Suitability of *Salicaceae* genotypes in a phytotechnological approach to industrial wastewater treatment and biomass production

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Abstract: Water circularity is a challenge which must be met to guarantee the sustainability of this resource. Woody biomass is another resource of interest for the bioeconomy, which has multiple uses and acts as a carbon sink. Combining both aspects involves establishing wastewater irrigated plantations, the so-called Vegetation Filters. The aim in this research was to contribute towards assessing the suitability of different *Salicaceae* genotypes for enhancing the efficiency of these simultaneous processes. Twenty-three genotypes of different species and hybrids of the genera *Populus* and *Salix* were irrigated using brewery wastewater under controlled conditions (in a greenhouse using hydroponic cultivation or in pots with substrate) and in the field. Although the application of wastewater reduced the overall production, relevant differences between the genotypes were detected. Growth, physiological activity and nitrogen attenuation efficiency provided good criteria for selection, although given the interaction with site conditions it is essential that plant material is selected based on its adaptation to the environment. The poplar hybrids '2000 Verde' and 'I-214' showed the highest rates of net photosynthesis and transpiration, with high percentages of N removal and moderate biomass production, these two initially being considered of interest for the purposes outlined above. The 'AF34' genotype showed the highest production in the field, followed by the 'Levante' willow hybrid. The white poplar 'PO-10-10-20', which presented moderate production in the field, is also of interest due to its autochthony, which can be advantageous in certain environments. The latter two also showed high attenuation percentages for the evaluated pollutants.

Keywords: plant material adequacy; multipurpose plantations; brewery wastewater; water circularity; biomass production; *Salicaceae*

1. Introduction

Salicaceae (*Populus* spp. and *Salix* spp.) are increasingly being considered for multipurpose plantations [1] with a variety of different objectives and end uses. Biomass production from plantations managed in short-rotation coppices is one of these choices, supplying a key raw material for the bioeconomy. Similarly, ecosystem services, phytoremediation at different scales (soil, water or air), or the direct obtaining of certain bio-based chemicals, are all well-known uses of *Salicaceae* [2–6]. Among them, the ability of the species to regenerate polluted water at the same time that biomass is produced is a matter of growing interest [7–10].

In a wider sense, phytoremediation is defined as the ability of plants, woody or herbaceous, to remove, destroy or sequester contaminants present in the soil or in the water [11]. Poplars and willows are excellent candidates for the task of wastewater phytoremediation. This is due to their rapid growth rate [12,13], high evapotranspiration capacity [14], high nutrient removal rate [15], the aptitude of their roots for water and nutrient uptake, both from deep and shallow soils [16], or their demonstrated capacity to degrade or bioaccumulate the compounds in different compartments [17,18]. In fact, *Salicaceae* species are those most commonly found in the composition of forest Vegetation Filters (VF) [19–22]. VFs are defined as a type of Land Application System where pre-treated or treated wastewater is used to irrigate an area of vegetated soil, commonly a forestry plantation [23]. In these systems, wastewater treatment is performed through the interplay of plants, soil and microbiota, involving different physical, chemical and biological processes, such as sorption, precipitation, biodegradation, and plant uptake.

Specific examples of water filtration have been highlighted, such as serving as barrier elements on riverbanks to avoid water contamination resulting from the management practices used for the adjoining agricultural crops [24,25], or in relation to municipal wastewater [26–28]. In fact, when phytotechnology is applied for the treatment of pollutants in a liquid phase, this botanical family is that which is most frequently employed.

Pollutants, both organic and inorganic, can be phytoremediated through extraction and immobilization processes in different compartments, or through different breakdown strategies based on degradation, metabolism or volatilization [29,30]. Specifically, high N and P retention have been reported in willows and to a lesser extent in poplars [19,31,32]. Removing N and P is crucial, as they are important contaminants of different environmental matrices when found in excess [33,34]. This capacity is magnified with poplar hybrids overexpressing a cytosol glutamine synthetase [35]. In relation to saline water, Smesrud et al. [36] pointed to the need for adequate selection of plant material as well as management practices to maintain a productive stand which is resilient to saline stress. Mirck and Zalesny [37] previously reported the potential of these species to recycle saline wastewater. Several authors have highlighted the wide variability of responses to this factor in the *Populus* genus [38,39].

The brewery production processes generate large amounts of polluted water effluent such as different organic components, sanitizing chemical as chlorine compounds or N and P dependent on the amount of yeast present in the effluent [40]. The attenuation of pollutants, characterized by the presence of several of the abovementioned compounds, was found to be satisfactory using vetiver-grass growing in hydroponic culture in Ethiopia [41]. As far as we know, there are no examples of cultivation of *Salicaceae* for this purpose. Water consumption per liter of beer produced varies greatly depending on the companies and their commitment to adopting good practices [42,43]. In any case, given the scarcity of the resource, the exploration of alternative uses for wastewater seems necessary.

Under Mediterranean conditions, irrigation is a necessary management practice for many months of the growing season [44]. The possibility of contributing to industrial wastewater reclamation while avoiding the use of clean water in the production of a necessary raw material, along with all the possible associated ecosystem services, poses a challenge centered on the notion of circularity.

In this regard, the choice of appropriate plant material is perhaps the starting point when defining the overall strategy. Phytoremediation as a new, additional objective in breeding is currently being considered [45,46]. In Midwestern USA, much effort has been channeled towards identifying suitable genotypes for these purposes, including traditional as well as new experimental genotypes [47–49]. These studies have identified broad variations, as well as specialist or generalist genotypes for a wide diversity of pollutants [47,50]. In Canada, at least seven clones originating from and cultivated in the country for the specific purpose of phytoremediation are included in the FAO checklist of *Populus* cultivars for ornamental and environmental uses [51].

In Europe, the use of plant material for phytoremediation purposes has been considered in different countries. Relevant clonal differences have been identified in wastewater-irrigated land polluted with trace metals in France [52]. In Serbia, Pilipovic et al [53] identified that the poplar and willow genotypes which show greater growth had a greater potential for the phytoremediation of nitrates. The N and P attenuation efficiency has also been evaluated in different scenarios by several authors, finding differences between genotypes, and highlighting the importance of clonal adequacy depending on the desired phytoremediation application [27,54,55].

As regards biomass production, a lot of research at global scale has focused on the selection of appropriate material, both for poplars [56,57] and willows [58,59]. The selection of material to produce biomass in short rotation, understood as adaptation to the environment, has also been evaluated under the specific conditions of the Mediterranean [60–65].

Variables associated with the physiological processes of the plant as well as classic traits related to plant growth and yield can be appropriate tools to determine the most suitable *Salicaceae* material for phytoremediation [53,55].

In this context, evaluating not only the productive capacity of this raw material under Mediterranean conditions but also its suitability for the remediation of a specific scenario has become a fundamental challenge. Thus, the main aim of this work was to identify different genotypes of the *Populus* and *Salix* genera with the dual function of producing biomass while at the same time treating wastewater from the agri-food industry, specifically from the brewing industry. Specific objectives were to: i) make an early selection of a large number of genotypes via hydroponic culture, ii) evaluate those of greatest interest on the substrate, through growth, production, physiological and biochemical variables, and finally iii) test the adequacy of these materials under field conditions, in a real scenario.

2. Materials and Methods

In order to achieve the objectives, three different experiments were conducted:

i) Pre-screening in hydroponic solution under greenhouse conditions

ii) Screening in substrate (pots) under greenhouse conditions

iii) Field plantation

2.1. Plant materials

Nineteen genotypes belonging to different species and hybrids of the *Populus* genus and four from the *Salix* genus were chosen to be tested in the different trials. All of them are listed in Table 1, as well as the species or hybrid group to which they belong and the type of trial in which they were included. Those belonging to *P. alba* and three of the four willows listed are autochthonous. Among the hybrids, some were included because of their strong performance for biomass production or because they were included in the Spanish Catalog of Base Materials and therefore their suitability for Mediterranean conditions had already been tested.

