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The determination of direct and indirect effects of carbon isotope discrimination (Δ), stomatal characteristics and water use efficiency on grain yield in wheat using sequential path analysis

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Abstract

The study was conducted on 20 wheat genotypes including four einkorn wheat (*T. monococcum* subsp. *monococcum*), eight durum wheat (*T. turgidum* subsp. *durum*), six hexaploid landraces and two developed modern varieties including Rooshan and Azar 2 to identify direct effects of stomatal characteristics, water use efficiency (WUE) and Δ on grain yield and also to discover the effects of stomatal characteristics on the character through affecting Δ and water use efficiency using sequential path analysis. The variations observed among the genotypes were highly significant for all the characters under study. Stomatal frequency indicated negative relationship with grain yield. While stomatal frequency on the either sides of leaves had negative association with grain yield (GY), stomatal area on the both sides indicated close positive correlations with GY. The only significant direct effect on GY was belonging to WUE and surprisingly neither stomatal characteristics nor Δ had significant direct effects on grain yield. The least significant direct effect was belonging to stomatal area on abaxial surface followed by stomatal area on adaxial one. Regarding to indirect effects, stomatal frequency on both sides of leaves had positive significant indirect effects on GY via WUE. In addition, stomatal frequency on the abaxial surface had a negative significant indirect effect on GY via Δ. A positive significant indirect effect on GY was also observed from stomatal area through carbon isotope discrimination.

Keywords*:* carbon isotope discrimination (Δ), sequential path analysis, wheat, WUE.
Abbreviations: GY: grain yield, SF: Stomatal frequency, SA: stomatal area, Δ: carbon isotope discrimination, WUE: water use efficiency.

