Agronomic and digital phenotyping evaluation of sweet sorghum public varieties and F_1 hybrids with potential for ethanol production in Spain

Verónica Codesido, Rubén Vacas, Bibiana Macarulla, Maria Pilar Gracia, Ernesto Igartua*

Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas, Avda de Montañana 1005, 50059, Zaragoza, Spain
*Corresponding author: E-mail: igartua@eead.csic.es

Abstract

Sweet sorghum is receiving a lot of attention as a potential crop for bioethanol production in the Mediterranean area. Its advantages are a combination of high productivity and good response to abiotic stresses. There is a lack of information on adaptation of sweet sorghum cultivars to Mediterranean conditions. This investigation was undertaken to explore the adaptation and agronomic traits of a group of international varieties of sweet sorghum, to identify the best candidates to initiate a breeding program for this species in Spain. Sixteen varieties, chosen based on passport, evaluation and genetic data from the USDA GRIN database, were sown in 2011 in an irrigated field plot in Zaragoza, Spain. Several agronomic traits, like fresh weight were determined in the field. Juice samples were analyzed for Brix and POL score of the juice of the stalks. Some of the varieties, particularly Sugar Drip, MN2826, Smith, Ramada and Dale, offer good prospects to initiate a breeding program for Spanish conditions, due to a combination of good agronomics, high sugar content and spread of flowering dates. At the same time, these varieties were used as pollinators to produce hybrids in crosses with either sweet A-lines or grain sorghum populations. The potential to use F_1 hybrids in this species was explored by analysing the growth of five of the most representative F_1 hybrids (three F_1 sweet sorghum hybrids and two crosses of grain sorghum by sweet sorghum) and their parents through a digital phenotyping analysis. Plant size was monitored on a daily basis. The sweet sorghum varieties apparently revealed different heterotic behaviour when crossed to sweet or grain female parents. Mid-parent heterosis for plant growth was detected, but suffered variations over time, which may be related to the experimental system.

Keywords: sweet sorghum, breeding program, sucrose content, flowering date, Brix, POL, digital phenotyping, heterosis

Introduction

Sweet sorghum is considered one of the best feedstocks for ethanol production, due to a favourable combination of agronomic and technological characteristics. It belongs to the same species as grain sorghum (*Sorghum bicolor* (L) Moench), but this denomination refers specifically to varieties that have a high concentration of soluble sugars in the stalk.

Sweet sorghum was traditionally used to make syrup for confectionery uses. After the 1970s, it gained reputation as a potential energy crop given its high potential of biomass and sugar production. It contains high soluble sucrose (43.6–58.2%), glucose and fructose in the stalk (Billa et al, 1997; Dolciotti et al, 1998; Amaducci et al, 2004; Antonopoulou et al, 2008), and 22.6-47.8% insoluble cellulose and hemicelluloses (Dolciotti et al, 1998; Rattunde et al, 2001; Antonopoulou et al, 2008). Juice content in the stems can be as high as 65-80%. Sugar content in the juice of the stems ranges around 9-15%, whereas sugar content in the fresh stem is between 7.9 and 12.0%. At harvest, sugar concentration in the stalks (on dry weight) may range between 20 and 45%, depending on the cycle length. Besides, the juice can be fermented very efficiently (up to 90%, according to Ratanavathi et al, 2004).

Like other sorghum types, sweet sorghum is a tropical crop, domesticated in Africa, and later disseminated to other tropical regions and, quite recently, to temperate zones. Breeding programs for temperate areas were first initiated in the US Sugar Crops Field Station, in Meridian, MS, with the conversion of tropical sweet sorghum into varieties adapted to temperate zones (mostly by removing the sensitivity to photoperiod), and carried out extensive breeding work for syrup production from 1940 until 1983. Other relevant breeding efforts were carried out at the universities of Texas A&M, Kansas State, Georgia and Nebraska, and thus much of the sweet sorghum germplasm for temperate zones comes from the USA. The Meridian centre also led the assembly of the first world collection of sweet sorghum, later transferred to the USDA in Griffin, GA (Freeman et al, 1973). The USDA currently holds 2,054 accessions

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of sweet sorghum in its active collection (http://www.ars-grin.gov), among them many of the varieties used or bred in the USA. Because of the richness of the collection and its focus (among others) on materials for temperate zones, it is the most interesting source of germplasm for Mediterranean countries.

Nowadays, sweet sorghum is experiencing a revival as an energy crop in places like the Mediterranean basin (Gnansounoua et al, 2005; Dolciotti et al, 1998). Its adaptation in temperate zones is limited by its low cold tolerance (Petrini et al, 1993), thus constraining its cultivation in Europe to the Mediterranean zone (Miller and Creelman, 1980). The interest in this crop is currently increasing for several reasons. On one hand, the use of biofuels is encouraged by a strong political will, as the EU has committed to increase the proportion of renewable energy from 9% in 2010 to 20% of total energy consumption by 2020 (EU, 2009). Also, EU Directives admit the cultivation of sweet sorghum in set-aside lands, therefore enhancing the economic significance of this crop in South-European regions. Further encouragement for the adoption of this crop comes from the European support of the Kyoto agreement, which spurred the enactment of Directives and national laws aimed to promote the use of biofuels and set indicative targets for their use in the transport industry (Fernández and Curt, 2005). This increase would contribute to reduce emissions of global warming gases and other particles harmful to human health and the environment. Biofuels, therefore, represent a fundamental element in preventing pollution and in supporting the use of renewable energy, as long as their adoption does not compromise food production or prices. On the other hand, sweet sorghum has been identified as the most promising annual crop for energy production in the Mediterranean region attending to agronomic reasons (Zegada-Lizarazu et al, 2012), in part due to its remarkable drought tolerance (Wayne and Frederiksen, 2005; Almodares and Mostafafi, 2006). Moreover, sweet sorghum has been reported as suitable for reclamation of contaminated soils and treatment of waste water (Fernando et al, 2010).

