OXYGEN ($\Delta^{18}O$) AND CARBON ($\Delta^{13}C$) ISOTOPE COMPOSITION IN PLANTS - AN APPROACH TO QUANTIFY TRANSPIRATION AND MESOPHYLL FACTORS ASSOCIATED WITH WATER USE EFFICIENCY

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Affectionately dedicated to:

A FRIEND, PHILOSOPHER AND GURU OF MY ENTIRE LIFE SAGA

MY FATHER (dady)

DEPARTMENT OF CROP PHYSIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE

CERTIFICATE

This is to certify that the thesis entitled **OXYGEN ($\Delta^{18}O$) AND CARBON ($\Delta^{13}C$) ISOTOPE COMPOSITION IN PLANTS - AN APPROACH TO QUANTIFY TRANSPIRATION AND MESOPHYLL FACTORS ASSOCIATED WITH WATER USE EFFICIENCY** submitted in partial fulfillment of ***Doctor of Philosophy*** in ***Crop Physiology*** to the University of Agricultural Sciences, Bangalore, is a record of research work carried out by **Mr. H. BINDU MADHAVA** under my guidance and supervision and that no part of the thesis has been submitted for the award of any other degree, diploma, associate ship, fellowship or any other similar titles.

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ABBREVIATIONS

ROS	*- Rain-out Shelter*
ML	*- Mini-lysimeter*
MLWD	*- Minilysimeter Weighing Device*
PWD	*- Pot Weighing Device*
IRGA	*- Infrared Gas Analyzer*
ASU	*- Air Supply Unit of IRGA*
FC	*- Field Capacity*
WUE	*- Water Use Efficiency*
TE	*- Transpiration Efficiency*
CWA	*- Cumulative Water Added*
CWT	*- Cumulative Water Transpired*
MTR	*- Mean Transpiration Rate*
SLA	*- Specific Leaf Area*
NAR	*- Net Assimilation Rate*
CGR	*- Crop Growth Rate*
DAS	*- Days After Sowing*
TDM	*- Total Dry Matter*
HI	*- Harvest Index*
CID	*- Carbon Isotope Discrimination*
LA	*- Leaf Area*
LAI	*- Leaf Area Index*
LAD	*- Leaf area duration (Functional leaf area)*
CER	*- Carbon Exchange Rate*
A	*- Assimilation rate*
g_s	*- Stomatal conductance*
g_m	*- Mesophyll efficiency/capacity*
dA/dCi	*- Initial slope of the CO_2 response curve*
Ca or Pa	*- Ambient CO_2 concentration or partial pressure*
Ci or Pi	*- Intercellular CO_2 concentration or partial pressure*
RuBisCO	*- Ribulose 1,5-BisPhosphate Carboxylase/Oxygenase*
VPD	*- Vapor pressure deficit*
E	*- Evaporation*
RWC	*- Relative Water Content*
T	*- Transpiration rate*
RH	*- Relative Humidity*
IRMS	*- Isotope Ratio Mass Spectrometer*
R	*- The molar ratio of heavy to the lighter stable isotope*
$\Delta^{13}C$	*- Discrimination against ^{13}C*
δ	*- Fractionation of stable isotopes*
$\Delta^{18}O$	*- Fractionation/enrichment of heavy isotope of oxygen*
‰	*- Parts per thousand (per mil)*
$\wp$	*- Péclet number/effect*
ε^*	*- Equilibrium fractionation*
ε_k	*- Kinetic fractionation*
ipt	*- Isopentyl transferase*
ABA	*- Abscissic Acid*
Z	*- Standardized normal distribution (transformed value)*

INTRODUCTION

INTRODUCITON

The basic source of water is precipitation in the form of rainfall or snowfall. Rainfall in India varies from place to place and from year to year. The country's average annual rainfall is about 119.4 cm, which when considered over the geographical area of 328 million ha amounts to 392 m.ha.m (Anon., 1998).

Out of the total of 1.4 billion km^3 of earth's water, 97 per cent is in the ocean and is saline. About 30 million km^3 (77%) of the remaining water exists in the form of ice caps and glaciers and other 4-5 million km^3 of ground water remains essentially inaccessible. Thus the supply of available fresh water for human needs depends very much on hydrological cycle. Therefore our existence is sustained on the remaining 23 per cent, of which the available forms are, ground water-22%, lakes and swamps-0.35%, atmosphere-0.4% and streams-0.01% (Shankar and Shivakumar, 2000). According to Swayne et al., (1990), human need of drinking water is about $1m^3$/year for survival. Nevertheless, domestic and recreational use of water accounts for only 6 per cent, while agriculture use accounts for 73 per cent of the world's fresh water usage.

The exploitable fresh water resource globally reached a plateau and there is an increasing demand for urban and industrial use. Therefore there is little scope to expand area under irrigation. In fact water will be the single most limitation to achieve or to improve crop productivity in future. Therefore, water harvesting, moisture conservation and increasing the water use efficiency of crop plants assume the greatest significance.

Plants continuously absorb and lose water. On a warm, dry sunny day a leaf will exchange upto 100 per cent of its water in a single hour. During the plant's lifetime, water equivalent to 100 times the fresh weight of the plant may be lost through the leaf surfaces. This shows that considerable amount of water is lost through transpiration (T). However, transpiration is indirectly relevant here since, stomata regulate the T and only when T occurs, carbon dioxide exchange takes place. So often a positive relation is seen between the T and biomass.

However, variations in carbon gain during transpiration can bring about differences in growth rates.

Hence it is important to increase the carbon gain per unit amount of water transpired which is termed as Water Use Efficiency (WUE). WUE is the ratio of photosynthetic efficiency in fixing the carbon to the amount of water lost in transpiration at single leaf level (A/gs at a given VPD). At whole plant level, it is the amount of dry matter produced over a period of time to the amount of water lost during the same period. The significance of these two traits, total T and WUE has been fairly well elucidated and the yield model proposed by Passioura (1986) largely explains the productivity components of a crop.

Yield = Water use (total transpiration) X Water Use Efficiency (WUE) X Harvest Index (HI)

This signifies the potential of WUE trait, and it has a dual applicability:

- *WUE increases the productivity under rainfed conditions*
- *WUE saves water under irrigated conditions*

The existing variability in WUE across the species (C_3 species- 1.5 to 3.5; Downes, 1969, C_4 species – 2.5 to 5.0; Caldwell, 1977) to a large extent determine their water requirement, signifies the importance of WUE.

For instance, in an area receiving 800mm rainfall, if one considers 40-45 per cent of water is available for uptake by plants, 0.1-unit increase in WUE enhances biomass to a tune of 0.345 tonnes/ha. If genetic variability in WUE is substantial, then selection for high WUE will be highly rewarding.

The existence of genetic variability in WUE was shown nearly five to six decades back (Briggs and Shantz, 1913) but little progress has been achieved due to quantification difficulties, despite gas exchange and gravimetric approaches are available.

However, large scale screening for the variability in WUE was possible with the advent of carbon isotope discrimination (CID) technique. $\Delta^{13}C$ is a potent tool that can be employed as a dependable, time averaged surrogate for WUE (Farquhar and Richards, 1984; Hubick, et al., 1984). Significant genetic variability has been reported using this approach paving way to exploit this trait for crop improvement.

However, exploitation of genetic variability in WUE was unsuccessful through breeding due to associated reduction in dry matter accumulation whenever attempts were made to increase WUE (Udaya Kumar, et al., 1998). This lack of success was primarily due to strong interdependency between transpiration and WUE. Since WUE and T directly influence the crop growth rates, interdependence between these traits is undesirable. It is therefore essential to identify genotypes/species in which this association is weak. This necessitates to identify types with high WUE despite relatively higher T. But the question is, is it theoretically possible to achieve higher WUE despite moderately high T.

The physiological traits that regulate WUE suggest such a possibility. Internal CO_2 concentration (Ci) determines the WUE. Ci infact is regulated by both stomatal (gs) and intrinsic mesophyll efficiency (g_m). If the change in Ci is more by g_m, then WUE and T will be less dependent on each other. Hence the question would be how to quantify g_m.

Quantification of gm is rather difficult. However, initial slope of CO_2 response curve (dA/dCi) (Van Caemerrer and Farquhar, 1981) and more recently the ratio of Ci to gs has been considered as a reflection of g_m (Sheshshayee et al., 1996). These are time instantaneous measurements; hence warrants time integrated estimates of both Ci and gs. $\Delta^{13}C$ has been well documented as a surrogate estimate of Ci integrated over time and there is a need to search for integrated surrogate for g_s.

These analyses indicate the need to quantify g_s and T in addition to WUE. Thus necessitating the development of a suitable approach for a time-integrated estimate of these traits.

Though T is an essential trait but the quantification of T integrated over time is rather cumbersome. Several studies have shown that stable oxygen isotopes (^{18}O) get enriched in water during evaporation due to its heavier mass and slower diffusivity than ^{16}O (Craig and Gordon, 1965). Since transpiration is an evaporative process one would expect the similar enrichment mechanism here also. The two important factors that regulate T, are VPD and gs. It is important to know how far ^{18}O enrichment is related to T irrespective of its regulation either by T or g_s. Ultimately it is necessary to establish that at a given VPD the differences in gs will bring about changes in T and hence the ^{18}O.

In view to test these hypotheses, the following objectives have been drawn out in the present investigation:

- ***To assess the relationship of ^{18}O enrichment in leaf water with both transpiration rate and stomatal conductance (g_s)***
- ***To study how far the transpiration rate integrated over time is related to $\Delta^{18}O$ in leaf biomass***

After assessing the relationship of transpiration rate with both leaf water as well as biomass ^{18}O composition, the next aspect would be to examine the stable isotope composition of both carbon and oxygen in categorizing and selection of crop genotypes for high WUE coupled with high T traits. We presume that such types will have high crop growth rates (CGR) and g_m. Therefore, the next objective here was:

- ***To identify genotypes with high $\Delta^{18}O$ and low $\Delta^{13}C$ and assess their growth and physiological traits.***

Yet another emphasis is to examine how far the ^{18}O, which regulate gs, and ^{13}C, which is an integrated estimate of Ci, can be used to identify types with high g_m. From this context, the other objective of this investigation was to

- ***Examine the relevance of dual isotope discrimination (^{13}C and ^{18}O) approach in identifying the desirable mesophyll capacity using linear resistance analysis***

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Plants have naturally evolved several adaptive mechanisms for survival under water limited conditions. However, from the agronomical point of view, the concept of drought resistance is linked to superior crop growth rates (CGR) under water limited conditions. Hence the water harvesting and its utilization assumes significant relevance. The latter is often referred to as Water Use Efficiency (WUE) i.e., the efficiency with which plants use water to produce biomass. In view of its importance in crop improvement, especially for rain-fed situations, significant progress has been made in recent years in understanding the physiological basis and the variability in WUE and its environmental regulation. Apart from WUE yet another important trait which play a significant role is transpiration. Transpiration rate has a direct relevance with productivity of crop plants. Many a times whenever it was attempted to improve WUE among crop plants there was a notable decrease in yield due to reduction in transpiration. This was perhaps the major reason for lack of success in breeding for high WUE. Hence several studies were carried out to improve transpiration along with WUE for better productivity. Though desirable, due to lack of rapid technique in determining transpiration integrated over crop growth period, did not yield appreciable outcome. In recent years quite a few workers have shown that stable isotope of oxygen (^{18}O), could be one such technique to measure the diffusion of water through stomata in leaves (transpiration). However, there are no unequivocal experimental evidences yet to prove this hypothesis. In this chapter, an elaborate review on these above-mentioned aspects has been done.

Water Use Efficiency (WUE) - Definition

The efficiency with which plant use water, is expressed as the ratio of biomass produced (g) to the amount of water transpired (kg). In other words, WUE is controlled by the rate of carbon assimilation and the rate

of water loss through transpiration. However, at a given level of transpiration, differences in WUE can contribute to large differences in crop growth rates among genotypes and species.

Different terminologies for WUE

WUE is computed from the ratio of various physiological processes as follows. The different terms used in this context are transpiration ratio or transpiration quotient, water use efficiency.

$$\text{Transpiration Ratio (TR or Transpiration Quotient (TQ)} = \frac{\text{Amount of water used in transpiration (ml)}}{\text{Amount of dry matter produced (g)}}$$

$$\text{Water Use Efficiency (WUE)} = \frac{\text{Dry matter produced (g)}}{\text{Water lost in transpiration (Kg)}}$$

Often many investigators use the term Transpiration Efficiency (TE) as a synonym to WUE and express as the ratio of amount of carbon fixed to the amount of water lost through transpiration over a period of time.

$$\text{Transpiration Efficiency (TE)} = \frac{\text{mmol of } CO_2 \text{ fixed}}{\text{mol of water transpired}}$$

Water Use Efficiency and its importance

Water Use Efficiency is one of the important components in the yield model proposed by Passioura (1986), which states:

Seed yield = Water Use (T) x Water Use Efficiency x Harvest Index (HI)

Where, Water use, which is synonym to transpiration, WUE is the efficiency of water use by the plant and HI is partitioning efficiency with which the metabolites translate to reproductive parts.

At a given level of T, differences in WUE can be contributed to large differences in crop growth rates among genotypes and species, since HI has more or less reached a plateau in several crop species. Therefore, seed yield is determined by WUE. Most yield improvements have achieved by increasing the transpirational component through management and breeding, and by increasing HI in certain grain crops (Austin, *et al.,* 1978). This suggests that improving WUE of our crop species has relevance.

Physiological parameters associated with WUE

1) <u>WUE at single leaf level</u>

At single leaf level, the carbon assimilation occurred at a given gs measured using gas exchange approach could be considered as intrinsic WUE (Osmond *et al.,* 1980). This ratio can be conveniently used to compute WUE of different species with special reference to tree crops. Estimation of intrinsic WUE is better technique because of its ease in computation and also for its comparability with other many reliable techniques (Ludow and Wilson, 1976).

Since, T is regulated by both VPD and gs, at a given VPD, Assimilation ('A') to conductance ratio (A/gs) reflects the intrinsic WUE. The carbon assimilation rate (A) responsible for biomass production and transpiration rate (T) are the two physiological traits associated with intrinsic WUE. Intrinsic functional ability of chloroplast (mesophyll efficiency) and stomatal diffusive characters (gs) regulate Pi. T is also controlled by gs at a given vapour pressure difference (VPD). This interrelationship in association to WUE can be described mathematically (Hubick, *et al.,* 1986).

WUE (A/gs) = (Pa-Pi)/1.6VPD or (1-Pi/Pa)/1.6VPD

Where Pa and Pi are the partial pressure of CO_2 in the ambient air and intercellular spaces, respectively. The factor 1.6 arises because of gs normally rafer to the diffusion of water vapour pressure rather than of CO_2. The vapour pressure of water is normally 1.6 times more than that of CO_2.

This equation implies that the WUE can be regulated either by altering the Pi/Pa ratio or the VPD. Any genetic or environmental factor that reduces the VPD, enhances WUE (Richards, 1991). Reducing the radiation load on plant canopy can reduce the tissue temperature there by decreasing the VPD. Breeding for increased waxy covering on the cuticle (Johnson *et al.*, 1993) and pubescence (Ghorashi *et al.*, 1971) results in increased WUE. Similarly active leaf movements which decrease radiation load and decreased stomatal sensitivity to drought may also reduce VPD.

However, CO_2 fixation and transpiration processes at single leaf level vary markedly both on diurnal and seasonal scales and also depending on leaf and plant age. Thus these instantaneous measurements do not integrate performance of the whole plant and can not assess the impact of morphological, physiological adaptations to drought that may influence season-long WUE and under water limited conditions (Sinclair *et al.*, 1984; Martin and Thorstenson, 1988). Further, these measurements have large co-efficient of variation and are thus usually not suitable for screening studies (Jones, 1989; Zeigher *et al.*, 1984).

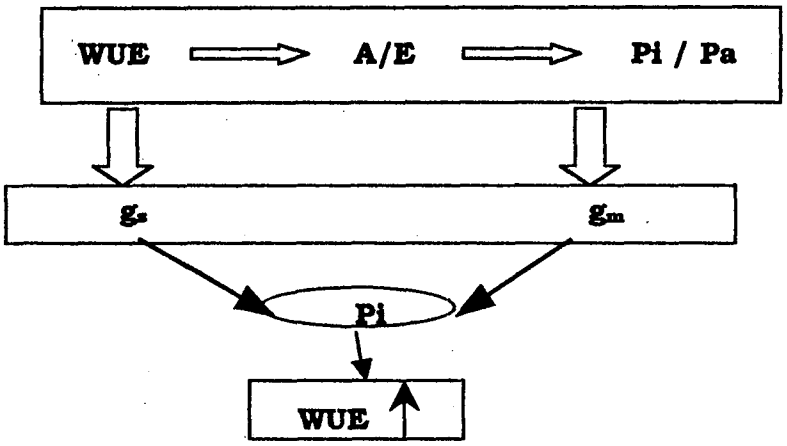
2) WUE at whole plant or canopy level

Since, gas exchange parameters are dynamic and instantaneous, *in situ* measurement of A/E values may not accurately represent and provide a comprehensive quantification of long-term WUE (integrated measure over crop growth stage). In this context, determination of WUE at canopy or whole plant level is noteworthy.

At the canopy/whole plant level it is the ratio of the total dry matter (including roots) per unit amount of water transpired. Because of the difficulty in measuring the root biomass in the field, WUE is generally computed on the basis of above ground biomass (Turner, 1986).

Measurement of ET under field conditions is difficult in many situations where drainage from the root zone, water uptake from saturated zones, and run and runoff from the area are difficult to measure both temporally and spatially. Gravimetric measurements can give reliable estimations of TE, as they allow accurate measurement of T and dry matter production including roots (Rao and Wright, 1994; Udaya Kumar, *et al.,* 1998b). However, these approaches are extremely laborious and are not practical for screening of germplasm or to carry out genetic studies associated with cultivar or clonal improvement.

In g_s dependent types the stomatal conductance is invariably lower, resulting in lower transpiration rate. This moisture conservation trait though relevant under stress, often results in a significant decline in total transpiration. Low g_s in these types also decrease the carbon flux and hence "A" will be less (Johnson and Tieszen, 1994). Therefore, in g_s-dependent types, total transpiration per unit leaf area will be low and so will be the net assimilation rate (NAR). Because of the relationship between total transpiration and total dry matter (TDM), a weak association between TDM and WUE can be expected in conductance types.



Physiological factors that determine inter-cellular CO_2 partial pressure (pi) and hence WUE

Variations in Pi/Pa ratio are largely dependent on Pi as the atmospheric CO_2 concentration is fairly constant. Pi/Pa is a complex character determined both by stomatal conductance and by photosynthetic capacity (Condon *et al.,* 1987). Reduced Pi/Pa and a consequent decrease in discrimination may reflect either reduced leaf conductance or greater photosynthetic capacity. An increase in g_s, though enhances CO_2 flux, results in higher T and lower WUE. However, at a given g_s, enhanced 'A' by virtue of high g_m will reduce the Pi and hence WUE will be higher (Farquhar *et al.,* 1989). Variations in Pi and hence WUE are determined by both g_s and g_m.

The atmospheric vapour pressure of CO_2 (Pa) is very stable over the season, while inter-cellular partial pressure of CO_2 (Pi) changes substantially, and the stomatal conductance (g_s), and carboxylation efficiency of RuBisCO of the leaf largely determine Pi. WUE therefore depends upon the changes in Pi. Increase in `A' relative to $g_s$ causes Pi to decrease and WUE to increase. Similarly decrease in $g_s$ for a small change in `A' will also reduce Pi and increases WUE (Farquhar *et al.,* 1989). This relation of WUE to Pi has been confirmed by Hubick *et al.,* (1988); Wright *et al.,* (1992). In this instance, they showed that decrease in Pi was due to increased `A' rather than decreased leaf conductance.

Recently, an inverse relationship RuBisCO content and Δ among groundnut genotypes was shown (Rao *et al.,* 1995). This suggests in groundnut, the variability in Δ and hence WUE could be brought about by the mesophyll capacity. Similar relationship was noticed when RuBisCO content was altered in transgenic plants with an anti-sense construct for the small sub-unit of RuBisCO (Hudson, *et al.,* 1992), or by increasing the leaf nitrogen status. Gutterrez and Meinzer (1994) explained the role of mesophyll efficiency in determining Δ and WUE.

