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Genotypic Variability in Carbon Isotope Discrimination and Water Use Efficiency among Recombinant Inbred Lines of Sunflower (*Helianthus annuus* L.)

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ABSTRACT

To evaluate genotypic variability in carbon isotope discrimination or CID and water use efficiency or WUE, recombinant inbred lines (RILs) population of sunflower (*Helianthus annuus* L.) was used. Seventy eight sunflower RILs were grown in greenhouse and 100 sunflower RILs were grown under field condition, and measured some morphological and physiological traits including leaf area at flowering (LA_f), net CO_2 assimilation rate (A) and transpiration per day at flowering (E_f). WUE, called "potential" WUE (WUEp), was calculated as the ratio of assimilation potential (Ap) to transpiration per day at flowering (E_f) where Ap was derived from the multiplication of A with LA_f. The CID was significantly varied among RILs and there was significant negative genetic correlation between CID and WUEp. Heritability of the CID was higher rather than the WUEp which reflected wide genetic variability of CID. The genetic correlation between CID and WUEp and the wide genotypic variability of CID indicated that CID can be proposed as an indicator to determine WUE in sunflower and open a way in understanding the genetic diversity of the RILs which could be used as a basic consideration before applying selection program in sunflower breeding.

INTRODUCTION

Sunflower is known as the highly important oil crops in worldwide (Chen et al., 2014; Flagella, Rotunno, Tarantino, Di Caterina, & De Caro, 2002) and is the fourth edible oil in the world behind rapeseed, soybean and palm oil (Adiredjo, Navaud, Lamaze, & Grieu, 2014; Gunstone, 2013). Upon this crop, study of genotypic variability affecting agronomic and economically important traits could assist in improving breeding techniques (Allinne, Maury, Sarrafi, & Grieu, 2009; Kiani et al., 2007). Traits such as yield components, yields as well as other quantitative characters are controlled by several genes. Thus, genotype x environmental interaction is the component of variance decreasing their heritability (Åkesson et al., 2008; Johnson, Agrawal, Maron, & Salminen, 2009)**.**

Targeting underlying agronomical, morphological and physiological traits could be able to contribute essentially to the improvements of yield. To be useful, the traits must have high heritability, not result in yield losses when conditions are favorable, and easy to measure, (Anyia, Slaski, Nyachiro, Archambault, & Juskiw, 2007; Richards, Rebetzke, Condon, & van Herwaarden, 2002). Performing genotypic increases in economically important trait, like yields, under rain fed environment has been a hard objection for plant breeders (Rauf, 2008; Richards, Rebetzke, Condon, & van Herwaarden, 2002). A main objection for plant breeders as well as sunflower scientists is to devise the efficient and effective strategy to optimize gained genetic. Essential work therefore goes into observing environments representative sets to assess yield and yield components of various breeding lines (Messina, Podlich, Dong, Samples, & Cooper, 2011).

Furthermore, identification of agronomical and physiological traits contributing to superior yield performance of crop plants has been a long term-goal of plant scientists (Allinne, Maury, Sarrafi, & Grieu, 2009; Blum, 1983; Jackson, Robertson, Cooper, & Hammer, 1996; Sternberg, 2012). In addition to management tillage, there are several agronomic options and so genetic improvements, to enhance the needs of water in rain fed condition in the capability of crops to use water more efficiently, namely water use efficiency (WUE) (Condon, Richards, Rebetzke, & Farquhar, 2002; Evett & Tolk, 2009; White, 2011).

Based on the scale of measurement and the units of exchange considered, WUE can be determined in many measures.The unit of production for plant physiologist might be moles of carbon gained in assimilation or photosynthesis (A) in exchange for water used in plant transpiration (T). The driving force for water loss will remain relatively unchanged and lead to an increase in water use efficiency, under any of particular set of conditions (Condon, Richards, Rebetzke, & Farquhar, 2002; Singh & Reddy, 2011). While the basic unit of production for agronomist and plant ecologist is the yield and yield components of harvested product achieved from the water consumption (Condon, Richards, Rebetzke, & Farquhar, 2004; White, 2011). However, there is lack of simple method of WUE estimation especially in field conditions. Nevertheless, the problem has been overcome since Farquhar, Ehleringer, & Hubick (1989) proved the negative correlation between WUE and CID in tissue of C3 plant species. CID during plant growth and development could be used as an excellent surrogate for determining WUE, which is usually directly measured in the field (Adiredjo, et al., 2014; Brendel et al., 2008; Brugnoli, Hubick, von Caemmerer, Wong, & Farquhar, 1988; Chen, Chang, & Anyia, 2011; Ebdon & Kopp, 2004).