In Spain, early studies on the use of sweet sorghum for energy purposes in Andalusia (Olalla et al, 1983a,b,c) and central Spain (Fernández, 1990) revealed a good potential of sweet sorghum for energy production purposes under favourable irrigation conditions. Also, good drought tolerance was confirmed under Spanish conditions (Curt et al, 1995). This feature will have to be included in breeding programs because water scarcity will be a common challenge in most irrigated lands in Spain for the foreseeable future (Fereres and Soriano, 2007), and water costs will tend to increase to approach cost recovery after the approval of the EU Water Framework Directive.

Companies aiming at the development of biofuel plants face the challenge of finding cultivars adapted to the local conditions, and also securing the seed supply by their own means, reducing dependence on external sources. All these considerations justify the interest of starting breeding activities for sweet sorghum in Spain, and also of the identification of suitable germplasm to serve as a starting point.

This study focuses on the evaluation of a set of selected lines from the USDA collection, to start a sorghum breeding program in Spain with the generation of hybrids from the most promising parents. Most of the area devoted to grain sorghum in the USA is sown with hybrids, to take full advantage of the high levels of heterosis found in this crop (20 to 60%, according to Axtell et al, 1999). However, most sweet sorghum cultivars are still inbred lines (Pfeiffer et al, 2010). The development and adoption of sweet sorghum hybrids could be fostered if they show the same level of heterosis as the grain types (Leo, 2005). As early as 1963, Quinby already described the existence of heterosis in sorghum for earlier blooming, increased height, larger stems, greater production of grain and biomass, and larger panicle heads with a higher threshing percentage. Since then, these features have been confirmed in abundant literature on the matter (for instance, Haussman et al, 1999; Makanda et al 2010). Earlier flowering, greater biomass from increased height and larger stems are all sought-after traits for sweet sorghum, and hybrid production may be a way to achieve them. There has been little research on heterosis in sweet sorghum so far (Pfeiffer et al, 2010), although this area is booming recently. Sorghum hybrids are usually produced using the A1 cytoplasmic male-sterility system because growers and breeders are familiar with it, and numerous lines restore fertility in the A1 system, making production of fertile hybrids possible (Pfeiffer et al 2010). However, fertility of hybrids is not a requisite for sweet sorghum, as the economic part of the plant is the stalks.

The specific objectives of this study were (1) to sample the genetic diversity of the USDA sweet sorghum collection, to select promising varieties for Spain, (2) a cursory evaluation of agronomic and quality traits of these varieties in Zaragoza (Spain), (3) to survey the relationship between stem sugar content and agronomic traits, particularly flowering date, (4) to identify parents with favourable traits to produce potentially superior sweet sorghum hybrids, and (5) to test the most promising hybrids and calculate heterosis for plant growth.

Materials and Methods

Plant material

Twenty lines were selected from the USDA GRIN database (USDA, 2013) that complied with the following criteria: high Brix grade, spread of flowering dates (particularly including the earliest material), good resistance to anthracnose (*Colletotrichum spp*, which affects grain sorghum in Spain), and elevated plant height or biomass. Also, the lines were selected to represent all four germplasm groups determined by Wang et al (2009), to ensure a wide sampling of genetic diversity. These groups represented US historic syrup type sweet sorghum accessions (G1), durra accessions from Asia (G2), landraces from Africa and a few modern US lines developed for sugar and energy production (G4) and a group of missed origins (G3).

Additionally, three male-sterile lines (AMP472, AMP493 and A3BTx3197), and two of their maintainer counterparts (BMP472 and BMP493) were also included in the experiment, as female parents to produce experimental hybrids crossed with the set of 20 lines. Also two grain sorghum populations obtained by EEAD (Zaragoza) were planted, to provide male sterile plants to do crosses with the sweet sorghum lines. These populations were selected locally for tolerance to abiotic stresses, one to low water availability (AD11B, Gracia et al, 1997), and the other for resistance to salinity (AD11RRP). These two grain sorghum populations segregated for a nuclear male sterility system and, therefore, a proportion of plants were male sterile and could be used as female parent in crosses. Grain sorghum populations like the two EEAD ones presented here are heterogeneous. The crosses of sweet sorghum lines (as male) with grain sorghum plants (as female) were done on one single female plant per pollinator. But each female plant was different and thus, all crosses on the grain sorghum populations are different, i.e. they are not half-sibs. Nevertheless, the crosses to these grain sorghum populations could be useful to assess the potential of grain x sweet crosses.

The experiment was carried out at the experimental field of the Aula Dei Research Station (EEAD-CSIC) of Zaragoza, in 2011. The UTM coordinates of the plot location referred to the MTN E 1:50.000 are: 30TXM814215; Z=225 m. The soil is typical xerofluvent, sandy loam, mixed (calcareous), with an effective depth of 120 cm (Farré, 2006). The same plots were used for crossing and for the determination of phenotypic traits.

