INTEGRATING BIOTREATED WASTEWATER REUSE AND VALORIZATION WITH ENHANCED WATER USE EFFICIENCY TO SUPPORT THE GREEN ECONOMY IN EU AND INDIA

Work Programme 2012 "COOPERATION"

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Water4Crops - EU

Work Package n°: 4
Improving WUE and drought tolerance of maize, sorghum, millet and tomato via genomics approaches and modelling

Deliverable 4.4
Sugar accumulation as a drought resistance mechanisms: New insights into sugar accumulation as a drought resistance mechanisms

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Participants: UNIBO
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Paragraphs

Introduction

One of the mechanisms utilized by plants to enhance the tolerance to water stress is osmotic adjustment (OA). The process of osmotic adjustment involves the reduction of water potential of a cell by the active accumulation of ions in the vacuole and/or of compatible solutes (ie. not interfering with cellular enzymatic processes in the cytoplasm). The accumulation of solutes enables the retention of water in the cell during episodes of low external water potential, thus limiting cell damage and cell and tissue turgor loss (Sanders and Arndt, 2012). Specific physiological processes such as such as stomatal opening, photosynthesis, and expansion growth can also be positively influenced (Blum 1996), with at least a theoretical positive effect on yield in crops.

Different types of molecules can lower the water potential of a cell and participate in OA: inorganic cations and anions, sugars and sugar alcohols, non-protein amino-acids, and organic acids. Inorganic ions may come to a low energetic cost for the cell and are usually confined in vacuole to avoid any damaging effect. Organic solutes are usually non associated with harmful effect to the cell and are present in cytoplasm and other compartments. Not always these organic molecules are actively synthesized. Indeed, some of these molecules may be available for OA because of slow down of metabolism due to stress events, or the breakdown of other molecules (eg storage molecules such as starch), or even catabolism.

Based on scientific literature maize capacity of osmotic adjustment is somewhat controversial. Preliminary analysis carried out by Inada et al. (1992) showed a lower osmotic adjustment in maize than in sorghum or millet, however the study was carried out by deriving all maize information from testing one single hybrid maize cultivar. Additional germplasm survey revealed little genetic variation for this trait, relatively low heritability and little response to selection (Bolanos and Edmeades, 1991; Guei and Wassom 1993). However, other studies showed the opposite. Chimenti et al. (1996) showed ample genetic variation for osmotic adjustment in maize. Lemcoff et al. (1998) analysed several maize hybrids for osmotic adjustment and concluded that genetic variation for this trait exist in maize and that high yield stability seems to be related, at least partially, to capacity for OA. In a follow-up study, Chimenti et al. (2005) analysed populations derived from high and low OA capacity lines and observed that, irrespectively of the timing of drought, the high OA derived population extracted significantly more water during the stress period, exhibited larger leaf area and grain yield components and greater harvest index than low OA population. They concluded that OA can contribute to drought tolerance in maize when exposed to water deficit before and during flowering and that plant materials selected for high OA capacity carry no yield penalty under well-watered conditions.

Sugars are a well-known component of OA in leaf tissues (Sanders and Arndt, 2012). Previous works addressed variability of sugar concentration in relation to drought and salinity tolerance in leaf tissues. Oligosaccharides like raffinose are ubiquitous in plant kingdom and contribute to stress tolerance likely by membrane stabilization and antioxidative functions (Van den Ende, 2013). Increased sucrose levels seem to protect wheat leaves from UVB stress (Pradhan et al., 2008) and have been identified as a major osmoprotectant (Miller, 2006). In maize, the role of sugar accumulation in OA has been relatively little explored. An early (3–4 days after initiation of water shortage) increase of the acid soluble invertase activity was observed in maize adult leaves. This increase correlated with fructose, glucose and to a lesser extent sucrose accumulation, however no evidence of osmotic adjustment was detected (Pelleschi et al. 1997). Valentinovic et al (2006) observed an increase in soluble sugars in roots, mesocotyl and leaves of young seedlings under moderate osmotic stress applied as 0.3 M sorbitol to a semi-hydroponic system. PEG-6000 mediated stress in hydroponics was applied to induce osmotic stress in young maize seedlings (Mohammadkhani and Heidari, 2008) and this resulted in an almost doubling of soluble sugars in roots and leaf tissue.

Objective of this study

Objective of this study was to explore the genetic variability for osmotic potential, osmotic adjustment and soluble sugars concentration in the leaf and their relationships with other drought-related traits, in the maize introgression line (IL) population B73 x Gaspé Flint. The population has been investigated in parallel (as described in other Reports of the Water4Crops project) for agronomic and drought tolerance traits. This study would offer the opportunity to get some preliminary insights into the possible positive role of sugar accumulation for drought tolerance in conjunction with other physiological and morphological mechanisms, and would provide the opportunity to identify chromosome regions and/or plant materials for subsequent characterization steps and information to be exploited in maize breeding.

Materials and methods

Plant materials

The plant material consisted of the B73 x Gaspé Flint introgression library (Salvi et al., 2011). B73 is an elite inbred line of the Iowa Stiff Stalk Synthetic heterotic group and is reference genome for maize. Gaspé Flint is a Canadian landrace belonging to the Northern Flint maize race group with a phenotype very different to B73. The IL population includes 75 lines originated by the cross B73 x Gaspé Flint followed by five cycles of marker-assisted backcross using B73 as recurrent parent, and two cycles of selfing. During backcross and selfing cycles, SSR markers were used to introgress in each line, and to fix as homozygous, a different portion of the Gaspé Flint donor genome (Salvi et al. 2011). In a different report of this project, we described the further high resolution genotyping of the same population with the maize 55K SNP array from ILLUMINA Inc.

Field experiment and sample collection

The collection of 73 (out of 75) IL lines was evaluated in replicated (3 reps) field trials at two water regimes (well watered -WW, and rain-fed or water-deficit - WD) in Ravenna (Italy) during summer 2013. One rep consisted in one plot of 15 plants seed-planted at 20 and 75 cm distance between hills on the row and between rows, respectively. For the purpose of this study, we utilized three consecutive competitive plants per plot (same plants utilized for root analysis with the 'Shovelomics' protocol, as described in the Water4Crops Deliverable 4.1 and 4.2). Leaf samples for osmotic potential and soluble sugar analysis were taken as the second leaf from the top of the plant, by counting, at tassel appearance. Samples from such leaf were collected as explained in the two protocols reported below. Because of the large variation in flowering time (and therefore in date of tassel emission) between different IL lines, plants were visually observed daily and sampling was distributed in time over approx. one week.

