UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in Scienze Biologiche e Molecolari

XXVIII Ciclo

Developing tools for accelerating the domestication of two promising biofuel crops: *Arundo donax* (giant reed) and *Panicum virgatum* (switchgrass)

Gaia Pigna

PhD Thesis

Scientific tutor: Piero Morandini

Academic year: 2015-2016

SSD: BIO/04; BIO/11

Thesis performed at - Department of Biosciences,
University of Milan
- Department of Plant Science,
University of Tennessee

Cover: *Arundo donax* canes in Monopoli (BA). Photo credit: Ettore Potente

A mia nonna Maria che mi ha insegnato a fare le moltiplicazioni
(To my grandma Maria who taught me multiplication)

Index

Part I

Part II

Pigna G., Morandini P., 2016 Domestication of New Species. *In* More Food: Road to survival, chapter 9, (pp. 206-259), *in press*.

Pigna, G., Dhillon, T., Dlugosz, E.M., Yuan, J.S., Gorman, C., Morandini, P., Lenaghan, S.C. and Stewart, C.N., 2016. Methods for suspension culture, protoplast extraction, and transformation of high biomass yielding perennial grass Arundo donax. Biotechnology Journal. *Published Oct 20.*
DOI: 10.1002/biot.201600486

Part III

Manuscript to be submitted:

Cryopreservation of switchgrass (*Panicum virgatum*) protoplasts
E.M. Dlugosz, G. Pigna, S.C. Lenaghan, and C.N. Stewart, Jr.

Abstract

The need for energy and the unsustainability in the long term of fossil fuels encouraged the quest for more environmental-friendly energy sources. One alternative is exploiting plants to produce biofuels. The screening of many perennial grasses suggested Miscanthus (*Miscanthus spp)*, Arundo (*Arundo donax*), and reed canary grass (*Phalaris arundinacea*) in Europe and switchgrass (*Panicum virgatum*) in both Europe and US as good candidates for bioenergy production. This thesis focuses on two of these plants: Arundo and switchgrass. Both plants already show a good agronomic performance, but they underwent little domestication, so there is room for their genetic improvement. On the contrary to switchgrass, breeding, genetic transformation and in vitro cultivation of Arundo are lagging behind for the sterility and the recalcitrance of this plant. We developed a method for *in vitro* cultivation of Arundo that is faster and more efficient than the one previously reported in literature. Calli are induced from stem segments on an MS-based medium containing 9 µM 2,4-D and then a suspension culture can be obtained using the same medium supplemented with 4.4 µM BAP, leading to a 5-fold increase in cell mass over 14 days. This medium was the best in terms of growth rate and final cell density among the four tested media. Protocols for protoplast isolation from suspension cultures and electroporation were also established, allowing a transformation

efficiency up to 3.3±1.5 %. The use of a novel vector with two distinct fluorescent protein reporters (GFP and RFP) driven, respectively, by the Cauliflower Mosaic Virus 35S and the ubi2 promoter of switchgrass, allowed a direct comparison of the strength of the two promoters in Arundo. The switchgrass ubi2 promoter provided a much higher expression compared with the 35S promoter. Lastly, as a further methodological advancement, we developed a method to cryopreserve mesophyll protoplast of switchgrass. Both vitrification and slow-freezing methods were tested. Slow freezing is more efficient than vitrification with a percent recovery of alive protoplast of 45.8% vs. 5.2%.

Abbreviations

2,4-D: 2,4-Dichlorophenoxyacetic acid

4CL: 4-Coumarate:coenzyme A ligase

AFEX: Ammonia Fiber Expansion

AFLP: Amplified Fragment Length Polymorphism

AtLOV1: *Arabidopsis LONG VEGETATIVE PHASE ONE*

BAP: 6-Benzylaminopurine

BFDP: Bioenergy Feedstock Development Program

BSA: Bovine Serum Albumin

BY-2: Bright Yellow 2

CAD: Cinnamyl Alcohol Dehydrogenase

Cg1: Corngrass 1

CIM: Callus Induction Medium

CRIPR-Cas9: Clustered Regularly Interspaced Short Palindromic Repeats

CRP: Conservation Reserve Program

CSLF: Lignocellulose Fractionation

DM: Dry Matter

DOE: Department of Energy

dsRNA: double-stranded RNA

EST-SSR: Expressed Sequence Tag-Simple Sequence Repeat

FSA: Fetal Serum Albumin

GA: Giberellic Acid

GHG: Green-House Gases

Hd3a: Heading date 3 a

ISSR: Inter-Simple Sequence Repeat

IVDM: *In Vitro* Dry Matter Digestibility

MYA: Million Years Ago

NO_x: Nitrogen Oxides

ORNL: Oak Ridge National Lab

PAR: Photosynthetic Active Radiation

PCD: Programmed Cell Death

PCV: Packed Cell Volume

PEG: Polyethylene Glycol

PL1: *Pectobacterium carotovorum* ectate lyase 1

***Pv*Ubi**: *Panicum virgatum* Ubiquitin

RAPD: Random Amplified Polymorphism DNA

RFT1: Rice Flowering Locus T 1

RID1: Rice Indeterminate 1

RNAi: RNA interference

RUE: Radiation Use Efficiency

siRNA: small interfering RNA

SRAP: Sequence-Related Amplified Polymorphism

TE: Transposable Elements

USDA: US Department of Agriculture

VOC: Volatile Organic Compounds

WRA: Weed Risk Assessment

ZmMPK5: *Zea mays* Mitogen-activated protein kinase

State of the art

1 *Arundo donax*: a promising biofuel crop

1.1 Origin

Arundo donax is a tall, perennial grass and a promising biomass crop candidate in Mediterranean countries. Arundo properties (tenacity, rigidity and vigor), were well known since ancient times in the middle East as mentioned in the Bible and by Pliny the Elder (**Perdue 1958**). Arundo was described by Linneaus (**1753**) as growing in Spain and south-eastern France, but today we know it is present worldwide, mostly in the Mediterranean area (**Hardion et al., 2014**) (**Figure1**). The origin of this plant was studied analysing 67 Mediterranean and 10 Asian clones combining two types of molecular markers: AFLP (Amplified Fragment Length Polymorphism) and ISSR (Inter-Simple Sequence Repeat). This study demonstrated the monophyletic origin of Arundo from Asia and the successive spread into the Mediterranean Basin thanks to human activities (**Mariani et al., 2010**). The plastid DNA sequencing and morphometric analysis of 127 specimens collected across sub-tropical Eurasia combined with the bioclimatic species distribution using 1221 Mediterranean localities confirmed the Asian origin. Specifically this study found an haplotype variation and phylogeographic structure across the Asian range of Arundo clones strongly suggesting a Middle East origin of the first clone reaching the Mediterranean (**Hardion et al., 2014**). This Asian origin is also supported by the difference of cytotype 2*n*=12 found in Thailand (**Larsen, 1963**), India (**Christopher and Abraham, 1971**) and Uzbekistan (**Bochantseva, 1972**) compared to the cytotype 2*n*=14 found in the Mediterranean area (**Hardion et al., 2012**).

After the spread in Southern Europe, Arundo has been then introduced in North America, starting from Southern California (**Bell et al., 1997**), and from there it colonized the US. In order to assess genetic variability among the US clones, molecular markers analysis have been performed. A collection of 185 clones of Arundo sampled across the country were analyzed using SRAP (Sequence-Related Amplified Polymorphism) and TE (Transposable Elements) markers. These analysis showed a low genetic variability suggesting that all these clones most probably derive from a single clone (**Ahmad et al., 2008**). This was in contrast with the moderate but existing genetic diversity found previously by Khudamrongsawa et al., (**2004**) with the RAPD (Random Amplified Polymorphism DNA) and isozymes analysis of 97 plants (31 phenotypes) of Santa Ana river area in California. It is important to consider that this analysis was based on a smaller and less representative collection and that, as Ahmad et al. (**2008**) pointed out, RAPD markers have limited reliability.

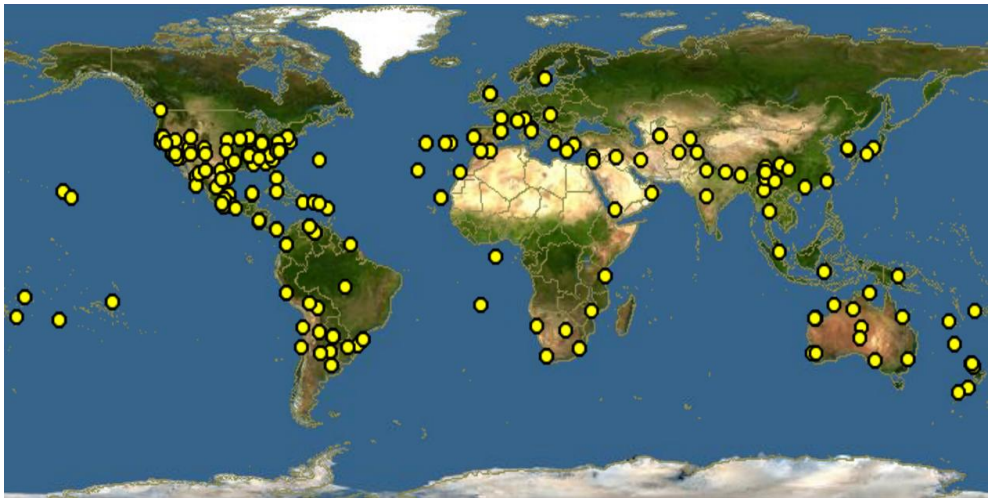

Figure 1: *Arundo donax* worldwide distribution. Yellow dots represent the location where Arundo was detected.
(http://www.discoverlife.org/mp/20m?kind=Arundo+donax)

1.2 Sterility and propagation mode

The lack of seed production in Arundo is well known and it has been shown by different studies (see **Boose & Holt 1999** for instance) but few have investigated its causes. An hypothesis is that its high level of polyploidy causes a failure in megasporogenesis (**Bhanwra et al., 1982**). According to Balogh and colleagues few cells display the archeosporial features of large nuclei and nucleoli, but these are confined to the hypodermis. None of the cells enters the microsporocyte stage and, although the ovule increases in size to resemble a seed, it does not develop to produce a female gametophyte and eventually the caryopsis (**Balogh et al., 2012**). Other studies indicated that the Italian Arundo has a normal megasporogenesis but the macrospore that remains vital (out of four) is the one close to the micropylar pole instead of the usual one at the chalazal pole. The macrospore enlarges and produces a large nucleolus, but the ovule fails to develop (**Mariani et al., 2010**). The same work also investigated the meiosis of the male gametophyte: the Italian *Arundo* apparently shows a normal meiosis, but then the pollen grains collapse and produce vacuoles indicating autolysis (**Mariani et al., 2010**). Another study showed that less than 10% of microsporocytes undergo meiosis and that, in the oldest anthers, there are several microspores but both the middle layer and the tapetum of the microsporangium in the anthers disintegrate (**Balogh et al., 2012**). A recent study supported the conclusions made by Mariani et al., 2010, though it shows that polyploidy is not necessarily the reason of the infertility in Arundo. In fact, other *Arundo* species (*plinii* and *donaciformis*) are fertile even if polyploids (**Hardion et al., 2015**).

Floral sterility represents an advantage for the economy of the plant since C (Carbon) and N (Nitrogen) are not allocated to seed filling and can hence be directed to vegetative growth. In addition floral sterility ensures plant

bioconfinement (**Ceotto et al., 2010**), restricting, even if not blocking invasiveness (see below). On the other side, floral sterility and lack of seed production, do not allow propagation by seed dispersal. Therefore alternative ways of multiplication are employed and namely: rhizome planting, stem cuttings and micro-propagation.

Rhizome propagation is the most traditional way to establish Arundo and it has been used for field studies in Southern and Central Italy (**Cosentino et al., 2006; Angelini et al., 2005**) but it is quite expensive, about $1.25 per plant (**Bezzi et al., 2006**), mostly due to rhizome collection and preparation which account for the 64% of the planting cost of Arundo (**Testa et al., 2016**). Although, one has to consider that sprouting is quite reliable using rhizomes, independently of the environmental conditions (**Decruyenaere and Holt 2001**), and hence, no extra care and related expenses are needed for subsequent cultivation. A less expensive alternative to rhizome planting is stem propagation. Stems cuttings regenerate with an efficiency up to 80% with a photoperiod of 16/8 hours at a temperature of 28/16 °C, but axillary buds integrity at the node is an essential prerequisite for the stem to regenerate. Also, a field study conducted in Northern Italy using samples collected in Central Italy showed that the best way to stem propagate Arundo is using shoot cuttings with lateral branches since this can improve uniformity and vigor of cane stand (**Ceotto et al., 2010**). Both rhizome and stem propagation are time consuming and labor intensive. Micro-propagation represents a valid alternative to these methods. Basically *in-vitro* plantlets are produced in a sterile environment in laboratory and then these are moved to the field . This approach is preferred also because it allows quick multiplication of interesting genotypes on a very large-scale (**Butt et al., 2015**). Cavallaro et al., for example, used sterilized axillary buds of Arundo to produce shoots, then they optimized shoot multiplication on solid and liquid media. Once the best condition was found,

shoots were used for field experiments. Micro-propagated plantlets showed better performances (about 1.3 fold) in term of dry biomass yield when compared to propagated rhizomes. This suggested that micro-propagation could be a better strategy for Arundo cultivation (**Cavallaro et al., 2011**).

1.3 Invasiveness

As abovementioned Arundo does not produce seeds, but, despite this, it shows a surprisingly high degree of invasiveness. It has received a weed risk assessment (WRA) score (**Pheloung et al., 1999**) of 4 "evaluate further" in 2005 in Florida (**Fox et al., 2005**) that basically indicated the need of answering more questions before introducing the plant in the target area. This evaluation was later reconsidered and a score of 8 "reject" (invasive species) was later granted (**Fox et 2007; Barney et al., 2008; Gordon et al., 2011**); this confirmed what previously reported by other authors (**Herrera et al., 2003**). The "reject" status is surely reinforced by the presence of Arundo in areas that are far and diverse from the native ones, but it is also due to the vigorous stem propagation in areas close to streams and rivers (**Virtue et al., 2010**) (**Figure 2**). Barney et al., in their discussion about the level of invasiveness of biofuel crops, described Arundo as a potential invasive biomass plant also because of its agronomic traits. These are: perenniality, rapid and high density growth rate, high aboveground biomass yield, drought and pests tolerance, tolerance to soil disturbance and reallocation of nutrients to perenniating structures (for further information see **Barney et al., 2008**). Some of these traits will be discussed later. Another analysis of Arundo invasiveness was conducted in California to explore its interaction with the wildlife of riparian areas, concluding that it puts in danger the arthropods that live in the area where Arundo is invasive, reducing their abundance and diversity (**Herrera et al., 2003**).

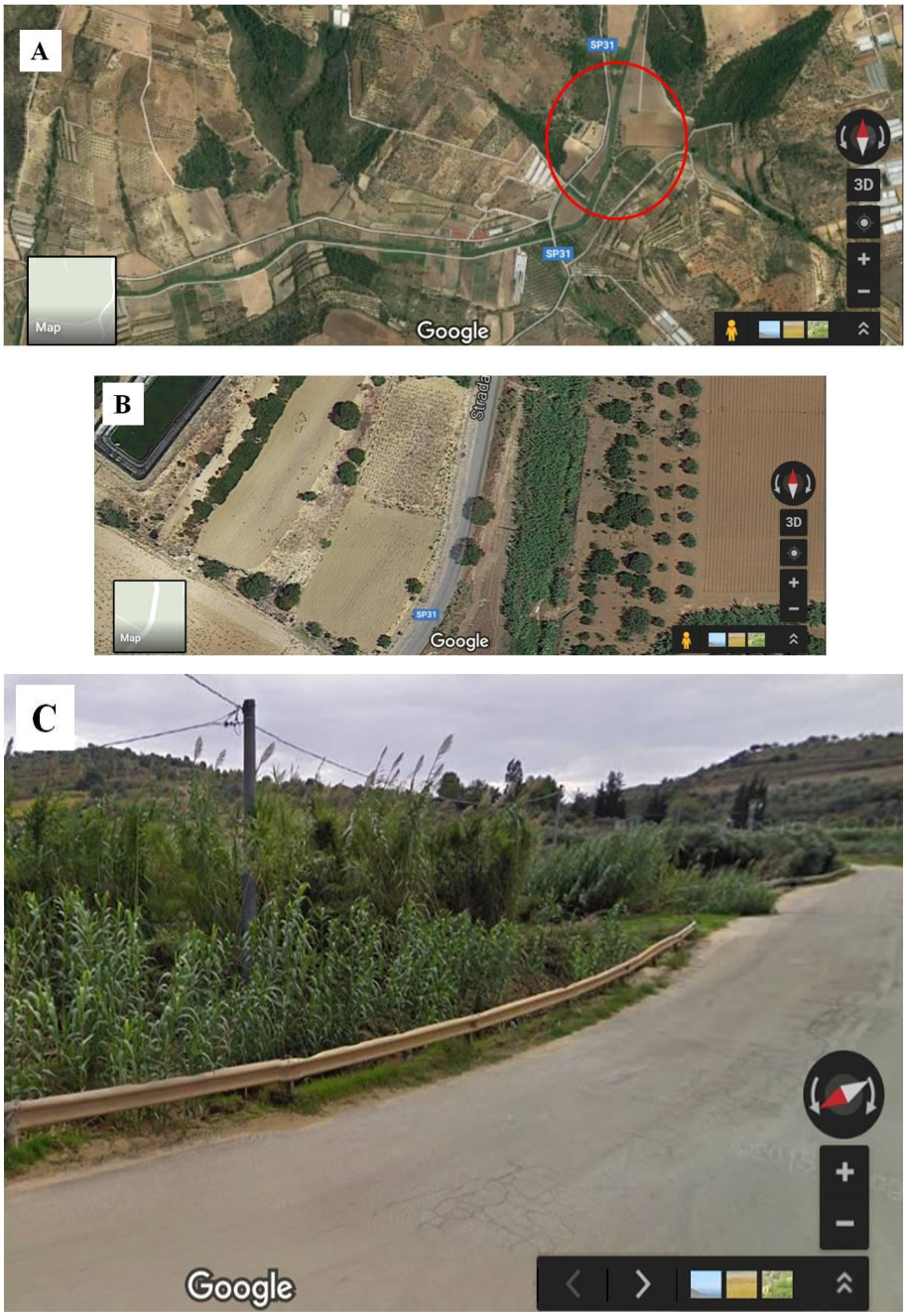

Figure 2: Arundo canes in Niscemi, Sicily (37.100629, 14.417700). Arundo colooy nized the bed of a dried river Visualization on Google maps **(A)**, zoom in **(B)**, picture took with Google view **(C)**.

1.4 Photosynthesis and Biomass yield

In a study conducted in Southern Portugal in unfertilized conditions (**Webster et al., 2016**), Arundo showed a high assimilation capacity in light-saturated, as well as light-limiting conditions. The former depends on high values of the maximum rates of carboxylation and of electron transport (these rates are twice the average values of other C3 plants and comparable to C4 plants), while, while the latter depends on a high efficiency in absorbing incident light and the maximum absolute quantum yield of CO_2 assimilation.

Similar studies on the photosynthetic ability of Arundo, found that the radiation-use efficiency (RUE) per photosynthetic active radiation (PAR) in Italy was greater than what observed for both *Miscanthus* and switchgrass (*Panicum virgatum*) in a similar study conducted in the US (**Ceotto et al., 2013**; **Heaton et al., 2009; Kiniry et al., 1999**).

All these studies indicated a high photosynthetic rate in Arundo that does not saturate at maximal natural photon density (**Webster et al., 2016; Rossa et al., 1998**). These high photosynthetic rates in Arundo are unaffected even by heavy metals (Cadmium and Nickel), in the soil (**Papazoglou et al., 2005**).

However, after reaching a very high RUE, Ceotto et al., (**2010**) in late spring, there is an unexpected decrease in the growth rate in subsequent months, called "summer slump". This term was used to describe the behavior of alfalfa at the end of the summer (**Sinclair and Randall, 1993**) but in Arundo the phenomenon is even more pronounced as shown by the sudden decrease of the RUE (**Figure 3**). Figure 4 shows that the biomass dry matter yields of Arundo are higher than sweet sorghum in all the three years of observation; however, in sweet sorghum the biomass production keeps increasing linearly until day 280 (at harvest). In Arundo, the biomass growth rate decreases and comes close to 0 at the end of the Summer (day 220-240). The reason of this behavior is unclear and one possibility could be a diversion of photosynthates to belowground

organs. However **Di Candilo and Ceotto, 2012** showed that the summer slump was detected for both above and belowground organs ruling out this possibility (**Ceotto et al., 2013**). Another interpretation is that there is some sort of regulation that slows Arundo vegetative growth (dependent on internal or external signals), independent of light availability.

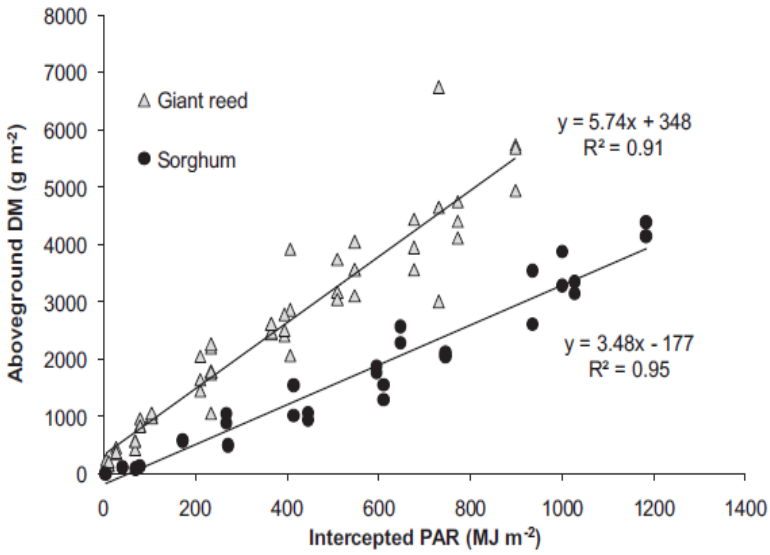

Figure 3: Relationships between accumulated shoot dry matter and intercepted PAR for giant reed and sweet sorghum. "The slope of regression lines (the RUE) is the estimated efficiency with which the crops convert intercepted PAR into aboveground biomass. For giant reed, data are limited to the first part of the growing season, until the first decade of August, because for the second part of the season no significant regression was found (data not shown). For sweet sorghum data for the entire growing season were included because the slope of regression is fairly constant until the end of the growing season". Original from Ceotto et al., (**2013**).

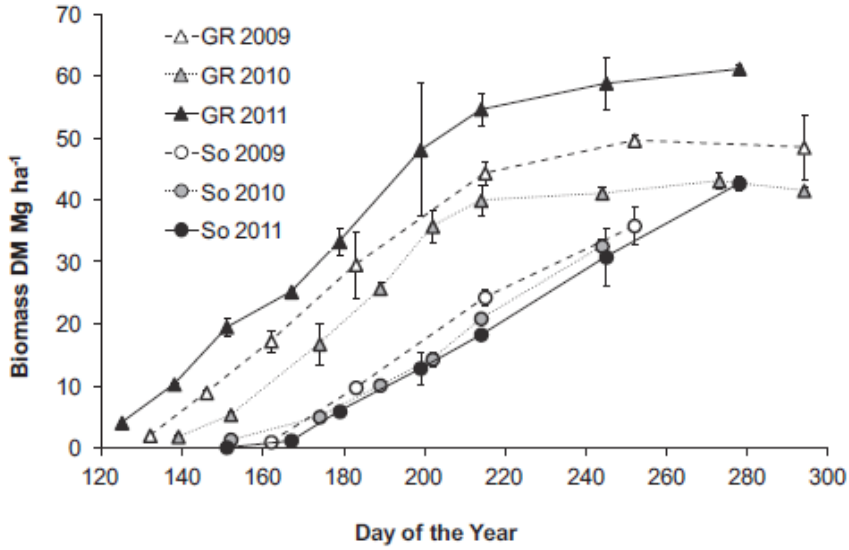

Figure 4. Total aboveground dry matter throughout the growing seasons. The graph shows the biomass yields expressed as Mg of Dry Matter per hectare in three years for Arundo/giant reed (GR) and sweet sorghum (So). Bars indicate ± standard error of the mean. Original from Ceotto et al., (2013)

One of the main reasons for choosing Arundo as biofuel crop is the remarkable high yield, despite it being a C_3 plant. Studies on yield of Arundo have been carried out worldwide, especially in the Mediterranean Basin, but most of the literature comes from Italy (**Ge et al., 2013**). In Central Italy, for example, Arundo displayed an average biomass production of 37.7 tDM ha $^{-1}$ year $^{-1}$ over 10 years of cultivation, a value 1.3 times higher than that obtained by Mischantus in the same work (**Angelini et al., 2009**). This value is higher than the one previously reported by Cosentino in a 2 years experiment (**Cosentino et al., 2006**). Interestingly, these studies demonstrated that during the second year of cultivation there is a yield increase of 50% compared to the year of the establishment (**Angelini et al., 2005**; **Foti e Cosentino 2001**). Most of the energy input is required during the 1st year of cultivation, mainly for rhizome sowing and adaptation, therefore it is not surprising that the yield of first year is

the lowest for the whole period (**Angelini et al., 2005**). The average energy output (determined as calorific value for dry yield) over 10 years of cultivation in unfertilized condition was 650 GJ/ha, 31% greater than *Miscanthus* (**Angelini et al., 2009**). It has been reported that Arundo accumulates high quantity of Nitrogen in unfertilized condition (**Ping et al., 2014**), meaning fertilization might not be necessary for cultivation, energy output and biomass yields are greater when fertilization is employed (**Angelini et al., 2005**; **Kering et al., 2012**). Biomass yield increases up to 15% and 40%, respectively in Italy and US, when cultivated for a period longer than 2 years (**Angelini et al., 2005**; **Kering et al., 2012**).