Genotypic variability of CID in C3 plant has received increasing attention of WUE (Adiredjo, et al., 2014; Condon, Richards, Rebetzke, & Farquhar, 2002; Ebdon & Kopp, 2004; Farquhar, Ehleringer, & Hubick, 1989; Rebetzke, Condon, Farquhar, Appels, & Richards, 2008). Plants discriminate toward the heavier carbon isotope (^{13}C), during assimilation processes, because the process depends on the intercellular and atmospheric carbon dioxide partial pressure (Pi and Pa). The process could be defined as the ratio of Pi/Pa. Higher Pi/Pa may result in higher CID due to higher the conductance of stomata. Thus, WUE is negatively related to both Pi/Pa and CID (Farquhar, Ehleringer, & Hubick, 1989; Singh & Reddy, 2011). A physiological way can balance breeding method and may improve the proportion of yield enhancement in the following approach. First, high seasonal variability in yield and genotype by environmental interactions will gain stagnant genetic for yield. Second, it could detect the important traits. There is insufficient genetic variability in plant breeders' populations. Specific aim of morphological and physiological traits that have a high value of heritability and limit yield could be more effective than direct selection for yield (Richards et al., 2010; Richards, Rebetzke, Condon, & van Herwaarden, 2002).

Therefore, a population of 78 RILs grown in greenhouse and a population of 100 RILs grown in field condition were used to evaluate genotypic variability and genetic correlations of heritable traits, CID and WUE. The WUE was an estimated-WUE, called "potential" WUE (WUEp), by using the traits of net CO_2 assimilation rate (A), leaf area at flowering (LA_f) and transpiration per day at flowering (E_f). The objectives of this study were: (i) to analyze genotypic variability of CID and (ii) to evaluate genetic correlations between WUE and CID in sunflower RILs. We are interested in investigating the possibility of CID as the potential trait to be used in the selection program of sunflower lines with high WUE.

MATERIALS AND METHODS

Plant Material and Traits Measurement

The sunflower genotypes in the experiment had been previously used by Kiani et al. (2007) and Kiani et al. (2009). The bulk of the phenotypic traits were presented in the previous study, but the CID reported here was not available yet. Two populations of RILs originated from a cross between 2 parental lines, PAC2 and RHA266, were generated as described previously by Kiani et al. (2009). The experiments were carried out in a greenhouse and in the field at the National Institute for Agricultural Research (INRA) Auzeville station, Toulouse, France ($43^0 31'46,94"$ N; $1^0 29'59,71"$ E), in spring 2010.

Briefly, for the RILs in greenhouse, plants were grown under controlled conditions. Temperature was considered at 25/18 ± 2 °C (day/night) with relative humidity 65–85 ± 5 %, and supplementary light at approximately 16-h light and 8-h dark period was maintained during experiment. Plants were individually planted in a mixture of soil, compost and sand with percentage of 40 %, 40 %, and 20 % respectively, by using plastic pots (4.0 l). For the RILs in field condition, the experiments were conducted in two water treatments, irrigated (IR) and non-irrigated treatments (NIR). Both IR and NIR treatments were put in the same field to have the same environment condition with adequate distance in order to avoid water to reach non-irrigated treatment. Split-plot design with three blocks was chosen to conduct the experiment. The main plot was IR and NIR water treatments while RILs were considered as sub-plot. Each block consisted of 2 rows 4.6-m long, with 25 cm between plants in rows and 50 cm between rows, giving a total number of about 32 plants per plot. Based on sunflower irrigation program determined by National Institute for Agricultural Research (INRA) France, irrigated field was watered two times at two stages, just before flowering and at about grain filling. For evaluation of the morphological and physiological traits, three plants per RIL per water treatment were randomly taken (Kiani et al., 2009).