Table 1. Poplar and willow plant material included in the trials.

Plant material				
Genotype	***Species/hybrid***	***Trials***		
		hydroponic	***pots***	***field***
'I-214'	*Populus* x *canadensis* Mönch	x	x	x
'MC'	*Populus* x *canadensis* Mönch	x	x	x
'2000 Verde'	*Populus* x *canadensis* Mönch	x	x	x
'AF34'	*Populus* x *canadensis* Mönch	x	x	x
'AF2'	*Populus x canadensis* Mönch	x		
'AF8'	*Populus x generosa* Henry x *P. trichocarpa* Torr. & A. Gray	x		x
'Viriato'	*Populus deltoides* W. Bartram ex Marshall	x		
'Guardi'	*Populus x canadensis* Mönch	x		
'Triplo'	*Populus x canadensis* Mönch	x		x
'Monviso'	*Populus x generosa* Henry *x P. nigra* L.	x		
'Luisa Avanzo'	*Populus x canadensis* Mönch	x		
'I-454/40'	*Populus x canadensis* Mönch	x		
'Branagesi'	*Populus x canadensis* Mönch	x		
'PO-10-10-20'	*Populus alba* L. autochthonous	x	x	x
'GU-1-21-29'	*Populus alba* L. autochthonous	x		x
'PO-9-16-25'	*Populus alba* L. autochthonous	x		
'J-1-3-18'	*Populus alba* L. autochthonous	x		
'S-18-5-22'	*Populus alba* L. autochthonous	x		
'111PK′	*Populus alba* L.	x		
'Levante'	*Salix matsudana* Koidz. x *Salix* spp.	x	x	x
	Salix atrocinerea Brot. autochthonous Ebro valley	x	x	
	Salix alba L. autochthonous Ebro valley	x		
	Salix eleagnus Scop. autochthonous Ebro valley	x		

Note: The plant material comes from fields of mother plants from the research center's own nurseries or, in the case of native willow genotypes, from official nurseries of the Spanish autonomous communities.

2.2. Experimental design and growing conditions

2.2.1. Hydroponic Culture Trial

The pre-screening tests in hydroponic culture (soilless) was carried out in a greenhouse under controlled conditions (max T: 25 ± 3ºC and min T: 10 ± 3ºC, humidity 65% and artificial lighting of 1000 μE m^{-2} s^{-1}). Unrooted cuttings of 30 cm in length were selected from lignified one-year-old stems. The upper cut of each cutting was performed ~ 1 cm above a bud.

Two trials were installed consecutively following identical procedures. The first of them included poplar material, both hybrids and autochthonous material. The second included all the willows, also including both hybrids and autochthonous genotypes. Both are listed in Table 1. In all cases, five replications per treatment and genotype were randomly installed in 55 l containers, inserting the cuttings in a foam slab above the water level to fix and prevent them from rubbing the bottom or walls of the container. Once the cuttings were established, a single dominant shoot per cutting was selected to facilitate comparison. Half of the containers contained secondary wastewater from the brewery, and the other half was filled with control solution. To avoid problems of biodegradation due to stagnation, 5 W pumps were incorporated into the containers and both treatments were renewed weekly. Trials were maintained for 2 months (64 days).

Throughout the experiment, different growth and physiological measurements referred to in section 2.4 were recorded.

2.2.2. Pots Trial

Under the same greenhouse conditions stated above, seven of the genotypes used in hydroponic culture were individually established in 15.5 l pots. These pots contained a TKS-2 peat substrate and river sand mixed at a ratio of 3:1.

Ten individual pots per genotype were randomly established in the greenhouse. Five of them were treated with secondary brewery wastewater and the remaining five with control solution for comparison. Therefore, each pot (combination of treatment and genotype) was considered as a replicate in a randomized design, with 5 replicates for each combination of genotype and treatment.

The inventoried parameters, referred to in 2.4, were quite similar to those of the hydroponic test. Additionally, the biomass of the different fractions was preserved for later analysis of the total N. The trial was maintained for 4 months (March to June).

2.2.3. Field Plantation

In an industrial field next to the Heineken beer factory (40°35'08.8"N 3°34'18.8"W), a 1000 m^2 plantation was established at a density of 10,000 cuttings ha^{-1} (2 x 0.5 m). An area of 60% of the whole plantation was dedicated to the experimental trial including different genotypes, while the remaining area was planted with the 'I-214' genotype, as it is the most widely planted in our country and is used in different urban wastewater Vegetation Filters [66].

Soil at the site was sampled systematically every 10 m lengthwise and 5 m widthwise of the total area, making a total of 16 samples composing the grid. A single compound sample was prepared by evenly mixing all the 16 samples for characterization (Table 2). Prior to the plantation, the area was tilled following the protocol established by Sixto et al. [44]. Cuttings of nine genotypes listed in Table 1 were manually planted. A design of three random blocks was established, including 15 trees for each genotype and block. Each genotype had its own border trees. In addition, the entire trial was surrounded by a row of the 'I-214' genotype.

A drip irrigation system with secondary wastewater from the anaerobic reactor at the factory's wastewater treatment plant was established. During the first stages of the plantation, weed control was carried out twice a week manually, although only in the row of poplars to allow their establishment. The grass between rows was removed twice to eliminate initial competition in the establishment phase of the crop [67], allowing its growth from that moment since it contributes to the attenuation of contaminants as part of the plant system of the Plant Filter [23]. Due to the abundance of leporidae in the area, a partially buried fence was installed around the plantation.

During the vegetative rest period in the first year of growth, the data collection described in 2.4 was carried out.

Table 2. Soil and climate characteristics at the field site.

Parameters	Methodology	Mean value
MT (ºC)		14.18
MMTW *(ºC)*		33.42
MMTC (ºC)		-0.42
pH	UNE ISO 10390:2012	8.48
EC (µS/cm)	UNE 77308:2001	172
Clay (%)		22.4
Lime (%)	UNE 103102:1995	31.5
Sand (%)		46.2
Bulk density (g/cm^3)	Undisturbed core sampling	1.58
Total N (mg/g)	Kjeldahl method	1.29
Assimilable P (mg/g)	Spectrophotometry	64.8
$CaCO_3$ (g/kg)	Bernard calcimeter	42.1
Na^+ (mg/kg)		93.8
K^+ (mg/kg)		258
Ca^{2+} (mg/kg)	ICP-MS	7188
Mg^{2+} (mg/kg)		539
CEC (cmol/kg)		19.8
Organic Matter (%)	LOI calcination	2.65

Climatic parameters values obtained from SIAR, Spanish government. MT, annual mean temp.; MMTW, mean maxim temp. of warmest month; MMTC, mean min. temp. of coldest month; EC, Electric conductivity; ICP-MS, Inductively coupled plasma mass spectrometry; LOI, Loss on Ignition; CEC, Cation Exchange Capacity.

2.3. Treatments

2.3.1. Hydroponic Culture Trials

For the broad pre-screening test under hydroponic conditions, secondary wastewater from the beer industry, this being the effluent form an Anaerobic Treatment (SW) was used. Additionally, and in order to calculate tolerance indices, a control solution (C), consisting of tap water with a commercial nutrient solution [68,69] at a concentration of 0.84 ml l^{-1} was employed.

The most relevant characteristics of SW are summarized in Table 3. Overall, chemical characterization shows tolerable pH values for poplar irrigation, but high amounts of nitrogen (in the form of organic and NH_4^+) and high electric conductivity (EC) values, derived from the high concentration of Na^+ and Cl^-. TP values do not seem problematic, as they are within the typical range for wastewaters and, from our experience, P is easily removed from water when using Vegetation Filters. SO_4^{2-} values are also far from being hazardous to the environment, and much lower than some natural mineral waters.