All but three sweet sorghum lines (Rio, M81E and Della, that were received later) were planted on May 4, 2011 in 2 row plots, 6 m long, 1 m row spacing, using 40 seeds per row, a single replication per genotype, due to scarcity of seed. The two grain sorghum populations were planted in plots of 20 rows, also 6m long, 1m row spacing. The whole experiment size was 22 x 25 m (including grain sorghum plots), was irrigated by a system of five sprinklers, four at each corner of the plot, and one at the centre. The 15 m wet radius ensured an appropriate sprinkler overlap and good irrigation of the whole plot. Irrigation was provided twice a week, following the schedule of commercial maize fields planted in the same farm. Emergence was noticed on May 23. On June 5, 2011, the three additional lines were hand-sown in single rows, adjacent to the previous ones.

The three A-lines and the grain sorghum populations were used as females. The rest of the lines were used as males. The three maintainer lines were crossed to their A-lines counterparts, to multiply them. All possible crosses were made in the summer of 2011 at the experimental farm of the EEAD-CSIC, in Zaragoza, Spain. Self-pollinations of parents were made to obtain seeds from the parental lines for future purposes.

Before anthesis, as the flower head was emerging from the flag leaf, the flower head was bagged with a sorghum pollinating bag (female lines, Broadhead, 1979). Plants were bagged on all plots to ensure that only received the pollen of the male parent chosen for us on each cross. Pollen was collected in paper bags from restorers (male lines) in the early morning hours (before 11:00 am) and dusted on to female earheads. Plots were harvested when the seeds on the male fertile pure-line varieties were at the hard-dough stage, stage 8 (Vanderlip, 1993).

Agronomic traits

Flowering date (number of days from emergence) was recorded when 50% of stems in the plot reached 50% flowering (determined as anther exertion occurring on 50% of the panicle). In September, when all the lines had reached at least 50% of flowering, plant height (measured from base to tip of the panicle in cm), number of nodes (counted from the top to the base) and panicle length (defined as the length from panicle neck node to the uppermost grain tip in cm) were measured on the main stems of all the plants at each plot.

Each line was harvested at soft dough stage, to guarantee that the lines were at a similar physiological stage and also to sample at the moment in which sugar accumulation reached an optimum level (Bian et al, 2006; Zhao et al, 2009). When the lines reached the soft dough stage, five random selected main stems per line were cut and weighed in the field, stripped of leaves and panicles, and weighed again to determine the percentage of stalk. The stems were then cut into three equal segments. The diameter of each segment was measured with a Vernier calliper at the middle internode of each part. The same five main stems were used to assess biomass.

Sugar and biomass yield measurements.

The fractionated stems were crushed by applying mechanical pressure with pliers in a top-down manner, and the juice collected in 50 ml vials. A composite juice sample was made, mixing equal amounts of juice from the three cut parts of stalks at physiological maturity, still at the field. Immediately, a drop of this composite sample was placed in a hand digital refractometer to measure degrees Brix (a measure of the mass ratio of soluble solids to water). This is a widely used approximation for sugar content of aqueous solutions. The rest of the composite juice sample was taken to the lab and frozen (-20°C), for later use in sucrose determination by saccharimeter (percentage of sucrose in the juice determined by polarimetry, POL) analyses. The percentage of sucrose was determined by using ADS 420 Saccharimeter (Bellingham and Stanley Ltd, Kent, United Kingdom) on centrifuged and filtered stalk juice. Juice preparation was done by first centrifugation (5,000 rpm for 5 min) and the supernatant was filtered (0.45 µm sieve) to remove plant residues. Purity was calculated as POL/Brix * 100 and it is interpreted as the quantity of sucrose that corresponds to a particular Brix score. Sucrose concentration in the juice (mg/100 ml) was calculated by using the formula

$$[\alpha]_{\lambda}^{T} = 100 * \alpha / C * l$$

where $[\alpha]_{\lambda}^{T}$ is the specific rotation for the sucrose (that corresponds with a value of 40.777 when the saccharimeter measurements were made with a wave length of 589 nm at 20°C of temperature), α is the rotation angle of the unknown solution (data obtained by the saccharimeter), C is the sucrose concentration (g/100 ml) and l is the length of the saccharimeter tube (mm). The sugar available is the percentage (in weight) of sorghum stem juice that would be recovered as pure sucrose from the total soluble solids contained in a sample. It was calculated using the Winter Carp - Geerling formula (Spencer and Meade, 1952) as modified by Mathur (1978):

Available sugar (%) = [S - 0.4 (B-S)] x F

in which B stands for Brix and S stands for POL, both in percentage of the juice extracted, and F is the value of the factor dependent on the fibre percentage of the sample. The fibre percentage in sweet sorghum is 16.1, and the corresponding F value is 0.74 (Reddi, 2006).

Digital phenotype evaluation

A large number of crosses between the three male-sterile lines and the grain sorghum populations as females, and the 20 sweet sorghum lines were attained in the 2011 field season. Seeds from five of the most promising hybrids, attending to their parent's characteristics (high biomass, high sucrose content and different flowering period to allow us prolonging the growing and harvesting cycle), were sent to Keygene N.V. (Wageningen, The Netherlands) for growth analysis using digital phenotype evaluation in 2012. Three of the hybrids were crosses of sweet sorghum male sterile line AMP472 by sweet sorghum varieties Juar, Ramada and Smith as pollinators. The other two hybrids were crosses of Ramada and Smith by male sterile plants of grain sorghum population AD11B (Gracia et al, 1997). The parents were also included, for a total of 10 accessions (5 hybrids and 5 parents).

About 10 seeds per accession were sown on February 27, 2012. Four seedlings per accession were transferred to 3 l pots (one plant per pot) with general purpose pot soil at March 13, 2012 and placed on the PhenoFab® system on March 20. The pots were irrigated at field capacity at the beginning of the experiment and water was replenished daily throughout the experiment, incorporating fertilizer (7-7-7, N-P-K, plus MgO + microspores).