Net CO_2 assimilation rate (A) measurement was determined at light-saturation of 1.500×10^{-6} mol m^{-2} s^{-1} photosynthetic photon flux density or PPFD, leaf temperature maintained at 25 ± 2 ^{0}C, relative humidity of 60 ± 5 % and ambient CO_2 concentration was approximately 400×10^{-6} mol mol^{-1}. Leaf area at flowering, called LA_f, was determined at flowering stage with the calculation following formula: $LA_f = \sum 0.7$ $L \times W$ (Alza & Fernández-Martínez, 1997). Potential of net CO_2 assimilation per plant (Ap) was derived from the multiplication of A with LA_f. Transpiration per day at flowering (E_f) was measured by weighing the pots every day during 12 days at flowering period, the difference of water loss between two days was considered as daily transpiration. The "potential" water use efficiency, called WUE_p, was calculated as the ratio of Ap to E_f. In addition, for relative water content (RWC), a trait that only measured for population in field condition, was determined by the formula: RWC = $F_W - D_W/T_W - D_W$, where F_W and D_W is fresh and dry weight (D_W is dry weight after oven drying for 24 h at 80 ^{0}C), and T_W is turgid weight. Tw was determined after 24 h rehydration at temperature of 4 ^{0}C in a dark room by positioning the petioles in a container with distilled water.

Carbon Isotope Discrimination (CID) Analysis

A little amount, 2-3 mg subsamples, of oven-dried leaves were balanced and placed in capsules (Elemental Microanalysis, UK). Before placing in capsules, those samples of each plant were ground into a homogenous fine powder. All capsules of subsamples were placed into a clean 96-well tray of a micro plate to be analyzed at UC Davis Stable Isotope Facility (California, USA), using a continuous flow Isotope Ratio Mass Spectrometer (Sercon Ltd., Cheshire, UK). Carbon isotope composition (delta^{13}C) was determined as: $delta_{plant} = (R_{sa} - R_{sd})/R_{sd} \times 1000[‰]$ where R_{sa} and R_{sd} are the ^{13}C : ^{12}C ratios of the sample and the standard respectively. The standard used is related to the international Pee Dee Belemnite (PDB) (Farquhar, Ehleringer, & Hubick, 1989). CID was calculated: CID = $(delta_{air} - delta_{plant})/(1 + delta_{plant}/1000)$ where $delta_{air}$ is the ^{13}C composition of CO_2 in the atmosphere which is assumed –8.0‰ (Farquhar, Ehleringer, & Hubick, 1989).

Statistical Analysis

In the present study, the PASW statistics software version 18 (IBM, New York, USA) was used. The test of normality was determined by a package of Kolmogorov-Smirnov test (lilliefors correction) completed with histogram and normal Q-Q plot graph. For each analysis, RIL and block were considered as fixed factors, while trait was considered as dependent factor. Tests were performed two-sided with a significance level of 5 %. Tukey-test was used to know the differences among genotypes.

Phenotypic (r_p) as well as Genetic (r_g) relationships between two traits were calculated using components of variance as follows: $r_p = Cov_p (xy) / (var_p (x) var_p (y))^{0.5}$ and $r_g = Cov_g (xy) / (var_g (x) var_g (y))^{0.5}$ where Cov_p is the covariance of phenotype between traits x and y and var_p is the variance of phenotype. Cov_g is the covariance of genotype (genetic) between traits x and y and var_g is the variance of genotype (Kearsey & Pooni, 1998). Heritability (h^2) value is derived from the ratio of genetic variance to phenotypic variance among genotypes ($h^2 = var_g/var_p$). Genetic variance (var_g) and phenotypic variance (var_g) can be estimated from the analysis of variance considering genotypic effects as random. Phenotypic variance was come from combination of genetic variance with environmental variance ($var_p = var_g + var_e$) where var_e is the environmental variance (Singh & Chaudhary, 1977).

RESULTS AND DISCUSSION

Genotypic Variability and Heritability (h^2) for CID and Related Traits in Greenhouse Condition

The CID values ranged from 18.54 to 26.58‰ and the mean value was 21.95‰. CID heritability estimation was high and more than 50 % (h^2 =0.82) (Table 1). The analysis of variance (ANOVA) summary or mean squares results for CID and other related traits are shown in Table 2.