1.5 Biomass conversion in Energy

The ultimate aim in cultivating biomass crops is to convert them into energy forms that are easy to integrate into modern lifestyles (e.g., liquid fuels). A plant dry biomass is composed mainly of cellulose (30-50%), hemicelluloses (15-35%) and lignin (10-30%) (**da Costa Sousa et al., 2009**).The biomass-to-energy conversion efficiency mostly depends on the relative abundance of the cell wall components and their conformation (for further information see **Dyk and Pletschke, 2012**). In **table 1** Arundo biomass properties and composition are reported.

Properties	Giant reed
Bulk density (t m^{-3})	0.1
Moisture content (%)	35–52
Heating value (MJ kg^{-1})[a]	17–24
Ash (% DW)	3–8
Lignin (% DW)	8–34
Cellulose (% DW)	21–42
Hemicellulose (% DW)	7–23
Extractives (% DW)	19–22
Crude protein (% DW)	4–8
C (% DW)	42.8–49.4
N (% DW)	0.2–0.3
H (% DW)	6.0–6.4
O (% DW)	42.0–45.1

Table 1. Heating value and composition of harvestable above ground biomass of giant reed. The red rectangle highlights the main components of the lignocellulosic biomass (**Ge et al., 2013**).

There are two main routes to convert a plant biomass into energy: bioconversion and thermochemical conversion.

- Bioconversion. The conversion of lignocellulosic biomass into fermentable sugars, using cellulolytic and hemicellulolytic enzymes, and the subsequent fermentation into ethanol. The bioconversion requires a pretreatment, which improves the composition and reduces the recalcitrance to enzymatic hydrolysis. Therefore this step influences the specificity of the enzymes used for the biomass hydrolysis step (**Dyk and Pletschke, 2012**). Studies have shown that lignin represents the major limiting factor to biomass hydrolysis by the enzymes. A partial delignification can be more effective than total delignification (lignin content below 5% g/g) since total delignification could cause cellulose microfibrills aggregation and therefore decrease cellulose digestibility (**Ishizawa et al., 2009**). Also hemicellulose removal by solubilization, increases cellulose hydrolysis (**da Costa Sousa et al., 2009**).

Since the pretreatment is the most crucial step, studies focused mainly on this phase and explored different alternatives. These can be:

- Physical: as milling procedures;
- Solvent-based: use organic solvents, phosphoric acids or ionic liquids. (this latter is considered the most promising procedure).
- Chemical: include acidic treatments (diluted-acid, steam explosion etc), alkaline treatments (as the ammonia fiber expansion (AFEX)), oxidative treatments (alkali wet oxidation)
- -Biological: non-energy intensive processes that involve also the use of fungi to remove lignin from biomass.

(For a review on pretreatments, see **Da Costa Sousa et al., 2009**).

Some of the pretreatments successfully performed in Arundo are solvent-based (**Ge et al., 2011**) and chemical (**Scordia et al., 2013**, **Shatalov and Pereira, 2012**), mostly associated with milling procedures. At the highest concentration of Oxalic Acid (5%), with a Severity Factor (a factor that considers temperature and residence time during the pretreatment) of 4.05, the xylan content was reduced by 99% although the percent of xylan recovered was not as high (**Scordia et al., 2013**). While 94% of xylose recovery was obtained with 1.27% of sulfuric acid (36.4 min at 141.6 °C) and cellulose digestibility went from 9% to 70% (**Shatalov and Pereira, 2012)**. Enzymatic hydrolysis is usually performed using commercial mixes of glucanases, hemicellulase and glucosidases (**Scodia et al., 2013**). In literature two strategies have been explored in Arundo. The first one is Lignocellulose fractionation (CSLF) technology, involving a pretreatment with phosphoric acid, that at a concentration beyond the critical value (>83%) removes the hemicellulose and part of the lignin (**Zhu et al., 2009**), then a fast enzymatic hydrolysis of the cellulose, and finally glucose fermentation (**Ge et al., 2011**). The second

strategy involves simultaneous saccharification and fermentation (**Scordia et al., 2013**). There is also an hybrid version of these strategies, where 48 hrs of enzymatic hydrolysis precede a sacharification/fermentation simultaneous phase. The role of this extra enzymatic step is to increase the glucan conversion, although no substantial differences have been observed with this hybrid strategy in Arundo in comparison with a non-hybrid one (**Palmqvist et al., 2014**). The studies report higher percent of glucan conversion for the traditional approach compared to the synergistic one, although different experimental conditions apply. It went from about 50% with the synergistic (**Palmqvist et al., 2014**) to almost 95% with the traditional strategy **(Scordia et al., 2013).**

- Thermochemical conversion. It is a performed at high temperature and/or high pressure to produce one or more products: heat, syn-gases, bio-oil, and biochar (a solid residue). Pyrolysis, the main type of thermochemical conversion, uses high temperature but in the absence of oxygen and has gained more attention than the other procedures because it is more versatile and convert biomass into more products (see **Goyal et al., 2008** for further information). In Arundo the biochar was produced by pyrolysis at 500 and 800°C of stems pretreated with phosphoric acid. Biochar yields were higher at lower temperature, confirming previous reports (**Horne et al., 1996**), although the pretreatment step made Arundo biochar more suitable as activated carbons than for fuel purposes (**Basso et al., 2006**). A study has investigated the effect of temperature (350-650 °C), heating ra*te (10*-40 °C/min) and sweeping gas (N_2) flow rate (50-250 ml/min) during the pyrolysis of Arundo stems for the production of bio-char and bio-oil. The bio-oil produced in maximum-yield conditions had a heating value of 24.70 MJ/Kg while bi*o-char* produced was highly basic and therefore

unsuitable as fuel but as liming material for correcting soil acidity (**Saikia et al., 2015**).

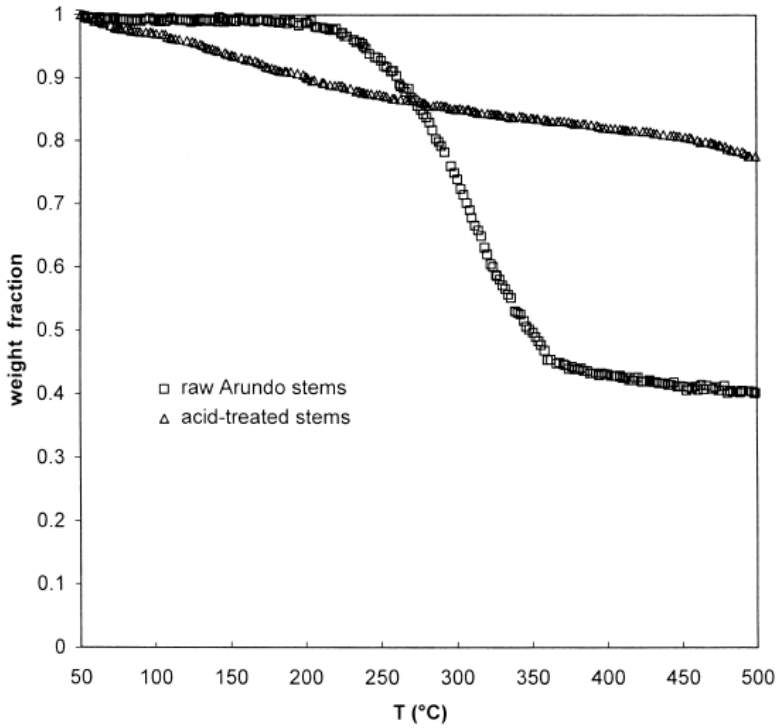

Figure 3. Thermogravimetric curves for pyrolysis of the raw and acid-treated Arundo stems under a N2 stream: weight fraction as a function of p*yrolys*is temperature (T). Original figure from Basso et al., (**2006**).

1.6 Biotechnological Approaches

As mentioned before, the sterility represents an advantage because limits the invasiveness of Arundo in non-riparian areas, but on the other hands prevents breed*i*ng. Alternative strategies are therefore mandatory for agronomic improvement, such as genetic transformation.

Although Arundo is a high performing biomass plant, some of its traits could be improved in order to make this plant the best choice for bioenergy production. Since it is sterile, flower development is dispensable in Arundo. Perhaps, re-

directing assimilates into a prolonged vegetative phase instead of producing reproductive organs could increase the biomass. One of the strategies to achieve this goal could be to delay the flowering by interfering with the internal clock. There is a complex mechanism behind flowering that reprograms the plant life style switching from vegetative growth to reproduction (**Komeda 2004**). This mechanism is regulated by several gene families in a complex network. A good example of a plant with an (almost) blocked floral transition is described by Komiya et al., (**2008**). They generated rice plants with a reduced or absent flowering by RNAi on the Hd3a (**Tamaki et al., 2007**) and RFT1 genes, also known as flowering locus-T like genes, responsible for the floral transition (**Komiya et al., 2009**). Similar results were achieved in rice by targeting the RID1 gene (**Wu et al., 2008**). A correlation between a delay in flowering and an increment of biomass was also shown in Miscanthus where a photoperiod greater than 12.5h was associated with a significant increase of biomass (**Jensen et al., 2013**). Delaying flowering does not always guarantee of a increase in biomass production. For instance in switchgrass the delaying the flowering implied a decrease in lignin content without affecting the biomass yield (**Xu et al., 2012**). Moreover, tuning the expression of plant hormones as giberellin, essential for many development processes in plant, could boost biomass production. For example the overexpression of GA (Giberellic Acid) 20-oxidase in hybrid aspen (Populus tremula × P. tremuloides) brought an increment of stem growth of about 30% in transgenic lines. The stems of these lines showed indeed a higher number of cells per internode, in comparison with the wildtype. The authors stated that the high content in giberellins induces mitotic activity in the subapical region of the stem, increasing cell division (**Eriksson et al., 2000**). Another strategy is to make the cell wall more accessible to physical and chemical pretreatments therefore increasing sugar release. In switchgrass, for example, the overexpression of Corngrass1 (Cg1), a

microRNA involved in promoting juvenile cell identity and morphology in corn, delayed flowering, but affected negatively the biomass yield. However it did increase cell wall digestibility and glucose release (**Chuck et al., 2011**). As described above, cell wall digestibility is strongly dependent on composition, hence, modifying the lignin content and structure, as demonstrated by the experiments performed with *Brachypodium distachyon*, could increase sugar release (**Marriott et al., 2014**). Perhaps it is possible to modify the cell wall by altering the lignin biosynthetic pathways. in *Arabidopsis* loss of function mutants of phenylpropanoid and monolignol pathway showed that those mutants with the highest reduction in lignin content had the highest cellulose conversion (**Van Acker et al., 2013**). Another way to improve cell wall digestibility is to express a gene that encodes for cell wall degrading enzyme as fungal ferulic acid esterase and b-1,4 endoxylanase in specific plant organelles (as apoplast or vacuole) and increasing the release of sugars and cell wall digestibility after harvesting (**Marcia et al., 2015**). Furthermore the expression of pectate lyase 1 (PL1) of *Pectobacterium carotovorum* under the control of a promoter expressed during the senescence, improves sacharification efficiency without affecting growth (**Tomassetti et al. 2015**). Also the expression of an inhibitor of the pectin methylesterase (PMEI) improves the saccharification efficiencyand provides the additional positive trait of microbial pathogen resistance (**Lionetti et al., 2011**)

None of these transgenic approaches were applied to Arundo because reliable methods for callus induction, stable transformation and plant regeneration are required. On the contrary to other biomass crops (see chapter 2 on switchgrass), few studies have been conducted on tissue culture in Arundo. Takahashi et al., (**2010**) is the only one describing callus induction and regeneration from axillary stem buds. This group investigated the effect of auxin (2,4-D) and

cytokinin (BAP) on callus induction and plant regeneration, on hormone free medium, of 10 genotypes collected in California (USA) and Japan. They identified a medium for callus induction that also predispose calli to regeneration and a suitable regeneration medium. Takahashi observed differences among the different genotypes and this is quite surprising considering the low genetic variability of Arundo, but also an important factor to consider when working with this plant. So far there is no protocol for stable genetic transformation, but just one study on transient genetic transformation of calli (**Dhir et al., 2010**) and another study by Takahashi (**2012**) where they reported the formation of hygromycin resistant calli, without regenerating any transgenic plant. In section II (**Pigna et al., 2016**) we propose a new protocol for transient transformation by particle gun of Arundo calli. The new method was partially derived from Takahashi et al., (**2012**). Agrotransformation has not been obtained so far (Dr Joshua Yuan personal communication) and thus highlight the recalcitrance to transformation and manipulation of Arundo.

A substitute method to traditional breeding is represented by protoplast fusion. This technique will be described later in the chapter 3 of the state of the art.

2 Switchgrass: the perennial grass from North America

2.1 The choice of switchgrass

At the end of the '70s, both the US and Europe started focusing on perennials grasses in their search for energy crops. This resulted in the choice of Arundo in the Mediterranean region, as discussed by **Lewandowski et al. (2003**). Thirty-five promising herbaceous species, 18 of which were perennials, were screened in different sites of the US under the Bioenergy Feedstock Development Program (BFDP) supported by the DOE (Department of Energy). This program started in 1978 at the Oak Ridge National Laboratory (ORNL) in Tennessee (**McLaughin et al., 1999**) (Figure 1) and when the screening ended in 1991, the perennial grass switchgrass (*Panicum virgatum* L.) was selected as the best performer (**Cherney et al., 1990, McLaughin et al., 1999**).

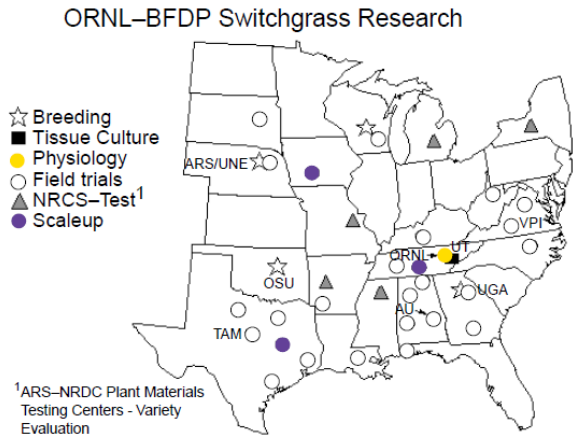

Figure 1. Map of research activities by location in the BFDP herbaceous energy crops research task in the US original from **McLaughin et al., 1999**).

English name	Latin name	Photo- synthetic pathway	Yields reported [t DM ha^{-1} a^{-1}][a]
Crested wheatgrass	*Agropyron desertorum* (Fisch ex Link) Schult.	C_3	16.3
Redtop	*Agrostis gigantea* Roth	C_3	Not available
Big bluestem	*Andropogon gerardii* Vitman	C_4	6.8–11.9
Smooth bromegrass	*Bromus inermis* Leyss.	C_3	3.3–6.7
Bermudagrass	*Cynodon dactylon* L.	C_4	1.0–1.9
Intermediate wheatgrass	*Elytrigia intermedia* [Host] Nevski	C_3	Not available
Tall wheatgrass	*Elytrigia pontica* [Podp.] Holub	C_3	Not available
Weeping lovegrass	*Eragrostis curvula* (Schrad.) Nees	C_4	6.8–13.7
Tall Fescue	*Festuca arundinacea* Schreb.	C_3	3.6–11.0
Switchgrass	*Panicum virgatum* L.	C_4	0.9–34.6
Western wheatgrass	*Pascopyrum smithii* (Rydb.) A. Love	C_3	Not available
Bahiagrass	*Paspalum notatum* Flugge	C_4	Not available
Napiergrass (elephant grass)	*Pennisetum purpureum* Schum	C_4	22.0–31.0
Reed canary grass	*Phalaris arundinacea* L.	C_3	1.6–12.2
Timothy	*Phleum pratense* L.	C_3	1.6-6.0
Energy cane	*Saccharum* spp.	C_4	32.5
Johnsongrass	*Sorghum halepense* (L.) Pers.	C_4	14.0–17.0
Eastern gammagrass	*Tripsacum dactyloides* (L.) L.	C_4	3.1–8.0

[a] t = Mg.

Table 1. 18 perennial grass species screened by the US herbaceous energy crops research program. Highlighted in light yellow switchgrass performance and characteristic (**Lewandowski et al., 2003**).

The rationale behind this choice was not only related to the highest biomass yield among the 35 tested species (**Table 1**), but also to many other favorable characteristics, which are hereafter summarized.

1) Switchgrass is native to North America and it is an ecological component of a wide variety of ecosystems from Canada to Mexico thanks to its ability to stand heat, cold and drought (**Casler et al., 2007**). Indeed it was already used as a forage since the ‘40s in the US (**Parrish et al.,2012**). It is able to adapt to poor-in-nutrients soils and it is amenable for cultivation through all the US territory (**Lewandowski et al., 2003**). This latter is a great advantage since it allows to unify the studies and production processes. However, considering the wide variation in photoperiod, climate, temperature,, rainfall, soil quality and so on, of the US territory, the idea of cultivating one and the same plant, even though with a high

environmental adaptability, in the entire area might not be a winning strategy. Perhaps it is the best choice only for the southern states of the US.

2) Conversely to Arundo, switchgrass allows traditional breeding and therefore offers the possibility to take advantage of the within-species genetic diversity to obtain yield improvement (**Lewandowski et al., 2003**). The use of EST-SSR molecular marker analysis showed a great genetic variability among switchgrass germplasm that represents a resource for domestication (**Narasimhamoorthy et al., 2008**).
3) Switchgrass can be easily included in traditional farming operations since, as already mentioned, it was already use as forage before it was rediscovered as an energy plant, hence the plant management can be assimilated to other hay plants.
4) It can be harvested only once a year without loss of biomass. This of course represents ad advantage in term of labor, money and time (**Turhollow report 1988**, **Lewandowski et al., 2003**).
5) Switchgrass can have a positive environmental impact. The conservation reserve program (CRP) was launched to reduce soil erosion caused by farming practices and particularly by tilling. Covering highly erodible cropland with perennial grasses is one strategy. Given the limited requirements for cultivation, switchgrass reduces, as expected, soil erosion by 95%. This is also due to the long root system that enhances soil preservation and carbon sequestration, improving soil quality compared to other grasses. Switchgrass improved also surface water quality and, last but not least, the conservation of grassland habitats (**Keshwani and Cheng, 2009**).

2.2 Origin and Genetic

Switchgrass belongs to the *Panicoideae* subfamily and its appearance is dated 2 MYA (**Parrish et al., 2012**). It is a cross-pollinated plant, mostly self-compatible, often tetraploid or hexaploid (**Lewandowski et al., 2003**). The analysis with SSR, studies of the physiology and morphology, and plastid DNA sequencing concluded that swichgrass can be subdivided into two groups or cytotypes: upland and lowland (**Moser et al., 1995, Parrish et al., 2012**). *Uplands* are often associated with the Northern Great Plains, usually finer stemmed, shorter, adapted to drier soils, tetra or octaploids (2n=8x=72). *Lowlands* belong to Southern USA, are more robust and resistant to rust, prefer wetter soils, grow in a bunch-type conformation, and are tetraploids (2n=4x=36) (**Lewandowski et al., 2003**, **Parrish et al., 2012**). The best performers in term of yields are the lowland type, especially two cultivars: "Alamo" (**Figure 2**) and "Kanlow" that are adapted to, respectively, deep South US and mid-latitudes. "Kave in Rock" is the best upland performer, well adapted to Northern US (**Lewandowski et al., 2003**).

Figure 2: Switchgrass lowland variety (Alamo) grown in greenhouse. **University** of Tennessee, Knoxville (TN), USA.

2.3 Physiology

Switchgrass is a tall perennial C4 grass, the stem reaching 3 m in length, while the root can reach 3.5 m. It is characterized by a big panicle (15-55 cm long) composed of many inflorescences with spikelets at the end. The spikelets are two-flowered: the first flower is fertile, the second sterile (**Lewandowski et al., 2003**). Leaf gas exchange measurements allow one to evaluate leaf physiological potential. Photosynthetic rates, as well as water use efficiency showed significant differences ($p<0.05$) among different genotypes. The first varied from 17.5 µmol m^{-2} s^{-1} to 30.8 µmol m^{-2} s^{-1}, the latter ranged from 2.08 to 3.77 µmol CO_2/mmol H_2O (**McLaughin et al., 1999**).

2.4 Ecological Demands

Switchgrass colonizes different soil types since is drought tolerant but tolerates wet areas as well (**Lewandowski et al., 2003**). Soil pH must be 5 or higher and P_2O_5 and K_2O concentration in soil determines if fertilization is needed or not

(**Wolf and Fiske, 2009**). It tolerates poor soils but does benefit from N fertilization. The amount of N fertilizer that needs to be applied depends on the quality of the soil, the cultivar potentiality and on field management (**Mitchell et al., 2008**). Seed dormancy is very common in switchgrass and can be broken by stratification (**Wolf and Fiske, 2009**). Generally switchgrass seeds germinate very slowly when soil temperature is below 15.5°C and seedlings generally appear after 3 days at 29 °C. Short days promote flowering,,however the inducing photoperiod can vary according to the ecotypes (**Lewandowski et al., 2003**). For instance one study showed that panicle production can be promoted in some lowland cultivars by exposing them to a photoperiod of 24 hours with low-irradiance of the daylight (**Castro et al., 2011**).

2.5 Biomass yield and quality

Switchgrass biomass yields can be very variable (0.9 to 34.6 t DM ha^{-1} a^{-1}). The highest yields, for small plots, have been reported for a lowland varieties in southern US reaching 36.7 Mg ha^{-1} yr^{-1}. Higher yields are possible (50 Mg ha^{-1} yr^{-1}), but require N fertilization (**Tubeileh et al., 2015**).

As already discussed the amount of cellulose, hemicelluloses and lignin in the cell wall influence the quality of the biomass and the conversion efficiency to ethanol. As for other morphological and physiological characteristics, there are differences in biomass composition among low and upland varieties. Lowlands have higher concentration of hemicelluloses compared to uplands, while cellulose content differs among cultivars but does not seem to be influenced by the morpho- or ecotype. The in vitro dry matter digestibility (IVDMD) is higher for lowlands indicating a lower lignin content and higher concentration of fermentable sugars compared to uplands. The same study found that the stemmy morphotype is preferable for energy use while the leafy morphotype has a proclivity for forage use (**Bhandari et al., 2014**). Therefore, beside the

ecotype, evaluating the morphotype could be important to determine the use of switchgrass for bioenergy production or forage consumption.

2.6 Bioethanol production

Theoretical bioethanol yields have been estimated to be 330–380 L of ethanol per Mg of dry switchgrass (**Keshwani and Cheng, 2009**) while Seepaul et al., (**2016**) reported that the greatest total ethanol yield was recorded in Alamo (165.8 L·Mg−1) and averaged 162.0 $L \cdot Mg^{-1}$ for other three tested genotypes (NF/GA001, NF/GA992 and Cave in rock) in an experiment conducted between 2006 and 2009. All switchgrass studies have reported that the amount of energy from ethanol produced from switchgrass biomass far exceeds the energy consumed for its production (**Wang et al., 2001**, **Pimentel and Patzek, 2005**) and specifically the energy produced with switchgrass is 540% greater than the energy spent for its production (**Schmer et al., 2007**).

Studies have investigate whether the ethanol produced from switchgrass could lead to production of greenhouse gases (GHG) and other pollutants such as volatile organic compounds (VOC) and nitrogen oxides (NO_x) during its life cycle (production, transportation, conversion of biomass into biofuel, transportation of the biofuel, and use). The average GHG emissions from the life cycle of ethanol produced from switchgrass were reported to be 94% lower than what estimated for the gasoline life cycle (**Schmer et al., 2007**). However, many studies have also explored the contribution of N fertilizer use for switchgrass cultivation. Fertilization above 56 kg N ha^{-1} indeed does not increase the production of biomass but increases the production of pollutants as GHG and this is of course undesirable. The great impact of fertilization on pollutants production is due to the fact that fertilizers represent a significant portion of the emissions for the switchgrass to ethanol conversion (**Mbonimpa et al., 2016**). Moreover, besides the amount of N fertilizer and the yield of

biomass, the quality of soil and the topography of the plots used for cultivation influence the gas emissions.

2.7 Biotech Approaches for Biomass Improvement

Being switchgrass a crop dedicated mainly to energy production, the efforts of biotechnologists have been directed to improve the biomass yield or its quality. Conversely to Arundo, swichgrass is endowed with all the tools and technologies for tissue culture production (**Gupta and Conger, 1999**, **Burris et al., 2009**, **Xu et al., 2012**) and stable transformation (**Li and Qu et al., 2011**, **Mann et al., 2011**). Modification of the cell wall is the main target to improve biomass quality and conversion efficiency. Most of the studies have concentrated the attention on lignin reduction and/or modification. One approach is to interfere directly with lignin biosynthesis. For example the 4-Coumarate:coenzyme A ligase (4CL) (**Xu et al., 2012**) or cinnamyl alcohol dehydrogenase (CAD) (**Fu et al., 2011**) are both involved in the monolignol biosynthesis. The downregulation by RNAi of switchgrass *Pv*4CL1, reduced extractable 4CL activity by 80%, leading to a reduction of lignin content of 22% in comparison with the WT, but leaving biomass yield uncompromised (**Xu et al., 2012**). Similarly, the reduction by RNAi of CAD, reduced lignin content and increased significantly sugar content and release, traits which are useful also when the plant is employed as forage (**Fu et al., 2011**).Another way to reduce lignin biosynthesis is to interfere with the gibberellins pathway. Gibberellins (or gibberellic acids, GA) have been implicated in lignin deposition in eudicots (**Biemelt et al., 2004**; **Zhao et al., 2010**). Gibberellin 2-oxidase (GA2ox) genes code for a group of 2-oxoglutarate-dependent dioxygenases that deactivate bioactive GA or its precursors. The overexpression of two members of this group *Pv*GA2ox5 and *Pv*GA2ox9 led to dwarf plants with more tillers but less lignin (**Wuddineh et al., 2015**).

