (< 45cm in length), class 2 (> 45 cm in length). Data were collected on 21/09/14, at the fifth year after transplantation.

Clone	Sampling location	Coordinates		Flowering stage	Inflorescence size class
		Latitude (N)	Longitude (E)		
Ad2	Cassina de Pecchi (MI)	45°31'	09°22'	1	1
Ad8	Cesano Maderno (MB)	45°37'	09°09'	2	1
Ad20	Leno (BS)	45°20'	10°16'	1	1
Ad22	Desenzano del Garda (BS)	45°26'	10°37'	1	1
Ad24	Ala (TN)	45°44'	10°58'	1	1
Ad25	Pescantina (VR)	45°29'	10°51'	2	1
Ad38	Cattolica (RN)	43°57'	12°42'	2	1
Ad42	Arenzano (GE)	44°23'	08°40'	1	1
Ad53	Orbetello (GR)	42°26'	11°10'	2	1
Ad68	Matera	40°39'	16°35'	3	1
Ad71	Margherita di Savoia (BT)	41°23'	16°06'	4	1
Ad82	Fluminimaggiore (CI)	39°25'	08°30'	4	2
Ad86	Bauladu (OR)	40°02'	08°41'	2	1
Ad91	Lignano Sabbiadoro (UD)	45°39'	13°04'	2	1
Ad202	Nizza di Sicilia (ME)	37°59'	15°24'	2	1

Table S5 – SSR primers used in this work. Primer sequences, annealing temperature, fragment size range, and GenBank accession number for each microsatellite primer pair. F forward; R reverse. Modified from Tarin et al., 2013.

Locus name	Primer sequences	GenBank accession No.	Size range bp	Annealing temperature °C
Ad_B7	B7-F: CTATGCATAAGTTTAGATCTACAACTAG	HQ224523	134–196	55
	B7-R: GGTTTTGGCGACAATAAGATAGTTC			
Ad_G8	G8-F: CCATGTAGAGACAAATCGGAAAG	HQ224521	132–155	55
	G8-R: CAATAGTTCCAATATCAAACGTATCC			
Ad_A3	A3-F: CACAGGCGCGTGATTGACC	HQ224519	102–149	55
	A3-R: CAGATCCGACAGGACGAGATG			
Ad_B1	B1-F: CATGGATGCCAACTCCTTCAAC	HQ224518	103–161	55
	B1-R: GGAAATACTTCATTCTAGTTAGAGATAGA			
Ad_F8	F8-F: TAGTGGTGACATGTGGCTTACC	HQ224527	98–142	55
	F8-R: GGTTGGGCATGCACACGG			
Ad4_13	Ad4-13F: TAGTAAGGCACCACGCAGCAC	HQ224526	90–125	55
	Ad4-13R: TTGGTCTAACTGAGAATGGGTTTC			
Ad_E10	E10-F: GCTTCATATGTTTTGTCCCAACTC	HQ224522	101–137	60
	E10-R : CAATCCTCGCCAGATCCAAGC			
Ad5_4	Ad5-4F: CGCGTATGATCGTCCACCG	HQ224525	90–144	55
	Ad5-4R: ACGACCACACGCATTGCTCTG			
Ad4_6	Ad4-6F:AGGAGTCCAATTTAACAGGAAAAGG	HQ224524	157–189	62
	Ad4-6R: GGGTTTCTCTCTAGTAGATTGATTAG			
Ad_H1	H1-F: GCTGGACATAGAAGCCGTTTTTG	HQ224520	121–159	62
	H1-R: GCTTCCTTGGAAGTGAGGCAG			

Table S6 – Contingence table regarding the molecular data collected.

Clones			AdB7							AdG8				AdA3			AdB1			AdF8			Ad4 13			AdE10			Ad5 4			Ad4 6			ADH1		N	E
AD2	1	1	2	0	0	0	0	0	1	2	0	0	1	2	0	1	2	0	1	2	3	1	2	3	1	2	1	2	3	1	2	0	1	2	45.31	9.22
AD8	2	1	2	0	0	0	0	0	1	2	0	0	0	2	0	1	2	3	1	2	0	1	2	3	1	2	1	2	3	1	2	0	1	2	45.37	9.09
AD20	3	0	2	3	0	0	0	0	1	2	0	0	1	2	3	1	2	0	1	2	0	1	2	3	1	2	1	2	3	1	2	0	1	2	45.2	10.16
AD22	4	0	2	0	4	0	0	0	0	0	3	4	1	2	0	1	2	0	1	2	0	1	2	3	1	2	1	2	3	1	2	0	1	2	45.26	10.37
AD24	5	0	2	0	0	5	0	0	1	2	0	0	0	2	3	1	2	3	1	2	0	1	2	3	1	2	1	2	3	1	2	0	1	2	45.39	13.04
AD25	6	0	2	0	4	0	0	0	0	0	3	4	1	2	0	0	2	0	1	2	0	1	2	3	1	2	1	2	3	1	2	0	1	2	45.44	10.58
AD38	7	0	2	3	0	0	0	0	1	2	0	0	1	2	0	0	2	3	1	2	0	1	2	3	1	2	1	2	3	0	0	3	1	2	45.29	10.51
AD42	8	0	2	0	4	0	0	0	1	2	0	0	1	2	0	0	2	3	0	2	3	1	2	3	1	2	1	2	3	1	2	0	1	2	44.23	8.4
AD53	9	0	2	3	0	0	0	0	1	2	0	0	1	2	0	0	2	3	1	2	0	1	2	3	1	2	1	2	3	1	2	0	1	2	43.57	12.42
AD68	10	0	2	0	0	5	0	0	1	2	0	0	1	2	0	0	2	3	1	2	0	1	2	3	1	2	1	2	3	0	2	0	1	2	42.26	11.1
AD71	11	0	2	0	0	5	0	0	1	2	0	0	1	2	0	0	2	3	1	2	0	1	2	3	1	2	1	2	3	0	2	0	1	2	40.39	16.35
AD82	12	0	2	0	0	0	0	0	1	2	0	0	0	2	0	1	2	3	1	2	0	1	2	3	1	2	1	2	3	1	2	0	1	2	41.23	16.06
AD86	13	0	2	0	0	0	6	0	1	2	0	0	0	2	3	1	2	0	1	2	0	1	2	3	1	2	1	2	3	1	2	0	1	2	39.25	8.3
AD91	14	0	2	0	0	0	0	7	0	0	3	4	1	2	0	1	2	0	1	2	0	1	2	3	1	2	1	2	3	1	2	0	1	2	40.02	8.41
AD202	15	0	2	0	0	0	0	7	1	2	0	0	0	2	0	1	2	0	1	2	0	1	2	3	1	2	1	2	3	1	2	0	1	2	37.59	15.24

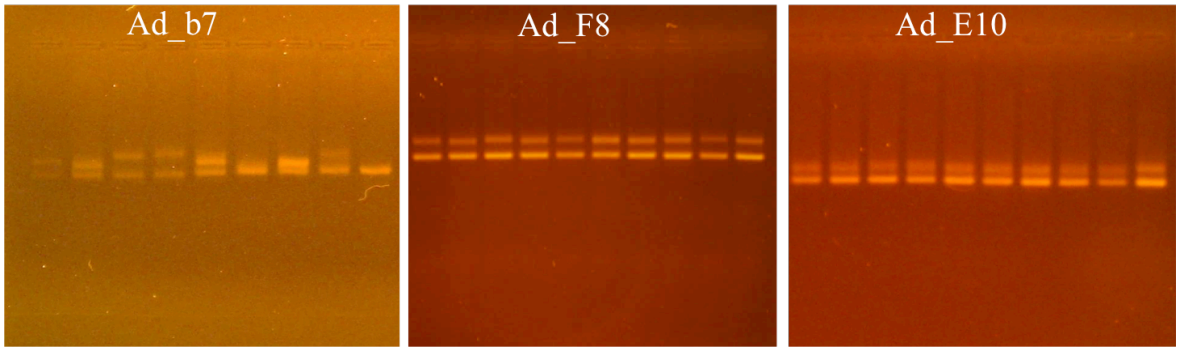

Figure S1 - **6.** Example of the amplified products obtained using Ad_B7, Ad_F8 and Ad_E10 SSR primers. The agarose gels were stained with ethidium bromide. Each band obtained by PCR was considered as an independent locus; a different number was attributed to each allele to generate the matrix (Online res 7.) used for statistical analyses. In each lane is loaded the PCR product obtained amplifying genomic DNA extracted from different clones (9/10 out of 15 analyzed).

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IV

Study of different *A. donax* propagation methods: influence of clonal variation

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Study of different *A. donax* propagation methods: influence of clonal variation

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Abstract

The recent growing interest for *Arundo donax* (Giant reed) as an energy crop is due to the great vegetative vigor and biomass production of this perennial rhizomatous plant. This species is able to grow in a wide range of pedoclimatic conditions and it has been employed by men for a great number of purposes, promoting its worldwide diffusion despite its sterility. Field establishment represents the most expensive phase of the whole cultivation cycle, because of the high cost of the propagules. In this work rhizomes transplantation, hydroponic cultivation and in vitro propagation were compared with the aim of improving the efficiency of these agamic propagation techniques, reducing costs and time required.

We focused our attention on the more promising in vitro and hydroponic cultivation, finding clones characterized by a high propensity to these propagation methods.

Despite the high costs of in vitro propagation, this technique remains the most widespread in large scale propagules production, even if according to our results hydroponic cultivation appears a valid propagation method too, making possible to obtain vigorous plants with lower costs and easy propagules finding. According to our observations a

longer growth in pot before transplantation in the open field seems to increase the yield at the first year favoring the propagules engraftment and increasing the survival rate. In particular we found a strong effect of the clone used with respect to the specific propagation method suggesting that clonal selection would take in consideration also this important parameter.

Keywords: Arundo donax; Energy crop; Agamic propagation; In vitro culture.

1. Introduction

Arundo donax (Giant reed) is a perennial rhizomatous plant belonging to the Poaceae family. This species is widespread almost all over the world (Dudley, 2000; Perdue, 1958) as it is able to grow in a wide range of pedoclimatic conditions (Quinn et al., 2007; Virtue et al., 2010; Lewandowski et al., 2003; Corno et al., 2014) and it has been employed by men for a great number of purposes thanks to its flexibility of usage, contributing to its diffusion (Perdue, 1958; Pilu et al., 2012; Pilu et al., 2013). Despite *A. donax* is a sterile species (Perdue, 1958; Mariani et al., 2010; Pilu et al., 2012; Decruyenaere and Holt, 2001; Ceotto and Di Candilo, 2010; Balogh et al., 2012; Else, 1996; Khudamrongsawat et al., 2009; Wijte et al., 2005; Pilu et al., 2013; Cavallaro et al., 2014; Cantaluppi et al., 2015), its diffusion reaches almost every temperate regions in the world representing sometimes an ecological problem especially where it was planted to avoid banks erosion. In recent years land movement by bulldozers further contributed to its diffusion (Boose and Holt, 1999; Boland, 2008).

Even if this species is often considered invasive (Perdue, 1958), its weed potential is limited by the absence of fertile seeds as previously mentioned: *A. donax* spreading beyond the margins of the plot can be easily impeded (Balogh et al., 2012; Williams et al., 2008) because rhizomes enlargement is very slow (Boland, 2006).

In the last years, giant reed became one of the most interesting energy crops because of the high yield and of the limited input required for cultivation, together with its tolerance to biotic and abiotic stresses (Angelini et al., 2005; Angelini et al., 2009; Corno et al., 2014). Thanks to its flexibility of usage, *A. donax*' biomass can be used to produce bioethanol, biogas (Corno et al., 2015), solid fuels for direct combustion (Dahl and Obernberger, 2004; Scordia et al., 2012; Pilu et al., 2013), textile fibers and for green chemistry (Corno et al., 2014).

A. donax yield is very high if compared with those of other crops traditionally used for energy biomass production, (e.g. corn); nevertheless yield greatly depend on several factors, such as soil moisture and others pedo-climatic conditions, temperatures, plant density and agronomics (Corno et al., 2014).

Even if the cultivation costs are very low, compared with others energetic crops (Pilu et al., 2013), the high cost of the propagules, due to the sterility of this species and to the labour required by the propagation techniques, still remains a limiting factor for its diffusion (Pilu et al., 2013).

Three main techniques are currently used to propagate this species: in vitro culture (Takahashi et al., 2010), hydroponic cultivation and rhizomes transplantation (Bucci et al., 2013; Ceotto and Di Candilo, 2010; Pilu et al., 2013; Corno et al., 2014; Boland, 2006). Difficulties in finding a high number of propagules, make the use of rhizomes almost not applicable on large scale (Pilu et al., 2013). Propagating plants from bud nodes could represent a possible solution to efficiently progate this plant reducing propagation and transportation costs (Ceotto and Di Candilo, 2010) whilst in vitro culture, although having high costs, permit to propagate *A. donax* without limits of quantity. After the production of seedlings, the transplantation of the propagules can be carried out using mechanical transplanters like those commonly used in horticulture. Plants are more susceptible to stress conditions during the first months after transplantation, for example water shortage during the first growing season seems to affect plants rooting and development, reducing yields at least during the first years of the cultivation cycle (Abichandani, 2007; Perdue, 1958).

Only few agronomic treatments (weeding and irrigation) are required by *A. donax* plants, at least during the first year after transplantation to avoid prolonged water stresses and to keep the ground spoilt of weeds. The number of culms quickly increases after transplantation in the open field, covering the surrounding area in a few years, depending on plant density, and reducing weeds competition. No treatments are usually needed after the first year (Pilu et al., 2013), except harvest, independently from the propagation method used, thanks to the plants vegetative vigor.

Although the limited input required (Pilu et al., 2013; Corno et al., 2014) makes possible to cultivate this species also on marginal lands usually left uncovered, the provision of fertilizers, especially nitrogen (Pilu et al., 2013; Angelini et al., 2009) even if not strictly necessary, increases the yield (Angelini et al., 2005; Christou et al., 2003; Gilbert et al.,

2010; Borin et al., 2013; Quinn et al., 2007; Corno et al., 2014), obviously increasing the cultivation costs. Considering that *A. donax* propagules cost is still a limiting factor for its diffusion, it is necessary to establish how to propagate this culture in the fastest and cheapest way and how the propagation techniques influence the yield.

In this paper different propagation methods were compared (rhizomes transplantation, hydroponic cultivation and in-vitro propagation), also considering their effect on plants development after their transplantation in the open field through different field trials (Fig. 1). Due to the fact that heritability of some traits like height and culm diameter has been reported to be linked to yield (Pilu et al., 2013; Cosentino et al., 2005), we studied the behavior of different clones sampled on the Italian territory with respect to different propagation methods focusing our attention on in vitro and hydroponic cultivation.

2. Materials and methods

2.1 Plant material

The plant material used in this study has been obtained from a collection of *A. donax* clones sampled all over the Italian territory between 38°26’ and 45°50’ of latitude and between 7°30’ and 17°54’ of longitude. The experimental field is situated in Landriano (N 45°18’, E 9°15’). (TAB. 1)

The clones collection was realized using rhizome fragments with at least one bud as propagules. Rhizome fragments were transplanted about 20 cm below the soil surface in rows 40 meters long, with a density of 2500 plants/hectare. Weeds competition was avoid covering the surrounding area with a mulching film or keeping the soil between rows spoilt of weeds. Plantlets were irrigated as needed during the first weeks after transplantation. The biomass was harvested every year at the end of the growing season.

2.2 Hydroponic cultivation of the cane nodes

Canes were sampled at the end of the growing season. The hydroponic cultivation of cane nodes was done cleaning canes from the leaf sheaths and cutting them to obtain fragments with at least one node, which were dip in tap water (about 300 cm^3 water each node) kept clean, at 20-25 °C.

After roots development the plantlets so obtained were separated from the canes and transplanted in pots 7 cm in diameter. Plantlets were finally transplanted in the open field about 3 weeks later.

2.3 Propagules storage

Cane nodes of the Ad40 clone sampled in late summer (30th August), autumn (23th October) and winter (22th December) were hydroponic cultivated 0, 30, 60, 90 and 180 days after sampling (n>20). Cane fragments were stored in plastic bags at 4°C in the dark after sampling.

2.4 Disinfectant treatment of cane nodes

118 cane fragments with at least one node were sampled in late summer from plants of the Ad40 clone at the third year of cultivation. 50 cane nodes were treated with 2.5% sodium hypochlorite for 5 minutes and hydroponic cultivated in comparison with 68 untreated cane nodes on December 31st, 2012.

The plantlets so obtained were measured on February 1st (2013).

2.5 Hydroponic cultivation: comparison among clones

Cane fragments of 9 different clones (Ad10, Ad11, Ad12, Ad14, Ad19, Ad20, Ad31, Ad34, Ad36) were sampled from plants of the collection at the end of the second year and hydroponic cultivated in tap water at 20-25°C on January 5th (2012). 466 cane nodes were used, obtaining 201 plantlets that were transplanted in pots until the end of February. 150 plantlets were transplanted in the open field in the early March (2012) with a density of 1 plant/4m^2 (2500 plants/ha). The surrounding area was kept spoilt of weeds during the following months. The survival rate of the plants in the open field was evaluated on July 17th, 2012.

2.6 In-vitro propagation: comparison among clones at different BA concentrations

Buds were removed from canes of 32 different clones (Ad1, Ad4, Ad5, Ad6, Ad7, Ad8, Ad9, Ad10, Ad11, Ad12, Ad13, Ad15, Ad16, Ad17, Ad18, Ad19, Ad20, Ad21, Ad43, Ad44, Ad64, Ad66, Ad68, Ad69, Ad72, Ad74, Ad76, Ad77, Ad78, Ad81, Ad82, Ad91) in spring and stored at 4°C. Tissues were previously treated to reduce fungal and microbial contamination, dipping them in pure ethanol and subsequently in sodium hypochlorite (4.5%), raising them both times with sterile water.

