



Research article

Bioethanol production using high density *Eucalyptus* crops in Uruguay

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ABSTRACT

Experimental scale crops for *Eucalyptus grandis*, *Eucalyptus benthamii*, *Eucalyptus dunnii* and *Eucalyptus tereticornis*, at 2,220, 4,440 and 6,660 trees ha⁻¹ were established in two soil units, at Paysandú and Tacuarembó, Uruguay. Wood samples were taken from twenty-two-months-old trees, and were used to produce bioethanol by pre-hydrolysis simultaneous saccharification and fermentation process (PSSF). Cellulose and lignin content was analyzed. Species and planting density affected biomass production at both sites; the highest value was obtained with *E. dunnii* at 6,660 trees ha⁻¹ at Paysandú. Cellulose content of wood varied between species at both sites, but only between planting densities at Tacuarembó. The site effect showed that the highest amount of cellulose (14.7 Mg ha⁻¹) was produced at Paysandú. *E. benthamii* and *E. tereticornis* wood showed higher lignin contents, conversely, the PSSF yields showed no differences, which led to a bioethanol average of 97 L Mg⁻¹. Bioethanol productivity was associated to the biomass productivity. It was possible to obtain 2,650 L ha⁻¹ of bioethanol using wood from *E. benthamii*, *E. dunnii* and *E. grandis* at 4,440 and 6,660 trees ha⁻¹ at Paysandú, and with *E. benthamii* at 4,440 and 6,660 trees ha⁻¹, and *E. dunnii* at 6,660 trees ha⁻¹ at Tacuarembó.

1. Introduction

The Paris Agreement has highlighted and confirmed the global goal for decarbonization of the energy sources that support society's development and lifestyle. In this sense, the development of renewable energy has become an important topic for developing national public energy policies. Before the Paris Agreement, Uruguay had reduced oil consumption by 25% and increased energy generation by 62% (Uruguay XXI, 2017): changes that were internationally recognized as an important to the decarbonization process (Ludeña and Ryfisch, 2015). The use of biomass as an energy source has been important to this process. However, several scientific gaps remain to be filled at both the levels of feedstock/biomass generation (agronomic phase) and conversion of the

biomass into energy or biofuel. Additionally, it is important to avoid the use of food crops for the development of biofuels.

The agricultural sector is a potential source of biomass for electric power generation (e.g. energy forest cultivation and forest residue) and for the production of second-generation bioethanol from starch (Carrasco-Letelier et al., 2012). Although the afforested area in Uruguay has grown markedly from 40,000 to 1,000,000 ha, since the forestry law provided legal and tax incentives to the sector in the 1990s, it represents only 25% of the total area of the soils prioritized for forestry (Dirección de Estadísticas Agropecuarias (DIEA), 2016). The alternative offered by energy afforestation has some advantages over the use of forest residues, including homogeneity of the material, greater energy content per unit of land surface area, and greater opportunities for nutrient recycling, using only the woody parts of the tree (Dias Müller and Couto, 2006). The use

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of forest crops for biomass production is based on the cultivation of fast-growing species, which are planted at a high density, in short rotation periods, and near power-generation plants, with the aim of obtaining high biomass volumes in a short period of time (Balan, 2014; Dias Müller and Couto, 2006; Rockwood et al., 2008; Volk et al., 2011).

Given its great adaptability to distinct site conditions, and its fast growing rate, the *Eucalyptus* genus has been shown to have good potential for use as an energy crop (Harwood, 2011). Despite their low tolerance to frost, some species have been shown to have the best performances for this purpose, including *Eucalyptus dunnii* Maiden, *Eucalyptus benthamii* Maiden & Cambage, *Eucalyptus tereticornis* Sm., and *Eucalyptus viminalis* Labill. Comparative studies with *Eucalyptus grandis* W. Hill ex Maiden, *Eucalyptus camaldulensis* Dehnhardt, *Acacia sp.*, *Salix sp.*, and *Populus sp.* in California (USA), showed that *E. grandis* and *E. camaldulensis* have the highest levels of growth (40 m³ ha⁻¹ yr) and 4,000 to 5,000 stems ha⁻¹ (Sachs and Low, 1983). However, in terms of the agro-industrial chain, some aspects of the production of biomass from forest crops require further analysis, especially those related to yield and environmental and economic issues (Balan, 2014).

From the productivity point of view, it is necessary to generate and/or adapt information about the potential of various energy commodities in terms of the volume of potential bioethanol production. The transformation of lignocellulosic biomass into bioethanol implies the hydrolysis of the polysaccharides, celluloses, and hemicelluloses that are part of the plant cell, into monosaccharides, which are then converted into ethanol by alcoholic fermentation. It is well known that the physical and chemical wood structures affect the substrate enzymatic digestibility (Please be informed that we have add Heliyon taxonomy term in the keywords section as per the journal standard style. Unfortunately, will not be able to make changes to the keywords as we require these terms to locate your paper on the website. Hope this is fine with you. Thank you for your understanding and apologies for any inconvenience caused in this regard., which requires the application of some type of treatment to improve the cellulose hydrolysis, by either increasing the cellulose surface area or removing the lignin (Leu and Zhu, 2013; Li et al., 2016; Meng and Ragauskas, 2014). Wood chemical composition varies between species (Salazar et al., 2016), crop life cycle (Moulin et al., 2015; Rencoret et al., 2011), planting density (Moulin et al., 2015; Ricardo et al., 2017; Rocha et al., 2016), soil quality, and environmental conditions (Downes et al., 2014; Ricardo et al., 2017). These differences mean that identical biomass treatment can lead to differences in the hydrolysis of cellulose (Balan, 2014; Salazar et al., 2016).