Table 3. Physicochemical characteristics of the secondary wastewater

Parameters	Methodology	Mean value and SD
pH	Electrometry	7.91 ± 0.18
EC (µS/cm)		6129 ± 1200
TN (mg/L)	Photometry	70.4 ± 14.9
TP (mg/L)	Photometry	15.4 ± 5.6
TOC (mg/L)	TOC analyzer	174.2 ± 95.0
COD (mg/L)	Photometry	657± 288
TSS (mg/L)	Filtration	220.2 ± 154.2
NH_4^+ (mg/L)	Ionic chromatography	50.1 ± 16.3
Na^+ (mg/L)		1661.9 ± 315.4
Cl^- (mg/L)		738 ± 326.6
SO_4^{2-} (mg/L)		17.5 ± 16.2

EC, Electric Conductivity; TN, Total Nitrogen; TP, Total Phosphorus; TOC, Total Organic Carbon; COD, Chemical Oxygen Demand; TSS, Total Suspended Solids.

2.3.2. Pots Trial

For the screening test on substrate, wastewater treatment effect was compared with a clean water treatment. In both cases, wastewater was collected weekly from a local brewery and transported to tanks located in the greenhouse. The application of the treatments was carried out manually, maintaining the field capacity according to the data from the humidity probes (ECH2O: mod. EC-5, METER Group, Pullman, WA, USA) and the observation of drainage in the pot saucers.

2.3.3. Field Plantation

For the field plantation, effluent water from the anaerobic reactor was conducted to a buffer tank to avoid solid blockages. This was the same outlet pipe from which the water was sampled for the tests under controlled conditions and therefore the composition is as previously described. The applied flow rate was always between 0.5 and 1 Potential Evapotranspiration (PET) and was adjusted to the vegetative activity.

2.4. Recorded parameters

Recorded variables in each type of trial are listed in Table 4.

Table 4. Variables recorded for each trial.

Type of trial	Growth, production and physiological variables
Hydroponic under greenhouse conditions	- Survival - Relative growth in height - Biomass in the different fractions (leaves, stems, roots) - Measurements related to gas exchange (A, E, gs)
Pots under greenhouse conditions	- Survival - Biomass in the different fractions (leaves, stems, roots) - Measurements related to gas exchange (A, E, gs) - Leaf and root total nitrogen content (TN)
Plantation in Field conditions	- Survival - Number of shoots - Total height and basal diameter (10 cm) of the dominant shoot - Woody biomass inferred from the variables recorded following biomass production models (Detailed in text)

Height measurements were recorded using a graduated rule or a pole in the case of the field test. A digital caliper was used for diameter measurements. Different fractions of the biomass (woody biomass, leaves, and roots) were collected in the trials performed under controlled conditions (hydroponic and pots) and then dried at 65ºC to constant weight. In the case of the root biomass obtained in the pot test, exhaustive dry and wet washing of the substrate was carried out. Since the field trial is part of the Vegetation Filter currently underway, the biomass production of the first year was estimated from models that take into account specific growth variables that have been measured directly. We inferred the biomass using the equations described in Oliveira et al. [65] for Mediterranean conditions.

Functional variables related to gas exchange were evaluated in three of the five replicates on fully expanded leaves in the upper third of the plant of each genotype/treatment combination, using a LICOR (LCPro+, ADC BioScientific Ltd. Hoddesdon, U.K.) using setting PAR of 1000 µmol m^{-2} s^{-1}. Measurements were taken monthly during the trial period. The net CO_2 assimilation rate (A, µmol m^{-2} s^{-1}), the stomatal conductance to water vapor (gs, mol m^{-2} s^{-1}), and the transpiration rate (E, mol m^{-2} s^{-1}) were determined.

Total N (TN) by elemental combustion was analyzed (CNS-2000, LECO, St Joseph, MI, USA), after grinding the leaves of three replicates that had been previously dried at 65ºC.

The percentage of TN and EC removal efficiency for each genotype was calculated with the input and output effluent values in the system (pots) in a similar way to that described by Worku et al. [41].

2.5. Data analysis

A factorial analysis was carried out to evaluate the relevance of the variables when differentiating the behavior of the genotypes that were grown in secondary wastewater under hydroponic conditions. For the target variables, and when normality was met, ANOVA analysis were performed and Duncan's mean separation test was used when necessary. If normality was not met, Kruskal-Wallis nonparametric tests were applied and Nemenyi's All-Pairs Rank

Comparison was used. Data analysis and visualization was performed using the Statistical package Statgraphics 19 X-64 and R software v.4.1.1 [70].

A tolerance index (TI), as proposed by Wilkins [71], was also calculated. We measured the ability of the plant to produce root or shoot biomass when growing in the secondary brewery wastewater in comparison to its growth in control water.

3. Results and Discussion

3.1. Pre-screening selection in hydroponic solution under greenhouse conditions

A factorial analysis was carried out to identify the traits with the most weight in the selection. Physiological variables (first factor) explained 43% of the variance (eigenvalue 258.175), with transpiration (E) and net photosynthesis rate (A) showing the highest load matrix values (0.97 and 0.83, respectively). The second factor (25.1% of the variation) identifies the root as well as the root:shoot ratio as the most relevant, both showing a high load matrix (0.97). Finally, the third factor (18.3% of the variance) identified the aerial biomass (leaves and stems) as relevant with a similar load matrix (0.97).

Physiological approaches using non-invasive techniques have provided good results when analyzing phytoremediation in the presence of heavy metals, for example [72,73]. Optimum root development is also key to ensuring absorption of wastewater, while the production of woody biomass is the desired final product. In fact, phytoremediation is focused on maximizing both yield and root growth [50], among other objectives. In this regard, the decision-making process in our research involved prioritizing the evaluation of both these traits.

Exploratory ANOVA analyses of the relevant variables were performed. Significance between the genotypes growing in the SW for almost all traits (p-value < 0.001) was detected (Table 5).

Table 5. Average and standard deviation of genotypes for each recorded variable in broad pre-screening hydroponic trials growing in wastewater.