Genotypes were equally distributed between and within a total of four belts in one greenhouse compartment. During the experiment, plants were imaged daily from four side angles (0, 90, 180, 270 degrees) and from the top with an RGB (visible light) camera.

The conversion of the images to digital phenotypes was executed through the use of image analysis algorithms. A pixel based value predictive of the size of the plant per side and top image per plant per day was calculated (exemplified in Figure 1), to provide an overall measure of the above ground fresh green shoot size (defined as the variable "Area"). It was derived integrating the four side view images taken per plant per day. Means and standard errors of final size of the plant (area, in pixels) were calculated per day of the experiment. An analysis of variance was performed per day of the experiment, taking "Genotype" as a fixed factor and assuming a completely randomized design. Least significant differences per day (P=0.05) were derived from these analyses of variance. Mid-parent heterosis (MPH) and high parent heterosis (HPH) for variable "Area" were calculated.

Figure 1 - Algorithm output example (in red the selected plant image is visible as an overlay).

Results

The selection of the lines was based on data stored at the USDA GRIN germplasm inventory. Most sweet sorghum lines in the USDA catalogue were late or very late flowering. Therefore, there were only a few that combined either medium or early flowering with good agronomic and quality characteristics that, a priori, might be suitable for Spanish conditions. A

Table 1 - Means and standard errors (between brackets) of agronomic and juice quality traits, for sixteen sweet sorghum varieties, measured in five main stems in Zaragoza, Spain.

Common name	PI number	Plant height (cm)	Flowering date (days)	Panicle Length (cm)	Nº of nodes	Stem diameter (cm)	Fresh stem weight (g)	Stripped stem fresh weight (g)	Brix	POL	Purity	Available Sugar (%)
INYANGENTOMBI	144134	272 (6)	90	22 (1.8)	10.8 (0.0)	1.51 (0.07)	960 (33)	741 (72)	16.9 (0.1)	12.68	74.81	8.12
SUGAR DRIP	146890	351 (12)	95	25 (0.8)	12.0 (0.8)	1.44 (0.06)	1107 (42)	870 (72)	17.2 (0.5)	15.12	87.75	10.56
MN2826	170787	283 (3)	76	23 (0.7)	7.4 (0.4)	1.14 (0.08)	630 (52)	428 (35)	18.8 (0.5)	14.02	74.53	8.96
JUAR	180348	184 (5)	70	9 (0.9)	6.0 (0.8)	0.74 (0.04)	369 (23)	129 (22)	17.0 (0.4)	13.16	77.37	8.59
HONEY SORGHUM	181080	211 (4)	70	22 (2.2)	4.2 (0.4)	0.61 (0.05)	232 (11)	102 (20)	16.5 (0.5)	12.18	73.68	7.73
SMITH	511355	239 (6)	75	17 (0.5)	9.8 (0.4)	1.33 (0.11)	634 (45)	398 (25)	20.9 (0.2)	16.82	80.59	11.25
N109	535794	105 (1)	102	20 (2.7)	7.7 (1.4)	1.33 (0.07)	450 (35)	180 (23)	12.7 (1.7)	8.92	70.29	5.48
N110	535795	225 (13)	76	25 (1.2)	10.3 (0.3)	1.73 (0.20)	760 (37)	600 (51)	17.1 (0.9)	13.60	79.72	9.07
N111	535796	237 (0)	96	25 (0.0)	8.7 (0.0)	1.74 (0.18)	675 (56)	550 (53)	18.3 (0.8)	13.64	74.40	8.68
SWEET SORGHUM	563917	132 (3)	70	22 (1.7)	5.0 (0.4)	1.01 (0.05)	303 (11)	89 (12)	12.2 (0.1)	6.96	56.86	3.59
TRACY	586541	251 (6)	70	11 (0.6)	7.8 (0.6)	1.69 (0.10)	1070 (103)	575 (56)	13.0 (0.4)	6.94	53.22	3.33
RAMADA	651493	316 (5)	117	19 (0.6)	15.7 (0.5)	2.40 (0.06)	2277 (133)	1782 (107)	18.9 (0.3)	15.96	84.56	10.95
DALE	651495	330 (4)	105	20 (0.5)	13.6 (0.6)	1.60 (0.06)	1525 (116)	1129 (104)	19.5 (1.3)	14.02	72.01	8.76
RIO*	563295	169 (1)	79	25 (0.6)	8.7 (0.6)	0.82 (0.04)	290 (23)	150 (16)	16.6 (0.4)	13.64	82.02	9.21
DELLA*	566819	283 (13)	82	22 (0.8)	10.0 (0.7)	1.31 (0.12)	748 (72)	542 (48)	18.1 (0.5)	10.68	58.84	5.69
M81E*	653411	292 (5)	91	30 (0.2)	15.2 (0.9)	1.45 (0.09)	938 (58)	635 (51)	10.0 (1.0)	8.42	64.72	4.87

*sown one month later than the other accessions

majority of the lines selected were from the USA, but some lines from Africa, Asia and Oceania were also selected, to provide ample genetic diversity, and coverage of all germplasm groups defined by Wang et al (2009).

Agronomic and quality traits

Three of the lines (Brawley, Keller, Wray) had to be discarded from the study, because their characteristics were too far apart from the USDA GRIN evaluation data, suggesting seed mistakes. This kind of accident is not uncommon when using seed straight from germplasm banks. Another one, N108, failed to germinate. Therefore, the study focuses on the 16 lines listed in Table 1.