Other studies in switchgrass focused on other aspects of the cell wall, like promoting the juvenile cell identity and morphology (**Chuck et al., 2011)** or promoting the elongation of the vegetative phase. The overexpression of the *Arabidopsis LONG VEGETATIVE PHASE ONE* (*AtLOV1*) in switchgrass led to an altered architecture of the plant, as well as altered lignin content and the monolignol composition of cell walls, and caused delayed flowering (**Xu et al., 2012**).

2.8 Switchgrass in Europe

Switchgrass was known in Europe for many years only as an ornamental grass. However since 1988, more than 20 varieties have been tested in small field trials (up to 2.5 ha) (**Elbersen et al., 2001**) within a EU-funded project. Switchgrass was selected as a promising bioenergy crop with great adaptation to different environments and a broad range of varieties because of the US studies (**Lewandowski et al., 2003**).

An Italian study explored the differences in cultivation between lowland and upland varieties and confirmed the US results. Lowland varieties produced a higher total dry matter (on average 14.9 and 11.7 Mg/ha, respectively). This study also concluded that two-cut per year produce more biomass than a single cut during the first 2 years of cultivation, but it reduces plant vigour and productivity in the following years (**Monti et al., 2008**).

The economic and environmental performance of switchgrass and Miscanthus have been analyzed in 5 countries of the EU25 (Poland, Hungary, UK, Italy and Lithuania) in an effort to lower GHG emissions by 2030 as demanded by the EU. With this long term view, the stability of these costs have also been explored. The cost of producing, storing and transporting Miscanthus biomass was lower than switchgrass and in particular in Poland, followed by Hungary and Lithuania. This study showed also that the cost of Miscanthus is expected

to decrease, while the cost of switchgrass to be stable in the next years (**Smeets et al., 2009**).

These studies suggest that switchgrass is probably not the best choice as an energy crop in Europe, and therefore other plants such as Mischanthus or Arundo are better candidates to produce bioenergy.

3 Protoplast: a new trend in plant cell biology

3.1 Protoplast as model system

Plant protoplasts have been used as a model system to study a variety of biochemical and genetic phenomena. One of the first protoplast isolation experiments was performed on root tips of tomato seedlings in the '60s. Vacuolated (**Figure 1A**) and non vacuolated protoplasts were indeed isolated from the most undifferentiated root zone of tomato seedlings using a cellulase isolated from *Myrothecium verrucaria* (**Cocking 1960**). Further studies achieved protoplasts regeneration in different monocot and dicot plants (**Figure 1B**) (**Takebe et al., 1971**). Later on, it was recognized the potentiality of protoplasts and a great number of studies started focusing on their genetic manipulation (**Hauptmann et al., 1987**). In the last 10 years, protoplasts have become popular again thanks to the introduction of technologies such as genome-editing and gene silencing (**Cao et al., 2014**).

Protoplast isolation and manipulation are well suited material for genome editing protocols thanks to their many advantages. They are easy to isolate, potentially totipotent, and therefore, theoretically able to eventually regenerate a plant (**Atanassov et al., 1984**). Transformation is simple, screening is easy and fast and does not necessarily imply the production of transgenic plants (**Burris et al., 2009**). Moreover, when transformed protoplast are regenerated, the production of chimeras is much reduced since the transformation occurs on single cells and hence every callus is homogeneous, as the regenerated plant derived from it. Another advantage is the easy visualization of fluorescent proteins (**Figure 1C**), which allows the study of basic plant processes. In maize, for instance, nucellus protoplast were used to show that MADS29 transcription factor could enhance the promoter activity of the Cys-protease, a key enzyme in

the programmed cell death (PCD) process (**Chen et al., 2015, Solomon et al, 1999**). Plant protoplasts facilitate subcellular localization studies, e.g. the Arabidopsis K+ channels and KAT2 in tobacco (**Nieves-Cordones et al., 2014**). Protoplasts are ideal for large scale studies as the analysis of the protein interactome such as, for instance, the work performed in rice (**Fujikawa et al. 2014**). In some cases, promoter screening are needed in the absence of genomic information before a stable transformation is attempted. **Nakashima et al.** (**2014**) and Pigna et al. (**2016**) showed how useful protoplasts are for preliminary studies in two monocots, rice and Arundo, respectively, especially for recalcitrant species.

Several factors that influence protoplasts yield must be considered prior to their isolation: the type and the age of the explants, the enzymes type and concentration, and the temperature used during the extraction. Several authors showed that protoplast yield can be effected by the choice of the plant material used for the extraction. In white lupin (*Lupinus albus*) and switchgrass for example, different sources of plant material have been compared for protoplast extraction: cotyledons, leaves, hypocotyls and roots (**Sinha et al., 2003, Mazarei et al., 2008**). These studies showed that better yields are obtained when protoplasts are isolated from leaves and cotyledons instead of roots and hypocotyls, even when different genotypes of the same species are compared. Another source for protoplast are suspension cell cultures. This source has the advantage of being aseptic, continuously sub cultured and easy to scale up or down according to the user's needs (**Figure 1D**). This is particularly helpful in sterile plants like Arundo where it is difficult to generate continuously new material. Another important factor to consider is the age of the explants. Burris and collaborators demonstrated that the age of both leaves and suspension cell culture influences protoplast yield in switchgrass (**Burris et al., 2016**). As expected, the younger the tissue, the higher is protoplasts yield. The enzymes

employed and their concentration are also important aspects to consider. The choice of the enzymes is obviously linked to the cell wall composition and therefore its digestibility. For example, the type of cellulase used to isolate protoplast from switchgrass, depends on the starting material: cellulase R-10 and RS are used for leaves and suspension cell culture, respectively (**Mazarei et al., 2008, Mazarei et al., 2011**). Other studies show the importance of enzyme types and their ratio/combination (Macerozymes and Pectolyase Y23 combined to Cellulase) in white lupin leaves and in *Gracilaria changii* thallus (**Sinha et al., 2003, Yeong et al., 2007**).

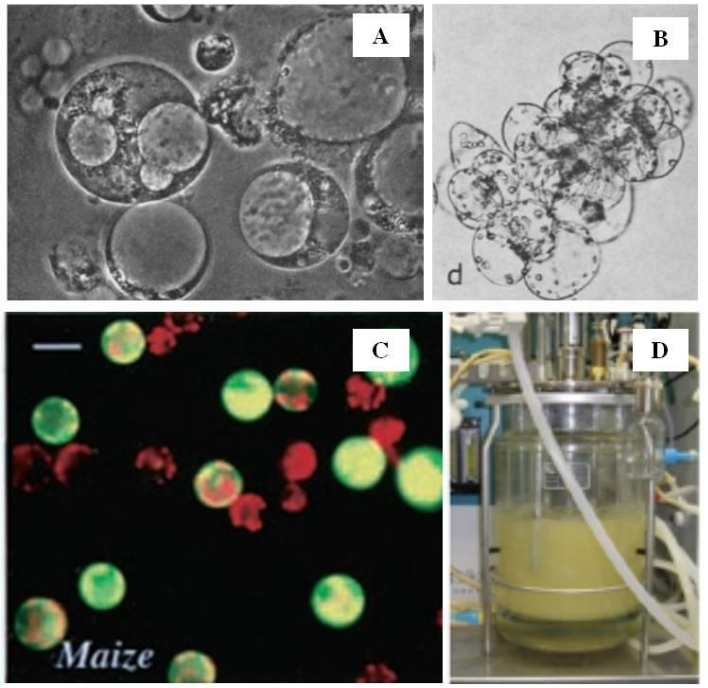

Figure 1: Early studies on plant protoplasts. (**A**) Vacuolated protoplast isolated from root tips of tomato seedlings (**Cocking 1960**). (**B**) Rice protoplasts forming a callus in liquid medium (**Takebe et al., 1971**). (**C**) GFP expressing protoplasts obtained with PEG-mediated transformation in Maize, scale bar = 25 µm (**Sheen et al., 2001**) (**D**) Tobacco BY-2 cells cultivation in a 5L fermenter before being harvested for protoplast isolation (**Buntru et al., 2014**).

The following sections in this chapter focus on protoplasts in monocots.

3.2 Protoplast Transformation in Monocot Plants

Protoplast transformation is a valid alternative to *Agrobacterium* and biolistic transformation of cells and calli. *Agrobacterium* mediated transformation has often low efficiency (**Rakoczy-Trojanowska, 2002**), sometime unreliable and dependent on the genotype and callus age, especially in monocots (**Burris et al., 2009**, **Li & Qu, 2011**). The biolistic method has the disadvantage of resulting in multiple inserts, which can lead to cosuppression or modified expression, beyond requiring an expensive instrumentation. The prevailing techniques used for transient and stable protoplasts transformation employ electroporation and Polyethylene glycole (PEG).

Protoplast electroporation was performed for the first time in maize by Fromm et al. (**1985**) who subsequently obtained kanamycin resistant protoplasts (**Fromm et al., 1986**). After these breakthroughs, electroporation was rapidly applied to other monocot plants, e.g. rice, that were considered quite recalcitrant, allowing the generation of transgenic plants (**Zhang et al., 1988**, **Tada et al., 1990**).

This technique has been abandoned in the following years, beside few exceptions **(Mohanty et al., 2000**, **Rakotondrafara et al., 2007**), and it has been replaced by PEG-mediated transformation.

PEG-mediated transformation was used to stably transform cereal (*Triticum monococcum*) protoplasts. With this method, kanamycin resistant protoplasts and successively microcalli were obtained (**Lorz et al., 1985**). This technique seems to be more versatile than electroporation and surely cheaper since it does not need a dedicated instrument (gene-pulser), but only PEG and a swinging-bucket centrifuge. This is probably the reason why it is still very popular especially among people working on monocot species. In rice, for example, it

has been used to perform gene-silencing by siRNA for the characterization of a large number of genes (**Bart et al., 2006**). PEG-mediated transformation was used in maize to introduce dsRNA that reduced the expression of the mitogen-activated protein kinase encoded by the ZmMPK5 gene (**Cao et al., 2014**).

3.3 Plant Regeneration from Protoplast in Monocot Plants

The biggest issue one may encounter when working with protoplast is plant regeneration, especially in recalcitrant plants such as many monocots. Many tried to overcome this problem and several approaches are now used to regenerate plants from protoplasts. One major aspect that has to be taken into consideration in the first growth phases after protoplast isolation is medium osmolarity. Unlike cells, protoplasts are not protected by the cell wall and hence are very susceptible to salts and sugars concentration. Their viability is strictly dependent on the osmolarity of the solution in which they are resuspended and this is therefore the first constraint to consider when discussing protoplast regeneration. For example, in maize, it has been reported that an osmolarity of 550 mOs enhances protoplast viability during isolation, electroporation and cultivation (**Huang et al., 1989**). A patch-clamp study on maize protoplast confirmed that to prevent membrane damage, the same osmolarity (510mOs) is required in all protoplast solutions (**Blom-Zandstra et al., 1995**). One popular strategy for regeneration is embedding the protoplasts in in gel matrices (e.g. agarose). A low percent (generally from 0.6 to 1%) agarose concentration protects “naked” cells before cell wall regeneration. Also, this procedure allows the monitoring of single or small groups of protoplasts over time. This technique was used for plant regeneration of rice protoplast after PEG transformation (with *Agrobacterium* spheroplasts and bacterial β-glucuronidase) (**Baba et al., 1986**, **Zhang & Wu 1988**). In maize it was used to generate sterile plants after performing somatic hybridization (**Szarka et al.,**

2002), in barley fertile transgenic plants were regenerated from mechanically isolated protoplasts (**Holm et al., 1994**, **Nobre et al., 2000**).

Another method applied to diverse dicot plants (*Catharanthus roseus*, Baily alicant, tobacco, Uchimiya and Murashige 1974)), but also to rice, combined protoplast isolation and immobilization (**Figure 2**). In this method, cells first undergo a degassing treatment using a vacuum pump and in the meantime they are incubated with protoplast isolation enzyme, then they are mixed with a sodium alginate solution and dropped into a $SrCl_2$ solution 30 mM containing the hydrolytic enzymes using a peristaltic pump. The beads are incubated in the same solution for 1h at 25°C and mixed with a magnetic stirrer. In this way protoplasts extraction and gel stabilization occur almost together. The degassing phase allows faster and efficient enzyme penetration into plant cells aggregate since it is considered that air bubbles reduce the enzymes transfer rate (**Uchimiya and Murashige, 1974**) The viability of the immobilized protoplasts is higher compared to the conventional method and, moreover, cell wall regeneration was about 70% after 10 days from isolation/ immobilization (**Aoyagi & Tanaka, 1999**).

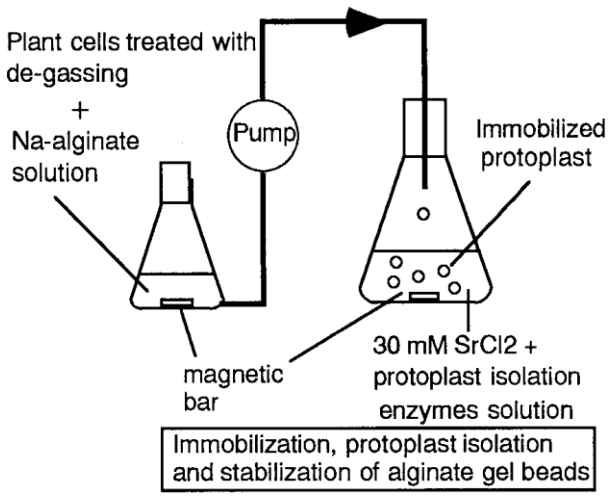

Figure 2: Schematic diagram of the method for immobilizing plant protoplasts in alginate gel beads. (**Aoyagi & Tanaka, 1999**).

An alternative method for protoplast regeneration not involving immobilization, envisages the addition of an artificial oxygen carrier (commercial hemoglobin) to stimulate mitotic division of rice protoplasts derived from cell suspension. The authors stated that a concentration 1:200 (v:v) of hemoglobin significantly improves microcalli formation and shoot formation (**Al-Forkan et al., 2001**).

3.4 Protoplast Cryopreservation

In recent years, the increased effort to study and/or modify plant cells and protoplasts has stressed the need for long term storage of living materials. Cryopreservation is a way to conserve living biological material at low temperature and, as such, it allows potentially indefinite storage of plant cells and protoplasts with low cost, minimal effort, and small space requirement. The two most common procedures for cryopreservation are slow-freezing and vitrification.

The slow-freezing technique consists in a slow cooling step to a temperature of - 40 °C and a quick step of further cooling in liquid nitrogen. The first phase is a dehydration step induced by water crystallization, which is confined outside of the cells, while the rest stays liquid. The concentration of sugars, salts and cryoprotectant (e.g. glycerol) increases in the liquid phase, because its volume decreases. During the second phase, as cooling continues, the viscosity of the unfrozen fraction (cells) is too high for any further ice crystallization. Thus cell contents solidifies (vitrifies) without ice crystal formation (**Meryman & Williams 1985**). The slow-freezing procedure has the advantage of cooling the protoplast slowly and, as a consequence, they are less damaged by the freezing process. On the other hand, the technique is time consuming. Direct vitrification is more commonly used to cryopreserve protoplasts. They are first mixed with a highly concentrated solution of cryoprotectants at non-freezing temperature that causes dehydration (loading step), then they are immersed in liquid nitrogen to allow vitrification to occur (**Steponkus et al. 1992**). When protoplast are thawed, they are immersed in a so called unloading solutions (osmotic solutions) to prevent cell breakage (unloading step). At sufficiently low temperatures, these cryoprotectant solutions are so viscous that protoplasts solidify without ice formation (**Rall and Fahy, 1985**). This procedure is faster than the slow-freezing and easier to perform, since it does not require specific devices for slow cooling, but, on the other hand, the high concentration of the cryoprotectant could be toxic or even lethal to some plant protoplasts. As mentioned before, plant protoplasts are crucially influenced by osmotic changes, and therefore they are very susceptible to changes that take place during cryopreservation. Langis (**1989**) stated that the reduction of osmotic stress and in particular the osmotic excursion during the loading/unloading phases could increase protoplast viability by 50% in rye (*Secale cereale*). It is worth mentioning that protoplast loss during the different steps of every

protocol lowers the total number of protoplasts and the percentage of living ones.

The percent of recovered protoplast is usually calculated as follows:

$$\frac{\text{[total protoplasts (after cryopreservation) * \% alive protoplasts (after cryopreservation)]}}{\text{[total protoplasts (initial) * \% alive protoplasts (initial)]}}$$

The number of total protoplasts surviving cryopreservation could also be increased by avoiding losses due to other causes. To this end, bovine or fetal serum albumin (BSA or FSA) and other animal proteins are usually applied to cryotubes to reduce the electrostatic interactions between protoplasts and the tube surface (**Langis et al., 1989**), limiting protoplasts loss. As expected, regeneration of cryopreserved protoplasts takes place with a greater lag in comparison with untreated protoplasts, as showed in the red alga *Porphyra yezoensis.* Cryopreserved protoplasts regenerated with a delay of 3 days compared to non-cryopreserved protoplasts. The authors speculate that this may be due to injuries caused by the low temperature (**Liu et al., 2004**).

3.5 Protoplast Fusion

Conventional breeding is the traditional way to improve agronomic traits of crops by crossing individuals within species or genus and selecting the best genotypic combinations in the progeny. Although very common, this depends on sexual reproduction and seed production, which are impossible for Arundo.

One way to circumvent the lack of sexual recombination is to perform somatic hybridization by protoplast fusion (**Mishra et al., 2015**). This technique is also widely used with fertile plants with a great economical relevance like rice, barley and wheat (**Wang et al., 2013; Xia et al., 2003; Kisaka et al., 1998**). The methods are similar to those used for genetic transformation since consist in the application of an electric field (electrofusion) or a chemical compounds,

as PEG, to fuse protoplasts of different or same species. In this way several intra genus hybrids were developed (**Sundberg and Glimelius et al., 1986**;), for instance between rice and barley (**Kisaka et al., 1998**) or between wheat and a forage grass (*Agropyron elongatum*) (**Xia et al., 2003)**. Asymmetric hybrid between monocot (rice) and dicot (carrot) plants have also been generated (**Kisaka et al., 1994**). In all cases the analysis of mithocondrial and chloroplastic DNA was used to confirm the hybrid nature of the resulting protoplasts. Most regenerated hybrid plants showed an intermediate phenotype between parents and genomic DNA analysis yielded bands specific for both parents, denoting nuclear genome fusion (Kisaka et al., 1998). Recombination between parental genomes (e.g. carrot and rice) could occur both at mitochondrial and nuclear genome levels, as shown by the novel mitochondrial sequence rearrangements detected in the hybrids that were absent in either of the parents (**Xia et al., 2003**; **Kisaka et al., 1994**).

Aim of the project

Arundo is sterile and, as such, does not produce viable seeds (**Hardion et al., 2012, 2014)**; as a consequence propagation through seeds as well as traditional breeding are not possible. Cultivation in greenhouse is also discouraged given the height of this plant (**Perdue 1958**), implying limited availability os fresh material throughout the year. The lack of seed is obviated by vegetative multiplication using culm or rhizome fragments or micropropagated plantlets. However, the lack of sexual reproduction is a serious impediment to plant improvement, which might be obviated by genetic transformation ans/or genome editing and the regeneration of whole plants. One of the aims of this project was therefore the development of a faster and efficient method for *in vitro* cultivation of Arundo, a prerequisite for both transformation and micropropagation. Just one work (**Takahashi et al., 2010**) showed callus induction from axillary stem buds, callus propagation and regeneration in Arundo but this system was unsuitable for the genotype employed ('Golden chain'), since calli grew very slowly on the solid medium described. We wanted to develop a system for rapid callus propagation that could be useful for further work (e.g. somatic embryogenesis and adventitious shoots production) of Arundo. Most importantly, another aim of this study was to develop a protocol for genetic transformation of Arundo. Again, only one work reported transformation by particle bombardment of calli (**Dhir et al.,2010**), but this system, besides requiring a specific equipment in order to be performed, also needs a good, fast and continuous production of calli that, as mentioned above, constitutes a constraint for Arundo. An alternative route is protoplasts transformation, advantages are that they are simple to obtain (from leaves or cell culture), to transform and to screen compared to methods involving the use

of calli (**Burris et al., 2016**). Protoplasts have already been used as plant cell material for gene silencing (**Cao et al., 2014**) or tuning gene expression (**Bart et al., 2006**). The transformation of Arundo protoplasts represents a way to introduce new traits and hence improve the characteristics of this plant. Obviously the generation of transgenic plants will still require the regeneration of plants from transformed protoplasts, which has not been achieved do far. A third aim was also to develop a system to cryopreserve protoplasts, in order to have available material for transformation all the year round, without the need for a constant supply of plant organs. This last aim was done on switchgrass, as the receiving laboratory had experience and protocols ready-to-use with this plant, while the other experimental work on Arundo described above was performed in parallel. Once that all the puzzle pieces will be available for Arundo, it should be possible to progress with its transformation and regeneration.

Main Results

Previous work on tissue culture of *Arundo donax* used solid callus induction medium (CIM) for callus induction and maintenance. Unfortunately, the callus derived from the Arundo genotype available in the US ('Golden chain') grew on this medium is relatively slowly, making it difficult and time consuming to generate enough tissue for further experiments. As it was not possible to get permission to use an original Arundo ecotype from Northern Italy due to the restriction of the US Department of Agricolture (USDA) to import alien vegetable material in the US, in order to solve this issue, we looked for an alternative way to induce callus from Arundo and cultivate it *in vitro*. We decided to established a suspension cell culture of Arundo from calli. At first we screened 4 liquid media and analyzed the growth of the suspension cell culture in each medium. After the suspension culture was filtered, growth was followed by measuring the packed cell volume (PCV) over the course of 21 days. The media used for screening are: CIM, MSDP, DBAP and B5G that differ for the basal salts and vitamins, the type of hormones and aminoacids used. Despite the fact that the original callus was grown on solid CIM medium, the liquid CIM version resulted in the poorest growth among the tested media. Over the first 7 days, the PCV of liquid CIM was below the limit of detection, and reached a measurable level only at day 10. Similar to CIM, suspension cultures initiated in MSDP medium showed no detectable growth over the first 10 days of culture, but reached a measurable level at day 14. Significant growth was observed in the B5G and DBAP media at the first time point, with the cultures reaching their peak density at 7-10 days and 10-14 days respectively. After 10 days in culture, the PCV of the suspension cultures in DBAP had increased 3.3-fold, while the B5G culture increased 2.8-fold compared with the

initial amount. Furthermore, comparison of the PCV at day 10 (the maximum for both DBAP and B5G) across all of the media showed a significant difference between DBAP and B5G compared to the other two media tested. While both DBAP and B5G were suitable for initiation and maintenance of Arundo suspension cultures, DBAP was chosen as the media for future experiments due to its higher PCV at 10 days of culture.Prior to establishment of the cultures, the majority of the cells in DBAP were kidney-shaped, with starch rich plastids, and evident nuclei. After establishment, the suspension cultures mainly consisted of cells devoid of vacuoles, with an elongated morphology. In addition, the established suspension cultures were a mix of individual embryogenic-like cells of various size classes. Once large (1-5 mm diameter) clusters were observable in the culture flasks, the cultures were considered suitable for further experiments. We compared the amount of tissue produced from either suspension cells or callus over the course of 21 days and we found that the suspension cell culture produces 2.5 times more tissue than callus propagation on solid medium. In addition, the established suspension cultures produce a maximum of 4.7 times the initial mass in 21 days. Based on these results, the established suspension cultures were used for the production of protoplasts and transformation. Protoplasts of Arundo were isolated from 4-7 days old suspension cell cultures, with an average of $3.8\times10^5 \pm 2.5\times10^5$ protoplasts/ml of PCV. Morphologically, the protoplasts ranged in size from 4.9 to 53.4 µm in diameter with an average size of 20.7±12.3 µm. The majority of the protoplasts ranged from 4 to 20 µm in diameter. Transformation of the isolated protoplasts was attempted using both PEG and electroporation. Initial transformation experiments were conducted using the pANIC10A-GFPstuffer, which expresses an improved RFP version (*pporRFP*) driven by the switchgrass ubiquitin promoter, *PvUbi1+3*. All attempts to transform Arundo protoplasts with PEG (varying DNA quantity, $MgCl_2$ concentration and PEG

concentration) failed, despite the switchgrass controls produced the expected signal. However, electroporation at 130 V and 1000 µF achieved a transformation efficiency of 3.3±1.5%. When the voltage was increased to 300 V, with the capacitance remaining the same, there was a decrease in protoplast viability from 67.7±3.0% to 11.9±4.5% and no transformation was detected. When the capacitance was decreased to 500 µF, with voltages of 130 and 300 V, the transformation efficiency was reduced to 1.0±0.2 and 0.3±0.4%, respectively. In these treatments, the viability remained high (82±1.5% at 130 V and 80±2.4% at 300 V), despite the lower transformation efficiency. Not surprisingly, the switchgrass *PvUbi2* promoter yielded higher marker gene expression than the *35S* promoter in Arundo protoplasts using the pTD-*PvUbi2*-*35S* binary vector. As expected, tobacco BY-2 protoplasts, used as a control, showed high expression of the mGFP5-ER fluorescent marker driven by the 35S promoter, but only weak expression of the pporRFP fluorescent marker driven by PvUbi2.