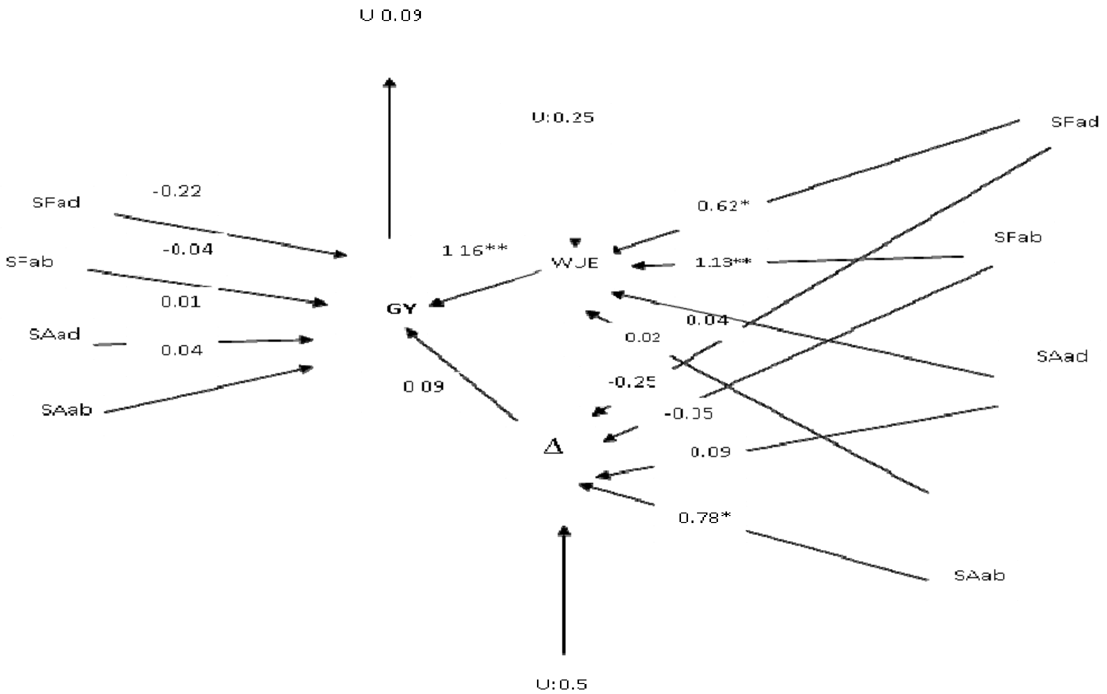
Introduction

Wheat is the most important world cereals and it has been cultivated within origin center of variation (southwest Asia) for over than 10000 years (Poehlman, 1995). The wild species of wheat are still under cultivation in many parts of the world. Carbon isotope discrimination (Δ) is a character indicating the amount of 13C depleted by photosynthesis mechanisms. Considerable variations have been observed between wheat cultivars and even among wheat wild relatives and landraces for Δ (Khazaei et al., 2009). Some tall plants in an experiment cultivated with a particular short variety are also reported to differ significantly from the mean of plants for Δ (Tokatlidis et al., 2004). This character is related to drought-tolerance indicators such as stomatal resistance and water use efficiency (Farquhar and Richards, 1984; Griffiths 1993; Taiz and Zeiger, 1998) and it has been widely studied in C3, C4 and CAM (Grassulacean Acid Metablolism) plants. Most of the studies are dealing with the relationship between this character and other agronomic traits. Ehdaie at al. (1991) reported strong negative correlation between Δ and dry matter production under pot well -watered conditions. Furthermore, Δ has indicated negative association with grain yield and water use efficiency under some drought conditions (Richards et al., 1998). Mohammady et al. (2005) also reported that drought tolerant variety Falchetto had more yield and lower Δ than drought susceptible variety Oxley under pre-anthesis water-stressed conditions. On the other hand, many researchers found positive significant correlation between Δ and grain yield (Sayre et al., 1995, Mehra et al., 2001; Tsialtas et al., 2001). Relationship between Δ and stomatal conductance is reported to be positive and significant under well-watered conditions (Ehdaie, 1995). In addition, carbon isotope discrimination is reported to be closely correlated with photosynthesis rate (PR) and water use efficiency (Condon et al., 1993). Since the characters such as grain weight and biomass in wheat have low heritability (Rebetzke et al., 2002), Δ has been considered as an indirect selection criterion in segregating generation of wheat during breeding programs to select for grain weight and biomass (Cooper et al., 1997; Rebetzke et al., 2006). Carbon isotope discrimination has also been proposed as an alternative selection criterion for drought tolerant cultivars (Ehdaie and Waines 1993; Mohammady et al., 2005). Stomata are the major gates for gas exchange of leaves and play an important role in the control of water evaporation and gas exchange in plant leaves (Maghsoudi and Maghsoudi moud, 2008). Transpiration and photosynthesis are affected by frequency and size of stomatal pores. In addition, the operation of the stomatal apparatus is influenced by plant environment (Heichel, 1971) and, therefore, stomatal regulation of transpiration is affected by both internal and external factors. Selection and variation for stomatal characteristics has been reported in bread wheat. Mohammady (2002) found significant differences for stomatal length in adaxial and abaxial surfaces among bread

Table 1. Variation observed among the genotypes for the characters under study

Characters	Range	Mean	S.E.	CV(‰)	F value
SFad (*no. mm-2*)	34.08-111.45	54.42	5.01	2.56	85.33**
SFab(*no. mm-2*)	45.59-110.60	70.53	4.84	3.25	43.52**
SAad (µ*m2 mm-2*)	541.00-1515.00	1075,695	68.73	3.50	26.77**
SAab(*µm2 mm-2*)	537.10-1557.80	1191,545	73.35	3.63	31.73**
WUE (gr mg-1)	0.41-1.13	0.84	0.04	3.99	44.63**
Δ(‰)	18.83-20.08	19.57	0.18	23.08	4.60**
GY (gr plant-1)	1.03-3.52	2.51	0.14	3.47	13.67**

SFad and SFab=Stomatal frequency on the adaxial and abaxial surfaces of flag leaves, respectively; SAad and SA ab=Stomatal area on the adaxial and abaxial surfaces of flag leaves, respectively; WUE=water use efficiency; Δ=Carbon isotope discrimination; GY=Grain yield. *,**: significant at 5% and 1% of probability levels, respectively