Field emergence was variable, as could be expected for seeds of different ages coming from a germplasm bank. Therefore, the crop stand was variable, and thus biomass determinations per unit of surface were not representative of uniform stands, and should be considered just as a rough assessment of potential of the lines (Table 1).

The flowering date for the 16 lines ranged from 70 to 117 days after emergence, confirming the ample variation for this trait shown by this set of lines. All sweet sorghum lines were later than the two adapted grain sorghum populations sown in a neighbour plot (65 days for AD11B and 67 days for AD11RRP). Therefore, we described the cycle of the sweet sorghum lines as medium (4 lines), late (6 lines) or very late (6 lines) for our region.

All sweet sorghum lines were almost completely free of symptoms of anthracnose, whereas the grain sorghum populations showed a moderate degree of infection, meaning that the disease was present in the plot, but the sweet sorghums were not infected. This fact is remarkable because, although anthracnose resistance was one of the criteria used to select the germplasm, we did not expect this good agreement between the USDA evaluation and our conditions, as there is no information available on the isolates or even species causing the disease in Spain. But it seems that they are not very different from the ones present in the trials performed by the USDA.

Plant height of most lines was remarkably high, as expected for sweet sorghums, and in good agreement with USDA data, with the exceptions mentioned in the first paragraph of this section. Height was highest for Sugar Drip (351 cm), whereas N109 was the shortest (105 cm). In general, later accessions grew taller and produced more biomass (Table 1) making them more suitable for ethanol production. There were also important differences for number of nodes (from 4.2 in Honey sorghum to 15.75 in Ramada), also related to cycle length and plant height (Table 1). There was also wide variation for panicle traits (Table 1). The stem diameter was thickest in Ramada (2.4 cm), whereas Honey Sorghum was the thinnest line (just 0.6 cm), with a majority of lines showing rather thick stems, between 1 and 2 cm.

Brix and POL values were rather high, in general, as expected given the previous selection of the lines.

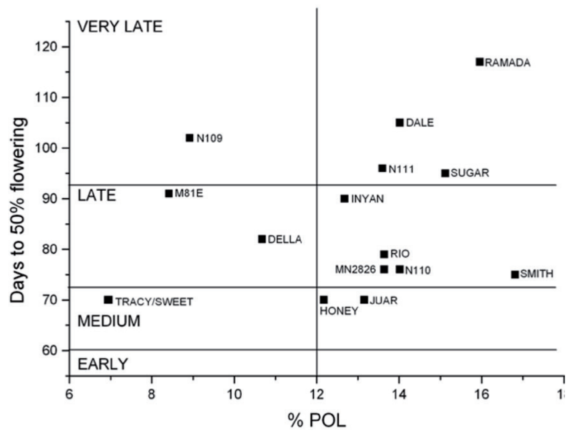

Figure 2 - Relation between flowering date and the % POL obtained for each line in the field experiment.

Table 2 - Pearson correlation coefficients among traits measured in the 16 sweet sorghum lines considered in the experiment

	Flowering date	Panicle length	Number of nodes	Stem diameter	Fresh plant weight	Fresh stem weight	Brix	POL	Purity
Plant height	0.41	0.19	0.71**	0.52*	0.72**	0.76**	0.40	0.43	0.23
Flowering date		0.26	0.74**	0.69**	0.73**	0.76**	0.15	0.28	0.33
Panicle length			0.33	0.07	-0.04	0.07	-0.15	0.04	0.16
Number of nodes				0.73**	0.82**	0.84**	0.13	0.31	0.28
Stem diameter					0.85**	0.86**	0.15	0.18	0.10
Fresh plant weight						0.99**	0.25	0.30	0.20
Fresh stem weight							0.33	0.40	0.29
Brix								0.88**	0.59**
POL									0.88**

These data, combined with the fresh weight of the stem, allowed a rough calculation of purity and percentage of available sugar (Table 1). There were also wide ranges of variation for Brix (from 10.1 in M81E to 20.87 in Smith), and for POL (from 6.94 in Tracy to 16.82 in Smith, Table 1).

Linear correlation coefficients between all traits (Table 2) revealed high and positive correlations between some agronomic traits as plant height, number of nodes, fresh plant weight and fresh stem weight (Table 2). Flowering date presented high positive correlations with all the other agronomic traits except with plant height. Interestingly, sugar traits were moderately, but not significantly correlated with agronomic traits. To illustrate the possibility of finding lines with good sugar production across a range of growth cycle durations, we plotted flowering date against sucrose percentage in the juice (POL) against each other (Figure 2), and found that it was possible to identify medium and medium late lines with high POL determinations, like Smith or Juar.

Digital phenotype evaluation

The experiment in the automated greenhouse was performed with the seed produced in the field experiment. It lasted 38 days, due to greenhouse space and time availability. Due to hardware errors, the images for day 8, 11, 12, 13, 14, 22, 23 and 37 were not available for analysis. In total 5920 images were generated for this experiment. Digital phenotyping was performed in a subsample of the crosses. We chose to analyse hybrids representing "sweet x sweet" and "grain x sweet" crosses with three sweet sorghum lines as male parents chosen for high Brix and POL values and different cycle (Juar as medium, Smith as late and Ramada as very late). The female parents were either the male-sterile line AMP472 or the grain sorghum population AD11B.

The number of tillers produced per plant varied from 3.3 for Ramada to 5.3 for Juar. The other three parents produced 4 tillers per plant. The five hybrids produced between 3.7 and 5 tillers per plant, with values intermediate between the two parents. The final size of the plant (area in pixels) on day 38 is presented in Supplementary Figure 1. Hybrids are presented between their parents. In general, hybrids presented larger area values than the parents, although these differences were not always significant (Table 3). The values of mid- and high-parent heterosis were positive in general, but rather low (Table 3).