How to quantify WUE?

Variability in WUE is mainly determined by any of the following three methods

1. At canopy level under field conditions using crop growth and yield model. This technique is more employed in perennial tree species
2. At the whole plant (canopy) level in small pots or big containers (field mini-lysimeter conditions) by adopting gravimetric technique
3. At a single leaf level by adopting the gas exchange approach

At single leaf level WUE is the ratio of carbon assimilation to water lost through transpiration and expressed as mg CO_2/g H_2O or mmol CO_2/mol H_2O. At the whole plant level it is the ratio of the total dry matter (including roots) per unit amount of water transpired. Because of the difficulty in measuring the root biomass in the field, WUE is generally computed on the basis of above ground biomass (Turner, 1986).

Does genetic variability in WUE exist?

For any trait to be successfully exploited through breeding programme, a sufficient genetic variability in that trait must exist. With the advent of this rapid technique, significant progress was achieved in establishing the genetic variability in WUE. Existence of such a variability both between

and with in species has been shown by Briggs and Shantz as early as in 1913. With the advent of isotope discrimination technique, rapid progress has been made to determine the genetic variability in WUE.

Considerable extent of genetic variability has been reported in crop species. Due to the existence of CO_2 enrichment mechanism coupled with higher photosynthetic efficiency, C_4 plants have at least 2-3 folds higher WUE compared to C_3 plants (Mabrouck, 1984). Range in WUE reported in C_3 and C_4 were 1.5 & 3.5 mg DM/g H_2O (Downes, 1969) and 2.9 & 4.3 mg DM/g H_2O by Codwell (1977) respectively.

Briggs and Shantz (1913) first documented genetic variability in WUE with in a species. Recent evidences confirm unequivocally the earliest work of Briggs and Shantz. Many demonstrated substantial genotypic/species variation in WUE in several crops (Table below).

Genetic variability in WUE across the crop species

Crop species	Reference
Groundnut	Hubick *et al.*, 1986, Wright, *et al.*, (1988, 1993), Rao, *et al.*, (1995), Hebbar, *et al.*, 1994; Roy Stephen, 1995,
Common bean	Ehlengier *et al.*, 1990, Ehlengier, 1991
Cowpea	Shashikumar, 1983; Ismail and Hall, 1992, 1993; Hall, *et al.*, 1992; Ashok, 1996; Ashok, *et al.*, 1999
Soybean	Arun, 1985
Wheat	Farquhar and Richards, 1984, Condon *et al.*, 1990, Ehdaie *et al.*, 1993, Ehdaie, 1995, Condon and Hall, 1997, Al-Hakimi *et al.*, 1997
Finger millet	Sashidhar, 1987; Uma, 1987
Barley	Hubick and Farquhar, 1989
Alfalfa	Ray *et al.*, 1998, 1999,
Sunflower	Ravishankar, 1988; Virgona *et al.*, 1990, Farquhar *et al.*, 1995, Aymen Shehada, 1999;
Tomato	Mervat, 1989, Martin *et al.*, 1999

Potato	Jefferies, 1995
Chickpea	Gangadhar, 1995
Pigeon pea	Deveraja Achar, 2000
Few tree species	Somashekar, 1988
Spur	Guy, *et al.*, 1996
Pine	Griffith, 1995
Cashew	Anil Koushik, 1999
Eucalyptus	Li, 2000

In groundnut, several studies were carried out for assessing the variation in WUE. In a container experiment, variability in WUE was ranged from 2.15 to 3.71 g DM/Kg of water (Wright, *et al.*, 1988). Hebbar (1990) reported a variation from 1.57 to 2.66 g/Kg in a field and from 1.5 to 3.5 in container experiments. In a minilysimeter experiment Wright, et al (1992) have showed a range in WUE of 2.96 to 3.7 g/Kg in well watered and 3.41 to 4.74 under terminal drought stress. Similar study was conducted by Roy Stephen (1995) and reported a range of 2.92 to 4.07 under 100% FC and 3.19 to 5.46 under 50%FC. These studies suggest the possibility of exploring this trait for crop improvement. The possibility of this trait could be used for breeding for drought resistance was shown way back in 1950's (De wit 1958) and subsequently reiterated by Fisher and Turner (1978); Tanner and Sinclair (1983).

WUE in C_4 plants

Carbon dioxide fixation in C_4 species is characterized by a CO_2-concentration mechanism that requires the coordinated function of mesophyll and bundle-sheath cells within a leaf. CO_2 is initially assimilated into C_4 acids by phospheenolpyruate (PEP) carboxylase in the mesophyll cells. These acids are transported to and decarboxylated in the bundle sheath, where the CO_2 is refixed by RuBisCO. CID in C_4 species reflects these two diferent caboxylations. It is dependent not only on the ratio of intercellular to ambient partial pressure of CO_2, Pi/Pa, but

also on leakiness (Farquhar, 1983), the proportion of CO_2 released into the bundle sheath by the C_4-acid cycle which is not fixed by RuBisCO but leaks back to the mesophyll. Since there is a limitation in leakiness, CID was as in C_3 species, linearly related to Pi/Pa. Hence the possibility exist that $\Delta^{13}C$ can be used as a tool for estimating WUE in C_4 species. Genetic variability in WUE and $\Delta^{13}C$ has been reported in separate studies of C_4 species (Owounube *et al.*, 1982 in S. bicolor; Hammer *et al.*, 1990 in barley). A greater number of studies have found genetic variation in Pi/Pa, CO_2 assimilation rate or g_s which, in turn, could result in genetic variation in WUE (for S. bicolor, Henzell *et al.*, 1976 and Krieg, 1983; Donatelli *et al.*, 1992 for Zea mays).

These results confirm the existence of considerable genotypic/species variability in WUE. Existence of such variation is useful in breeding programs to improve crop productivity.

Factors affecting WUE

The factors that influence the WUE can be grouped as:

I. Environmental factors
II. Plant factors/characters
III. Nutrient factors

I. Environmental factors affecting WUE

WUE is influenced by several environmental factors. They are:

Vapour Pressure Deficit (VPD)

VPD is the driving force for transpiration, hence an increase in leaf to air vapor pressure difference substantially increases the transpiration, thereby decrease in WUE (Fischer and Turner, 1978). The prevailing

ambient RH and leaf temperature influence the VPD, therefore, vapour saturation gradient in air has a major effect on WUE (Ong and Simmonds, 1987). Several studies have shown a substantial increase in T by VPD without an effect o photosynthetic rate thereby, decreasing WUE (Stanhill, 1986 and Turner, 1986).

Richards (1991) suggested two different means to improve WUE that relay on minimizing the VPD. When the VPD is low, crop growth rates are higher and lead to an increase in WUE. This effect of VPD on WUE can be achieved by planting the crops early in the season, when VPD is generally lower (Keatinge and Cooper, 1983).

Rawson and Begg (1977) and Jones *et al.*, (1985) reported that apparent photosynthesis were sharply reduced by an increase in VPD above 1.8 to 2 KPa in both stressed and non stressed plants thus reduced the intrinsic WUE. The intrinsic WUE calculated for a constant VPD decreased with an increase in VPD (Turner *et al.*, 1984)

Shulze *et al.*, (1985, 1986) demonstrated the stomatal response to change in humidity and showed a decrease in transpiration rate from 4.5 mmol. $m^{-2}.s^{-1}$ to 2.7 mmol. $m^{-2}.s^{-1}$ in response to VPD change from 1.0 to 3.0 Kpa due to decrease in conductance.

Light and temperature

The solar radiation has a vital role to play in determining WUE. There is an optimum irradiance for maximum efficiency of water use. Several instances of diurnal regulation of WUE by irradiation in a few crop species was reported (Singh, *et al.*, 1993). Fischer and Turner (1978) reported the importance of irradiance in increasing the leaf temperature and reducing the stomatal resistance, and suggested that there is an optimum irradiance for maximum WUE. An increase in leaf temperature, whether because of radiation load or indirectly because of

reduced water uptake may have a profound effect on water use and WUE (Craufurd *et al.,* 1999). Heat stress reduces WUE through deleterious effects on carboxylation and respiration and increases water use via effect of increased vapour pressure deficit associated with higher temperature and leaf to air temperature difference.

Soil Volume

Ismail *et al.,* (1994) have found that when cowpea accessions and hybrids were grown under field condition and subjected to wet and dry treatments in three different pot size. The pot size treatment altered the values of both Δ and WUE. In dry condition, Δ was high and WUE was low in big pots compared to the small and medium pots.

Moisture stress

Drought stress is a complex combination of stress because of both water deficit and high temperature. The degree of stomatal closure induced by water stress depends on the level of stress and the ability of the crop to meet evapotranspirational demands (Guy *et al.,* 1988). Direct measurements of TE using whole plant carbon and water balance have shown that moderate drought can cause an increase in TE of up to 100 per cent, while extreme drought could substantially decrease TE (McCree and Richardson, 1987). A common response to water stress is a simultaneous decrease in A and T and an increase in leaf temperature (Farquhar and Sharkey, 1982). If T decreases faster than A, then Pi will decrease (Cowan, 1982). This response results in water saving to the plant and subsequent increase in TE. The increase in WUE of genotypes under moisture stress was associated with higher A/gs & low Pi (Farquhar and Richards, 1984, Rensbery et al, 1993). Moisture stress induced increase in WUE is often associated with greater reduction in gs than in `A' (Uma, 1987; Sashidhar 1987; Wright et al. 1993; Mayland *et al.,* 1993).

In long term studies in both growth chamber and field conditions, plants under water deficit had lower Pi, as indicated by ^{13}C discrimination analysis (Farquhar and Richards, 1984; Ehleringer and Cooper, 1988). Water stress resulted in about two per cent lower Δ than in well-irrigated plants of chickpea (Winter, 1982). Under severe water deficit TE decreased, because leaves become less efficient with respect to water and CO_2 exchange; water can still be lost through the cuticle but CO_2 entry through stomata is severely restricted, causing reduced TE (Wenkert, 1983). In groundnut, severe stress decreases both Δ and TE, which could be related to increased respiration losses of carbon (Wright .*et al.,* 1993). A similar response has been reported for sunflower (Virgona *et al.,* 1990). Respiratory losses of Carbon can be as much as 40 per cent under severe drought conditions (McCree .and Richardson, 1987).

II. Plant characters influencing the WUE

Among the plant characters affecting WUE, the importance of leaf size was studied by Nobel (1980), through its effect on leaf's boundary layer. WUE decreases as the ratio of the internal assimilatory surface to its external transpiration surfaces increase (Gupta *et al.,* 1989; Rao and Wright, 1994). Fischer and Turner discussed the importance of leaf movements; leaf rolling etc., as an adaptation to increase WUE.

Canopy cover plays a considerable role in determining the stomatal diffusive trait which in turn has an effect on WUE. Boundary layer resistance gets altered if resistance of canopy to heat and vapour pressure is large there by lowering the VPD, WUE would increase (Farquhar, et al 1980, Read, et al 1991). Canopy architecture (like leaf size, orientation, growth habit & canopy height) has a profound effect on radiation leading to alteration in WUE. (Jarvis & Mc Naughton, 1986).

leaf movement and surface reflectance

The optimum irradiance for TE is usually less than the irradiance incident upon a leaf-oriented normal to the radiation (Jones, 1976; Downes, 1970). This is primarily because transpiration normally shows a positive relationship with increasing irradiance. Leaf movement and surface reflectance pattern provide a means for optimizing the energy load on the leaf for the maximization of WUE. This has an advantage under water deficit environments (Berg, and Henchelin, 1990). Leaf pubescence helps in controlling the leaf temperature and water balance (Ghorashy *et al.,* 1971; Ehleringer, 1980). In a study with isogenic lines of soybean the pubescent leaves had significantly lower T than normal iso-lines (Baldocchi *et al.,* 1985). Considerable genetic variability exits in crop species for epicuticular wax levels and cuticle thickness, resulted in increase in WUE and significant reduction in T (Clarke, *et al.,* 1989; Castonguay and Markhart, 1991). Araus *et al.,* (1997) have shown the effect of leaf structure and water status on WUE and delta in field grown durum wheat. The orientation of leaves directly to incident radiation resulted in relatively higher loss of water and hence increasing the WUE.

Root System

The distribution of roots, its density and hydraulic resistance can influence water use in space and time. Thus the rate of growth and spread of roots can affect WUE, particularly during early stages of crop growth. When two genotypes growing together are subjected to an increasing soil moisture deficit, both will show an increase in WUE. If one genotype with deeper roots were able to extract more soil moisture from deeper profiles and be less water-stressed, that genotype would display a relatively lower WUE, while it may produce a little more yield than the other. This concept has been confirmed experimentally in wheat, where the most drought-susceptible (low yielding) genotypes had

high WUE, while the most drought-resistant genotypes had the low WUE (high yielding) when subjected to moisture stress (Morgan *et al.,* 1993).

III. Influence of nutrients on WUE

The dependence of WUE on water and nutrient supply has been demonstrated for various C_2 plant species including wheat (Farquhar and Richards, 1984), barley (Hubick and Farquhar, 1989), peanut (Hubick *et al.,* 1986) and sunflower (Virgona and Farquhar, 1996). Although less pronounced, WUE of C_4 plants also appear to be affected by water and nutrient supply (Payne *et al.,* 1992 and 1995).

Changes in WUE reflects the changes in gs and or internal capacity for CO_2 fixation, latter being affected by enzyme activity and plant nutrient status (Payne *et al.,* 1992; Ranjith *et al.,* 1995). Phosphorus supply had significant effects on yield, WUE and delta. Yield wat reduced by 48 per cent by low P, however there was no effect on WUE was observed. But increase in P supply in combination with water stress increased WUE to a tune of 37 per cent (Bruck *et al.,* 2000). Payne *et al.,* (1995) have showed that WUE along with Nutrient Use Efficiency (NUE) increased to added nitrogen and phosphorus nutrients in pearl millet. The increase in WUE was also linked to water stress and varied inversely with phosphorus use efficiency (PUE). In barley, silicon content was shown to correlate with both WUE and delta (Walker and Lance, 1991). In another study, Onken and Wendt (1989) obtained significant increase in WUE of sorghum due to high nitrogen level coupled with water stress. Similarly Parameswarn *et al.,* (1981, 1984) observed that an increase in WUE in wheat cultivars was due to applied nitrogen and water stress. Merah *et al.,* (1999) while working with 21 durum wheat genotypes for their genetic variability in grain yield, $\Delta^{13}C$, mineral and silicon content, reported a strong positive correlation between silicon content and $\Delta^{13}C$ and mineral ash. The result obtained in this study confirms the variability of kernel $\Delta^{13}C$ as a predictive criterion for grain yield under water stress.

The existence of genotype by environment (G x E) interactions for grain yield in crops has complicated selection and breeding strategies for many years. G x E interactions is noticeable when genotypes being evaluated rank differently among trails conducted in different locations and seasons (Wright *et al.,* 1996).

For any trait to be successfully exploited in breeding program, in addition to significant genetic variability, a low G × E interaction is preferred. Low G × E for TE has, however been reported in legume crops such as peanut (Wright *et al.,* 1993; Rao and Wright, 1994), cowpea (Hall *et al.,* 1993) and soybean (white *et al.,* 1998). Ashok *et al.,* (1999) found a significant relationship between the K (WUE adjusted for the VPD) of cowpea in two experiments conducted in two entirely different seasons and soil volumes (pot and minilysimeter). Thus indicating a low G × E interaction when WUE was adjusted for the prevailing VPD and the relative ranking of the genotypes between pot and minilysimeter also showed similar trend. Significant variation among peanut cultivars in WUE under well-watered and water-limited conditions has been shown in isolated plants in the glasshouse and in small canopies in the field. Further, G x E interaction for WUE, Δ and SLA was shown to be very low, while heritability of Δ was high, indicating that these traits could be used for selecting high WUE in peanut breeding programs (Wright *et al.,* 1993). Similarly Ray, *et al.,* (1999) have demonstrated the broad sense heritibility among the physiological traits viz., WUE, delta, canopy temperature and yield in alfalfa cutivars grown in water stressed conditions in two cropping seasons. Heritibility in WUE traits and delta was reported in cowpea (Menendez *et al.,* 1995).

Selection of any genotype for the trait Δ as a surrogate estimate of WUE to be useful in breeding should entirely depends on its inheritance,

heritability and any genetic associations with other desirable traits (Hall *et al.,* 1993). Under natural soil conditions all of the F1 hybrids had Δ values similar to the high Δ parents, suggesting partial dominance for high Δ (Ismail and Hall, 1993). They also reported that nuclear genes control the WUE and high WUE exhibited the partial dominance (Hubick *et al.,* 1988).

Why breeding for high WUE has not been successful?

One needs to adequately analyze the relative significance of physiological traits that regulate WUE. Though WUE is an important component of yield in the model proposed by Passioura (1986), the existing genetic variability in WUE could not be exploited through breeding. Many such attempts were not successful since any improvement in WUE was often associated with reduction in dry matter accumulation and yield (Matus *et al.,* 1995). Because most often plants have evolved to maximize the WUE through a reduction in transpiration that is tightly linked. Since dry matter production is strongly associated with total transpiration (T), any reduction in T results in reduced CGR. Since g_s is associated with T and internal CO_2 partial pressure (Pi), WUE and T become strongly interdependent. This inter-dependency seems to be the major reason for the lack of success in breeding for increased WUE. Martin *et al.,* (1993) described that the improvements of crop water use efficiency has not been successful because evaluation for this component of drought resistance is unreliable in field grown plants.

How to achieve breeding success for high WUE?

As mentioned in the earlier section, if the variation in Pi and hence WUE is brought about by g_m and selection for WUE in such types will result in high CGR, thereby one can achieve the breeding success. Here, the inter dependency between T and WUE will be less (Udaya Kumar *et al.,* 1998). In other words, if genotypes/species are identified which are having high

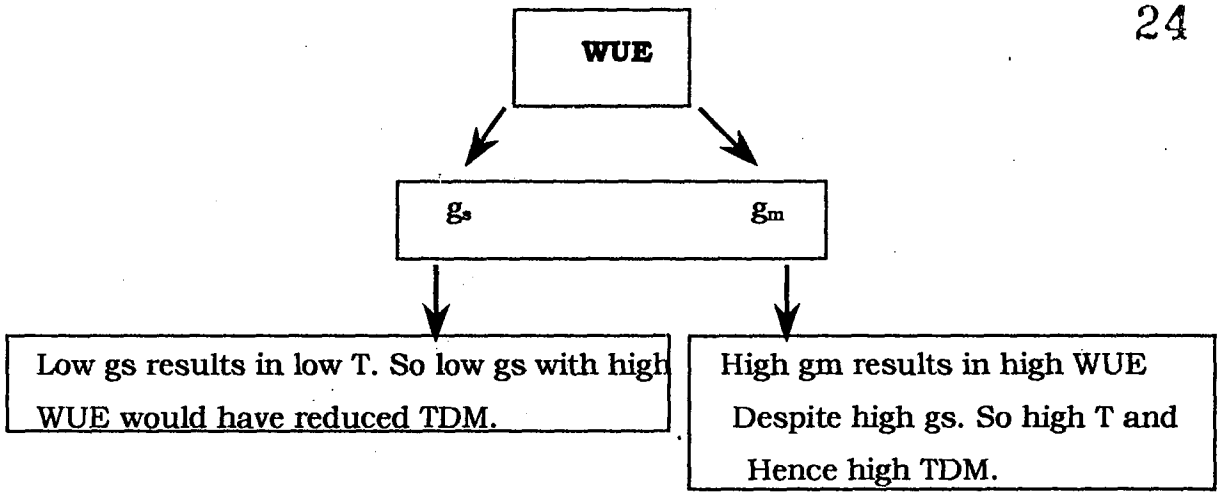
WUE by virtue of high mesophyll efficiency (g_m) will have an advantage in crop improvement programme.