The information mentioned above led us to hypothesize that changes in tree species, planting density, and soil can affect the biochemical characteristics of the wood produced and the performance of bioethanol production. To test this hypothesis, we assessed the biomass and bioethanol production of four species of *Eucalyptus* planted with three densities in two kinds of forest soils. The short crop rotation period used in these experiments was 22 months and was based on the mean values used for afforestation plantations for energy-production purposes.

2. Material and methods

2.1. Trial characteristics

Two trials were performed in spring (October–November 2010), in two regions in Uruguay: Tacuarembó (32°13'33" S and 55°54'43" W); and Paysandú (32°24'6" S and 57°31'1"W) on a forestry company estate. These zones have a temperate subtropical climate and a mean annual temperature of 18 °C (12–24°C) and mean annual rainfall of 1300–1400 mm (Castaño et al., 2011).

The experimental design was a split-plot design in randomized blocks involving three replications of the four species (main plot): *Eucalyptus grandis*, *E. dunnii*, *E. benthamii*, and *E. tereticornis*, and three planting densities (2,220, 4,440 and 6,660 trees ha⁻¹ (sub-plots).

E. grandis seeds were obtained from the seed orchard of the National Institute of Agriculture Research (INIA) Uruguay, *E. dunnii* seeds from Moleton West (Coffs Harbour, Australia), *E. benthamii* seeds from APS Pinhão, (Paraná, Brazil), and *E. tereticornis* seeds from the seed orchard of the Faculty of Agronomy (Universidad de la República, Uruguay). Each plot comprised six rows with 25 plants each planted at distances of 3 × 1.5, 3 × 0.75, and 3 × 0.5 m, respectively.

The planting area was subsoiled, using a ripper with a single tine, to a depth of more than 60 cm, and soil clods were broken up using a spring harrow and culta-mulcher to provide a more level surface for planting. The fertilization rates per hectare were 150 kg ha⁻¹ using an N–46% P–0% K mixture at Tacuarembó and 180 kg ha⁻¹ using a 14% N–30% P–12% K mixture plus 6% S, and 0.2% B at Paysandú. The types and doses of fertilizer were determined according to the expected differences in the chemical properties of both types of soils.

The average areas of the plots were 706, 350, and 234 m², and the areas evaluated were 470, 234, and 156 m² at densities of 2,220, 4,440, and 6,660 trees ha⁻¹, respectively. The plot surface area was calculated as the product of the length and width, both measured using a tape measure. The area of each planting density by species combination was determined as the average of the three replications.

2.2. Tree sampling and measurements

In 2012, diametric classes of 2 cm width were established for each plot, and the tree frequency was established for each class. According to the proportion of trees in each class relative to the total number of trees, five trees per plot with an average diameter at breast height (dbh) and height were measured in each diametric class (low –, middle –, and high). The wet weight of the stem-wood with bark was then measured. From the basal portion of the stem, discs of 2 cm thickness were extracted at 50% and 75% of the commercial height, and these discs were used to measure the wet weights of the wood and bark separately.

2.2.1. Analysis of biomass

Using the wet and dry weights, the proportion of each was determined to estimate the wet weight of the debarked stem-wood and bark. The wet weight for these two fractions of each tree was estimated as the weighted average multiplied by the surface area of the extracted samples. The wood and bark were dried in an oven with forced ventilation (Thermo Scientific, Waltham, MA, USA) at 60 °C to avoid loss of nutrients such as N. The constant weights reached were used to estimate the percentage of dry matter. The dry weight of each individual tree fraction was estimated as the product of the wet weight and the respective percentage of dry matter. The average individual tree biomass (kg tree⁻¹) of each plot multiplied by the number of trees was used to estimate the biomass per hectare (Mg ha⁻¹) of each species-by-planting density combination. Using the samples taken from the harvested trees from each plot, a sample comprising a weighted tree was formed according to the surface of each disc of the different heights sampled. The samples of the two fractions of this tree were milled (Retsch mill, model SR 200, Haan, Germany) to obtain a particle size of 40–60 mesh, for determination of the nutrient content.

2.2.2. Analysis of cellulose, lignin, and ash

The dry analytical matter and ash content were measured in wood samples from 22-month-old trees of each species, crop density, and site (AOAC Internacional, 1990). Lignin content was measured using the method of Goering and Van Soest (Goering and Van Soest, 1970). Acid detergent fibre (ADF) was measured using ANKOM Technology Method 12 (Macedon, NY, USA). Cellulose content (CC) was calculated as shown in Eq. (1)

$$CC (\%) = ADF (\%) - (Lignin (\%) + Ash (\%)) \quad (1)$$

2.2.3. Bioethanol production

Wood samples from trees of each species, crop density, and site were evaluated. A wood physical treatment method was chosen for this study (Balan, 2014), and the samples were milled to 12 mm in a continuous flow mill (Marconi MA 600, Brazil). Bioethanol production was performed using a pre-hydrolysis simultaneous saccharification and fermentation (PSSF) process that ensures the optimal temperature for cellulase enzyme activity, which is an advantage over the traditional simultaneous saccharification and fermentation (SSF) process (McIntosh et al., 2016; Trevorah et al., 2018). For this purpose, 15 g of wood in 300 mL (5% w/v) of pH 4.8 acetate buffer was sterilized separately for 15 min at 121 °C, and 1 atm, and then pre-hydrolysed with a commercial cellulase complex (36.6 Filter Paper Units per gram of dry biomass; Sunson, China), at 50 °C at 150 rpm for 72 h in a shaker incubator.