Relevant traits							
		Parameter	*Root biomass*	*Woody Biomass*	*Root:Shoot ratio*	*E*	*A*
		units	*mg*	*mg*	-	*mol m^{-2} s^{-1}*	*mol m^{-2} s^{-1}*
Trial 1	Poplar hybrids	'2000 Verde'	27.0 ± 14.0 *a*	232.0 ± 60.5 *a*	0.13 ± 0.09 *ab*	0.51 ± 0.1 *bcde*	1.87 ± 0.63 *bcde*
		'AF2'	3.3 ± 0.1 *b*	108.4 ± 31.7 *bcde*	0.03 ± 0.01 *b*	0.47 ± 0.12 *cde*	1.53 ± 0.97 *cde*
		'AF34'	11.3 ± 12.2 *ab*	128.6 ± 32.9 *bc*	0.08 ± 0.07 *ab*	0.41 ± 0.12 *de*	1.32 ± 0.69 *de*
		'AF8'	9.3 ± 8.3 *ab*	73.3 ± 28.3 *cdef*	0.10 ± 0.08 *ab*	0.66 ± 0.37 *abcd*	2.67 ± 2.39 *abcd*
		'Branagesi'	4.9 ± 3.9 *b*	128.0 ± 24.6 *bc*	0.04 ± 0.02 *b*	0.78 ± 0.30 *abc*	2.61 ± 1.79 *abcd*
		'Guardi'	8.8 ± 8.2 *ab*	128.0 ± 50.3 *bc*	0.08 ± 0.08 *ab*	0.41 ± 0.09 *de*	1.30 ± 0.38 *de*
		'I-214'	3.3 ± 0.1 *b*	68.5 ± 24.5 *cdef*	0.06 ± 0.03 *b*	0.64 ± 0.22 *abcd*	2.49 ± 0.98 *abcd*
		'I-454/40'	3.0 ± 0.6 *b*	63.4 ± 13.4 *def*	0.05 ± 0.02 *b*	0.55 ± 0.23 *bcde*	1.60 ± 1.43 *bcde*
		'Luisa Avanzo'	14.5 ± 9.1 *ab*	101.1 ± 27.8 *cde*	0.16 ± 0.10 *ab*	0.5 ± 0.19 *bcde*	1.65 ± 1.21 *bcde*
		'MC'	26.8 ± 21.3 *a*	159.3 ± 13.6 *b*	0.14 ± 0.14 *ab*	0.32 ± 0.08 *e*	0.53 ± 0.52 *e*
		'Monviso'	4.9 ± 2.1 *b*	50.4 ± 11.5 *ef*	0.09 ± 0.02 *ab*	0.45 ± 0.11 *de*	1.63 ± 0.94 *bcde*
		'Triplo'	6.1 ± 4.6 *b*	68.4 ± 32.3 cdef	0.10 ± 0.06 *ab*	0.56 ± 0.3 *bcde*	2.31 ± 1.57 *bcd*
		'Viriato'	8.4 ± 7.3 *ab*	98.9 ± 58.9 cde	0.10 ± 0.06 *ab*	0.55 ± 0.08 *bcde*	1.90 ± 0.71 *bcde*
	Populus alba L.	'111PK'	10.6 ± 4.5 *ab*	106.3 ± 22.4 *bcde*	0.10 ± 0.04 *ab*	0.56 ± 0.22 *bcde*	1.73 ± 1.29 *bcde*
		'GU-1-21-29'	4.7 ± 3.1 *b*	78.4 ± 37.6 *cdef*	0.07 ± 0.04 *b*	0.89 ± 0.36 *a*	3.94 ± 2.19 *a*
		'J-1-3-18'	12.5 ± 11.0 *ab*	63.3 ± 30.5 *def*	0.18 ± 0.16 *ab*	0.81 ± 0.45 *ab*	2.73 ± 0.84 *abcd*
		'PO-10-10-20'	27.6 ± 44.8 *a*	125.3 ± 31.0 *bc*	0.23 ± 0.31 *a*	0.65 ± 0.21 *abcd*	2.64 ± 1.69 *abcd*
		'PO-9-16-25'	3.9 ± 2.2 *b*	30.2 ± 19.4 *f*	0.15 ± 0.16 *ab*	0.64 ± 0.13 *abcd*	3.15 ± 0.86 *abc*
		'S-18-5-22'	6.1 ± 5.3 *b*	99.2 ± 66.5 *cde*	0.08 ± 0.07 *ab*	0.81 ± 0.43 *ab*	3.25 ± 1.95 *ab*
Trial 2	*Salix* spp.	'Levante'	66.6 ± 19.0 *a*	47.6 ± 14.10 *b*	1.43 ± 0.08 *a*	0.49 ± 0.32 *ab*	1.80 ± 1.27 *a*
		S. alba	20.8 ± 13.5 *c*	130.0 ± 47.6 *a*	0.16 ± 0.09 *c*	0.82 ± 1.20 *ab*	1.56 ± 1.60 *a*
		S. atrocinerea	28.3 ± 15.5 *bc*	64.2 ± 46.6 *b*	0.54 ± 0.14 *bc*	0.87 ± 0.07 *b*	2.53 ± 0.44 *a*
		S. eleagnus	47.4 ± 5.4 *b*	57.9 ± 18.8 *b*	0.94 ± 0.05 *b*	0.53 ± 0.28 *a*	1.99 ± 1.73 *a*

Means within each parameter and trial (labeled with different letters) were significantly different at $p < 0.05$ in the Duncan tests or Nemenyi's All-Pairs Rank Comparison in the case of root:shoot ratio.

Note: Woody biomass is referred to all the woody biomass 10 cm above the soil. Root biomass did not include the plant original cutting.

The tolerance index (TI) proposed in this study (Figure 1) allowed to define three tolerance ranges: tolerant (TI≥66); moderately tolerant (TI= 33-66) and sensitive (TI≤33), very similar to those described by Lux et al. [74] in relation to the response of willows to the presence of Cd (Figure 1).

Among the tested willow genotypes, two different approaches were considered. On the one hand, we focused on the genotype that presented the lowest biomass losses when growing in wastewater compared to the control (TI), in total biomass (shoots and root). In this regard, the autochthonous genotype *S. atrocinerea*, had the highest tolerance index (Figure 1). On the other hand, we identified the genotype that presented the highest root biomass when growing in wastewater, while maintaining a good aerial biomass and a high root:shoot ratio (Table 5). The latter was observed in the hybrid genotype 'Levante' of *S. matsutdana* x *Salix* spp. Furthermore, this genotype ('Levante') showed one of the significantly highest transpiration rates and the highest net photosynthesis rate in absolute terms although this was not significantly different. In addition, the wide use of this genotype in Italy for phytoremediation purposes is well known, making it potentially interesting [75,76].

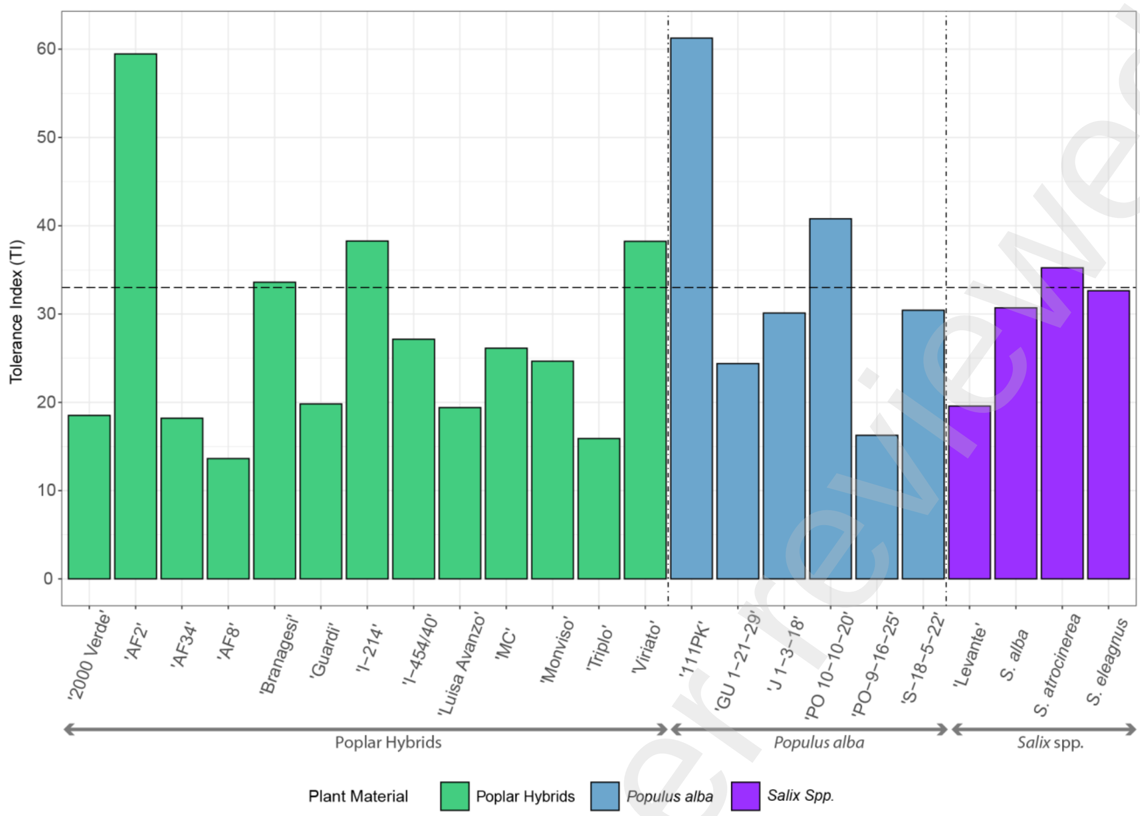


Figure 1. Tolerance index (TI) calculated for each genotype. Dash-dotted lines separate poplar hybrids, autochthonous poplars and willows, respectively. Dashed line marks the limit between the sensitive and the moderately tolerant fields.