Conclusions and Future Prospective

Despite the significant growth of the suspension cultures of Arundo in this work, it was still low compared to cultures of other biofuel crops like sugarcane (*Saccharum officinarum*) (**Ho and Vasil, 1983**) or switchgrass (**Gupta et al., 1999**). This suggests that further optimization of the culture media may be possible for Arundo, as other bioenergy crops were studied more extensively in this direction. Also, the adaptability to *in vitro* manipulation of several plant species is strongly genotype-dependent (e.g.: **Tomita et al., 2013; Gurel et al., 2008; Hoque et al., 2004; Duncan et al., 1985**), therefore more efforts are needed to screen different accessions and identify genotypes more amenable to *in vitro* cultivation. One characteristic differentiating Arundo from other bioenergy crops is the lack of sexual reproduction, and therefore, seed production (**Hardion 2012, Hardion 2015**). As such, Arundo is not amenable to standard breeding practices. The lack of seed production, however, is an advantageous trait when considering bioconfinement and restriction of gene flow (**Barney et al., 2008**). In either case, genetic improvement of Arundo requires a reliable method for transformation to introduce novel genes, or inhibit/overexpress native genes. An effective transformation method is also essential for testing the strength and the suitability of heterologous promoters for gene expression, gene silencing or genome-editing, and general cell and molecular assays. Considering that only some RNA sequencing (**Fu et al., 2016; Barrero et al., 2015**) and no genome information is currently available for Arundo, the tools developed in this work should facilitate screening at early stages of useful sequences (e.g. promoters signal sequences), prior to the developing of transgenic plants. Currently, transformation of Arundo callus has only been achieved in one work with particle bombardment (**Dhir et al., 2010**).

As an alternative, PEG-mediated transformation and electroporation of Arundo protoplasts was evaluated in this work. High molecular weight PEG solutions have proven toxic to protoplasts isolated from some plant species (**Kao et al., 1986**). In addition, the presence of Ca^{2+} or Mg^{2+} and the ratio of cations to PEG are critical to determining the success of protoplast transformation (**Lazzeri et al., 1991; Negrutiu et al., 1987**). Despite varying these parameters while attempting PEG-mediated transformation of Arundo protoplasts, we were unable to transform Arundo protoplasts using PEG. However, while PEG-mediated transformation failed to yield any transformant, electroporation was successful in achieving transient expression of the fluorescent reporter genes. Even though electroporation requires a specialized equipment, its throughput can be modified to plate-based assays as with PEG transfection; in addition, the screening after electroporation can be conducted in less than 24 hours, providing a rapid assessment of gene expression. To demonstrate the functionality of the Arundo protoplast transformation system, the expression of two fluorescent reporters driven by a monocot- (PvUbi2) and a dicot-specific (35S) promoter was evaluated using the pTD-*PvUbi2-35S* vector developed in this work. The results demonstrated that the 35S showed very limited expression in Arundo protoplasts. This finding was at odds with previous work using particle bombardment to transform Arundo callus (**Dhir et al., 2010**) but the discrepancy could be due to differences in genotype or growth conditions. However, protoplast systems are much more sensitive for measuring fluorescent protein expression compared with callus-based systems (**Cole et al., 2013**) and this highlights the importance of screening promoters/gene expression in a rapid protoplast system. The identification of PvUbi2 as a strong promoter for Arundo has implications in the development of future vectors aimed at the generation of stably-transformed Arundo. For instance, PvUbi2 or promoters of similar strength should be chosen to drive the plant resistance cassette in order

to allow a strong selection and reduce false positives/escapes (**Cao et al., 1992; Christou et al., 1991**). Assays such as the one described in this work may help to overcome current limitations in the generation of transgenic Arundo, by providing data on promoter activity prior to regeneration (**Takahashi et al., 2012**). Further, identification of a strong promoter functional in Arundo may help to overcome problems with the selection of transformed cells. As a matter of fact, transformation of Arundo needs to be optimized and, once this is achieved, new genes could be introduced or endogenous genes be tuned by genome editing (using for instance CRIPRS-Cas9 (**Schaeffer and Nakata, 2015**) or RNA interference (**Casacuberta et al., 2015**).

Some examples of desirable modifications in Arundo are mentioned below:

- Interfering with flowering, for example by knocking down genes involved in floral transition (**Komiya et al., 2008**; **Tamaki et al., 2007**, **Wu et al., 2008**) in order to prolong the vegetative phase thereby increasing biomass production.
- Tuning the production of plant hormones (e.g., gibberellin), essential for many development processes in plant leading once again to boost biomass production (**Eriksson et al., 2000**).
- Affecting the cell wall structure making it more accessible to physical and chemical pretreatments therefore increasing saccharification efficiency (**Chuck et al., 2011**). In several cases this was achieved by modifying the lignin content or structure in the cell wall (**Liu et al., 2008**, **Marriott et al., 2014**, **Pauly et al., 2008**, **Srivastava et al., 2015**) orexpressing genes that encode for cell wall degrading enzyme (**Marcia et al., 2015; Lionetti et al., 2011**)

The results obtained in this work, together with a future advancements in molecular and cell biological tools and knowledge of Arundo genome sequence will provide the critical mass of technologies and sequences able to unlock the

full potential of Arundo improving the agronomic traits of this plant to achieve full domestication for bioenergy production.

References

Ahmad, Riaz, Pui-Sze Liow, David F. Spencer, and Marie Jasieniuk. "Molecular evidence for a single genetic clone of invasive Arundo donax in the United States." Aquatic Botany, 88, no. 2 (2008): 113-120.

Al-Forkan, M., P. Anthony, J. B. Power, M. R. Davey, and K. C. Lowe. "Haemoglobin (Erythrogen™)-enhanced microcallus formation from protoplasts of indica rice (Oryza sativa L.)." Artificial Cells, Blood Substitutes, and Biotechnology, 29, no. 5 (2001): 399-404.

Angelini, L. G., L. Ceccarini, and E. Bonari. "Biomass yield and energy balance of giant reed (Arundo donax L.) cropped in central Italy as related to different management practices." European journal of agronomy 22, no. 4 (2005): 375-389.

Angelini, Luciana G., Lucia Ceccarini, Nicoletta Nassi o Di Nasso, and Enrico Bonari. "Comparison of Arundo donax L. and Miscanthus x giganteus in a long-term field experiment in Central Italy: analysis of productive characteristics and energy balance." Biomass and bioenergy 33, no. 4 (2009): 635-643.

Aoyagi, Hideki, and Hideo Tanaka. "Development of simple methods for preparation of yeast and plant protoplasts immobilized in alginate gel beads." Biotechnology techniques, 13, no. 4 (1999): 253-258.

Atanassov, Atanas, and Daniel CW Brown. "Plant regeneration from suspension culture and mesophyll protoplasts of Medicago sativa L." Plant Cell, Tissue and Organ Culture, 3, no. 2 (1984): 149-162.

Baba, Akiko, Seiichiro Hasezawa, and Kunihiko Syōno. "Cultivation of rice protoplasts and their transformation mediated by Agrobacterium spheroplasts." Plant and cell physiology, 27, no. 3 (1986): 463-471.

Balogh, Erika, John M. Herr, Mihály Czakó, and László Márton. "Defective development of male and female gametophytes in Arundo donax L.(POACEAE)." biomass and bioenergy, 45 (2012): 265-269.

Barney, Jacob N., and Joseph M. DiTomaso. " Nonnative species and bioenergy: are we cultivating the next invader?." Bioscience, 58, no. 1 (2008): 64-70.

Barrero, R.A., Guerrero, F.D., Moolhuijzen, P., Goolsby, J.A., Tidwell, J., Bellgard, S.E. and Bellgard, M.I., 2015. Shoot transcriptome of the giant reed, Arundo donax. Data in brief, 3, pp.1-6.

Bart, Rebecca, Mawsheng Chern, Chang-Jin Park, Laura Bartley, and Pamela C. Ronald. "A novel system for gene silencing using siRNAs in rice leaf and stem-derived protoplasts." Plant methods, 2, no. 1 (2006): 1.

Basso, María C., Elsa G. Cerrella, Enrique L. Buonomo, Pablo R. Bonelli, and Ana L. Cukierman. "Thermochemical conversion of Arundo donax into useful solid products." Energy sources, 27, no. 15 (2005): 1429-1438.

Bell, Gary P. "Ecology and management of Arundo donax and approaches to riparian habitat restoration in southern California." (1997): 103-1.

Bezzi G, Monti A, Venturi G. Colture da energia: tecniche di coltivazione e gestione economica. Agricoltura 2006;6(30): 24-30 [in Italian].

Bhandari, H.S., Walker, D.W., Bouton, J.H. and Saha, M.C., 2014. Effects of ecotypes and morphotypes in feedstock composition of switchgrass (Panicum virgatum L.). GCB Bioenergy, 6(1), pp.26-34.

Bhanwra, R. K., S. P. Choda, and S. A. N. J. I. V. Kumar. "Comparative embryology of some grasses." Proceedings of the Indian National Science Academy, 48, no. 1 (1982): 152-62.

Biemelt, S., Tschiersch, H. and Sonnewald, U., 2004. Impact of altered gibberellin metabolism on biomass accumulation, lignin biosynthesis, and photosynthesis in transgenic tobacco plants. Plant Physiology, 135(1), pp.254-265.

Blom-Zandstra, M., H. T. M. Koot, J. Van Hattum, and S. A. Vogelzang. "Isolation of protoplasts for patch-clamp experiments: an improved method requiring minimal amounts of adult leaf or root tissue from monocotyledonous or dicotyledonous plants." Protoplasma, 185, no. 1-2 (1995): 1-6.

Bochantseva, Z. P. "Ochislakh chromosom."Introd. Akkli. Rasteny Akad. Nauk. UZ. SSR (1972): 44-53.

Boose, A., and J. Holt. "Environmental effects on asexual reproduction." Weed Res, 39 (1999): 117-127.

Buntru, Matthias, Simon Vogel, Holger Spiegel, and Stefan Schillberg. "Tobacco BY-2 cell-free lysate: an alternative and highly-productive plant-based in vitro translation system." BMC biotechnology, 14, no. 1 (2014): 1.

Burris, J.N., Mann, D.G., Joyce, B.L. and Stewart Jr, C.N., 2009. An improved tissue culture system for embryogenic callus production and plant regeneration in switchgrass (Panicum virgatum L.). BioEnergy Research,2(4), pp.267-274.

Burris, Kellie P., Elizabeth M. Dlugosz, A. Grace Collins, C. Neal Stewart Jr, and Scott C. Lenaghan. "Development of a rapid, low-cost protoplast transfection system for switchgrass (Panicum virgatum L.)." Plant cell reports, 35, no. 3 (2016): 693-704.

Butt, S.J., Varis, S., Nasir, I.A., Sheraz, S. and Shahid, A., 2015. Micro propagation in advanced vegetable production: a review. Advancements in Life Sciences, 2(2), pp.48-57.

Cao, J., Duan, X., McEIroy, D. and Wu, R., Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Reports 1992, 11, 586-591.

Cao, Jianmei, Dongmei Yao, Fan Lin, and Mingyi Jiang. "PEG-mediated transient gene expression and silencing system in maize mesophyll protoplasts: a valuable tool for signal transduction study in maize." Acta physiologiae plantarum, 36, no. 5 (2014): 1271-1281.

Casacuberta, J.M., Devos, Y., Du Jardin, P., Ramon, M., Vaucheret, H. and Nogue, F., 2015. Biotechnological uses of RNAi in plants: risk assessment considerations. Trends in biotechnology, 33(3), pp.145-147.

Casler, M.D., Vogel, K.P., Taliaferro, C.M., Ehlke, N.J., Berdahl, J.D., Brummer, E.C., Kallenbach, R.L., West, C.P. and Mitchell, R.B., 2007. Latitudinal and longitudinal adaptation of switchgrass populations. Crop Science, 47(6), pp.2249-2260.

Castro, J.C., Boe, A. and Lee, D.K., 2011. A simple system for promoting flowering of upland switchgrass in the greenhouse. Crop science, 51(6), pp.2607-2614.

Cavallaro, Valeria, Simona Tringali, and Cristina PatanÈ. "Large-scale in vitro propagation of giant reed (Arundo donax L.), a promising biomass species." The Journal of Horticultural Science and Biotechnology, 86, no. 5 (2011): 452-456.

Ceotto, Enrico, and Mario Di Candilo. "Shoot cuttings propagation of giant reed (Arundo donax L.) in water and moist soil: The path forward?." Biomass and Bioenergy 34, no. 11 (2010): 1614-1623.

Ceotto, Enrico, Mario Di Candilo, Fabio Castelli, Franz-W. Badeck, Fulvia Rizza, Carlo Soave, Antonio Volta, Giulia Villani, and Vittorio Marletto. "Comparing solar radiation interception and use efficiency for the energy crops giant reed (Arundo donax L.) and sweet sorghum (Sorghum bicolor L. Moench)." Field Crops Research, 149 (2013): 159-166.

Chen, Jiang, Qiang Yi, Qiaoheng Song, Yong Gu, Junjie Zhang, Yufeng Hu, Hanmei Liu, Yinghong Liu, Guowu Yu, and Yubi Huang. "A highly efficient maize nucellus protoplast system for transient gene expression and studying programmed cell death-related processes." Plant cell reports, 34, no. 7 (2015): 1239-1251.

Christopher, J., and A. Abraham. "Studies on the cytology and phylogeny of South Indian grasses " Cytologia 36, no. 4 (1971): 579-594.

Christou, P., Ford, T.L. and Kofron, M., Production of transgenic rice (Oryza sativa L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Nature Biotechnology 1991, 9, 957-962.

Chuck, George S., Christian Tobias, Lan Sun, Florian Kraemer, Chenlin Li, Dean Dibble, Rohit Arora "Overexpression of the maize Corngrass1 microRNA prevents flowering, improves digestibility, and increases starch content of switchgrass." Proceedings of the National Academy of Sciences108, no. 42 (2011): 17550-17555.

Cocking, E. C. "A method for the isolation of plant protoplasts and vacuoles." (1960): 962-963.

Cole, M., Jacobs, B., Soubigou-Taconnat, L., Balzergue, S., Renou, J.P., Chandler, J.W. and Werr, W., 2013. Live imaging of DORNRÖSCHEN and DORNRÖSCHEN-LIKE promoter activity reveals dynamic changes in cell identity at the microcallus surface of Arabidopsis embryonic suspensions. Plant cell reports, 32(1), pp.45-59.

Cosentino, Salvatore Luciano, Venera Copani, Giuseppina Marina D'Agosta, Emanuele Sanzone, and Mariadaniela Mantineo. "First results on evaluation of Arundo donax L. clones collected in Southern Italy." Industrial Crops and Products 23, no. 2 (2006): 212-222.

da Costa Sousa, L., Chundawat, S.P., Balan, V. and Dale, B.E., 2009. "Cradle-to-grave'assessment of existing lignocellulose pretreatment technologies." Current opinion in biotechnology, 20(3), pp.339-347.

Decruyenaere, Joseph G., and Jodie S. Holt. "Seasonality of clonal propagation in giant reed." Weed Science 49, no. 6 (2001): 760-767.

Dhir, Sarwan, Kaye Knowles, C. Livia Pagan, and Justin Mann. "Optimization and transformation of Arundo donax L. using particle bombardment." African Journal of Biotechnology, 9, no. 39 (2010): 6460-6469.

Di Candilo, E. Ceotto Seasonal dynamic of above and belowground dry matter accumulation in giant reed (Arundo donax L.). Proceedings of 20th European Biomass Conference and Exhibition, 18–22 June 2012, Milan, Italy (2012), pp. 507–510.

Duncan, D.R., Williams, M.E., Zehr, B.E. and Widholm, J.M., 1985. The production of callus capable of plant regeneration from immature embryos of numerous Zea mays genotypes. Planta, 165(3), pp.322-332.

Elbersen, H.W., Christian, D.G., El Bassem, N., Yates, N.E., Bacher, W., Sauerbeck, G., Alexopoulou, E., Sharma, N., Piscioneri, I., De Visser, P. and Van den Berg, D., 2001. 5 Switchgrass variety choice in Europe4.Switchgrass (Panicum virgatum L.) as an alternative energy crop in Europe Initiation of a productivity network Final Report for the period from 01-04-1998 to 30-09-2001, p.33.

Eriksson, M.E., Israelsson, M., Olsson, O. and Moritz, T., 2000. Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. Nature biotechnology, 18(7), pp.784-788.

Foti, S., and S. L. Cosentino. "Annual and perennial energy crops."Tecnica Agricola (Italy) (2001).

Fox, Alison M., Doria R. Gordon, Joan A. Dusky, Linda Tyson, and Randall K. Stocker. "IFAS assessment of the status of non-native plants in Florida's natural areas." http://plants. ifas. ufl. edu/assessment/pdfs/finalassessjun05. pdf) Accessed: October 1 (2005): 2007.

Fox, Allison. "A giant reed conundrum." Wildland Weeds. Florida Exotic Pest Plant Council (2007).

Fromm, Michael E., Loverine P. Taylor, and Virginia Walbot. "Stable transformation of maize after gene transfer by electroporation." (1986): 791-793.

Fromm, Michael, Loverine P. Taylor, and Virginia Walbot. "Expression of genes transferred into monocot and dicot plant cells by electroporation."Proceedings of the National Academy of Sciences, 82, no. 17 (1985): 5824-5828.

Fu, C., Xiao, X., Xi, Y., Ge, Y., Chen, F., Bouton, J., Dixon, R.A. and Wang, Z.Y., 2011. Downregulation of cinnamyl alcohol dehydrogenase (CAD) leads to improved saccharification efficiency in switchgrass. BioEnergy Research, 4(3), pp.153-164.

Fu, Y., Poli, M., Sablok, G., Wang, B., Liang, Y., Porta, N., Velikova, V., Loreto, F., Li, M. and Varotto, C., 2016. Dissection of early transcriptional responses to water stress in Arundo donax L. by unigene-based RNA-seq. Biotechnology for biofuels, 9(1), p.1.

Fujikawa, Y., Nakanishi, T., Kawakami, H., Yamasaki, K., Sato, M.H., Tsuji, H., Matsuoka, M. and Kato, N., 2014. Split luciferase complementation assay to detect regulated protein-protein interactions in rice protoplasts in a large-scale format. Rice, 7(1), p.1..

Ge, Xumeng, David M. Burner, Jianfeng Xu, Gregory C. Phillips, and Ganapathy Sivakumar. "Bioethanol production from dedicated energy crops and residues in Arkansas, USA." Biotechnology journal, 6, no. 1 (2011): 66-73.

Ge, Xumeng, Fuqing Xu, Juliana Vasco-Correa, and Yebo Li. "Giant reed: A competitive energy crop in comparison with miscanthus." Renewable and Sustainable Energy Reviews 54 (2013): 350-362.

Gordon, D. R., K. J. Tancig, D. A. Onderdonk, and C. A. Gantz. "Assessing the invasive potential of biofuel species proposed for Florida and the United States using the Australian Weed Risk Assessment." Biomass and Bioenergy, 35, no. 1 (2011): 74-79.

Goyal, H.B., Seal, D. and Saxena, R.C., 2008. Bio-fuels from thermochemical conversion of renewable resources: a review. Renewable and sustainable energy reviews, 12(2), pp.504-517.

Gupta, S.D. and Conger, B.V., 1999. Somatic embryogenesis and plant regeneration from suspension cultures of switchgrass. Crop science, 39(1), pp.243-247.

Gurel, E., Gurel, S. and Lemaux, P.G., 2008. Biotechnology applications for sugar beet. Critical reviews in plant sciences, 27(2), pp.108-140.

Hardion, Laurent, Régine Verlaque, Alex Baumel, Marianick Juin, and Bruno Vila. "Revised systematics of Mediterranean Arundo (Poaceae) based on AFLP fingerprints and morphology." Taxon, 61, no. 6 (2012): 1217-1226.

Hardion, Laurent, Régine Verlaque, Kristin Saltonstall, Agathe Leriche, and Bruno Vila. "Origin of the invasive Arundo donax (Poaceae): a trans-Asian expedition in herbaria." Annals of botany (2014): mcu143.

Hardion, Laurent, Régine Verlaque, Marcela Rosato, Josep A. Rossello, and Bruno Vila. "Impact of polyploidy on fertility variation of Mediterranean Arundo L.(Poaceae)." Comptes rendus biologies 338, no. 5 (2015): 298-306.

Hauptmann, R. M., P. Ozias-Akins, V. Vasil, Z. Tabaeizadeh, S. G. Rogers, R. Bo Horsch, I. K. Vasil, and R. T. Fraley. "Transient expression of electroporated DNA in monocotyledonous and dicotyledonous species."Plant Cell Reports, 6, no. 4 (1987): 265-270.

Heaton, Emily A., Frank G. Dohleman, and Stephen P. Long. "Seasonal nitrogen dynamics of Miscanthus× giganteus and Panicum virgatum." GCB Bioenergy, 1, no. 4 (2009): 297-307.

Herrera, Angelica M., and Tom L. Dudley. "Reduction of riparian arthropod abundance and diversity as a consequence of giant reed (Arundo donax) invasion." Biological Invasions 5, no. 3 (2003): 167-177.

Ho, W.J. and Vasil, I.K., 1983. Somatic embryogenesis in sugarcane (Saccharum officinarum L.): Growth and plant regeneration from embryogenic cell suspension cultures. Annals of Botany, 51(6), pp.719-726.

Holm, Preben B., Soren Knudsen, Peter Mouritzen, Diana Negri, Finn L. Olsen, and Catherine Roue. "Regeneration of Fertile Barley Plants from Mechanically Isolated Protoplasts of the Fertilized Egg Cell." The Plant Cell6, no. 4 (1994): 531-543.

Hoque, M.E. and Mansfield, J.W., 2004. Effect of genotype and explant age on callus induction and subsequent plant regeneration from root-derived callus of Indica rice genotypes. Plant cell, tissue and organ culture, 78(3), pp.217-223

Horne, P.A. and Williams, P.T., 1996. Influence of temperature on the products from the flash pyrolysis of biomass. Fuel, 75(9), pp.1051-1059.

Huang, Yu-Wen, and Elizabeth S. Dennis. "Factors influencing stable transformation of maize protoplasts by electroporation." Plant Cell, Tissue and Organ Culture, 18, no. 3 (1989): 281-296.

Ishizawa, C.I., Jeoh, T., Adney, W.S., Himmel, M.E., Johnson, D.K. and Davis, M.F., 2009. “Can delignification decrease cellulose digestibility in acid pretreated corn stover?” Cellulose, 16(4), pp.677-686.

Jensen, E., Robson, P., Norris, J., Cookson, A., Farrar, K., Donnison, I. and Clifton-Brown, J., 2013. “Flowering induction in the bioenergy grass Miscanthus sacchariflorus is a quantitative short-day response, whilst delayed flowering under long days increases biomass accumulation.” Journal of experimental botany, 64(2), pp.541-552.

Kering, M.K., Butler, T.J., Biermacher, J.T. and Guretzky, J.A., 2012. "Biomass yield and nutrient removal rates of perennial grasses under nitrogen fertilization". Bioenergy Research, 5(1), pp.61-70.

Keshwani, D.R. and Cheng, J.J., 2009. Switchgrass for bioethanol and other value-added applications: a review. Bioresource technology, 100(4), pp.1515-1523.

Kiniry, J. R., C. R. Tischler, and G. A. Van Esbroeck. "Radiation use efficiency and leaf CO 2 exchange for diverse C 4 grasses." Biomass and Bioenergy, 17, no. 2 (1999): 95-112.

Kisaka, H., H. Lee, M. Kisaka, A. Kanno, K. Kang, and T. Kameya. "Production and analysis of asymmetric hybrid plants between monocotyledon (Oryza sativa L.) and dicotyledon (Daucus carota L.)."Theoretical and applied genetics 89, no. 2-3 (1994): 365-371.

Kisaka, H., M. Kisaka, A. Kanno, and T. Kameya. "Intergeneric somatic hybridization of rice (Oryza sativa L.) and barley (Hordeum vulgare L.) by protoplast fusion." Plant cell reports 17, no. 5 (1998): 362-367.

Komeda, Yoshibumi. "Genetic regulation of time to flower in Arabidopsis thaliana." Annu. Rev. Plant Biol. 55 (2004): 521-535.

Komiya, Reina, Akiko Ikegami, Shojiro Tamaki, Shuji Yokoi, and Ko Shimamoto. "Hd3a and RFT1 are essential for flowering in rice."Development 135, no. 4 (2008): 767-774.

Komiya, Reina, Shuji Yokoi, and Ko Shimamoto. "A gene network for long-day flowering activates RFT1 encoding a mobile flowering signal in rice." Development, 136, no. 20 (2009): 3443-3450.

Langis, R., B. Schnabel, E. D. Earle, and P. L. Steponkus. "Cryopreservation of Brassica campestris L. cell suspensions by vitrification." Cryo-Letters (United Kingdom), (1989).

Larsen K. 1963. Studies in the flora of Thailand 14. Cytological studies in vascular plants of Thailand. Dansk Botanisk Arkiv 20: 211–275.