The CID was significantly different among RILs ($p < 0.01$). The CID values varied from 18.54 to 26.58 $^0/_{00}$. This result is in line with several previous studies (Centritto, Lauteri, Monteverdi, & Serraj, 2009; Jones, 1993) that the range value of CID for C_3 plant material was from 13 to 28 $^0/_{00}$. A large range of CID variation (8.04 $^0/_{00}$) was observed in our study, exceeding the range 2.5 $^0/_{00}$ – 4.4 ‰ investigated in greenhouse studies of sunflower (Adiredjo, et al., 2014; Lambrides, Chapman, & Shorter, 2004). The large ranges and the significant difference of CID among RILs in the present study

indicated that there is possibility to conduct the selection program for sunflower genotypes by using CID as a trait.

In the present study, a high heritability of CID indicated that the assay developed for pots grown sunflower was robust, and the genetic effect for CID was bigger than the environmental effect. The similar results have been published by Anyia, Slaski, Nyachiro, Archambault, & Juskiw (2007) and Lambrides, Chapman, & Shorter (2004) where the heritability was high among 87 genotypes and 20 inbred lines of sunflower. Several works showed that CID had higher heritability compared to the WUE and yield, for example as reported in wheat (Ehdaie & Waines, 1994), cotton (Stiller, Read, Constable, & Reid, 2005), and rice (This et al., 2010). Hence, the higher heritability compared to WUEp suggested that CID can be used as a selection tool in breeding strategies to improve WUE. Results of this study confirm that the genetic variation associated with CID in sunflower is inherited polygenically (Adiredjo, et al., 2014).

Table 1. Range of genotypic variability and heritability (h^2) for CID and related traits of 78 RILs in greenhouse condition

Trait	CID	WUEp	A	Ap	Ef	LAf
Mean	21.95	206.17	19.56	50551.38	246.46	2563
Minimum	18.54	114.76	14.83	27521.48	147.89	1528
Maximum	26.58	274.51	25.03	76583.89	324.48	3505
Std.dev.	2.15	36.49	2.72	12845.23	49.52	529.86
h2	0.82**	0.01	0.01	0.28*	0.68**	0.58**

Remarks: CID = carbon isotope discrimination ($^0/_{00}$), WUEp = "potential" water use efficiency (10^{-6} mol CO_2 .cm^2. m^{-2} s^{-1} ml^{-1} day^{-1}), A = net CO_2 assimilation rate (10^{-6} mol CO_2 m^{-2} s^{-1}), Ap = potential of net CO_2 assimilation per plant (10^{-6} mol CO_2 .cm^2. m^{-2} s^{-1}), E_f = plant transpiration per day at flowering period (ml per day), LA_f = leaf area at flowering (cm^2). **) high value of heritability ($0.5 < h^2$): the genetic effect was dominant rather than the environmental effect. *) medium value of heritability ($0.2 < h^2 < 0.5$): the genetic and the environmental effects were equally involved.

Table 2. Result of ANOVA, mean squares for CID and related traits of 78 RILs in greenhouse condition

SV	d.f.	CID	WUEp	A	Ap	Ef	LAf
Genotype	77	11.62**	3996 ns	22.15 ns	489727937**	7357**	842264ns
Block	2	11.94**	58218**	302.06**	7082396692**	28791**	6019183**
Error	154	1.04	3787	21.30	225765657	1008	224591
Total	233						

Remarks: SV = source of variance, d.f = degree of freedom. *) significant at $P < 0.05$, **) significant at $P < 0.01$, ns) not significant. CID = carbon isotope discrimination ($^0/_{00}$), WUEp= "potential" water use efficiency (10^{-6} mol CO_2 .cm^2. m^{-2} s^{-1} ml^{-1} day^{-1}), A = net CO_2 assimilation rate (10^{-6} mol CO_2 m^{-2} s^{-1}), Ap = potential of net CO_2 assimilation per plant (10^{-6} mol CO_2 .cm^2. m^{-2} s^{-1}), E_f = plant transpiration per day at flowering period (ml per day), LA_f = leaf area at flowering (cm^2).