In the case of *P. alba*, the genotypes '111PK' and 'PO-10-10-20' were those that exhibited the highest index, both being moderately tolerant (Figure 1). The autochthonous genotype `PO-10-10-20´ was also the one with the highest root and aerial biomass production as well as having a significantly higher root:shoot ratio (Table 5). This genotype previously showed a tolerant behavior to high salinity conditions [39], which also makes it of potential interest.

In relation to the genotypes of productive hybrids of the *Populus* genus, the aerial biomass of the genotypes '2000 Verde', 'AF34' or 'AF2' was significantly greater. The first two, together with 'MC' and 'Luisa Avanzo', also presented significantly higher root production, while 'AF2' displayed very scarce root biomass. Thus, despite having very good aerial production and a moderate tolerance index (the highest of the hybrid poplars), genotype 'AF2' would not be a good candidate. The rest of the above-mentioned genotypes also displayed statistically similar root:shoot ratios (Table 5); although all of them had tolerance indexes in the sensitivity range. Among the poplar hybrids, the other genotypes that presented a moderate tolerance index were 'Viriato', 'Branagesi' and 'I-214', the latter being the most widely planted under Mediterranean conditions. In relation to physiological variables, 'I-214' showed high rates of net photosynthesis as well as transpiration.

The trial under hydroponic allowed us to identify genotypes with different response capacities. In any case, forest plant cultivation is only one of the components in the complex system that constitutes the VF, in which other factors such as the composition and structure of the soil itself, the rhizo-microbiata, or the associated spontaneous vegetation also play important roles [20,23].

3.2. Screening in substrate (pots) under greenhouse conditions

Under similar controlled conditions, although this time using soil substrate as described in section 2.2, the response to the application of wastewater was evaluated in seven of the previously tested genotypes which had exhibited the best responses in terms of physiological and/or production traits. The number was restricted to seven for reasons of space. We wanted to include genotypes from all the groups tested: willows, native white poplars, and productive poplar hybrids. The reasons for this selection is based on the results stated in the previous section, but academic reasons were also considered. For example, 'I-214' and 'MC' represent at least 80% of the area of poplars planted in our country [77,78], therefore determining their particular response may be of interest in the Mediterranean area.

P-values obtained from the ANOVA tests performed on every of the above mentioned traits related to biomass production and physiological parameters are shown in table 6. Overall, significant differences were found between treatments and also between genotypes. Concerning physiological traits, these differences were not present at the first measurements, and they appeared during the trial.

Table 6. Observed significance levels for effects of genotype, treatment and their interaction from ANOVA test for the different parameters in pots trial.

Parameters			Factors		
			Genotype	**Treatment**	**G*T Interaction**
Biomass	Root Biomass		< 0.0001	< 0.0001	0.0726
	Aerial Woody Biomass		< 0.0001	< 0.0001	0.7386
	Root:Shoot Ratio[1]		< 0.0001	0.01569	-
Physiological	22 days	A	0.0668	0.1294	0.8640
		E	0.5048	0.7935	0.9827
		gs	0.0792	0.4190	0.8050
	50 days	A	0.0022	0.7418	0.0376
		E	0.0123	0.6093	0.1617
		gs	0.0511	0.0244	0.0781
	64 days	A	0.0009	0.7607	0.1329
		E	0.0023	< 0.0001	0.3836
		gs	0.1834	< 0.0001	0.5660

[1] p-values obtained using the non-parametrical Kruskal-Wallis Test.

3.2.1. Biomass Production

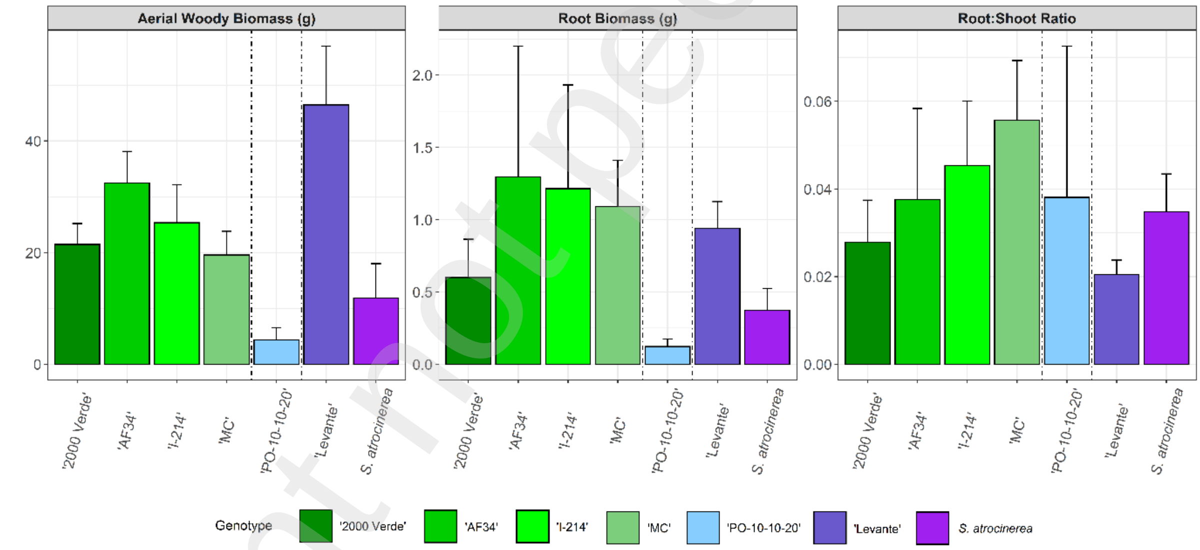
As regards biomass production, significant differences between the wastewater and tap water (control) were detected, both for woody and root biomass. For both fractions, production was higher in the control pots, with a global decrease in wastewater of 33% and 61% for woody and radical biomass, respectively.

These decreases were contrary to what has been stated and found by other authors, who talked about the fertilizing effect of wastewater or polluted water application [50,79,80]. This decrease could be explained by the high N concentration in the wastewater, which can lead to decreased growth as a consequence of a certain phytotoxicity effect [81,82]. The salinity of the wastewater is also a key factor that probably contributed to this drop in production. In general, values of up to 4 dS m^{-1} are considered tolerable for *Salicaceae* [83], the concentration in this wastewater being up to two times higher, within a range considered moderately saline [39]. Despite the decrease in biomass, the usual foliar burn symptoms were not observed and the general development of the plants was not affected. The survival rates were 100%, except for the genotype *P. alba* ‘PO-10-10-20’, for which the rate survival was 80% (1 out of 5 replicates). This was probably due the poor ability of the white poplar for rooting, which has been well documented for many years [12].

The root:shoot ratio also differed significantly between treatments, according to the non-parametric Kruskal-Wallis test (Table 6). Root:shoot ratios were 33% lower in pots irrigated with wastewater than in control pots. The lower values of the ratios for plants growing with wastewater are probably due to the previously reported effect caused by high levels of N promoting greater aerial than root growth [84] or to the increased polluting effect on the roots [85].

The evaluation of the genotype behavior under wastewater irrigation, which is encouraging for the selection, showed relevant differences among genotypes both for above- and belowground biomass (Figure 2). The willow genotype ‘Levante’ was that which had the highest aerial woody production, followed by the poplar hybrids ‘AF34’ and ‘I-214’. The autochthonous genotypes *P. alba* ‘PO-10-10-20’ and *Salix atrocinerea* were those which produced less woody biomass. With respect to roots, the poplar hybrid ‘AF34’ also presented the highest values, while the lowest values again corresponded to the genotypes ‘PO-10-10-20’ and *S. atrocinerea*.

Figure 2. Aerial woody biomass, root biomass and root:shoot ratios for the genotype growing



in the wastewater in the pots test. Dash-dotted lines separate poplar hybrids, autochthonous poplars and willows, respectively.