Shoot tissues were cultivated on a MS (Murashige & Skoog) w/vitamins medium (4.4g/l), adding 100 mg/l Inositol, 30 g/l sucrose, 1 ml/l plant preservative medium and 2.5 g/l

gelrite as a gelling agent. The pH of the medium was 5.6. 6-benzylaminopurine was added to the medium in different concentrations: 1, 3 and 5 mg/l.
Explants were kept in growth chamber, at a temperature of 26°C, 18 hours in the light and 6 hours in the dark, using cool white fluorescent lamps (F36T12/CW/HO) by GTE SYLVANIA (Lighting Products Group, Danvers, MA).
The shoots were separated during the explants, depending on their branching ability. The number of shoots/buds was evaluated 45 days after the first explant on the medium.

2.7 Field establishment: comparison between rhizomes and hydroponic cultivated plantlets

Rhizomes fragments with at least one bud and cane fragments of the Ad20 clone were sampled from the collection situated in Landriano, at the end of the growing season (2011). Rhizomes fragments were stored at 4°C till their transplantation.
Cane fragments with at least one node were hydroponic cultivated in tap water. The plantlets so obtained were transplanted in pots (7 cm in diameter). Both rhizomes and hydroponic propagated plantlets were transplanted in the open field on March 28th (2012) and at the end of the first growing season (October 18th 2012), were measured height, diameter and number of culms.

2.8 Field establishment: comparison between hydroponic cultivated and in-vitro propagated plantlets

Canes of the Ad20 clone were sampled from the experimental field at the end of the growing season (2012). Cane fragments were hydroponic cultivated at 20°C. After roots emission, the plantlets were transplanted in pots 7 cm in diameter. Buds of the same clone were in vitro cultivated and transplanted in pots.
24 hydroponic cultivated plantlets and 6 in-vitro propagated plantlets were randomly transplanted in the field (density of 1plant/4m^2) on June 7th, 2013. Plants were measured at the end of the growing season on October 16th (2013), considering canes diameter and length, culms number and biomass production. Culms number and biomass production were measured also at the end of the second growing season (October 2nd, 2014).
Two field trials were performed during the growing season 2015 in Formigara (CR, Italy, N 45°12', E 9°46') and Landriano. Cane fragments were sampled during winter, and hydroponic cultivated since December 23th, 2014 or February 16th, 2015, respectively for the two field trials.

The hydroponic cultivated plantlets were transplanted in pots, 7 cm in diameter, between January 20th and February 12th, 2015, while in vitro propagated plantlets were transplanted in pots between January 20th and March 23rd, 2015. The plantlets obtained from both methods were transplanted in the open field on April 22nd, 2015, with a plant density of 1 plant/4m^2, and measured on September 4th, 2015. 5 in vitro propagated and 18 hydroponic cultivated plants were measured considering culms number, culms height and culms diameter; biomass production was evaluated considering 5 in-vitro propagated and 24 hydroponic cultivated plants.

The experiment regarding the duration of stay in pot before plants transplantation was conducted using 16 hydroponic cultivated plantlets (Ad20 clone) transplanted in pots, 7 cm in diameter, between January 20th and March 11th, 2015. The plantlets obtained were randomly transplanted in the experimental field situated in Landriano (PV) on April 29th, 2015; with a plant density of 1plant/4m^2. The plants were measured on September 4th, 2015, considering biomass weight, culms number, culms height and culms diameter.

2.9 Informatic tools used

Microsoft Excel® and SPSS were used to elaborate the collected data.

3. Results

3.1 Hydroponic cultivation: propagules storage and disinfectant treatment of cane buds

Cane nodes sampled from Ad40 clone in late summer (August/ September) or early autumn (October) are able to growth in hydroponic with a germination rate of 100 % whilst if sampled later in winter (22th December) showed a lower germination rate of 20% (Table 2). We found that cane buds sampled in summer/early autumn and stored in plastic bags at 4°C in the dark maintained the capacity of germinate at the same ratio of T0 sample until 90 days whilst at 180 days the germination resulted decreased to 58-38 %. In the case of cane buds sampled in winter (December-January) and stored 180 days in the same conditions we observed the loss of ability to germinate. (Table 2).

Rhizomes of about 400-500g, sampled at the end of the growing season and stored in the same conditions, resulted to maintain after 6 months good germination percentages of about 70-80% (data not shown).

With the aim of improving the hydroponic cultivation propagation method, the effect of a treatment able to reduce microbial and fungal contamination of cane fragments was

evaluated. 50 cane nodes sampled from the Ad40 clone were treated with 2.5% sodium hypochlorite for 5 minutes, and their growth was compared to 68 untreated cane nodes. The comparison didn't show significant differences in shoots development and rooting (data not shown, even if treated cane fragments seemed to show a higher germination rate of their buds (+13%), and a higher rooting ability of the shoots so obtained (+15%) compared to the untreated ones. (TAB.3).

3.2 Hydroponic cultivation: comparison among clones

466 cane nodes from 9 different clones (Ad10, Ad11, Ad12, Ad14, Ad19, Ad20, Ad31, Ad34, Ad36) were subjected to hydroponic cultivation in tap water at 20-25°C on January 5th (2012). Water was kept clean and changed frequently, to avoid algae proliferation. 201 plantlets endowed with both roots and leaves were obtained until the end of February (43.1% of the total number of the cane nodes). Among the compared clones, the higher values percentage were reached by the clones Ad20 (71.7%), Ad19 (54.8%) and Ad31 (55.0%) whilst the lower by Ad 12 (19.6%) and Ad 36 (22.5%) (FIG. 2).

The plants obtained through hydroponic cultivation were transplanted in pots and 150 among them were transplanted in the open field about a month later (March, 2012), with a density of 1 plant/4m^2. Plants survival rate (92%, corresponding to 138 plants) was evaluated in mid-July. The survival rate was found to be different for different clones plants: 6 out of the 9 clones provide a survival rate of 90% or higher, instead the clones Ad34, Ad36 and Ad11 showed a lower survival rate (between 75% and 60%) (FIG. 3).

3.3 In-vitro propagation: comparison among clones at different BA concentrations

32 *A. donax* clones, sampled from the Landriano clones collection were compared considering their propensity to in vitro propagation. The bud tissue was removed from cane nodes, treated with ethanol (70%) and sodium hypochlorite (4.5%) and rinsed with deionized water, to reduce fungal and microbial contamination.

The effect of different BA (6-benzylamninopurine) concentrations in the medium on plants growth was evaluated with the aim of finding the optimal BA concentration. Differences among clones were observed considering the number of shoots after 45 days from the explantantation with 1, 3 and 5 mg/l BA (FIG. 4). The better results were obtained with a 5 mg/l BA concentration. A strong correlation was observed between the average number of shoots and BA concentration (r= 0.973).

Among the 32 clones compared, Ad11, Ad13, Ad15, Ad17, Ad19, Ad20, Ad21, Ad44, Ad68, Ad81 and Ad91 gave the better results, producing more than 40 shoots in 45 days with a 5 mg/l BA concentration whilst the worst result were register with Ad1, Ad64, Ad72, Ad77 and Ad78, producing less than 10 shoots (Fig. 3).

3.4 Field establishment: comparison between rhizomes and hydroponic cultivated plantlets

In 2012 the use of rhizome fragments and hydroponic cultivation were compared using the Ad20 clone, with the aim of verifying the effect of these propagation methods on plants development. Both rhizomes and hydroponic propagated plantlets were randomly transplanted in the open field on March 28th (2012) and at the end of the first growing season (October 18th, 2012) plants obtained using both propagation methods (20 rhizome fragments and 20 hydroponic cultivated plants) were measured, considering culms number, height and diameter (TAB. 4). The average culms height in plants obtained by hydroponic cultivation resulted to be 2.57 m, similar to the one of plants obtained through rhizomes transplantation (2.78 m). Canes average diameter also resulted very similar in plants obtained through hydroponic cultivation and rhizome fragments transplantation (12.7 mm and 13.4 mm respectively). The average number of culms resulted higher in plants obtained through hydroponic cultivation (8.3) compared to the ones obtained from rhizomes fragments (7.5). Differences between the two propagation methods were not found to be significant.

3.5 Field establishment: comparison between hydroponic cultivated and in-vitro propagated plantlets

Hydroponic cultivation and in-vitro propagation were also compared: plantlets obtained with these two techniques were randomly transplanted in the experimental field situated in Landriano (PV, Italy, N 45°18', E 9°15') on June 7th, 2013 (24 hydroponic cultivated plants and 6 in-vitro propagated plants). Culms height, number and diameter were measured at the end of the first growing season, on October 16th, 2013. Differences were observed among the plants obtained with these different techniques, but they were not found to be significant (P=0.05) considering these parameters. (TAB. 5)

Culms average height was higher in hydroponic cultivated plants compared to the in vitro propagated ones (1.43 m and 1.10 m). Hydroponic cultivated plants also resulted to have

culms with a larger average diameter, instead the average number of culms resulted higher in in vitro propagated plants: 12 against 6 (TAB. 5).

Biomass average production (fresh weight) resulted to be significantly higher in hydroponic cultivated plants compared to the in vitro propagated ones. At the second year (October 2nd, 2014) hydroponic cultivated plants produced a higher average number of culms compared to the in vitro propagated ones (21.2 against 13.5) and also a higher average amount of biomass: 10.69 Kg and 4.50 Kg fresh weight respectively.

A further comparison was performed in the 2015 growing season between these two techniques in two different experimental fields. In the experimental field situated in Formigara (CR, Italy, N 45°12', E 9°46'), 24 hydroponic cultivated plants and 11 in vitro propagated plants were compared. Plants were transplanted in the field on April 22nd, 2015, with a plant density of 2500 plants/ha and measured on September 4th, 2015.

Among the differences observed considering biomass average weight and the average values of culms number, culms height and culms diameter, only the differences regarding biomass production and the average culms diameter resulted to be significant (TAB. 5). Hydroponic cultivated plants produced a higher average amount of biomass (1.34 kg fresh weight/plant) compared to the in vitro propagated ones (0.88 kg fresh weight/plant).

In the field situated in Landriano we observed also a strong effect of the duration of the period of growth in pot before plants transplantation in the field on the fresh biomass at the end of the first year of cultivation, with a Spearman correlation of 0.73 (FIG. 5).

4. Discussion

A. donax appears to be the best perennial energy crop in Mediterranean regions, as attested by comparative studies on biomass yield (Lewandowski et al., 2003). The economic interest for this species has recently increased because its cultivation represents a cheap and efficient way to produce bioenergy.

A. donax is a sterile plant (Perdue, 1958; Di Tomaso and Healey, 2003; Dudley, 2000; Mariani et al., 2010; Pilu et al., 2012; Balogh et al., 2012; Else, 1996; Khudamrongsawat et al., 2009; Wijte et al., 2005; Pilu et al., 2013; Pilu et al., 2013; Cavallaro et al., 2014; Cantaluppi et al., 2015) able to propagate itself only agamically (Decruyenaere and Holt, 2001; Ceotto and Di Candilo, 2010; Cantaluppi et al., 2015). For this reason it cannot be sown but it is necessary to transplant rhizome fragments or plantlets already endowed with a photosynthetic apparatus. The absence of seeds can be considered an advantage, as it

limits *A. donax* spontaneous spreading outside the cultivated areas, (Balogh et al., 2012; Williams et al., 2008), but it also represents a problem because of the high cost of the propagules that makes transplantation the most expensive phase of the whole cultivation cycle. Propagation using rhizome fragments was used for the first time to realize a large scale cultivation in Italy (Torviscosa, UD), with the aim of producing cellulose for industrial purposes: in 1938, 25 millions of rhizomes were transplanted on 1200 hectares. Despite this, this technique doesn't appear realy adapt to be applied on large scale because of the cost, and of the difficulties related to propagules finding, as a great surface is needed to produce them. At maturity it is possible to obtain 20-30 rhizomes fragments per square meter, losing all the plants of this area (Pilu et al., 2013).

Plantlets with leaves and roots can be obtained by in vitro or hydroponic cultivation. In vitro propagation is a technique that can be adopted on large scale (Takahashi et al., 2010) as it allows to obtain a great number of plantlets in less time compared to the other methods. About 1200 plantlets can be obtained from a single bud every six multiplication cycles, in about 6 months, (Cavallaro et al., 2014), increasing plants establishment rate in comparison to the use of rhizomes (Cavallaro et al., 2014) as they already have a photosynthetic apparatus. Like the in vitro propagation method, hydroponic cultivation of the cane nodes produces plantlets already adapted to be transplanted in the open field, also using a transplanter. Propagules production through hydroponic cultivation requires a much lower cultivated surface compared to the use of rhizomes: every cane bears 20 to 40 nodes, this means that using cane fragments it is possible to obtain dozens of propagules from a single square meter of cultivated surface, without losing the plants of this area (Ceotto and Di Candilo, 2010; Pilu et al., 2014). The plantlets obtained are ready to be transplanted in pots in only 20-30 days, after being separated from the cane, and they can be further transplanted in the open field 30-40 days later.

Hydroponic cultivation and in vitro propagation seem to be the best techniques to be applied on large scale. In this paper both these techniques were compared taking in consideration the effect due to the clone used, with the aim to increase their efficiency and competitiveness. Different clones from our collection (TAB. 1) were cultivated with both techniques because of the well known influence of genotype on the propagation efficiency with the aim to find among different genotypes the ones characterized by increased frequency of plant regeneration (Thomas and Ravindra, 1997; Arnold et al., 2002; Nair

and Gupta, 2005; Mikula and Rybczynsky, 2001; Kikkert et al., 1997; Kintzios and Taravira,1997; Litz and Gray, 1995; Meurer et al., 2001; Tomlin et al., 2002).

Propagules conservation could represent an important issue in large scale propagation, in particular considering cane fragments, whose buds are used as rough material for hydroponic and as starting point of in vitro cultivation.

Cane fragments sampled in late summer or autumn showed a germination rate of 100% whilst sampling them in winter only 20% is able to germinate. After a 6 months storage at 4°C, canes sampled in summer showed a higher germination rate (60%) compared to the ones sampled in autumn (40%), instead canes sampled in winter were found to be unable to germinate at all (TAB. 2). These values can be improved by a disinfectant treatment using sodium hypoclorite at 2.5% (TAB. 3). Rhizomes stored in the same conditions for a long time, resulted to maintain a germination rate of 70-80% after 6 months (data not shown).

Considering the use of cane nodes as propagules, the possibility of preserving them for a long time seems to be useful for a large scale application of the hydroponic cultivation method, to produce plantlets according to market needs. Even if rhizomes could be stored for a long time too, their use as propagules doesn't appear to be particularly suitable to be applied on large scale because of their greater volume the production cost.

9 different clones were compared with the aim of verifying clones propensity to hydroponic propagation. They showed significant differences in the ability of producing propagules, both considering shoots emission and roots development. Among the compared clones (Ad10, Ad11, Ad12, Ad14, Ad19, Ad20, Ad31, Ad34, Ad36), the clones Ad20, Ad31 and Ad19 gave the better results originating plants from the 71.1%, 55.0% and 54.8% of the cane nodes respectively (FIG. 2). Hydroponic cultivated plants gave good results also considering their survival rate in the open field (92%, three months after the transplantation), even if differences among clones were noticed. 6 out of the 9 clones compared provided a survival rate of 90% or more, but the clones Ad34, Ad36 and Ad11 showed a lower survival rate, between 75% and 60% (FIG. 3).

Good results could be obtained using the clones more adapt to this propagation method, increasing the efficiency of this technique and reducing costs. To our knowledge this is the first report regarding the influence of genotype on the capacity of Arundo to produce new plant from the bud by hydroponic technique. 32 different *A. donax* clones from the whole

Italian territory were compared to assess if clonal selection could be useful to improve in vitro propagation efficiency.

Different BA (6-benzylaminopurine) concentrations in the medium (Murashige & Skoog) were also considered to verify the effect on plant development and to find the optimal BA concentration. Gelrite (2.5 g/l) was used as gelling agent in this study, shoots were observed 45 days after the explantation (FIG. 4). In most cases 5 mg/l BA gave the better results, compared with 1 and 3 mg/l BA as confirmed by a strong correlation (r=0.973) between the average number of shoots and BA concentration in the medium. These data confirm that high BA concentrations could increase in vitro propagation effectiveness as showed by Cavallaro and coauthors in 2014.

As showed in FIG. 4, our results suggest the existence of big differences among clones, considering their propensity to the in vitro propagation technique, as observed for hydroponic cultivation. Among the 32 clones compared, only 11 (Ad11, Ad13, Ad15, Ad17, Ad19, Ad20, Ad21, Ad44, Ad68, Ad81 and Ad91) were able to produce more than 40 shoots in 45 days with the highest BA concentration (5 mg/l BA). Although this species reproduces itself only agamically, the existence of phenotypic and genetic differences among clones has been pointed out by several studies (Haddadchi et al., 2013; Pilu et al., 2013; Cosentino et al., 2005; Tarin et al., 2013; Cantaluppi et al., 2015). Genotype dependent differences in the ability to form regenerable callus and to regenerate plants from embryogenic callus were observed considering the related species Miscanthus sinensis (Petersen et al., 2003).

Of course some of the observed differences might be due to epigenetic phenomena, so they could be lost during the succeeding years, making clonal selection ineffective in the long term (Pilu et al., 2013). However results obtained by 4 years in vitro experiment confirmed that the differences observed were conserved among clones even restarting the in vitro culture from a new explant (data not shown).

In this work another aspect taken in consideration was the effect of different propagation techniques on plants in field development in the first year. During the 2012 growing season, the use of rhizome fragments and hydroponic cultivation were compared: plants were measured at the end of the first growing season (October 18th). The differences observed considering culms number, height and diameter, were not found to be significant (TAB 4). Similar results were obtained by Boersma and Heaton (2014) considering the similar species Mischanthus × giganteus. Also in that case limited but persistent

differences in the morphology of plants generated from stem segments and from rhizomes (rhizome propagated plants had larger basal circumferences and a lower number of stems on average) suggested that the two propagation techniques result in plants that may achieve similar yields. Nevertheless different growing conditions could increase the observed differences (Boersma and Heaton, 2014).

Hydroponic and in vitro cultivation were compared during the growing season 2013 and 2015, considering plants development after the transplantation in the open field. In both the experiment at the end of the growing season plants obtained by hydroponic technique had a higher biomass with higher values for culms height and diameter whilst the number of culms was higher using in vitro propagation (TAB 5).