The intrinsic mesophyll efficiency (g_m) and the CO_2 diffusive process associated with the stomata (g_s) regulate `A'. Transpiration rate on the otherhand is predominantly controlled by the differences in gs at a given vapor pressure difference (VPD). These two physiological traits determine the CO_2 partial pressure in the mesophyll inter-cellular spaces (Pi) which is directly influences the changes in WUE. Depending on the extent of contribution of g_s or gm to Pi and hence WUE, species and genotypes can be classified as gs-dependent (conductance types) or gm dependent (capacity types) (Table-&).

How do g_m types facilitates high breeding success for WUE?

WUE is predominantly regulated by the stomatal diffusive factor (g_s) and /or mesophyll efficiency (g_m) that regulated carbon assimilation. Therefore, the variability in WUE brought about by intrinsic differences in g_m is desirable. In such cases WUE and T will be less dependent on each other (Table below represents the category of crop species based on the stomatal and mesophyll characters).

In capacity types, the mesophyll factors associated with A will determine the variability in WUE. So, in these types, WUE is independent of gs and hence T will not be associated with WUE. Selection for high WUE from these types will result in high CGR (Hubick and Gibson, 1993). WUE as well as yield levels can be enhanced only when capacity types selected and are used in breeding program.



Inter relationship between WUE, T and mesophyll conductance in determining biomass production

WUE traits associated with TDM in two contrasting groups of genotypes

	Capacity (g_m) types		**Conductance (g_s) type**
Author	**Crop**	**Author**	**Crop**
Wright et al.1988	Groundnut[d]	Condon et al.1993	Wheat[ac]
Condon et al.1990	Wheat[d]	Farquhar et al.1988	Wheat[c]
Hubick et al.1988	Groundnut[d]	Martin and Thorstenso.1993	Tomato[c]
Hubick et al.1996	Groundnut[d]	White et al.1985	Phaseolus[c]
Wright et al.1993	Groundnut[d]	Acevedo et al.1993	Barley[c]
White et al.1993	Beans[e]	Ehdaie et al.1991	Wheat[b]
Johnson etal.1986	Grasses[d]	Richards and Codon,1993	Wheat[c]
Matus et al.1995	Canola[d]	Meinzer et al.1990	Coffee[a]
Sun et al.1996	Spruce[b]	Lu et al.1996	Cotton[a]
		Gutterrez and Meinzer,1994	Coffee[a]

Index

[a] - Δ is positively related to gs

[b] – TDM is negatively related to WUE

[c] – TDM is positively related to Δ

[d] – TDM is inversely related to Δ

[e] – TDM and Δ have no relationship

Unlike stomatal conductance (g_s), determination of g_m is difficult because of the complexity in its regulation. Often gm is indirectly estimated by determining the initial slope of the CO_2 response Curve (dA/dCi) (Von Caemmerer and Farquhar, 1981) or by determining certain mesophyll components such as RuBisCO content and its activity (Hudson, *et al.*, 1994; Mate, *et al.*, 1998). Yet another approach to arrive the carboxylation efficiency is to assess the change in Pi at a given g_s. Since Pi is a function of g_s and g_m, variation in Pi at a given g_s should reflect the g_m. That is, at a given gs, Pi will be less if the mesophyll efficiency is higher. In the recent past, it was shown that the ratio of Pi to g_s significantly correlates with mesophyll efficiency and hence can be considered as a good reflection of g_m (Sheshshayee *et al.*, 1996;Krishna Prasad *et al.*, 1996).

However, dA/dCi or Ci/gs ratios being gas exchange measurements can not be used as estimates of mean g_m integrated over a period of time. Therefore, a time-averaged determination of Pi and g_s becomes essential.

All the methods employed to estimate WUE which were described elsewhere have their own limitations. In view of the importance of WUE as a useful physiological trait, attempts have been made to develop alternative methods to quantify WUE. One such approach has been to assess the significance of $\Delta^{13}C$ discrimination in determining the variability in WUE. Plants discriminate against the heavy isotope of carbon ($\Delta^{13}C$) during the process of photosynthesis. However the extent of discrimination depends on the Pi and hence ^{13}C content in the plant samples has been emerged as a potential to quantify Pi and WUE. Several reports confirm the close relationship between Pi and Δ , therefore Δ could be a time integrated estimate of Pi.

Several workers have demonstrated the importance of stable oxygen isotope (^{18}O) in assessing the water evaporation during transpiration (Flanagan and Farquhar, 1993; Wang and Yakir, 1991; Yakir *et al.*, 1990). So, oxygen isotope composition can reflect the variations in transpiration and hence gs at a given VPD.

In view of the significance of these two isotopes (carbon and oxygen), in the following sections we describe the theory of isotope discrimination.

Stable isotopes of Carbon and Oxygen and their relevance

I) Carbon Isotopes

There are two naturally occurring stable isotopes of carbon, ^{12}C and ^{13}C. Most of the carbon is ^{12}C (98.9%) with 1.1% being ^{13}C. The isotopes are unevenly distributed among and within different compound and this isotopic distribution can reveal an information about the physical, chemical and metabolic processes involved in carbon transformations.

The overall abundance of ^{13}C relative to ^{12}C in plant tissue is commonly less than in the carbon of atmospheric CO_2, indicating that carbon isotope discrimination occurs during the incorporation of CO_2 into plant biomass. Because the isotopes are stable and non-radioactive in nature, the information is inherent in the ratio of abundance of carbon isotopes, presented by convention as $^{13}C/^{12}C$, is invariant as long as carbon is not lost.

The $\Delta^{13}C$ in plant samples is generally determined using a sophisticated analytical instrument called Isotope Ratio Mass Spectrometer (IRMS) specially designed for high precision measurements of the ratio R, defined as:

$R = {}^{13}CO_2/{}^{12}CO_2$

The plant material is converted to CO_2 by combustion to determined the isotope composition. In general R is low in organic sample. The atmosphere has a relatively higher fractionation value of around –7.8 per mil (‰) which is in comparison with a standard PDB (Pee Dee Belemnite, from North Carolina, USA). The R in this standard is 0.0124 and in many of the plant material it is approximately 0.012, suggesting a very minor changes in the R value, and hence R in a sample can be compared with that of standard and expressed as ^{13}C in units per mil or parts per thousand (‰)

$$\delta^{13}C = R_{sample} - R_{standard}/R_{standard} * 1000$$

Since the organic sample has less R value than the standard, $\delta^{13}C$ of organic material is more negative, i.e., less ^{13}C content hence more discrimination and vice versa (O'Leary, 1981).

Importance and biochemical basis of $\Delta^{13}C$ and the relationship of Δ with WUE have extensively been studied (Farquhar *et al.,* 1982; Hubick, *et al.,* 1988; Condon *et al.,* 1990; Read, *et al.,* 1991). The range of $\Delta^{13}C$ values across the crop types, having different photosynthetic pathways, is given below:

Range of $\delta^{13}C$ composition	
1. Atmosphere (Air)	= -6.4 to –7.0‰
2. C_3 plants	= -22 to –44‰
3. C_4 plants	= -9 to –19‰
4. CAM plants	= -11‰(approximate)

Carbon isotope discrimination ($\Delta^{13}C$) at different steps during photosynthesis

The fractionation of carbon isotope during photosynthesis involves several distinct biochemical and physical processes. These processes have different tendencies to discrimination between ^{12}C and ^{13}C, and the overall discrimination of a particular plant will be a function of the mechanism it uses for CO_2 fixation and the relative balance of the processes that participate in photosynthesis. During photosynthesis, CO_2 must diffuse from the atmosphere to the chloroplast stroma. Since $^{12}CO_2$ diffuses faster than $^{13}CO_2$, several fractionation processes occurs along this diffusion path, so that the CO_2 available at the sites of carboxylation is always significantly depleted in ^{13}C compared to the atmosphere (Table-below) (Farquhar, *et al.*, 1989; Brugnoli, *et al.*, 1998).

Isotope effects of steps leading to CO_2 fixation in C_3 plants

Process	Discrimination (‰)	Reference
Diffusion of CO_2 in air through the stomatal pore	4.4	Craig, 1954
Diffusion of CO_2 in air through the bondary layer to the stomata	2.9	Farquhar, 1983
Diffusion of dissolved CO_2 through water	0.7	O'Leary, 1984
Fixation of gaseous CO_2 by RuBisCO	29	Guy *et al.*, 1987

The principal components of photosynthesis that influence discrimination are diffusion of CO_2 through the stomata and the carboxylation process mediated by RuBisCO (O'Leary, 1988; 1993). Irrespective of the photosynthetic sub component-determining discrimination, Δ is related to Pi as following relationship suggests:

$$\Delta = \{a+(b-a)Pi/Pa - d\}$$

Where, **a, b** are constants for the discrimination against $^{13}CO_2$ during diffusion of CO_2 into the leaf and carboxylation, respectively and **d** is a component contributed from respiration, diffusion of dissolved CO_2 and Pi & Pa are the intercellular and ambient CO_2 partial pressures.

$\Delta^{13}C$ and WUE - relationship

Since, Pi/Pa ratios predominantly determine the variations in WUE and Δ, therefore, a strong relationship between $\Delta^{13}C$ and WUE can be expected and explained by the following equation (Farquhar *et al.*, 1989; Vogel, 1993).

$$\mathbf{WUE = \{(1 - \theta)(b - d - \Delta)\}/1.6V(b - a)}$$

Where, θ is the proportion of fixed CO_2 lost in respiration, V is the leaf-air vapor pressure gradient.

An inverse relationship between A/g_s and $\Delta^{13}C$ (Meinzer *et al.*, 1990; Richards and Tieszen, 1993) and a positive relationship between Pi/Pa and Δ signify that Pi determines the variability in Δ (Hubick *et al.*, 1988 ; Gutierrez and Meinzer, 1994).

Although WUE and Δ are related trough the ratio of Pi/Pa, as well as with A/g_s (Condon *et al.*, 1990), because of diurnal and seasonal fluctuations in 'A' and g_s, these parameters will not give an integrated estimate of WUE over a period of time (Hall *et al.*, 1993 and Udaya Kumar and Prasad, 1994). From, this context, $\Delta^{13}C$ is a dependable parameter as it is a reflection of time integrated estimate of carbon gain per unit transpiration, especially in C_3 plants. $\Delta^{13}C$ in whole plant dry matter appears to be reliable indicator of plant WUE in pot grown sunflower and negative relationship was obtained between these two traits in structural carbon both in well watered and drought conditions (Johnson, *et al.*, 1993). In wheat, as in other C_3 species, genetic variability in $\Delta^{13}C$ is reflected in

variation in WUE at both the leaf and at the whole-plant level (Condon and Richards, 1993).

Relationship between $\Delta^{13}C$ and WUE in a few crop species

Species	Reference
Wheat	Farquhar and Richards, 1984; Condon *et al.*, 1987
Peanut	Hubick *et al.*, 1986, 1988; Wright *et al.*, 1988,1993; Roy Stephan, 1995, Rao *et al.*, 1993, 1995, Crauford, *et al.*, 1999
Cowpea	Ismail and Hall, 1995; Ashok, 1996; Ashok *et al.*, 1999
Tomato	Matin and Thorstenson, 1988, Martin *et al.*, 1999
Potato	Jefferies, 1995
Barley	Hubick and Farquher, 1989; Crauford, *et al.*, 1991
Sunflower	Virgona, *et al.*, 1990, Farquhar, et al. 1995, Aymen shehada, 1999
Chickpea	Gangadhara, 1995
Cashew	Koushik, 1999
Tree species	Saurer, *et al.*, 1993, 1995; Guy, et al. 1995
Coffee	Meinzer, et al. 1991

Such a relationship between $\Delta^{13}C$ and WUE in several crop species as depicted (Table-above) was not altered even when plants were subjected to abiotic stresses. Maintenance of the relative ranking of genotypes in control and stress implies that for WUE and Δ, genotype and environment interaction is low and the broad sense heritability is high (Hubick *et al.*, 1988; Acevedo, 1993 and Wright *et al.*, 1993). Due to these distinct advantages, Δ appears to be a very reliable parameter for the identification of variability in WUE. This led to the initiation of several breeding programs to improve WUE using carbon isotope discrimination technique (Hall *et al.*, 1993; White, 1993).

In the following section a brief review on origin of oxygen isotope concept, its composition in different sources and relevance of oxygen isotope in plant physiological processes has been described.

II) Stable isotopes of Oxygen

Historical origin

The existence of natural isotopes of oxygen and hydrogen has been known for more than half a century. Giaque and Johnson discovered the oxygen isotopes ^{17}O and ^{18}O in 1929 and Uray and his associates later identified deuterium in 1932. Since then the description of the relative ratios of these isotopes in various geochemical and biological systems has been the major focus of scientific research. Thus, there is a rather large body of literature documenting variations in the natural abundances of oxygen and hydrogen in different chemicals and in water derived from various sources. These variations result from fractionations caused by phase transitions, chemical or biological reactions and transport processes (Gat, 1982).

The earliest references for the measurements of $\delta^{18}O$ in organic matter were way back in 1970s. At the leaf however, the oxygen isotope ratio of leaf water increases because of VPD effect (Ferhi and Letolle, 1997). The extent of enrichment of oxygen isotope in water in leaf surfaces depends on, among other factors a few specific physiological traits of the species. Thus, for instance, enrichment of ^{18}O in water from CAM plants doesn't occur during the day, but it does in C_3 plants (Epstein *et al.*, 1977).

There are three different stable isotopes of oxygen present in nature and are available at a definite proportion (Dansgaard, 1964)

^{16}O – 99.759%

^{17}O – 0.037%

^{18}O – 0.204%

Theory on enrichment of heavy isotope of oxygen during evaporation

Craig and Gorden (1965) originally developed a model of isotopic fractionation for process occurring during the evaporation of water from the water bodies. They discovered that during evaporation, water molecules with the lighter isotope of oxygen ($H_2{}^{16}O$) tend to diffuse and evaporate faster than their heavier isoform ($H_2{}^{18}O$). This, they showed, resulted in an enrichment of $H_2{}^{18}O$ at the evaporating surface of the water body. As an explanation, they studied the isotopic effects of oxygen in water.

Enrichment occurs at the evaporating surface because of the following two-fractionation process.

a) An equilibrium fractionation effect resulting from the phase change from liquid water to water vapour in air. The equilibrium fractionation (α^*) is defined as:

$$\alpha^* = Rl/Rv,$$

where, **R**-molar ratio of heavy to lighter isotope of oxygen ($H_2{}^{18}O/H_2{}^{16}O$) **l**-liquid and **v**-water vapour in air. This equilibrium fractionation is very sensitive to the water surface and air temperature. The α^* is 9.2‰ at 20°C and it increases to 9.6‰ at 25°C (Botinga, 1969).

b) Kinetic fractionation factor (α_K): defined as the ratio of diffusion co-efficient for water vapour molecules containing light and heavy isotopes.

$$\alpha_K = g/g_1$$

Where, **g** and g_1 are the diffusivity of water vapour molecules containing light and heavy isotopes respectively.

For a very long time the model developed by Craig and Gordon was used for various ecological studies. Farquhar, *et al.,* (1988) have stated that the potential application of the leaf water evaporative enrichment model in plant physiological and ecological studies is in studies of the leaf air VPD. Since, VPD is proportional to ratio of air to leaf vapour pressures, measurement of isotopic composition of leaf water could be applied to field studies to estimate VPD (Sternberg *et al.,* 1989; Zeigler, 1988).

Theory on enrichment of ^{18}O during the process of transpiration

While studying the isotopic composition of atmospheric oxygen, Urey, 1947 observed that it was 6‰ more enriched than the sea water. More systematic studies of Dole, *et al.,* 1954, suggested that the atmospheric oxygen was infact 24‰ more enriched than the seawater. Since the atmospheric oxygen predominantly originates from the photooxidation of water during photosynthesis, it is predictable that the leaf water is significantly enriched with ^{18}O. This was perhaps the beginning of extending ^{18}O enrichment concept in understanding a plant physiological process.

Transpiration being an evaporative process, early workers tried to study the effect of transpiration on ^{18}O enrichment in leaf water ((Sternberg, 1988 and Ziegler, 1988; Zundel, 1978). However, more systematic studies were conducted relatively recently. Flanagan et al. (1991) developed mechanistic models to predict the ^{18}O enrichment at the evaporative site of a leaf as a function of transpiration. Water vapour molecules containing the lighter isotopes of oxygen escape from the leaf more readily than do molecules containing ^{18}O, so that during T leaf water becomes enriched in heavy isotope molecule.

The isotope composition of leaf water can be estimated based on a model of isotopic fractionation during transpiration. There is some controversy about the assumption that chloroplast water has the same isotopic

composition as water at the sites of evaporation within leaves. However, Yakir *et al.*, (1994) have suggested that the isotopic signature of water in chloroplasts is closer to that of total leaf water, than to water at the sites of evaporation. Perhaps the most direct way to estimate $\delta^{18}O_2$ has been to use isotopic analysis of photosynthetically produced oxygen. Since there is no isotopic fractionation in the water splitting reaction during photosynthesis (Guy *et al.*, 1987; 1993), the $\delta^{18}O$ value of the photosynthetically produced oxygen directly reports that of the water in chloroplasts, from which it was produced. Laboratory experiments with sunflower plants, showed the potential of this approach and indicated that, under the experimental conditions, $\delta^{18}O$ of chloroplast was indistinguishable from the $\delta^{18}O$ of bulk leaf water (Yakir *et al.*, 1994a).

Oxygen isotope compositions are also expressed as parts per thousand (per mil) as that of carbon isotopes, i.e.,

$$\delta^{18}O = (R_{sample}-1)/R_{standard} * 1000$$

Where, R is molar ratio of heavy to light isotope. All water sample isotopic compositions are expressed relative to SMOW (Standard Mean Oceanic Water). The absolute ratio for SMOW used in the calculations were $^{18}O/^{16}O = 0.0020052$ (Ehleringer and Osmond, 1989).

Oxygen isotope fractionation in soil water

Effect of leaching on the isotopic composition of soil water

Leaching, the downward movement of water through the soil zone, is generally considered to be a non-fractionating process for oxygen isotopes in soil water. If fractionation does occur, it would probably be the result of reactions with soil minerals or organic matter (Amundson, 1998). Savin (1980) has pointed out that at the temperature that exist in

soil, isotopic exchange reactions between water and soil minerals occurs extremely slowly.

Effect of transpiration on the isotopic composition of soil water

During transpiration, plants act as a conduit for water transport from the soil to the atmosphere. Water travels across the root boundary, through the stem and to the leaf, where it eventually evaporates. The transfer of water from the soil to the root appears to be isotopically non-fractionating in most cases (Zimmerman *et al.,* 1967). Similar to the case of hydrogen isotope ratios, fractionation of oxygen doesn't occur during uptake of soil water through root and the stem up to the leaf (Gonfiantine *et al.,* 1965; Zundel *et al.,* 1978).

Allison and Hughes (1983) conducted a study on enrichment of stable oxygen isotopes from moist and dry soil. They have suggested that in dry soils, fractionating may occur during transpiration. As soils dry out, root shrinkage may occur as a result of water stress, creating a gap between the root surface and the soil matrix. Vapour in this zone, produced by the evaporation of soil water, could be taken up by the roots, and the remaining soil water would become enriched in ^{18}O. thus, under conditions of water stress, it is theoretically possible for soul water to become isotopically heavier as a result of transpiration.

Saurer, *et al.,* (1997) have expressed that, no significant fractionation seems to occur during uptake of water by roots and the subsequent transport of water from the roots to the leaves. However the water in leaf lamina was found to enrich significantly. The Craig and Gordon model predicts that the enrichment is possible only when there is a phase change between liquid water to water vapour. Therefore, transpiration can presumably cause the observed enrichment in the leaf water. The source of water taken up by plants is through precipitation and /or ground water. Since, no fractionation occurs during water uptake by

most plants, the isotope ratio of water in plant roots is the same as that of the water available in the soil (Ehleringer and Dawson, 1992; Flanagan *et al.,* 1992).