Analysis of osmotic potential and osmotic adjustment

Osmotic adjustment was estimated as the difference in osmotic potential between water deficit and control plants (Chimenti et al. 2006; Turner, 1981) and is provided in MPa and as non-dimensional value. For osmotic potential, three leaf discs (3 x 0.5 cm diametr, c. 22 mg fresh weight) were punched out of the medial part of the sampled leaf for each of the three selected plants. All disks from plants of the same plot were collected in plastic bag, and immediately frozen with liquid nitrogen. In the lab, samples were thawed and osmotic potential was measured using an automatic freezing point depression osmometer (Roebling, Germany), essentially as described in Pelleschi et al. (2006). Values are expressed as MPa.

Soluble sugars determination

Soluble Sugars was determined using an Anthrone Reagent protocol. The anthrone procedure is based on the reaction of the anthrone (9,10-dihydro-9-oxoantraceno) with furfural conformation of carbohydates (treatment of carbohydrate in strong sulfuric acid) to give a colored hemiacetal which is determined spectroscopically at 630 nm (Galicia et al. 2009). The protocol was optimized using 96-well plates. A standard curve was produced by serial dilutions of sucrose in water as follows (0, 6, 12, 18, 24, 30, 40, and 60 ug/ml dilutions).

Besides leaf samplings for osmotic potential and sugar concentration, we continuously monitored plant water status, by periodically controlling relative water content (RWC): [(fresh weight_dry weight)/(re-hydrated weight_dry weight) * 100], rehydration lasted 24 h in the dark at 4 °C in a 50 ml Falcon tube containing 10 ml of water.

Statistical analyses were carried out in MS Excel and in Past v. 3.0 (Hammer et al. 2001). Normality of phenotypic distributions were tested using Shapiro-Wilk test in Past 3.0. Heritability (h^2) was estimated following Falconer and Mackay (1996) and as described in Salvi et al. 2016.

Results and discussion

Osmotic potential and osmotic adjustment in the B73 x Gaspé Flint introgression library (IL) collection.

Osmotic potential (OP) of leaf samples was measured across the IL collection grown at two water regimes, well-watered (OP WW) and water-deficit (OP WD) and results are analytically presented in Table 1 and Figure 1. RWC of plants in WD experiment was approx. 60%, so the severity of the induced stress can be considered of medium type.

Analysis of variance for both OP WW and OP WD resulted highly significant ($P < 0.01$) (Fig. 2), suggesting the presence of genetic difference for osmotic potential in both WW and WD conditions acting within the B73 x Gaspè Flint introgression line.

In WW, leaf OP ranged from −1.63 MPa to −0.21 MPa and averaged at −0.83 MPa (Table 1. Figure 1). B73, the population reference line, was characterized by an intermediate leaf OP (−1.20 MPa). As expected, under WD conditions the leaf osmotic potential distribution slightly shifted to lower values, and showed a mean value of −1.12 MPa, and a distribution ranging between −1.60 MPa to −0.48 MPa. The decrease in OP was expected in WD as compared to WW, as this is what is usually observed under regimes of mild to mid water deprivation in plant leaf samples. B73 value was similar in WW and WD. In addition, the histogram distribution under WD (Fig. 1, OP WD) was suggestive of two modal values, one close to the mean value (at −1.10 MPa) and one at −0.5 MPa. This distribution could indicate the presence in the collection of lines of two partially alternative types of response to dehydration, as far as leaf osmotic potential is concern. In the first more frequent type, lines would accumulate solutes under WD and would consequently tend to lower their osmotic potential. In the second, less frequent, group of genotypes (about ten IL lines), plants do not modify their osmotic potential accordingly with the water regimes. A visual investigation of possibly common Gaspé Flint chromosome introgressions shared between these 10 IL lines was carried out, however no common chromosome introgressions were found (not shown, discussed later); based on this investigation, it is more likely that two or three different and independent chromosome regions are involved in the control of the observed phenotypic difference. A more open distribution of OP values was observed for the WW experiment (Fig. 1), however, the range of the values was comparable between WD and WW. Heritability (h^2) values between WD and WW were again similar and ranged from 0.78 to 0.81 (Table 1). Absolute OP values in both WW and WD conditions were in line with those identified previously by other authors (eg. Chimenti et al. 2006).

Osmotic adjustment (OA) of each IL line was computed as the difference between their mean leaf osmotic potentials in WW and WD. The distribution of OA values ranged between 0 MPa, that is equivalent to absence of OA (ie. no change in leaf osmotic potential between water regimes of the plant) and 0.84 MPa, with a mean value of 29 MPa. Sixteen IL lines, including the reference line B73, had a mean OA <0.05 MPa, making the ‘0’ class in the histogram distribution the modal value (Fig. 1, OA). The line characterized by the highest OA was IL100-2-5-3 (Table 1).

Because the underlying hypothesis was that the leaf OP can increase, at least in a some IL lines, as induced by an active unknown mechanism in WD conditions, emphasis in the following discussion will be given to OP data collected in WD and the correlation with OA. Further hypothesis is that variation in OP WD and OA would be associated with variation in sugar content in the leaf and this would provide protection from drought stress (further phenotypic data on yield and drought tolerance indexes were collected in the same experiment and are being presented in detail and discussed in Water4Crops Deliverable 4.2). For OP WD, no IL lines was shown to statistically differ from the reference line B73. However, several lines showed relatively extreme low OP values. Least significant difference (LSD) based on Tukey's test was computed (LDS Tukey = ± 1.0 MPa and the differentiation of OP WD by LSD was taken as criterion to identify lines with more extreme OP values in the OP WD tail distribution. These lines resulted IL121-6-6-6, IL3-3-2, IL29-1-4, IL5-6-1 and IL159-7-2-1. Among these lines, four were characterized by a sizeable OA (ie. specific increase of osmotic potential in WD vs WW conditions). More specifically, OA was maximum for IL121-6-6-6 and IL159-7-2-1 (OA = 0.45 MPa for both lines).

Sugar content

Leaf sugar content, analysed and expressed as soluble sugars (SS), was obtained from the same leaf samples and plants utilized for osmotic potential and osmotic adjustment. Lines SS mean values, standard deviations, minimum and maximum values are reported in Table 1, and distributions are reported in Fig. 2. SS content is expressed as mg of sugar per g fresh weight (FW). In WW condition, SS minimum, mean and maximum values were 15.4, 25.0 and 35.9 mg, respectively. The distribution shifted to higher SS content in WD condition. In such condition SS minimum, mean and maximum values were 21.2, 33.4 and 47.9 mg, respectively. Both distributions (WW and WD) were not statistically distinct from normality (not significant. Shapiro-Wilk test). The value of the reference line B73 were close to the distribution center (modal and mean values) 25.2 and 29.4, in WW and WD conditions, respectively. The line IL121-6-6-6, previously identified as top value OP WD and also characterized by a relatively high OA value, also in this case showed a relatively high SS content in WD condition.