Lazzeri, P.A., Brettschneider, R., Lührs, R. and Lörz, H., " Stable transformation of barley via PEG-induced direct DNA uptake into protoplasts". Theoretical and applied genetics 1991, 81, 437-444.

Li, Ruyu, and Rongda Qu. "High throughput Agrobacterium-mediated switchgrass transformation." Biomass and bioenergy, 35, no. 3 (2011): 1046-1054.

Linnaeus, C.V., 1753. Species plantarum, 2 vols. Salvius, Stockholm.

Liu, Hongquan, Wengong Yu, Jixun Dai, Qianhong Gong, Kunfeng Yang, and Xinzhi Lu. "Cryopreservation of protoplasts of the alga Porphyra yezoensis by vitrification." Plant science, 166, no. 1 (2004): 97-102.

Lörz, Horst, Barbara Baker, and Jeff Schell. "Gene transfer to cereal cells mediated by protoplast transformation." Molecular and General Genetics MGG, 199, no. 2 (1985): 178-182.

Mann, D.G., King, Z.R., Liu, W., Joyce, B.L., Percifield, R.J., Hawkins, J.S., LaFayette, P.R., Artelt, B.J., Burris, J.N., Mazarei, M. and Bennetzen, J.L., 2011. Switchgrass (Panicum

virgatum L.) polyubiquitin gene (PvUbi1 and PvUbi2) promoters for use in plant transformation. BMC biotechnology,11(1), p.74.

Marcia, M. de O., Sue Dalton, Tim Langdon, Emma Timms-Taravella, Erica A. Shearer, and Phillip Morris. "Functional co-expression of a fungal ferulic acid esterase and a β-1, 4 endoxylanase in Festuca arundinacea (tall fescue) modifies post-harvest cell wall deconstruction." Planta 242, no. 1 (2015): 97-111.

Mariani, C., R. Cabrini, A. Danin, P. Piffanelli, A. Fricano, S. Gomarasca, M. Dicandilo, F. Grassi, and C. Soave. "Origin, diffusion and reproduction of the giant reed (Arundo donax L.): a promising weedy energy crop." Annals of Applied Biology 157, no. 2 (2010): 191-202.

Marriott, Poppy E., Richard Sibout, Catherine Lapierre, Jonatan U. Fangel, William GT Willats, Herman Hofte, Leonardo D. Gómez, and Simon J. McQueen-Mason. "Range of cell-wall alterations enhance saccharification in Brachypodium distachyon mutants." Proceedings of the National Academy of Sciences 111, no. 40 (2014): 14601-14606.

Mazarei, Mitra, Hani Al-Ahmad, Mary R. Rudis, and C. Neal Stewart. "Protoplast isolation and transient gene expression in switchgrass, Panicum virgatum L." Biotechnology Journal, 3, no. 3 (2008): 354.

Mazarei, Mitra, Hani Al-Ahmad, Mary R. Rudis, Blake L. Joyce, and C. Neal Stewart. "Switchgrass (Panicum virgatum L.) cell suspension cultures: Establishment, characterization, and application." Plant science, 181, no. 6 (2011): 712-715.

McLaughlin, S., Bouton, J., Bransby, D., Conger, B., Ocumpaugh, W., Parrish, D., Taliaferro, C., Vogel, K. and Wullschleger, S., 1999. Developing switchgrass as a bioenergy crop. Perspectives on new crops and new uses,282.

Meryman, Harold T., and Robert J. Williams. "Basic principles of freezing injury to plant cells; natural tolerance and approaches to cryopreservation."Cryopreservation of plant cells and organs (1985): 13-47.

Mishra, Tanmayee, Arvind Kumar Goyal, Malay Bhattacharya, Pallab Kar, and Arnab Sen. " Polyethylene glycol mediated protoplast fusion of medicinally important Canna." Research in Plant Biology, 5, no. 1 (2015).

Mitchell, R., Vogel, K.P. and Sarath, G., 2008. Managing and enhancing switchgrass as a bioenergy feedstock. Biofuels, Bioproducts and Biorefining, 2(6), pp.530-539.

Mohanty, Amitabh, Monendra Grover, Ashok Chaudhury, Quazi Rizwan-ul-Haq, Arun K. Sharma, Satish C. Maheshwari, and Akhilesh K. Tyagi. "Analysis of the activity of promoters

from two photosynthesis-related genes psaF and petH of spinach in a monocot plant, rice." Indian Journal Of Biochemistry And Biophysics, 37, no. 6 (2000): 447-452.

Monti, A., Bezzi, G., Pritoni, G. and Venturi, G., 2008. Long-term productivity of lowland and upland switchgrass cytotypes as affected by cutting frequency. Bioresource technology, 99(16), pp.7425-7432.

Moser, L.E. and Vogel, K.P., 1995. Switchgrass, big bluestem, and indiangrass. Forages, 1, pp.409-420.

Nakashima, Kazuo, Asad Jan, Daisuke Todaka, Kyonoshin Maruyama, Shingo Goto, Kazuo Shinozaki, and Kazuko Yamaguchi-Shinozaki. "Comparative functional analysis of six drought-responsive promoters in transgenic rice." Planta, 239, no. 1 (2014): 47-60.

Narasimhamoorthy, B., Saha, M.C., Swaller, T. and Bouton, J.H., 2008. Genetic diversity in switchgrass collections assessed by EST-SSR markers.Bioenergy Research, 1(2), pp.136-146.

Negrutiu, I., Shillito, R., Potrykus, I., Biasini, G. and Sala, F., 1987. Hybrid genes in the analysis of transformation conditions. Plant Molecular Biology 1987, 8, 363-373.

Nieves-Cordones, Manuel, Alain Chavanieu, Linda Jeanguenin, Carine Alcon, Wojciech Szponarski, Sebastien Estaran, Isabelle Chérel, Sabine Zimmermann, Hervé Sentenac, and Isabelle Gaillard. "Distinct amino acids in the C-linker domain of the Arabidopsis K+ channel KAT2 determine its subcellular localization and activity at the plasma membrane." Plant physiology, 164, no. 3 (2014): 1415-1429.

Nobre, J., M. R. Davey, P. A. Lazzeri, and M. E. Cannell. "Transformation of barley scutellum protoplasts: regeneration of fertile transgenic plants." Plant Cell Reports, 19, no. 10 (2000): 1000-1005.

Palmqvist, Benny, and Gunnar Lidén. "Combining the effects of process design and pH for improved xylose conversion in high solid ethanol production from Arundo donax." AMB Express 4, no. 1 (2014): 1.

Papazoglou, E. G., G. A. Karantounias, S. N. Vemmos, and D. L. Bouranis. "Photosynthesis and growth responses of giant reed (Arundo donax L.) to the heavy metals Cd and Ni." Environment International, 31, no. 2 (2005): 243-249.

Parrish, D.J., Casler, M.D. and Monti, A., 2012. The evolution of switchgrass as an energy crop. In Switchgrass (pp. 1-28). Springer London.

Pauly, Markus, and Kenneth Keegstra. "Cell-wall carbohydrates and their modification as a resource for biofuels." The Plant Journal, 54, no. 4 (2008): 559-568.

Perdue, Robert E. "Arundo donax—source of musical reeds and industrial cellulose." Economic Botany 12, no. 4 (1958): 368-404.

Pheloung, P. C., P. A. Williams, and S. R. Halloy. "A weed risk assessment model for use as a biosecurity tool evaluating plant introductions." Journal of Environmental Management, 57, no. 4 (1999): 239-251.

Pigna, G., Dhillon, T., Dlugosz, E.M., Yuan, J.S., Gorman, C., Morandini, P., Lenaghan, S.C. and Stewart, C.N., 2016. Methods for suspension culture, protoplast extraction, and transformation of high-biomass yielding perennial grass Arundo donax. Biotechnology Journal.

Pimentel, D. and Patzek, T.W., 2005. Ethanol production using corn, switchgrass, and wood; biodiesel production using soybean and sunflower. Natural resources research, 14(1), pp.65-76.

Ping, Huang, David I. Bransby, and Edzard van Santen. "Long-term biomass yields of giant reed, mimosa and switchgrass in Alabama." Biofuels, Bioproducts and Biorefining, 8, no. 1 (2014): 59-66.

Rakoczy-Trojanowska, Monika. "Alternative methods of plant transformation-a short review." Cellular and molecular biology letters, 7, no. 3 (2002): 849-858.

Rakotondrafara, Aurelie M., Jacquelyn R. Jackson, Elizabeth Pettit Kneller, and W. Allen Miller. "Preparation and electroporation of oat protoplasts from cell suspension culture." Current protocols in microbiology, (2007): 16D-3.

Rall, W.F. and Fahy, G.M., 1985. Ice-free cryopreservation of mouse embryos at −196 C by vitrification.

Rossa, B., A. V. Tüffers, G. Naidoo, and von DJ Willert. "Arundo donax L.(Poaceae)—a C3 species with unusually high photosynthetic capacity." Botanica Acta, 111, no. 3 (1998): 216-221.

Saikia, R., Chutia, R.S., Kataki, R. and Pant, K.K., 2015. Perennial grass (Arundo donax L.) as a feedstock for thermo-chemical conversion to energy and materials. Bioresource technology, 188, pp.265-272.

Schaeffer, S.M. and Nakata, P.A., 2015. CRISPR/Cas9-mediated genome editing and gene replacement in plants: transitioning from lab to field. Plant Science, 240, pp.130-142.

Schmer, M.R., Vogel, K.P., Mitchell, R.B. and Perrin, R.K., 2008. Net energy of cellulosic ethanol from switchgrass. Proceedings of the National Academy of Sciences, 105(2), pp.464-469.

Scordia, Danilo, Salvatore L. Cosentino, and Thomas W. Jeffries. "Enzymatic hydrolysis, simultaneous saccharification and ethanol fermentation of oxalic acid pretreated giant reed (Arundo donax L.)."Industrial Crops and Products, 49 (2013): 392-399.

Seepaul, R., Macoon, B., Reddy, K.R. and Evans, W.B., 2016. Nitrogen Application Rate and Genotype Effects on Switchgrass Production and Chemical Characteristics. American Journal of Plant Sciences, 7(03), p.533.

Shatalov, Anatoly A., and Helena Pereira. "Biorefinery of Energy Crop Cardoon (Cynara cardunculus l.)-Hydrolytic Xylose Production as Entry Point to Complex Fractionation Scheme." Journal of Chemical Engineering & Process Technology, 2011 (2012).

Sheen, Jen. "Signal transduction in maize and Arabidopsis mesophyll protoplasts." Plant Physiology, 127, no. 4 (2001): 1466-1475.

Sinclair, T. R., and H. C. Randall. "Nitrogen and biomass accumulation by alfalfa under high temperatures of late summer." Field Crops Research, 31, no. 3 (1993): 287-294.

Sinha, A., A. C. Wetten, and P. D. S. Caligari. "Optimisation of protoplast production in white lupin." Biologia plantarum, 47, no. 1 (2003): 21-25.

Smeets, E.M., Lewandowski, I.M. and Faaij, A.P., 2009. The economical and environmental performance of miscanthus and switchgrass production and supply chains in a European setting. Renewable and sustainable energy reviews, 13(6), pp.1230-1245.

Solomon, Mazal, Beatrice Belenghi, Massimo Delledonne, Ester Menachem, and Alex Levine. "The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants." The Plant Cell, 11, no. 3 (1999): 431-443.

Srivastava, Avinash C., Fang Chen, Tui Ray, Sivakumar Pattathil, Maria J. Peña, Utku Avci, Hongjia Li et al. "Loss of function of folylpolyglutamate synthetase 1 reduces lignin content and improves cell wall digestibility in Arabidopsis." Biotechnology for biofuels, 8, no. 1 (2015): 1.

Steponkus, P. L., R. Langis, and S_ Fujikawa. "Cryopreservation of plant tissues by vitrification." Advances in low temperature biology, 1 (1992): 1-61.

Sundberg, E. and Glimelius, K., 1986. A method for production of interspecific hybrids within Brassiceae via somatic hybridization, using resynthesis of Brassica napus as a model. Plant science, 43(2), pp.155-162

Szarka, B., I. Göntér, M. Molnár-Láng, S. Mórocz, and D. Dudits. "Mixing of maize and wheat genomic DNA by somatic hybridization in regenerated sterile maize plants." Theoretical and Applied Genetics, 105, no. 1 (2002): 1-7.

Tada, Yuichi, Masahiro Sakamoto, and Tatsuhito Fujimura. "Efficient gene introduction into rice by electroporation and analysis of transgenic plants: use of electroporation buffer lacking chloride ions." Theoretical and applied genetics, 80, no. 4 (1990): 475-480.

Takahashi, Wataru, and Tadashi Takamizo. "Molecular breeding of grasses by transgenic approaches for biofuel production". INTECH Open Access Publisher, 2012.

Takahashi, Wataru, Tadashi Takamizo, Makoto Kobayashi, and Masumi Ebina. "Plant regeneration from calli in giant reed (Arundo donax L.)."Grassland science, 56, no. 4 (2010): 224-229.

Takebe, I., Gudrun Labib, and G. Melchers. "Regeneration of whole plants from isolated mesophyll protoplasts of tobacco." Naturwissenschaften, 58, no. 6 (1971): 318-320.

Tamaki, Shojiro, Shoichi Matsuo, Hann Ling Wong, Shuji Yokoi, and Ko Shimamoto. "Hd3a protein is a mobile flowering signal in rice." Science, 316, no. 5827 (2007): 1033-1036.

Testa, Riccardo, Mario Foderà, Anna Maria Di Trapani, Salvatore Tudisca, and Filippo Sgroi. "Giant reed as energy crop for Southern Italy: An economic feasibility study." Renewable and Sustainable Energy Reviews 58 (2016): 558-564.

Tomassetti, S., Pontiggia, D., Verrascina, I., Reca, I.B., Francocci, F., Salvi, G., Cervone, F. and Ferrari, S., 2015. "Controlled expression of pectic enzymes in Arabidopsis thaliana enhances biomass conversion without adverse effects on growth". Phytochemistry, 112, pp.221-230.

Tomita, K.I., Hiura, S. and Tamagake, H., 2013. Evaluation of the potential for somatic embryogenesis in sugar beet (Beta vulgaris L.) breeding lines and improvement of regeneration efficiency. Plant Biotechnology, 30(5), pp.479-487.

Tubeileh, A., Rennie, T.J. and Goss, M.J., 2016. A review on biomass production from C 4 grasses: yield and quality for end-use. Current opinion in plant biology, 31, pp.172-180.

Turhollow AF, Cushman JH, Johnston JW. Herbaceous Energy Crops Program: Annual Progress Report for FY, Report ORNL-6639, 1990, Oak Ridge National Laboratory: Oak Ridge, TN 37831-6285, 1988. 76pp.

Uchimiya, Hirofumi, and Toshio Murashige. "Evaluation of parameters in the isolation of viable protoplasts from cultured tobacco cells." Plant Physiology54, no. 6 (1974): 936-944.

Van Acker, R., Vanholme, R., Storme, V., Mortimer, J.C., Dupree, P. and Boerjan, W., 2013. ”Lignin biosynthesis perturbations affect secondary cell wall composition and saccharification yield in Arabidopsis thaliana”. Biotechnology for biofuels, 6(1), p.1.

Van Dyk, J.S. and Pletschke, B.I., 2012. “A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-factors affecting enzymes, conversion and synergy.” Biotechnology advances, 30(6), pp.1458-1480.

Virtue, John G., Tim Reynolds, Jenna Malone, and C. Williams. "Managing the weed risk of cultivated Arundo donax L." In Seventeenth Australasian Weeds Conference, Christchurch, New Zealand, pp. 176-179. 2010.

Wang, Juan, Jinjin Jiang, and Youping Wang. "Protoplast fusion for crop improvement and breeding in China." Plant Cell, Tissue and Organ Culture (PCTOC), 112, no. 2 (2013): 131-142.

Wang, Z., Hopkins, A. and Mian, R., 2001. Forage and turf grass biotechnology. Critical Reviews in Plant Sciences, 20(6), pp.573-619.

Webster, Richard J., Steven M. Driever, Johannes Kromdijk, Justin McGrath, Andrew DB Leakey, Katharina Siebke, Tanvir Demetriades-Shah et al. "High C3 photosynthetic capacity and high intrinsic water use efficiency underlies the high productivity of the bioenergy grass Arundo donax."Scientific reports, 6 (2016).

Wolf, D.D. and Fiske, D.A., 2009. Planting and managing switchgrass for forage, wildlife, and conservation.

Wu, C., You, C., Li, C., Long, T., Chen, G., Byrne, M.E. and Zhang, Q., 2008. “RID1, encoding a Cys2/His2-type zinc finger transcription factor, acts as a master switch from vegetative to floral development in rice.” Proceedings of the National Academy of Sciences, 105(35), pp.12915-12920.

Wuddineh, W.A., Mazarei, M., Zhang, J., Poovaiah, C.R., Mann, D.G., Ziebell, A., Sykes, R.W., Davis, M.F., Udvardi, M.K. and Stewart, C.N., 2015. Identification and overexpression of gibberellin 2-oxidase (GA2ox) in switchgrass (Panicum virgatum L.) for improved plant architecture and reduced biomass recalcitrance. Plant biotechnology journal, 13(5), pp.636-647.

Xia, Guangmin, Fengning Xiang, Aifen Zhou, Huai Wang, and Huimin Chen. "Asymmetric somatic hybridization between wheat (Triticum aestivum L.) and Agropyron elongatum (Host) Nevishi." Theoretical and Applied Genetics107, no. 2 (2003): 299-305.

Xu, B., Escamilla-Treviño, L.L., Sathitsuksanoh, N., Shen, Z., Shen, H., Percival Zhang, Y.H., Dixon, R.A. and Zhao, B., 2011. Silencing of 4-coumarate: coenzyme A ligase in switchgrass

leads to reduced lignin content and improved fermentable sugar yields for biofuel production. New phytologist, 192(3), pp.611-625.

Xu, B., Sathitsuksanoh, N., Tang, Y., Udvardi, M.K., Zhang, J.Y., Shen, Z., Balota, M., Harich, K., Zhang, P.Y.H. and Zhao, B., 2012. "Overexpression of AtLOV1 in switchgrass alters plant architecture, lignin content, and flowering time". PLoS One, 7(12), p.e47399.

Xu, W.Z., Zhang, X.Q., Huang, L.K. And Ma, X., 2012. Callus induction and differentiation of switchgrass Panicum virgatum seeds [J]. Pratacultural Science, 1, p.011.

Yeong, Hui-Yin, Norzulaani Khalid, and Siew-Moi Phang. "Protoplast isolation and regeneration from Gracilaria changii (Gracilariales, Rhodophyta)." Journal of applied phycology, 20, no. 5 (2008): 641-651.

Zhang, H. M., H. Yang, E. L. Rech, T. J. Golds, A. S. Davis, B. J. Mulligan, E. C. Cocking, and M. R. Davey. "Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts." Plant Cell Reports, 7, no. 6 (1988): 379-384.

Zhang, W., and R. Wu. "Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants." Theoretical and Applied Genetics, 76, no. 6 (1988): 835-840.

Zhao, X.Y., Zhu, D.F., Zhou, B., Peng, W.S., Lin, J.Z., Huang, X.Q., He, R.Q., Zhuo, Y.H., Peng, D., Tang, D.Y. and Li, M.F., 2010. Over-expression of the AtGA2ox8 gene decreases the biomass accumulation and lignification in rapeseed (Brassica napus L.). Journal of Zhejiang University Science B,11(7), pp.471-481.

Zhu, Z., Sathitsuksanoh, N., Vinzant, T., Schell, D.J., McMillan, J.D. and Zhang, Y.H.P., 2009. Comparative study of corn stover pretreated by dilute acid and cellulose solvent-based lignocellulose fractionation: Enzymatic hydrolysis, supramolecular structure, and substrate accessibility. Biotechnology and bioengineering, 103(4), pp.715-724.

Acknowledgment

Oggi sono uscita dall'università e ho pensato: ancora poche settimane e non sarò più una studentessa, mai più. Lo so, non si smette mai di studiare e gli esami non finiscono mai, ma formalmente non sarò più una studentessa. Ora sono Gaia Pigna e basta. E adesso la paura mi assale, sarò in grado di farcela? Sarò capace di mettere a frutto tutti gli insegnamenti ricevuti ? Questo ancora non lo so, ma sono sicura che quello che sono diventata lo sono anche grazie alle persone che mi hanno accompagnato in questi 4 lunghi anni di dottorato, o più in generale in questi quasi 10 anni di università).

Grazie sicuramente a Piero, perché non mi ha solo insegnato tantissimo sulla scienza ma mi ha fatto sempre tanto coraggio nei momenti più difficili.
Piero è una di quelle persone che nella scienza e nella ricerca ci crede davvero ed essere stata sua studente è stato un onore. Piero insegna la precisione dell'analisi del dato, a sviluppare il senso critico verso quello che si legge senza mai dare nulla per scontato. Ricordo ancora una sua lezione nella quale ci ha dato come compito quello di trovare un errore di concetto in una pagina di un illustre libro di biochimica. Piero insegna a ragionare a imparare che anche i libri, anche i più grandi possono sbagliare, che non bisogna mai smettere di studiare e pensare attivamente
Grazie per avermi reso una persona migliore.

Grazie ovviamente a chi è stato in lab con me e che mi ha fatto ridere quando c'era solo da piangere, grazie soprattutto a Sonia, Elia per avermi sempre tirato su di morale anche quando ero oltreoceano. Grazie anche a Nadia per le pause, le riflessioni e Giovanni Truppi.

Grazie alla mia compagna di disavventure e ansia, CdG.

Grazie ai miei amici che non sempre hanno capito le mie scelte ma mi hanno sempre sostenuta. C e F sono sempre un faro nel mio porto.

Grazie a chi mi ha sempre spronato a fare del mio meglio, a non abbattermi, a superare le critiche e a sfogare le mie ansie ballando, camminando in montagna, tirando a canestro o scalando la parete di una palestra. È stato importante conoscerli e averli con me (Il mondo è un posto bellissimo).

Un grazie speciale va ai miei genitori che sono i veri custodi della mia sanità mentale. Mi dispiace di essere stata così dura con loro in questi ultimi anni ma la verità è che questo titolo è più loro che mio. Non mi capacito di come siano stati comprensivi con me anche quando l'unica cosa logica e razionale da fare era mandarmi a quel paese. Soprattutto mia madre che ha avuto la pazienza di una santa.

Grazie a tutti

Gaia

Part II

Book chapter in press:

Pigna et al., 2016

Domestication of New Species

Gaia Pigna[1,2], Piero Morandini[1*]

[1]*Università degli Studi di Milano, Department of Biosciences, Milano, Italy*

[2] *Present address: The University of Tennessee, Department of Plant Sciences, Knoxville, TN, USA*

Abstract: Domestication is the process through which a wild plant becomes a crop. The process is the result of the selection, either deliberate or as a byproduct of agricultural practices, of characteristics favorable to human beings. The sum of such characteristics is usually described as the 'domestication syndrome' because the types of traits selected are often shared among many different species. The most commonly selected traits are loss of seed dispersal, reduced seed dormancy, changes in growth habit, flowering time, and gigantism, all of which have an impact on morphology, reproductive strategies and, most importantly, production (yield and harvest index). Depending on the plant and its use, other traits could be selected, such as reduction or loss of toxic compounds, winter hardiness, nutritional quality, etc. Most domestication took place in ancient times, but there are a few examples of recent and accelerated domestication, for instance sugar beet. It is now possible to achieve the domestication of new species, based on the deliberate induction and combination of traits, using a set of approaches: classical plant breeding *via* hybridization and selection (including wide area crosses, hybrid seeds and plant cell culture), coupled with molecular tools such as Marker Assisted Selection, transgenesis, and site directed mutagenesis. Examples of interesting traits as well as candidate crops are discussed. Thus, we have the means to repeat the achievements of the early domestication wave and do even better, but this requires drastic changes in international and national regulations impacting on plant biotechnology and novel breeding techniques.

Details: in press in More Food: Road to Survival R. Pilu and G Gavazzi (Eds), Bentham science, Bejing, China.

Research Article

Methods for suspension culture, protoplast extraction, and transformation of high-biomass yielding perennial grass *Arundo donax*

Gaia Pigna[1,2]

Taniya Dhillon[1]

Elizabeth M. Dlugosz[1]

Joshua S. Yuan[4]

Connor Gorman[4]

Piero Morandini[2,3]

Scott C. Lenaghan[5,6]

C. Neal Stewart, Jr.[1]

[1]Department of Plant Sciences, University of Tennessee, Knoxville, USA

[2]Department of Biosciences, University of Milan, Milano, Italy

[3]National Research Council, Institute of Biophysics, Milano, Italy

[4]Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX, USA

[5]Department of Food Science, University of Tennessee, Knoxville, USA

[6]Department of Mechanical, Aerospace, and Biomedical Engineering, University of Tennessee, Knoxville, USA

Correspondence: C Neal Stewart, Jr., Department of Plant Sciences, University of Tennessee, 2431 Joe Johnson Dr., Knoxville, TN 37996, USA

E-mail: nealstewart@utk.edu

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/biot.201600486.