The genotypes exhibited notable differences in the root:shoot ratios. The willow hybrid 'Levante' and the white poplar ‘PO-10-10-20’ were the ones with the lowest R:S ratio. Thus, ‘MC’ more than doubled the ratio of the willow hybrid ‘Levante’ (Figure 2), evidenced by the different patterns, with both genotypes showing similar root production while the willow exhibited much greater aerial development.

This seems to indicate the importance of considering the ratio when selecting plant materials for a specific purpose, since high aerial production is not always matched by good radical development. Therefore, this parameter alone may not always be a reliable indicator when evaluating adaptation. Tree growth is a complex system in which both roots and shoots as well as the relationship between the two must be taken into account to understand the physiology of this system [86].

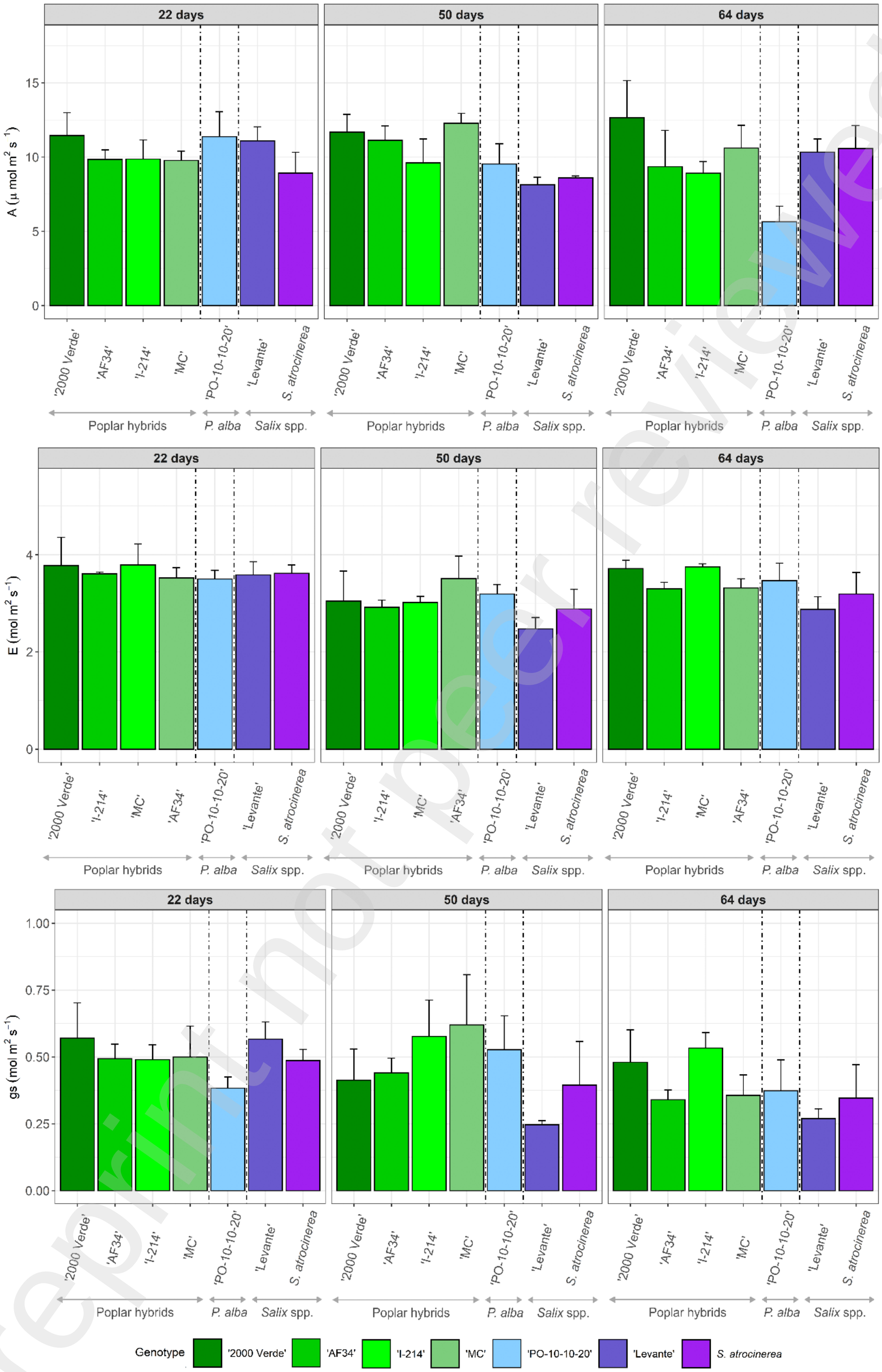
3.2.2. Physiological parameters

Growth reductions due to pollutants are frequently accompanied by reductions in the rate of net photosynthesis, transpiration, and other physiological parameters [87,88]. Significant differences between treatments (Table 6) in the transpiration rate (E) were only detected at the end of the trial (64 days); the control pots exhibiting a rate 15 % higher than those irrigated with wastewater. Significant differences were also observed between genotypes growing in the wastewater from the second measurement date onwards (the poplar hybrids ‘I-214’ and ‘2000 Verde’ being the genotypes which had the highest values, while the willow genotypes ‘Levante’ and *S. atrocinerea* had the lowest.

Differences in stomatal conductance (gs) were only significant for treatments from 50 days of exposure until the end of the experiment (64 days) (Table 6), the stomatal opening being 69% higher in the control plants (overall). The effect of contaminants in wastewater, such as increased salinity, induces stomatal closure.

Finally, photosynthesis rates (A) was the only physiological trait not significantly affected by the application of wastewater at any time during the experiment (Table 6), although there was a small percentage decrease. However, significant differences were found between genotypes from the second measurement in the wastewater treatment. The genotype presenting the highest A values at the end of the trial was the poplar hybrid ‘2000 Verde’, followed by the hybrid ‘MC’ and the willows ‘Levante’ and *S. atrocinerea*, while the lowest values were recorded for the autochthonous poplar *P. alba* ‘PO-10-10-20’ (Figure 3). Intraspecific and interspecific differences in the rate of photosynthesis in this family have previously been reported [89,90]. In summary, physiological measurements show that the use of secondary wastewater from the brewing industry significantly affects both transpiration rate and stomatal conductance after a given time of exposure, although it does not appear to affect the rate of photosynthesis. Therefore, it seems that the genotype effect must be taken into account, with ‘2000 Verde’ and ‘I-214’ being those that exhibit higher rates of photosynthesis and higher levels of transpiration, respectively.

In general, the N increase in the medium affects gas exchange traits, stimulating the rate of photosynthesis and finally causing an increase in growth in numerous C3 species [91]. In our experiment, no stimulation of gas exchange was observed as a result of irrigation enriched in nitrogen, which is probably due to the high values, higher than normal fertilization [92], but also to other water characteristics such as high salinity.



409 **Figure 3**. Photosynthesis (A), transpiration (E) and stomatal conductance (gs) for each
410 genotype growing under the wastewater at different times. Dash-dotted lines separate poplar
411 hybrids, autochthonous poplars, and willows, respectively.

3.2.3. Nitrogen content and phytoremediation potential

The N concentrations in the genotypes irrigated with wastewater were significantly different for both roots and leaves ($p < 0.0001$ in both cases), indicating different location dynamics from one genotype to another (Figure 4). In all cases, the total nitrogen content (TN) was on average 40% higher in the leaves than in the root. This distribution was similar to that described by Bhati and Singh [93] for *Eucalyptus camaldulensis* irrigated with municipal effluents.