Environmental and biological effects on leaf water isotopic composition

Warnburg and Smith, way back in 1934, explained that the evapotranspirtion from leaves is associated with enrichment of heavy isotopes (^{18}O and Dueterium) in leaf water. Dongmann *et al.,* (1974) later explained that this was due to preferential loss of water molecules containing the lighter isotopes (^{16}O and ^{2}H). Later isotopic composition leaf water was found to vary over the course of the day. This variation was often cyclic and isotopic fractionation was related to changes in the leaf temperature and RH of the canopy (Allison, *et al.,* 1985). The overall fractionation varies due to transpiration rate influencing the behavior of leaf water. The enrichment of oxygen isotopes in leaf water is also influenced by atmospheric turbulence, transpiration rate and isotopic composition of feed water and atmospheric water vapour concentration (Walker et al., 1989). In barley, the changes in natural abundance of oxygen and hydrogen isotopes in water occur as a result of transpiration (Walker and Lance, 1991).

At steady state (controlled environmental) conditions, the observed leaf water isotopic compositions in Phaseolus was enriched above the stem (source) water, with the extent of enrichment dependent on VPD and the isotopic composition of atmospheric water vapour (AWV) (Flanagan *et al.,* 1993). The inferences drawn from their study were:

a) larger the VPD, higher the heavy isotopic composition of leaf water

b) At a constant VPD, the observed leaf water isotopic composition was relatively depleted in ^{18}O when exposed to AWV that had a low isotope content (i.e., when T is high)

c) Leaf water was relatively enriched in heavy isotope when exposed to AWV that had a large heavy isotope content (i.e., when T is low).

As noted earlier, isotopic fractionation does occur in the leaf during transpiration. So water in aboveground plant tissues can have an oxygen isotope ratio substantially different from that of source water, depending on the rate of evaporation relative to the in-flow of unfractionated source water in that tissue (Farquhar and Lloyd, 1993; Flanagan, 1993). Most non-green stem tissue shows no evaporative enrichment of ^{18}O (Dawson and Ehleringer, 1992). In contrast green, unsuberised stem tissue can have water with a substantially different ^{18}O content than source water. However, usually it is only the leaves that have water enriched in ^{18}O (Farris and Strain, 1978; Brenninkmerjier, 1983; Flanagan *et al.*, 1991; Yakir, *et al.*, 1990). The stable isotopic composition of leaf and chloroplast water is not normally constant, but is altered by fractionation processes that occur during T (Flanagan, 1993). Flanagan, *et al.*, (1994) have tested the Craig and Gorden isotopic model and did measurements of carbon and oxygen isotope in leaves at varying level of VPD and observed increase in $C^{18}O^{16}O$ and leaf water $^{18}O/^{16}O$.

On the contrary, Yakir, *et al.*, (1993) have measured the isotopic composition of O_2 involved during photosynthesis in order to directly determine the oxygen isotopic composition of chloroplast water and reported a less enrichment of ^{18}O in chloroplast water compared to bulk leaf water. Such an approach is based on the observation of no isotopic fractionation during the water splitting reaction of photosystem-II (Guy, *et al.*, 1993). The application of enrichment model to leaves is difficult, since water in leaves is not well mixed isotopically (Yakir, *et al.*, 1993). Large variations in the isotopic composition of water in different sections of leaf were observed (Lue and Sternberg, 1992). When T is high, lighter isotope of oxygen having high diffusion gradient coupled with greater vapour pressure escapes early out of leaf surface. The heavy isotope of oxygen lags behind farming an enrichment zone at sub-stomatal cavity of mesophyll cells of leaves (Sternberg, *et al.*, 1984).

Although several workers (Yakir, 1998; Wang and Yakir, 1995 and Yakir, *et al.,* 1994) have discussed the theory explaining the occurrence of ^{18}O enrichment in leaf during T, Fraquhar and Lloyd (1993) opined that it might not be a straightforward approach for the determination of T. They coined a term Péclet effect for dilution of enriched water by the convection stream. Farquhar, *et al.,* (1998) recently provided an experimental evidences for the existence of péclet effect (Barbour and Farquhar, 2000a). The péclet effect could be possible under ISS, a condition when the isotopic composition of transpired water stream is same as that of the xylem water. An ISS has been shown to be achieved only under controlled growth conditions (Flanagan, *et al.,* 1991).

However, under natural conditions, relative humidity changes dynamically on both diurnal and seasonal scales, hence delaying the attainment of the ISS till later after noon (Harwood, *et al.,* 1998). Even though, the leaf water volume is small compared to the convection stream, a progressive enrichment of ^{18}O in leaf water could still be expected as long as T increases. More recently, it has been reported that the attainment of ISS is not mandatory for adopting the ^{18}O technique for the determination of T (Roden and Ehleringer, 1999).

Mechanism of oxygen isotope incorporation in plant cellulose/biomass

Why are we interested in the oxygen isotope ratio of organic matter (cellulose)?

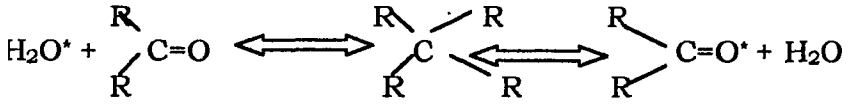
Measurement of the oxygen isotope ratio of plant organic matter (cellulose in tree rings) assist in reconstruction of paleoclimatic data. For example, they help in establishing time-series for oxygen isotope composition (the $\delta^{18}O$) of the rain water supplying moisture to the tree, which in turn can be used as a palaeo-thermometer (Farquhar, *et al.,* 1998). It also aids identification of the origin of the plant material thereby implying the forensic and other studies of the origins of paper

and cloth (DeNiro, *et al.*, 1988). There are also physiological and genetic influences on the the $\delta^{18}O$ of plant organic matter, and these should prove useful in, for example understanding differences in yield potential among lines of cereals (Farquhar, *et al.*, 2000). Measurement of the $\delta^{18}O$ also aids interpretation of differences in carbon isotope discrimination among individuals growing in the same environment.

<u>^{18}O enrichment in leaf water determines ^{18}O content in leaf cellulose</u>

Oxygen isotope ratio of plant cellulose is determined by isotopic exchange occurring during hydration of carbonyl groups of the intermediates of cellulose synthesis (Sternberg, *et al.*, 1986).

Chemically,



(Carbonyl oxygen exchange with water)

Oxygen in other functional groups, such as hydroxyl, carboxyl and phosphate are not exchangeable under temperature and pH ranges found in plants (Sternberg *et al.*, 1986). The fractionation factor of carbonyl oxygen exchange with water has been measured in acetone to be 1.028 at 25°C (Sternberg and DeNiro, 1983).

The incorporation and isotope labeling of oxygen in cellulose synthesized at the leaf could theoretically be attributed to three different sources:

1. Oxygen from carbon dioxide
2. Oxygen from water and,
3. Oxygen input via photorespiration (negligible)

However, the potential input has not been extensively evaluated but studies with labeled oxygen gas showed the effect of oxygen via photorespiration may be lost during the Calvin cycle (Berry, *et al.*, 1978). Although, oxygen from CO_2 is incorporated into carbohydrate during the

carboxylation reaction, its oxygen isotope ratio is of no consequence in the isotopic labeling of cellulose.

Several evidences indicated that carbonyl exchange of oxygen might be correct (DeNiro and Epstein, 1981; Sternberg and DeNiro, 1983b). First evidence was that the $\delta^{18}O$ value of carbonyl oxygen of acetone is 27‰ higher that of water with which it in equilibrium. This result indicates that the carbonyl hydration reaction sufficient to render the $\delta^{18}O$ value of cellulose 27‰ higher than that of water at the site of synthesis. The second evidence was, carbonyl oxygen of metabolic inter-mediates exchange with water *in-vivo*. This was demonstrated by growing carrot tissue cultures, Acetobacter xylinum (a cellulose producing bacteria) on germinating castor beans in the dark with water having different quantities of ^{18}O enrichment (Sternberg, *et al.*, 1987). Further the oxygen atoms of carbohydrate and water exchange during cellulose synthesis the $\delta^{18}O$ values of cellulose from these cultures showed a strong relationship with the water available for the growth. Thus substantiating the exchange with water is similar to the percentage of carbonyl oxygen in the substrate and subsequent intermediates during the pathway of cellulose synthesis. DeNiro and Epstein (1979) have shown that the oxygen isotope ratios of cellulose for two sets of wheat plants grown with water having similar oxygen isotope ratios, but with CO_2 having largely varied ratios, did not differ significantly. The conclusion here is, water is the principal labeling agent governing oxygen isotope ratios of cellulose.

These observations suggest that oxygen isotope ratios of cellulose may be determined by the carbonyl hydration reaction occurring at the 3-carbon sugar level, possibly with Phosphoglyceraldehyde.

MATERIAL AND METHODS

MATERIAL AND METHODS

Plants have naturally evolved several adaptive mechanisms for survival under water-limited conditions. However, from agronomical point of view, the concept of drought resistance is linked to superior crop growth rates (CGR) under water-limited conditions. Accordingly, water harvesting and its utilisation assume greater importance as per the biological yield model of Passioura (Yield = WUE x T x HI). At a given T, WUE determines the CGR and hence any improvement in this trait will have a substantial impact in improving productivity both under irrigated and rainfed situations. It is evident from the model that, both WUE and transpiration are important in determining the crop yield. Hence there is a need to determine the variability in both these parameters. Several approaches are available to quantify WUE either at single or at whole plant level.

The carbon isotope discrimination ($\Delta^{13}C$) technique has been well established as a powerful surrogate for WUE. Using this technique, significant genetic variability has been shown in WUE among several crop species. However no such technique is available to quantify T integrated over time. We hypothesise that the enrichment of ^{18}O ($\Delta^{18}O$) in leaf water that occurs during transpiration and hence subsequently in the biomass could serve as a potential measure of T. We tested this hypothesis using a few systems where stomatal conductance (g_s) and T were altered.

Several laboratory, pot and field experiments were conducted to show that ^{18}O enrichment does occur during evaporation and also during transpiration. The relationship of $\Delta^{18}O$ with g_s, intrinsic transpiration rate and transpiration integrated over time (mean transpiration rate) was examined. Further, significance of the composition of stable isotopes of carbon and oxygen in assessing the physiological parameters associated with WUE was also investigated in a few crop species.

Various methods adopted and material used in this investigation are described under the following two parts:

Part-I: General descriptions of the experimental protocols followed and materials used in this investigation.

Part-II: Descriptions on individual experiments conducted.

Part-I

Location

All the experiments to assess the genetic variability in transpiration rate, WUE and associated physiological traits at whole plant canopy level (pot as well as field conditions) were conducted at GKVK farm, University of Agricultural Sciences, GKVK, Bangalore. The site is situated at 12° 58' North latitude, 77° 35' East longitude and at an altitude of 930m above Mean Sea Level (MSL).

Materials used

The different systems used in this study were:

a) Excised leaves – sunflower leaves of pot grown plants
b) Pot grown plants
c) Field (minilysimeter) grown plants

Crop species selected for the study:

- Cowpea
- Sunflower
- Groundnut
- Amaranthus
- Soybean
- Tobacco

Planting material

In these investigations several genotypes of cowpea, sunflower and groundnut were used. Seeds for these selected genotypes were procured from ACIRP- Pulses,

AICRP- Sunflower, University of Agricultural Sciences, Bangalore and ICRISAT, Asia center, Hyderabad, respectively.

Pot culture study

Several pot experiments were conducted in this study. Pot plants were used to quantify WUE using gravimetry at whole plant level. In addition, gas exchange technique was also employed to assess the WUE and associated traits at single leaf level in plants grown in pots.

I. Gravimetric determination of WUE and associated physiological traits

Preparation of pots

Battery containers made of carbonized rubber of 0.3(L) x 0.15(B) x 0.45m(H) with capacity to hold 20 Kg of soil were used. The pots were filled with potting medium (mixture of red loamy soil + organic matter, in the proportion of 3:1). Grips to hold the containers were provided by tying nylon ropes after drilling holes to facilitate lifting of pots during weighing.

Crop management

The required dosage of fertilizers to be applied was calculated based on soil volume in the pots considering the top 5 inches of one acre soil weighs one million kg. At the time of sowing recommended dosage of fertilisers was applied. About 6-8 seeds were sown in each pot and the plant population was thinned down to maintain two plants per pot depending on the species selected. All the pots were irrigated daily twice to maintain the soil at 100 per cent field capacity until 30-35 days after sowing (DAS). Prophylactic plant protection measures were taken to contain damage of crop by pests and diseases by spraying plant protective chemicals periodically as per the recommended package of practices.

On 30th DAS, all the containers were saturated with water and left for 5-6 hours to bring the soil to 100 per cent FC. The drainage holes of the containers were closed using cement paste. The exposed soil surface in the containers was mulched by spreading plastic pieces (around 500 g) to reduce soil evaporative losses. The weight of individual container with soil at 100 per cent field capacity, plastic pieces and plant was recorded with help of a mobile electronic load cell balance of 50 kg capacity with a resolution of 100 g (Plate 1). The load cell balance was fixed on a mobile gantry system with a provision for movement along the rails horizontally to access every pot.

A high-density polythene feeder pipe, of 50 cm length, 50 mm inner diameter, with perforations of 7.5 cm intervals and one end sealed, was buried to a depth of 30cm. The required amount of water was added manually through the feeder pipe after weighing. Irrigating the pots through feeder pipes ensures water availability at the root zone. The containers were weighed along with feeder pipe once daily between 9 to 11 am to record the amount of transpirational losses. After weighing, the required amount of water was added to each pot to bring back the soil to 100 per cent FC. The detailed procedure adopted is as follows:

$$WFC_{100} = X + Y + Q_{100}$$

Where, WFC_{100} is the container weight at 100 per cent FC

X is dry soil weight + carbonised rubber container

Y is the weight of plastic pieces spread on the soil surface and feeder tubes

Q_{100} is the quantity of water present at 100 per cent FC

Therefore,

$$Q_{100} = WFC_{100} - (X+Y)$$

ML weighing Device

ML in field

Mobile ROS (field facility)

Plate-1. Field facility for gravimetric determination of WUE and associated traits

The required amount of water (RW) for individual container on each day was calculated as follows:

$$RW_{100} = WFC_{100} - X_{100}$$

Where, X_{100} is the daily weight of container.

The daily water added (RW_{100}) was summated once the entire experimental period (30 to 55DAS) to arrive at the cumulative water added (CWA) to pots with plants. Though necessary care was taken to reduce the direct surface evaporational losses, some amount of water would still be lost. To give correction to this, a set of empty containers without plants (with same amount of soil and plastic pieces as that of planted pots) were maintained and weighed daily to measure daily evaporation which were also brought back to 100 per cent FC.

The cumulative water added to these empty containers was noted as CWA* and used to correct the evaporation loss. This corrected cumulative water added for each container (CWA-CWA*) was taken as the cumulative water transpired (CWT) during the experimental period).

The containers were placed in an open area and protected from any external moisture entry (rain interruption) by using a mobile rain out shelter (ROS). Whenever required and during nights the ROS was drawn over the experimental area, thus the containers were maintained at specified water regime. This portable ROS designed and fabricated at ICRISAT, Asia Centre, was modified further to improve the efficiency (Udaya Kumar et al., 1998b)

Rain Out Shelter (ROS)

The structure of ROS was supported by three trusses (Plate-1) each with a span of 6 m, the trusses are made of MS pipe and inter-connected with purling at a spacing of 3 m. The vertical supports of each truss were attached with an

extension of rod, which was clamped, to the ROS frame to provide an overhang on each side. This arrangement not only prevented drift of rain from sides but also helps to drain the rainwater off the experimental plot effectively. The end supports were mounted on rubber wheeled castors to enable the structure to move along the two C-section channels on the plot boundary. The C-section railings were fixed on elevated reinforced concrete blocks at a height to facilitate easy movement of ROS. A galvanized iron wire is woven on all the bays of ROS over which a high-density silpaulin sheet was covered. The silpauline sheet was fastened to the ROS with a nylon rope running through eyelets all round the edge of the sheet. To prevent the ballooning of ROS during strong winds, the ROS cover was anchored firmly to the ROS frame by running nylon ropes on the top as well.

Pot weighing device (PWD)

Specialised mobile weighing device used both in pot and minilysimeter experiments consists of a mobile gantry, which moved on the C-channel on which the rainout shelter was also placed. The PWD was fabricated using a frame of `I' section and supported by two vertical `I' sections at both ends. A lever and a fulcrum, was attached to the lower plate of the mobile platform, which moved along the horizontal `I' beam with the help of castor wheels. A 50 kg $\pm$ 100g capacity load cell was attached to one end of the lever to which the container was suspended using nylon rope. The gantry could be manually moved over the C-section channel and the platform moves along horizontal `I' beam to access each pot. The pots with plants could be lifted clear off the ground by depressing the lever by a foot-operated pedal (Plate 2). The load cell with a digital read out was mounted on upper plate of the mobile platform and powdered by 12V dry lead sealed rechargeable battery.

Plate-2. Foot operated peddle to lift the pots clear off the ground using a pot weighing device

II Determination of WUE and associated parameters under field (minilysimeter) experiments

Two field experiments were conducted in this investigation. First experiment with five genotypes of cowpea and the second with four hybrids of sunflower.

Installation of minilysimeters:

Minilysimeters (ML) were prepared with cylindrical reinforced cement concrete tubes of 0.3 m inner diameter and 0.9 m height, with bottom end sealed to make them water-tight. While fabrication of the ML two strong MS loops were built into the rim of open end to serve as handles to facilitate lifting of the ML. Soil from site adjacent to the experimental block was excavated systematically in layers of 30 cm, to a depth of one meter. The MLs were filled with soil in 30cm layers and compacted to simulate natural soil profile of the experimental site. Circular pits of 1m depth and 0.35 m diameter were dug where MLs were placed. A galvanised iron sheet riveted to make a 1m long x 0.35m diameter cylinder was inserted into each pit as a sleeve to prevent collapsing of soil from the sides of the pit. Bottom of the pits were provided with stone jelly to avoid wet mud sticking to the minilysimeter.

A mobile ROS as described earlier, was also erected in the field. The entire ROS shield an area of 7.2 x 15 m from intervention of rainwater.

Raising the crop

Lay-out

The area under the ROS was divided into four blocks, which contained 8 MLs. Each block serves as a replicate. Seeds of a given crop species were sown in four rows with each row having MLs, including one row of two MLs in all the blocks. Two plants were ultimately maintained in each ML. Recommended dosage of FYM and fertilisers were applied to both field and MLs. Plants were maintained

at a specific water regime of 100% FC. Two MLs without plants were maintained to give correction for evaporation losses.

During the experimental period, each MLs was weighed once in two days using the mobile ML-weighing device (MWD). MWD was mounted on a mobile gantry system as in case of Pot weighing device (Plate-1).

On either side of the experimental site long rows of maize plants were grown to avoid the convection and advection effect of heat energy on main crop.

The following observations were recorded both in pot as well as in minilysimeter conditions:

- Cumulative Water Added (CWA)
- Whole plant leaf area at the beginning and end of the experiment
- Total dry matter accumulated at the beginning and end of the experiment

Based on these primary observations the following parameters were computed:

- Cumulative Water Transpired (CWT)
- Water Use Efficiency (WUE)
- Leaf Area Duration (LAD)
- Mean Transpiration Rate (MTR)
- Net Assimilation Rate (NAR)

Total dry matter accumulation (TDM):

Entire plants including roots were harvested and oven dried at 70 °C for three days. The biomass accumulated during the experimental period was computed as the difference in initial and final dry matter and expressed as gram per pot.

Functional leaf area (LAD):

The LAD, a reflection of functional leaf area for assimilation during the experimental period LAD was calculated. Leaf area was measured using the leaf area meter (MK-2, Delta-T devices, England), at the beginning and end of the experiment.

$$LAD = \frac{L_1 + L_2}{2} \text{ days}$$

where, L_1 – initial leaf area
L_2 – leaf area the end of experiment (final leaf area)

Cumulative Water Transpired (CWT):

The amount of water added daily to each container after weighing to bring back to 100 per cent field capacity was summated individually for each pot during the experiment period (30 to 60 DAS), and was expressed as cumulative water added (CWA*). The daily soil evaporation loss was determined by weighing the empty pots. The soil evaporation was removed from the CWA* to arrive at the cumulative water transpired (CWT).

$$CWT = CWA - CWA^*$$

Mean transpiration rate (MTR):

The rate of transpiration over the entire experimental period was measured as the mean transpiration rate. This parameter was arrived at by computing the ratio of cumulative water transpired to the functional leaf area and is expressed as gram or ml of water per $dm^{-2}LA.day^{-1}$. The MTR is considered as the time integrated measure of T.

$$MTR = CWT/LAD$$

Water Use Efficiency (WUE):

Measurement of Water use efficiency by gravimetric approach involves the measurement of dry matter accumulated over a specific period of time and the total water transpired by the plant during the same period. The ratio of the TDM during the experimental period to the total water transpired was computed to arrive at the whole plant WUE, and expressed as g DM/kg of water transpired.