Submitted: 15-Aug-2016
Revised: 13-Oct-2016
Accepted: 19-Oct-2016

Keywords: *Arundo*, Electroporation, Protoplast, Suspension Culture, Transformation

Abbreviations: **PEG**, polyethylene glycol; **CIM**, callus induction media; **2,4-D**, 2, 4-dichlorophenoxyacetic acid; **BAP**, benzylaminopurine ; **PCV**, packed cell volume

Abstract

Arundo donax L. is a promising biofuel feedstock in the Mediterranean region. Despite considerable interest in its genetic improvement, *Arundo* tissue culture and transformation remains arduous. We developed methodologies for cell- and tissue culture and genetic engineering in *Arundo.* A media screen was conducted, and a suspension culture was established using callus induced from stem axillary bud explants. DBAP medium, containing 9 µM 2,4-D and 4.4 µM BAP, was found to be the most effective medium among those tested for inducing cell suspension cultures, which resulted in a 5-fold increase in tissue mass over 14 days. In contrast, CIM medium containing 13 µM 2,4-D, resulted in just a 1.4-fold increase in mass over the same period. Optimized suspension cultures were superior to previously-described solidified medium-based callus culture methods for tissue mass increase. Suspension cultures proved to be very effective for subsequent protoplast isolation. Protoplast electroporation resulted in a 3.3±1.5 % transformation efficiency. A dual fluorescent reporter gene vector enabled the direct comparison of the CAMV 35S promoter with the switchgrass ubi2 promoter in single cells of *Arundo.* The switchgrass ubi2 promoter resulted in noticeably higher reporter gene expression compared with that conferred by the 35S promoter in *Arundo*.

1 Introduction

In recent years there has been significant interest in harvesting renewable fuels and value-added chemicals from plant feedstocks, especially from perennial grasses such as *Arundo donax* L. [1,2,3]. Some reasons why perennial grasses are desirable as next-generation bioenergy feedstock are: 1) their low soil management requirements and growth on marginal land; 2) their ability to prevent soil erosion and increase soil organic carbon; 3) few natural pests; and 4) the capacity to reliably produce biomass [4]. In the U.S., switchgrass *(Panicum virgatum*) is a widely adapted biomass feedstock whereas in Europe, *Arundo* may fulfill an important role as a perennial grass feedstock [5,6]. Compared with switchgrass, *Arundo* has a much higher photosynthetic capacity, despite being a C3 plant [7,8], leading to the production of significant biomass [9]. In addition, *Arundo* is salt tolerant, and has been used for phytoremediation of soils contaminated with heavy metals [10,11]. Further, the cell wall composition of *Arundo* [12,13], along with thermochemical conversion into biofuel [14], has been characterized. The lignin present in the stems and leaves has a HGS-type structure, but is primarily composed of G subunits. Delignified stems have a highly heterogenous population of hemicelluloses in terms of sugar composition, molecular weight, and structure [13].

Despite the potential of *Arundo* as a bioenergy feedstock in Southern Europe [1], there are very few publications on tissue culture and transformation of this species. At present, *Agrobacterium*-mediated transformation of *Arundo* has not been demonstrated and limited success has been achieved using biolistics to transiently transform *Arundo* callus cultures [15]. A transient transformation protocol could provide crucial insight into gene expression [16], promoter screening [17], protein-protein interactions [18], transcriptional regulatory networks [19], and protein subcellular localization [20], as well as providing a necessary first step for the development of a stable transformation protocol for crop improvement [21]. As such, the goal of this work was to develop an *in vitro* culture system for *Arundo* to rapidly generate axenic protoplasts for subsequent transformation and analysis.

2 Materials and methods

2.1 Induction and maintenance of *Arundo* callus

Callus was induced using the protocol reported by Takahashi et al., [22] with some modification. Briefly, stem explants of *A. donax* were cut into approximately 30 cm long segments and surface sterilized with 70% (v/v) ethanol for 30 s and then transferred to a 4% (v/v) sodium hypochlorite solution, with one drop of Tween 20 (Sigma Aldrich, St. Louis, MO, USA), for 10 min prior to washing 3 times in sterilized distilled water. The cane internodes were then excised and placed on a basal Murashige and Skoog (MS) medium [23] supplemented with 0.05% Plant Preservative Mixture (PPM) (Plant Cell Technology, Washington, DC, USA) and 0.3% (w/v) Gelzan (Sigma Aldrich, St. Louis, MO, USA). The excised internodes were placed in a growth chamber at 25^{o}C under standard cool white fluorescent tubes with a flux rate of 35 µmol $s^{-1}m^{-2}$ and a 16-h photoperiod. Excised internodes were transferred onto CIM medium, MS medium supplemented with 3% sucrose (Fisher Scientific, Fair Lawn, NJ, USA), 9 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.7 g/L casein hydrolysate, 0.5 g/L L-glutamine, 1 g/L L-proline, and 0.4% Gelzan. These were placed in a growth chamber in the dark at 25°C for a month to induce calli. Once induced, the calli were maintained on the same medium, with 13 µM 2,4-D [22].

2.2 Establishment and maintenance of *Arundo* suspension cultures

Cell suspension cultures were initiated from 10 month old embryogenic callus by adding 500 mg of calli to a 125 ml Erlenmeyer flask containing 25 ml of MS or B5 medium supplemented with 3% sucrose and various hormones. In order to test the effect of different media on initiation of suspension cultures, the hormone type and concentration were varied, along with the basal media as follows. DBAP medium was composed of MS salts, B5 vitamins, 9 µM 2,4-D and 4.4 µM 6-benzylaminopurine (BAP) (Sigma Aldrich, St. Louis, MO, USA) [24]. MSDP media was composed of MS salts and vitamins supplemented with 9 µM 2,4-D and 100 nM phytosulfokine-α (PSK-α) (PolyPeptide Group, Torrance CA, USA) [25]. B5G medium was composed of B5 salts and vitamins [26] supplemented with 0.7 g/L glutamine (Sigma Aldrich, St. Louis, MO, USA) [27]. After transfer of the calli to flasks, cultures were incubated in the dark at 28°C for 14 days on a gyratory

shaker at 80 rpm. Cultures were then filtered through a 100 µm Fisherbrand™ Cell Strainer (Fisher Scientific, Fair Lawn, NJ, USA), brought up to a volume of 25 ml with fresh medium, and transferred to a 125 ml flask. Three flasks, initiated separately from 3 different calli, were used for each medium and represented 3 independent biological replicates. Five milliliters of fresh medium was added to the cultures every 7 days for 6-8 weeks to compensate liquid evaporation. At the end of 8 weeks, cultures were considered to be established, as indicated by the presence of large cell clusters [29, 29] and the growth was assayed for another 21 days by measuring the packed cell volume (PCV) of the cultures [30]. Briefly, 5 ml of culture was transferred to a 15 ml conical centrifuge tube (Corning Incorporated) and allowed to settle for 10 minutes, at which point the PCV was measured. At the end of the experiments, the cultures were used to generate protoplasts for subsequent experiments.

2.3 Tissue culture compared with cell suspension culture

In order to determine the best strategy for rapidly attaining large amounts of *Arundo* tissue, we tested four culture conditions, with three independent replicates for each condition, all of which were initiated with 500 mg (fresh weight) of callus or cells. Treatment 1 (liquid DBAP to liquid DBAP) was initiated with 500 mg of an established suspension culture added to 25 ml of DBAP medium in an Erlenmeyer flask. Each replicate was initiated from a different flask of suspension cell culture. Treatment 2 (liquid DBAP to solid CIM) was initiated the same as Treatment 1, with the exception of the established suspension culture being added to solid CIM media. Treatment 3 (solid CIM to liquid DBAP) was initiated from callus grown on solid CIM media transferred directly into 25 ml of DBAP medium in an Erlenmeyer flask. Finally, Treatment 4 (solid CIM to solid CIM) was initiated from callus grown on solid CIM media transferred to solid CIM media. To determine the tissue mass at 1, 3, 5, 7, 10, 14, 18, and 21 days, the suspension cultures were filtered through a 70 µm Fisherbrand™ Cell Strainer (Fisher Scientific, Fair Lawn, NJ, USA) and the contents weighed, accounting for the mass of the cell strainer, and then returned to the culture. The mass of the calli grown on solid media, at the same time points, was measured by transferring calli to a Petri dish and taking fresh weight.

2.4 Protoplast isolation from suspension cell culture

Protoplasts of *Arundo* were isolated using a previously established method for isolation of protoplasts from switchgrass suspension cultures [31] with slight modifications. In addition to using switchgrass as a control, tobacco (*Nicotiana tabacum* L.) cv. Bright Yellow 2 (BY-2) suspension culture, cultured as previously described, was used as a positive control to ensure functionality of the enzymes [31,32,33]. Briefly, a 10 ml PCV of *Arundo* suspension culture collected during the exponential growth phase was used. Cell walls were digested with 20 ml of a solution (0.6 M mannitol, 10 mM MES pH 5, 1 mM $CaCl_2$, 0.1% BSA, 5 mM 2-mercaptoethanol) containing food grade enzymes (Rohament CL 15820 ECU, Rohapect 10L 10080 ADJU, and Rohapect UF 0.078 ADJU) (AB Enzymes, Darmstadt, Germany) was added to the cells and incubated at 37°C, for 2-3 hours with gentle shaking. After incubation, the solution was filtered through a 40 µm Fisherbrand™ cell strainer to remove cellular debris and centrifuged at 150 g for 10 min. Five volumes of W5 wash solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7) [34,35] was then added to the protoplasts. After resuspension, protoplasts were imaged using an Olympus IX 81 inverted microscope and counted using a hemocytometer. Protoplast diameter was measured using the ImageJ software package (US National Institutes of Health, Bethesda, Maryland, USA) calibrated with an ocular micrometer. The size distribution of the protoplasts was generated by grouping protoplasts in different size classes [36].

2.5 Vector construction

Vector pTD-PvUbi2-35S was derived from pANIC10A [37] after several modifications. First, a unique restriction site, *Mlu*I, was inserted between the switchgrass polyubiquitin 1+3 (*PvUbi1+3*) promoter and the orange fluorescent protein, *pporRFP*. Since *PvUbi2* is a stronger promoter compared to *PvUbi1+3* [38], the former was PCR amplified from pANIC10A and used to replace *PvUbi1+3* using *Psp*XI and *Mlu*I-HF restriction enzymes. To compare the strength of the constitutive cauliflower mosaic virus *35S* promoter relative to *PvUbi2* in *A. donax*, a *35S-mGFP5ER-nos* cassette was PCR amplified from pBIN m-gfp5-ER [39] using the forward primer 5'-

taatctgcttggtaaccagattagccttttcaat-3' and reverse primer 5'-ttacttgttggcgcgcctcccgatctagtaacataga-3' (restriction sites are underlined) and inserted in pANIC10A using the restriction enzymes *Bst*EII and *Asc*I. The primers used for PCR amplification were designed in SnapGene and synthesized by Integrated DNA Technologies (Coralville, IA, USA). PCR was performed using Platinum *Taq* DNA polymerase (ThermoFisher Scientific, Waltham, MA, USA). To improve the efficiency of protoplast transformation, the size of the original vector was reduced by deleting unneeded sequences including 1) a 3,837 bp fragment encompassing 85% of the total length of ZmUbi1-Gateway-compatible ccdB cassette-OCS, using *Mfe*I, 2) a 3,405 bp sequence containing DNA required for *Agrobacterium*-mediated transformation, which included the right T-DNA border, the stability region, and 652 bp of the origin of replication, using *Fsp*I, and 3) a 2,977 bp sequence spanning *PvUbi2* promoter and hygromycin selectable marker, using *Pac*I and *Psp*XI. All PCR amplification and restriction digestion products were gel purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and quantified on a NanoDrop-1000 spectrophotometer (ThermoFisher) prior to cloning. Following *Pac*I and *Psp*XI digestion, the vector was blunt-ended and 5' phosphorylated using the Quick Blunting Kit (New England Biolabs, Ipswich, MA, USA). All ligation reactions were carried out using T4 DNA Ligase (New England Biolabs). Ligated constructs were transformed into either “One Shot ccdB survival 2 T1R” chemically competent cells or “One Shot Mach1 T1 phage-resistant” chemically competent cells (ThermoFisher) as per the manufacturer’s instructions and sequence confirmed. The final vector, pTD-PvUbi2-35S contains 8,690 bp (**Figure 1**) and features two independent fluorescent protein cassettes: the orange fluorescent protein *pporRFP* under the control of *PvUbi2* promoter and the green fluorescent protein *mGFP5-ER* under the control of CAMV *35S* promoter.

2.6 Polyethylene glycol (PEG)-mediated transformation

PEG-mediated DNA transformation was performed as previously described [35,21], with switchgrass protoplasts serving as a positive contol, with slight modifications. Protoplasts of *Arundo* were resuspended in MMg (0.4 M mannitol, 25–150 mM $MgCl_2$, 4 mM MES (pH 5.7)) at a concentration of 1×10^6 protoplasts/ml. Ten micrograms of pANIC10A-GFPstuffer [38] plasmid DNA

was then mixed with 200 µl of protoplasts (approximately 2×10^5 protoplasts). A 0–40 % PEG solution (0.6 M mannitol, 100 mM $CaCl_2$, 0–50 % PEG 4000 (Sigma–Aldrich, St. Louis, Missouri, USA) was added to the protoplasts to a final PEG concentration of 0, 10 or 20 %. After 20 min of incubation at room temperature (RT), protoplasts were washed twice with 1 and 4 ml of W5 and collected by centrifugation at 100 × g for 6 min. Protoplasts were then resuspended in 1 mL of WI (0.6 M mannitol, 4 mM KCl, 4 mM MES, pH 5.7), transferred to a 12-well plate (Corning Incorporated, Corning, NY, USA) and incubated at 28 °C in the dark for 20 h. As a positive control, switchgrass mesophyll protoplasts were transformed using PEG as previously described [31]. The transformation efficiency (%) was calculated by counting the number of protoplasts expressing pporRFP divided by the total number of protoplasts using a hemocytometer and multiplied by 100 as shown in the formula below:

$$\left(\frac{\#\ protoplasts\ expressing\ pporRFP}{total\ \#\ protoplasts}\right)\times 100 = \%\ transformation\ efficiency$$

To ensure the detection of all positive protoplasts, samples were collected from individual wells, centrifuged at 100 × g prior to resuspension in a minimal volume (~100 µl) and counted on a hemocytometer. Three independent biological replicates were analyzed for each tested condition.

2.7 Protoplast electroporation-mediated transformation

As an alternative to PEG-mediated transformation, electroporation was performed as previously described for tobacco BY-2 protoplasts with slight modifications [40]. One hour after isolation, protoplasts were centrifuged at 100 x g for 2 min and the W5 wash solution was removed. Protoplasts were then washed twice with electroporation buffer (0.4 M sucrose, 2.4 g/l HEPES, 6 g/l KCl, 600 mg/l $CaCl_2$ H_2O, pH 7.2), at 80 x g for 10 min without deceleration. A 500 µl aliquot containing 5-6×10^5 protoplasts in electroporation buffer was then placed into 0.4 cm gap electroporation cuvette (Bio-Rad Laboratories, Hercules, CA, USA). Forty micrograms of pTD-PvUbi2-35S DNA was then added to *Arundo* and tobacco BY-2 protoplasts and incubated at RT for 10-15 min, placing the cuvette horizontally. After incubation, electroporation of *Arundo* was conducted using a Bio-Rad Gene Pulser™ (Bio-Rad Laboratories, Hercules, CA, USA) at 130-300 V with a capacitance of 500 or 1000 µF. Tobacco BY-2 protoplasts were used as a positive control,

at 300 V with a capacitance of 1000 µF. The time constant was recorded after each pulse to ensure the samples were uniform, and the pulse was delivered. After electroporation, samples were incubated for 40 min at RT in the cuvette, and then moved into a single well of a 12-well plate, followed by the addition of 2 ml of protoplast culture medium (4.3 g/l MS salts, 0.4 M sucrose, 2.5 mM MES hydrate, 5.4 mM $CaCl_2 \cdot H_2O$, 3.1 mM NH_4NO_3, pH 5.7). The remaining solution in the cuvette was mixed with 1 ml of protoplast culture medium and transferred into a second well. An additional 1 ml of protoplast culture medium was finally used to wash the cuvette and poured into the second well. The protoplast viability was monitored after electroporation using propidium iodide, working solution (1 mg/50 ml, Sigma–Aldrich, St. Louis, Missouri, USA) [31]. Similarly to PEG-mediated transformation, the transformation efficiency was measured at 14-16 hours after transformation by counting the number of protoplasts expressing mGFP5-ER and pporRFP and dividing by the total protoplast number. For this method efficiency of transformation was estimated directly in the well by counting 3 random fields of view. The efficiency of each field was calculated separately as described previously. Using this strategy, an average of about 300 protoplasts were counted per well. Three independent biological replicates, each the result of independent protoplast isolations and transformation, were analyzed for all conditions tested.

2.8 Statistical analysis

A completely random experimental design was used for media screening, growth analysis, and transformation. All experiments included at least three independent biological replicates, and were analyzed using mixed model ANOVA (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY, USA). Least significant differences (LSD) were used to determine significant differences among means when the ANOVA results were statistically significant (P<0.05).

3 Results

3.1 Establishment of suspension cultures of *Arundo*

To test various media, the PCV of cultures were analyzed over the course of 21 days (**Figure 2A**). Despite using solidified CIM medium for culturing the donor callus, liquid CIM resulted in the

poorest growth among the media tested (**Table 1**). Over the first 7 days, the PCV of the liquid CIM was below the limit of detection (50 µl), and reached a measurable level only at day 10. Moreover, there was no significant growth observed for the CIM cultures from day 10 to day 21 ($p=0.71$). Similar to CIM, suspension cultures initiated in MSDP medium showed no detectable growth over the first 10 days of culture, but reached a measurable level at day 14. From day 14 to the completion of the experiments at day 21, there was no significant growth ($p=0.13$). Significant growth was observed in the B5G and DBAP media, with the cultures reaching their peak growth at 7-10 days and 10-14 days respectively (**Table 1**). In B5G and DBAP, the PCV was measurable after only 1 day in culture, as opposed to 3 and 7 days for MSDP and CIM respectively. Between DBAP and B5G, there was no significant difference in growth over the first 3 days of culture ($p=0.65$); however, the PCV of the B5G culture was significantly greater at day 7 compared to DBAP ($p=0.024$). After 10 days in culture, the PCV of the suspension cultures in DBAP had increased 3.3-fold, while the B5G culture increased 2.8-fold compared with the initial amount. At this point, the DBAP culture surpassed the maximum PCV observed for the B5G culture. Furthermore, comparison of the PCV at day 10 (the maximum for both DBAP and B5G) across all of the media showed a significant difference between DBAP and B5G compared to the other two media tested (**Table 1**). After 10 days in culture, the PCV of the B5G culture decreased markedly. A similar trend was observed in the DBAP culture, but the decrease occurred after 14 days in culture. In both cases, the rapid cell growth in these cultures led to an observable decrease in the amount of liquid media in flasks. While we conclude that both DBAP and B5G were suitable for initiation and maintenance of *Arundo* cell suspension cultures, DBAP was chosen as the media for future experiments because of its higher PCV at 10 days of culture, indicating more biomass would be available sooner for follow-on experiments.

Prior to establishment of the cultures, the majority of the cells in DBAP were kidney-shaped, contained starch-rich plastids, and clearly-discernable nuclei (**Figure 2B**). After establishment, the suspension cultures mainly consisted of elongated cells devoid of vacuoles (**Figure 2C**). In addition, the established suspension cultures were a mix of individual embryogenic-like cells of

various size classes (**Figure 2E**). Once large (1-5 mm diameter) clusters were readily observable in the culture flasks, the cultures were considered suitable for subsequent experiments.

3.2 *Arundo* performs better in liquid culture than on solidified medium

A comparison of the mass of suspension cells and callus was also evaluated over the course of 21 days (**Table 2**, **Figure 2F**). At 14 days of culture, there was no significant difference in the amount of tissue generated from *Arundo* grown on solid media (maintained on solid CIM), a freshly incubated liquid culture (callus moved from solid CIM to liquid DBAP medium), or an established liquid culture transferred to solid media (liquid DBAP to solid DBAP) (**Figure 2D**). However, there was significantly more tissue generated from the established suspension cultures that were transferred to fresh liquid DBAP. In fact, 2.5 times more tissue was generated by cultures maintained in liquid DBAP compared to all other treatments at 14 days (**Table 2**). In addition, the established suspension cultures reached a maximum of 4.7 times the starting tissue mass over 21 days, whereas no other treatment exceeded 2 times the cell mass over the same time period. Based on these results, the established suspension cultures were used for the generation of protoplasts and transformation.

3.3 Protoplast preparation and transformation.

Protoplasts of *Arundo* were isolated from 4 to 7 day old suspension cell cultures (**Figure 2G**), with an average of $3.8\times10^5 \pm 2.5\times10^5$ protoplasts/ml of PCV. Morphologically, the protoplasts ranged in size from 4.9 to 53.4 µm in diameter with an average size of 20.7±12.3 µm. The majority of the protoplasts ranged from 4 to 20 µm in diameter (**Figure 2H**). PEG- and electroporation transformation experiments were performed on protoplasts. Initial transformation experiments were conducted using the pANIC10A-GFPstuffer [31], which expresses *pporRFP* driven by the switchgrass ubiquitin promoter, *PvUbi1+3* [38]. All attempts to transform *Arundo* protoplasts with PEG (varying DNA quantity, $MgCl_2$ concentration, PEG% solution) failed, despite the success with the switchgrass positive controls. However, electroporation at 130 V and 1000 µF attained a transformation efficiency of 3.3±1.5%. When the voltage was increased to 300 V, with the

capacitance remaining the same, there was a decrease in protoplast viability from 67.7±3.0% to 11.9±4.5% and no transformation was detected. When the capacitance was decreased to 500 µF, at 130 and 300 V, the efficiency of transformation was reduced to 1.0±0.2 and 0.3±0.4%, respectively. In these treatments, the viability remained high, 82.±1.5% at 130 V and 80±2.4% at 300 V, despite the lower transformation efficiency. Not surprisingly, the switchgrass *PvUbi2* [38] promoter yielded higher marker gene expression than the *35S* promoter in *Arundo* protoplasts using the pTD-*PvUbi2*-*35S* binary vector (**Figure 3A-D)**. As expected, tobacco BY-2 protoplasts, used as a control, showed high expression of the mGFP5-ER fluorescent marker driven by the *35S* promoter, but only weak expression of the pporRFP fluorescent marker driven by the *PvUbi2* promoter (**Figure 3E-H**).

4 Discussion

Previous work on tissue culture of *Arundo donax* used solid CIM medium for callus induction and maintenance [22]. Unfortunately, *Arundo* callus grown on this medium is relatively slow-growing, making it difficult and time-consuming to generate enough tissue for subsequent transformation. Of particular importance, *Arundo* callus growth on CIM appears to be suboptimal compared with other relevant plant species. For example, *Sorghum* callus grow 1.7 times faster than *Arundo* on solidified CIM [41]. For these reasons, a suspension culture system for *Arundo* was pursued as a mean to increase the amount of tissue produced. The results from the *Arundo* suspension cultures established in DBAP demonstrate that cultivation in liquid medium produces far more tissue than solid culture with CIM. Similar results have been found in other species, such as cotton (*Gossypium hirsutum* L.) and broccoli (*Brassica oleracea*) [42-43]. The difference in *Arundo* growth in liquid culture may be the result of greater nutrient availability, a more gradual pH change, or lower production of toxic compounds [42].

Despite the significant increase in tissue mass for the suspension cultures of *Arundo*, the amount obtained was still relatively low when compared to suspension cultures of other biofuel crops. Suspension cultures of sugarcane (*Saccharum officinarum*) have been shown to increase by 6

times the starting inoculum in only 10 days [44]. Similarly, switchgrass suspension cultures have been shown to increase 8.6 times over 14 days of culture [24]. In both of these systems, the increase in fresh weight is ~2 times greater than *Arundo*. These data suggest that further optimization of the culture media may be necessary for *Arundo*, as other bioenergy crops have placed extensive effort in this direction [45,46,47]. Also, the ability of plant species to be manipulated *in vitro* is strongly genotype-dependent [48,49], therefore more effort is needed to screen different accessions and identify genotypes more amenable to *in vitro* cultivation.

One unique characteristic of *Arundo* compared with other bioenergy crops is that it does not sexually reproduce; therefore there is no seed production [50,51]. As such, *Arundo* is not amenable to improvement through standard breeding practices. The lack of seed production, however, is a highly advantageous trait when considering bioconfinement and restriction of gene flow, especially transgene flow [52]. In either case, genetic improvement of *Arundo* requires a reliable method for transformation to introduce novel genes, or inhibit/overexpress native ones [53,54]. An effective transformation method is also essential for screening of gene expression, validation of gene silencing or genome-editing vectors, and general cell and molecular assays. Considering that only there have been few RNA sequencing studies [55,56] and no whole genome information is presently available for *Arundo*, it is essential that screening be conducted at early stages, prior to developing transgenic plants. Currently, transformation of *Arundo* callus has only been achieved in one study, in which particle bombardment was used to introduce exogenous DNA into callus [15]. While effective, particle bombardment requires specialized equipment and is costly with regards to consumables. Further complicating transient-, and to a greater extent, stable transformation, callus growth is very slow, leading to a long lag time prior to when screening can be performed. As an alternative, PEG-mediated transformation and electroporation of *Arundo* protoplasts was evaluated in our project. High molecular weight PEG solutions have proven to be toxic to protoplasts isolated from some plant species [57]. In addition, the presence of Ca^{2+} or Mg^{2+} and the ratio of cations : PEG are critical factors for optimizing protoplast transformation [58,59]. While we did vary these parameters, we never achieved PEG-mediated transformation of *Arundo*

protoplasts. However, while PEG-mediated transformation failed, electroporation was successful in generating transient expression of the fluorescent reporter genes. While, similar to particle bombardment, electroporation requires specialized equipment, its throughput can be markedly increased using plate-based assays. In addition, screening after electroporation can be conducted in less than 24 hours, providing a rapid assessment of gene expression. To demonstrate the functionality of the *Arundo* protoplast transformation system, the expression of fluorescent reporters driven by monocot (*PvUbi2*) and dicot (*35S*) promoters were evaluated using the pTD-*PvUbi2*-*35S* vector developed in this work. The results demonstrated the utility of screening promoters/gene expression in a rapid protoplast system: *35S* promoter imbued low expression in *Arundo* protoplasts. This finding was in contrast to previous work using particle bombardment to transform *Arundo* callus [15]. However, protoplast systems are much more sensitive for measuring fluorescent protein expression compared with callus-based systems [29]. The identification of *PvUbi2* as a strong promoter for *Arundo* has implications in the development of future vectors aimed at the generation of stably-transformed *Arundo*. For instance, a strong promoter should be chosen to drive the plant resistance cassette in order to impose a strong selection and eliminate the majority of false positives/escapes [60,61]. Assays such as the one conducted in this work may help to overcome current limitations in the generation of transgenic *Arundo*, by providing data on promoter activity prior to regeneration [62].