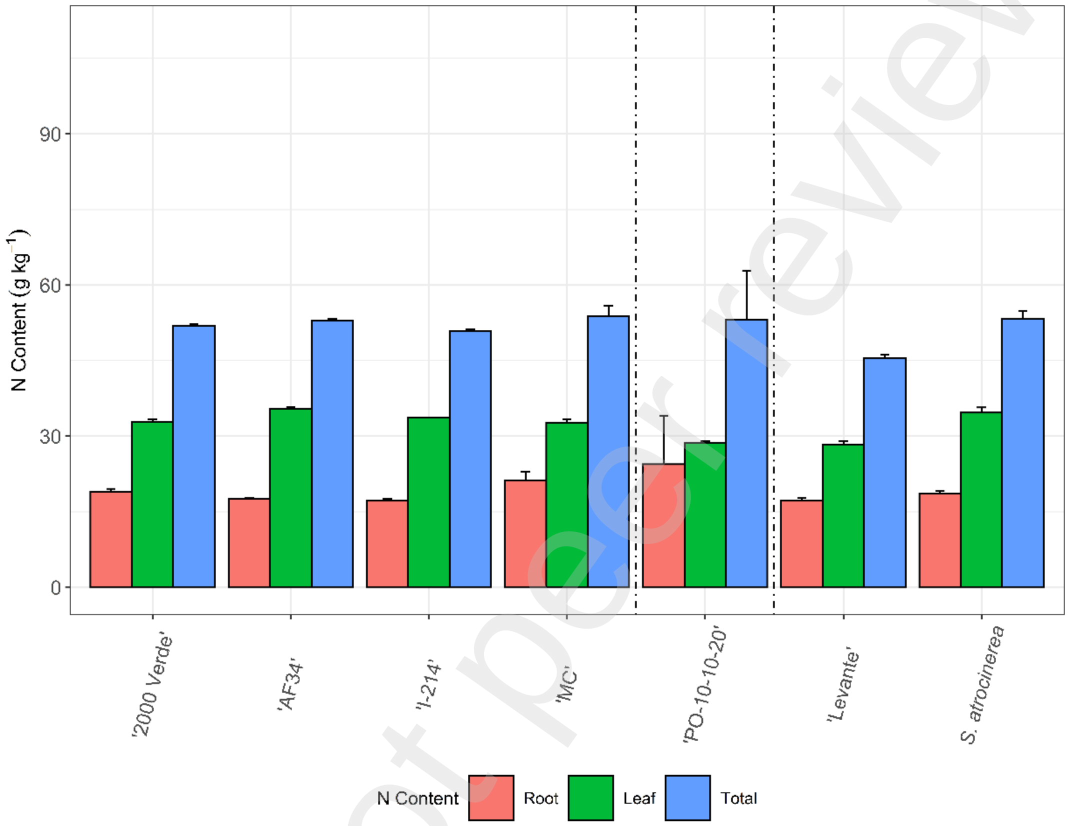


Figure. 4. Roots, leaves and total nitrogen contents for each genotype when irrigated with wastewater. Genotypes labelled with different letters were significantly different at $p < 0.05$ according to Duncan tests in the wastewater treatment, considering each fraction independently.

The autochthonous poplar 'PO-10-10-20' was the genotype that had the highest N contents in roots when growing with wastewater, while the poplar hybrid 'I-214' had the lowest values. On the other hand, the poplar hybrid 'AF34' presenting the highest N in leaves values, followed by the autochthonous willow *S. atrocinerea*. The willow hybrid 'Levante' and the autochthonous poplar 'PO-10-10-20' had the lowest values. As regards the aggregate root and leaf N content, only the values for the willow hybrid 'Levante' were significantly lower than the rest of the genotypes (Figure 4).

On average, the N in water attenuation is around 57%, with notable differences between genotypes, although all of them showed a greater or lesser degree of aptitude for N removal (Table 7). The poplar hybrid '2000 Verde', the autochthonous white poplar 'PO 10-10-20' and the willow hybrid 'Levante' showed the highest attenuation percentages (above 60%), being around the average for 'I-214' or *S. atrocinerea*. The poplar hybrids 'MC' and 'AF34' showed the lowest attenuation percentages. The suitability of the willow hybrid 'Levante' for phytoextraction of metals in contaminated soils has been repeatedly demonstrated [73,76].

Table 7. Nitrogen and Electric Conductivity attenuation percentages for each tested genotype between the beginning and the end of the experiment (T= 4 months) in the pots trial.

Genotype		TN attenuation (%)	EC attenuation (%)
Poplar hybrids	'2000 Verde'	72.4 ± 18.6 (55.3 – 90.7)	80.0 ± 13.7 (62.8 – 94.1)
	'AF34'	39.5 ± 32.7 (0.74 – 82.3)	74.0 ± 18.7 (47.0 – 91.7)
	'I-214'	57.5 ± 36.5 (4.76 – 96.6)	79.8 ± 20.3 (49.9 – 99.9)
	'MC'	51.2 ± 30.5 (12.9 – 84.8)	76.6 ± 18.4 (49.6 – 93.4)
Populus alba	'PO-10-10-20'	62.7 ± 27.5 (24.1 – 100)	83.4 ± 9.1 (66.2 – 90.3)
Salix spp.	'Levante'	60.7 ± 21.1 (25.0 – 89.9)	80.2 ± 16.0 (58.0 – 94.3)
	S. atrocinerea	57.2 ± 27.2 (12.9 – 94.7)	85.1 ± 11.9 (66.4 – 96.2)

Values shown are the means calculated ± standard deviation, using the weekly % attenuation. The values in brackets are minimum and maximum, respectively.

The fact that the total N values in the plant irrigated with wastewater (leaves and roots) were only 10% higher than in control pots, together with the N removal capacity of the soil-plant system in all the genotypes, would appear to indicate that, in all cases, the elimination of N is taking place to a greater or lesser extent, probably via nitrification-denitrification processes. However, it would be necessary to determine the N contents both in the soil and in the wood to better understand the differences among the studied genotypes.

Regarding the attenuation of electrical conductivity, the percentages were high in all cases (greater than 70%) (Table 7) with the best results corresponding to the autochthonous *S. atrocinerea* and the white poplar genotype 'PO-10-10-20'. Although high intraspecific variability exists in relation to the ability to exclude sodium from the roots as well as differences in the regulation of ion transport through the leaf cell membranes [94], the greater suitability of white poplars for growth under saline conditions, especially this particular genotype, has previously been mentioned in the literature [39,95]. Nevertheless, and as stated above, the role played by the soil and the microbiota should be considered and assessed.

3.3. Field plantation

The same genotypes used in the pot trial were used in the plantation. However, since two more positions were available in the plantation design, two more genotypes were added. These were the autochthonous *P. alba* 'GU 1-21-29', which had shown a salt-tolerant behavior in the past [39] and the productive hybrid 'AF8', considered very promising for biomass production [63], both of these genotypes having displayed high rates of A and E in the hydroponic trial. The poplar hybrid 'Triplo', despite not being especially outstanding for any of the variables analyzed under hydroponic conditions, is widely cultivated in our country for wood production, and especially in Catalonia region where it is the most planted genotype [96]. With this in mind, we decided to include this genotype in place of *S. atrocinerea* to prioritize the plantation of poplars over willows, as poplars are more suitable for Mediterranean conditions [23,26,97].