Genetic Correlation between CID and WUEp and Other Related Traits

There was significant negative genetic correlation between CID and WUEp as well as between CID and *A* and/or Ap (Table 3). A negative genetic correlation between CID and WUEp suggests the possibility of employing WUEp as surrogate of WUE at whole-plant level. Thus, negative genetic correlation is in accordance with previous report (Adiredjo, et al., 2014; Lambrides, Chapman, & Shorter, 2004) that observed a negative genetic correlation (r_g= -0.92**) between CID and WUE of sunflower genotypes in greenhouse experiment at well-watered condition. Indeed, this result is consistent with an argument from Farquhar, Ehleringer, & Hubick (1989) that studied on wheat, and is in accordance with numerous authors (Carelli, Fahl, Trivelin, & Queiroz-Voltan, 1999; Centritto, Lauteri, Monteverdi, & Serraj, 2009; González-Martínez, Huber, Ersoz, Davis, & Neale, 2008; Zhao, Kondo, Maeda, Ozaki, & Zhang, 2004; Zhu, Li, Liang, Xu, & Li, 2010). This significant genetic correlation indicated that CID is a potential indicator to determine WUE in sunflower genotypes under optimal plant growth (non-limited soil water availability).

Table 3. Genetic correlation (r_g) between CID and WUEp and related traits of 78 RILs in greenhouse condition

Trait (x)	Trait (y)	rg(xy)
CID	WUEp	-0.30*
	A	-0.54*
	Ap	-0.27*
	Ef	-0.22
	LAf	-0.18

Remarks: *) Significant at $P < 0.05$, values without * are not significant. CID = carbon isotope discrimination ($^0/_{00}$), WUEp = "potential" water use efficiency (10^{-6} mol CO_2 .cm^2. m^{-2} s^{-1} ml^{-1} day^{-1}), *A* = net CO_2 assimilation rate (10^{-6} mol CO_2 m^{-2} s^{-1}), Ap = potential of net CO_2 assimilation per plant (10^{-6} mol CO_2 .cm^2. m^{-2} s^{-1}), E_f = plant transpiration per day at flowering period (ml per day), LA_f = leaf area at flowering (cm^2).

The *A* (net CO_2 assimilation) and the Ap (potential of net CO_2 assimilation) were genetically associated with CID, since the negative genetic correlations were observed among these traits. Another study on *Coffea* species grown in greenhouse and watered daily with enough water has shown a strong negative correlation between CID and *A* (Carelli, Fahl, Trivelin, & Queiroz-Voltan, 1999). This result might be explained that high amount of carbon isotope composition (delta^{13}C) which cause little discrimination of ^{13}C was significantly maintained the rates of photosynthetic CO_2 fixation. De Miguel, Sánchez-Gómez, Cervera, & Aranda (2012) noted that to sustain reasonable rates of CO_2 fixation, plant must make an enormous amount of Rubisco.

Study of genetics for the traits that have significant genetic correlation in this paper is an important aspect throughout breeding, physiological and agronomical approach in sunflower development. The CID, WUEp, *A*, Ap and LA_f are polygenic traits, since any gene that affects either stomatal conductance or photosynthesis rate per unit leaf area can have an effect. Farquhar, Ehleringer, & Hubick (1989) proposed that the genetic evaluation of polygenic trait like *A* may enable plant breeders to follow the results of crossing material with desirable *A* into commercial variety but in parallel with conducting study on the genetic control of CID by the plant. In fact, the variation of CID and its related traits are considered by the influences of genetic and environmental effects.

Genotypic Variability for CID and Related Traits in Field Condition

Table 4 presented the range of genotypic variability of CID and related traits. For CID under irrigated, the mean was 21.05 $^0/_{00}$ and the range of CID values was from 19.41 $^0/_{00}$ (minimum) to 22.84 $^0/_{00}$ (maximum), whereas for CID values under non-irrigated the mean was 21.17 $^0/_{00}$ with the minimum was 19.25 $^0/_{00}$ and the maximum was 23.26 $^0/_{00}$.