Net Assimilation Rate (NAR):

The net carbon gain, a measure of photosynthetic rate integrated over time was gravimetrically determined using the following formula

$$NAR = \frac{(\ln L_2 - \ln L_1)(W_2 - W_1)}{(L_2 - L_1)(T_2 - T_1)}$$

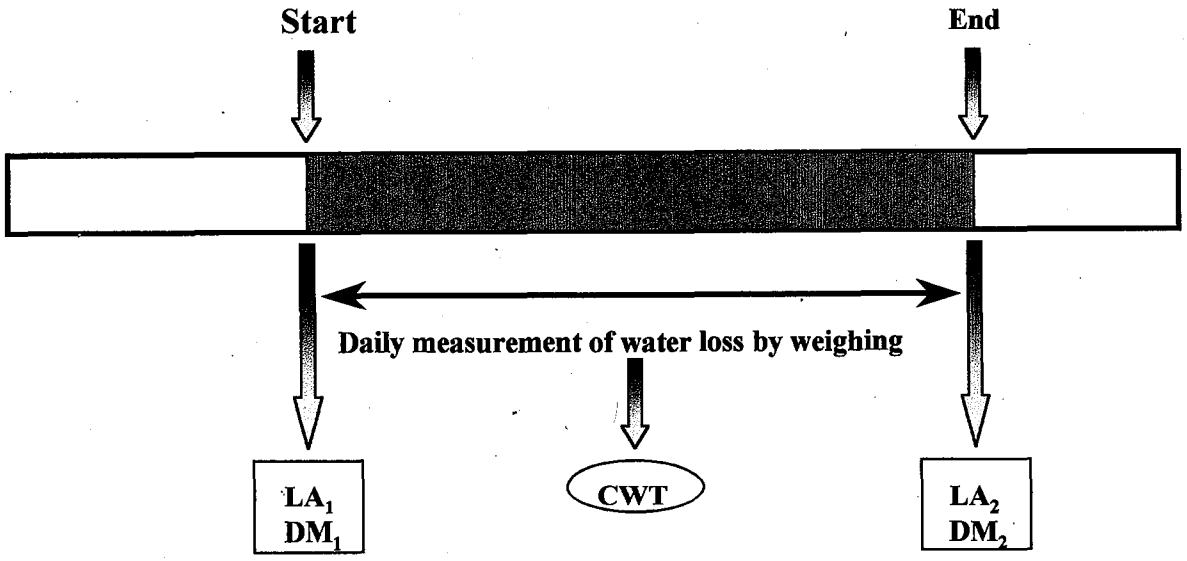
Where,

W_1, L_1 and W_2, L_2 are dry matter and leaf area per pot at sampling days T_1 (initial) and T_2 (final), respectively.

The NAR is a good measure of the assimilation capacity per unit leaf area. This was computed using the dry matter accumulated during the experimental period and functional leaf area of that period, and expressed as mg. dm^{-2} day^{-1}.

The major feature of the gravimetric approach standardized at our centre is that, the genetic variability in WUE as well as the physiological traits like MTR and NAR could be determined simultaneously (Flow-chart. A)

Flow chart. A: Schematic representation of the experimental protocol for the gravimetric determination of WUE and the associated physiological parameters



$WUE = (DM_2-DM_1)/CWT$	$NAR = (DM_2-DM_1)/LAD$
$LAD = \{(LA_1+LA_2)/2\} \times days$	$MTR = CWT/LAD$

Gas exchange characteristics:

Gas exchange traits such as net CO_2 assimilation rate ('A'), stomatal conductance (g_s), intercellular CO_2 concentration (Ci) etc., were measured using portable photosynthetic systems.

In this investigation, we used three different portable photosynthetic systems (IRGA-Infra-Red Gas Analyser) to measure gas exchange parameters. They are:

1. LICOR-6200, USA
2. LCA4-ADC, UK
3. CIRAS-I, PP-Systems, UK

All these three systems basically perform the measurement of photosynthetic rate and stomatal conductance. The description of each system has been given below:

1. LICOR-6200:

This system operates on a closed system; where in the CO_2 in the atmosphere once drawn will be utilized at different intervals of time. So there is a gradual depletion of CO_2 concentration in the leaf chamber.

The system comprises of two components. One is the main console, which house the CO_2 IRGA inside, and another being the leaf chamber (LC). A broad leaf chamber connected to the IRGA was used in the present investigation. The LC has quantum and RH sensor attachments and thermocouple junction for measuring leaf temperature.

<u>Instrument records these following primary values:</u>

a. Leaf area
b. CO_2 concentration
c. Light intensity (PAR)
d. Relative Humidity and chamber temperature

Using these primary values the following parameters will be computed

i. Stomatal conductance (g_s) and Stomatal resistance (r_s)
ii. Transpiration rate
iii. Photosynthetic rate
iv. Vapour pressure difference (leaf to air)
v. Leaf temperature (°C)

2. LCA4-ADC:

LCA4 operates in an open system, and hence it was possible to maintain steady state CO_2 concentration and relative humidity in the leaf chamber. The instrument is composed of two distinct components.

Leaf Chamber Analyser (LCA): This component consists of in-built IRGA coupled with Air Supply Unit (ASU)- (contains a peristaltic pump that pumps air at a constant rate ranging from 200 to 600 $ml.min^{-1}$). LCA has two IRGAs namely a reference to which air is pumped straight from ASU and the analysis chamber to which air from leaf chamber is pumped. An actively photosynthesising leaf would deplete CO_2 from the air and hence the analysis chamber CO_2 concentration would always be less compared to reference CO_2. The in-house data logger uses this differential CO_2 level to compute the photosynthetic rate.

Parkinson's Leaf Chamber (PLC): It accommodates a leaf area of 6.25 cm^2 and is equipped to measure the light intensity in the PAR range by a quantum sensor, relative humidity by a thermocouple and temperature of the air by a thermostat. Butyl rubber tubing is used to carry air from the leaf chamber into the IRGA in LCA.

LCA4 measures these following basic parameters:

- CO_2 concentration
- Relative Humidity
- Air temperature
- Light intensity
- Flow rate

Based on these primary values required gas exchange parameters will be computed by the software provided inside the system.

3. CIRAS-I (Combined Infra-Red Analysis System)

The Combined Infrared Gas Analysis System (CIRAS-1) is a portable photosynthetic system and it is designed to operate as an open system to measure several gas exchange parameters. It consists of a main console that harbours seperate CO_2 and H_2O IRGAs, an internal air supply unit and the necessary software for the computation of the gas exchange parameters. CIRAS uses four independent infrared gas analyzers, 2 each for CO_2 and H_2O. One pair of CO_2 and H_2O analyzers have a common inlet/outlet and are defined as the reference and similarly second pair as analysis. Measurements are expressed as absolute concentrations for the reference, and as the difference between the reference and the analysis concentrations.

Parkinson's Leaf Chamber (PLC): It accommodates a leaf area of 6.25cm^2 and is equipped to measure the light intensity in the PAR range by a quantum sensor, relative humidity by a thermocouple and temperature of the air by a thermostat. Butyl rubber tubing is used to carry air from the leaf chamber into the IRGA in LCA.

The leaf chamber air to leaf VPD can be regulated by altering the chamber Relative Humidity (RH) and temperature. The RH is normally regulated by altering the flow rates of dry air into the cuvette or by regulating the RH of the input air.

CO_2 control

A CO_2 catridge normally carrying 8 g of pure CO_2 in liquid form was used get the requisite CO_2 concentration in the leaf chamber. With some minor replacement of scrubbers, ambient air could also be conveniently be used for measuring photosynthetic traits at ambient CO_2 concentration.

The instrument measures the following primary parameters.

1. Volume flow rate of dry air into the cuvette ($cm^3 . s^{-1}$)
2. Leaf area (cm^2)
3. Boundary layer resistance to water vapour (m^2 s. mol^{-1})
4. Photon flux density incident on cuvette (μ moles. m^{-2} s^{-1})
5. CO_2 concentration and water vapour pressure of air entering and leaving the leaf cuvette.

Using several physical constants and measured parameters, the equipment computes the following gas exchange parameters.

1. Stomatal conductance to water vapour (mmol. m^{-2} s^{-1})
2. Rate of Photosynthetic rate [μmol. m^{-2} s^{-1}]
3. Transpiration Rate (mol. m^{-2} s^{-1})
4. Sub-stomatal cavity CO_2 concentration (ppm)
5. Leaf temperature (°C)
6. Saturated vapour pressure at leaf temperature (bars)
7. Vapour pressure of water entering the cuvette (bars)
8. Vapour pressure of water in the cuvette

<u>CO_2 response curve:</u>

Photosynthetic rates were recorded at different CO_2 concentrations ranging from 0 to 1000 ppm. The Assimilation rate was plotted against the intercellular CO_2 concentration (Ci) and a best-fit polynomial function was fitted to obtain the CO_2 response curves. The initial high response region of the curve is normally linear. This linear portion was extended by drawing a tangent to this portion of the curve. The slope of this line was determined as the dAdCi, which is often regarded as an estimate of carboxylation efficiency (Caemerrer and Farquhar, 1981)

<u>Recording gas exchange parameters</u>

The gas exchange parameters were determined using these different portable photosynthetic systems in different experiments. The top fully expanded leaf

was clamped to the leaf chamber and the observations were recorded when A , gs and Ci reached a stable value (Plate 3). All gas exchange parameters were recorded between 9 AM and 12 noons on bright sunny days. The leaf chamber of CIRAS-1 is equipped with a peltier cooling system that can maintain the chamber temperature. The operational option provided with the system also maintains a constant chamber RH around that of the ambient air.

Quantification of $\Delta^{13}C$ composition in leaf samples:

The Carbon Isotope Discrimination ($\Delta^{13}C$) was determined with an objective to study how far $\Delta^{13}C$ is associated with the observed genetic variability in WUE in a few crop species.

1. Third fully expanded leaves that were developed during the experimental period were collected (5-6 for each genotype)
2. Dried at 80°C for 3 days
3. Dried samples were powdered in a mortar and pestle. Care was taken to prevent any mixing of different samples by washing the pestle and mortar with alcohol after grinding each sample
4. One gram of powdered leaf sample was put in glass vial, properly labelled and sent to three different laboratories (our overseas collobarators) namely, Dr. Steeve Brooks, at PDZ Europa Ltd, UK, Dr. Peter Watkins, Finnigan Mat (ThermoQuest), Germany and Dr. Mathias Saurer , PSI, Switzerland, for $\Delta^{13}C$ analysis.

Mass spectrometric analysis:

One mg of the leaf powder was taken in tin capsules and dropped on to the combustion column using an autosampler.

Combustion (oxidation) column contains chromium oxide in quartz tubes heated to 1050°C. The oxidation product mainly consists of CO_2, CO, N_2O and H_2O. These gases were swept into the reduction furnace using helium carrier gas. The reduction furnace contains reduced copper sandwiching copper oxide in quartz

Plate-3. Recording gas exchange parameters using a portable photosynthesis system (CIRAS-1)

tubes heated to 680°C. The N_2O is reduced to N_2 gas in the column. The resultant gases are then flushed through scrubbers to trap CO and water. The pure CO_2 and N_2 gas after passing through a GC column (5°A molecular sieve) and a thermal conductivity detector (TCD) into the ion source of the IRMS. An appropriate standard of known isotopic composition (Craig-corrected against PDB) was introduced at regular intervals to check the linearity and standard deviation of the run.

Based on the fractionation (isotopic composition with respect to PDB), the ^{13}C discrimination ($\Delta^{13}C$) in the plant sample was computed as follows:

$$\Delta^{13}C\ (‰) = \frac{\delta_a{}^{13}C - \delta_p{}^{13}C}{1 + \delta_p{}^{13}C/1000}$$

Leaf Mineral Ash content (%)

The mineral ash content was determined in leaves of crop species. After harvest of the crop, leaves were oven dried (80°C for 3 days). The dried leaf samples were ground using pestle and morter. A known quantify of dried leaf powder (1g) was taken in a silica dish. Initial weight of the dish was taken before adding 1g of sample. The dried samples along with silica dish was subjected to ashing in a muffle furnace (600°C for 8hrs) as described by Power (1966). After 8 hours the dish is allowed to cool for 6 hours and the final weight along with ash was recorded. The difference in initial and final weights indicates the mineral ash content, which was expressed either in percentage or $g.kg^{-1}$.

Methodology for estimation for stable isotope of oxygen (^{18}O) *In leaf water*.

a. Extraction of leaf water:

Fresh leaf punches were taken in stoppered plastic tubes with sealed tapering ends. The tubes with leaf pieces were purged with pure nitrogen gas to avoid any interference of atmospheric oxygen and the tubes were frozen to liquid nitrogen

temperature. After rapidly thawing the tubes in a hot water bath, the tubes were centrifuged at 4000 rpm for 5 minutes. The leaf water that collected at the bottom of the tapering end was taken out by introducing a syringe. About 150-200 μ l of the leaf water was injected into vaccutainer tube (Beckmen, USA)

b. Equilibration of leaf water:

The leaf water collected in vaccutainers was equilibrated overnight (12-15 hours) on a mechanical stirrer by injecting the pre calibrated CO_2 gas (i.e., CO_2 gas whose ^{18}O isotopic composition is know) (Epsten et al, 1965 and Scrimgrover, 1996). The equilibrated CO_2 was then introduced into a continuous-flow isotope ratio mass spectrometer (Tracer Mass, Europa Scientific Ltd, UK) facility available at Nutrition Research Lab, St. John Medical College, Bangalore for the determination of ^{18}O composition (Plate 4).

Protocol followed for leaf water extraction and ^{18}O analysis

I Extraction of leaf water

Fresh leaf punches taken in a tapering plastic tube (airtight)

↓

(purged with pure nitrogen gas)

Frozen to liquid nitrogen temperature for 30 minutes

↓

Thawed in hot water for 15-20 minutes

↓

Centrifuged the contents at 4000rpm for 5 minutes

↓

Leaf water was collected in syringe

↓

150-200 ul leaf water introduced to vaccutainer tubes (Beckmen, USA)

↓

Stored at 5°C until use for ^{18}O analysis

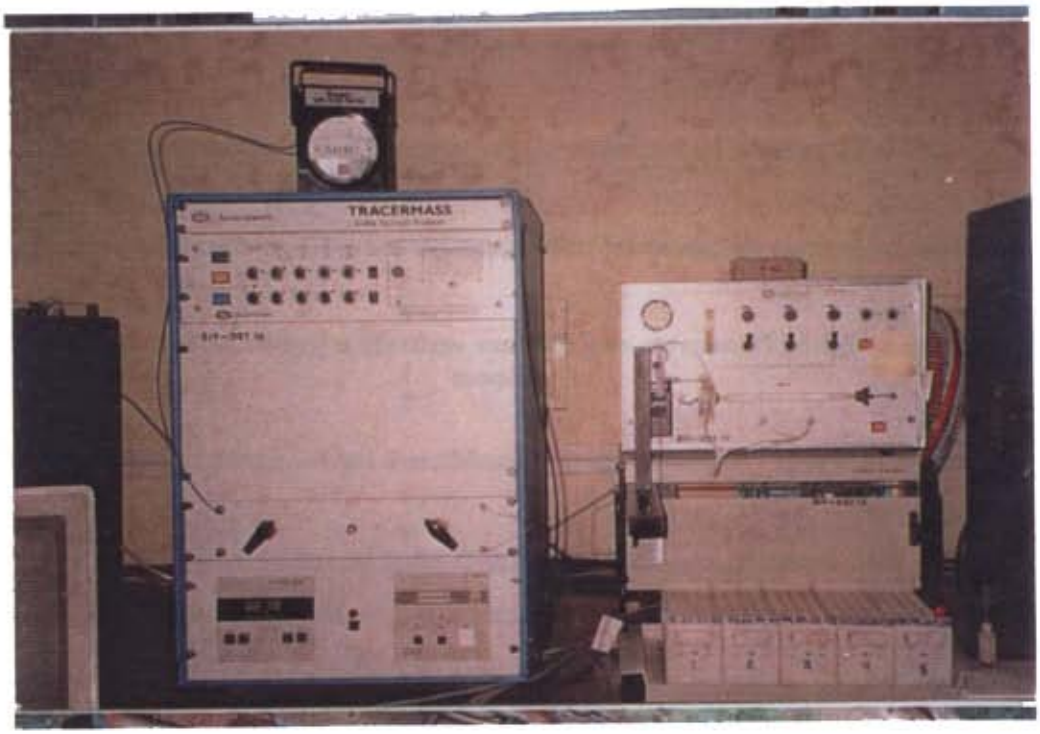


Plate-4. Table-top Isotope Ratio Mass Spectrometer (Tracermass, Europa Ltd. UK) along with the head space gas auto-sampler facility for determination of $\Delta^{18}O$ in liquid samples at Nutrition Research Center, St. John's medical college, Bangalore.

II ^{18}O analysis

10ml of pure CO_2 gas of known isotopic concentration was injected to these vaccutainer tubes

↓

Equilibrated for 12-15 hours on an automated shaker at 28°C

↓

After equilibration, CO_2 would carry the isotopic signature of water

↓

CO_2 gas is drawn along a Helium carrier gas stream through a septum needle

↓

Passed through Magnesium Perchlorate trap to scrub traces of moisture

↓

Dry CO_2 gas introduced into IRMS (Tracermass, Europa Ltd, UK)

(the m/z for 44 and 46 mass was determined to compute the $\delta^{18}O$ against vSMOW)

Estimation of ^{18}O composition in leaf biomass/cellulose:

The estimations were done at four different institutes by our overseas collaborators following the procedure outlined below.

Dr. Matthias Saurer Department of Chemistry Paul Scherrer Institute, PSI Switerzland	Dr. Steeve Brookes Chemical Laboratory, PDZ-Europa Ltd. Crewe, United Kingdom.
Dr. M.S. Sheshshayee Visiting Fellow, Environmental Biology Group Research School of Biological Sciences Australian National University, Canberra Australia.	Dr. Dan Yakir Dept. of Environment and Ecology Weizmann Institute of Biological Sciences Weizmann, Israel.

Method

The oxygen isotopic composition of biomass is generally determined ofter quantitative pyrolysis of the sample at high temperature in the complete absence of oxygen. The dried leaf powder (1 - 1.5 mg) was taken in tin or silver capsule and dropped sequentially into a pyrolysis column by an auto sampler. The pyrolysis column contains either glassy carbon or nicklised carbon in a quartz or ceramic column heated to 1080°C. The pyrolysis of organic matter results in the production of carbon monoxide (CO) and nitrogen (N_2) gas. CO_2 is generally <5 per cent. These gases are swept by a helium (He) carrier gas (purity >99.996%), through an absorbent column to scrub for the traces of CO_2 and water vapour respectively. Since the mass to charge ratio (m/z) of CO and N_2 is same, it is essential to separate these two gases before introducing into the ion source of for isotopic ratio determination. These gases were passed through a GC-column containing 5°A molecular sieve heated to 60°C.

The CO travels slower than N_2 gas through the molecular sieve hence the two gas species can be quantitatively separated. The m/z ratio for 28 and 30 masses corresponding to $C^{16}O$ and $C^{18}O$, respectively was determined by the IRMS. An appropriate standard (Craig-corrected against vSMOW) was also introduced in the run to determine the accuracy of mass detection and standard deviation of the run. The result obtained from the Continuous-Flow (CF)-IRMS analysis (Plate 5) was used to determine ^{18}O enrichment over and above the source water as follows:

$$\Delta^{18}O\ (‰) = \frac{\delta_p{}^{18}O - \delta_s{}^{18}O}{1 + \delta_p{}^{18}O/1000}$$

Plate-5. Isotope Ratio Mass Spectometer (Isochrome, Micromass, UK) facility for pyrolytic determination of $\Delta^{18}O$ in solid biomass samples at the Research School of Biological Sciences, ANU, Canberra, Australia.