ANOVA analysis for both SS WD and SS WW did not find statistically significant differences between IL lines (Fig. 3). However, the F-test *P* value from the ANOVA table was not far from significance in the WD experiment (*P* = 0.2), therefore we additionally analysed the data in order to search for potentially signal suggestive of the presence of genetically controlled difference in soluble sugar accumulation among IL lines.

Table 1. Summary of data collected for osmotic potential (OP), osmotic adjustment (OA) and soluble sugar content (SS) in water deficit (WD) and well-watered (WW) conditions. OP and OA are reported as MPa, while sugar levels are reported in mg/g fresh weight (FW). *sd* = standard deviation.

Line		**OP WD**		**OP WW**		**OA**	**SS WD**		**SS WW**	
		MPa	*sd*	MPa	*sd*	MPa	mg gFW^{-1}	*sd*	mg gFW^{-1}	*sd*
B73	1	-1.20	0.30	-1.20	0.52	0.00	29.42	9.25	25.15	9.12
2-4-1	2	-0.91	0.14	-0.74	0.53	0.17	25.76	14.27	23.52	16.36
4-6-1	3	-1.24	0.41	-1.03	0.04	0.21	32.42	3.80	28.87	9.63
5-6-1	4	-1.55	0.28	-1.22	0.37	0.03	32.66	10.16	26.81	4.06
6-7-1	5	-1.18	0.16	-1.22	0.16	0.04	26.57	12.68	27.69	9.43
7-11-1	6	-1.21	0.40	-0.77	0.36	0.44	28.79	6.26	17.50	8.27
8-5-1	7	-0.48	0.40	-0.51	0.56	0.01	38.00	9.85	23.16	7.36
9-1-1	8	-1.30	0.35	-0.79	0.48	0.51	36.53	17.62	22.50	8.44
10-7-1	9	-1.12	0.30	-0.58	0.42	0.53	39.26	6.37	21.00	5.99
3-3-2	10	-1.59	0.12	-1.17	0.40	0.13	30.22	9.77	28.34	8.68
4-4-2	11	-1.07	0.21	-0.51	0.16	0.56	44.48	9.00	28.82	4.71
6-11-3	12	-1.23	0.67	-0.98	0.55	0.25	26.68	1.55	23.30	10.86
3-6-4	13	-1.35	0.06	-1.32	0.52	0.03	38.27	8.61	32.31	8.77
100-2-1-19	14	-1.04	0.15	-0.61	0.40	0.71	33.44	4.73	24.04	6.75
100-2-5-13	15	-0.62	0.47	-0.73	0.22	0.11	27.91	7.06	20.25	8.11
100-2-5-3	16	-1.24	0.17	-0.60	0.38	0.84	39.58	4.74	25.64	11.41
100-5-8-5	17	-0.58	0.23	-0.54	0.23	0.37	26.50	4.00	24.95	12.07
121-6-6-6	18	-1.60	0.10	-1.15	0.33	0.45	40.85	9.59	16.75	3.83
130-2-8-2	19	-1.11	0.32	-0.84	0.45	0.27	35.46	4.81	19.02	2.16
130-2-8-5	20	-1.10	0.39	-0.95	0.13	0.15	32.48	2.17	16.73	8.87
13-7-4	21	-1.17	0.47	-0.70	0.48	0.48	36.13	9.67	33.76	7.52
13-7-5	22	-0.76	0.07	-0.65	0.36	0.40	33.36	3.86	20.38	3.51
140-8-4-4	23	-1.10	0.63	-1.07	0.17	0.03	24.24	3.70	20.54	11.46
140-8-4-5	24	-1.03	0.33	-0.58	0.43	0.45	35.36	10.05	26.27	12.69
14-11-3	25	-1.28	0.31	-0.95	0.18	0.34	36.09	4.43	29.99	18.33
142-8-4-2	26	-1.30	0.22	-1.07	0.45	0.23	32.85	9.45	31.28	10.48
145-3-1-10	27	-1.07	0.26	-0.67	0.16	0.39	31.79	3.87	24.25	4.20
155-4-3-12	28	-1.16	0.24	-0.97	0.26	0.20	29.63	5.52	35.91	7.17

159-7-2-1	29	-1.51	0.69	-1.06	0.73	0.45	33.46	12.52	22.06	9.53
16-1-1	30	-1.05	0.44	-0.66	0.41	0.66	47.92	14.76	24.10	7.60
163-5-1-16	31	-0.98	0.04	-0.76	0.20	0.22	42.18	3.85	27.88	5.04
163-5-5-3	32	-0.68	0.08	-0.58	0.61	0.02	35.82	7.58	17.98	7.44
18-4-1	33	-0.60	0.12	-0.59	0.21	0.07	25.70	3.52	26.28	10.17
189-7-1-2	34	-0.58	0.15	-0.65	0.50	0.19	22.46	5.63	25.55	9.93
19-2-2	35	-1.27	0.09	-0.67	0.29	0.60	38.60	5.53	34.21	7.61
192-5-5-2	36	-1.24	0.09	-0.94	0.42	0.30	34.18	3.84	24.70	5.07
20-2-1	37	-1.11	0.63	-0.77	0.17	0.34	33.36	10.89	29.00	2.41
20-2-2	38	-1.08	0.08	-0.74	0.25	0.34	32.44	6.63	22.09	6.86
20-3-2	39	-1.02	0.11	-0.50	0.37	0.62	38.76	9.43	33.11	5.17
21-7-1	40	-1.18	0.01	-0.52	0.15	0.65	33.53	7.32	32.29	11.13
22-11-2	41	-0.59	0.38	-0.53	0.30	0.06	28.18	3.72	27.51	11.93
22-1-3	42	-0.67	0.23	-0.56	0.42	0.30	31.45	10.75	25.92	2.00
22-4-1	43	-0.73	0.12	-0.53	0.19	0.41	32.65	11.25	21.03	5.42
23-6-1	44	-1.15	0.29	-0.63	0.30	0.82	33.76	6.33	23.50	4.93
25-1-1	45	-1.02	0.22	-0.87	0.11	0.15	30.61	15.77	24.00	16.54
26-3-1	46	-1.38	0.16	-0.86	0.46	0.51	32.32	8.07	22.90	5.37
27-10	47	-1.02	0.23	-0.69	0.19	0.33	33.45	2.87	25.31	8.88
27-16-4	48	-1.13	0.17	-0.82	0.29	0.32	35.87	3.64	30.29	10.23
28-7-1	49	-1.16	0.62	-0.69	0.61	0.60	41.36	5.53	26.05	0.06
29-1-4	50	-1.56	0.12	-1.18	0.45	0.38	30.53	3.12	29.94	10.84
30-5-1	51	-0.99	0.10	-0.89	0.19	0.10	34.23	2.35	25.11	12.14
33-5-1	52	-1.29	0.28	-0.72	0.49	0.57	39.28	13.35	27.50	4.90
34-1-1	53	-1.20	0.13	-1.07	0.70	0.13	26.88	3.25	21.11	10.64
35-1-2	54	-1.17	0.40	-0.89	0.51	0.28	33.04	5.93	24.63	8.90
35-12-6	55	-1.43	0.15	-1.15	0.37	0.27	28.83	5.19	30.39	7.23
36-6-1	56	-1.29	0.13	-1.32	0.13	0.03	29.41	10.22	28.36	8.15
37-9-1	57	-1.24	0.15	-1.19	0.35	0.05	29.66	7.09	21.84	2.48
38-3-4	58	-1.19	0.24	-1.22	0.69	0.03	29.18	3.06	19.64	5.56
39-1-2	59	-1.27	0.28	-0.87	0.21	0.41	39.41	10.73	23.59	7.85
41-13-1	60	-1.05	0.15	-0.85	0.28	0.20	37.33	7.67	24.35	9.30
42-6-1	61	-1.16	0.28	-0.76	0.54	0.39	33.90	8.00	25.14	7.85
42-8	62	-1.48	0.38	-1.13	0.44	0.15	37.16	9.83	19.26	3.54