Fermentation was undertaken with using the yeast strain SacSV-10, which was obtained from the culture collection of the Biochemistry and Biotechnology Laboratory (CIN, UdelaR, Uruguay). This strain was obtained by irradiation of the M522 *Saccharomyces cerevisiae* strain (Vázquez et al., 2012), and was maintained at -20°C in 10% v/v low-fat milk. The inoculum of SacSV-10 was prepared in liquid YPD medium (yeast extract, peptone and dextrose), at a concentration of 106 cells mL⁻¹ and incubated at 37 °C for 72 h, without shaking. After 72 h of wood pre-hydrolysis, the temperature was cooled to 37 °C to optimize the yeast performance, and the inoculum of 106 cell mL⁻¹ (10% v/v inoculum) was added to begin the fermentation to a 4.5% w/v solids concentration in a final volume) of 330 mL of medium. The PSSF process was maintained for 24 h at 37 °C in a shaker incubator at 130 rpm.

Quantification of total reducing sugars was performed using the dinitrosalicylic acid technique (Bonifacino-Buttiglione, 2012; Chaplin and Kennedy, 1986) at the end of the PSSF. The ethanol concentration at the end was measured using the potassium dichromate technique (Bonifacino-Buttiglione, 2012; Isarankura-Na-Ayudhya et al., 2007) after simple distillation of the samples. All analyses were conducted in duplicate. The yield of the PSSF process for each *Eucalyptus* species at each crop density and site was calculated as the percentage of the mass of ethanol obtained relative to its theoretical mass (Eq. 2) according to Dowe and McMillan (2008). The bioethanol productivity for each species, planting density, and site was calculated relative to the megagrams (Mg) of biomass (Eqs. 3 and 4) and per hectare of crop (Eq. 5).

$$\text{PSSF yield (\%)} = ([\text{Ethanol}]_f / 0.51 \times (f \times [\text{Biomass}] \times 1.111) \times 100 \quad (2)$$

$$\text{Bioethanol mass per Mg of wood (kg Mg}^{-1}\text{)} = 1000 \text{ kg} \times f \times 0.51 \times 1.111 \times (\text{PSSF yield} / 100) \quad (3)$$

$$\text{Bioethanol volume per Mg of wood (L Mg}^{-1}\text{)} = \text{Bioethanol mass per Mg of wood} / 0.798 \quad (4)$$

$$\text{Bioethanol per hectare (L ha}^{-1}\text{)} = \text{Bioethanol volume per Mg of wood} \times \text{Mg of biomass per hectare} \quad (5)$$

2.3. Statistical analysis

The normality of the residuals of the data was verified using the Shapiro-Wilks test and the homogeneity of the variance using the Levene test (Sokal and Rohlf, 1998). The effects of species, and plantation density, and their interactions, were assessed. If the parametric assumptions were satisfied, the variables were compared using one- and two-way analysis of variance (ANOVA), with a significance level of 0.05 and Duncan's post hoc analysis. The effects of species were compared by examining the interaction between species and blocks, and the effects of planting density and the interaction were compared using the mean squared error. When the parametric assumption was not satisfied, the means of different treatments were compared using the Kruskal-Wallis test by ranks and Dunn's post hoc test. In such cases, the effects of the

species and of the planting density were analysed separately. These statistical analyses were performed using Statistix 10 and R software (R Core Team, 2016).

3. Results

3.1. Biomass

In the ANOVA, species and planting density, and their interaction, differed significantly according to biomass yield at Paysandú (23 Mg ha⁻¹) and Tacuarembó (15.8 Mg ha⁻¹), although the differences between sites were not significant (Table 1 and Figure 1). The highest biomass yield was observed for *E. dunnii* (41.6 Mg ha⁻¹) at the Paysandú site, and for *E. benthamii* (26.8 Mg ha⁻¹) and *E. dunnii* (24.5 Mg ha⁻¹), both with 6,660 trees ha⁻¹, at the Tacuarembó site. At both sites, *E. tereticornis* had the least growth. For all species, the highest levels of productivity were obtained with the smallest spacing.

3.2. Chemical composition of wood

The wood cellulose content was higher in samples from Paysandú (62.0% w/w) than from Tacuarembó (59.4% w/w) (Figure 1). Higher cellulose content was observed in samples from the Paysandú site: *E. benthamii* (60.8%–63.7%), *E. dunnii* (61.3%–62.4%), and *E. grandis* (62.8%–65.9%). In samples from the Tacuarembó site, the values were as follows: *E. benthamii* (56.4%–60.7%), *E. dunnii* (58.9%–61.1%), and *E. grandis* (60.6%–64.0%) (Figure 1). The wood lignin content was lower in samples from the Paysandú site (14.7%) than from the Tacuarembó site (16.1%) (Figure 1). At Paysandú, the lowest lignin content was found in *E. dunnii* (12.9%–13.7%) and *E. grandis* (12.4%–12.8%). At Tacuarembó, the lowest lignin content was found in *E. dunnii* (13.8%–15.2%) and *E. grandis* (13.4%–14.7%).

Table 1. P-values of statistical comparison of 22-months old wood's characteristics of dry matter of biomass. Woods samples from Tacuarembó and Paysandú sites.