[a]**Fig 1.** Sequential path diagram showing direct and indirect effects of stomatal characteristics on grain yield. *a*: * and **: the effects are significant at P<0.05 and P<0.01, respectively; Δ:carbon isotope discrimination; GY: grain yield; SAab and SAad: stomatal area at abaxial and adaxial surfaces, respectively; SFab and SFad: stomatal frequency on abaxial and adaxial surfaces, respectively;: U: residual effects on the characters; WUE: water use efficiency surface;

wheat cultivars. He also reported that stomatal length is more effective than stomatal width on water transpiration. It has been suggested that wheat cultivars having wider stomatal aperture produce higher yields without consuming more water (Khazaie et al., 2010). Wang and Clarke (1993b) reported that stomatal frequency positively correlated with the rate of water loss. This indicates breeding for smaller and fewer stomata may lead to reduction in water loss. Gaskell and Pearce (1983) found that stomatal density negatively correlated with grain yield and with stomatal size. Stomatal frequency in wheat was shown to be greater on the adaxial than on the abaxial surface. Path coefficient analysis has been extensively utilized to investigate the direct and indirect effects of yield related traits on grain yield and assist in identifying traits that are useful as selection criteria to improve crop yield (Milligan et al., 1990). This allows partitioning the correlation coefficient into its components, one component being the path coefficient that measures the direct effect of a predictor variable upon its response variable, and the second component being the indirect effect(s) of a predictor variable on the response variable through another predictor variable (Dewey and Lu, 1959). Recent applications of the method to the analysis of yield in wheat include studies from Garcia del Moral et al., (2003), Zečević et al., (2004), Aycicek and Yildirim (2006), Fagam et al., (2006) and Ali et al. (2008). Most of these studies however emphasized the relationship between grain yield and its components among modern cultivars or breeding lines, and very few involve physiological traits. Among the traits that have been proposed to explain yield variation in cereals, carbon isotope discrimination has been quite intensively analyzed (Condon et al., 2002; Monneveux et al., 2005). However, reported correlations between grain yield and Δ vary from strongly positive to strongly negative (Condon et al., 2002; Monneveux et al., 2005). Negative correlations between grain yield and Δ were generally found in plant parts

Table 2. Pair-wise simple Pearson correlation coefficients among characters. Abbreviated terms are described in the legend of the Table 1.

	SF_{ad}	SF_{ab}	SA_{ad}	SA_{ab}	WUE	Δ	GY
SF_{ad}(*no. mm^{-2}*)	1.0						
SF_{ab}(*no. mm^{-2}*)	0.915**	1.0					
SA_{ad}(μm^2 mm^{-2})	-0.885**	.-0.81**	1.0				
SA_{ab}(μm^2 mm^{-2})	-0.92**	-0.939**	0.93**	1.0			
WUE(gr mg^{-1})	-0.781**	-0.768**	0.860**	0.82**	1.0		
Δ(‰)	0.539*	0.523*	-0.45*	-0.44	-0.59*	1.0	
GY(gr plant^{-1})	-0.668**	-0.65**	0.77**	0.710**	0.94**	-0.512*	1.00

*,**: significant at 5% and 1% of probability levels, respectively

sampled at early stages of development (Condon et al., 2002; Rebetzke et al., 2002) and were rarely observed when organs were sampled at maturity (Araus et al., 1998; Merah et al., 2001; Misra et al., 2006). As mentioned above, correlations between grain yield and morpho-agronomical traits have been widely studied using path analysis in wheat. Considerable numbers of the studies include correlations between grain yield and yield components or between grain yield and morpho-agronomical traits. The meeting point of the all studies was to bring grain yield in centre of consideration and then consequently almost try to provide incomplete information on the relative importance of the direct and indirect effects on grain yield. Although carbon isotope discrimination is a characters inherited simpler compared with grain yield (Rebetzke et al., 2006) but still under polygenic control (Rebetzke et al., 2008) and almost all characters involved in gas exchange process in plants can affect Δ, directly or through affecting other characters (Taiz and Zieger, 2002). Among these characters stomatal characteristics have not been sufficiently studied regarding their influence on yield and carbon isotope discrimination. The objectives of this study were to identify direct effects of stomatal characteristics on grain yield and also to discover their indirect effects on the characters through affecting Δ and water use efficiency using sequential path analysis.