In vitro propagated plants showed a high number of culms, characterized by a small diameter: this behavior, observed also in Mischanthus x giganteus (Płażek et al., 2015), could be linked to a juvenile phase of in vitro propagated plants as shown for different species (Read and Bavougian 2013).

In this work we considered also the effect of the period of stay in pot before the transplantation on the plant biomass at the end of the first year.

A correlation between the duration of the growth in pot period before transplantation in the open field (days) and biomass production (kg fresh weight) at the end of the first year, was observed considering hydroponic cultivated plants (Spearman correlation coefficient r = 7.37), confirming the importance of plantlets size in obtaining a good field engraftment (FIG. 5). Even if this correlation was obtained considering only hydroponic cultivated plantlets, preliminary results indicated that it could be valid also for in vitro propagated plants. Of course further studies will be required to assess if this difference will be maintained in the following years, although preliminary indications seem to confirm the influence of the well establish first year on the productivity of the field for years to come.

In bad growing conditions hydroponic cultivated plants could be favored respect to in vitro propagated ones by their bigger dimensions and by their more developed rhizome and root system, able to confer them a competitive advantage; this problem could probably be overcome by leaving in vitro propagated plants longer in pots, so they can better develop their root system, becoming more resistant to water shortage and to other abiotic stresses.

To obtain good results the transplanting period is very important too, as it can influence yields as attested by Cavallaro et al., (2014): whatever the propagation method, plants transplanted in November showed at the end of the first growing season a lower biomass

production (3.63 Mg/ha) compared to the ones transplanted in March (9.19 Mg/ha). For this reason at 40-45 degrees of latitude, the optimal transplanting period seems to be early spring, independently from the propagation method used; however, in Mediterranean semi-arid environment, in order to avoid water shortage, autumn could be the best period for the establishment (Cavallaro et al., 2014). As suggested by our observations, suboptimal conditions during the first year could compromise plants development and yields also in the following ones.

4. Conclusions

The result showed in this work suggest, for the first time, that different *A. donax* genotypes influence the efficiency of agamic reproduction clearly showing the importance of clonal selection in optimizing this crucial step.

Further studies will be necessary to better explore *A. donax* variability, considering a higher number of clones, with the aim of finding clones characterized by a high propensity to in vitro and hydroponic propagation methods, increasing the efficiency of these techniques, through the reduction of the costs and of the time required.

Despite the high costs of in vitro propagation, this technique remains the most widespread in large scale propagules production, because of the good adaptability of this species and of the high multiplication rates observed. Hydroponic cultivation of the cane nodes appears a valid propagation method too, as it is possible to obtain vigorous plants at low propagation costs. Both methods can be further ameliorated to increase efficiency and reduce costs.

The labor that is currently required, independently from the propagation method used, represents a limiting factor in *Arundo donax* propagules production, hence a mechanization of somatic embryogenesis procedure seems to be the key point to reduce costs of mass propagules production.

The study of the plants obtained through hydroponic cultivation and in vitro propagation, before and after their transplantation in the open field showed that in vitro propagated plants were characterized by a higher number of culms whose diameter was quite lower compared to the ones obtained through hydroponic cultivation. Independently from the propagation method used, leaving the plantlets in pots for a longer time before their transplantation in the open field seems to favor their engraftment and their survival rate, increasing yields at the first year and most likely for all the duration of the culture cycle.

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Table 1 – *A. donax* clones collected in Landriano (PV) Italy (N 45°18', E 9°15'), used in this study.

Clones	Sampling place	Latitude	Longitude
Ad1	Buccinasco (MI)	45°24'	9°06'
Ad4	Sforzesca (PV)	45°17'	8°52'
Ad5	Cisliano (MI)	45°26'	8°58'
Ad6	Marudo (LO)	45°16'	9°18'
Ad7	Rozzano (MI)	45°22'	9°08'
Ad8	Cesano Maderno (MB)	45°37'	9°09'
Ad9	Boffalora Sopra Ticino (MI)	45°27'	8°49'
Ad10	Vittuone (MI)	45°28'	8°57'
Ad11	Aulla (MS)	44° 18'	10°03'
Ad12	Piacenza (PC)	45°01'	9°38'
Ad13	Gazzola (PC)	44°58'	9°34'
Ad14	Rodano (MI)	45°29'	9°20'
Ad15	Civesino (MI)	45°23'	9°15'
Ad16	Alessandria (AL)	44°56'	8°37'
Ad17	Bergamo (BG)	45°40'	9°38'
Ad18	Scaldasole (PV)	45°07'	8°54'
Ad19	Brescia (BS)	45°32'	10°10'
Ad20	Leno (BS)	45°20'	10°16'
Ad21	Calcinato (BS)	45°28'	10°24'
Ad31	Sozzano (BS)	45°39'	10°12'
Ad34	Castelnuovo Scrivia (AL)	44°59'	8°53'
Ad36	Fidenza (PR)	44°52'	10°05'
Ad40	Landriano (PV)	45°19'	9°15'
Ad43	Montale (SP)	44°11'	9°37'
Ad44	Corrodano (SP)	44°14'	9°38'
Ad64	Palma Campania (NA)	40°51'	14°32'
Ad66	Balvano (PZ)	40°36'	15°32'
Ad68	Matera (MT)	40°39'	16°35'
Ad69	Bari (BA)	40°56'	16°38'
Ad72	Foggia (FG)	41°28'	15°40'
Ad74	Bellano (LC)	45°59'	9°15'
Ad76	Laterza (TA)	40°37'	16°48'
Ad77	Sternata (LE)	40°13'	18°14'
Ad78	Tricase (LE)	39°57'	18°24'
Ad81	Iglesias (CI)	39°18'	8°33'
Ad82	Fluminimaggiore (CI)	39°25'	8°30'
Ad91	Lignano Sabbiadoro (UD)	45°39'	13°04'

Table 2 – Effect of sampling period and long-term storage on cane buds germination rate.Axillary buds germination rate (%) in hydroponic cultivated cane fragments was monitored T_0, 30, 60, 90 and 180 days after sampling. Cane fragments were stored at 4°C in the dark.

	Canes sampled in summer	Canes sampled in autumn	Canes sampled in winter
Days after sampling	Cane buds germination rate (%)	Cane buds germination rate (%)	Cane buds germination rate (%)
T_0	100	100	20
30	100	100	20
60	100	100	14
90	100	100	14
180	60	40	0

Table 3 – Comparison between hydroponic cultivated cane nodes treated with 2,5% sodium hypoclorite and untreated cane nodes, considering their ability of emitting shoots and roots. Confidence intervals at 95% are shown.

	Shoots emission (%)	Roots emission (%)
Untreated cane nodes	47.06 ± 11.95	36.76 ± 11.55
Treated cane nodes	60.00 ± 13.72	52.00 ± 13.99

Table 4 – Comparison among plants obtained using rhizome fragments (n=20) and hydroponic cultivated plantlets (n=20) as propagules, considering culms number, culms height and culms diameter. Plants were transplanted in the open field on March 28th, 2012, and measured on October 18th, 2012. Confidence intervals at 95% are shown.

Propagule	Average culms number	Average culms height (m)	Average culms diameter (cm)
Rhizome fragments	7.50 ± 0.92	2.78 ± 0.17	1.34 ± 0.08
Hydroponic propagation	8.29 ± 0.90	2.57 ± 0.18	1.27 ± 0.08

Table 5 – Comparison among plants obtained by in vitro propagation and hydroponic cultivation, transplanted in the open field on June 7th, 2013, (Landriano, PV) and on April 22nd, 2015, (Formigara, CR), considering the average production of biomass at the first year (Kg fresh weight), the average culms height and diameter and the average number of canes at the end of the season. Confidence intervals at 95% are shown.

Clone	Average biomass (kg fresh weight)	Average culms number	Average culms height (m)	Average culms diameter (cm)
Landriano, 2013 growing season				
In-vitro propagation	1.28 ± 0.20	12.00 ± 2.65	1.10 ± 0.22	0.71 ± 0.19
Hydroponic propagation	2.17 ± 0.27	6.04 ± 0.94	1.43 ± 0.13	0.99 ± 0.10
Formigara, 2015 growing season				
In-vitro propagation	0.88 ± 0.22	11.80 ± 2.37	1.61 ± 0.17	0.88 ± 0.23
Hydroponic propagation	1.34 ± 0.10	9.00 ± 0.99	1.76 ± 0.08	1.33 ± 0.08

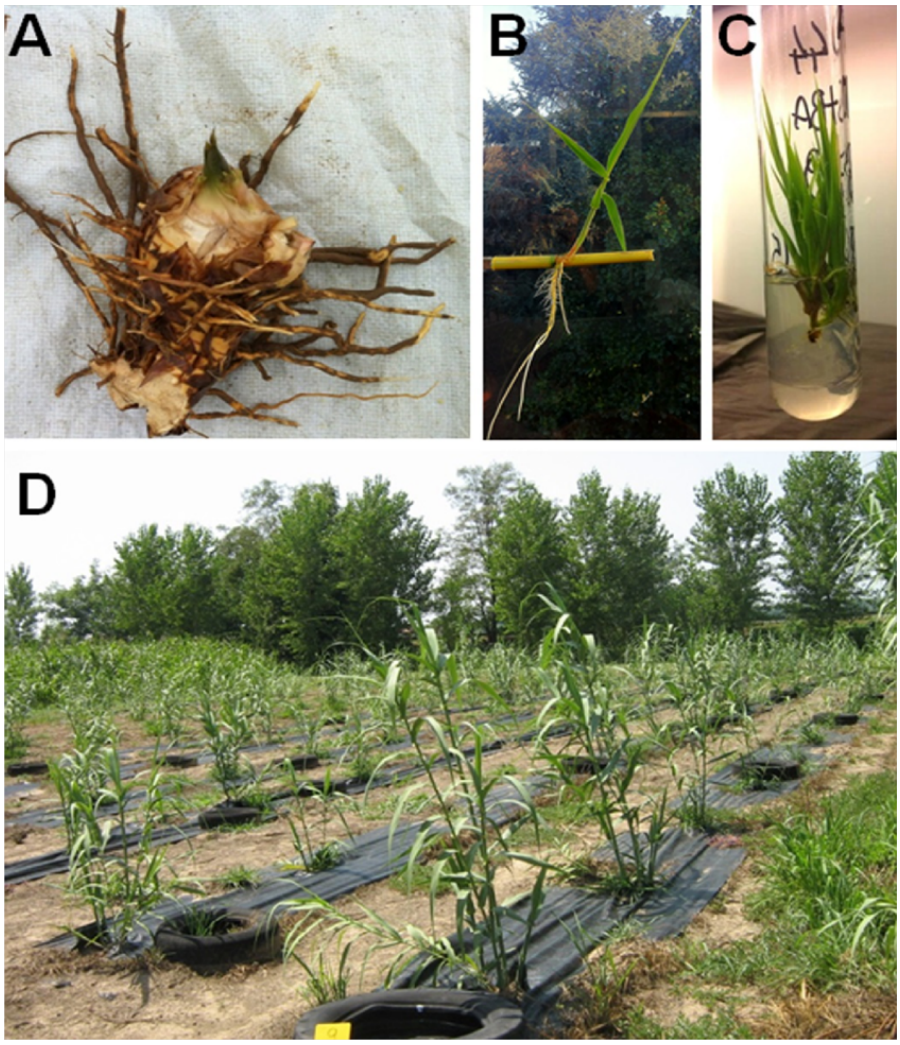

Figure 1 – Propagules obtained using different agamic propagation methods. Rhizome fragments (A), hydroponic cultivated plantlets (B) and in vitro propagated shoots (C). Experimental field situated in Landriano (PV): *A. donax* plants obtained through rhizome transplantation at the end of the first growing season (late summer).

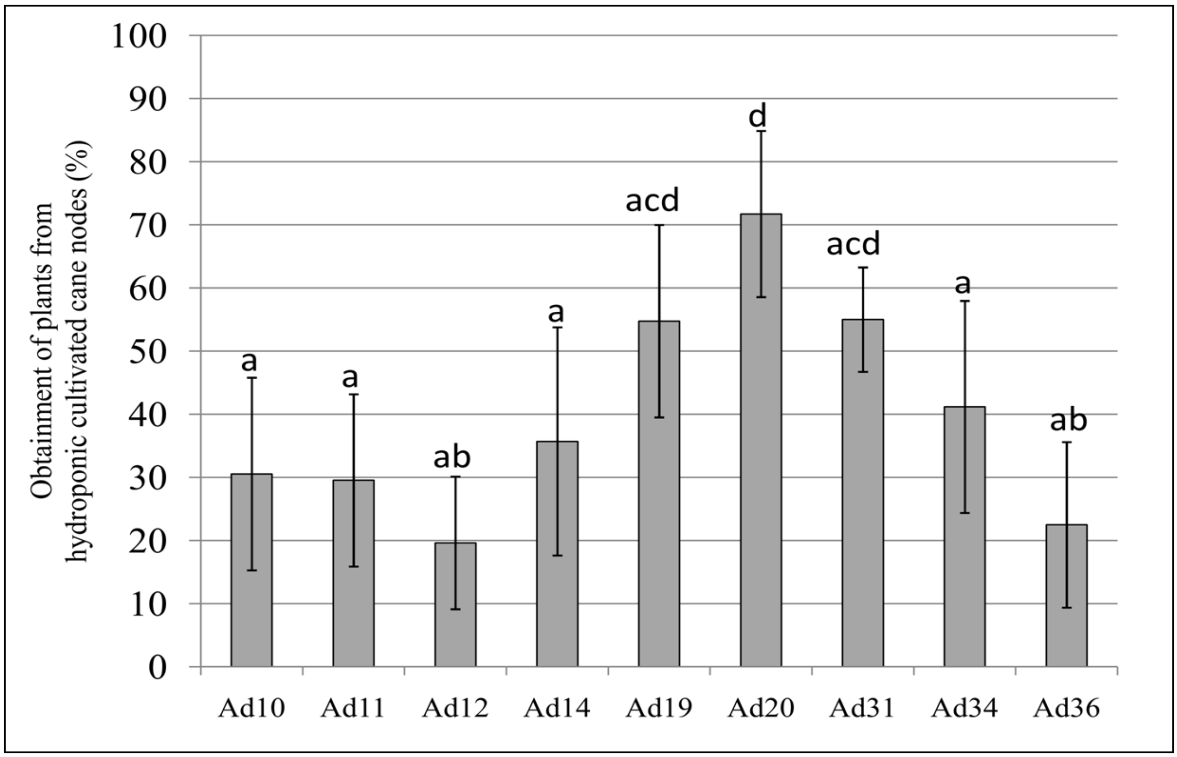

Figure 2 – Comparison among 9 hydroponic cultivated *A. donax* clones considering their ability to produce plantlets. Plantlets (with both shoot and root) were obtained from the buds of the clones Ad34, Ad20, Ad12, Ad10, Ad19, Ad14, Ad36, Ad11, Ad31. Confidence intervals at 95% are shown.

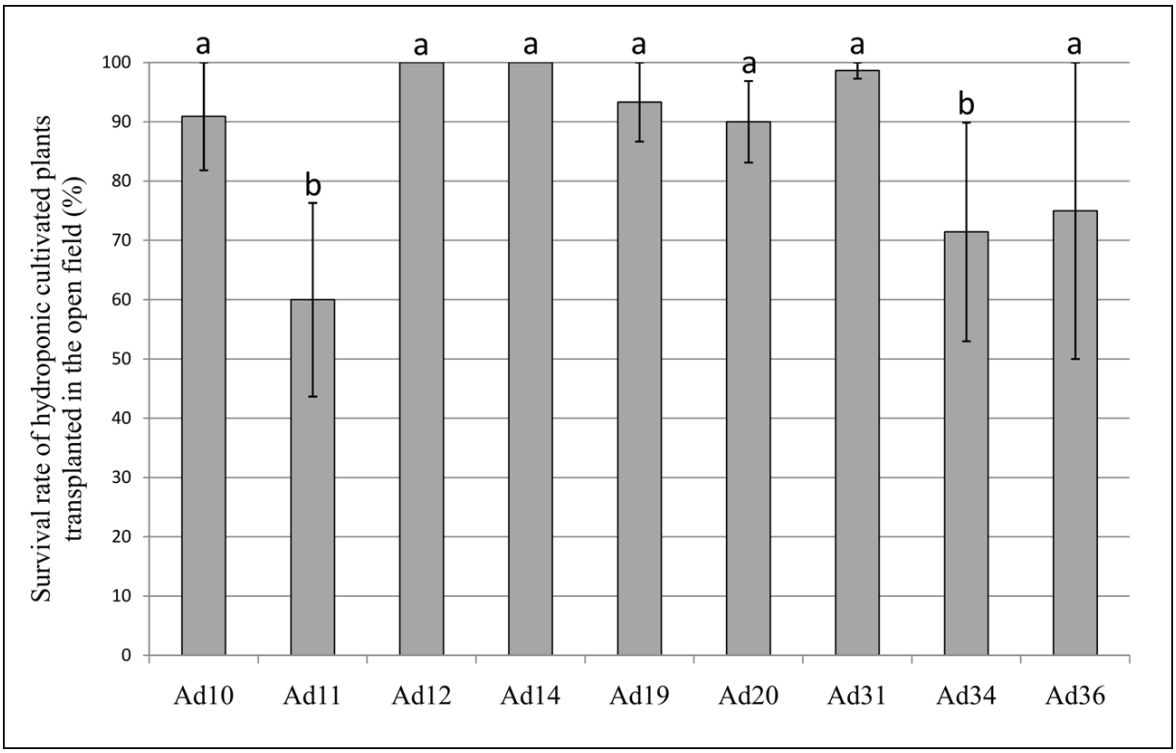

Figure 3 – Comparison among 9 *A. donax* clones considering the survival rate of hydroponic cultivated plants. 150 hydroponic cultivated plants of the clones Ad34, Ad20, Ad12, Ad10, Ad19, Ad14, Ad36, Ad11, Ad31 transplanted in the open field in March, 2012, were compared considering the survival rate observed on July 17th, 2012. Confidence intervals at 95% are shown.