Effects	Variables	Site	
		Paysandú	Tacuarembó
Species	Biomass yield (Mg ha ⁻¹)	<0.0001	<0.0001
	Cellulose (%)	0.0001	0.0490
	Lignin (%)	0.0015	<0.0001
	Reducing sugar/biomass (mg g ⁻¹)	<0.0001	0.0003
	PSSF yield (%)	0.6190	0.4640
	Bioethanol yield (L Mg ⁻¹)	0.4577	0.5140
	Bioethanol yield (L h ⁻¹)	0.0026	0.0016
Planting density	Biomass yield (Mg ha ⁻¹)	<0.0001	<0.0001
	Cellulose (%)	0.6160	0.0420
	Lignin (%)	0.4970	0.3350
	Reducing sugar/biomass (mg g ⁻¹)	0.7232	0.3152
	PSSF yield (%)	0.4935	0.9401
	Bioethanol yield (L Mg ⁻¹)	0.4931	0.9165
	Bioethanol yield (L ha ⁻¹)	0.6877	<0.0001
Species x Planting density	Biomass yield (Mg ha ⁻¹)	0.0007	0.0135
	Cellulose (%)	-	0.7080
	Lignin (%)	0.1530	0.8750
	Reducing sugar/biomass (mg g ⁻¹)	-	-
	PSSF yield (%)	0.2657	0.9609
	Bioethanol yield (L Mg ⁻¹)	0.2652	0.9390
	Bioethanol yield (L ha ⁻¹)	0.1026	0.0227

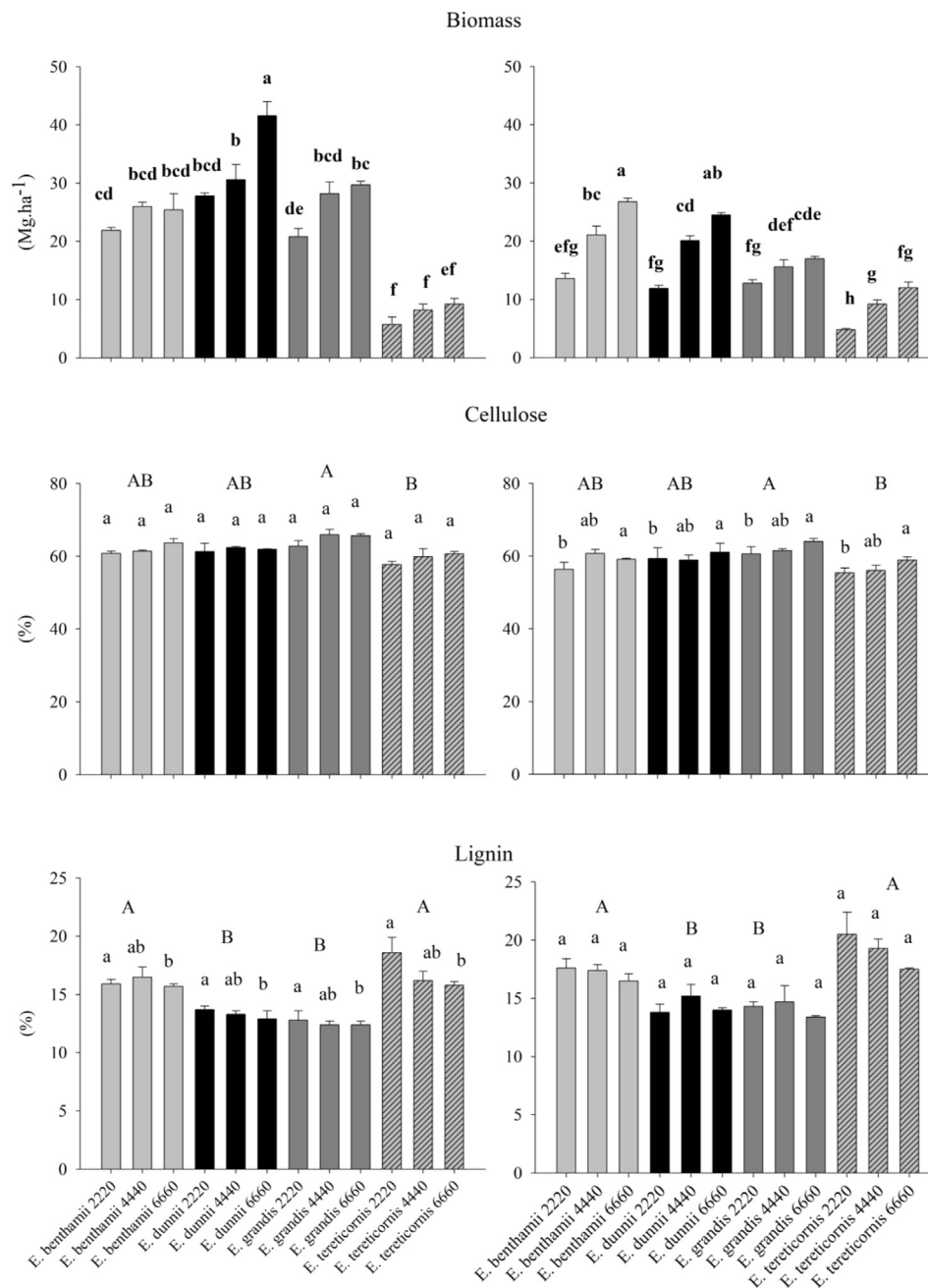


Figure 1. Means (standard error) of biomass, cellulose and lignin of 22-months-old woods at Paysandú (left) and Tacuarembó (right) sites. Different superscripts indicate significant statistical differences of planting density (lowercase) and species (capital letter). Different bold letters indicate differences due to the interaction between species and planting densities.