A large range of CID variation for RILs in field condition of about 3.5 $^0/_{00}$ (irrigated) and 4 $^0/_{00}$ (non-irrigated) are exceeding from the range 2.5 $^0/_{00}$ of sunflower genotypes in field studies reported by Virgona & Farquhar (1996). The large ranges of CID variations in the present study suggest that there is a potential to select sunflower lines by using CID as a trait.

There was significant difference of comparing means differences by *paired t-test* between CID irrigated (i) and CID non-irrigated (ni) of 100 RILs (Table 5).

Table 4. Range of genotypic variability in CID and morphological and physiological traits of 100 RILs in field condition

	CID		LAf		RWC	
	ir	Nir	ir	nir	Ir	Nir
Mean	21.05	21.17	4317.11	3932.46	78.45	77.22
Minimum	19.41	19.25	1988.35	2479.12	64.53	52.00
Maximum	22.84	23.26	7739.40	6949.06	95.86	89.51
Std.dev.	0.70	0.69	1173.81	888.39	5.06	7.30

Remarks: ir = irrigated, nir = non-irrigated; CID =carbon isotope discrimination ($^0/_{00}$), LA_f = leaf area at flowering (cm^2), RWC = relative water content (%)

Table 5. Result of *paired t-test* for CID between irrigated and non-irrigated (*r*) among 100 RILs in field condition

Traits	Paired T-test (i-ni)	
	t (99) ; p value	
CID	-2.137*	0.035

Remarks: i = irrigated, ni = non-irrigated, t (99) = value of student t-test with the number of degree of freedom was 99. *) Significant at the 0.05 level.

The significance result of differences between irrigated and non-irrigated (t (99) = -2.137, $p < 0.05$), means that the trend of CID values were significantly decreased from irrigated to non-irrigated treatment. The trend of decreasing CID under water deficit for all lines indicates that environmental effect influenced CID especially when there is a lack supply of water availability. According to Kumar & Singh (2009), plants under water stress may be attributed to cause diffusion limitation for both CO_2 and H_2O and partial or complete stomatal closure condition. The consequences of stomatal closure are lower reactivity with Rubisco during C fixation and little discrimination against ^{13}C (Serraj, 2006) which will cause smaller CID of the plants. In addition, Kumar & Singh (2009) noted that low in Rubisco activity may cause the decrease in assimilation under severe drought condition. The CO_2 supply may be limited under drought stress either by stomatal closured or by stomatal tissue shrinkage. Our result supported the work of Frank & Berdahl (2001) who observed CID values were significantly decreased from well-watered to water-stressed treatment in diploid and tetraploid of Russian wildrye. Another previous study was reported by Zhao, Kondo, Maeda, Ozaki, & Zhang (2004) where CID under water stress significantly decreased of all analyzed plant parts measured at tillering in two cultivars of upland rice. Furthermore, the general trend of decreasing CID under water stress support the idea that there is negative relationship between CID and WUE, as noted by previous authors (Centritto, Lauteri, Monteverdi, & Serraj, 2009; Chen, Chang, & Anyia, 2011) that WUE increased when water deficit due to higher conductance reduction than assimilation reduction and extended drought can substantially increase WUE.

Our result in the present work indicated that the variation of CID in greenhouse was not correlated to the variation of CID in field condition. This result suggests an interpretation, despite the RILs were used in greenhouse represent the same RILs in field condition, separately each RIL had not consistent to show variation of CID which may be caused by different level of genotype x environment (G X E) interaction. Physiologically, discrimination of ^{13}C through diffusion and carboxylation that happened in leaf of RILs were influenced by different factors whether in greenhouse or in field condition. It can be analyzed that different scales of water deficit treatment had been proven different availability of water in pots and in the soil. The phenomenon of this result is related to the observation that initially noticed by Farquhar, Ehleringer, & Hubick (1989) and reported by several authors (Brendel et al., 2008; Marguerit, Brendel, Lebon, Van Leeuwen, & Ollat, 2012; Rengel et al., 2012; Tardieu & Tuberosa, 2010), accordingly CID variation in the patterns of water use will likely be dependent on whether adjacent plants within a community are competing for the same limiting resource. It can be argued that efficient use of a resource, such as water, may only be adaptive if plants exert some control over the rates of soil water extraction from the soil volume in which their roots are located. If plants are competing for the same limit water resources, there may be selection against conservative use of this resources, and capture the resources as fast as possible.