Materials and methods

Plant Materials

The study was conducted on 20 wheat genotypes including four einkorn wheats (*T. monococcum* subsp. *monococcum*), eight durum wheats (*T. turgidum* subsp. *durum*), six hexaploid landraces and two developed modern varieties including Rooshan and Azar 2. All the genotypes were provided by Iranian wheat gene banks situated in Karaj. Azar 2 is a drought tolerant variety and Rooshan is reported to be susceptible to drought (Golestani Araghi and Asad, 1998)

Experimental conditions

Seeds were germinated in Petri plates and transferred into a growth chamber (2-4 °C) for 5 weeks to meet vernalisation required for flowering initiation. Three seedlings from each genotype were then planted in plastic pots filled with 1.1 kg of soil containing 42% sand, 36% silt and 22% clay. The water holding capacity of the soil was 25% by weight. Pots were arranged in a randomized complete-block design with three replications, in a greenhouse, at the University of Shahrekord, Iran (latitude, 50° 49', longitude; 32° 21'). Eight days after transplanting, two seedlings were removed from each pot leaving the most vigorous one. Then each pot was brought to water-holding capacity by adding 250 ml of water. Fifteen grams of perlite was added to the top of each pot to reduce soil evaporation. Pots were weighted every 3 days and amounts of water equal to the loss in weight were added to the pots until ripening (Ehdaie et al., 2003).

Measurements

When the flag leaves fully emerged Zadoks' scale 58 (Zadoks et al., 1974), the flag leaf of the main tiller of each plant was considered for determination of stomatal size, stomatal frequency and Δ analysis. Stomatal frequency and stomatal size (length and width) were measured in the middle part of the adaxial and abaxial surfaces by impression method (Wang and Clarke, 1993a). The number of stomata was counted from seven different microscopic fields of view at 160x magnification. To estimate stomatal frequency, the number of stomata per field of view was converted to the number of stomata per mm2 of leaf using a standard scale. Stomatal length and stomatal width were measured on the both surfaces from the impressions using a scaled eyepiece of microscope and then stomatal size was converted to µm. Stomatal area per unit leaf area (SA) (µm of stomata mm-2) was calculated using modified method of Wang and Clarke (1993a) as the product of SF × SL × SW. The above measurements were made randomly on 20 stomata of each impression and the mean values of the 20 measurements were used for statistical analyses. Carbon isotope discrimination was measured on the dried flag leaves. Leaves were dried at 80˚C for 2 days and finely ground to a powder to ensure homogeneity (Boutton, 1991) and 1 ± 0.05 *mg* samples were taken for analysis. The carbon isotope composition (δ13C) of samples was determined using an elemental analyzer isotope ratio mass spectrometer (ANCA-SL, Iso-Analytical Limited, Cheshire, UK). Carbon isotope composition (δ13C) was calculated by comparing 13C to 12C composition of a sample (R sample) relative to the composition of the Pee Dee Belemnite (PDB) standard (R PDB): δ13C (‰)= [(R sample / R PDB) −1] ×1000. The Δ value of the samples was obtained according to the formula: Δ (‰) = (δa−δp)/(1+δp), where δp is the δ13C of the plant sample and δa, the δ13C of the atmospheric CO2. In calculating Δ values of a sample, we assumed the δa to be −8 ‰ (Farquhar et al., 1989). Each sample was analyzed twice to assure accuracy of measurements.