3.3. Bioethanol production

In wood from both sites, the content of total reducing sugars per unit of biomass at the end of the pre-hydrolysis differed significantly between species (Figure 2). The content of reducing sugars differed significantly between the woods from the Paysandú site (49.9 mg g^{-1}) and Tacuarembó site (50.5 mg g^{-1}). The highest values for reducing sugars were for *E. benthamii* ($53.0\text{--}61.4 \text{ mg g}^{-1}$), *E. dunnii* ($49.4\text{--}56.6 \text{ mg g}^{-1}$), and *E. grandis* ($48.8\text{--}53.9 \text{ mg g}^{-1}$) in the Paysandú samples (Table 2). The highest values for reducing sugars were for *E. benthamii* ($55.0\text{--}58.5 \text{ mg g}^{-1}$), *E. dunnii* ($38.9\text{--}57.6 \text{ mg g}^{-1}$), and *E. grandis* ($48.9\text{--}66.8 \text{ mg g}^{-1}$) in the Tacuarembó samples (Figure 2). On average, the wood samples produced 50.2 mg g^{-1} of total reducing sugars per unit of biomass after 72 h of pre-hydrolysis. The PSSF yields did not differ significantly

between the Paysandú ($19.2\%\text{--}33.1\%$) and Tacuarembó ($25.0\%\text{--}30.8\%$) samples. The assessment of bioethanol yield per unit of wood mass did not differ significantly between the Paysandú ($57.3\text{--}117.7 \text{ L Mg}^{-1}$) and Tacuarembó ($94.7\text{--}110.8 \text{ L Mg}^{-1}$) sites (Figure 3). However, if the area required to produce wood is considered, the bioethanol production per hectare was significantly higher for Paysandú woods ($2,268 \text{ L ha}^{-1}$) than for Tacuarembó woods ($1,616 \text{ L ha}^{-1}$). In wood grown at the Paysandú site, the highest bioethanol production was observed in *E. benthamii* ($2,032\text{--}3,042 \text{ L ha}^{-1}$), *E. dunnii* ($1,869\text{--}4,156 \text{ L ha}^{-1}$), and *E. grandis* ($2,118\text{--}2,688 \text{ L ha}^{-1}$). In wood grown at the Tacuarembó site, the highest bioethanol production per area unit was observed in *E. benthamii* 4,440 trees ha^{-1} ($2,318 \text{ L ha}^{-1}$), *E. benthamii* 6,660 trees ha^{-1} ($2,971 \text{ L ha}^{-1}$), *E. dunnii* 4,440 trees ha^{-1} ($2,012 \text{ L ha}^{-1}$), *E. benthamii* 6,660 trees ha^{-1} ($2,337 \text{ L ha}^{-1}$), and *E. grandis* ($1,629 \text{ L ha}^{-1}$).

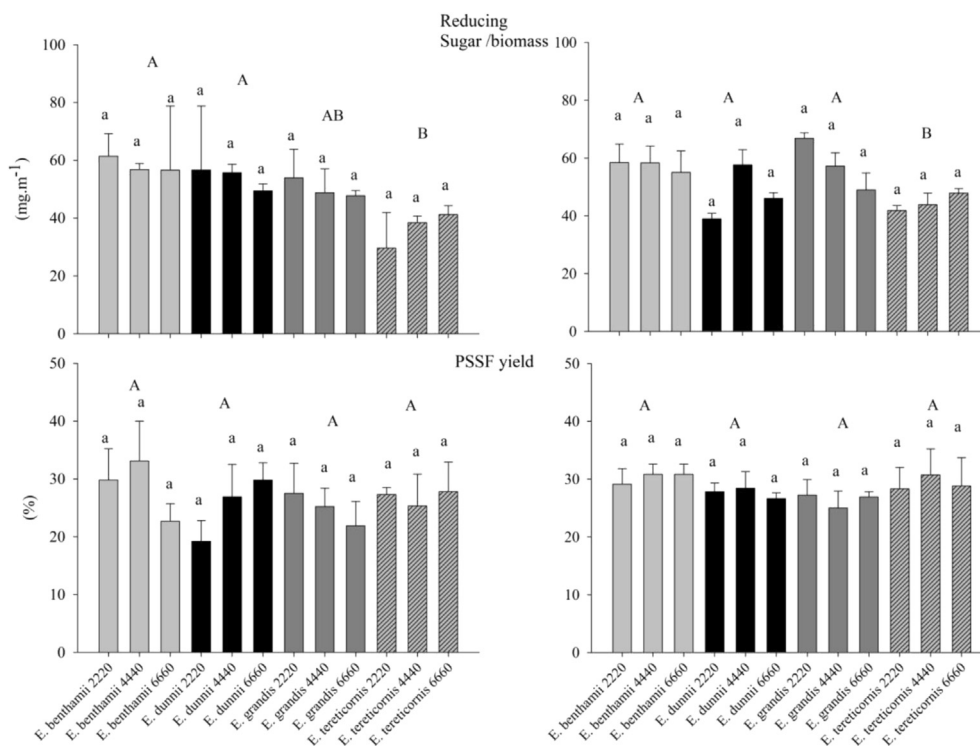


Figure 2. Mean (standard error) of final reducing sugars after pre-hydrolysis step and PSSF yield at Paysandú (left) and Tacuarembó sites (right) are for 22-month-old crops of species and planting densities. Different superscripts indicate significant statistical differences of planting density (lowercase) and species (capital letter). Different bold letters indicate differences due to the interaction between species and planting densities.