An outstanding feature if this system is the possibility of monitoring growth very closely. We focused particularly on the evolution of heterosis over time. The evolution of the advantage of hybrids over the parents during the experiment was not homogeneous. In general, hybrids started the experiment with larger size than parents (though not significantly), then this advantage in size peaked during the second and third week of the experiment, then waning at the end of the third week, and remaining low and non-significant until the end of the experiment (Figure

Table 3 - Averages for digital area and number of tillers for five sweet sorghum hybrids and their parents at the end of the digital phenotyping experiment. Mid-parent heterosis (MPH) and high-parent heterosis (HPH) are also presented.

	Area (pixel count)			Percentage	
	F1	Parent 1	Parent 2	MPH	HPH
AMP472/Juar	254259 a	235668 ab	229540 b	9.3	7.9
AMP472/Ramada	239938 a	235668 a	216184 a	6.2	1.8
AMP472/Smith	268028 a	235668 b	250883 ab	10.2*	6.8
AD11B/Ramada	234372 a	202411 b	216184 ab	12.0*	8.4
AD11B/Smith	226576 b	202411 c	250883 a	0.0	-9.7
	Number of tillers			Percentage	
AMP472/Juar	5.0 ab	4.0 b	5.2 a	8.1	-4.8
AMP472/Ramada	4.5 a	4.0 a	3.3 b	22.7*	12.5
AMP472/Smith	4.0 a	4.0 a	4.0 a	0.0	0.0
AD11B/Ramada	3.7 a	4.2 a	3.3 a	-1.1	-11.8
AD11B/Smith	4.7 a	4.2 a	4.0 a	15.2	11.8

*significantly different from 0 (P<0.05)

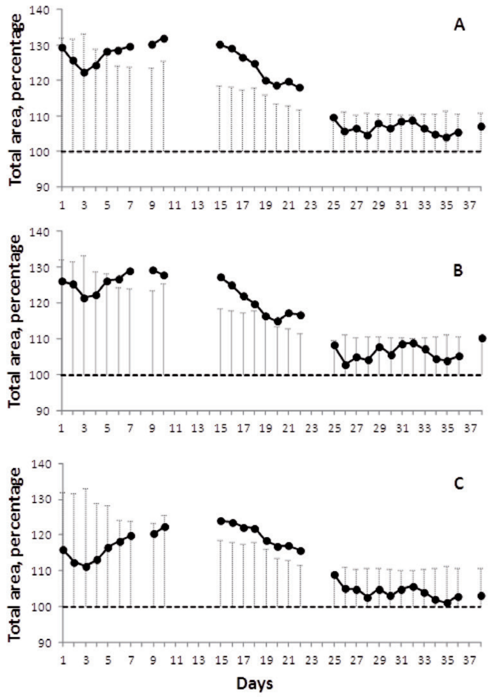

Figure 3 - Daily progress of heterosis for digital area of the plants. The average of the lines was set to 100 at each date, and the average of all hybrids is expressed as percentage of the area of the lines. Vertical bars indicate the LSD (P<0.05) between genotypes. The difference between averages at each day is the overall mid-parent heterosis. A) all genotypes (5 parents, 5 hybrids. B) genotypes involved in sweet x sweet crosses (4 parents, 3 hybrids). C) genotypes involved in grain x sweet crosses (3 parents, 2 hybrids).

3). This featured was evident for every hybrid tested in the experiment (data not shown).

Discussion

The success of any breeding program relies on the existence of sufficient genetic variation for the target traits. Germplasm collections are one of the main sources of genetic variation for breeders, particularly if the crop is essentially new for the targeted area, as is the case for sweet sorghum in Spain. This study represents a first step towards implementing a breeding program for sweet sorghum in Spain, aiming at the selection of superior lines per se, and for hybrid production.

There are over 36,000 sorghum accessions in the USA (Reddy et al, 2006) and at the International Crops Research Institute for the Semiarid Tropics (Saballos et al, 2008). Among these, 2054 entries in the GRIN database (Pederson and Spinks, 2006) carry the identifiers 'MN' and 'MER', which represent the historic US sweet sorghum collection (GRIN, 2008). From them, we have selected sixteen sweet sorghum lines with a combination of desirable traits for ethanol production, and for growth under Spanish conditions.

The experimental design used for the field trial was unreplicated, due to lack of seeds. For the agronomic traits, we used individual plants as repetitions. These plants were all in the same plot, and thus their errors are more correlated than with plants in different plots. For this reason, we do not have a good estimate of the experimental error that may have occurred as an effect of heterogeneity across the whole plot. For this reason also, even if the field experiment was quite homogeneous (based on the appearance of the large grain sorghum plots), the data collected can be considered only as a rough description of the variability found in this set of lines.

There was a good amount of diversity among lines, for flowering date, plant height, stem diameter, panicle size and shape. In general, later accessions grew taller, and produced more biomass, making them better candidates for ethanol production, as was observed before in studies carried out in several countries (Bapat et al, 1983; Palanisamy and Prasad, 1984; Meli, 1989; Reddy et al, 2005). Regarding available sugar percentage, the figures obtained for some lines in our experiment were similar to those obtained by Tew et al (2008), for Rio, M81E and Dale, and by Channapagoudar et al (2007) for Rio.