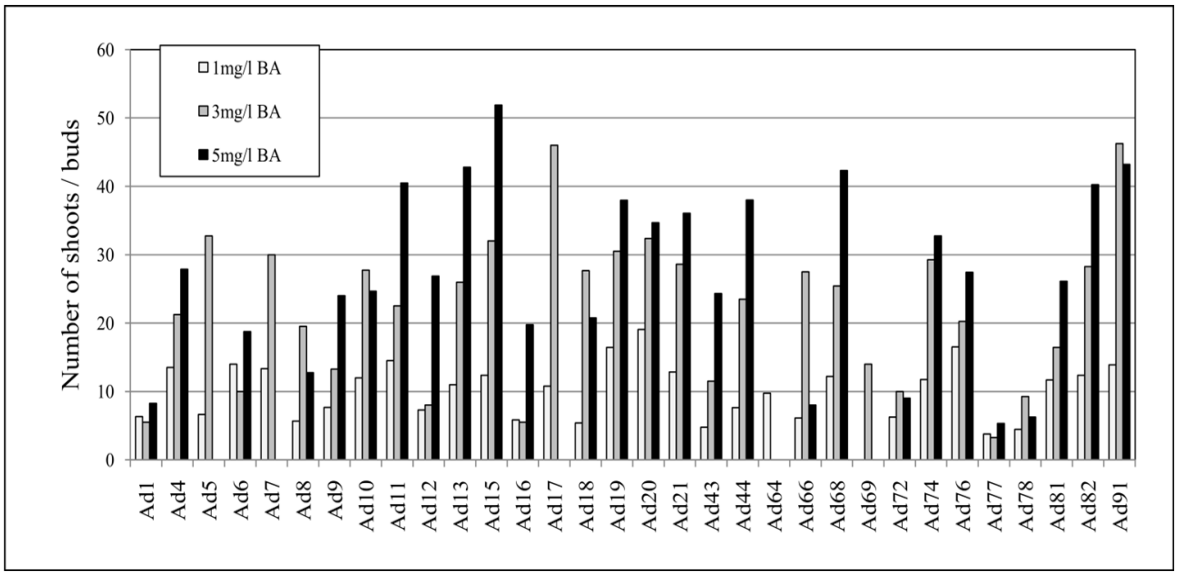

Figure 4 – Comparison among in vitro cultivated clones at different BA concentrations. 32 different clones: Ad1, Ad4, Ad5, Ad6, Ad7, Ad8, Ad9, Ad10, Ad11, Ad12, Ad13, Ad15, Ad16, Ad17, Ad18, Ad19, Ad20, Ad21, Ad43, Ad44, Ad64, Ad66, Ad68, Ad69, Ad72, Ad74, Ad76, Ad77, Ad78, Ad81, Ad82, Ad91, were compared considering in vitro shoots/buds development in 45 days at different BA concentrations (1.3 and 5 mg L^{-1}).

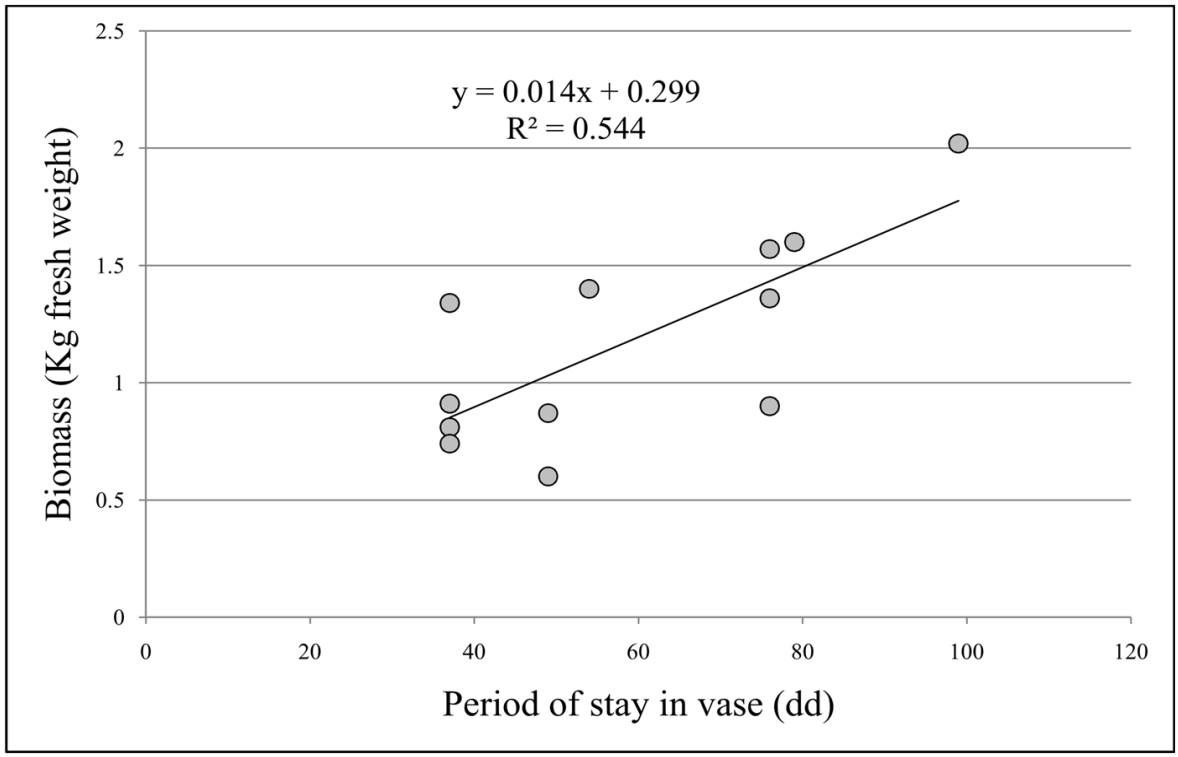

Figure 5 – Effect of the growth in pot period on biomass production. Correlation between the duration of the growth in pot period before transplantation in the open field (days) and biomass production (kg fresh weight, dry matter 23%) at the end of the first year, considering 12 hydroponic cultivated plants. Plants were transplanted in the open field on April 29th, 2015, and measured on September 4th, 2015.

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V

Giant cane (*Arundo donax* L.) for biogas production: the effect of ensilage on biomass characteristics and biogas potential

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Giant cane (*Arundo donax* L.) for biogas production: the effect of ensilage on biomass characteristics and biogas potential

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Abstract

Arundo donax L. is a perennial plant that can substitute traditional energy crops to produce biogas, reducing cost because of high biogas yield per Ha cultivated and low agronomic and energetic inputs. Nevertheless, *Arundo donax* biomass needs to be ensilaged to, be preserved and used. Because no full scale data exist about *A. donax* ensilage and the effect of this process on potential biogas production, in this work two different ensilage techniques, i.e. trench and silo-bag silo, were performed at full scale and processes studied for 200 days. Results obtained indicated that *A. donax* could be successful ensilaged by using the two approaches. Ensilage proceeded by fermentation of organic acids already present in the biomass, i.e. malic and oxalic acids that were degraded giving volatile fatty acid accumulation. This was different from corn ensilaging, characterized by starch fermentation to lactic acids. Biological processes determined a loss of the potential biomethane production i.e. -20.1 % and -7.6 % for trench and silo-bag, respectively. Taking into consideration biomethane yield per Ha and biomethane losses after ensilage, a potential income loss coming from electric energy generation was estimated to be of 5,000 € Ha^{-1} and 2,000 € Ha^{-1}, respectively. Nevertheless, taking into consideration the higher biomass and biomethane yields Ha^{-1} than the other energy crops *A. donax* still remained more valid and cheaper than traditional energy crops in producing biogas.

Keywords: Arundo donax; Biomethane; Bioenergy; Ensilage; Trench silo; Silo-bag silo.

1. Introduction

Biogas production by anaerobic digestion is one of the most interesting biotechnology producing electricity/biomethane to be used as substituting of fossil fuels (Weiland, 2010), and digestate to be used as fertilizer (Ledda et al., 2013). Anaerobic digestion can use different substrates, being energy crops the most employed biomass in the agricultural sector (Weiland, 2010). Nevertheless, energy crops can conflict with food/feed production and they are costly so that bioelectricity/biomethane need benefit to be produced (Schievano et al., 2014). In this way, low cost biomasses needs to be found and investigated reducing biogas cost and making the anaerobic digestion competitive with fossil fuels.

Giant cane (*Arundo donax* L.) has been recently proposed as an interesting energy crop because of its advantages with respect to traditional energy crops, i.e. high biomass yields, low agronomical inputs, great adaptability to different soils and low production cost (Lewandowski et al., 2003; Corno et al., 2014). The potential exploitation of giant cane in the energy sector are related to the production of biogas, bioethanol, and biomass for combustion (Corno et al., 2014), although its use for green chemistry has been, also, reported as part of integrated biorefineries (Corno et al., 2014; Manenti et al., 2016).

Giant cane for biogas seems to be a promising application because of its ability to substitute traditional energy crops (Ragaglini et al., 2014). Recently, Corno et al. (2015) showed, by lab-scale approach, that giant cane could substitute integrally corn in mix with pig slurries, reducing total cost for electricity/biomethane production.

Energy crops need to be ensiled in order to assure biomass availability along all the year (Neureiter et al., 2005). If many studies have been dedicated to crop energy behavior during anaerobic digestion (Weiland, 2010), less effort has been dedicated to study the effect of biomass storage (ensilage) on biogas potential preservation (Emery and Mosier, 2012). Ensilage process is a technique that exploits the natural fermentation operated by bacteria, allowing crops to be stored for long periods (Weiland, 2010; Pakarinen et al., 2008). Crop silage characteristics are affected by biomass chemical composition (Amon et al., 2007), as well as by fermentation products created during ensilage (Herrmann et al., 2011). The silage technique, also, affects silage characteristics (Weiland, 2010) because of different environment created that lead to the production of different amount and kinds of acids (Pakarinen et al., 2008) and biomass degradation (Pakarinen et al. 2008; Emery and Mosier, 2012); the latter affects biomass energetic content (Herrmann et al., 2011).

Trench and silo-bag are the most employed techniques used to ensilage energy crops (Bacenetti and Fusi, 2015); trenches silos are characterized by a three-wall structure with floor in which the biomass is accumulated, compressed by tractor, and covered by plastic covers (González Pereyra et al., 2011). The silo-bag solution does not require stable structures and *ad hoc* facilities but consists in the storing of biomass in plastic bags of variable dimensions (González Pereyra et al., 2011).

Literature data agree that silo-bag ensilage is better than trench ensilage because it reduces nutrients and organic matter losses (Ashbell et al., 2001; Bacenetti and Fusi, 2015), and environmental impact (Bacenetti and Fusi, 2015), increasing silage quality (Gonzalèz Pereyra et al., 2011).

By our knowledge, no full-scale data exist in the literature about *A. donax* ensilage, with particular reference to the silage characterization and to the biogas potential preservation after ensilage process. Nevertheless, recently Liu et al. (2015[a,b]) by lab-scale approach indicated that giant cane can be ensilaged successful without inoculum addition.

The scope of this work was to evaluate the ensilage process of giant cane biomass by adopting the two most used silage techniques, i.e. trench silo and silo-bag, at full-scale. In particular, this work aims to investigate the suitability of ensilage in preparing giant cane biomasses to be stored for long periods and to verify the effect of the two different techniques on the preservation of the biogas potentials, being this knowledge a requisite to allow giant cane becoming a widespread and low cost energy crop.

2. Materials and methods

2.1. Giant cane experimental field

Giant cane cultivation was performed at the experimental farm A. Menozzi of the University of Milan, located in Landriano, Pavia, Italy (N 45°18', E 9°15'). The experimental field was established on a soil previously used for corn cultivation in 2010, transplanting rhizomes of 400-500 g at 20 cm depth with a distance of 2 x 2 m as reported by Pilu et al. (2014). For the first year, plants were kept free from weeds by the application of glyphosate in surroundings. A first irrigation was performed to facilitate plant engraftment and every time during the dry season when water lack was substantial. During the successive years no irrigation, no fertilization and no chemical treatments were performed. The biomass growth was constantly monitored during the years collecting data about the height, weight and number of canes per plant. These data were processed in order

to get estimation of the biomass yields per hectare.

2.2. Silage process

At the end of October 2012, giant cane biomass was harvested and ensiled in trench silo. Harvesting was performed at the end of the growing season in order to achieve the maximum amount of biomass. The harvesting was carried out with the same technique employed for corn; 0.5-1 cm shredding dimension was set. In a trench silos usually used for corn silage, bale wrapper isolated the biomass from the floor and walls, and it was used to cover the silage. No enzymes or inoculum were added to the biomass before silage. Tractor was used to press the biomass and to facilitate the establishment of anaerobic condition before covering it with the bale wrapper. The trench was kept closed for all the monitoring period. Samples were collected perforating the biomass at a ≈80 cm depth with a sampler device in different points of the surface getting a representative sample of 4 kg of fresh matter.

The biomass for silo-bag trial was harvested at the end of October 2013 by adopting the same procedure used during 2012; shredded biomass was immediately used to fill the silo-bag by using a bagger normally employed for ensiling corn or sorghum. No enzymes or inoculum were added to the biomass before ensilage. The silage was sampled with the help of a sampler, in different positions of silo-bag with the same procedure used for trench silo. Both silages were sampled after 100 and 200 days from the trench and bag closures; during samplings, three subsamples were collected from the silos; subsamples were then mixed getting a representative sample. On-site inspection was performed every time silos were sampled; in particular, biomass color and smell (González Pereyra et al., 2011), moisture content, pH and the presence of molds and leachate were performed. Temperature was detected in the middle of the mass (more than 70 cm depth), to avoid the effect of environment temperature (Bartosik, 2012).

2.3. Chemical characterization of biomasses

The silages were characterized for the principal chemical parameters. The determination of total solids (TS), volatile solids (VS), total organic carbon (TOC), total nitrogen (TN) and ammonia nitrogen ($N\text{-}NH_4^+$) was conducted according to the standard procedures (APHA, 1998). Volatile fatty acids (VFA) and total alkalinity (TA) were determined by the titration method (APHA, 1998; Lahav et al., 2002). Organic acids extraction and quantification

were determined by HPLC analysis according to Canale et al., (1984). A 300x7.8 mm Aminex HPX-87H column and a UV detector (Waters 2410, Milford, MA, USA) at 210 nm were used for separation and quantification of acids. Analyses were performed isocratically with an operational temperature of 25°C, injection volume of 20 μL, flow rate of 0.6 ml min^{-1}, a 0.0025 Mol L^{-1} H_2SO_4 mobile phase and a running time of 30 min. Chromatograms were elaborated with the software Breeze (Waters, Milford, MA, USA).

The characterization of fibers, i.e. cell soluble, hemicellulose, cellulose and lignin contents was performed by the determination of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) (Van Soest et al., 1991). Anaerobic biogas potential and biomethane potential (ABP and BMP) tests were performed on all samples according to Schievano et al. (2009); a gas chromatograph (Micro GC 3000, Agilent Technology, Palo Alto, CA, USA) was used for determining CH_4 concentration in biogas (v/v).

One-way ANOVA, using the Tukey test to compare means with a level of significant differences set at $p<0.05$, was performed on all data. SPSS software (SPSS Statistics v21.0, IBM, Armonk, NY, USA) was used for statistical analyses.

3. Results and discussion

3.1. Practical comparison on field of the two silages

The two silos were opened and on-site inspection and the determination of simple parameters were performed (Table 1). Silages colors were those typical of an ensiled biomass (Figure S1); no molds were observed on both biomasses and the pHs were between 3.5 and 4; in addition no percolate was noted for both the silages. Different smell characterized the two silages, i.e. trench silage smelled of acetic acid, contrarily to silo-bag silage that smelled of alcohol, suggesting that different fermentation occurred during ensilages. Even if the raw biomasses before silages had comparable moisture content, after ensilage the trench biomass was characterized by higher moisture content than silo-bag silage (Table 1). The different techniques used to prepare silage influenced also the structure of the biomass silages, i.e. the trench biomass was more compact than the silo-bag one.

3.2 Chemical characterization of silages

Raw biomasses employed for silage tests were harvested in two different years, i.e. 2012 and 2013. Both biomasses were chemically characterized in order to evaluate if silage processes were affected by the raw material composition. Biomasses were quite similar (Table 2): pH, conductivity, ammonia, VFA, organic acids profile, TA, TKN, TS, VS, ashes and fiber composition were not statistically different.

A. donax silage obtained by trench silo approach showed (Table 2) a pH around four for both silage samples took after 100 days and 200 days that were much lower than that measured for the raw biomass. pH lowering was due to the accumulation of VFAs (Linke et al., 2015) which content was constant along all silage period and, as average, of 8.23±0.29 g CH_3COOH kg^{-1} TS and of 8.64±0.7 g CH_3COOH kg^{-1} TS, respectively for 100 d and 200 d samples (Table 2). VFAs and organic acids speciation indicated that different biological fermentations occurred during silage process (Herrmann et al., 2011). In particular, organic acids speciation indicated that biomass silages were characterized after 100 d of ensilage for the strong presence of Krebs cycle-derived acids such as oxalate (above all) but also citrate, malate and succinate. On the other hand, malate acid that was present in large concentration in the raw material was practically completely degraded just after 100 d of ensilage process.

Krebs cycle acids, especially malate and oxalate, are normally produced by plant cells as protection against foraging animals and insects and for pH regulation and osmoregulation; these acids, because of their toxicity, are normally stored in the cell as salt (Libert and Franceschi, 1987). The fermentation of both sugar and Krebs cycle acids determined after 100 d of ensilage, the presence in the silage of lactate, and above all acetate and other VFAs (Whittenbury et al., 1967). Ensilage then continued consuming organic acids so that after 200 days of ensilage, Krebs cycle organic acid were strongly reduced (oxalate above all) as well as lactate, leading to an increase in the silage of acetate, propionate, butyrate, N-butyrate and iso-valerate (Whittenbury et al., 1967).

Other chemical parameters also changed during ensilage: conductibility and ammonia contents increased because of polymers hydrolysis and protein degradation, producing monomers and ammonia, respectively. The fiber composition of final silage changed as well, i.e. hemicelluloses content decreased and cellulose (above all) and lignin increased. Volatile solid did not change during ensilage indicating that no-appreciable organic matter

degradation occurred. This data was confirmed by TOC content that did not change as well.