Table 2. Nonparametric test results of the effect of the site on cellulose and lignin content, biomass, PSSF yield, bioethanol per Mg of biomass and per hectare for 22-months-old crops from Paysandú and Tacuarembó sites.

Cellulose (%)		Lignin (%)		Biomass (Mg ha ⁻¹)		PSSF yield (%)		Bioethanol (L Mg ⁻¹)		Bioethanol (L ha ⁻¹)	
H	p-value	H	p-value	H	p-value	H	p-value	H	p-value	H	p-value
13.8	0.0001	6.55	0.0098	18.8	<0.0001	2.49	0.116	2.47	0.117	7.1	0.007

All significant results are indicated in bold.

These results show significant differences for all parameters analysed except for the volume of bioethanol per megagram of biomass (Table 1). The average cellulose content was higher for woods from Paysandú; however, the lignin content was higher for woods from Tacuarembó. No significant differences were observed between the PSSF yields obtained for wood grown at both sites. This meant that the volume of bioethanol obtained per megagram of biomass was the same for woods from both sites. The highest biomass productivity was obtained at Paysandú, and this difference was reflected in the production of a higher volume of bioethanol per hectare.

Nonparametric analysis of the effects of site showed that the site affected the cellulose and lignin contents of wood, biomass, and bioethanol produced per hectare for the 22-month-old crops.

However, the site did not affect the PSSF process and bioethanol production per Mg of biomass (Figure 4).

4. Discussion

4.1. Biomass of species and planting density

Analysis of the results for the production of biomass at both sites showed an interaction between species and planting density, which indicates that the amount of biomass depends on the combined effects of both variables. In this sense, for 22-month-old crops, the tendency to increase biomass productivity by increasing the crop density seems to

depend on the species, and, the effects of the species on productivity depend on the planting density. The highest biomass productivity was obtained at the Paysandú site mainly because of the higher initial survival compared with the Tacuarembó site as reported earlier (Resquin et al., 2019). Those authors considered that the lower survival at the Tacuarembó site was caused by less soil preparation before planting and less weed control during the initial growth phase.

The temperature and rainfall regime during the first 2 years of growth were similar for both sites, which would not explain the differences observed in the mortality rate (INUMET, 2018). *E. tereticornis* had health problems caused by the presence of the leaf spot (*Teratosphaeria pseudoecalypti*), which led to the lowest levels of biomass production at both sites. In all species, a reduction in spacing leads to higher productivity, which has been widely reported in the literature (Bernardo et al., 1998; Goulart et al., 2003; Rocha, 2011; Eufraide Junior et al., 2016; Lopes et al., 2017). Although the growth levels can vary greatly between *Eucalyptus* species, planting density, and climate and soil conditions at a site, the length harvest in short rotation systems are close to 4–6 years (Seixas, 2008). This means that the productivity levels could be higher than those registered, so far considering the high rates of increase that this type of crop presents in the first years of growth.

In this study, the increases in productivity as a function of reduced spacing differed between sites (Figure 1). The average increase in biomass production for extreme planting densities (6,660 vs 2,220 trees per hectare) at Paysandú and Tacuarembó was 39 and 86%, respectively.

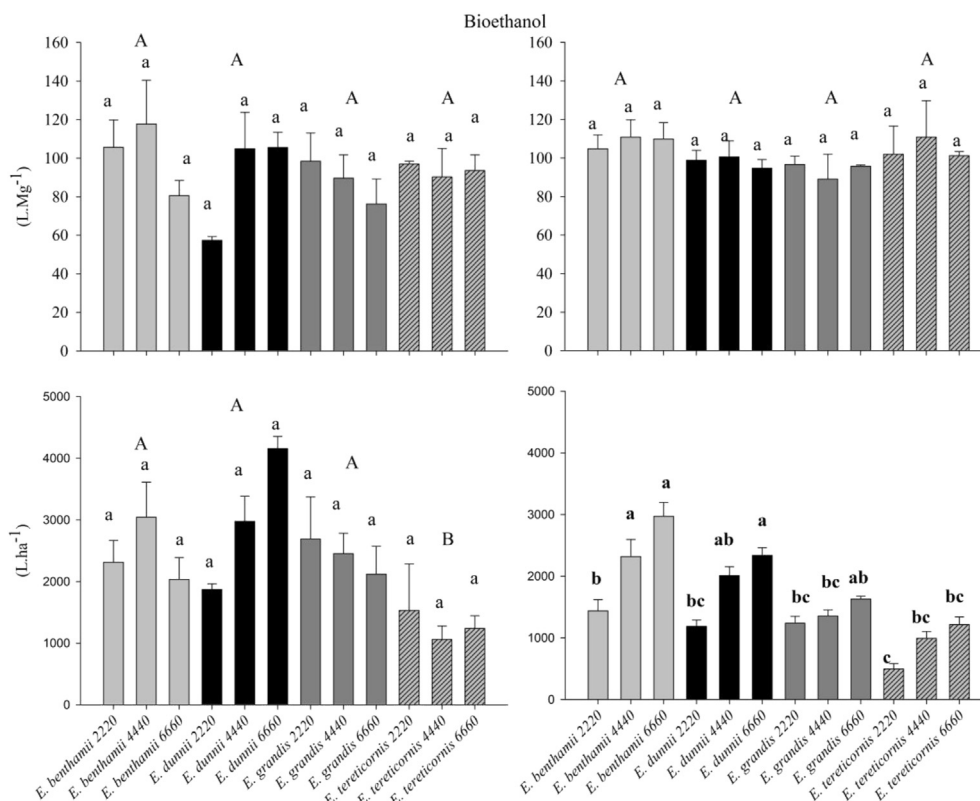


Figure 3. Mean (standard error) of bioethanol per biomass and per hectare for 22-month-old crops at Paysandú (left) and Tacuarembó (right) sites of species and planting densities. Different superscripts indicate significant statistical differences of planting density (lowercase) and species (capital letter). Different bold letters indicate differences due to the interaction between species and planting densities.