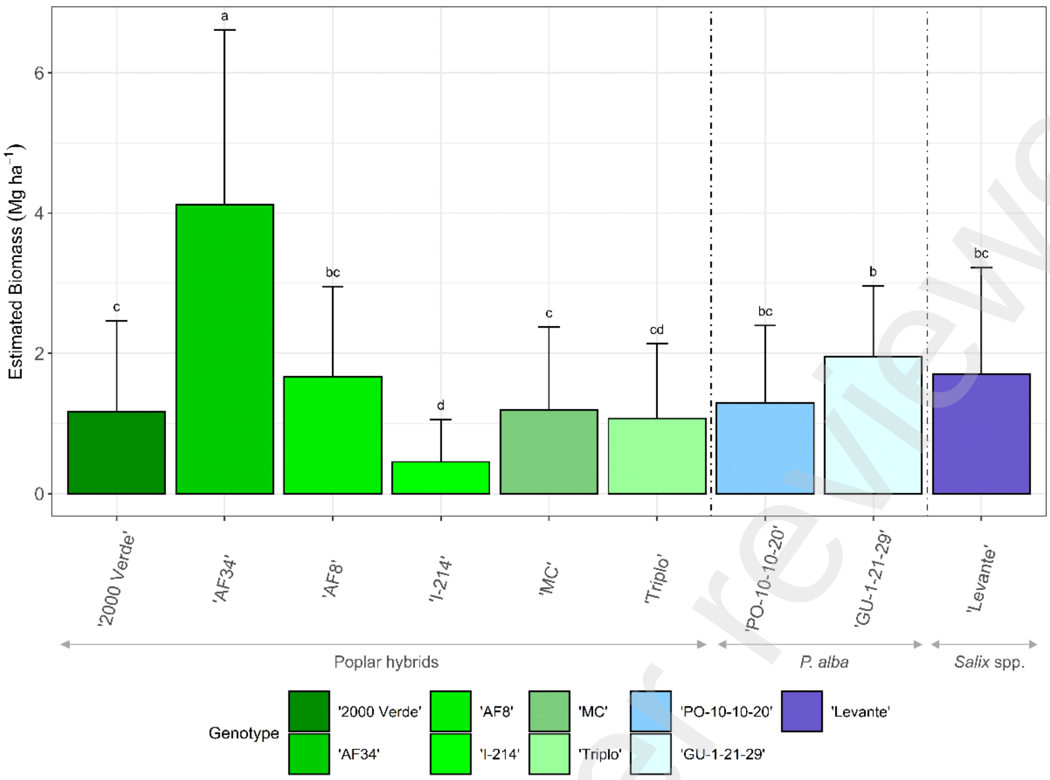


Figure 5. Estimated biomass production at the end of the 1st year of rotation under wastewater irrigation. Genotypes labelled with different letters were significantly different at $p < 0.05$ according to Duncan tests. Dash-dotted lines separate poplar hybrids, autochthonous poplars, and willows, respectively.

The mortality of the plantation was 4.1%, the genotypes 'GU 1-21-29' and '2000 Verde' showing the highest percentage (11%) and 'AF34', 'AF8' and 'MC' the lowest (0%). This overall value is in line with the accepted normal mortality rate in high-density plantations, which is around 10% [44]. This is a very promising result as regards the viability of the plantation as a Vegetation Filter.

The overall estimated production of dry biomass in the first year of the rotation was 1.62 Mg ha^{-1}. The values ranged from 4.12 Mg ha^{-1} for the hybrid genotype 'AF34' to 0.45 Mg ha^{-1} for the autochthonous white poplar 'GU-1-21-29' (Figure 5). This yield is in line with that obtained under other scenarios in which the *Salicaceae* is used as a phytotechnological tool, such as that obtained under irrigation with landfill leachate (from 0.51 to 2.5 Mg ha^{-1}) as reported by Zalesny et al. [48] or even under irrigation with clean water and fertilization (100 kg ha^{-1} of total NPK fertilizer applied twice a year [98]). However, these levels of production are far from those obtained under Mediterranean conditions for plantings with a similar design when the irrigation water comes from a clean source and the soil is more suited to the demands of the species [62,99]. However, studies have pointed to the fact that first year poplar cuttings require significant investment in the root, which is why growth is usually lower than that obtained in subsequent years of the rotation; with production often doubling once the crop is established [98,100].

The soil conditions were not optimal for poplar cultivation. Nevertheless, the site was selected because of its proximity to the factory, since it is a requirement for this type of plantations. This is to be expected on land adjoining an industrial zone and probably contributes to the detriment of optimal yields, affecting root development, soil properties and stability. Despite this, genotypes with yields that may be of interest by modifying management were identified. In this regard, a possible management option would be to extend the rotation in such a way that production is maximized against the costs of cultivation, particularly if payment for ecosystem services such as carbon sequestration is taken into account, this currently being set at eight years in our country [101].

In the field, the 'AF34' genotype exhibited a significantly higher production than the other genotypes (Fig. 5). The improved productive performance in the field of the autochthonous white poplar 'PO-10-10-20' compared to controlled conditions is also worthy of note, with yields not differing significantly from the hybrids 'Levante', 'Triplo' or 'AF8'. This is likely due to the increasing difference in the yield of the autochthonous material versus the hybrids over time, previously detected in other field trials [102,103] and which has occurred in this case as this field trial was longer (1 year) than those carried out under controlled conditions. This difference has frequently been attributed to the greater difficulty of the white poplars to emit roots from the cuttings [12]. It should be noted that the standard deviation of the data was very high, given the previously mentioned nature of the soil. In any case, longer rotations will probably be necessary to maximize production, although more research is needed in this respect. Furthermore, when considering production, industrial land should not only be evaluated from the purely economic aspect of the production but also from the perspective of the ecosystem services that are generated.

Although hydroponic cultivation and, in general, trials under controlled conditions allowed us to make a good assessment of the behavior of a large number of genotypes, the response in the field, where soil and climate interacted, was not always in line with what was expected, as previously reported by other authors [55].

4. Conclusions

Secondary treated wastewater from the production of beer, used as a substitute for irrigation water, allowed the establishment and growth of different genotypes of *Salicaceae* (poplars and willows) with acceptable percentages of failed plants, both in pots under controlled conditions and in the field, which is initially very promising. However, in all cases, production losses were observed compared to the control pots irrigated with tap water under controlled conditions, as well as lower production than normal in the field for these plants in the Mediterranean area. Given the reasonably good percentages of attenuation obtained, on average, both for TN and EC, this decrease in overall production is probably attributable to the low suitability of the land too.

Furthermore, clear differences were revealed as regards the response of the genotypes to the different variables studied under wastewater irrigation in greenhouse conditions. Thus, the willow hybrid 'Levante' exhibited very high production and a very high percentage efficiency in N attenuation, despite the low transpiration rates observed. 'AF34', also highly productive, exhibited a high rate of photosynthesis as well as moderate transpiration, although the percentage N removal efficiency was the lowest in this case. Given that both genotypes exhibited the highest productivity, they are of potential interest for inclusion in plantations irrigated with this type of wastewater, despite large differences between the two in terms of N removal efficiency.

The poplar hybrids '2000 Verde' and 'I-214' showed the highest rates of net photosynthesis and transpiration, with very high percentages of N removal efficiency and moderate woody biomass production. Therefore, both genotypes should initially be considered of interest for this purpose.

Also of interest is the native white poplar ('PO-10-10-20'), which exhibited a high capacity for the attenuation of the evaluated pollutants, even though it was not among the high yielding genotypes.

Finally, the autochthonous willow (*S. atrocinerea*), which is not very productive and has a low nitrogen attenuation capacity, would therefore be of little interest for this use.

Preliminary results for production using irrigation with wastewater under field conditions reveal a production pattern, which is very similar to that observed under controlled conditions. The best growth response corresponded to the 'AF34' genotype while the 'Levante' willow hybrid also exhibited notable production. Additionally, the white poplar genotype 'PO-10-10-20' is of interest because of its autochthonous character despite its not so high productivity.

Although the different productive, physiological and nutrient removal efficiency criteria served their purpose for the early selection of a large number of genotypes, the importance of interaction with site conditions and therefore the adaptation capacity of the different genotypes became apparent in the field trials. The fact that it is a land that is not very suitable for cultivation but necessary due to its proximity to the wastewater source must be considered.

In this specific scenario, it will probably be necessary to modify the management techniques applied, extending the rotation period while also taking into consideration the ecosystem services provided, such as carbon sequestration.

The results reveal the intra- and inter-specific variability of *Salicaceae* when grown using wastewater from the brewing industry and highlight the necessity for more in-depth research into the suitability of irrigation with wastewater under Mediterranean conditions. Promoting the circularity of water, not just the potential improvement of water quality, is an essential factor in the push towards sustainability.

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Data Availability Statement: In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics. If the study did not report any data, you might add "Not applicable" here.

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