45-15-1	63	-1.45	0.33	-1.20	0.27	0.25	39.38	12.23	22.61	15.19
46-2-1	64	-1.38	0.28	-0.97	0.10	0.41	40.56	5.52	21.61	3.94
55-14-1-3-7	65	-0.63	0.04	-0.59	0.16	0.34	29.46	3.18	25.19	4.01
62-3-2-14	66	-1.15	0.27	-1.21	0.41	0.06	23.78	16.58	15.38	8.26
62-8-8-4	67	-1.20	0.09	-0.58	0.17	0.63	36.55	7.51	27.88	4.04
65-2-4-2	68	-1.48	0.23	-1.15	0.53	0.07	30.70	7.53	17.41	6.81
80-1-4-1-14	69	-1.38	0.25	-0.83	0.22	0.55	33.86	5.95	22.44	2.76
80-1-4-1-6	70	-1.11	0.48	-1.10	0.09	0.00	28.15	10.72	31.29	6.75
80-1-4-2-4	71	-1.19	0.12	-0.69	0.31	0.49	44.46	2.13	30.79	4.10
94-10-5-13	72	-1.22	0.09	-1.19	0.34	0.03	21.19	16.49	22.21	4.71
94-12-1-11	73	-1.26	0.11	-0.81	0.47	0.45	36.83	8.29	27.08	6.01
94-6-16	74	-0.58	0.14	-0.57	0.39	0.11	32.93	12.02	24.02	1.74
Mean		*-1.12*	*0.27*	*-0.83*	*0.33*	*0.29*	*33.37*	*5.35*	*25.04*	*4.50*
Min		*-1.60*	-	*-1.63*	-	*0.84*	*21.19*	-	*15.38*	-
Max		*-0.48*	-	*-0.21*	-	*0*	*47.92*	-	*35.91*	-
LSD		*1.00*	--	*1.11*	-	-	*na.*	-	*na.*	-
h^2		*0.78*	-	*0.81*	-	-	*0.56*	-	*0.63*	-

h^2: heritability (in the broad sense). LSD: least significant difference based on Tukey test.

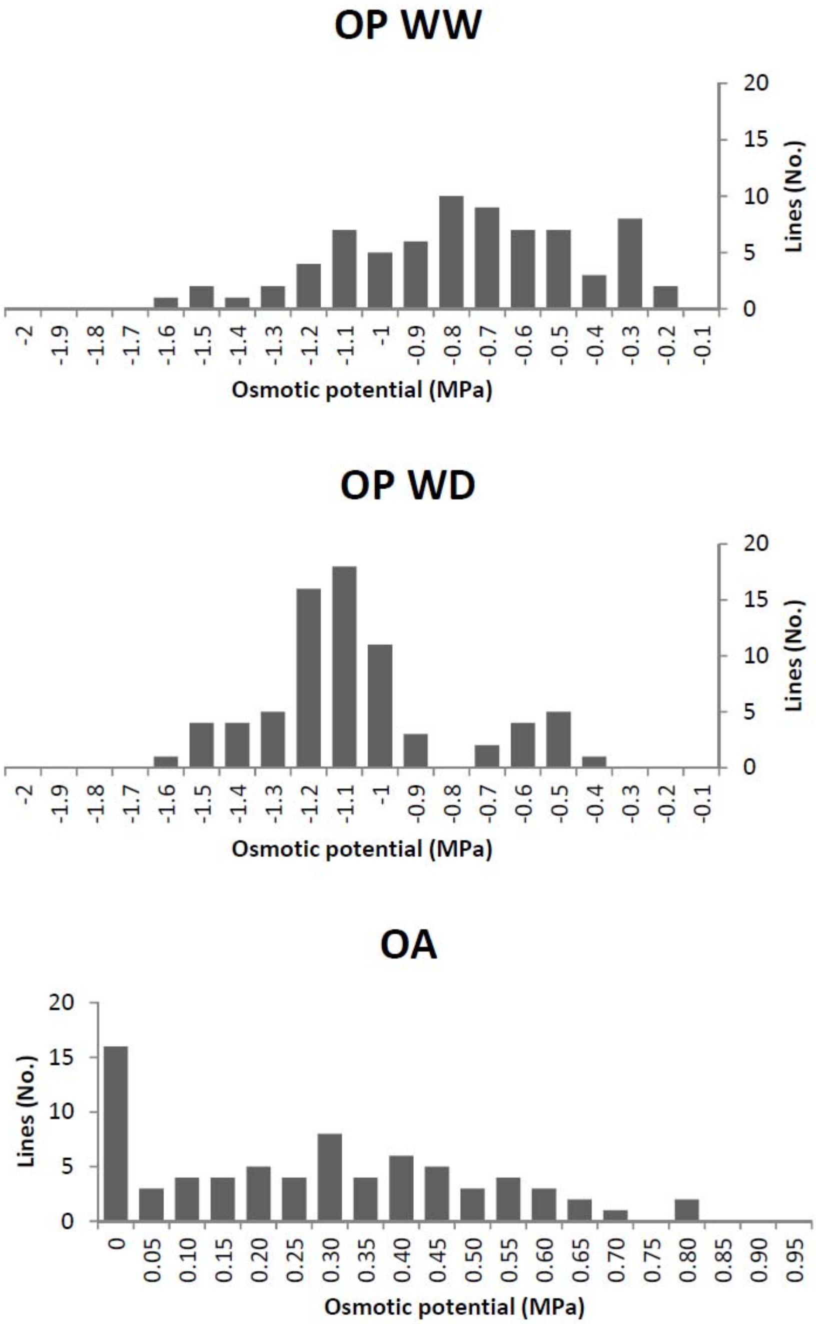


Figure 1. Frequency distribution of Osmotic Potential (OP) in well- watered and water-deficit conditions (WW and WD, respectively) and Osmotic Adjustment (OA). Samples were collected from the field grown B73 x Gaspé Flint introgression library population. Total number of IL lines tested was 74 (including the IL reference line B73).