Statistical analyses

Simple pair-wise Pearson correlation analysis, ANOVA and sequential path analysis were all performed on a Microsoft XP computer using MINITAB V.14 Software package. In path analysis, grain yield was considered as the predicted, WUE and Δ as intermediates and stomatal characteristics as predictor variables, respectively. Thus, direct effects of all characters and indirect effects of stomatal characteristics via WUE and Δ were analyzed on grain yield.

Table 3. Direct effects of water use efficiency, Δ and stomatal characteristics along with the indirect effects of stomatal characteristics through water use efficiency and Δ on grain yield

characters	Direct effect	Indirect effects through:		Total correlation with grain yield
		WUE	Δ(‰)	
WUE (gr mg^{-1})	1.16**	-	-	0.94**
Δ(‰)	0.09	-	-	-0.512*
SF_{ad}($n\ mm^{-2}$)	-0.22	0.62*	-0.25	-0.668**
SF_{ab}($n\ mm^{-2}$)	-0.045	1.13**	-0.5*	-0.65**
SA_{ad}($um^2\ mm^{-2}$)	0.04	.04	0.09	0.77**
SA_{ab}($um^2\ mm^{-2}$)	0.01	0.02	0.78*	0.710**

*,**: significant at 5% and 1% of probability levels, respectively

Results

The variations observed among the genotypes were highly significant for all the characters under study (F values in Table 1). This implies that the plant materials used in the current study are suitable sources for being used in programs dealing with wheat improvement worldwide. The highest and the lowest values of each character are also included in Table 1 under the range column. As can be seen from the table, the differences are huge in each case. The highest coefficient of variation (CV) was shown by Δ followed by water use efficiency and the lowest CV was belonging to stomatal frequency on the adaxial surface of leaves. The overall phenotypic correlations among the traits are presented in Table 2. Almost all the characters had significant correlation to each other. Stomatal frequency and stomatal area indicated diverse relationships with grain yield. While stomatal frequency on the either sides of leaves had negative association with GY, stomatal area on the both sides indicated close positive correlations with GY. Such a trend was observed in the case of correlation between stomatal characteristics and WUE. WUE and Δ were positively and negatively correlated with GY, respectively. It also was revealed that Δ and WUE correlate negatively to each other. Stomatal frequency on the both sides of flag leaves showed positive correlation with Δ. These correlations were significant at $P<0.05$. As it was expected, stomatal frequency on the upper and lower surfaces of leaves indicated negative correlation with stomatal area on the respected area. Looking at the both sides of leaves, it was revealed that the mean of stomatal frequency on the abaxial surface was higher than stomatal frequency on the adaxial surface. A similar trend was also observed in the case of stomatal area (Table 1). Stomatal frequency and stomatal area on the adaxial surface were closely correlated with those on abaxial one (Table 2). To sum up, it was revealed that all stomatal characteristics indicated significant correlations with GY and WUE and all except one (stomatal area on the abaxial surface) with Δ. As it was presented earlier, all stomatal characters, WUE and Δ had significant correlations either negative or positive with GY. These correlations were analyzed further by the sequential path procedure. This procedure involves a method by which direct and indirect effects of characters via intermediate characters or pathways are differentiated. In the current study, direct effects of all stomatal characteristics, WUE and Δ were identified and indirect effects of stomatal characteristics through WUE and Δ were also measured. The result of sequential path analysis was summarized in Table 3. As can be seen from the Table, GY being a complex and important commercial trait was considered as independent variable and other characters including Δ, WUE and stomatal characteristics as causal variables. The only significant direct effect on GY was belonging to WUE and surprisingly neither stomatal characteristics nor Δ had significant direct effects on grain yield. The least significant direct effect was belonging to stomatal area on abaxial surface followed by stomatal area on adaxial one. Regarding to indirect effects, stomatal frequencies on both sides of leaves had positive significant indirect effects on GY via WUE. In addition, stomatal frequency on the abaxial surface had a negative significant indirect effect on GY via Δ. A positive significant indirect effect on GY was also observed from stomatal area through carbon isotope discrimination.