Silo-bag ensilage gave different patterns with respect to trench ensilage: the pH registered was of 3.96±0.04 after 100 days to increase to 4.72±0 after 200 days of silage; although they were both lower than that measured for raw material, they were higher than those registered for the trench silo.

Such as registered for the trench silo, after 100 days of ensilage oxalic acid was the most represented organic acid followed by others Krebs cycle acids and VFAs. The successive silage sampling after 200 d indicated that Krebs cycle organic acids were strongly reduced and VFAs accumulated (Table 2). Probably the organic acid reduction (oxalate above all) (Table 2) was responsible for higher pH at the end of ensilage with respect sample took after 100 days. In particular moving from the first to the second sampling, the Krebs cycle acids (oxalate, malate and succinate) were consumed or totally degraded (succinate) so that very low concentrations of these organic acids were detected after 200 days of silage. Lactate was above all produced during the first 100 days of ensilage, while its concentration decreased at the end of the ensilage. On the other hand, acetate and VFAs in general, gradually increased during ensilage (Table 2) because of fermentation of organic acids (Whittenbury et al., 1967). Conductivity and ammonia concentrations were higher than those measured for raw biomass because, such as showed for trench silo, organic matter hydrolysis and protein degradation occurred. The fiber composition changed during the ensilage, i.e. cell soluble decreased determining a relative increased of both cellulose and hemicelluloses; these trends agreed with organic acid degradation, indicating that, above all, oxalate fermentation and Krebs cycle organic acids to VFA were responsible for cell soluble increasing. Again, both VS and TOC did not change during ensilage indicating that no appreciable organic matter degradation occurred.

3.3. Comparison of the two silage techniques and biomass preservation

The chemical characterization of raw materials revealed that biomasses obtained in two different crop year (2012 and 2013) had chemical characteristics that were not statistically different so that a tentative in comparing different ensilage techniques can be made.

From the Table 2, it can be seen that the most remarkable differences between the two biological processes consisted in the final silage-pH, VFA and organic acids accumulation and speciation (Table 2).

Both silages showed lower pH values than those of raw materials and in line with those reported for corn silage (Linke et al., 2015) and other ensiled energy crops (Herrmann et al., 2011). Nevertheless, trench silage showed lower pH than silo-bag silage. Being alkalinity and VFAs (on fresh matter) registered for the two silages were similar, pH difference was due to the higher degradation of organic acids (mainly oxalate) occurred in the silo-bag, so that their concentrations at the end of the ensilage were much lower than those of the trench silo (Table 2).

Trench silage revealed also the higher presence of acetate (about double, on a dry matter basis) than silo-bag, being reported short chain acids to be indicator of clostridia activity and consequently of biomass putrefaction (Whittenbury et al., 1967).

Fiber composition behavior showed different trends: trench silo was characterized by hemicelluloses decrease in agreement with other works (Herrmann et al., 2011) and cellulose increase (on a relative basis) differently by silo-bag that was characterized for cellular soluble fraction decreased and both cellulose and hemicellulose increased (on a relative basis). These differences seems to indicate that in the trench silo fiber was also involved in the biological process, i.e. hemicelluloses were hydrolyzed and fermented producing VFAs, as well as Krebs cycle acid (Table 2). The fact that cellular soluble fraction did not change along ensilage process, although organic acid were strongly degraded, suggested that new cellular soluble fraction was continuously produced from hemicelluloses fermentation replacing organic acid degradation, i.e. both organic acid and hemicelluloses were fermented producing VFAs (Pérez et al., 2002). Fiber degradation could be due to possible air infiltrations during ensilage (Bacenetti and Fusi, 2015).

Contrarily silo-bag silo was above all characterized for VFAs production because of organic acid fermentation, being fiber fraction not involved in any biological process.

In conclusion, presented data indicated that ensilage processes proceeded by fermentation of already present plant-organic acids (malate and oxalate) producing above all VFAs. Trench silage was characterized also by the partial degradation of hemicelluloses.

Arundo donax silages were compared with data collected for corn silage (Table 2). Corn silage, as average, was characterized by lower pH, TA and VFA contents and higher lactate concentrations than *A. donax* silages. Differences were due to both different chemical biomass composition and fermentation occurred, i.e. the cornstarch was fermented producing lactic acid (Haag et al., 2015).

3.4. Evaluations on silages conversion to biogas and bioenergy

Anaerobic biogas potential (ABP) were performed on silage samples and results reported in Table 2. Silage processes determined a reduction of the ABP in comparison to values measured for raw materials. In particular, ABPs measured for both trench and silo-bag silages were statistically lower than those of corresponding raw material, i.e. 18.4 % and 10.9%, respectively. As CH_4 contents of the biogas (Table 2) were not statistically different between and within different silages with an average of 61.3±1.2 %, speculations made for ABPs resulted valid also for biomethane potentials (BMP), i.e. BMP reduction of 20.1% and 7.6% for trench and silo-bag silage, respectively. BMP reduction for trench silage occurred, above all, during first ensilage stage (after 100 d) (Table 2), being this data identical to the one detected after 200 days of ensilage.

These results agree with literature that reported how the preservation and the quality of biomass is generally more assured by silo-bag technique (Bacenetti and Fusi, 2015), because of better isolation of biomass from environment (Gonzaléz Pereyra et al., 2011) that allow the preservation of organic acids against further fermentation processes (Ashbell et al., 2001).

Potential methane reductions obtained were in contrast with previous works that reported an increase of the biogas production after ensilage (Herrmann et al., 2011; Pakarinen et al., 2008; Neureiter et al., 2005). Nevertheless, it was also reported that the assumption that the silage process could promote the biogas and biomethane yields sometimes is not supported by significant results, especially when lab-scale and full-scale data are compared (Neureiter et al., 2005).

Taking into consideration biomass yields of 71.8 Mg TS Ha^{-1} and of 63.2 Mg TS Ha^{-1} for the 2012 (trench silage) and 2013 (silo-bag ensilage) respectively, the total amounts of methane producible per hectare were calculated and reported in Table 3. From these data, it can be deduced that ensilage processes determined a potential methane reductions of 5,000 Nm^3 CH_4 Ha^{-1} for trench silage and of 2,000 Nm^3 CH_4 Ha^{-1} for silo-bag silage. Less methane per Ha means less electricity production per Ha that was estimated as 17.8 MW h_{EE} Ha^{-1} for trench ensilage and 6.9 MW h_{EE} Ha^{-1} for silo-bag silage respectively. Considering the benefit accorded to the biogas plant in Italy (0.28 € kW h_{EE}^{-1}) (Riva et al., 2014) and the potential electricity producible per Ha (Table 3), the potential income losses due to the less potential biogas because of ensilage, were estimated as 4,985 € Ha^{-1} and 1,927 € Ha^{-1} for trench and silo-bag silages, respectively.

In any case, if both silages and potential biomethane productions were considered in comparison with data for other energy crops such as corn, sorghum, rye and triticale (Corno et al., 2014; Schievano et al., 2014) the total amount of biomethane producible per Ha in 2012 and 2013, were much higher than those of other energy crops (Table 4). By comparison with other biomethane losses, giant cane silage from silo-bag showed losses that were more comparable to sorghum silage i.e. 4.1 % (Herrmann et al., 2011; Schievano et al., 2014) while reductions of trench silage were not comparable with all the reported crops. Corn, rye and triticale silages showed increased methane yields after the storage. Consequently, if for sorghum the income loss was not remarkable, for the other cultures a potential profit ranging between 523 € Ha^{-1} and 1,203 € Ha^{-1} was estimated. Even if in both analyzed cases the giant cane silage was affected by an economical loss, the net potential income from electric energy production was in any case higher in comparison to all the other energy crops.

4. Conclusions

Giant cane represents and valid alternative to traditional energy crops to produce biogas, reducing cost and increasing biogas yield per Ha. Full-scale ensilage trials indicated that, although giant cane does not contain starch, it can be correctly ensiled and so stored for a long period. Both trench and silo-bag techniques seems to be useful for *A. donax* ensilage, although silo-bag stands out to be more helpful in preserving biogas potential, in agreement with literature. In any case, taking into consideration biogas potential after ensilage and total biomass produced per Ha, giant cane resulted strongly more productive than traditional energy crops; this fact, in joint with the low cost to produce this crop, makes *A. donax* a valid alternative to traditional energy crops in producing biogas.

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Table 1 - Practical evaluation on site.

	Trench silage		Silo-bag silage	
	100 days	200 days	100 days	200 days
Color	Bright color. Yellow-green or yellow-brown	Bright color. Yellow-green or yellow-brown	Bright color. Yellow-green or yellow-brown	Bright color. Yellow-green or yellow-brown
Mold	No	No	No	No
Smell	Acetic acid	Acetic acid	Alcoholic	Alcoholic
Moisture	65.8 %	65.4 %	57.4 %	57.4 %
T	6°C	28°C	18 °C	24 °C
Percolation	Low	Low	No	No
pH	3.5-4	3.5-4	4	4
Dimension (cm)	0.5-1	0.5-1	0.5-1	0.5-1
Structure	Material is compact but crumbles easily.	Material is compact but crumbles easily.	Material crumbles easily and is dry.	Material crumbles easily and is dry.

Table 2 – Principal chemical parameters, structural composition of biomasses and biogas and biomethane potentials.

		Trench silage			Silo-bag silage			Corn silage[a]
		Raw biomass	100 days	200 days	Raw biomass	100 days	200 days	
pH		5.21±0.18 B,c	4.00±0.02 A,a	3.99±0.03 A,a	5.18±0.14 C,c	3.96±0.04 A,a	4.72±0 B,b	3.73±0.24
Conductibility	mS	0.63±0.02 A,a	1.36±0.08 B,b	1.80±0.03 C,c	0.59±0.01 A,a	3.09±0.10 B,d	2.98±0.10 B,d	2.09±0.69
N-NH_4	g kg^{-1} TS^{-1}	0 A,a	0.53±0.11 B,ab	0.81±0.08 B,b	0 A,a	0.52±0.03 B,ab	0.7±0.15 B,b	0.97±0.48
Oxalate	g kg^{-1} TS^{-1}	63.3±11.7 B,b	54±3.2 B,b	21.6±1.4 A,a	62.5±10.9 B,b	22±10.4 A,a	4.88±0.24 A,a	1.4[b]
Citrate	g kg^{-1} TS^{-1}	14.6±3 B,b	1.3±0.2 A,a	0 A,a	11.3±1.1 B,b	0 A,a	0 A,a	0.57[b]
Malate	g kg^{-1} TS^{-1}	102±6 B,b	2.65±0.26 A,a	0 A,a	100±1 B,b	4.42±1.89 A,a	0.78±0.05 A,a	1.31[b]
Succinate	g kg^{-1} TS^{-1}	n.d. A,a	7.37±0.89 B,ab	0 A,a	0 A,a	11.4±5.9 B,b	0 A,a	1.53[b]
Lactate	g kg^{-1} TS^{-1}	0 A,a	9.1±1.26 C,d	3.73±0.3 B,c	0 A,a	3.5±0.52 C,c	0.17±0.01 B,ab	9.42[b]
VFA	g CH_3COOH kg^{-1} FM^{-1}	0 A,a	8.23±0.18 B,c	8.64±0.7 B,c	0 A,a	6.39±0.51 B,b	10.3±0.2 C,d	4.74±6.71
Alkalinity	g $CaCO_3$ kg^{-1} FM^{-1}	0 A,a	7.59±0.1 B,c	8.59±0.27 C,d	0 A,a	4.51±0.36 B,b	8.44±0.34 C,cd	3.73[b]
Acetate	g kg^{-1} TS^{-1}	0 A,a	7.83±0.94 B,d	10.7±0.8 B,d	0 A,a	2.1±0.7 B,b	5.24±0.13 C,c	2.87±2.57
Propionate	g kg^{-1} TS^{-1}	0 A,a	0 A,a	0.58±0.05 B,ab	0 A,a	0.96±0.36 B,b	0.49±0.07 AB,ab	0.21±0.29
Isobutyrate	g kg^{-1} TS^{-1}	0 A,a	0 A,a	1.02±0.07 B,b	0 A,a	0.76±0.3 B,b	1.88±0.04 C,c	2.09±2.95
Nbutyrate	g kg^{-1} TS^{-1}	0 A,a	0 B,c	1.68±0.14 A,b	0 A,a	0.75±35 AB,ab	2.11±0.49 B,b	-
Isovalerate	g kg^{-1} TS^{-1}	0 A,a	0 A,a	3.02±0.3 B,c	0 A,a	1.08±0.36 AB,b	2.29±0.49 B,c	-
TS	% (w/w)	40.7±0.1 B,b	34.2±0.5 A,a	34.6±0.5 A,a	40.0±0.3 A,b	42.6±0.4 B,c	40±0.3 A,b	35.1±3.7
VS	% (w/w)	94.5±0.1 A,ab	94.4±0.8 A,ab	93.8±0.7 A,a	96.2±0.6 A,b	96.0±0.6 A,ab	96.2±0.4 A,b	95.2±2.5
Ash	% (w/w)	5.5±0.1 A,ab	5.6±0.8 A,ab	6.2±0.7 A,b	3.8±57 A,a	4±0.64 A,ab	3.8±0.37 A,a	4.8±2.5
TOC	g kg^{-1} TS^{-1}	433±5 A,a	436±4 A,a	459±29 A,a	436±11 A,a	424±7 A,a	485±23 A,a	-
TKN	g kg^{-1} TS^{-1}	8.87±0.12 C,d	6.80±0.26 A,b	7.92±0.09 B,c	6.05±0.07 A,a	7.39±0.07 B,c	6.08±0.12 A,a	10.7±4.9
Cellular soluble	% (w/w)	31.9±0.8 B,c	25.1±0.1 A,ab	30.4±1.2 B,bc	32±1.3 B,c	26.5±0.9 AB,bc	20.5±2.6 A,a	52.9±12.5
Hemicellulose	% (w/w)	21.3±1.7 A,a	21.2±0.4 A,a	17.9±1.7 B,a	23±2.9 A,ab	21.4±0.6 A,a	27.6±0.9 A,b	15.1±2.2
Cellulose	% (w/w)	35.3±0.2 A,a	39.5±0.4 A,a	39.4±3.6 A,a	34.7±0.7 A,a	38.5±0.4 AB,a	40.0±1.7 B,a	24.6±5.8
Lignin	% (w/w)	11.5±2.1 A,a	14.2±0.6 A,a	12.3±2.1 A,a	10.3±1.0 A,a	13.6±0.7 B,a	11.9±0 AB,a	7.2±4.8
ABP	Nm^3 Mg^{-1} TS^{-1}	581±2 B,c	473±2 A,a	470±6 A,a	610±3 A,d	596±11 B,cd	544±5 A,b	653±**57**
ABP	Nm^3 Mg^{-1} VS^{-1}	613±1 B,c	501±6 A,a	500±6 A,a	634±0 B,d	622±7 B,cd	565±3 A,b	688±65
CH_4	% (v/v)	61.6±2.5 A,a	59.8±4.9 A,a	59.9±2.3 A,a	61.4±0.4 A,a	62.9±2.6 A,a	62.1±1.2 A,a	64.9±4.2
BMP	Nm^3 Mg^{-1} TS^{-1}	354±14 B,b	285±16 A,a	281±12 A,a	366±3 C,b	369±17 B,b	338±7 A,b	425±63
BMP	Nm^3 Mg^{-1} VS^{-1}	374±1 B,b	302±4 A,a	299±21 A,a	380±14 B,b	385±5 B,b	351±2 A,b	448±67

[a] Average corn silage values calculated from Schievano et al. (2009), Santi et al. (2015), Linke et al. (2015) and Authors data about trench silage (unpublished data).

[b] Data reported only by Authors unpublished data.

Values followed by the same letter in the same line are not statistically different with a p-value<0.05 (Tukey test); uppercases refer to the analysis of the same silage technique, lowercases to both the silage techniques.

Table 3 – Efficiencies of different silage techniques on preserving biomethane potentials in comparison with other crops and evaluation of potential income loss due to ensiling process.

		Biomass[a] yield	BMP	TS losses during ensilage	BMP loss[b]	BMP per hectare[c]	Potential electric energy per hectare[d]	Potential electric energy loss	Potential electric energy income[e]	Potential income loss
		Mg TS Ha^{-1}	Nm^3 CH_4 Mg^{-1} TS^{-1}	% starting TS	% starting BMP	Nm^3 CH_4 Ha^{-1}	MW h_{EE} Ha^{-1}	%	€ Ha^{-1}	€ Ha^{-1}
Giant cane (in trench)	Raw material	71.8	374±1b[fg]	n.d.[h]		26,853	94.4		26,442	
	Silage		299±21a	0	-20.1	21,468	75.5	-18.9	21,139	-5,302
Giant cane (in silo-bag)	Raw material	63.2	380±14b	n.d.		24,016	84.5		23,648	
	Silage		351±2a	0	-7.6	22,183	78	-6.4	21,843	-1,805
Corn	Raw material	19.7	330±7a	n.d.		6,499	22.9		6,399	
	Silage		373±10b	5.7	6.6	6,931	24.4	1.5	6,825	425
Sorghum	Raw material	19.4	317±12a	n.d		6,152	21.6		6,057	
	Silage		333±14a	7.6	0	5,975	21	-0.6	5,883	-174
Rye	Raw material	8.7	293±3a	n.d.		2,552	9		2,513	
	Silage		346±8b	9.1	7.3	2,738	9.6	0.7	2,696	183
Triticale	Raw material	16.5	340±5a	n.d.		5,585	19.6		5,499	
	Silage		365±10b	8.6	-1.7	5,488	19.3	-0.3	5,404	-95

[a]Energy crop yield as in the following: giant cane: this work; corn, sorghum, rye and triticale: from Schievano et al. [3].
[b]BMP losses assumed equal to zero when non statistical differences were detected.
[c]Calculated taking into consideration BMP and total solid losses during ensilage and total solid produced Ha^{-1}.
[d]Calculated taking into consideration the methane lower heating value and the electrical generation yield as reported by Schievano et al. [3].
[e]Estimated with the biogas benefit accorded to Italian law (0.28 € kW h_{EE}^{-1}).
[f]Values followed by the same letter are not statistically different within the same energy crop, with a p-value<0.05.
[g]Potential biomethane productions as in the following: giant cane: this work; corn, sorghum, rye and triticale: from Herrmann et al. [13].
[h]Not detectable.