This difference in response could be explained by the greater survival recorded at Paysandú (Resquin et al., 2019), which may reflect greater competition between individual trees for all the spacings evaluated. In all cases, tripling the tree population (6,660 vs 2,220 trees ha⁻¹) led to disproportionate increases in productivity. This suggests that the reduction in individual growth because of increased competition is larger than the increase in the number of trees (Rodríguez et al., 2013).

4.2. Chemical composition of wood

The highest content of cellulose was obtained from wood from *E. grandis*, *E. benthamii*, and *E. dunzii* grown at Paysandú, where the average amount of cellulose was 14.7 Mg ha⁻¹. At Tacuarembó, the cellulose content of the wood was affected by both the species and planting density; the wood from all species had the lowest cellulose content in trees grown at the lowest planting density (2,220 trees ha⁻¹). The effect of planting density on the composition of eucalypts woods has been reported by other authors. Rocha et al. (2016) observed significant effects of planting density on insoluble lignin and holocellulose (cellulose and hemicellulose) contents but not on extractives and soluble lignin contents. Moulin et al. (2015) suggested that the relationship between the chemical compositions of *Eucalyptus* woods and planting densities depends on the age of the plants and their genetic material.

Although the lignin content was higher in wood from *E. benthamii* and *E. tereticornis* than in wood from *E. dunzii* and *E. grandis* at both study sites, the yields of fermentable sugar cellulose from pre-hydrolysis were higher in *E. benthamii*, *E. dunzii*, and *E. grandis* than in *E. tereticornis*. According to Salazar et al. (2016) the greatest influences on the production of high amounts of cellulose are the amount and type of associations between the polymers that form the cell wall. In this sense, the slightly lower values obtained for *E. tereticornis* may be associated with morphological differences in cellulose fibres and the higher pentosan

content (Dutt and Tyagi, 2011; Sharma et al., 2011). Related to the lignin content, and taking into account that no chemical pre-treatment was applied in this study; the differences in the PSSF yields were expected (Salazar et al., 2016; Sun and Cheng, 2002).

4.3. Bioethanol production

The transformation of the fermentable sugars to bioethanol by the PSSF process was equal for all species and both study sites, which suggests that the differences in the contents of cellulose and lignin of woods had no effects on the PSSF process. The bioethanol production per biomass unit was the same for 22-month-old trees of the four *Eucalyptus* species grown at all planting densities in both sites. The average yield of the PSSF process was 27%, which corresponds to a bioethanol production of 97 L Mg⁻¹ (77.4 kg Mg⁻¹), of eucalypts wood, a value close to that obtained by treating *E. globulus* wood with 30 min of steam explosion at 180 °C (Romaní et al., 2013); or by treating *E. obliqua* wood with 42.5% (w/w) gamma-valerolactone at 120 °C for 1.25 h (Trevorah et al., 2018).

The values obtained in this study may have been improved by prior delignification (Bonifacino-Buttiglione, 2012; Rico et al., 2014; Trevorah et al., 2018), but the physical treatment applied allowed for the analysis of a large number of samples in a short period of time, which was important for comparing the levels of bioethanol production as the crop age increases. The methodology used in this study may be a starting point for further research about pre-treatment and optimization of the hydrolysis and fermentation processes. We note that, at the end of the PSSF process (96 h), all samples contained reducing sugars (data not shown) that could have been fermented by increasing the processing time. The PSSF process should be improved with the aim of taking advantage of the high amounts of cellulose obtained.

Finally, the volume of bioethanol obtained per hectare of crop was affected by the *Eucalyptus* species, crop density, and site, and could be