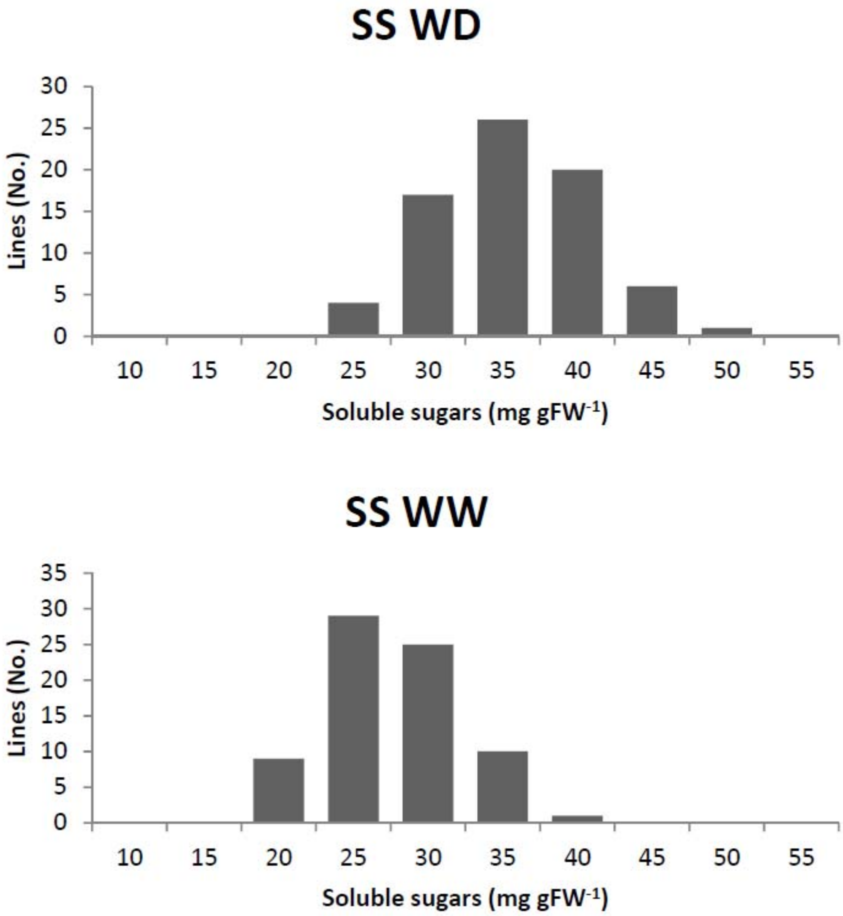
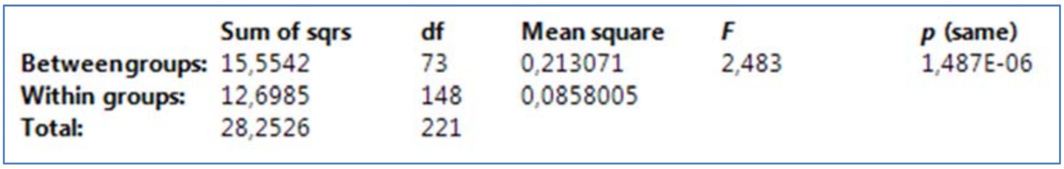


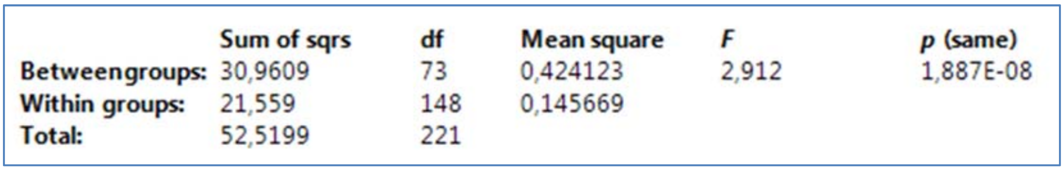
Figure 2. Frequency distribution of B73 x Gaspé Flint introgression library (IL) lines for leaf soluble sugar (SS) content collected from water-deficit and well-watered experiments (SS WD and SS WW, respectively). Total number of IL lines tested was 74 (including the IL reference line B73).

Osmotic Potential, WD experiment



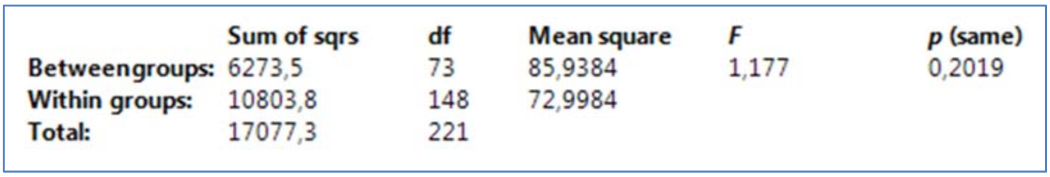
	Sum of sqrs	df	Mean square	F	p (same)
Betweengroups:	15,5542	73	0,213071	2,483	1,487E-06
Within groups:	12,6985	148	0,0858005		
Total:	28,2526	221			

Osmotic Potential, WW experiment



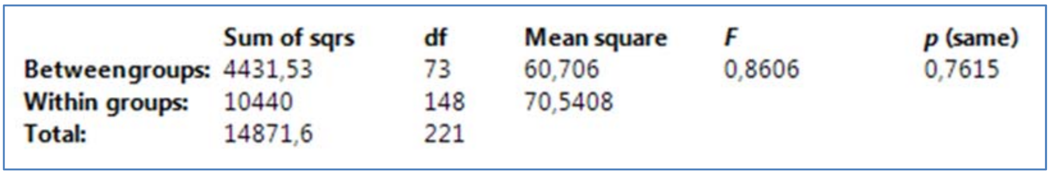
	Sum of sqrs	df	Mean square	F	p (same)
Betweengroups:	30,9609	73	0,424123	2,912	1,887E-08
Within groups:	21,559	148	0,145669		
Total:	52,5199	221			

Soluble sugars, WD experiment



	Sum of sqrs	df	Mean square	F	p (same)
Betweengroups:	6273,5	73	85,9384	1,177	0,2019
Within groups:	10803,8	148	72,9984		
Total:	17077,3	221			

Soluble sugars, WW experiment



	Sum of sqrs	df	Mean square	F	p (same)
Betweengroups:	4431,53	73	60,706	0,8606	0,7615
Within groups:	10440	148	70,5408		
Total:	14871,6	221			

Figure 3. Summary of results of ANOVA for osmotic potential and soluble sugar contents collected in the two field-based experiments (Water-deficit, WD, and well-watered, WW) with the B73 x Gaspé Flint IL population.