Statistical analysis:

The results obtained from several pot and field experiments were analyzed statistically for their significance using standard ANOVA technique (Sunderraj, et al., 1972).

Grouping of the genotypes for the differences in dry matter accumulation, WUE and stable isotope composition was done. The measured values were transformed and a standard normal distribution of these parameters was plotted.

$$Z = \frac{\bar{X} - \bar{X}_i}{\sigma}$$

where, X - General mean of the parent
Xi – Mean for the genotype
σ - Standard deviation of the experiment

Part-II

Methods of individual experiments conducted:

The experiments conducted in this study has been categorised into following three sections:

Section-I

3.1. ^{18}O enrichment during evaporation and transpiration

3.1.1. Influence of VPD on $\Delta^{18}O$ in water during surface evaporation

To assess the influence of VPD on $\Delta^{18}O$ in water during surface evaporation, a known amount of water (10 ml) was taken in a metallic cup, specially designed for this purpose (3.5cm diameter and 2cm depth) (Plate. 6). These cups were placed in a humidity chamber maintained at a specific VPD which was obtained by altering the RH level in the chamber at a constant temperature of 28 °C. After 2 hours of exposure, the amount of water evaporated from each cup was

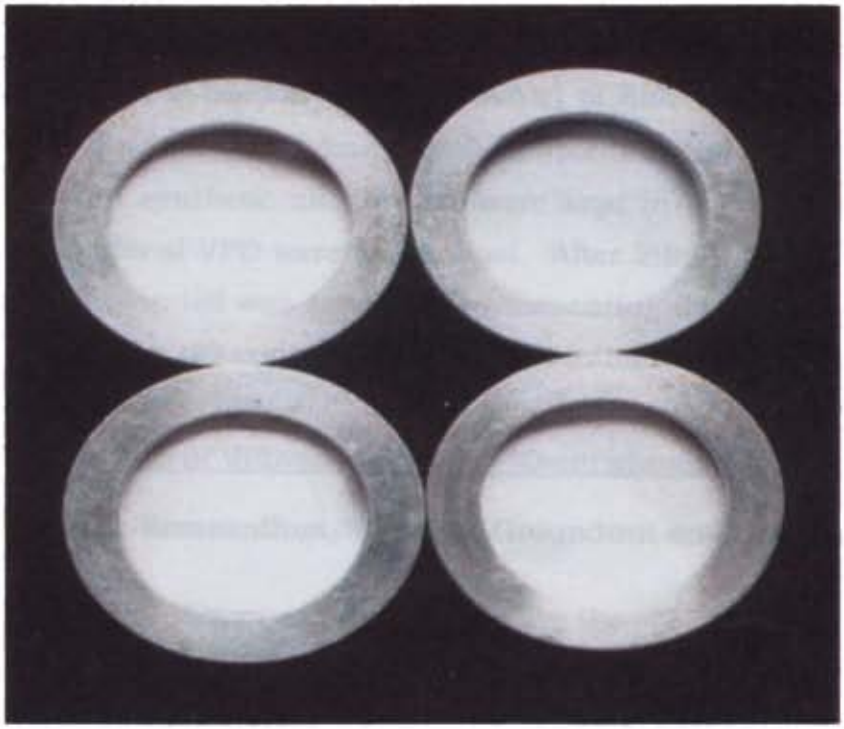


Plate-6. **Specially designed metallic cups (covered with synthetic membranes) used to examine the pattern of ^{18}O enrichment during evaporation through the pores of synthetic membranes.**

measured and the water retained in the cup was collected in vaccutainer tubes and analysed for $\Delta^{18}O$.

3.1.2. ^{18}O enrichment in water as affected by VPD and membrane pore size

The membranes (Isopore poly-carbonate) of different porosity (0.2, 0.45, 0.8 and 1.22 μ) were covered on a metallic cup containing 10 ml water. These cups covered with synthetic membranes were kept in a humidity chamber wherein different levels of VPD were maintained. After 2 hours of exposure, the amount of water evaporated was estimated by measuring the volume of water remaining in the cup. The ^{18}O content was analysed in the water that remained.

3.1.3. Influence of VPD on leaf water ^{18}O enrichment in different crop species.

Species used: Amaranthus, Soybean, Groundnut and Cowpea

The objective of this experiment was to see the effect of VPD induced differences in transpiration rate on ^{18}O enrichment in leaf water. Well-established container grown plants (30 day old) of different species were exposed to different VPD levels (7.2, 12.5 and 18 mbar) to alter transpiration rate. Specific VPD level was achieved by changing the RH at a constant air temperature of 28 °C in a growth chamber (Plate 7). Plants were exposed to a given VPD for a period of 3 hours under saturating light intensity of 1500 $\mu mol.m^{-2}.s^{-1}$. The soil surface of the pots was covered with small plastic pieces to minimise the surface evaporation. The total water loss from the each pot was determined gravimetrically by weighing the pots before and after exposing them to a given VPD. The transpiration rate was calculated and expressed on a leaf area basis (the leaf area was measured non-destructively by measuring the length and breadth of all the leaves). At the end of the exposure, leaf water was extracted, collected in vaccutainers and analysed for $\Delta^{18}O$.

A couple of similar experiments were conducted to reconfirm the results obtained in previous experiment. One with three species (Sunflower, soybean

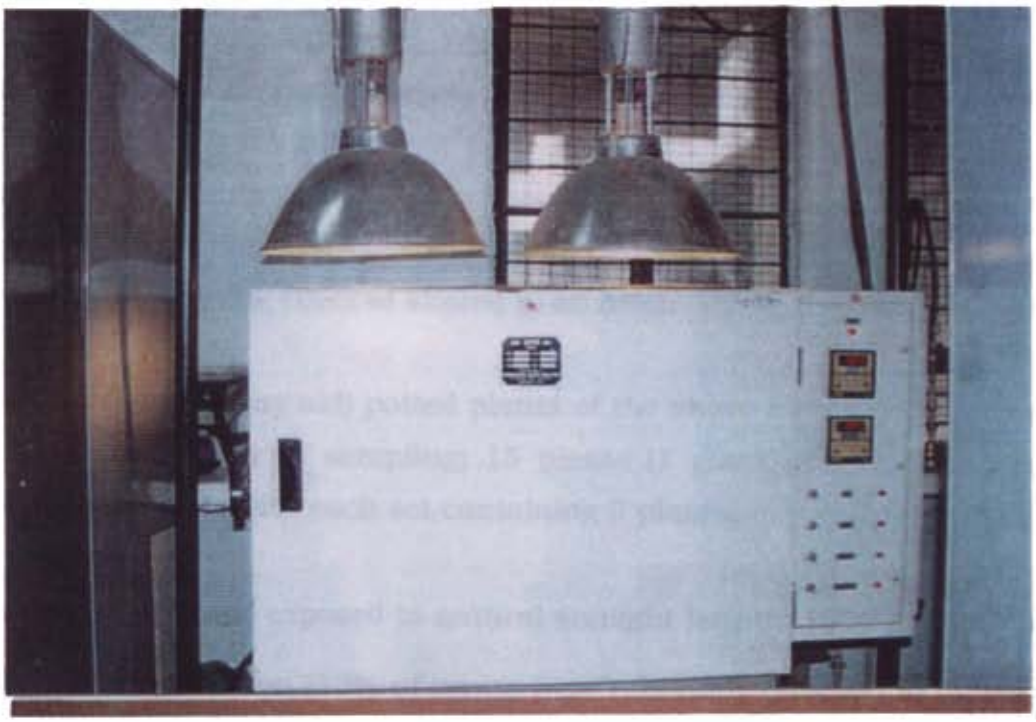


Plate-7. Growth chamber with temperature and RH control options used to maintain specific VPD to alter the transpiration rate.

and groundnut). And the other with two sunflower genotypes viz, IHM-306 and IHM-318.

3.1.4 Stomatal conductance and $\Delta^{18}O$ in leaf water

Species used: Amananthus, Cowpea, Soyabean , Groundnut and Sunflower.

From the experiments conducted previously at out centre, it has been shown that partial defoliation and partial shading can vary the g_s. Experiments were conducted to study the effect of altered g_s on $\Delta^{18}O$.

Well-established (30 day old) potted plants of the above-mentioned species were selected. On the day of sampling; 15 plants (1 plant/pot) were chosen and considered as 3 sets with each set containing 5 plants.

Set I: Control – plants exposed to natural sunlight (around1500 $\mu mol.m^{-2}.s^{-1}$).

Set II: Partial defoliation (50% of leaves were randomly removed to increase the root to leaf area ratio. The plants were kept under natural sunlight.

Set III: Partial shading – plants were shifted to a shaded condition with a light intensity was approximately between 400-500 $\mu mol.m^{-2}.s^{-1}$.

After 24 hours of treatment imposition, water from leaf tissues was extracted and analysed for $\Delta^{18}O$.

3.1.5. Effect of ABA induced reduction in g_s on ^{18}O enrichment in leaf water

As yet another approach, the g_s was altered by using the plant hormone ABA. Fully expanded sunflower leaves were excised under water and the cut end of petioles was dipped in beakers containing ABA solution of different concentrations (10^{-4} to 10^{-7} M). The leaves were allowed to transpire in a growth chamber at 28° C, 60%RH and 800 $\mu mol.m^{-2}s^{-1}$ light intensity. The time taken by leaves to transpire 15ml of water was determined and transpiration rate was

computed based on leaf area. The leaf water was collected and analysed for $\Delta^{18}O$.

3.1.6. Effect of g_s on leaf water ^{18}O enrichment in transgenic tobacco plants

Cytokinin, a plant hormone induces stomatal opening under natural condition. Genetic engineering techniques have been developed to alter the cytokinin levels by over-producing ***ipt*** (isopentanyl transferase) a rate limiting enzyme of iosynthetic pathway. Over expression of ***ipt*** results in increased cytokinin level and the levels vary in different transgenic lines. Such transgenic lines of tobacco were used in this experiment. The g_s was measured on fully expanded top leaves exposed to light intensity of 1300 $\mu mol.m^{-2}s^{-1}$ using a portable photosynthetic system (LCA4, ADC UK). After assessing g_s in both transgenic and wild plants, leaves were harvested and $\Delta^{18}O$ was determined in leaf water.

3.1.7. Species variability in Mean transpiration rate, g_s and their influence on $\Delta^{18}O$

Crop species selected: Amaranthus, Cowpea, Soybean and Sunflower

Seedlings of all the four species were allowed to establish in pots for 30 days. The MTR was determined gravimetrically (30-50DAS) on the basis of total T on a day and the functional leaf area present during the experimental period. Along with MTR, stomatal conductance was also recorded on 50th DAS using a portable photosynthesis system (LCA-4). After the measurement the leaves were collected and analysed for $\Delta^{18}O$ in leaf water.

3.1.8. Genetic variability in stomatal conductance (g_s), mean transpiration rate and $\Delta^{18}O$ in leaf water in a few crop species

A. Sunflower

3.1.8.1. Pot grown:

Genotypes used: IHM-306 and IHM-318

Well-established (35 day old) sunflower genotypes were selected and the daily water loss was computed gravimetrically for 20 days (35 to 55 DAS) to arrive at the MTR. On the day of final sampling (ie., 55 days), g_s was measured on fully expanded top leaves using photosynthesis system (LCA4). The tissue leaf water was extracted from both the genotypes and analysed for $\Delta^{18}O$.

3.1.8.2. Field grown:

Genotypes selected: IHM-318, IHM-312, IHM-314 and IHM-306

The genotypes were allowed to establish in minilysimeters for a period of 35 days. The daily water loss was determined gravimetrically between 35 to 55 DAS to assess MTR. On the day of final harvest, g_s was measured on top fully expanded 3rd leaf using a portable photosynthesis system (LICOR 6200). Water was extracted from the leaf used for gas exchange and ^{18}O content was quantified.

B. Cowpea

3.1.8.3. Pot culture

Genotypes used: APC 40-GC-20, APC-123V-683, V-585, APC-4125 and APC-121- P132.

Genetic variability in transpiration rate was determined gravimetrically based on the amount of water transpired on a specific day (50th DAS). The total water transpired on that day was divided by the whole plant leaf area to arrive at the

transpiration rate. The leaf area was measured by determining the length and breadth of all the leaves in the plant. g_s was also measured on 50th day on a top fully expanded leaf using a portable photosynthesis system (LICOR-6200). Leaf water was extracted from these leaves for $\Delta^{18}O$.

Observation recorded:

MTR

Stomatal conductance (g_s)

and $\Delta^{18}O$ in leaf water.

Following relationships were examined:

-g_s and MTR

- g_s and $\Delta^{18}O$ leaf water

-MTR and $\Delta^{18}O$ leaf water

Section-II

3.2. Stable isotope ($\Delta^{13}C$ and $\Delta^{18}O$) composition in leaf biomass: an approach to assess the genetic variability in transpiration rate and water use efficiency in crop species

I COWPEA (field grown)

Genotypes used: APC-982, APC-478, APC-370, APC-580 and APC-229

Genetic variability in transpiration rate, WUE and associated physiological traits in selected cowpea genotypes was quantified under minilysimeter condition by gravimetric approach between 35 to 65 DAS.

Observation recorded in these genotypes:

- Leaf area and biomass at the beginning and at the end of the experimental period
- Cumulative Water Added to pots with and without plants.
- Functional Leaf area during the experiment (LAD)

- Mean Transpiration Rate
- Water Use efficiency

At the end of the experiment, the leaf samples were collected, and dried for carbon and oxygen isotope analysis using an IRMS.

In addition, the results were transformed to plot a standard normal distribution (Z) graph to identify contrasting genotypes for their dry matter accumulation and WUE characters. A similar exercise was done with $\Delta^{13}C$ and $\Delta^{18}O$ as well.

II. Sunflower:

Genotypes used

KBSH-42; KBSH-1; IHM-313; IHM-306; IHM-318; RHC-1; IHM-321

A pot culture study was conducted to assess the genetic variability in MTR, WUE and associated traits using gravimetry. CWT was determined by weighing the pots daily, MTR was computed by the ratio of total water transpired to the functional leaf area retained. In one set of leaf samples, tissue water was extracted and analysed for ^{18}O content. In another set of leaf samples, carbon and oxygen isotope composition was quantified in leaf biomass.

The genotypes were grouped based on TDM and WUE traits ; $\Delta^{13}C$ and $\Delta^{18}O$. Contrasting genotypes were identified.

Gas exchange parameters were quantified on 60th DAS in fully expanded top 3rd leaves using photosynthesises system (LICOR-6200).

Observations recorded:

- Assimilation Rate (A)
- Stomatal conductance (g_s)
- Inter-cellular CO_2 concentration (Ci)

Using these primary values following intrinsic physiological traits were computed:

- A/g_s (intrinsic WUE)
- Ci/g_s (intrinsic mesophyll efficiency)

III Groundnut

Following genotypes were used:
ICGS-44; ICGV-86031; TMV-2; ICGS-76; ICG-476

Mean transpiration rate, WUE and associated traits were determined in five genotypes of groundnut between 30 and 55 DAS using gravimetric approach. At the end of the experiment (55th day), the leaves were harvested for the quantification of carbon and oxygen isotope composition in leaf biomass.

Parameters recorded:

Leaf area and TDM on 30th and on 55th DAS.
Cumulative Water Transpired
Leaf Area Duration
Net Assimilation Rate, Mean Transpration Rate and WUE
$\Delta^{13}C$ and $\Delta^{18}O$ composition in leaf biomass
and Mineral ash content

Standardized normal distribution (Z-analysis):

The genotypes of groundnut were grouped based on their relative performance *w.r.t* physiological as well as stable isotope traits. On this basis, contrasting genotypes were selected with desirable physiological characters.

Gas exchange traits

The gas exchange parameters (A, g_s, Ci etc) were measured to arrive at intrinsic WUE (A/g_s) on top fully expanded 3rd leaf in each genotype using a portable photosynthesis system (CIRAS-I).

Section-III

Quantification of mesophyll efficiency based on carbon ($\Delta^{13}C$) and oxygen ($\Delta^{18}O$) composition in leaf biomass

3.4. COWPEA (pot grown)

Genotypes used:

APC-20 GC-40; APC 123-V 683; V-585; APC-4125; APC 121-P-132

Water Use Efficiency and associated physiological traits at whole plant level were quantified between 35 and 60 DAS by gravimetry in the above cowpea genotypes grown in pots.

Observations recorded:

- Initial and final leaf area and biomass at the beginning and at the end of the experimental period
- Cumulative Water Added
- Leaf Area Duration
- Mean Transpiration Rate
- Water Use efficiency

Carbon and oxygen isotope composition in leaf biomass

Leaves that developed during the experimental period were collected for the4 determination of carbon as well as oxygen isotope composition in biomass as explained earlier.

Determination of Water Use Efficiency at single leaf level (A/g_s)

Assimilation rate to stomatal conductance ratio (A/g_s) is often considered as an instantaneous or intrinsic WUE at single leaf level. In this experiment, intrinsic WUE and other gas exchange parameters were measured on top fully expanded 3rd leaves using gas exchange equipment (LICOR-6200).

Observations recorded:
Assimilation rate (A)
Stomatal Conductance (g_s)
Inter-cellular CO_2 concentration (Ci)
A/g_s and Ci/g_s

Grouping of genotypes

The genotypes were classified under specific groups after transforming the TDM and WUE and $\Delta^{13}C$ and $\Delta^{18}O$ into standardized normal distribution values.

Z-analysis was done for grouping of genotypes for their TDM and WUE traits coupled with stable isotope signatures of carbon and oxygen to identify contrasting genotypes.

Intrisic carboxylation efficieny (dA/dCi) using CO_2 response curve

CO_2 response curves (A/Ci) were developed in selected cowpea genotypes using the CIRAS-1 to deduce dA/dCi from the initial slope of the curve.

Mineral ash content

Leaves of the cowpea genotypes that developed during the experimental period of 35-60 DAS were collected and analysed for plant mineral ash content as explained earlier.

EXPERIMENTAL RESULTS

EXPERIMENTAL RESULTS

It has been fairly well documented in the literature that during evaporation from the open water bodies (Ocean, Lake Etc,) water gets enriched with heavy isotope of oxygen (^{18}O). Since transpiration is an evaporative process, the enrichment process should occur in plant system also. Several experiments were conducted to examine this concept.

The theory of enriched ^{18}O molecule gets incorporated (finds its path) into biomass/ cellulose has been worked out in recent years (Sternberg, et al., 1986). In the present investigation, we have used a few model crop systems to understand the relevance of $\Delta^{18}O$ in transpiration process.

Yet another study has also been conducted to quantify the physiological traits associated with transpiration and water use efficiency in a few selected crop species at whole plant level using carbon and oxygen isotope composition in leaf biomass across the crop species.

In addition, we also emphasize the significance of plant mineral content in assessing the genetic variability in transpiration rate and its relationship with $\Delta^{18}O$ in leaf biomass.

The results are presented in the following three sections.

I. *^{18}O enrichment during evaporation and transpiration.*

II. *Stable isotope ($\Delta^{13}C$ and $\Delta^{18}O$) composition in leaf biomass: an approach to assess the genetic variability in transpiration rate (T) and water use efficiency (WUE) in crop species*

III. *Quantification of mesophyll efficiency based on carbon ($\Delta^{13}C$) and oxygen ($\Delta^{18}O$) composition in leaf biomass.*

Section-I

4.1. *^{18}O enrichment during evaporation and transpiration*

4.1.1. Influence of Vapour Pressure Dificit (VPD) on ^{18}O enrichment in water during the surface evaporation.

Surface evaporation from the water bodies is influenced by the vapour pressure concentration across the two medium. The prevailing RH and temperature regulates the vapour concentration of water. With an increase in VPD, there was an increase in evaporation, resulted an increase in $\Delta^{18}O$ in water (Fig. 1). The maximum enrichment of 29.95 per mil was seen in the water sample kept at 18 mbar and minimum of 26.94 per mil was observed in water sample kept at VPD of 6.5 mbar.