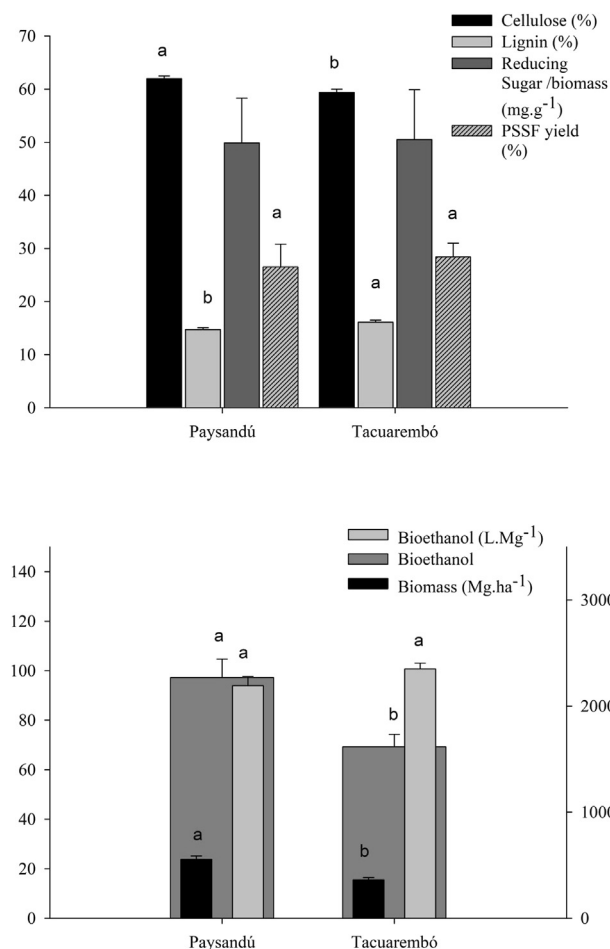


Figure 4. Means (standard error) of biomass, cellulose, lignin, final reducing sugars after pre-hydrolysis step, PSSF yield, bioethanol per biomass and per hectare for 22-month-old crops of both sites. Different letters indicate significant statistical differences of sites.

explained mainly by the biomass production. It was possible to obtain 2,650 L of bioethanol per hectare using the PSSF process and wood from *E. benthamii*, *E. dunnii*, and *E. grandis* planted at 4,440 and 6,660 trees ha⁻¹ at Paysandú. At Tacuarembó, this volume was obtained using wood from *E. benthamii*, planted at 4,440 and 6,660 trees ha⁻¹ and *E. dunnii* planted at 6,660 trees ha⁻¹.

An interaction between species and planting density on the volume of bioethanol per hectare was observed at Tacuarembó, which suggests that the volume of bioethanol depends on the combined effects of both variables. As for biomass production, the tendency to increase bioethanol productivity by increasing the crop density depends on the species, and, the effects of the species on productivity depend on the planting density. Given that it was possible to obtain [14.7–9.2] Mg ha⁻¹ of cellulose from 22-month-old crops of *E. benthamii* or *E. dunnii* planted at high density in a short rotation period, and that the PSSF yield can be improved, in theory, it may be possible to obtain more than 9,000 L ha⁻¹ of bioethanol.

In addition to this study, a parallel study was conducted in which wood samples from *E. benthamii*, *E. dunnii*, and *E. grandis* were used to produce acetic acid and isopropanol (Lopretti et al., 2016). These processes performed together would provide a more sustainable bioethanol production from these energy crops (Balan, 2014; Cebreiros et al., 2017).

Maximization of bioethanol production per hectare was found in *E. benthamii*, *E. dunnii*, and *E. grandis* planted at Paysandú and *E. benthamii* at Tacuarembó. Paysandú and Tacuarembó departments contain a group of soils that are not yet totally forested, and there may be space for the

further development of afforestation focused on bioethanol generation. The preliminary survey by Carrasco-Letelier et al. (2012) showed that a main question needs to be answered before further development of biofuel: when and how do the energy and economic profits occur of each biofuel production system. Answering this question requires more than developing the feedstock production because most of the energy profit gained in the agriculture phase can be lost in the conversion to biofuel. A holistic view of the entire biofuel production system is needed to identify the sustainable technological options. This idea is backed by the modern assessment criteria for biofuels, such as the European Union norm (2009/28), which defines as sustainable those biofuels with a Green House Gas emission lower than 40% of the reference values for petrol. This has also been highlighted by Cavalett and Cherubini (2018) in terms of the sustainability of jet fuel generated from forest residues in Norway. In this context, the results of this study complement the research on the use of biomass to produce biofuels and whether wood is an effective component for biochemical ethanol production.

5. Conclusions

The biomass production from 22-month-old *Eucalyptus* crops was affected by species, planting density, and site. The highest biomass productivity was obtained with *E. dunnii* and *E. benthamii* at 6,660 trees ha⁻¹ at Paysandú and Tacuarembó, respectively. These results suggest that biomass productivity basically depends on the planting density and the species. The cellulose content of wood was affected by the species and by the planting density in trees grown at Tacuarembó, where the wood from all species had the lowest content of cellulose in trees grown at the lowest planting density. The lignin content of woods varied between species. However, the differences in wood composition did not result in differences in the PSSF process or in the volume of bioethanol obtained per unit of biomass.

The volume of bioethanol obtained per hectare of crop was affected by the *Eucalyptus* species, crop density, and site, and these differences were explained mainly by the biomass production. The interaction between species and planting density affected the production of bioethanol per hectare at Tacuarembó, which indicates that the volume of bioethanol obtained from wood grown at that site depended on the combined effects of both variables.

The species and planting densities associated with maximum production of bioethanol per hectare were *E. benthamii*, *E. dunnii*, and *E. grandis* planted at 4,440 and 6,660 trees ha⁻¹ at Paysandú, and *E. benthamii*, planted at 4,440 and 6,660 trees ha⁻¹ and *E. dunnii* planted at 6,660 trees ha⁻¹ at Tacuarembó. The results obtained in this study may be of interest to the regions of southern Brazil and the eastern coast of Argentina, where the evaluated species have adapted well and are widely used commercially.

Declarations

Author contribution statement

Silvana Andrea Bonifacino: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Fernando Resquín: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mary Isabel Lopretti: Conceived and designed the experiments; Performed the experiments.

Luciana Buxedas, Sylvia Vázquez, Mariana González, Alejandra Sap-olinski, Andrés Hirigoyen, Javier Doldán: Performed the experiments.

Cecilia Rachid: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Leonidas Carrasco-Letelier: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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