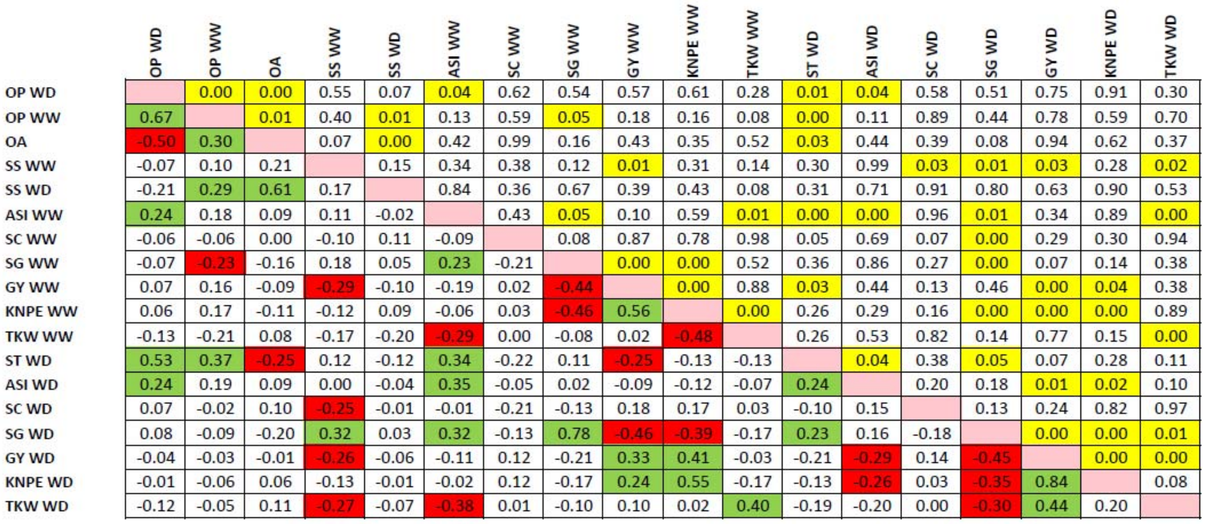
Analysis of correlation between Osmotic potential (OP), osmotic adjustment (OA), and soluble sugar content (SS)

In Table 2 we reported all correlations (Pearson *r*) values and statistical significance (*P*) between pairs of traits, including OP and SS from WW and WD experiments and OA, with all major traits involved in maize productivity (yield and yield components) and response to drought tolerance, as discussed in details in Water4Crops Deliverable 4.1 and 4.2. All correlation values discussed in the following were statistically significant. OP WW and OP WS resulted highly correlated ($r = 0.67$). Additionally, OA resulted positively correlated with OP WD ($r = 0.50$) and negatively correlated OP WW ($r = -0.30$). These results were expected given the way OA is computed (OA represents the change in osmotic potential between WW and WD conditions, therefore any line with a remarkably high potential in WD will contribute to an increase in OA while lines with low potential will contribute to a reduction of OA values).

Soluble sugar level (SS) collected in WW did not correlate with OP values and OA. However, SS WD did correlate positively with OP WW ($r = 0.29$), and mildly with OP WD, and with OA ($r = 0.61$). The latter highly significant correlation is important for the purposes of testing our starting hypothesis because it suggests the existence of a link between the observed OA in maize leaves (induced by drought stress) and the accumulation of sugars in the same leaves.

In order to better visualize the above correlations between osmotic adjustment (OA) and soluble sugar content (SS), we displayed detailed correlation graphs for the OA - SS WW and OA – SS WD (Fig. 4; Fig. 5). While OA and SS WW were not correlated (Table 2, Fig. 4), an increased in OA was linearly associated with an increase in SS content in WD condition ($r = 0.61$ from Table 2, which is equivalent to $R^2 = 0.37$ as obtained from the linear regression computed and reported in Fig. 4. The more soluble sugar in the leaf, the higher the osmotic adjustment of the same leaf. This is more clearly shown in Figure 5. Lines with progressively higher OA (toward the right end of the distribution. Fig. 5) tended to accumulate higher amount of sugars under WD conditions ($b = 0.15$, $p < 0.05$, from linear regression fitting the WD distribution), whereas no difference was present in WW conditions ($b = 0.04$, ns, for linear regression fitting the WW data distribution).

Table 2. Correlation analysis between Osmotic potential, Osmotic adjustment and Soluble Sugar contents and the main agronomic traits related with drought tolerance, in the B73 x Gaspè Flint maize introgression library collection. Correlation values (Pearson r) are reported below the diagonal. Green and red boxes indicate positive and negative correlation values, respectively. Statistical significance of correlation values is reported above the diagonal, yellow boxes indicate P <0.05. Acronym list is reported below.