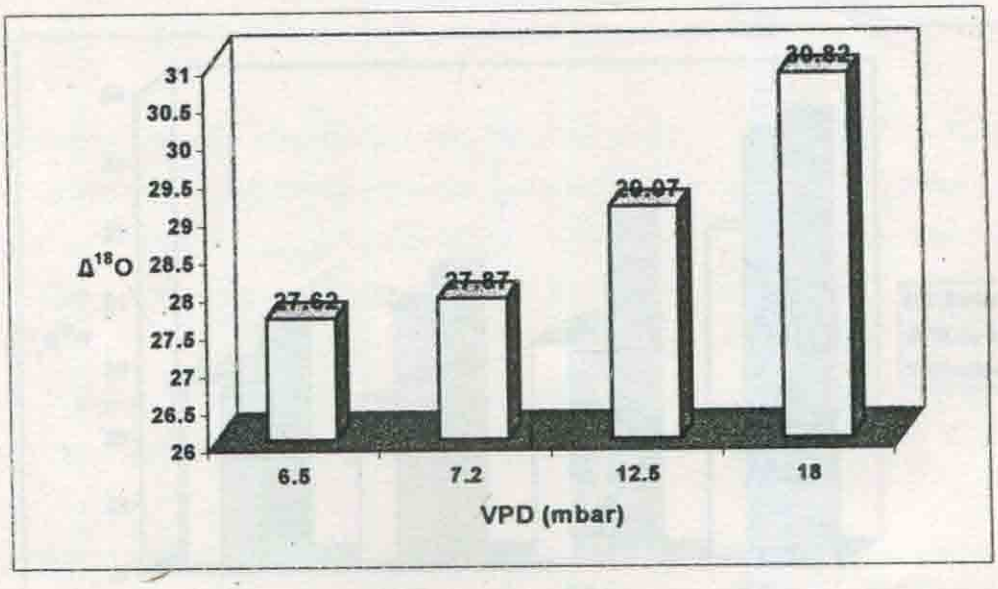
4.1.2. ^{18}O enrichment in water as affected by VPD and membrane pore size

In a biological system, water escapes from the plant through stomata (a tinny opening on leaf surface). To simulate similar effect artificially, synthetic membranes of different porosity were used to study the pattern of evaporation through these membranes and the ^{18}O enrichment.

Marked difference in the pattern of $\Delta^{18}O$ in water was seen across the membranes of different pore size irrespective of the VPD level. At any given level of VPD, evaporation was high as pore size increased from 0.22 μ to 1.22 μ (Fig. 2).

This experiment reveals that ^{18}O enrichment was higher when evaporation was high at higher VPD or with larger pore size of the membrane.

Fig. 1: Effect of VPD on $\Delta^{18}O$ (‰) in water during surface evaporation



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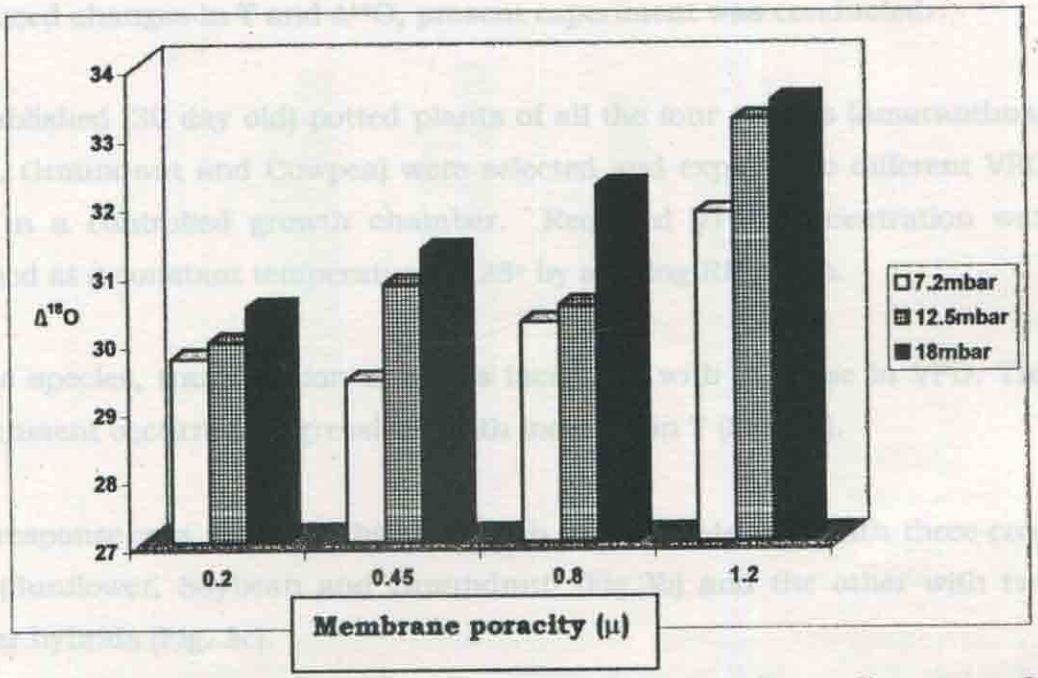
Ten ml of water was taken in a metalic cup (3.5 cm diameter x 2 cm depth) and placed in a humidity chamber maintained at different VPD. The chamber temperature was kept at 28°Cand different VPD was achieved by altering the RH inside. After 2 h of exposure the amount of water evaporated from the cup was measured and the water retained was analyzed for $\Delta^{18}O$.

Fig. 2: $\Delta^{18}O$ (‰) in leaf water as affected by VPD and membrane pore size



Ten ml of water was taken in a metallic cup (3.5 cm diameter x 2 cm depth) covered with a synthetic membrane (isopore polycarabonate) of specific poracity. The cups were placed under different levels of VPD in a controlled humidity chamber. After 2 h of exposure, the amount of water evaporated was calculated by measuring the amount of water retained in the cup. In the retained water $\Delta^{18}O$ was analyzed.

Volume of water retained (ml) in each cup of having different membrane porosity at different VPD level is as follows:

Pore size	7.2mbar	12.5mbar	18mbar
0.2μ-	9.8	9.2	8.3
0.45μ	9.4	8.5	8.0
0.80μ	8.8	8.3	7.2
1.2μ	8.7	8.2	7.4

4.1.3. Influence of VPD on $\Delta^{18}O$ in leaf water in different crop species

Influence of VPD on transpiration has been well established. To examine the VPD induced changes in T and $\Delta^{18}O$, present experiment was conducted.

Well-established (30 day old) potted plants of all the four species (Amaranthus, Soybean, Groundnut and Cowpea) were selected and exposed to different VPD regimes in a controlled growth chamber. Required VPD concentration was maintained at a constant temperature of 28° by altering RH levels.

In all the species, transpiration rate was increased with increase in VPD. The ^{18}O enrichment occurred progressively with increase in T (Fig. 3a).

Similar response was seen in other two such experiments, one with three crop species (Sunflower, Soybean and Groundnut) (Fig.3b) and the other with two sunflower hybrids (Fig. 3c).

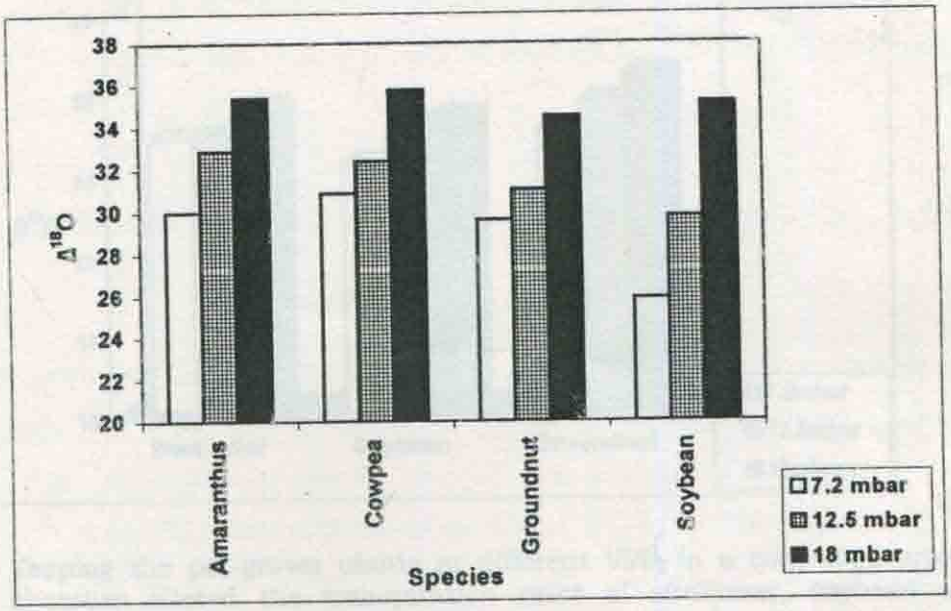
These experiments provide convincing evidences that ^{18}O enrichment in the leaf water occurs during transpiration and the extent of enrichment is related to T.

4.1.4. Stomatal conductance and $\Delta^{18}O$ in leaf water

The objective of this experiment is to access the effect of altered g_s on variations in $\Delta^{18}O$. To address this issue a pot culture experiment was conducted. g_s was altered either by exposing the plants to low light (shading treatment), thus decreasing the g_s or by increasing the root to leaf area ratio (defoliation treatment) thus increasing the g_s.

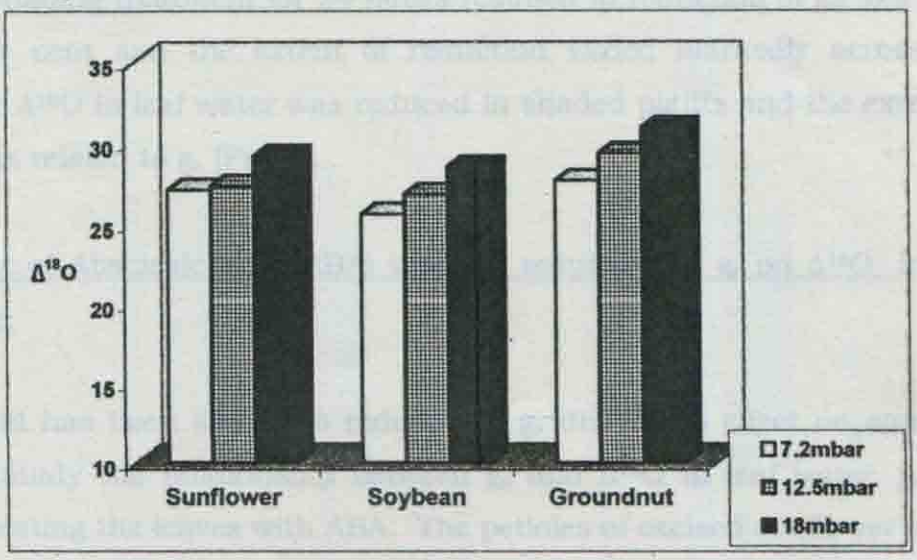
The partial defoliation (50%) treatment increased the g_s substantially (18-25%) due to its effect on altered root to leaf area ratio. The extent of g_s increase differed significantly across the species. The $\Delta^{18}O$ in leaf water also increased in response to partial defoliation in all the species compared to un-defoliated control plants.

Fig. 3a: Influence of VPD on $\Delta^{18}O$ (‰) in leaf water in different crop species



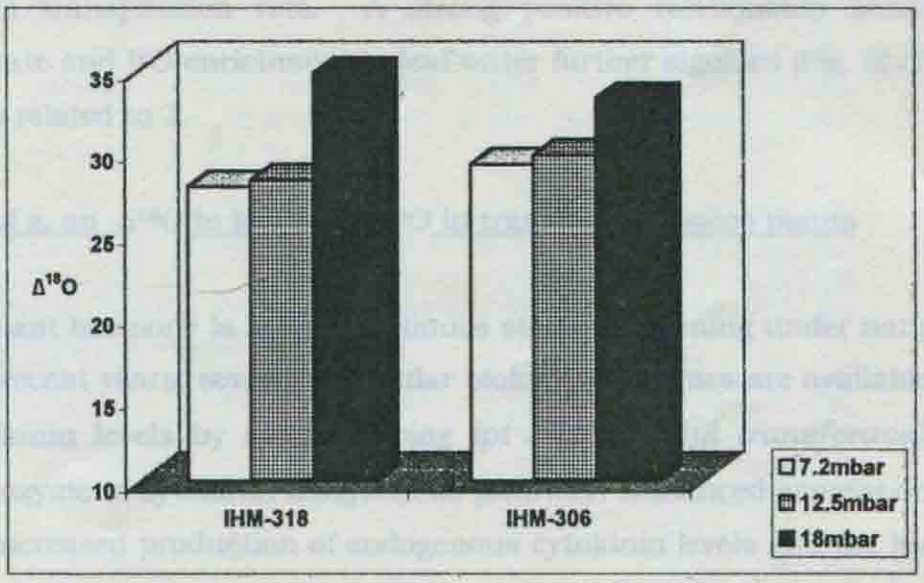
Different crop species were grown in containers. Transpiration rate of these species was altered by keeping them at different VPD levels in a growth chamber. At the end of 3 h of exposure, leaf water was extracted and analyzed for $\Delta^{18}O$.

Fig. 3b: Influence of VPD on $\Delta^{18}O$ (‰) in leaf water in different crop species



Keeping the pot-grown plants at different VPD in a controlled growth chamber altered the transpiration rates of sunflower, soybean and groundnut. Light intensity maintained inside the chamber was around 800μmol.m^{-2}.s^{-1}. At the end of 3 h of exposure, the leaf water was extracted and analyzed for $\Delta^{18}O$.

Fig. 3c: Influence of VPD on $\Delta^{18}O$ (‰) in leaf water in two sunflower genotypes



Well-established potted plants (35days old) of two sunflower genotypes were kept under different levels of vapour pressure deficit in a controller growth chamber. Light intensity maintained inside the chamber was around 800μmol.m^{-2}.s^{-1}. After 3 h of exposure, the leaf water was extracted and analyzed for $\Delta^{18}O$.

Imposing a shading treatment for 24 hours resulted in reduction of gs to a tune of 20-40 per cent and the extent of reduction varied markedly across the species. The $\Delta^{18}O$ in leaf water was reduced in shaded plants and the extent of reduction was related to g_s (Fig. 4).

4.1.5. Effect of Abscissic acid (ABA) induced reduction in g_s on $\Delta^{18}O$ in leaf water:

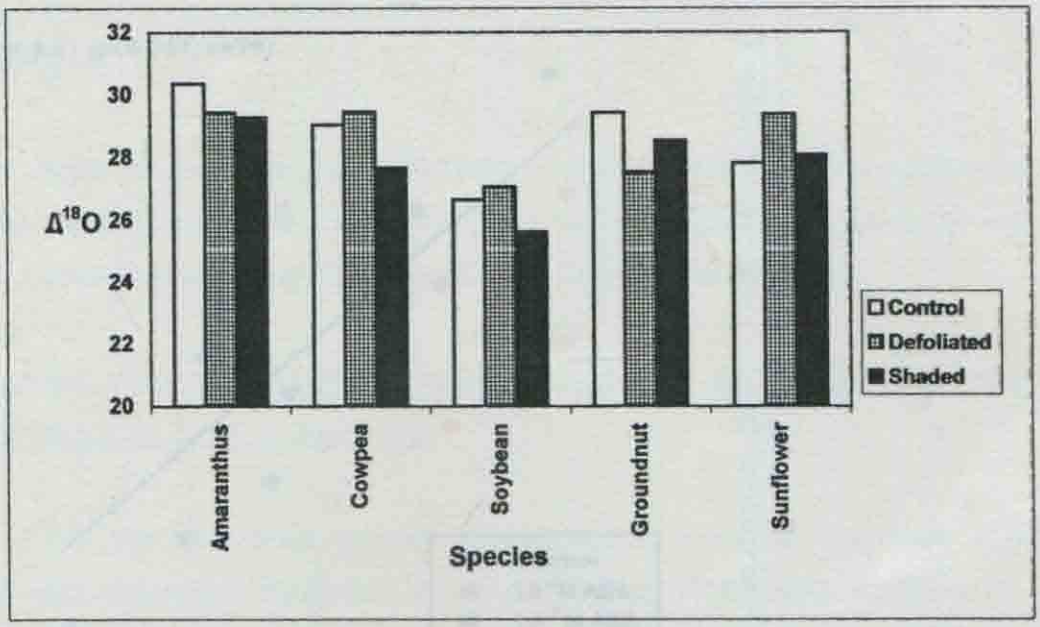
Abscissic acid has been shown to reduce the g_s due to its effect on stomatal closure. To study the relationship between g_s and $\Delta^{18}O$ in leaf water, g_s was altered by treating the leaves with ABA. The petioles of excised sunflower leaves were incubated in different concentrations of ABA (10^{-4} to 10^{-7}M) to reduce the g_s.

This experiment suggests that, as ABA concentration increased, significant decrease in transpiration rate was seen due to reduction in g_s. ^{18}O enrichment was high in control (untreated) and decreased progressively whenever there was a reduction in transpiration rate. A strong positive relationship between transpiration rate and ^{18}O enrichment in leaf water further signifies (Fig. 5) $\Delta^{18}O$ in leaf water is related to T.

4.1.6. Effect of g_s on $\Delta^{18}O$ in leaf water ^{18}O in transgenic tobacco plants

Cytokinin, a plant hormone is known to induce stomatal opening under natural condition. In recent years, several molecular biology techniques are available to alter the cytokinin levels by overproducing *ipt (Isopentanyl transferase),* a rate-limiting enzyme in cytokinin biosynthetic pathway. Enhanced expression of *ipt* results in increased production of endogenous cytokinin levels and the levels vary in different transgenic lines. One such transgenic line of tobacco was used in this experiment to assess the effect of g_s on leaf water ^{18}O enrichment.

Fig. 4: Influence of altered g_s on $\Delta^{18}O$ (‰) in five different crop species



Three sets of well-established (30 day old) potted plants (1plant/pot) were selected for this study. On the day of sampling:

1. I-set (5 pots) was kept as untreated (control) plants under normal light condition of 1300μmol.m^{-2}.s^{-1}
2. II-set: partial defoliation (50%) was effected in order to increase stomatal conductance and kept under normal light (1300μmol.m^{-2}.s^{-1})
3. III-set: kept under partial shade (light intensity was around 400μmol.m^{-2}.s^{-1}) for 24 hours

After 24 hours of imposing the treatment, transpiration rate was measured and leaf water was extracted from all the three sets and analyzed for $\Delta^{18}O$. (Reduction in transpiration rate in shaded plants was ranged from 20-40 per cent, similarly in defoliated plants an increase of 18-25 per cent was observed)

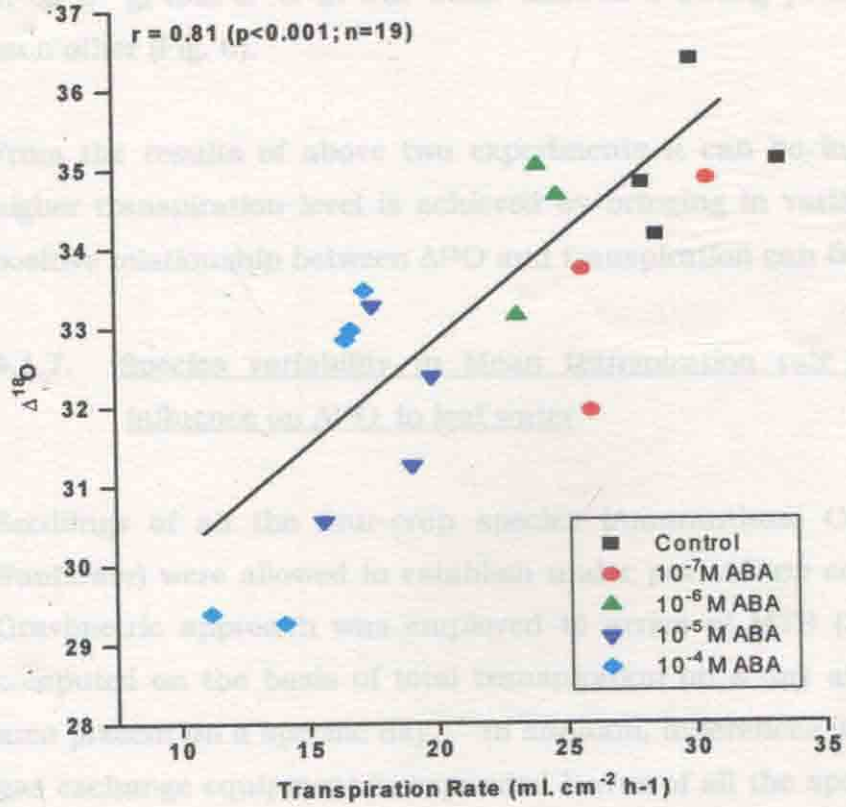


Fig. 5. Changes in $\Delta^{18}O$ (‰) in leaf water and transpiration rate in sunflower with induced differences in stomatal conductance by different concentrations of ABA

Fully expanded sunflower leaves were excised under water and the cut end of the petioles were placed in vials containing ABA solutions of different concentrations (10^{-4} to 10^{-7} M). The leaves were allowed to transpire in a growth chamber. The time taken by the leaves to transpire 15 ml of water was noted and transpiration rate was computed on a leaf area basis. The leaf water was collected and analyzed for $\Delta^{18}O$.