Figure S1 – Trench silage (2012-2013) and silo-bag silage (2013-2014) and the detailed picture of both the silages during sampling.

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VI

New energy crop giant cane (*Arundo donax* L.) can substitute traditional energy crops increasing biogas yield and reducing costs

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New energy crop giant cane (*Arundo donax* L.) can substitute traditional energy crops increasing biogas yield and reducing costs.

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Abstract

Giant Cane is a promising non-food crop for biogas production. Giant cane and corn silages coming from full-scale fields were tested, in mixtures with pig slurry, for biomethane production by a continuous stirred tank lab-scale-reactor (CSTR) approach. Results indicated that giant cane produced less biomethane than corn, i.e. 174±10 Nm^3 CH_4 Mg^{-1} TS^{-1} and 245±26 Nm^3 CH_4 Mg^{-1} TS^{-1}, respectively. On the other hand, because of its high field biomass production, the biogas obtainable per Ha was higher for giant cane than for corn, i.e. 12,292 Nm^3 CH_4 Ha^{-1} and 4,549 Nm^3 CH_4 Ha^{-1}, respectively. Low energetic and agronomic inputs for giant cane cultivation led to a considerable reduction in the costs of producing both electricity and biomethane, i.e. 0.50 € Nm^{-3} CH_4^{-1} and 0.81 € Nm^{-3} CH_4^{-1}, and 0.10 € kWh_{EE}^{-1} and 0.19 € kWh_{EE}^{-1} for biomethane and electricity production, and for giant cane and corn mixtures respectively.

Keywords: Arundo donax; Biogas; ^{13}C CPMAS NMR; CSTR approach; Energy crop.

1. Introduction

World biogas production was reported for 2012 to be of about 17,200 ktoe y^{-1} (Raboni and Urbini, 2014), with most of it concentrated in the EU, i.e. about 60% of the total production, while the contribution of North America is limited to approximately 22% and with much lower contributions by the Asian-Pacific (about 11%), Latin American (6%) and the Middle East-African regions (about 1%) (Raboni and Urbini, 2014).

Biogas production has been rapidly diffused in the last 10 years because of the supportive politics of central governments towards renewable energy production. In Italy, for example, for the period 2009-2012 electricity from biogas had a value of 0.28 € $kWhe^{-1}$. High benefits pushed agricultural producers in the direction of using energy crops to produce biogas as they are more productive in terms of methane produced per kg of biomass, allowing medium size biogas plants to be established (1 MW). The reduction in the subsidy benefit to 0.16 € $kWhe^{-1}$ (referring to plants using more than 30% wet weight – ww energy of energy crops) started in 2013 (Schievano et al., 2014). It has had the effect of causing a new look at biogas plant feedstock because energy crops were no longer sustainable in terms of biomass cost. The impact of this cost on the total biogas production cost (1 MW plant) was of 47%-66% (Schievano et al., 2014). All these facts stimulated, in Italy, the shifting of the feeding of biogas plants with energy crops to the use, above all, of solely animal slurry, since the use of municipal solid-organic waste is banned from agriculture and agro-industrial wastes are not always available. Since animal slurry is much less productive than energy crops, biogas plant size is shifting from medium sizes (1MW) to small sizes (0.05-0.25 MW). If the use of animal slurry has the benefit of reducing biomass cost and soil use (Schievano et al., 2014), low biogas productivity, by reducing plant size, increases the costs of transforming biogas into electricity (Schievano et al., 2014). As a logical consequence, the integration of animal slurry with energy crops remains important, as long as the costs of producing biomass can be considerably reduced.
Reducing the costs for energy crop production means increasing crop yields per Ha and/or reducing inputs to produce the biomass.
Recently Corno et al., (2014) reviewed *Arundo donax* L. (giant cane or giant reed), as an interesting emerging crop for energy production and/or green chemistry. Giant cane is a perennial and herbaceous plant widespread all over the world in different mainly frost-free environments. It grows preferentially along lakes, ponds and rivers, but it also easily grows in different types of ecosystems with lower water availability; giant cane cultivation can also be established on different kinds of soils (Lewandowski et al., 2003). One of the most important and distinguishing characteristics of this plant is the high biomass production per Ha (Angelini et al., 2009). Angelini et al. (2009) reported an average biomass production on a 12 years length crop of 30-40 Mg Ha^{-1} of dry matter. This biomass is produced with low agronomic input, e.g. low or no use of irrigation, fertilizers, pesticides and low agronomic interventions with machinery (Lewandowski et al., 2003): these mean both low

costs for its cultivation (Soldatos et al., 2004) and low environmental impacts (Riffaldi et al., 2012). Thanks to these characteristics, giant cane has recently been proposed as an energy crop for producing biogas, and a full field crop campaign started in Italy in 2013 on about 25 farms. Although many data exist about production of the crop (Corno et al., 2014), no data exist regarding the biogas production, or rather, data exist solely for anaerobic biogas potential (ABP), through tests performed by the batch approach (Corno et al., 2014; Ragaglini et al., 2014). These data suggested that giant cane produced less biogas than traditional energy crops (Corno et al., 2014). The lower biogas production was associated with the composition of the fibers, characterized by high cellulose and lignin content (Komolwanich et al., 2014) and to the absence of starch because the sterile cane plants produce no grains (Wijte et al., 2005). On the other hand the higher biomass yield per unit area of giant cane compared with other energy crops (Corno et al., 2014) allows cane to produce much more biogas per cultivated unit area. As reported by Corno et al. (2014) and Ragaglini et al. (2014), by using ABP data, the obtainable methane yield can be more than 9,000 Nm^3 CH_4 Ha^{-1} (Corno et al., 2014; Schievano et al., 2014).

Until now, the *Arundo donax* derived biomass has never been tested in a continuous test (continually stirred tank reactor – CSTR) and so, there are no exit data about the performance of this energy crop in producing biogas.

The aim of this work was to test the giant cane biomass produced and ensiled at full field scale, in producing biogas in a lab-scale CSTR in comparison with corn. In particular we wanted to obtain biogas production data in combination with data relative to the costs of biomass production and transformation into final products, we aimed to obtain total costs of producing electricity and biomethane to be compared with those of corn, other traditional energy crops and organic wastes.

We also aimed to carry out more in depth investigations about anaerobic processes, biogas production performance, biogas composition and organic matter transformation in order to offer for the first time a complete picture of the use of *Arundo donax* to produce biogas.

2. Materials and methods

2.1. Field experiment and silage

The biomasses used for this work were cultivated and prepared at the experimental farm A. Menozzi of the University of Milan, located in Landriano, Pavia, Italy (N 45°18', E 9°15'). Corn was sown in April 2012; the soil surface of 40 Ha was plowed, and dimethenamide-P

(700 g Ha^{-1}) plus terbutylazide (625 g Ha^{-1}) solution was used as pre-emergence herbicide. After corn emergence fluroxipir (100 g Ha^{-1}) plus nicosulforon (40 g Ha^{-1}) solution was used for weed control. Corn was fertilized with bovine slurry (80 m^3 Ha^{-1}) and urea (110 kg N Ha^{-1}). Surface irrigation was employed for plant water supply by two interventions with 2100 m^3 Ha^{-1} of water. Corn was treated with 25 g Ha^{-1} of a deltamethrin solution against the European Corn Borer (*Ostrinia nubilalis*). In July the corn biomass was harvested and ensiled. Total production was of 63.7 Mg fresh matter Ha^{-1} and of 19.8 Mg dry matter Ha^{-1}.

Giant cane cultivation took place at the same experimental station and was established in 2010 by using 100 different clones coming from all the Italian national territory. Rhizomes of 400-500 g were planted at 20 cm depth with a distance of 2 x 2 m, on an area of 3,000 m^2. For the first year, plants were kept free from weeds by the application of glyphosate in the surroundings. A first irrigation was performed to facilitate young plant establishment; in the subsequent years no irrigation, no fertilization and no chemical treatments were used. At the end of October 2012, giant cane biomass was harvested and ensiled. Total biomass production registered was of 210 Mg fresh matter Ha^{-1} and of 71.8 Mg dry matter Ha^{-1}.

Both ensiled biomasses were sampled on February 2013 for subsequent use.

2.2. Lab-scale anaerobic digesters

Biogas production was carried out by using a continuous flow stirred tank reactor (CSTR) of 3 L in volume using a total biomass volume of 2 L; the anaerobic digestion process was performed in mesophilic conditions at 38 °C.

The feeding mixtures were prepared by mixing the energy crop (corn or giant cane) with pig slurry at the ratio (on wet weight/ wet weight) of 36.3 % and 63.7 % for corn and of 32.8% and 67.2 % for Giant cane (Table 1). Total solid content chosen was of approximately of 130 g kg^{-1} w/w, which is that normally used in full-scale CSTR plants.

Trials started using digestate coming from a full-scale plant that used energy crops plus pig slurries as feeding mixtures. Then CSTR rectors were fed daily with the studied and prepared mixtures for 40 days, i.e. the washout-phase, in order to substitute completely the digestate with the mixtures tested. After the washout phase, CSTR trials continued for another 15 days during which they were completely monitored for process parameters (more data in Table S2). Trials were performed in duplicate.

Biogas production was monitored every day and chemical parameters, i.e. total solids (TS), pH, conductibility, total organic carbon (TOC), total nitrogen (TKN), total ammonia (N-NH_4^+), volatile fatty acid (VFA), alkalinity (ALK), VFA/ALK ratio, organic acids, specific oxygen uptake rate (OD_{20}), anaerobic biogas potential (ABP) and biomethane potential (BMP), were determined on samples taken directly from the reactors every 3 days, for a total of 4 samples for each treatment studied.

2.3. Biogas analysis

Qualitative analysis of biogas was performed with a gas-chromatograph (Micro GC 3000, Agilent Technology) for determining the CH_4 concentration (v/v). Qualitative and quantitative biogas analyses were conducted, also, to identify the presence of siloxanes. GC-MS procedure was used as previously reported by Orzi et al. (2010). A manual SPME device and divinylbenzene (DVB)/Carboxen/polydimethylsiloxane (PDMS 50-30 µm fiber – Supelco, Bellefonte, PA, USA) were used. Fibers, preconditioned for 3 h at 250°C as suggested by the supplier, were exposed to a 20 mL aliquots of digestates. A solution of perdeuterated *p*-xylene in methanol was used as the internal standard for quantitative analysis. Siloxanes analysis was performed using Agilent 5975C Series GC/MSD. Siloxanes were separated using a capillary column for VOC (Meta.VOC, Teknokroma, Sant Cugat del Vallès, Barcelona, Spain) 30 m x 0.32 mm ID, with a film thickness of 3.0 µm. The carrier gas was helium at a flow rate of 1 ml min^{-1}. Siloxanes were desorbed exposing the fiber in the GC injection port for 3 min at 250°C. A 2 mm glass liner was used and the injection port was in a splitless mode. The temperature program was isothermal for 3 min at 35°C, raised to 200°C at a rate of 8°C min^{-1}. The transfer line to the mass spectrometer was maintained at 250°C. The mass spectra were obtained by electronic impact at 70 eV, with a multiplier voltage of 1294 V and collecting data at a *m/z* range of 33–300. Siloxanes were tentatively identified by comparing their mass spectra with those contained in the NIST (USA) 98 library. Semi-quantitative analysis for all the identified compounds was performed by direct comparison with the internal standard. Results were expressed as g m^{-3} headspace.

2.4. Chemical and spectroscopic analysis on raw material, feeding mixtures and digestates

A complete chemical characterization was performed on ensiled energy crops, pig slurry, feeding mixtures and digestates. The determination of TS, VS, TOC, TN and N-NH_4^+,

were conducted according to the standard procedures (APHA, 1998). In brief, TS were determined by drying the biomass at 105°C until constant weight, while VS by OM igniting at 550°C in muffle furnace. TN and N-NH_4^+ were determined by titration applying Kjeldahl method, and in the last TOC was determined by titration method after the oxidation of the OM by dichromatic acid by using Springer-Klee method. VFA and ALK were determined by the titration method (APHA, 1998). Organic acids extraction and determination were determined by HPLC analysis according to Canale et al., (1984). A 300 x 7.8 mm Aminex HPX-87H column and a UV detector (Waters 2410) at 210 nm were used for separation and quantification of acids. Analyses were performed isocratically with a temperature of 25°C, injection volume of 20 µL, flow rate of 0.6 ml min^{-1}, a 0.0025 M H_2SO_4 as mobile phase and a running time of 30 min. Chromatograms were analysed with the software Breeze (Waters).

Short-term degradability of biomasses was performed by the Oxygen Demand test (OD_{20}) as suggested by Adani et al. (2003). The estimation of hemicellulose, cellulose and lignin contents was carried out by the determination of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) applying the Van Soest method (Van Soest et al., 1991). The anaerobic biogas potential and the biomethane potential (ABP and BMP) tests were determined on all samples according to Schievano et al. (2009). Microporosity detection was performed on both ingestate and digestate of giant cane, as reported by Adani et al., (2011). Data were not reported for corn as the presence of starch does not allowed reliable data to be obtained.

Carbon distribution in macromolecular compounds was determined by using the ^{13}C CPMAS NMR spectroscopy, such as previously reported by Tambone et al., (2010). For a semi-quantitative approach, the ^{13}C CPMAS NMR spectra were subdivided into four regions: i) alkyl-C (0-47 ppm); ii) O-alkyl-C (47–113 ppm); iii) aromatic carbon (113–160 ppm); iv) carbonyl carbon (160–210 ppm). Spectra were elaborated using TOPSPIN 1.3 software (Bruker BioSpin GmbH, Rheinstetten, Germany).

Absolute C-fraction contents (g) were calculated taking into consideration relative C-fraction content (% of total C), absolute TS weight (g) and TOC content (g kg^{-1} TS^{-1}).

2.5. Anaerobic digestion performance

Anaerobic digestion performance was evaluated in terms of biomethane yield (BMY) calculated as reported by Schievano et al., (2011) with the following equation:

$$\text{BMY}\ (\%) = \frac{\text{SMP}}{\text{BMP}_{\text{in}}} \times 100 \quad (1)$$

in which BMP_{in} represents the biomethane potentials of inputs mixtures ($Nm^3\ CH_4\ Mg^{-1}\ TS^{-1}$) and SMP is the specific methane production obtained during CSTR trials ($Nm^3\ CH_4\ Mg^{-1}\ TS^{-1}$).

2.6. Total costs of producing biomass, biomethane and electricity

Total costs for producing biomethane and electricity were performed by considering the costs of producing biomasses and costs of transforming biomasses into products, i.e. biomethane and electricity. By doing so, biomasses' costs were evaluated directly in the field, taking into consideration costs for agronomic operations: plowing, harrowing, sowing or plant transplanting, weeding, chemical treatments, irrigations, fertilizations, harvesting, silage process and digestate use, when necessary, and costs to buy materials, i.e. seeds or plants, herbicides, insecticides, chemical fertilizers, water, when necessary, and cost to rent land (Schievano et al., 2014). For the giant cane some of these costs were necessary only for the first year (plowing, harrowing, sowing or plant transplanting, irrigation, weed control and plant buying); in all these cases costs were shared over a period of 12 years (Angelini et al., 2009). The data referring to the biomass yields were corrected with respect to losses in mass which occurred during the silage process, to consider the correct amount of biomass available per Ha^{-1} for producing biomethane or electricity.

A lower methane heating value of 31.6 MJ Nm^{-3} (8.79 kWh $Nm^{-3}\ CH_4^{-1}$) was employed for energy content determination (Schievano et al., 2014). The biogas and biomethane unit cost and electric energy unit cost (cost due to biomass production) were calculated as reported by Schievano et al., (2014). Costs to transform biomasses into electricity were those reported by Schievano et al., (2014) and Riva et al., (2014) for biogas plants of 0.5-1 MW, located in the area in which this work was done. The total cost for biomethane production was calculated taking into consideration the same data used to calculated the cost of producing electrical energy, but avoiding the costs in transforming methane into electricity and adding a cost for biogas upgrading, i.e. 0.176 € $m^{-3}\ CH_4^{-1}$ (Browne et al., 2011).

2.7. Statistical analyses

Results were processed by one-way ANOVA using the Tukey test to compare means. Statistical analyses were performed by using SPSS software (SPSS v19.0, IBM). The level of significant difference was set at $p<0.05$.

3. Results and discussion

3.1. Biomasses characteristics

After 100 days of the silage process about 6 % and 1.8 % of the initial TS contents, for corn and giant cane respectively, were lost; the final TS contents for the two biomasses studied were very similar (Table 1). pH registered and VFA contents were of 4±0 mg CH_3COOH L^{-1} and of 8,226±177 mg CH_3COOH L^{-1}, and of 3.6 ± 0.1 and of 17,221 ± 1,973 mg CH_3COOH L^{-1} for giant cane and corn silages, respectively (Table 1). Corn silage was characterized, above all, by volatile organic compounds, such as acetic acid (the most abundant VFA present), butyric acid and propionic acid; on the other hand oxalic acid characterized, above all, giant cane organic acid composition, reflecting different biomass composition (see later) and ensilage conditions, i.e. giant cane silage was more porous, probably allowing more air penetration.

Biomass compositions were different for the two energy crops studied: corn was preferentially characterized by cellular soluble fraction (59.2±3.8 % TS) (starch) rather than fibers; on the other hand giant cane was composed, prevalently, by fiber fractions, with more than 50 % TS represented by cellulose. The VS and ash percentage between the two biomasses were very similar (Table 2). Different composition determined different ABPs: corn ABP was higher than that of giant cane, i.e. 583 ± 21 Nm^3 Mg^{-1} TS^{-1} and of 473 ± 2 Nm^3 Mg^{-1} TS^{-1}.