In conclusion, in our project, a cell suspension culture system using DBAP medium was established for *Arundo*, which yielded up to 6-fold tissue-mass increase relative to callus culture systems. In addition, we developed a low-cost protoplast isolation system from suspension cultures that appear to have utility for assays for gene expression, and promoter screening. They could be useful for genome editing and stable transformation. Finally, the transient transformation of *Arundo* protoplasts using electroporation was achieved and used to screen a representative monocot and dicot promoter. These experiments indicated that *PvUbi2* was a strongly-active promoter in *Arundo*, whereas 35S was a weak promoter, thus informing the design of future vectors for *Arundo* biotechnology.

Acknowledgement

This work was supported by ARPA-E award number DE-AR0000313. The authors thank Robert N. Trigiano for the use of the Bio-Rad Gene Pulser™.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

5 References

[1] Lewandowski, I., Scurlock, J.M., Lindvall, E. and Christou, M., The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe. *Biomass Bioenergy* 2003, *25*, 335-361.

[2] Corno, L., Pilu, R., Tran, K., Tambone, F.,et al., Sugars Production for Green Chemistry from 2nd Generation Crop (Arundo donax L.): A Full Field Approach. *ChemistrySelect* 2016, 1, 2617-2623.

[3] Corno, L., Pilu, R., Cantaluppi, E. and Adani, F., Giant cane (*Arundo donax* L.) for biogas production: The effect of two ensilage methods on biomass characteristics and biogas potential. *Biomass Bioenergy* 2016, 93, 131-136..

[4] Angelini, L.G., Ceccarini, L., o Di Nasso, N.N. and Bonari, E., Comparison of *Arundo donax* L. and *Miscanthus x giganteus* in a long-term field experiment in Central Italy: analysis of productive characteristics and energy balance. *Biomass Bioenergy* 2009, *33*, 635-643.

[5] Mantineo, M., D'agosta, G.M., Copani, V., Patanè, C. and Cosentino, S.L., Biomass yield and energy balance of three perennial crops for energy use in the semi-arid Mediterranean environment. *Field Crop Res.* 2009,*114*, 204-213

[6] Scordia, D., Testa, G. and Cosentino, S.L., Perennial grasses as lignocellulosic feedstock for second-generation bioethanol production in Mediterranean environment. *Ital. J. Agron.* 2014, *9*, 84-92.

[7] Rossa, B., Tüffers, A.V., Naidoo, G. and Willert, V.D., *Arundo donax* L. (Poaceae)—a C3 species with unusually high photosynthetic capacity. *Bot. Acta*, 1998, *111*, 216-221.

[8] Ceotto, E., Di Candilo, M., Castelli, F., Badeck, et al., Comparing solar radiation interception and use efficiency for the energy crops giant reed (*Arundo donax* L.) and sweet sorghum (*Sorghum bicolor* L. Moench). *Field Crop. Res.* 2013, *149*, 159-166.

[9] Beale, C.V. and Long, S.P., Can perennial C4 grasses attain high efficiencies of radiant energy conversion in cool climates? *Plant Cell Environ.* 1995, *18*, 641-650.

[10] Papazoglou, E.G., Karantounias, G.A., Vemmos, S.N. and Bouranis, D.L., Photosynthesis and growth responses of giant reed (*Arundo donax* L.) to the heavy metals Cd and Ni. *Environ. Int.* 2005, *31*, 243-249.

[11] Mirza, N., Mahmood, Q., Pervez, A., Ahmad, R., et al., Phytoremediation potential of *Arundo donax* in arsenic-contaminated synthetic wastewater. *Bioresour. Technol.* 2010, *101*, 5815-5819.

[12] You, T.T., Mao, J.Z., Yuan, T.Q., Wen, J.L. and Xu, F., Structural elucidation of the lignins from stems and foliage of *Arundo donax* Linn. *J. Agric. Food Chem.* 2013, *61*, 5361-5370.

[13] Peng, F., Bian, J., Peng, P., Xiao, H., Ren, et al., Separation and characterization of acetyl and non-acetyl hemicelluloses of *Arundo donax* by ammonium sulfate precipitation. *J. Agric. Food Chem.* 2012, *60*, 4039-4047.

[14] Basso, M.C., Cerrella, E.G., Buonomo, E.L., Bonelli, P.R. and Cukierman, A.L., Thermochemical conversion of *Arundo donax* into useful solid products. *Energ. Sources* 2005, *27*, 1429-1438.

[15] Dhir, S., Knowles, K., Pagan, C.L. and Mann, J., Optimization and transformation of *Arundo donax* L. using particle bombardment. *Afr. J. Biotechnol.* 2010, *9*, 6460-6469.

[16] Chen, J., Yi, Q., Song, Q., Gu, Y., et al., A highly efficient maize nucellus protoplast system for transient gene expression and studying programmed cell death-related processes. *Plant Cell Rep.* 2015, *34*, 1239-1251.

[17] Nakashima, K., Jan, A., Todaka, D., Maruyama, K., et al., Comparative functional analysis of six drought-responsive promoters in transgenic rice. *Planta* 2014, *239*, 47-60.

[18] Fujikawa, Y., Nakanishi, T., Kawakami, H., Yamasaki, K., et al., Split luciferase complementation assay to detect regulated protein-protein interactions in rice protoplasts in a large-scale format. *Rice* 2014, *7*, 1.

[19] Lin, Y.C., Li, W., Sun, Y.H., Kumari, S., et al., SND1 transcription factor–directed quantitative functional hierarchical genetic regulatory network in wood formation in *Populus trichocarpa*. *Plant Cell* 2013, *25*, pp.4324-4341

[20] Nieves-Cordones, M., Chavanieu, A., Jeanguenin, L., Alcon, C., et al., Distinct amino acids in the C-linker domain of the *Arabidopsis* K+ channel KAT2 determine its subcellular localization and activity at the plasma membrane. *Plant Physiol.* 2014, *164*, 1415-1429.

[21] Masani, M.Y.A., Noll, G.A., Parveez, G.K.A., Sambanthamurthi, R. and Prüfer, D., Efficient transformation of oil palm protoplasts by PEG-mediated transfection and DNA microinjection. *PLoS One* 2014, *9*, e96831

[22] Takahashi, W., Takamizo, T., Kobayashi, M. and Ebina, M., Plant regeneration from calli in giant reed (*Arundo donax* L.). *Grassl. Sci.* 2010, *56*, 224-229.

[23] Murashige, T. and Skoog, F., A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 1962, *15*, 473-497.

[24] Gupta, S.D. and Conger, B.V., Somatic embryogenesis and plant regeneration from suspension cultures of switchgrass. *Crop Sci.* 1999, *39*, 243-247.

[25] Eun, C.H., Ko, S.M., Matsubayashi, Y., Sakagami, Y. and Kamada, H., Phytosulfokine-α requires auxin to stimulate carrot non-embryogenic cell proliferation. *Plant Physiol. Biochem.* 2003, *41*, 447-452.

[26] Gamborg, O.L., Miller, R. and Ojima, K., Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 1968, *50*, 151-158.

[27] Finer, J.J. and Nagasawa, A., Development of an embryogenic suspension culture of soybean (*Glycine max* Merrill.). *Plant Cell Tiss. Org.* 1988, *15*, 125-136.

[28] Savoure, A., Magyar, Z., Pierre, M., Brown, S., et al., Activation of the cell cycle machinery and the isoflavonoid biosynthesis pathway by active *Rhizobium meliloti* Nod signal molecules in *Medicago* microcallus suspensions. *EMBO J.* 1994,*13*, 1093.

[29] Cole, M., Jacobs, B., Soubigou-Taconnat, L., Balzergue, S., et al., Live imaging of DORNRÖSCHEN and DORNRÖSCHEN-LIKE promoter activity reveals dynamic changes in cell identity at the microcallus surface of *Arabidopsis* embryonic suspensions. *Plant Cell Rep. 2013*, *32*, pp.45-59.

[30] Mustafa, N.R., de Winter, W., van Iren, F. and Verpoorte, R., Initiation, growth and cryopreservation of plant cell suspension cultures. *Nature Protoc.* 2011, *6*, 715-742.

[31] Burris, K.P., Dlugosz, E.M., Collins, A.G., Stewart Jr, C.N. and Lenaghan, S.C., Development of a rapid, low-cost protoplast transfection system for switchgrass (*Panicum virgatum* L.). *Plant Cell Rep.* 2016, *35*, 693-704.

[32] Buntru, M., Vogel, S., Spiegel, H. and Schillberg, S., Tobacco BY-2 cell-free lysate: an alternative and highly-productive plant-based *in vitro* translation system. *BMC Biotechnol.* 2014, *14*, 1.

[33] Buntru, M., Vogel, S., Stoff, K., Spiegel, H. and Schillberg, S., A versatile coupled cell-free transcription–translation system based on tobacco BY-2 cell lysates. *Biotechnol. Bioeng.* 2015, *112*, 867-878.

[34] Sheen, J., Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. *Plant Physiol.* 2001, *127*, 1466-1475.

[35] Yoo, S.D., Cho, Y.H. and Sheen, J., *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature Protoc.* 2007, *2*, 1565-1572.

[36] Kim, G.H., Klotchkova, T.A. and Kang, Y.M., Life without a cell membrane: regeneration of protoplasts from disintegrated cells of the marine green alga *Bryopsis plumosa*. *J. Cell Sci.* 2001, *114*, 2009-2014.

[37] Mann, D.G., LaFayette, P.R., Abercrombie, L.L., King, Z.R., et al., Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass (*Panicum virgatum* L.) and other monocot species. *Plant Biotechnol. Jo.* 2012, *10*, 226-236.

[38] Mann, D.G., King, Z.R., Liu, W., Joyce, B.L., et al., Switchgrass (*Panicum virgatum* L.) polyubiquitin gene (PvUbi1 and PvUbi2) promoters for use in plant transformation. *BMC Biotechnol.* 2011, *11*, 74.

[39] Haseloff, J., Siemering, K.R., Prasher, D.C. and Hodge, S., Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc. Nat. Acad. Sci.* 1997, *94*, 2122-2127.

[40] Miao, Y. and Jiang, L., Transient expression of fluorescent fusion proteins in protoplasts of suspension cultured cells. *Nature Protoc.* 2007, *2*, 2348-2353.

[41] Hagio, T., Blowers, A.D. and Earle, E.D., Stable transformation of sorghum cell cultures after bombardment with DNA-coated microprojectiles. *Plant Cell Rep.* 1991, *10*, 260-264.

[42] Gawel, N.J. and Robacker, C.D., Somatic embryogenesis in two *Gossypium hirsutum* genotypes on semi-solid versus liquid proliferation media. *Plant Cell Tiss. Org.* 1990, *23*, 201-204.

[43] Yang, J.L., Seong, E.S., Kim, M.J., Ghimire, B.K., et al., Direct somatic embryogenesis from pericycle cells of broccoli (*Brassica oleracea* L. var. italica) root explants. *Plant Cell Tiss. Org.* 2010, *100*, 49-58

[44] Ho, W.J. and Vasil, I.K., Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.): Growth and plant regeneration from embryogenic cell suspension cultures. *Ann. Bot.* 1983, *51*, 719-726.

[45] Perera, D., Barnes, D.J., Baldwin, B.S. and Reichert, N.A., Direct and indirect in vitro regeneration of *Miscanthus* × *giganteus* cultivar Freedom: effects of explant type and medium on regeneration efficiency. *In Vitro Cell. Dev. Biol.Plant* 2015, *51*, 294-302.

[46] Liu, Y.R., Cen, H.F., Yan, J.P., Zhang, Y.W. and Zhang, W.J., Inside out: high-efficiency plant regeneration and *Agrobacterium*-mediated transformation of upland and lowland switchgrass cultivars. *Plant Cell Rep.* 2015, *34*, 1099-1108.

[47] Birch, R. G. Sugarcane Biotechnology: Axenic Culture, Gene Transfer, and Transgene Expression in: P. H. Moore and F. C. Botha (Ed.), *Sugarcane: Physiology, Biochemistry, and Functional Biology*, John Wiley & Sons Ltd 2013, pp. 645-681

[48] Tomita, K.I., Hiura, S. and Tamagake, H., Evaluation of the potential for somatic embryogenesis in sugar beet (*Beta vulgaris* L.) breeding lines and improvement of regeneration efficiency. *Plant Biotechnol.* 2013, *30*, 479-487.

[49] Gurel, E., Gurel, S. and Lemaux, P.G., Biotechnology applications for sugar beet. *Crit. Rev. Plant Sci.* 2008, *27*, 108-140.

[50] Hardion, L., Verlaque, R., Rosato, M., Rossello, J.A. and Vila, B., Impact of polyploidy on fertility variation of Mediterranean *Arundo* L.(Poaceae). *C. R. Biol.* 2015, *338*, pp.298-306.

[51] Hardion, L., Verlaque, R., Baumel, A., Juin, M. and Vila, B., Revised systematics of Mediterranean *Arundo* (Poaceae) based on AFLP fingerprints and morphology. *Taxon* 2012, *61*, 1217-1226.

[52] Barney, J.N. and DiTomaso, J.M., Nonnative species and bioenergy: are we cultivating the next invader?. *Bioscience* 2008, *58*, 64-70.

[53] Biswal, A.K., Hao, Z., Pattathil, S., Yang, X., et al., Downregulation of GAUT12 in *Populus deltoides* by RNA silencing results in reduced recalcitrance, increased growth and reduced xylan and pectin in a woody biofuel feedstock. *Biotechnol. Biofuels* 2015, *8*, p.1.

[54] Jung, J.H. and Altpeter, F., TALEN mediated targeted mutagenesis of the caffeic acid O-methyltransferase in highly polyploid sugarcane improves cell wall composition for production of bioethanol. *Plant Mol. Biol.* 2016, DOI:10.1007/s11103-016-0499-y

[55] Fu, Y., Poli, M., Sablok, G., Wang, B., et al., Dissection of early transcriptional responses to water stress in *Arundo donax* L. by unigene-based RNA-seq. *Biotechnol. Biofuels* 2016, *9*, p.1.

[56] Barrero, R.A., Guerrero, F.D., Moolhuijzen, P., Goolsby, J.A., et al., 2015. Shoot transcriptome of the giant reed, *Arundo donax*. *Data Brief 2015*, *3*,1-6.

[57] Kao, K.N. and Saleem, M., Improved fusion of mesophyll and cotyledon protoplasts with PEG and high pH-Ca 2+ solutions. *J. Plant Physiol.* 1986, *122*, 217-225.

[58] Lazzeri, P.A., Brettschneider, R., Lührs, R. and Lörz, H., Stable transformation of barley via PEG-induced direct DNA uptake into protoplasts. *Theor. Appl. Genet. 1991*, *81*, 437-444.

[59] Negrutiu, I., Shillito, R., Potrykus, I., Biasini, G. and Sala, F., 1987. Hybrid genes in the analysis of transformation conditions. *Plant Mol. Biol. 1987*,*8*, 363-373.

[60] Cao, J., Duan, X., McElroy, D. and Wu, R., Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. *Plant Cell Rep.* 1992, *11*, 586-591.

[61] Christou, P., Ford, T.L. and Kofron, M., Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Nature Biotechnol.* 1991, *9*, 957-962.

[62] Takahashi, W. and Takamizo, T., *Molecular breeding of grasses by transgenic approaches for biofuel production*. INTECH Open Access Publisher, 2012.

Table 1. Media screen for the establishment of suspension cultures over a 21-day culture period. Cell growth was monitored for 21 days after filtration through a 100 µm cell strainer by measuring the packed cell volume (PCV). Three independent replicates were measured for each medium.

Media	Packed cell volume (µl) measured by day					
	1	3	7	10	14	21
DBAP	900 ±10	100 ±20	130 ±30	280 ±30	280 ±30(a) [(a)]	90 ±10
CIM	N.D.[(b)]	N.D.	N.D.	60 ±20	60 ±10 (b)	60 ±40
MSDP	N.D.	N.D.	N.D.	N.D.	60 ±20 (b)	200 ±100
B5G	80	90 ±10	220 ±30	220 ±30	120 ±30 (b)	90 ±10

[(a)]Letter designations at 14 days indicates the results of Tukey's HSD analysis, with common letters among treatments representing no significant difference from 3 biological replicates.

[(b)] N.D. indicates that the packed cell volume was below the detection limit (50 µl).

Table 2. Comparison of various cell- and tissue culture regimes to generate tissue mass over a 21-day culture period. The starting material for each replicate was 500 mg with the treatment time course (day 0) beginning with the second medium in each treatment. Three independent replicates were measured for each treatment.

Treatments	Fresh weight (mg) per day							
	1	3	5	7	10	14	18	21
Liquid DBAP to liquid DBAP	500 ±10	700 ±70	730 ±50	940 ±70	1500 ±130	2400 ±380 (a) [(a)]	2300 ±570	2000 ±600
Solid CIM to solid CIM	510 ±10	540 ±30	580 ±40	630 ±10	730 ±70	720 ±20 (b)	720 ±210	730 ±200
Solid CIM to liquid DBAP	510 ±10	720 ±30	740 ±150	780 ±180	750 ±230	740 ±240 (b)	830 ±290	660 ±280
Liquid DBAP to solid CIM	500	660 ±50	720 ±40	820 ±20	860 ±90	940 ±140 (b)	980 ±70	970 ±80

[(a)] Letter designations at 14 days indicates the results of Tukey's HSD analysis, with common letters between treatments representing no significant difference from 3 biological replicates.

Figure and table legends

Table 1. Media screen for the establishment of suspension cultures over a 21-day culture period. Cell growth was monitored for 21 days after filtration through a 100 µm cell strainer by measuring the packed cell volume (PCV). Three independent replicates were measured for each medium.

Table 2. Comparison of various cell- and tissue culture regimes to generate tissue mass over a 21-day culture period. The starting material for each replicate was 500 mg with the treatment time course (day 0) beginning with the second medium in each treatment. Three independent replicates were measured for each treatment.

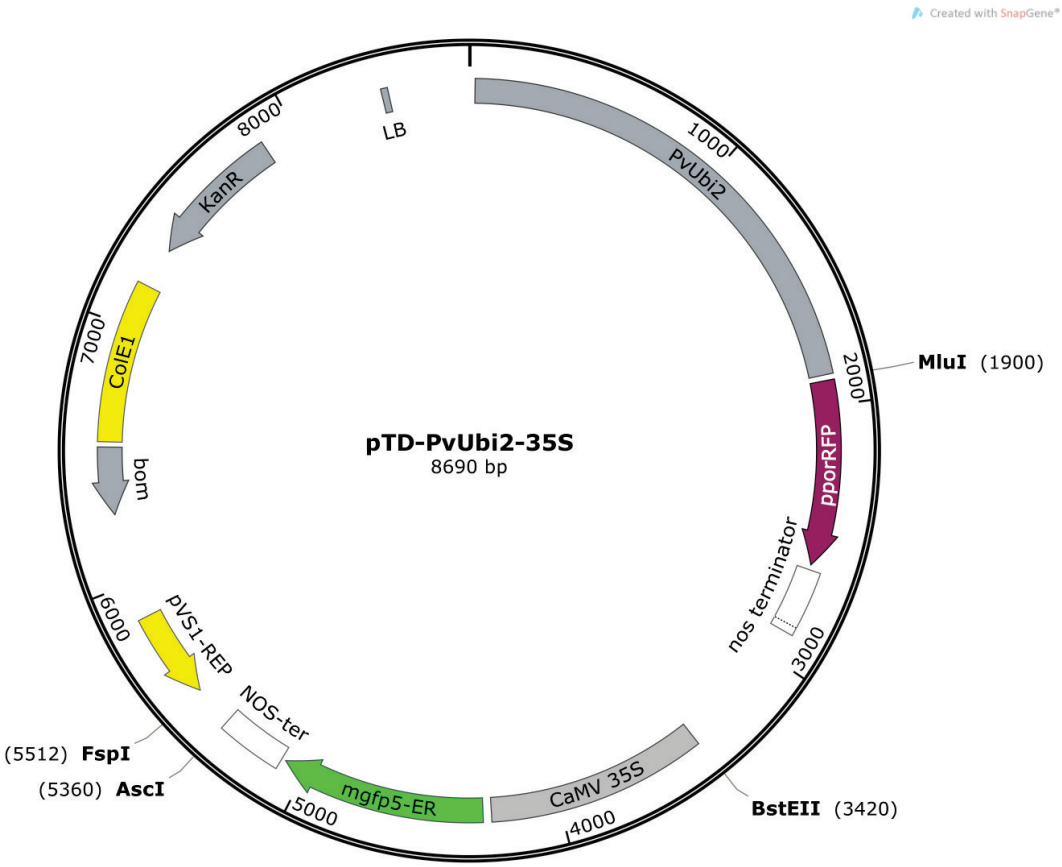

Figure 1. Map of pTD-*PvUbi2-35S vector*. Abbreviations: *PvUbi2* (*Panicum virgatum* polyubiquitin *2* promoter and intron), *pporRFP* (*Porites porites* red fluorescent protein coding region), *nosT* (*Agrobacterium tumefaciens* nopaline synthase terminator), CaMV 35S (cauliflower mosaic virus 35S promoter), mgfp5-ER (modified green fluorescent protein with an endoplasmic reticulum targeting sequence), ColE1 (origin of replication in *E. coli*), bom (origin of transfer in *E. coli*), KanR (kanamycin bacterial resistance marker), LB (T-DNA left border). Unique restriction sites used for vector construction are also indicated.

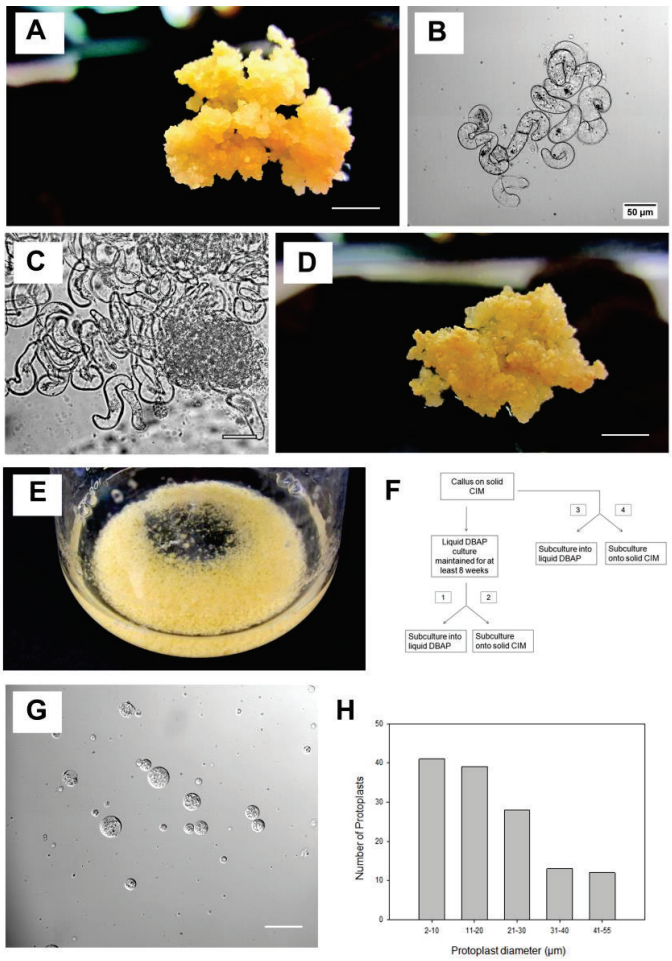

Figure 2. Suspension cell culture establishment and protoplast isolation. (A) One month-old callus grown on solid CIM medium. (B) Cells from seven day-old filtrated suspension cell culture in liquid DBAP medium. (C) Cells from fourteen day-old established suspension cell culture in DBAP medium. (D) Callus from one month-old suspension cell culture regrown on CIM solidified medium. (E) Large cell clusters (1-5 mm) after 1 week of subculture of 5 ml of PCV in 20 ml of fresh DBAP medium in a 125 ml Erlenmeyer flask. (F) Experimental scheme for comparing tissue culture and suspension cell culture. All treatments were initiated from callus on solid CIM: (1) liquid DBAP to liquid DBAP (2) liquid DBAP to solid CIM (3) solid CIM to liquid DBAP (4) solid CIM to solidified CIM medium. (G) Protoplasts isolated from a 5 day-old suspension cell culture. (H) Distribution of protoplast size in 5 samples of isolated protoplasts. Scale bar in A and D is 500 µm, scale bar in B, C and G is 50 µm.