	OP WD	OP WW	OA	SS WW	SS WD	ASI WW	SC WW	SG WW	GY WW	KNPE WW	TKW WW	ST WD	ASI WD	SC WD	SG WD	GY WD	KNPE WD	TKW WD
OP WD		0.00	0.00	0.55	0.07	0.04	0.62	0.54	0.57	0.61	0.28	0.01	0.04	0.58	0.51	0.75	0.91	0.30
OP WW	0.67		0.01	0.40	0.01	0.13	0.59	0.05	0.18	0.16	0.08	0.00	0.11	0.89	0.44	0.78	0.59	0.70
OA	-0.50	0.30		0.07	0.00	0.42	0.99	0.16	0.43	0.35	0.52	0.03	0.44	0.39	0.08	0.94	0.62	0.37
SS WW	-0.07	0.10	0.21		0.15	0.34	0.38	0.12	0.01	0.31	0.14	0.30	0.99	0.03	0.01	0.03	0.28	0.02
SS WD	-0.21	0.29	0.61	0.17		0.84	0.36	0.67	0.39	0.43	0.08	0.31	0.71	0.91	0.80	0.63	0.90	0.53
ASI WW	0.24	0.18	0.09	0.11	-0.02		0.43	0.05	0.10	0.59	0.01	0.00	0.00	0.96	0.01	0.34	0.89	0.00
SC WW	-0.06	-0.06	0.00	-0.10	0.11	-0.09		0.08	0.87	0.78	0.98	0.05	0.69	0.07	0.00	0.29	0.30	0.94
SG WW	-0.07	-0.23	-0.16	0.18	0.05	0.23	-0.21		0.00	0.00	0.52	0.36	0.86	0.27	0.00	0.07	0.14	0.38
GY WW	0.07	0.16	-0.09	-0.29	-0.10	-0.19	0.02	-0.44		0.00	0.88	0.03	0.44	0.13	0.46	0.00	0.04	0.38
KNPE WW	0.06	0.17	-0.11	-0.12	0.09	-0.06	0.03	-0.46	0.56		0.00	0.26	0.29	0.16	0.00	0.00	0.00	0.89
TKW WW	-0.13	-0.21	0.08	-0.17	-0.20	-0.29	0.00	-0.08	0.02	-0.48		0.26	0.53	0.82	0.14	0.77	0.15	0.00
ST WD	0.53	0.37	-0.25	0.12	-0.12	0.34	-0.22	0.11	-0.25	-0.13	-0.13		0.04	0.38	0.05	0.07	0.28	0.11
ASI WD	0.24	0.19	0.09	0.00	-0.04	0.35	-0.05	0.02	-0.09	-0.12	-0.07	0.24		0.20	0.18	0.01	0.02	0.10
SC WD	0.07	-0.02	0.10	-0.25	-0.01	-0.01	-0.21	-0.13	0.18	0.17	0.03	-0.10	0.15		0.13	0.24	0.82	0.97
SG WD	0.08	-0.09	-0.20	0.32	0.03	0.32	-0.13	0.78	-0.46	-0.39	-0.17	0.23	0.16	-0.18		0.00	0.00	0.01
GY WD	-0.04	-0.03	-0.01	-0.26	-0.06	-0.11	0.12	-0.21	0.33	0.41	-0.03	-0.21	-0.29	0.14	-0.45		0.00	0.00
KNPE WD	-0.01	-0.06	0.06	-0.13	-0.01	-0.02	0.12	-0.17	0.24	0.55	-0.17	-0.13	-0.26	0.03	-0.35	0.84		0.08
TKW WD	-0.12	-0.05	0.11	-0.27	-0.07	-0.38	0.01	-0.10	0.10	0.02	0.40	-0.19	-0.20	0.00	-0.30	0.44	0.20	

OP: leaf osmotic potential, OA: osmotic adjustment, SS: soluble sugars, ASI: anthesis silking interval, SC: stomatal conductance, SG: stay green, GY: grain yield, KNPE: kernel number per ear TKW: thousand kernel weight, ST: visual stress index, WD: water deficit experiment, WW: well watered experiment.

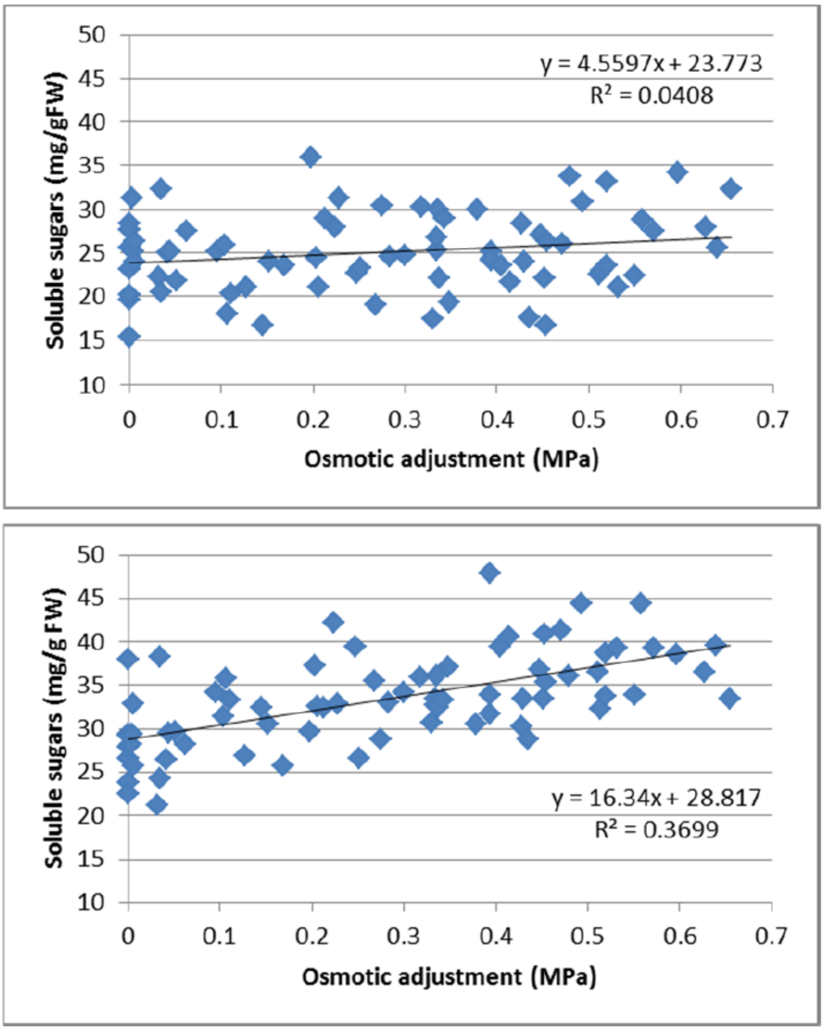


Figure 4. Graphical visualization of correlations between Osmotic Adjustment (OA) and content of soluble sugars (SS) in maize leaves of the B73 x Gaspé Flint IL population in well watered (WW. Upper) and water-stressed (WD. Lower) conditions.