Chemical compositions of mixtures prepared with corn and giant cane plus pig slurry were very similar each other (Table 1). The main effect of the addition of pig slurry to the biomasses was an increase of pH, TN and $N\text{-}NH_4^+$, and a decrease of organic acid contents. ABPs were slightly affected by pig slurry addition when referred to the TS units. Short-term degradability test, i.e. OD_{20} (Table 1), indicated that the corn mixture was more easily degradable than the giant cane mixture because of the presence of starch in the corn biomass (Table 2).

3.2. Anaerobic digestion: CSTR trials

Process parameters indicated that anaerobic digestion proceeded correctly (Table 1, corn mixture and giant cane mixture digestates). The TS contents diminished from 13.6 ±0.5 % and 13.9±0.9 % for corn and giant cane mixtures, to 4.35±0.30 % and 6.98±1.26 % for corn and giant cane digestate, respectively. The pH values were alkaline for both the digested mixtures, 8.23±0.34 for corn digestate and 8.32±0.21 for giant cane digestate, because of the accumulation of ammonia during anaerobic processes. The detected ammonia concentrations of about 1 g L^{-1}, were comparable with those previously reported for well performed anaerobic processes and not inhibitory for microbial biomasses (Schievano et al., 2012). VFAs were drastically reduced by the digestion processes, indicating that VFAs produced by OM fermentation were readily transformed into CH_4. These data demonstrated that the biological processes were in equilibrium (Schievano et al., 2012), such as indicated, also, by VFA/ALK ratios below 0.4 (Schievano et al., 2012).

The proper functioning of the most relevant chemical and physical parameters allowed a biogas production of 2.98±0.31 L day^{-1} for the corn mixture and of 2.02±0.11 L day^{-1} for the giant cane mixture. These daily volumetric yields corresponded to 399±41 Nm^3 biogas Mg^{-1} TS^{-1} (426±44 Nm^3 biogas Mg^{-1} VS^{-1}) and 272±15 Nm^3 biogas Mg^{-1} TS^{-1} (288 ± 16 Nm^3 biogas Mg^{-1} VS^{-1}), for corn and giant cane mixtures respectively (Table 3). The detected CH_4 contents in biogas were of 61.3±2 % v/v for corn mix and of 64.1±1.6 % v/v for giant cane mix. Therefore the biomethane yields were of 245±26 Nm^3 CH_4 Mg^{-1} TS^{-1} (261±28 Nm^3 CH_4 Mg^{-1} VS^{-1}) and of 174±10 Nm^3 CH_4 Mg^{-1} TS^{-1} (185±11 Nm^3 CH_4 Mg^{-1} VS^{-1}) for corn and giant cane biogas, respectively (Table 3). The biogases were mainly composed of CH_4 and CO_2 with relative percentages as shown in Table 3; H_2S concentrations were almost lower than 0.2 % v/v.

Biogas quality was investigated, also, for the presence of silicon-derived organic compounds, since these molecules can give problems (abrasive action) to the mechanical structure of reactors and motors (Accettola and Guebiz, 2008). Literature reported that giant cane contains more silicon than that in other energy crops, ranging from 6.2 g kg^{-1} TS^{-1} in stems to 17.2 g kg^{-1} TS^{-1} in leaves (Monti et al., 2008).

Results of this work indicated that for both biogas samples, the most abundant molecules were represented by cyclotrisiloxane, hexamethyl-D3, cyclotetrasiloxane, octamethyl-D4 and cyclopentasiloxane, decamethyl-D5, respectively, in agreement with Rasi et al., (2013). The total amount of these molecules was of 0.203 g m^{-3} headspace for giant cane

digestate, and of 0.522 g m^{-3} headspace for corn digestate, and so were more concentrated for corn mixture despite the lower ash content in the plant (Table 2). This result was probably due to both the greater total degradation of corn with respect to giant cane (see later), and to the fact that giant cane accumulates silicon as phytoliths, an amorphous, stable and non-degradable Si-deposition product (Chauhan et al., 2011).

3.3. Anaerobic digestion performance and Mass balances

Anaerobic digestion performances measured by the BMY index indicated that corn was able to produce 76.6 % of the potential biomethane (BMP) and giant cane only 65.7 % of BMP. Previous data on full-scale processes indicated BMY values that ranged from 84 % to 93 % (Schievano et al., 2011) and so were higher than those reported in this work. Probably the HRT used (40 days) was not sufficient to degrade all degradable OM because of the high presence of fiber, unlike the previous mixtures studied by Schievano et al., (2011) in which energy crops were minimum or not considered as feed. This work seems to confirm the fact as the BMY obtained agree with the fiber contents that was much higher for Giant cane than corn (Table 2). These results agree, also, with mass balance of TS that indicated a TS reduction of 72.6 % for corn and 54.8 % for giant cane.

3.4. Organic matter evolution vs. biomass recalcitrance

^{13}C CPMAS NMR provides qualitative and quantitative information on the composition of the ingestates and digestates by identifying the main C-type of which the organic matter consists (Tambone et al., 2010). The relative changes in the levels of C-containing groups are presented in Table 2 (see also Figure S1).

The spectra region of 0-47 ppm indicates the presence of alkyl-C, i.e. fatty acids and lipids in general, but also, proteins (Dignac et al., 2000). The presence of different peaks in this region indicated the presence of both long chain linear structures and branched and/or short chains (Figure S1). In particular the peak detected at 21 ppm could be attributed to volatile fatty acid that effectively composed starting mixtures (Table 1) (Tambone et al., 2010); peaks at 30-33 ppm can be related to the aliphatic carbon of long polymethylenic chains (Pichler et al., 2001) such as cutine and waxes contained in both corn and giant cane silages. Degradation processes led to a relative increase in the alkyl-C area (Table 3). On the other hand, absolute data indicated that the alkyl-C fraction was degraded extensively, i.e. 81% total C and 64% total C, for corn and giant cane mixtures, respectively.

Degradation was due, above all to VFA degradation, as the strong reduction of peaks at 21 ppm suggested. On the other hand the relative increase of a peak at about 30 ppm indicated that cutin and wax-like molecules were preferably preserved (Pereira et al., 2005).

The area of 47-115 ppm, indicated, mainly the presence of O-alkyl-C (Tambone et al., 2010), but also protein and lignin: the peak at 56 ppm, in fact, corresponded to carbon atoms substituted by amino groups, i.e., in peptides and amino acids, but also it indicated the $–OCH_3$ of both lignin and hemicelluloses. The peak at 72 ppm was due to O-alkyl C of C-2, C-3 and C-5 atoms of polysaccharides (starch, cellulose and hemicelluloses). A peak at 105 ppm represented the anomeric carbon atoms (C-1) of cellulose and a peak at 65 ppm was due to C-6 in hexose and/or C-5 in pentose. The decreasing of this area for both corn and giant cane indicated that anaerobic digestion provided mainly for the degradation of carbohydrate-like molecules and that the degradation was generally correlated with the biogas production. In particular, it can be seen that the degradation of these compounds was more marked for corn compared with giant cane mixtures because of the presence in the former of starch.

Absolute data indicated that the O-alkyl fraction was degraded for 75.4 % of total C and for 55.1 % of total C, for corn and giant cane mixtures, respectively. Differences in the O-alkyl degradation for the two mixtures studied, were due to the presence of starch in corn, as indicated before, that was more degradable than the giant cane carbohydrates forming the fiber fraction, i.e. cellulose and hemicelluloses (Table 2).

The aromatic-C area (115-160 ppm) was represented by lignin and lignin-derived molecules; these molecules are generally indicated to have recalcitrant properties and so become concentrated during the anaerobic digestion process (Tambone et al., 2010). On the other hand absolute data (Table 2) indicated that aromatic-C was degraded extensively during the anaerobic digestion, i.e. 72% and 68% of total C for corn and giant cane mixtures.

The 160-210 ppm area and in particular the peaks at 173 ppm (carboxyl group of the aliphatic chain of VFA, organic acid and cutin) relatively increased after anaerobic digestion; on the other hand absolute data indicated reductions of 83% and 65% of total C, for corn and giant cane mixtures, respectively.

C-fraction degradation and the final composition of digestates reflected the different starting composition, i.e. corn's starch content determined an extensive reduction of the O-alkyl-C which led to a relative increase of alkyl-C, aromatic-C and keto-C, that in any case

were, also, on an absolute basis, extensively degraded. However, the quite similar degradation which occurred for the fractions contained in the giant cane, determined a final composition (on a relative basis) of the digestate that was not so much different from that of the starting mixture (Table 2), in that it still contained an O-alkyl-C fraction (cellulose plus hemicelluloses) that represented the 75.6 % of the total C.

Biomass recalcitrance depends upon the 3D structures of cell wall in which cellulose fibrils are protected by the presence of hemicellulose and lignin which together form a microporous structure that prevents the cellulase enzymes from coming into direct contact with the cellulose, as their sizes exceed the cell wall pore size (Adani et al., 2011). Pretreatment is needed to remove recalcitrance, i.e. if hemicellulose and lignin can be removed, this increases cellulose accessibility (Adani et al., 2011). Pretreatments are necessary to produce bioethanol as cellulose need to be hydrolyzed in a short time (hours) by adding enzymes producing glucose to be subsequently fermented. On the other hand, as biogas production acts over a much longer time (months), pretreatment need not be such as recently recommended for corn stover and switchgrass (Papa et al., 2015). In that work it was demonstrated, in fact, that ionic liquid pretreatment to remove both hemicelluloses and lignin had effects on bioethanol production, but not on the potential biogas production (Papa et al., 2015). Microporosity detection (i.e. porosity of 0.3-1.5 nm of diameter) on giant cane before and after the anaerobic digestion performed in this work gave very similar data, i.e. 45.6 ± 2.1 m^2 g^{-1} TS and 47.4 ± 0.7 m^2 g^{-1} TS, for ingestate mix and digestate respectively. These values agree with those reported by Adani et al., (2011) and indicate that, substantially, 3D structures of cell walls did not change during the anaerobic process. This fact seems to suggest that giant cane degradation proceeded by a simultaneous and non-selective degradation of macromolecules forming giant cane, carried out by the complex microbial consortium acting during anaerobic digestion.

Therefore it can be concluded that the complete degradation of giant cane depends upon the time available and that longer HRT should allow increased biomethane production. In effect BMP performed under batch conditions for 60 d (Table 1) allowed the production of more biomethane than the CSTR test performed for 40 d. So, taking into consideration that agricultural biogas plants operating with energy crops consider HRT periods of longer than 40 d, we consider that the results reported in this work are an underestimation of the biogas-production potential of giant cane.

3.5. Energetic and economic evaluations

The collected data from CSTR trials were evaluated in term of energy production and costs. Taking into consideration full-scale biomass yields and the production of methane obtained during the anaerobic fermentations, data were developed in order to calculate the m^3 of bio-methane produced per surface area of land (Ha) and consequently the related producible energy.

Biomethane obtainable from one hectare of corn (4,549 Nm^3 CH_4 Ha^{-1}) was much less than that producible by giant cane (12,292 Nm^3 CH_4 Ha^{-1}) (Table 4) despite the fact that methane producible per TS units was higher for the former (Table 4). This result was due to the higher biomass production per Ha^{-1} of giant cane (71.8 Mg TS Ha^{-1}, i.e. 70.5 Mg TS Ha^{-1}, if TS losses after ensilage were considered) compared with that of corn (19.8 Mg TS Ha^{-1}, i.e. 18.6 Mg TS Ha^{-1}, if TS losses after ensilage were considered). As consequence of that, the total obtainable energy (GJ Ha^{-1}) per cultivated Ha, was much more for the giant cane than for corn, i.e. 389 GJ Ha^{-1} and 144 GJ Ha^{-1}, respectively.

The total costs of producing both biomethane (€ Nm^{-3} CH_4^{-1}) and electric energy (€ kWh_{EE}^{-1}) are other two important parameters used to compare the two energy crops. These parameters are principally related to the cost of producing the biomass and to subsequent costs due to the conversion of biogas into electricity or biomethane. The total cost to produce giant cane biomass was lower than that of corn, i.e. 1,000 € Ha^{-1} versus 1,800 € Ha^{-1} (Table 4); this means costs of 0.40 € Nm^{-3} CH_4^{-1} and 0.08 € Nm^{-3} CH_4^{-1} for biomethane and 0.12 € kWh_{EE}^{-1} and 0.02 € kWh_{EE}^{-1} for electricity production, for corn and giant cane mixtures, respectively. Taking into consideration the costs to transform biomasses into biomethane or electricity (see M & M), the total costs for biomethane were 0.81 € Nm^{-3} CH_4 and 0.50 € Nm^{-3} CH_4, and for electricity 0.19 € kWh_{EE}^{-1} and of 0.10 € kWh_{EE}^{-1} for corn and giant cane mixtures, respectively.

Literature indicated for giant cane a trend in biomass production rising from the first to the 3^{rd}–4^{th} years, then decreasing later in the cycle (Angelini et al., 2009; Corno et al., 2014). In this work, the study referred to biomass harvested in the third year and characterized by a very high biomass yield with respect to the average data reported in the literature (Corno et al., 2014). Therefore, an average biomass production of 37.7 Mg TS Ha^{-1}, such as that obtained by a long-term study of 12 years (Angelini et al., 2009) was also considered for calculating the economic considerations. By doing so, biomethane production for giant cane, taking into consideration the specific biomethane production obtained in this work,

was of 6,573 Nm^3 CH_4 Ha^{-1} and the obtainable energy was of 208 GJ Ha^{-1}, figures which are, in any case, still both much higher than the data obtainable with corn (Table 4). Taking into consideration the long-term biomass production data (Angelini etc al., 2009), the total biomethane and electricity costs were calculated to be 0.57 € Nm^{-3} CH_4^{-1} and 0.12 € kWh_{EE}^{-1}, respectively.

The results obtained can be compared with previous data reported by Schievano et al., (2014) who studied different energy crops in the same area, and reported costs for producing electricity of 0.17-0.20 € kWh_{EE}^{-1} using traditional energy crops, in line with data reported in this work for corn (0.19 € kWh_{EE}^{-1}). Similar evaluations can be carried out for biomethane production as well. Cost reported in Table 4 for electricity production with giant cane, when compared with those reported by Schievano et al., (2009) and Riva et al., (2014) for organic wastes, agro-industrial by-products and residues of animal manures, i.e. 0.096-0.159 € kWh_{EE}^{-1}, indicated that giant cane, at 0.10 € kWh_{EE}^{-1}, was competitive as well.

As costs to produce both electricity and biomethane using different energy crops do not change, the lower costs calculated for giant cane were related to its agronomic peculiarities, i.e. low agronomic and energy inputs and high biomass yield.

4. Conclusions

Giant cane, if compared with corn, produced less methane when specific methane production was considered (L CH_4 kg^{-1} TS). This was due to the crops' chemical composition, i.e. the presence of starch in corn and the higher presence of fiber in giant cane. On the other hand, because of its very high biomass production, the biogas yield per Ha was much higher for giant cane than for corn. Low energetic and agronomic inputs for giant cane, led to a considerable reduction in the costs of producing electricity and biomethane from it, in comparison with traditional energy crops.

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Table 1 – Principal physical and chemical characterization of corn and giant cane silages, pig slurry, mixtures of corn and giant cane with the pig slurry and corn and giant cane digestates.

		Corn silage	Giant cane silage	Pig slurry	Corn mixture	Giant cane mixture	Corn mixture digestate	Giant cane mixture digestate
Energy crop	% w/w	-	-	-	36.3	32.8	-	-
Pig slurry	% w/w	-	-	-	63.7	67.2	-	-
TS	% w/w	31.1±0.57e	34.2±0.54f	1.8±0.1a	13.6±0.5d	13.9±0.9d	4.3±0.3b	6.98±1.26c
pH		3.6±0.1a	4±0b	7.24±0.03e	4.74±0.01c	5.29±0.01d	8.23±0.34f	8.32±0.21f
Conductibility	mS	1.69±0.05b	1.36±0.08a	6.22±0.06c	1.30±0.07a	1.4±0ab	12.5±0.9d	15.1±0.2e
TOC	g kg^{-1} TS^{-1}	443±8bc	436±4bc	360±24a	462±26c	445±31a	400±38b	428±24bc
TN	g kg^{-1} TS^{-1}	10.8±0.1b	6.8±0.2a	54.4±1.2e	16.5±0.4c	15.5±0.5c	61.8±0.7f	32.3±0.2d
$N\text{-}NH_4^+$	g L^{-1}	0.91±0.03b	0.53±0.11a	34±0c	0.56±0.01a	0.5±0.1a	1.11±0.01b	1.06±0.01b
VFA	mg CH_3COOH L^{-1}	17,221±1,973e	8,226±177d	287±55a	5,750±677c	4,645±64.6b	507±331a	158±52a
ALK	mg $CaCO_3$ L^{-1}	8,744±7483a	7,592±98a	2,526±112a	5,079±224a	4,689±99a	4,935±216a	5,757±232a
VFA/ALK		1.97	1.08	0.11	1.13	0.99	0.10	0.03
Oxalic acid	g L^{-1}	-	184,789±1,086	-	-	15,984±1,578	-	-
Citric acid	mg L^{-1}	-	443±68	-	-	-	-	-
Malic acid	mg L^{-1}	-	908±88	-	-	-	-	-
Succinic acid	mg L^{-1}	32,337±3148	2,519±304	-	19,992±435	-	-	-
Lactic acid	mg L^{-1}	8,455±751	3,111±430	-	3,804±21	2,614±96	-	-
Acetic acid	mg L^{-1}	12,390±1,287	2,677±322	600±238	5,573±965	1,806±443	396±75	444±79
Propionic acid	mg L^{-1}	3,808±1127	-	-	-	-	-	-
Isobutirric acid	mg L^{-1}	1,689±730	-	-	966±494	-	-	-
N-butirric acid	mg L^{-1}	9,613±1974	-	-	1,084±332	-	-	-
C/N		-	-	-	33.6	35.3	7.35	10.5
Total P	g kg^{-1} TS^{-1}	-	-	-	6.73±0.33	3.01±0.15	16.6±0.8	6.1±0.3
Total K	g kg^{-1} TS^{-1}	-	-	-	12.4±0.6	9.27±0.4	39.2±1.9	23.5±1.1
N/P		-	-	-	2.45	5.15	3.72	5.31
N/K		-	-	-	1.33	1.67	1.58	1.37
OD_{20}	mg O_2 g^{-1} TS^{-1}	-	-	-	174±2	84.7±12.3	36±1	18.3±0.9
ABP	Nm^3 Mg^{-1} TS^{-1}	583±21f	473±2d	195±9b	551±11e	428±28c	138±22a	182±22b
Methane	% v/v (biogas)	59.7±3.7a	59.8±4.9a	65.9±3.5bc	58.1±1.9a	61.9±4.6b	69.4±3.7c	67.6±5.1c
BMP	Nm^3 CH_4 Mg^{-1} TS^{-1}	348±25c	283±23b	128±9a	320±12c	265±26b	96±16a	123±18a

Values followed by the same letter in the same line are not statistically different with a p-value<0.05; Tukey test.