Discussion

Variations among the genotypes and correlations among the characters

Variation observed among the genotypes for the characters under study indicated the possibility of selection between the genotypes for wheat improving purposes. Such variations for Δ and grain yield (Condon et al., 2004, Khazaei et al., 2009, Mohammady et al., 2009, Rebetzke et al., 2006) and for stomatal characteristics (Mohammady et al., 2005) were also previously reported among wheat genotypes. Almost all the variations reported in the literature for the above characters were observed among wheat cultivated varieties. Since Iran is a part of diversity centre for wheat (Poehlman, 1995) and the land races and wild genotypes used in the current study were all collected from Iran, they can be studied further in order to find suitable physiological characters enhancing wheat grain yield in different environments. It is theoretically expected that varieties with higher number of stomata per unit area and greater length and width of stomata lose more water during the growth period. This happens if stomata remain open during the water-stress period. Reduction in water loss from leaf surfaces during periods of severe water-stress is an important drought tolerance indicator. Low rate of cuticle transpiration, therefore, may reduce leaf dehydration and promote leaf survival (Wang and Clarke, 1993b). When water-stress develops, the response of stomata to water-stress seems to be of a great importance in reducing water loss comparing with stomatal characteristics. Thus genotypes with lower number of stomata may be useful for breeding drought tolerant varieties under some drought conditions. Mohammady (2002) studied the relationships between stomatal characteristics and water status in 2 wheat varieties named Falchetto (water-stress tolerance) and Oxley (water-stress susceptible). He reported that stomatal frequency of Falchetto was significantly higher than Oxley, but Falchetto had smaller stomata. On the other hand, his results revealed that Falchetto had a higher Leaf Relative Water Content (LRWC) and Stomatal Resistance (SR) than Oxley. These results indicated that SF is not always correlated with plant water status. This is because stomatal size, response of stomata to environmental stress and even cuticle resistance are also involved in determining plant water status particularly under water-stress conditions. The results of other workers concerning the relationship between stomatal characteristics and plant water status are inconsistent. Wang and Clarke (1993b) reported that SF was not correlated with relative water loss and leaf water content in field experiments. However, their results indicated that SF was positively correlated with the rate of water loss but not with leaf water content under growth room experiments. The inconsistency of this relationship is possibly due to the influence of other characteristics of stomata rather than SF and due to negative relationships between stomatal size and frequency as observed in the current experiment (Table 2). In addition to SF and stomatal size, the stomatal responses to water-stress and cuticle resistance are other factors which influence water status of plants under water-stress conditions. Thus, the results explained above indicate that stomatal characteristics are affecting water status of plants as a complex, and every component of this complex should be studied in relation to other components and with other factors which influence water status of plants.A negative correlation was found between stomatal frequency and stomatal area. This implies that increase or decrease in transpiring area may not be achieved by selecting for high or low SF due to the negative correlation between SF and stomatal size (Venora and Calcagno 1991). For this reason, it seems that SA as a combination of SF, SL and SW is a better determination of water status in plants. The relationship between stomatal resistance and stomatal characteristics is also important in determining water status of crops under water-stress conditions. In a study carried out by Mohammady (2002), SR and SA were investigated in Varieties Falchetto (water-stress resistant) and Oxley (water-stress susceptible). He reported no significant differences between the two varieties for SA on the both surfaces of leaves but highly significant difference for stomatal resistance was observed between the two varieties. These results indicated that higher SR of Falchetto on the adaxial surface is not due to smaller SA but is possibly due to either differences in stomatal response to water-stress or differences in cuticle resistance. The most important issue regarding water-stress tolerance is that the characteristics of stomata of the crop must match the pattern of water supply (Passioura, 1996). When the water supply is insufficient from the onset of growth, less number of stomata and low stomatal area can lead to a conservative consumption of water and thus can be considered as a suitable adaptive trait. On the other side, when there is a small shortage of water supply happens at the end of growth cycle, low stomatal frequency or area, or even low stomatal transpiration, have no benefit to the crops due to low photosynthesis and thus enhancing yield reduction in crops. In general, wild species of wheat and landraces used in the present study indicated variation for different aspects of stomatal characteristics and therefore they can be used in wheat breeding programs aiming to manipulate stomatal characteristics. There is also a need to evaluate these genotypes for other traits related to water statue in order to come to a conclusion about their promising for being involved in wheat breeding programs aiming to improve wheat cultivars for water statues in particular in dry regions. In the most experiments carried out under irrigated conditions, Δ values were found to be high and the relationship between grain yield and Δ non significant (Ehdaie et al., 1991; Ehdaie and Waines, 1994; Monneveux et al., 2005; Misra et al., 2006; Xu et al., 2007) or even negative (Condon et al., 2002). In general, measuring Δ does not provide information on whether its variation is being driven by variation in stomatal conductance or photosynthetic capacity (Farquhar and Sharkey, 1982; Ehdaie et al., 1991). In the present study, grain yield was negatively related to Δ in a good agreement with Waines et al., (1993). Carbon isotope discrimination **(Δ)**, which is negatively correlated to transpiration efficiency at the leaf level (Farquhar et al., 1982), was found in the present study to be positively correlated to stomatal frequency. In contrast, other researchers found a negative correlation between stomatal frequency and Δ. Waines et al., (1993), and Khazaei et al., (2009) reported that higher Δ is associated with less number and bigger size of stomata. In general, carbon isotope composition of a plant may be a useful criterion to assess water use efficiency in C3 plants, and therefore, an efficient method to screen genotypes for improved drought resistance.