Different studies of trait relationships have pointed out the need of a combination of good quality traits with appropriate agronomic traits, particularly biomass production, to optimize ethanol yield (Seetharama et al, 1987; Tsuchihashi and Goto, 2004). Ganesh et al (1995) reported that the cane yield, juice yield, Brix, total sugars, and sucrose content, all presented high and positive linear correlations with alcohol yield, whereas girth of the stem showed just a moderate correlation. In our case, we found the correlations between agronomic traits and sugar content (Brix and POL) were only moderate. Previous studies have shown that plant height, harvested stem length and number of nodes were positively and significantly correlated with sugar concentration of stalk juice, suggesting that genetic improvement in these three traits could improve the total biomass and sugar content (Murray et al, 2008; Zou et al, 2011). The rough estimates of our study, on the other hand, though the data are rather limited, indicate a certain independence of sugar and biomass traits, that should be confirmed with further experimentation.

One of the goals of this work was to find out whether we could find enough good lines from the point of view of sugar production that covered the widest possible range of flowering dates, to provide alternatives to extend the period of crop harvest as much as possible during the year. The relationship of flowering dates with POL illustrates that there exists a variety of lines which can provide good sugar production potential across different cycle durations. Several sweet sorghum lines with high Brix values, high percentage of fresh stem weight and promising sugar and theoretical ethanol yields were identified. None of the four "medium" genotypes showed high biomass potential, though Juar and Honey Sorghum were the best among them. Among the late lines, Smith was the best of all, and it was remarkably earlier in our experiment than Ramada and Dale which, *a priori*, had the same cycle length. MN2826 and Sugar Drip, were also late genotypes with good potential of sugar production. Ramada and Dale were the best among the very late genotypes. The composition of the extracted juice of those five best lines was close to or above 18 Brix and 14 POL, well above the minimum necessary values for ethanol production reported by Blum et al (1975) and also by Woods (2001), who reported minimum values of 9 POL and 12 Brix. These genotypes may be used directly as varieties, providing a spread of cycle lengths of close to 50 days from the earliest to the latest, or introduced as parents to produce F_1 hybrids suitable for the Spanish conditions.

Regarding growth cycle, our conditions of temperature are intermediate between those of other Spanish regions where sweet sorghum is a potential crop (Southern Spain and the Central Plateau). Based on a parallelism with maize cultivation, we can extrapolate the cycle duration at these regions based on the cycle determined in Zaragoza. For instance, a line whose cycle is medium in Zaragoza would be considered early in the South (Andalusia, Murcia) and late in the Central Plateau.

The simple methodology adopted here allowed us to characterize the sweet sorghum productivity under non-limiting water conditions. We did not have a good estimation of biomass or juice yield, and thus cannot be sure of the performance of these lines in commercial stands. But the data reported here are good indications of the potential of these lines and, by extension, of other USDA sweet sorghum lines to be used for production or breeding in Spain. For the 16 lines analysed, the agronomic and quality data were in good agreement, in general, with the evaluation data provided in the GRIN database (not shown). Therefore, the USDA GRIN database is a reliable source of information to select sweet sorghum lines for Spain.

As mentioned before, biomass production is essential to produce a good ethanol yield (Seetharama et al, 1987; Tsuchihashi and Goto 2004). Given the high levels of heterosis reported for grain sorghum (Haussmann et al, 1999; Makanda et al, 2010; Ben-Israel et al, 2012), it is interesting to assess if the heterosis in sweet sorghum is also high enough to justify the production of F_1 hybrid cultivars. The studies performed with sweet sorghum have reported that hybrids produce greater stalk yield due to both taller plants and greater stem diameter. This greater stalk yield translated into a greater juice amount even though there was no hybrid vigour for juice fraction (Pfeiffer et al, 2010). Nevertheless, Sangwan et al (1972) found high heterotic effects for total sugar yield and Kamala et al (1986), Naik (1993), and Selvi and Palanisamy (1987) found higher sugar content in hybrids compared to parents. In the last study, the authors reported up to 82.6 and 28.4 percent positive heterosis for sucrose and total sugar, respectively. Many recently published works report promising heterosis levels for both biomass and sugar-related traits, among them Makanda et al (2009), Umakanth et al (2012) and Vinaykumar et al (2012).

The hybrids' final size in the digital phenotyping experiment indicated a moderate mid parent heterosis and low high parent heterosis. We have not found reports of heterosis during vegetative growth in the literature neither for grain or sweet sorghum and, therefore we cannot make proper comparisons of our results. Reports of mid-parent heterosis for grain yield in grain sorghum vary between 13% and 88% (Haussmann et al, 2000, and references cited therein). Digital phenotyping offers good prospects for germplasm evaluation. The insufficient duration of the experiment and the limitation of pot size in the digital phenotyping experiment both possibly hindered a good estimation of plant growth throughout its entire duration. The hybrids showed great growth potential early in the experiment and levelled off in the second part, possibly because their growth was limited earlier by pot limits, found allowing the other lines to catch up. On the other hand, this technology presents an enormous potential to unravel the dynamics of plant growth in relation to heterosis at a scale that was not possible before, provided the system is fine tuned to the biological needs of each species.

Although the number of hybrids is not enough to assess general or specific combining ability, some patterns arised. On one hand, the two grain x sweet crosses showed lesser growth overall than the sweet x sweet types, probably caused by the lower growth potential of the grain parent AD11B. On the other hand, it is interesting to note that the two sweet sorghum parents used in both sweet x sweet and grain x sweet crosses (Ramada and Smith), presented different heterotic behaviour. Ramada produced rather large heterosis (MPH and HPH) crossed to AD11B, and low heterosis when crossed to AMP472. For Smith it was the opposite. Ramada and Smith were placed in the same germplasm group by Wang et al (2009), who placed them in group G4, and Murray et al (2009), who placed these two cultivars in the group of "modern, sugar and energy types, MN landraces". However in these two studies, as well as in Ali et al (2008), the genetic distance between them, calculated with molecular markers, is quite large, and may explain their different heterotic behaviour.