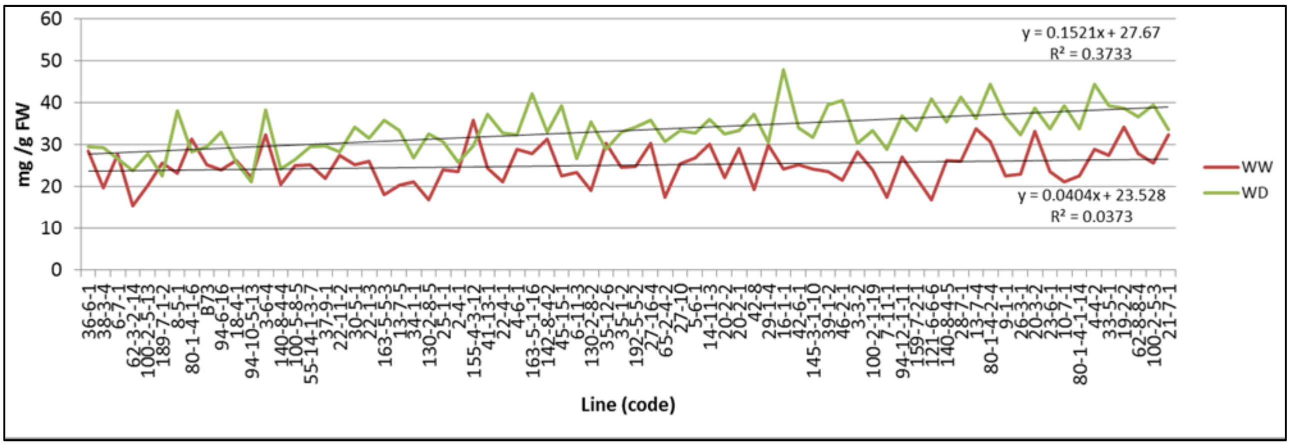


Figure 5. Content of leaf soluble sugars in the B73 x Gaspé Flint maize introgression line (IL) collection. Sugars content is expressed as mg per g of leaf tissue fresh weight (FW). Green and red lines represent IL lines sugar content levels from water deficit (WD) and well-watered (WW) experiments. Lines are ordered based on Osmotic Adjustment (OA) values as recorded in the same experiment (from least OA, IL36-6-1, to maximum OA, IL21-7-1).

Analysis of correlation between OP, OA, and SS traits with other agronomic traits

All main correlations between OP, OA and SS with all other traits (more detailed analysed in Deliverable 4.2) are reported in Table 2. Besides the correlation with OP and SS WD as described above, OA correlated with just one additional trait, Visual Stress index in WD condition, ST WD, (*r* = −0.25). This correlation indicates that an increase in OA was associated with a reduction in visual stress in the WD experiment, suggesting a possible involvement of OA mechanism in protecting this maize materials from the damage induced by drought.

As far as SS content is concern, it was interesting to see that SS WW negatively correlated with grain yield (GY) and its main component 'thousand kernel weight' (TKW) (*r* values ranged from −0.26 to −0.29). Such negative correlation likely reflects a trade-off between soluble sugars and starch in the kernels. In short, the higher the sugars left free in the leaves, the lower the sugar captured in starch in the grains. It is intriguing that such negative correlations were not present when SS content were collected in WD, where SS levels were higher. This likely indicates the positive effect of higher SS content in the leaf in water deficit condition.

Association of OP, OA and SS phenotypes with performance in the field and with molecular markers

The lines identified with remarkably extreme values of OP under WD (IL121-6-6-6, IL3-3-2, IL29-1-4, IL5-6-1 and IL159-7-2-1) did not share a unique chromosome segment, therefore no final conclusions could be drawn at the moment about the presence and the map positions of the genes involved in the control of OP (and therefore OA) in this population. However, it should be noted that a portion on chrom 1 (bin 1.06-07) was shared between IL121-6-6-6 and IL5-6-1, both characterized by very low OP values in WD conditions.

The results of this experiment point to IL121-6-6-6 as an interesting line for further analysis. As reported above, IL121-6-6-6 was the top line as far as OP WD is concern and one of the top lines for OA and leaf SS content. Very intriguingly, this line was the only line with a highly significant low score for visual stress index in the field in WD conditions. The score was carried out independently (ie. by other colleagues) on the same plant materials utilized here for OP, OA and SS analysis. Additional visual observations carried out in subsequent year (2014, 2015, not shown) confirmed a propensity of this line to withstand drought episodes. The not particularly high performance of this line in terms of yield might be due to the its relatively early phenotype, which, in fertile environments, represents a constraint toward top production.

IL121-6-6-6 contains two Gaspé Flint introgressions (substitutions), a short one on chrom. 1 (bin 1.06-07) and a longer one encompassing a large portion of chrom. 9. None of these substitutions are unique within the IL collection, so, as noted above, it is currently impossible to assign the position of the putative gene/QTL controlling the phenotype observed for this line. Experimental crosses are being carried out in order to increase the isogenicity of this line (as compared to B73) and move toward fine mapping and cloning of the underlying genes.

Conclusions

Our study aimed at testing for the presence of osmotic adjustment (OA) in maize in conditions of mid water deprivation, under the hypothesis that OA could be an interesting mechanism to be exploited in breeding programs. Under this research line, we checked soluble sugars (SS) content in leaf samples from the same plants which undergone OA analysis, in order to look for an association between OA and sugar concentration, as already found in other species. Finally, information on OA and SS could be compared with the large amount of genetically and phenotypic data as collected in other sections of the Water4Crops project.

Our results suggest the presence of (genetically controlled) difference in the capacity of changing leaf osmotic potential (OP) when maize plants growing in well-watered conditions undergo drought stress. This is in line with the report of Chimenti et al. (2006), who found the same genetic difference in OA, positive association of OA and yield and no penalty when plants were grown under irrigation. On the other hands, our results are in contrast to what proposed by Bolanos and Edmeades (1991), who excluded a major role of OA in maize.

Soluble sugar (SS) content in the leaf was, at least in our conditions, a less heritable trait, and it was not possible to find statistically significance differences between the IL line population tested here. However, interesting statistically significant correlations were identified between SS and OP or OA, and with other traits, suggesting that physiological mechanisms are in place in maize, linking sugar content in the leaves, osmotic potential and tolerance to drought. More specifically, SS content in WD positively correlated with OA. Overall, our correlation results strongly suggest that soluble sugar content plays a role in OA and drought tolerance, however the complexity of the SS accumulation prevented us to pick up a precise genetic signal in terms of lines and markers associated with the trait. Our results indicate that in order to address the genetic control of leaf sugar accumulation, a more powerful (ie. with more replicates) experiment than the one carried out here should be planned.

The combination of phenotypic results and correlations, including both the traits (OP, OA and SS) described here and the ones reported in Deliverable 4.2 strongly suggests that the line 121-6-6-6 is characterized by a relatively unique genetic settings. This line was shown here to be the top OP performer in water deficit, and was characterized by high OA and SS content. At the same time, it was shown to be the line with the highest score in terms of drought tolerance (Deliverable 4.2).

Thanks to the knowledge gained in this part of the Water4crops project, and the plant materials characterized (eg. IL121-6-6-6), it is now possible to plan additional more focused investigations towards a full understanding of the molecular mechanisms in play in the response of maize to drought.

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