Table 2 – Determination of fibers composition, volatile solids content and determination of ^{13}C CPMAS NMR.

		Corn	Giant cane	Pig slurry	Corn mixture	Giant cane mixture	Corn digestate	Giant cane digestate
Cellular soluble	% TS	59.2±3.8d	21.8±0.3a	54.9±1.9cd	50.5±2.4c	27.5±1.5b	52.8±1.1c	28±5b
Hemicellulose		20±4bc	19.9±0.5bc	11.5±3.3a	24.6±1.1c	16.4±2.2b	8.3±0.6a	22.5±7.5bc
Cellulose		12.2±1.7a	53±1f	25.6±1.8d	16.5±1.6b	36.4±1.3e	22±1c	22.7±0.9c
Lignin		8.8±1.6a	5±0a	8.1±0.4a	8.4±0.4a	20±2b	16.4±0.7b	37.8±3.4c
Volatile solids		95.2±0.8d	94.4±0.8d	73.1±0.1a	93.8±0.2d	94.3±0.1d	81.3±1.5b	88.4±2.3c
Ash		4.8±0.8a	5.6±0.8a	27.0±0.1d	6.15±0.2a	5.7±0.1a	18.7±1.5c	11.6±2.3b
	ppm area	^{13}C CPMAS NMR % C (g of C)						
Alkyl-C bonded to other aliphatic chain or to H	0-47	-	-	-	12.4 (5.9)	9.7 (5.6)	22.3 (1.1)	11.7 (2)
O-alkyl C di-O-alkyl C O-CH_3 or N-alkyl	47-115	-	-	-	75.6 (35.9)	78.4 (34.5)	58.5 (8.8)	74.6 (15.5)
Aromatic-C phenol or phenyl ether-C	115-160	-	-	-	6.9 (3.3)	7.9 (3.2)	10.3 (0.9)	9.6 (1.6)
Carboxyl-C keto-C	160-210	-	-	-	5.1 (2.4)	4 (2.3)	8.9 (0.4)	4.1 (0.8)

Values followed by the same letter in the same line are not statistically different with a p-value<0.05; Tukey test.

Table 3 – Biogas productions, biomethane yields (BMY) and principal biogas composition. (Values followed by the same letter in the same line are not statistically different with a p-value<0.05; Tukey test).

		Corn biogas	Giant cane biogas
Biogas production	Nm^3 biogas Mg^{-1} TS^{-1}	399±41b	272±15a
	Nm^3 CH_4 Mg^{-1} TS^{-1}	245±26b	174±10a
	Nm^3 biogas Mg^{-1} VS^{-1}	426±44b	288±16a
	Nm^3 CH_4 Mg^{-1} VS^{-1}	261±28b	185±11a
BMY	% v/v	76.6±8.6	65.7±7.5
CH_4	% v/v	61.3±2a	64.1±2b
CO_2	% v/v	38.4±2.2b	35.7±2.1a
H_2S	% v/v	0.2±0.4a	0.1±0.1a
H_2	% v/v	0.1±0.2a	0.1±0.2a

Table 4 – Energetic and economic evaluations of the anaerobic digestion of giant cane in comparison with energy crops (corn and succession corn+triticale) and organic wastes.

		Corn	Giant cane	Giant cane (average of 12 years)	Corn (1st crop)[a]	Triticale +Corn[a]	Organic Wastes[b]
Biomass yield	Mg DM Ha^{-1}	18.6 [c]	70.5 [c]	37.7 [d]	21.5	34.1	-
CH_4 production	Nm^3 CH_4 Mg^{-1} TS^{-1}	245	174	174	382 [e]	347 [e]	184 [f]
CH_4 percentage	% v/v	61.3	64.1	64.1	55	55	65
BMY [g]	% v/v	-	-	-	87	87	87
CH_4 Lower Heating Value [h]	MJ m^{-3} CH_4^{-1} (kWh_{EE} Nm^{-3} CH_4^{-1})	31.6 (8.79)	31.65 (8.79)	31.65 (8.79)	31.65 (8.79)	31.65 (8.79)	-
Biomethane per hectare	m^3 CH_4 Ha^{-1}	4,549	12,292	6,573	7,145 [i]	10,294 [i]	-
Energy per hectare	GJ Ha^{-1}	144	389	208	226	326	-
Electric Energy per hectare [j]	$MWh_{EE}Ha^{-1}$	15.6	42.1	22.5	25.5	35.3	-
Total biomass cost	€ Ha^{-1}	-1,800 [k]	-1,000 [k]	-1,000 [k]	-2,106	-3,346	+45 (€ Mg^{-1})
Biomethane Unit Cost (biomass) [l]	€ Nm^{-3} CH_4^{-1}	-0.40	-0.08	-0.15	-0.29	-0.33	+0.28 [m]
Electric energy Unit Cost (biomass) [l]	€ kWh_{EE}^{-1}	-0.12	-0.02	-0.04	-0.09	-0.09	+0.07 [m]
Total costs of biomethane [n]	€ Nm^{-3} CH_4^{-1}	-0.81	-0.50	-0.57	-0.71	-0.74	-0.45 [o]
Total costs of Electric Energy [p]	€ kWh_{EE}^{-1}	-0.19	-0.10	-0.12	-0.16	-0.17	-0.09 [o]

[a] biomass productions yields, CH_4 percentage and total biomass costs reported by Schievano et al. (2014).
[b] CH_4 percentage and total biomass benefits (€ Mg^{-1}) reported by Schievano et al. (2009).
[c] biomass yields after the TS losses during silage process (6.1 % for corn; 1.8 % for giant cane).
[d] average of biomass yield in a temperate climate (43°40'N, 10°19'E, Pisa, Italy) (Angelini et al., 2009).
[e] CH_4 production calculated from Schievano et al. (2014).
[f] CH_4 rate calculated from Schievano et al. (2009).
[g] biomethane yield reported by Schievano et al. (2014).
[h] CH_4 lower heating value reported by Schievano et al. (2014).
[i] biomethane yield calculated from Schievano et al. (2014).
[j] calculated with an electrical generation yield of 39 % (Schievano et al., 2014).
[k] biomass costs evaluated in the experimental farm of Landriano (PV, Italy).
[l] costs depending only on the biomasses costs.
[m] biomethane unit cost and electric energy unit cost of organic wastes calculate from Schievano et al. (2009).
[n] total cost of biomethane calculated taking into consideration the same data used to calculate the total cost of electric energy, avoiding the cost transforming methane in electricity and adding cost for biogas upgrading, i.e. 0.176 € m-3 CH4 (Browne et al. 2011).
[o] organic wastes costs considering the management/maintenance costs and depreciation charges reported by Riva et al, (2014) with a CH4 content of 65 %. The Browne et al. (2011) cost for biomethane upgrading was used (0.176 € m-3 CH4).
[p] total cost of electric energy taking into consideration the management/maintenance costs of 0.029 € kWhEE-1 and the depreciation charges of 0.046 € kWhEE-1 of 1 MW plant treating energy crops, manure, agricultural residues and industrial organic by-products (Riva et al., 2014).

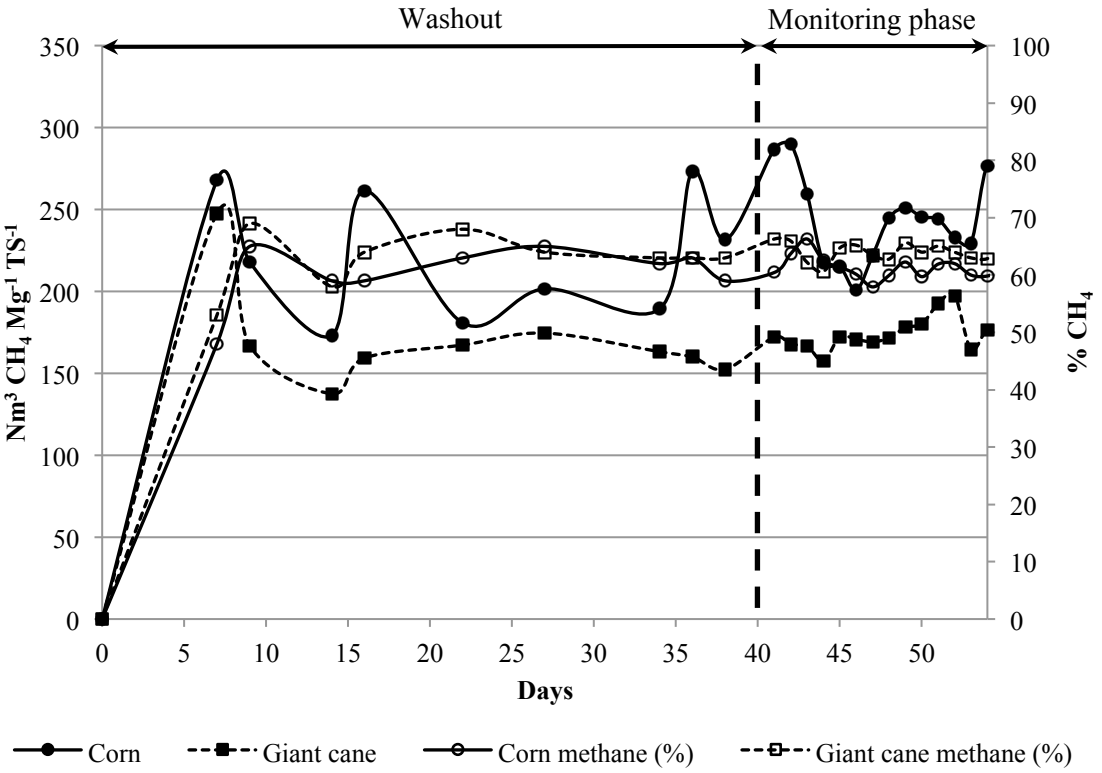

Figure 1 – Trend of methane production (Nm^3 CH_4 Mg^{-1} TS^{-1}) and amount of methane in biogas (%) during the washout phase and monitoring phase.

Table S1 – Principal chemical and physical characteristics of soils where corn and giant were cultivated (Experimental farm A. Menozzi of the University of Milan, Landriano, PV, Italy).

Parameter		Corn	Giant cane
pH		7±0.6	5.83±0.13
Sand	%	60.7±4.8	51±4.6
Silt	%	33.5±4.4	44.1±2.2
Clay	%	5.6±0.5	4.9±0.3
Total organic carbon	g kg^{-1} TS^{-1}	10.7±2.1	12±0.2
Organic matter	g kg^{-1} TS^{-1}	18.4±2.5	20.7±0.3
Total Kjeldahl nitrogen	g kg^{-1} TS^{-1}	1±0.2	1.43±0.03
C/N ratio		10.8±3	8.39±0.2
P Olsen	mg kg^{-1} TS^{-1}	115±13	66.1±1.5
Cation Exchange Capacity	$cmol^{+}$ kg^{-1} TS^{-1}	19.2±4	24.4±0.8

Table S2 – Process details of both the lab-scale anaerobic digestion trials.

Type of process		CSTR
Volume	L	2
Hydraulic Retention Time (HRT)	days	40
Temperature	°C	38
Total Solids Input	g TS day^{-1} (corn)	6.80
	g TS day^{-1} (giant cane)	6.95
Volatile Solids Input	g VS day^{-1} (corn)	6.38
	g VS day^{-1} (giant cane)	6.55
Organic Loading Rate	g VS L^{-1} day^{-1} (corn)	3.05
	g VS L^{-1} day^{-1} (giant cane)	3.06

Table S3 – Siloxanes compositions of corn and giant cane digestates. Data are reported as grams of detected siloxanes per m^{-3} headspace.

		Corn digestate	Giant cane digestate
Cyclotrisiloxane, hexamethyl-D3	g m^{-3}	0.35±0.01	0.18±0.23
Cyclotetrasiloxane, octamethyl-D4	g m^{-3}	0.11±0.03	0.02±0.02
Cyclopentasiloxane, decamethyl-D5	g m^{-3}	0.06±0.01	u.d.l.
Cycloheptasiloxane, tetradecamethyl-	g m^{-3}	-	u.d.l.
Cyclooctasiloxane, hexadecamethyl-	g m^{-3}	u.d.l.	u.d.l.
Hexasiloxane, tetradecamethyl-	g m^{-3}	u.d.l.	-
Total siloxanes	g m^{-3}	0.52±0.33	0.20±0.32

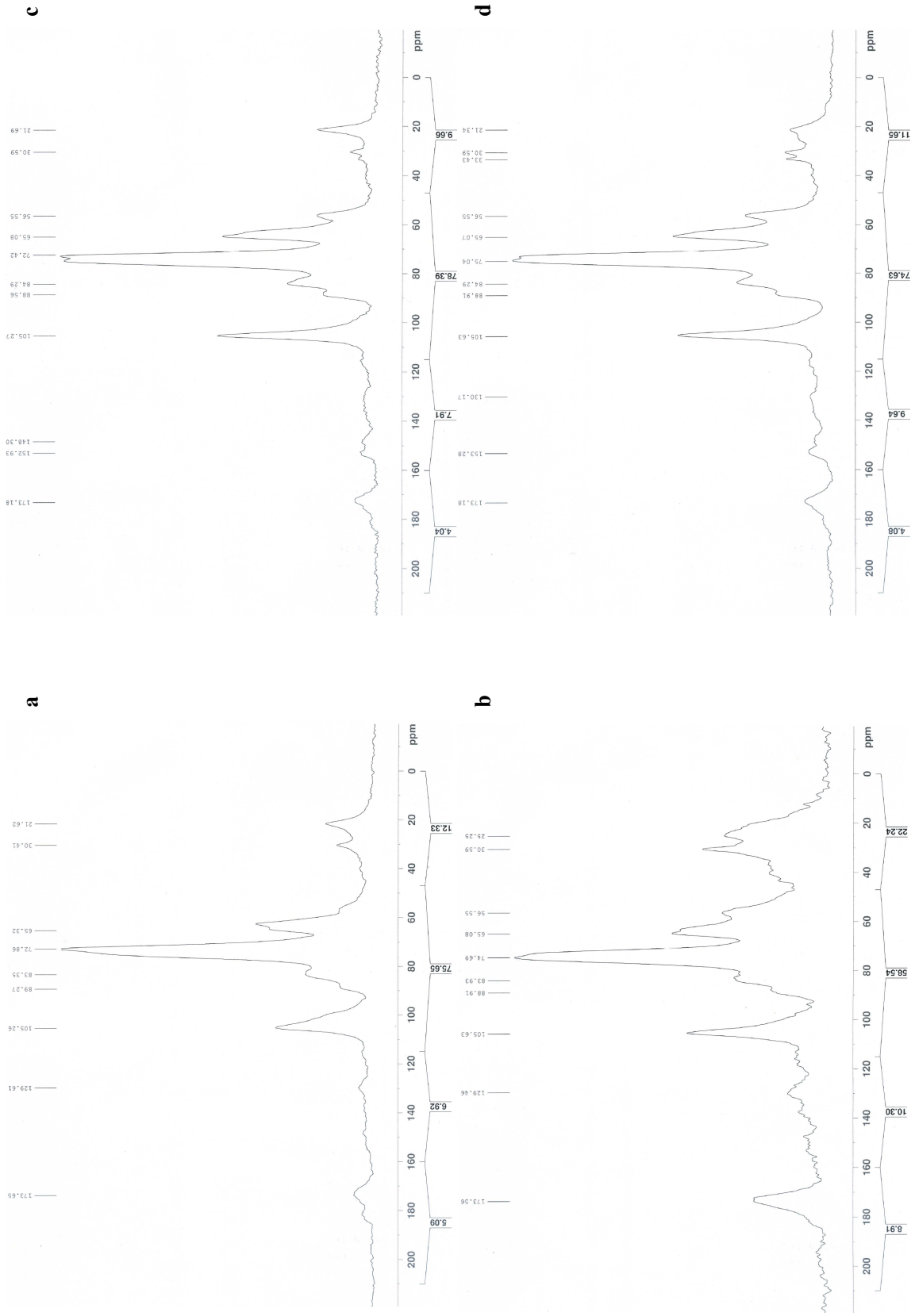

Figure S1 – ^{13}C CPMAS NMR spectra of corn mixture (a) and digestate (b); giant cane mixture (c) and digestate (d).

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Conclusions

Arundo donax L. is a second-generation crop that can efficiently substitute the traditional energy crops in both energetic and green chemistry sectors.

In this work, the many advantages of *A. donax* culture were highlighted making this crop competitive against the other energy crops. The higher biomass yields were achievable with few agronomic interventions; this, allowed to a lower final cost of biomass and a less impact on the environment. Moreover, the sterility of the plant reduced its weed potential but could also be an issue for further genetic improvments that will be limited to the clonal selection. The feasible clonal selection could be exploited for the choice of the easier and cheaper propagation technique, i.e. the hydroponic propagation.

Considering *A. donax* as a potential feedstock for bioenergy and green chemistry, preservation and conversion of biomass is a crucial point in a biorefinery concept. The *A. donax* biomass could be stored for a long period as silage with techniques already used for energy crops, such as trench and silo-bag, and without using inoculum. The silo-bag technique resulted more efficient in preserving biomass and its biogas potential than the trench technique. The exploitation of *A. donax* silage for bioga3s production was found sustainable and cheaper than others energy crops thanks to the higher yields and the lower biomass costs. In particular, the costs of methane and electric energy resulted lower than both energy crops and crop rotations.

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