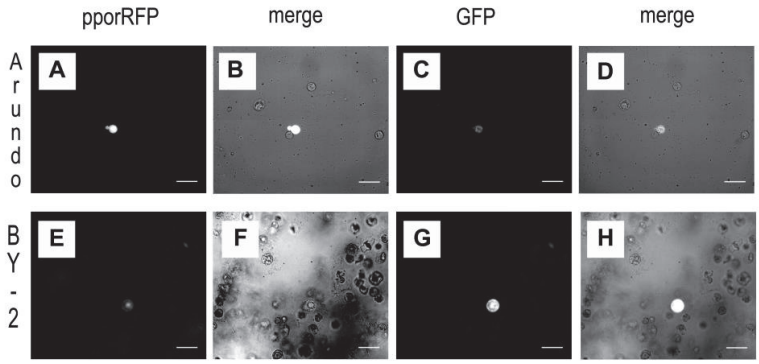

Figure 3. Transient expression of GFP and pporRFP fluorescent proteins in *Arundo* and tobacco BY-2 protoplasts transformed with pTD-*PvUbi2-35S* by electroporation. (A, B, C, D) *Arundo* protoplast. (E, F, G, H) tobacco BY-2 protoplast. Visualization of transformation using a TdTomato filter set: 545/30 nm excitation and 605/50 nm band pass emission (A, B, E, F) and GFP filter set: 470/30 nm excitation and 525/50 nm band pass emission (C, D, G, H). (B, D, F, H) represent the merge of fluorescent and bright field images. Exposure time was 300 ms and the scale bar is 50 µm in all pictures.

Part III

Manuscript to be submitted:
Dlugosz et al.,

Cryopreservation of switchgrass (*Panicum virgatum*) protoplasts

E.M. Dlugosz[1],

G. Pigna[1,3],

S.C. Lenaghan[2],

and C.N. Stewart, Jr.[1]

[1]Department of Plant Sciences, The University of Tennessee, Knoxville, Tennessee

[2]Center for Renewable Carbon, The University of Tennessee, Knoxville, Tennessee

[3]Department of Biosciences, Università degli Studi di Milano

Abstract

In recent years, the increased effort in genetic modification of plant cells has required methods for effective long term storage of materials with a reduced probability of unwanted mutations. Cryopreservation allows the indefinite storage of plant cells and protoplasts with low cost, minimal effort, and a small space requirement. The two most common procedures for cryopreservation are slow-freezing and vitrification, both of which reduce the formation of ice crystals during freezing through application of a cryoprotectant. In this work,

vitrification and slow-freezing methods were evaluated for cryopreservation of switchgrass mesophyll protoplasts. In total, ten vitrification solutions were tested, along with four slow-freezing solutions. Slow freezing resulted to be more efficient than vitrification, as demonstrated by the higher percent recovery of alive protoplast, 45.8% instead of 5.18%. The large difference between the two methods could depend on the toxicity of the components or on the different rate of temperature change. These results pave the way to further experiments of transformation and regeneration of cryopreserved protoplasts.

Introduction

Cryopreservation is a process where biological material is preserved by cooling it down to very low temperature, close to -200 °C, and kept alive thanks to the presence of cryoprotectants that ensure cell integrity [1]. Thanks to this technique, aseptic material can be stored at low temperature (generally -80 °C), potentially indefinitely, in sterile conditions, and used when needed after thawing. Cells, organs, tissues or embryos can be blocked in time and hence kept free of spontaneous genetic changes due to growth and DNA replication . There are two ways to perform cryopreservation: vitrification or slow-freezing [2,3]. These two methodologies differ by the composition of the solutions employed and by the physical steps involved in the process. During vitrification cells are immerged in very viscous solutions and quickly brought to low

temperatures while slow-freezing involves less viscous and toxic solutions but requires special freezers or specific tools that slowly decrease sample temperature. This technique is widely used in medicine in support of medical therapies. For instance, cryopreservation of ovarian tissue is used to guarantee fertility to patients under aggressive chemotherapy [4], while the same technique applied to human umbilical cord blood fractions provides an alternative source of mesenchimal stem cells for therapeutic applications[2]. In plants cryopreservation is less popular, although its potential in plant science is easily appreciable. It is important for the maintenance of wild and rare species [5] or more in general it allows genotype banking, for instance of pollen[6]. Moreover this technique has a great potential in preserving lines, created by breeding or genetic transformation, that could not be kept in a laboratory space, that are sterile (e.g. do not produce flowers), or as a back-up of plant material [7]. This powerful tool is particularly delicate when protoplasts are involved. Plant protoplasts are an important source for a wide variety of experiments. They are rapidly obtained from leaves or cell suspension cultures and can be transformed more easily than cell cultures with different techniques [8]. Cryopreservation of protoplasts has been reported in few works [2,3] and requires a dedicate protocol that takes into consideration cell osmolarity and requires physical manipulation, beside cold temperature tolerance.

The need of renewable fuel sources had exploded in the last decade, therefore more and more plants have been tested for bioenergy production. Switchgrass (*Panicum virgatum*) is a perennial grass native to North America, chosen as the most promising biofuel crop in the US [9]. The aim of this study was to develop a protocol for cryopreservation of switchgrass protoplast using vitrification or slow-freezing for the long term storage of transgenic lines.

Material and Methods

Plant material

Panicum virgatum cv. Alamo seeds were obtained from Bemert Seed Company (Muleshoe, Texas, USA). For initial optimization, Alamo seeds were planted at an approximate density of 20 mg/cm2 in Fafard 3B soil mix (Sun Gro Horticulture, Agawam, Massachusetts, USA), and grown with a 16 h light, 8 h dark cycle at 22 °C to generate lawns of switchgrass plants in nursery flats.

Protoplast isolation

Protoplasts were isolated form leaf tissue as reported in Burris et al. **[8]**. Four-hundred mg of leaves were cut into 2 cm long pieces and incubated with shaking at 80 rpm for 2 hours in 20 ml of buffer solution (0.6 M mannitol, 10 mM MES, 1 mM $CaCl_2$, 5 mM 2-mercaptoethanol, and 0.1 % BSA, pH 5) containing food-grade enzymes at the manufacturer's suggested concentrations

(Rohament CL 1320 ECU, Rohapect 10L 840 ADJU, and Rohapect UF 0.0065 ADJU) (AB Enzymes, Darmstadt, Germany) and filtered through a 0.22 µm syringe filter (Millipore Express PES Membrane, Merk Millipore Ltd, Tullagreen, Carrigtwohill Co. Cork, Ireland). Following incubation, the suspension was filtered through a 40 µm filter (Fisherbrand, Fisher Scientific, Hampton, New Hampshire, USA). Five milliliters of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7) was then passed through the same filter to dilute the enzyme solution and maximize protoplast recovery. Protoplasts were collected by centrifugation at 150 × g, 22 °C for 10 min and the enzyme solution discarded. Protoplasts were then resuspended in W5 solution, counted, and viability assessed with propidium iodide (PI) staining (working solution: 1 mg/ 50 mL, Sigma–Aldrich, St. Louis, Missouri, USA). Each protoplast preparation gave about 10^6 protoplast/ml. Protoplasts were kept on ice following isolation and prior to further experiments.

Vitrification Technique

For each vitrification solution (**Table 1**), a loading solution was prepared (loading solution = 25% vitrification solution with a supposed osmolarity of 550mOs). Vitrification methods were similar to those used in Liu et al. [2]. Around $1 \cdot 10^6$ freshly-isolated protoplasts (1 ml) were dispensed in cryovial (Salimetrics, Carlsbad, CA, USA) and spun at 500 × g for 5 min, the

supernatant was removed, and the pellet was re-suspended in 1 ml loading solution. Protoplasts were incubated on ice for 5 minutes in the loading solution and then spun at 500 × g for 5 min. The supernatant was removed, and the pellet was re-suspended in 1 ml of the vitrification solution. Protoplasts were thoroughly mixed in the vitrification solution and the vials were quickly submerged in liquid nitrogen. Protoplasts were stored in liquid nitrogen overnight. Thawing tests were performed by removing the vials from the liquid nitrogen and immediately submerging them in a 37^{o} C water bath. When the solution was completely thawed (approximately 2-3 minutes), the protoplasts were transferred to 10 ml of unloading solution (same as base media; 0.7 M sorbitol in MS). Prior to adding the unloading solution, tubes used for unloading were coated with 5 % Fetal Bovine Serum (FBS) (Gibco, Thermo Fisher) to prevent protoplasts from sticking to the walls of the tube during unloading. After 20 minutes on ice, the unloading solution with the protoplasts was spun at 500 × g for 5 min. The supernatant was carefully removed and the protoplast pellet was re-suspended in 1 ml base media (0.7 M sorbitol in MS). The protoplast solution was thoroughly mixed and vials were kept in the refrigerator or on ice until counted.

Slow-freezing technique

Four different slow-freezing solutions were used (**Table 2**). Around $1 \cdot 10^6$ protoplasts (1 ml) were spun at 500 × g for 5 min, the supernatant was removed, and the pellet was re-suspended in 1 ml of slow-freezing solution. The protoplasts were thoroughly mixed with the slow-freeze solution by pipetting and the solution was transferred to a cryovial. Protoplasts were incubated approximately 5 minutes at room temperature and mixed again by inverting the vial. Vials were added to a CoolCell (Biocision, Menlo Park, CA) and placed in -80^{o}C freezer overnight. Thawing tests were performed by removing the vials from the -80^{o}C freezer and 0.5 ml base media (0.7 M Sorbitol + MS, warmed to 37^{o}C) was immediately added to the cryovial. The vial was closed again and immediately submerged in a 37^{o}C water bath for 2-3 minutes until the protoplast solution was completely thawed. The vial was mixed well and protoplasts were counted.

For both techniques protoplasts were counted twice: once right before cooling and once after thawing.

Control samples of protoplasts were maintained in a solution of 0.7 M sorbitol (Sigma–Aldrich) in MS [10] media. Protoplast viability was assessed using propidium iodide stain. Percentage of recovered protoplasts after cryopreservation was calculated as follows:

$$\frac{\textbf{[total protoplasts (after cryopreservation) * \% live protoplasts (after cryopreservation)]}}{\textbf{[total protoplasts (initial) * \% live protoplasts (initial)]}}$$

Chemical

(DMSO: Sigma-Aldrich; Ethylene glycol: Thermo Scientific, Waltham Massachussetts; Glycerol: Thermo Scientific; Sucrose: Sigma-Aldrich, Polyethylene glycol 6000: Sigma-Aldrich; Acetamide: Sigma-Aldrich; Propylene Glycol: Thermo Scientific).

Results

Vitrification technique

All vitrification solutions had a significantly higher percent recovery than controls. The number of protoplast counted after cryopreservation was 26% of the initial one, meaning that most protoplasts exploded due to harsh cooling or lost during other steps (e.g. during centrifugation). Living protoplasts were still round in shape while dead ones, detected with propidium iodide, were mostly deflated or showed membrane damage. The percent recovery of protoplasts subjected to vitrification ranged from 0.72 to 5.18% (**Figure 1**) and the results also show no statistical difference among the different treatments, ANOVA ($p>0.05$).

Slow-freezing technique

The number of protoplasts recovered after cryopreservation by slow-freezing was higher compared to the vitrification technique. Living protoplasts were round in shape and presented well distinguishable chloroplasts inside (**Figure 2A**), while dead protoplasts had lost turgor, presented clumps of chloroplast and collapsed nuclei (**Figure 2B**). Also, less chloroplasts were detected outside the cells as compared to vitrified samples. All slow-freezing methods yielded higher percent recovery of protoplasts (**Figure 3**) as compared to vitrification methods. Slow-freezing methods may result in better recovery of protoplasts as a result of either the slow cooling rate or the lower toxicity of solutions. Compared to recovery in a control solution (13.16% +/- 0.008), all four slow-freezing solutions showed a significant increase in protoplast recovery ($p < 0.002$). Recovery after slow-freezing in 12% DMSO + 10% FBS was not significantly different from 12% ethylene glycol + 10% FBS and 12% DMSO (no FBS) solutions ($p = 0.0987$ and 0.1694, respectively). However, compared to 12% ethylene glycol (no FBS), there is a significant increase in protoplast recovery using the 12% DMSO +10% FBS solution ($p = 0.0419$).

Discussion

Ten vitrification and four slow-freezing solutions were evaluated for the cryopreservation of switchgrass mesophyll protoplasts with the aim of storing and recovering material for future experiments. Although Liu et al. [2] reported

percent recovery over 40% for *Porphyra yezoensis* protoplast using the vitrification solution VS6, none of the ten vitrification solutions tested in switchgrass yielded a percent recovery higher than 5.18%. Overall, slow-freezing methods yielded better results in terms of viable protoplast recovery following cryopreservation. There are a number of explanations that may account for the relatively low percent recovery of protoplasts after the use of vitrification solutions. First, the vitrifications methods (e.g. plunging cryopreservation tubes into liquid nitrogen) may be too harsh for switchgrass mesophyll protoplasts, as the cooling rate is higher with vitrification and, therefore, the temperature leap could be detrimental to switchgrass protoplast. Secondly, the vitrification solutions themselves may be too toxic for switchgrass mesophyll protoplasts. Conversely to Liu et al. [2], we did not test the toxicity of the solutions to switchgrass protoplasts in non-freezing condition, therefore we cannot exclude this as an explanation. This represents one aspect worth of further investigation, but the important conclusion is that vitrification is definitely inferior to slow-freezing. The sucrose, presents in most of the vitrification solutions, is an osmotic stabilizer that helps to maintain membrane (and therefore protoplasts) integrity by substituting for water on the membrane surface and thus stabilizing membrane under dry and freezing conditions [11]. Sorbitol, in this case, may be more efficient in keeping cell osmolarity of switchgrass protoplast compared to sucrose. Sugar alcohols such

as sorbitol or mannitol are considered inert and tend to enter less easily into the cells compared to sucrose. Sorbitol, therefore keeps a high solute concentration outside the cell and maintains the osmolarity[12] by providing conditions where cells tend to shrink[13]. A useful experiment to test the beneficial effect of sorbitol on switchgrass protoplast would be to prepare a vitrification solution with sorbitol instead of sucrose and prepare the slow-freezing solution with sucrose instead of sorbitol. This would clarify if sorbitol has a relevant influence during cryopreservation. Another aspect to consider is that all the slow-freezing solutions contain BSA that, as reported by Langis et al. [3], helps reducing the loss in protoplast due to the physical manipulation during the procedures (such as centrifugation, pipetting etc) impeding or at least reducing the electrostatic interactions between the protoplast surface and the containers [3]. Slow-freezing may be more ideal for switchgrass mesophyll protoplast cryopreservation as a results of the slow, controlled rate of cooling and/or the lower toxicity of the solutions employed as opposed to vitrification. A much higher percent of recovery (90%) has been observed for cell suspension cultures of Arabidopsis and tobacco with slow-freezing, but the protecting effect of the cell wall [13] might also explain why vitrification is preferred to slow freezing systems when suspension cell culture are cryopreserved [14]. More studies are obviously required to improve cell storage of unique samples with this procedure. The limited literature on protoplast cryopreservation makes this

work a valuable starting point, even if preliminary. Cell density is of great importance for regeneration step and it is obvious that in this case it depends on the number of protoplasts surviving cryopreservation. Therefore, increasing this number is a primary goal of future experiments. It would also be important to improve the transformation efficiency of switchgrass protoplasts that, as reported by Burris et al. (**2016**), is below 25% in the best cases with PEG transient transfection. A stable transformation method is required to obtain transformed plants. Therefore, the key achievement that is still lacking is plant regeneration from switchgrass protoplasts. This will be very important to complete the cycle of transformation, cryopreservation and regeneration. In conclusion we propose a method for protoplast cryopreservation of switchgrass that represents a step forward for plant storage.

Vitrification Solution	Composition
VS1	30% DMSO, 15% ethylene glycol, 30% glycerol, 0.15M sucrose
VS2	40% ethylene glycol, 0.25M sucrose
VS3	20% DMSO, 8% polyethylene glycol 6000, 15% acetamide
VS4	5% DMSO, 15% ethylene glycol, 30% glycerol, 0.4M sucrose
VS5	5% DMSO, 15% ethylene glycol, 30% glycerol, 0.4M sucrose
VS6	10% DMSO, 30% glycerol, 10% sucrose
VS7	19% glycerol, 13% ethylene glycol, 13% propylene glycol, 6% DMSO, 0.5M sorbitol
VS8	30% glycerol, 15% ethylene glycol, 7.5% propylene glycol, 7.5% DMSO, 0.4M sucrose
VS9	30% glycerol, 15% ethylene glycol, 15% propylene glycol, 0.4M sucrose
VS10	50% glycerol, 50% sucrose

Table1: Vitrification Solutions. All vitrification solutions are reported in Liu et al., 2004.

Slow-freezing Solution	Composition
SF1	12% ethylene glycol, 1% BSA, 0.7M sorbitol + MS
SF2	12% ethylene glycol, 1% BSA, 10% FBS, 0.7M sorbitol + MS
SF3	12% DMSO, 1% BSA, 0.7M sorbitol + MS
SF4	12% DMSO, 1% BSA, 10% FBS, 0.7M sorbitol + MS

Table 2: Slow freezing solutions. All solutions were partially adapted from (Menges et al., 2004).

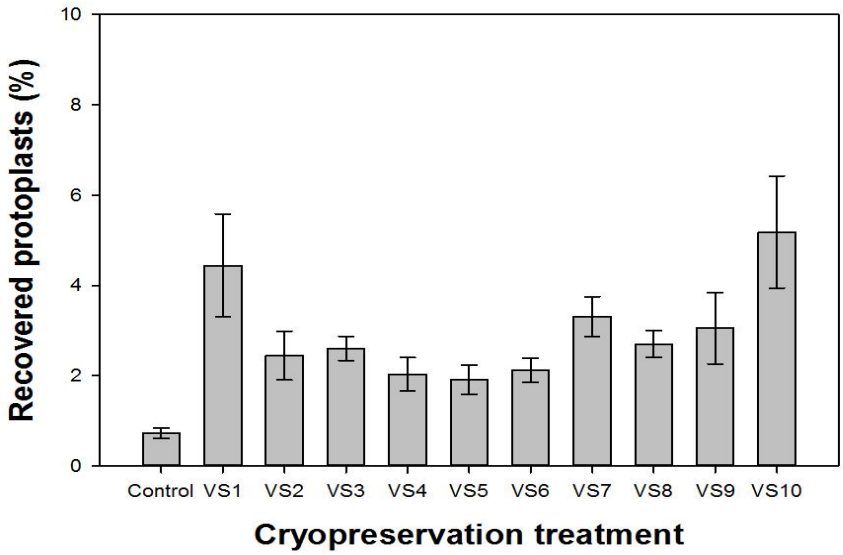

Figure 1. Protoplast recovery (% of initial counts) using vitrification on switchgrass mesophyll protoplasts. The experiments are the result of 4 biological replicates corresponding to 4 different protoplast preparations. Bars represent standard error (SE). No statistical difference was observed among the vitrified samples ($p>0.05$).

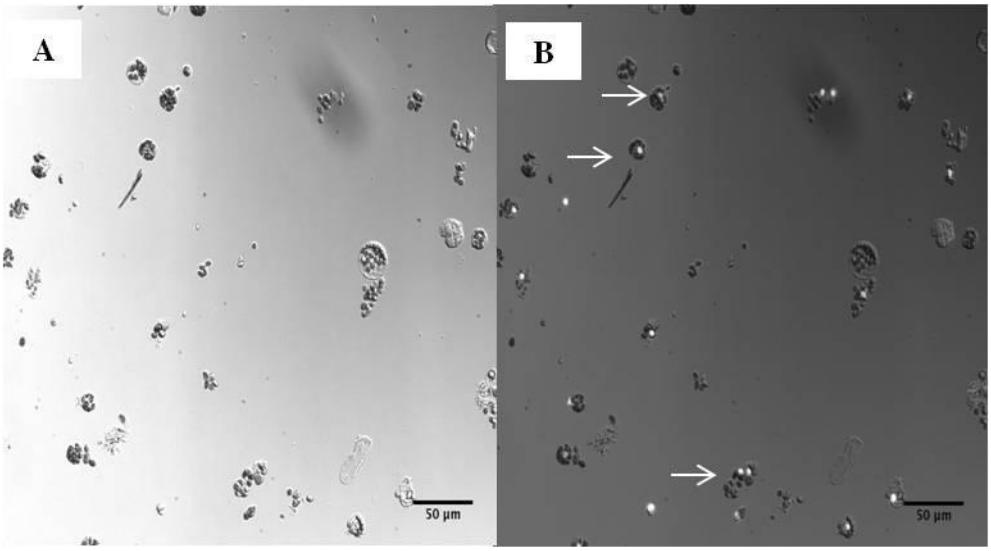

Figure 2. Recovered protoplasts using slow freezing solution SF4. Protoplast in bright field (A) and the bright field image with the fluorescence signal from propidium iodide visualized using a tdTomato filter set: 545/30 nm excitation and 605/50 nm band pass emission, arrows indicate dead protoplasts following the slow-freezing procedure (B).

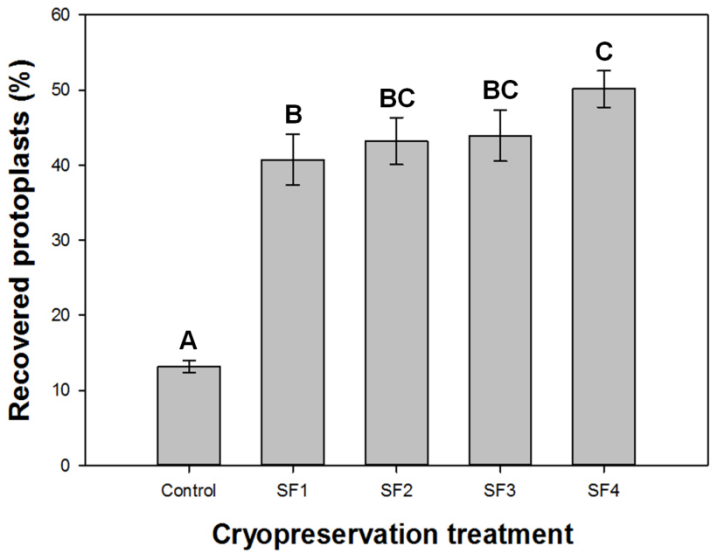

Figure 3. Protoplasts recovery (% of initial counts) using slow-freezing to cryopreserve switchgrass mesophyll protoplasts. All the experiments are the result of 4 biological replicates corresponding to 4 different protoplast preparations. Bars represent SE and letters indicate statistical differences ($p<0.05$).

References

[1] Yokoyama, W.M., Thompson, M.L. and Ehrhardt, R.O., 2012. Cryopreservation and thawing of cells. Current protocols in immunology, pp.A-3G.

[2] Liu, H., Yu, W., Dai, J., Gong, Q., Yang, K. and Lu, X., 2004. Cryopreservation of protoplasts of the alga Porphyra yezoensis by vitrification. Plant science, 166(1), pp.97-102.

[3] Langis, R. and Steponkus, P.L., 1990. Cryopreservation of rye protoplasts by vitrification. Plant physiology, 92(3), pp.666-671.

[4] Practice Committee of the American Society for Reproductive Medicine, 2014. Ovarian tissue cryopreservation: a committee opinion. Fertility and sterility, 101(5), pp.1237-1243.

[5] Lee, M.W., Choi, J., Yang, M.S., Moon, Y.J., Park, J.S., Kim, H.C. and Kim, Y.J., 2004. Mesenchymal stem cells from cryopreserved human umbilical cord blood. Biochemical and biophysical research communications, 320(1), pp.273-278.

[6] Senula, A. and Keller, E.R.J., 2013, August. Pollen cryopreservation to support maintenance of a wild species collection of the genus Allium. In II International Symposium on Plant Cryopreservation 1039 (pp. 289-296).

[7] Xu, J., Li, B., Liu, Q., Shi, Y., Peng, J., Jia, M. and Liu, Y., 2014. Wide-scale pollen banking of ornamental plants through cryopreservation. CryoLetters, 35(4), pp.312-319.

[8] Uragami, A., Sakai, A., Nagai, M. and Takahashi, T., 1989, June. Cryopreservation by vitrification of cultured cells and somatic embryos from mesophyll tissue of asparagus. In VII International Asparagus Symposium 271 (pp. 109-116).

[9] Burris, K.P., Dlugosz, E.M., Collins, A.G., Stewart Jr, C.N. and Lenaghan, S.C., 2016. Development of a rapid, low-cost protoplast transfection system for switchgrass (Panicum virgatum L.). Plant cell reports, 35(3), pp.693-704.

[9] Lewandowski, I., Scurlock, J.M., Lindvall, E. and Christou, M., 2003. The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe. Biomass and Bioenergy, 25(4), pp.335-361.

[10] Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia plantarum, 15(3), pp.473-497.

[11].H. Crowe, L.M. Crowe, J.F. Carpenter, C.A. Wistrom, Stabilization of dry phospholipid bilayers and proteins by sugars, Biochem. J. 242(1987) 1–10.

[12] Evans, D.A. and Bravo, J.E., 1983. Protoplast isolation and culture. Handbook of plant cell culture (USA).

[13] Menges, M. and Murray, J.A., 2004. Cryopreservation of transformed and wild-type Arabidopsis and tobacco cell suspension cultures. The Plant Journal, 37(4), pp.635-644.

[14] Reinhoud, P.J., Schrijnemakers, E.W., van Iren, F. and Kijne, J.W., 1995. Vitrification and a heat-shock treatment improve cryopreservation of tobacco cell suspensions compared to two-step freezing. Plant cell, tissue and organ culture, 42(3), pp.261-267