Path analysis

The correlation coefficients between various characters were partitioned into direct and indirect effect using path analysis technique. Stomatal characteristics indicated no direct effect on grain yield. Comparison between overall correlations between these characters with GY and those observed in path analysis revealed that the effect of stomatal characteristics on GY come to operation through gas exchange related characters such Δ and WUE. Stomatal frequency on the abaxial surface had a negative significant indirect effect on GY via Δ. A positive significant indirect effect on GY was also observed from stomatal area through carbon isotope discrimination. These results indicate the ability of Δ and WUE to be used as indirect selection criteria for GY during wheat breeding programs. According to Araus et al., (2003), carbon isotope discrimination (Δ), and transpiration efficiency are negatively related. Δ has also largely been recommended as a selection criterion for transpiration efficiency (Farquhar and Richards, 1984), which holds true when the amount of captured water is the same for all genotypes. According to Zâmeĉnik and Holubec (2005), strong negative correlations between transpiration efficiency and carbon isotope discrimination in wheat (*Triticum aestivum* L.) suggest that selection of progeny with low Δ may increase transpiration efficiency and aerial biomass under water-limited conditions (Rebetzke et al., 2002). Condon et al., (2004) also pointed out that it is possible to use Δ as a selection criterion for genotypic improvement in transpiration efficiency and productivity in rice. In addition to direct and indirect effects, uncorrelated residual values (U) were estimated in the path analyses on GY, WUE and Δ (Fig 1) so while these residuals were low and non significant indicating that the path analysis explained the majority of variation in the traits studied. WUE similar to GY is a complex oligogenic trait but simpler than GY (Hui et al., 2008). In this investigation it indicated direct and indirect effect on GY. On the other hand, it also indicated negative significant correlation with Δ. Thus any stomatal factors which influence WUE have a certain effect on GY and Δ. Since a path coefficient is a measure without dimension and can eliminate the effects of different variances for the physiological traits (Hui et al., 2008), the implementation of path analysis can objectively evaluate the relative importance of WUE and Δ to GY. Furthermore, intra plant variations and variations observed within pure varieties for stomatal size and frequencies (Khazaie et al., 2010) have indicated that inconsistency exist toward stomatal size and frequency in wheat. Hence, measuring WUE and Δ is much feasible and reliable rather than stomatal size and/or stomatal frequency and we propose selection for WUE or /and Δ can improve grain yield in wheat cultivars and landraces.

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