In addition, other factors may be ascribed from the different rooting pattern and temperature as well as humidity. As pointed by Pinheiro, DaMatta, Chaves, Loureiro, & Ducatti (2005), differences in leaf delta[13]C and WUE may be related to rooting pattern. In this case, it can happen for the RILs in greenhouse had shallow root because the plants were planted in the pot which were not happened for the RILs grown in field condition. Consequently, the respond of RILs to water deficit treatment were not the same and gave the effect to the stomatal conductance which will cause different composition of carbon isotope (delta[13]C) of the plant. Besides, the RILs grown in different condition (greenhouse and field) were predicted had different temperature and humidity which influenced the variation of CID in these two different conditions. As noted by Cernusak, Arthur, Pate, & Farquhar (2003) and Farquhar, Ehleringer, & Hubick (1989) that within the constraints set by minimal and maximal stomatal conductance for a particular leaf type, response stomata to temperature and humidity would result in maximal daily CO_2 assimilation rate for a specific daily water consumption. It has been clearly explained (in previous discussion) that net CO_2 assimilation (A) was genetically associated with CID.

Prospect of Genetic Studies in CID and WUE

Study of genetics for the traits that have significant genetic correlation in the present work is an important aspect especially related to the context of integration among breeding, physiological and agronomical approach in sunflower development (Condon, Richards, Rebetzke, & Farquhar, 2004; Richards, Rebetzke, Condon, & van Herwaarden, 2002). The CID and related traits of RILs in greenhouse as well as in field condition are most likely to be polygenic, since any gene that affects either photosynthesis or stomatal conductance can have an influence (Barbour, Warren, Farquhar, Forrester, & Brown, 2010; Comstock, 2002; Tardieu & Simonneau, 1998).

Evaluating the genotypic variation for understanding the genetic basis of CID and WUE is essential for crop improvement (breeding) (Chen, Chang, & Anyia, 2011). It has been known that in breeding populations, with sufficient attention to sampling scenarios, CID is a highly heritable and reliable trait which is relatively easy to be manipulated (Condon, Richards, Rebetzke, & Farquhar, 2002; Rebetzke, Condon, Richards, & Farquhar, 2002). All these observations indicated CID as a potential trait in breeding for greater agronomic WUE (Condon, Richards, Rebetzke, & Farquhar, 2004). In sunflower, genetic quantitative study has been conducted to analyze the genotypic variability of WUE and the potential use of leaf CID as an indicator to determine WUE (Lambrides, Chapman, & Shorter, 2004). However, because of the complexity and difficulty of determining WUE in large number of genotypes under field conditions, determination and improvement of WUE through classical breeding techniques are not practical (Mian, Ashley, & Boerma, 1998; Rajabi, Ober, & Griffiths, 2009). Thus, in searching for an alternative to the classical approach for the WUE breeding of field crops, it is needed an effort to apply the advance genetic or genomic studies. Genomic approaches conditioning WUE for crops by applying indirect selection for high WUE may prove to be a useful approach in this respect (Adiredjo, et al., 2014; Brendel et al., 2008; Chen, Chang, & Anyia, 2012; Mian, Ashley, & Boerma, 1998).

CONCLUSION AND SUGGESTION

Direct measurement of WUE is difficult under natural field conditions with high labor requirements. On the other hand, the "intrinsic" of water use efficiency (WUE_i) has been clearly established. It is still evident to consider an estimation of WUE as an alternative method to predict WUE in plant scale. In our study, we proposed the measurement of WUE estimation from some appropriate physiological traits, so called water use efficiency "potential" (WUE_p).

Our work proved that there were wide genetic variabilities of CID whether for the RILs in greenhouse condition or in field condition. The result of our study has proved that genetic variation of CID for RILs in greenhouse was significantly associated with WUE_p under controlled treatment. A negative genetic correlation between CID and WUE_p suggested that CID as a potential trait to be used in sunflower improvement program. In addition, CID is very considerable trait to be used as selection criterion in plant breeding program to determine WUE due to the heritability of CID was higher than WUE_p.

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