The genotype Ramada showed a slower growth than the other parents of the hybrids in the PhenoFab experiment, but it reached large height and stalk diameter under field conditions. This may be explained by the much longer growing period of this genotype in the field, which allows it to reach large final size (Table 1) even though the growth rate might not be as high as others.

In this study, we included entries with a large range of variation for cycle duration, from medium (like Juar, 70 days until flowering) to very late maturing materials (Ramada, 117 days). The varieties evaluated and the hybrids produced will probably show a wide range of flowering and harvesting dates, something desirable from the production point of view, to ensure supply of raw material to ethanol factories over the longest period possible. We do not know if the hybrids produced are fertile or not, as the plants did not reach flowering in the digital phenotyping experiment. But seed set is not a relevant issue for this crop, as the economic produce are the stems, and seed should be produced every year by seed companies, as for other hybrid crops like maize.

Conclusions

Overall, these results demonstrate that this germplasm has potential for direct use and also for the development of sweet sorghum hybrid cultivars for Mediterranean environments. The best lines identified in this study show good promise, and should be further tested per se and in testcrosses in a systematic manner. Appropriate experimental designs and multi-location trials have to be carried out to obtain genetic parameters and estimates of genotype by environment interactions. To start a breeding program, specific experiments should be carried out to study the combining ability effects (GCA and SCA) of the most promising lines, the magnitude of heterosis for biomass, juice yield and grain yield, and to identify stable genotypes and responses of sweet sorghum under a variety of conditions, including limited water availability.

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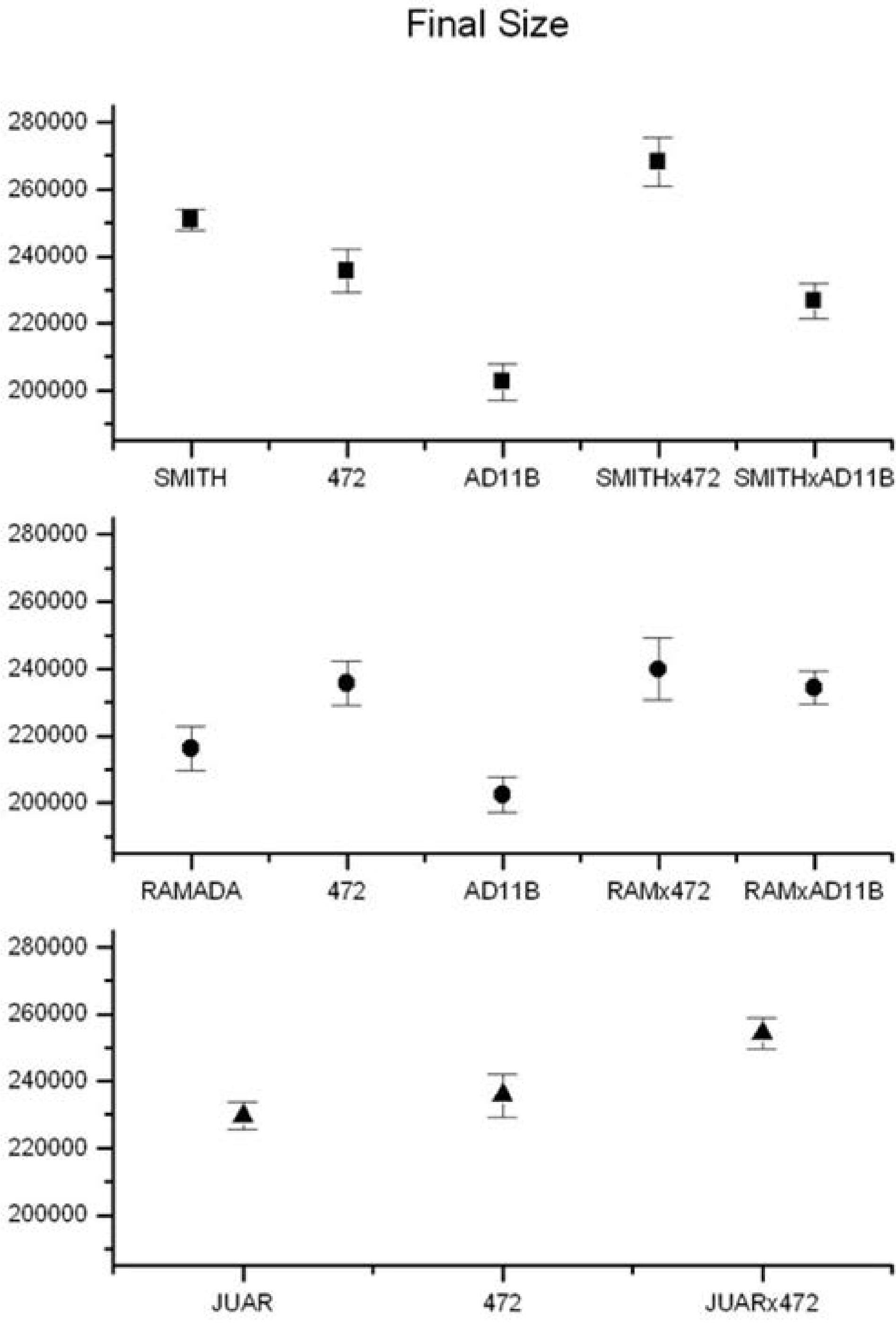
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