In pot grown wild and transgenic tobacco plants, gs was measured by gas exchange equipment on top fully expanded leaves (light intensity >1300μmoles. $m^{-2}.s^{-1}$). g_s and $\Delta^{18}O$ in leaf water showed a strong positive relationship with each other (Fig. 6).

From the results of above two experiments it can be inferred that whenever higher transpiration level is achieved by bringing in variations in gs, a strong positive relationship between $\Delta^{18}O$ and transpiration can be observed.

4.1.7. Species variability in Mean transpiration rate (MTR), g_s and their influence on $\Delta^{18}O$ in leaf water

Seedlings of all the four-crop species (Amaranthus, Cowpea, Soybean and Sunflower) were allowed to establish under pot culture conditions for 30 days. Gravimetric approach was employed to arrive at MTR (30-50DAS). MTR was computed on the basis of total transpiration on a day and the functional leaf area present on a specific day. In addition, differences in gs was quantified by gas exchange equipment in expanded leaves of all the species on 50th day. On the same day leaf samples were collected for $\Delta^{18}O$.

The result presented in Figure-7a, indicated considerable species variability in both stomatal conductance and MTR. The maximum MTR was recorded in sunflower (88.3 $ml.dm^{-2}.day^{-1}$) followed by soybean (62.55) and lowest in amaranthus (48.55).

The variation in gs followed a similar path as that of MTR (sunflower showed relatively high g_s of 560$mmol.m^{-2}.s^{-1}$ and low g_s of 145$mmol.m^{-2}.s^{-1}$ was recorded in amaranthus). The variation in $\Delta^{18}O$ was substantial when either g_s or MTR was altered. A strong positive relationship was observed between g_s and $\Delta^{18}O$ as well as MTR and $\Delta^{18}O$.

Species variability in MTR, g_s and $\Delta^{18}O$ exists. The extent of ^{18}O enrichment is related to changes in both g_s and MTR. Though a positive trend was seen

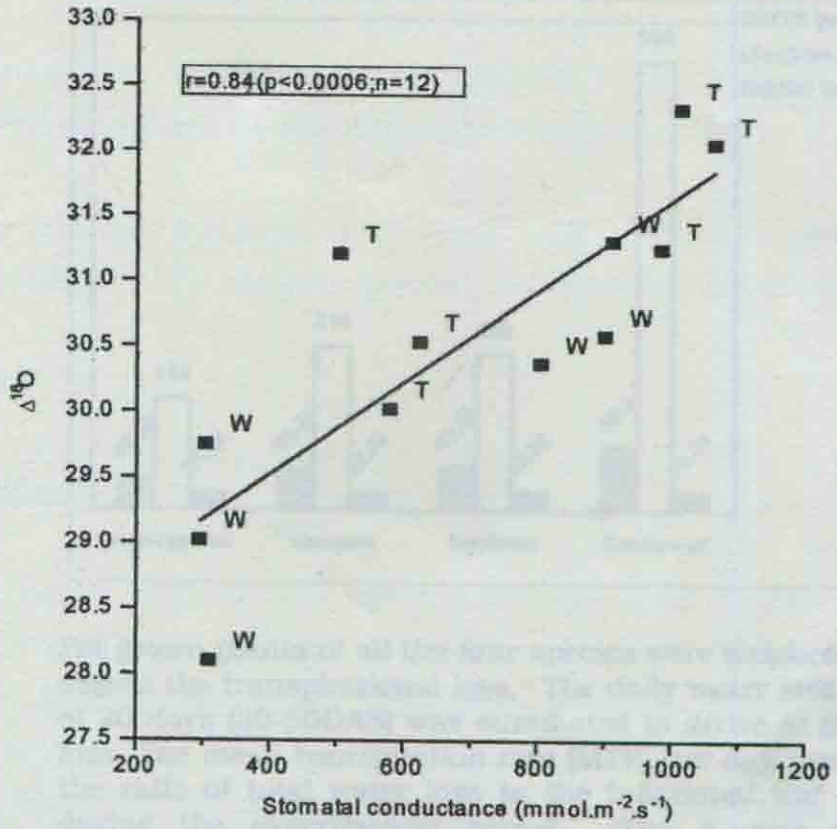
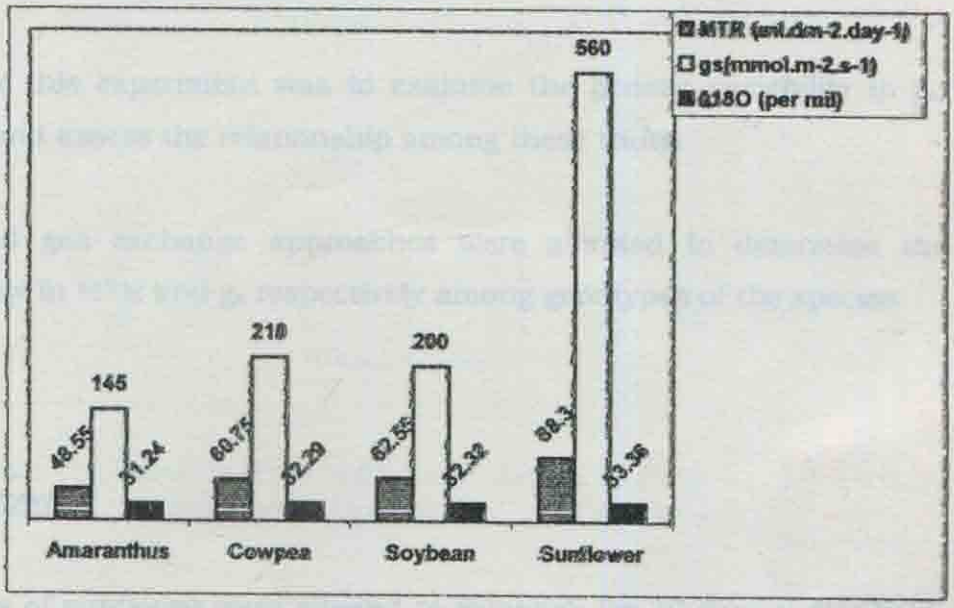


Fig. 6: Relationship between g_s and $\Delta^{18}O$(‰) in leaf water in wild (W) and transgenic (T) plants of tobacco

In pot grown wild and transgenic (cytokinins overproducing) tobacco plants, the g_s was measured in different leaves exposed to light of 1300μmol.m^{-2}.s^{-1} using gas exchange equipment. After determining the g_s, the leaf water was collected and analyzed for $\Delta^{18}O$.

Fig. 7a: Variation in mean transpiration rate, g_s and their influence on $\Delta^{18}O$ (‰) in leaf water in different crop species



Pot grown plants of all the four species were weighed once daily to assess the transpirational loss. The daily water loss over a period of 20 days (30-50DAS) was summated to arrive at the total water loss. The mean transpiration rate (MTR) per day was arrived from the ratio of total water loss to the functional leaf area retained during the experimental period. The g_s was measured by employing gas exchange equipment. At the end of the experiment, leaf water was extracted and analyzed for $\Delta^{18}O$.

between g_s and $\Delta^{18}O$ (Fig. 7b), the relationship was significant between MTR and $\Delta^{18}O$ (Fig. 7c).

4.1.8: Genetic variability in stomatal conductance (g_s), Mean transpiration rate (MTR) and $\Delta^{18}O$ in leaf water in few crop species

The objective of this experiment was to examine the genetic variability in g_s, MTR and $\Delta^{18}O$ and assess the relationship among these traits.

Gravimetric and gas exchange approaches were adapted to determine the genetic variability in MTR and g_s respectively among genotypes of the species.

A) Sunflower

4.1.8.1. Field grown

Selected hybrids of sunflower were allowed to establish for 30 days under field-minilysimeter conditions. Cumulative Water Transpired (CWT) was measured by weighing the minilysimeter grown plants once in two days for a period of 20 days using the electronic weighing scale fitted on a mobile gantry system, which moves on metal rails. The MTR was computed based on the primary values of CWT for the last two days of experimental period based on the functional leaf area present on these two days. Leaf water extracted from the leaves developed during this period for $\Delta^{18}O$.

Considerable genetic variability in MTR and gs was noticed among sunflower genotypes (Table-1). IHM 306 recorded maximum MTR of 36.6ml.dm^{-2}.day^{-1} compared to IHM-318 (23.65ml.dm^{-2}.day^{-1}).

The g_s and $\Delta^{18}O$ also showed a significant variation. Positive relationships were observed between MTR and $\Delta^{18}O$ ($r=0.90$); g_s and $\Delta^{18}O$ ($r=0.86$), though the correlation was not significant at $p=0.05$ but significant at $p=0.08$ level.

Fig. 7b: Relationship between MTR and $\Delta^{18}O$ (‰) in leaf water in different crop species

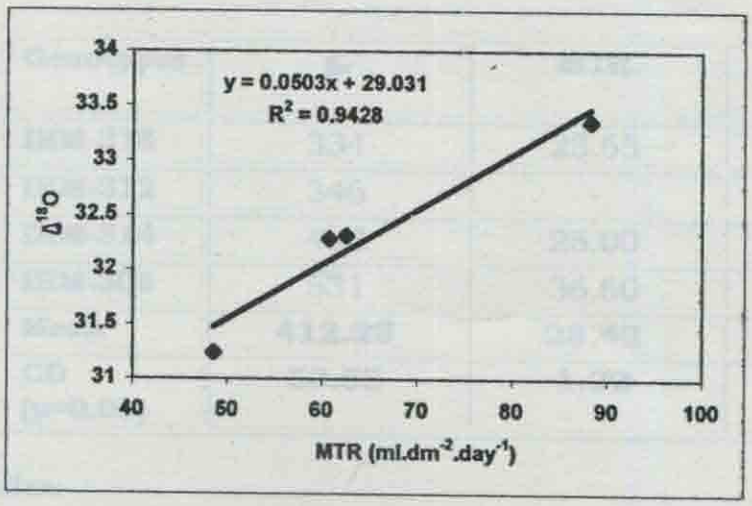
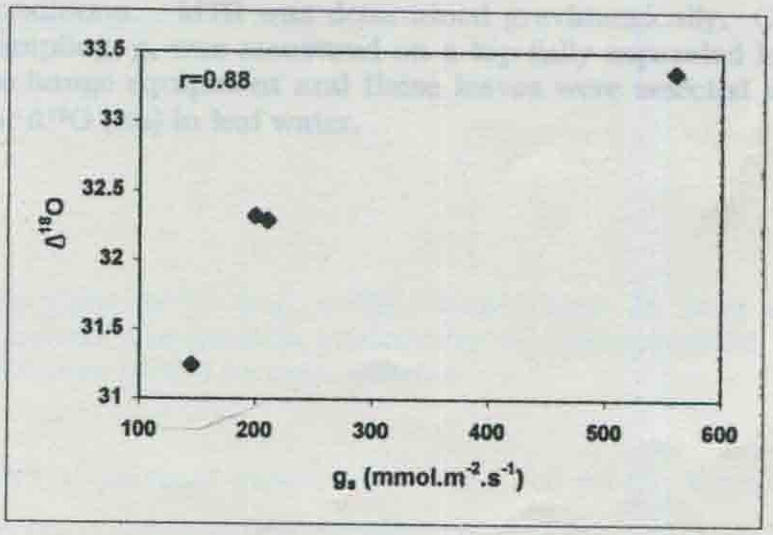


Fig. 7c: Relationship between g_s and $\Delta^{18}O$ (‰) in leaf water in different crop species



By adopting both gravimetric and gas exchange approaches, MTR and g_s were determined in four different crop species. After the measurement the leaf water was extracted and analyzed for $\Delta^{18}O$

Table-1: Genetic variability in stomatal conductance (g_s), MTR and $\Delta^{18}O$ (‰) in leaf water in field grown sunflower

Genotypes	g_s	MTR	$\Delta^{18}O$ (‰) in leaf water
IHM-318	334	23.65	32.16
IHM-312	346	-	33.11
IHM-314	438	25.00	32.71
IHM-306	531	36.60	34.96
Mean	**412.25**	**28.42**	**33.24**
CD (p=0.05)	**53.55**	**1.22**	**0.92**

Index:
g_s – stomatal conductance ($mmol.m^{-2}.s^{-1}$)
MTR – Mean Transpiration Rate ($ml.dm^{-2}.day^{-1}$)

Four genotypes of sunflower were grown in field minilysimeter conditions. MTR was determined gravimetrically. On the day of sampling, g_s was measured on a top fully expanded leaf using gas exchange equipment and these leaves were selected and analyzed for $\Delta^{18}O$ (‰) in leaf water.

4.1.8.2. Pot grown

Genetic variability in MTR, g_s was examined in two genotypes of Sunflower grown in pots. Among the genotypes, there was a marked difference in MTR, g_s and $\Delta^{18}O$. Though the absolute values of MTR varied, the ranking of hybrids remained same across the sampling dates. Hence the MTR for the entire experimental period was compared with the $\Delta^{18}O$ of leaf water. The hybrid, IHM-318, which had high MTR also showed high $\Delta^{18}O$ compared to other hybrid, IHM-306 (Fig. 8).

B) Cowpea

4.1.8.3. Pot grown

A pot experiment was conducted to determine MTR, g_s and leaf water ^{18}O and their inter-relationships among few genotypes. The result indicated a strong positive relationship of $\Delta^{18}O$ in leaf water with both g_s (Fig. 9) and MTR (Fig. 10) respectively.

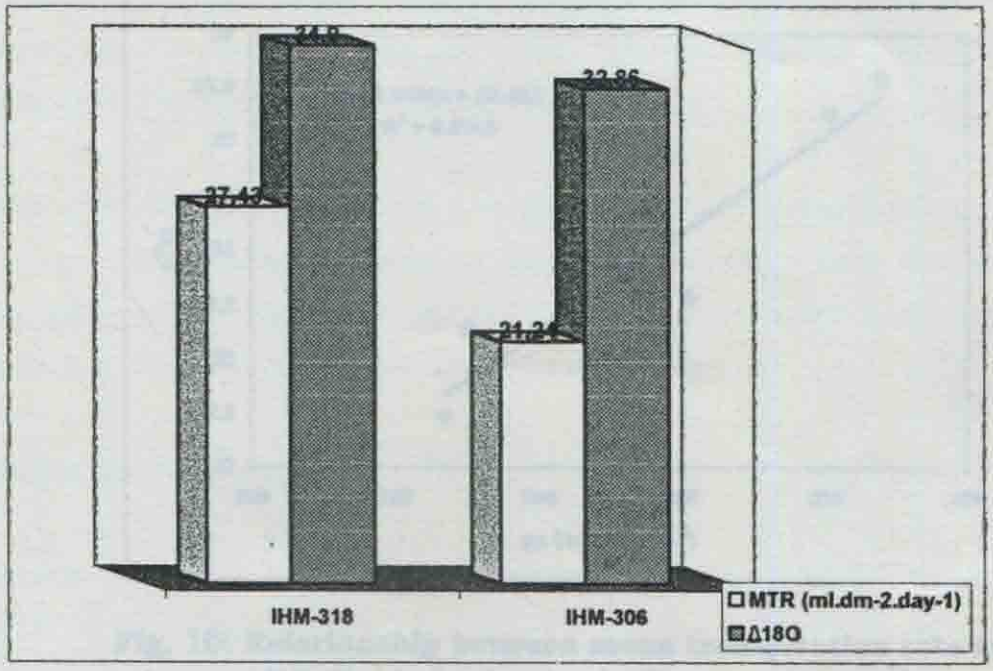
Section-II

4.2. *Stable isotope ($\Delta^{13}C$ and $\Delta^{18}O$) composition in leaf biomass: an approach to assess the genetic variability in transpiration rate (T) and water use efficiency (WUE) in crop species*

From the results of previous experiments explained so far, it was evident that strong positive relationship exists between transpiration rate and $\Delta^{18}O$ in leaf water. In other words, leaf water gets enriched with $\Delta^{18}O$ during transpiration and the extent of enrichment varies as transpiration increases.

Few other evidences are also available which explains that, during photosynthesis there will be a continuous hydration and dehydration reaction of CO_2 mediated by carbonic anhydrase (CA). Because of this, an exchange of oxygen atom between water and CO_2 constantly occurs. Depending on ^{18}O

Fig. 8: Variation in mean transpiration rate (MTR), g_s and $\Delta^{18}O$ (‰) in leaf water in two genotypes of sunflower



Transpiration rate ($ml.dm^{-2}.day^{-1}$) was determined on a specific day during the experimental period. The leaves were collected and analyzed for $\Delta^{18}O$ in biomass in two genotypes of sunflower grown in pots. The g_s of IHM-318 was $545mmol.m^{-2}.s^{-1}$ and that of IHM-306 was $480mmol.m^{-2}.s^{-1}$.

Fig. 9: Relationship between g_s and $\Delta^{18}O$ (‰) in leaf water in five genotypes of cowpea

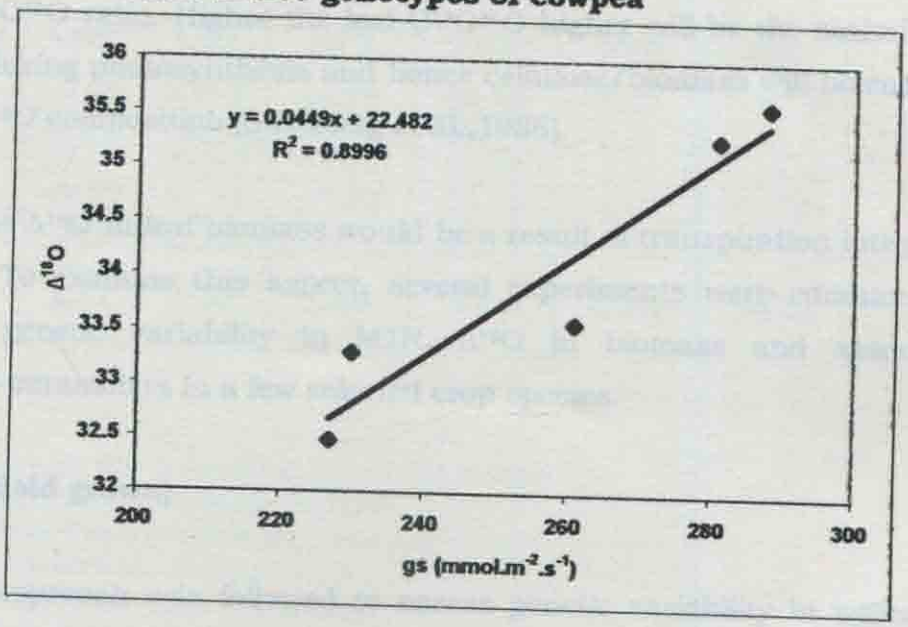
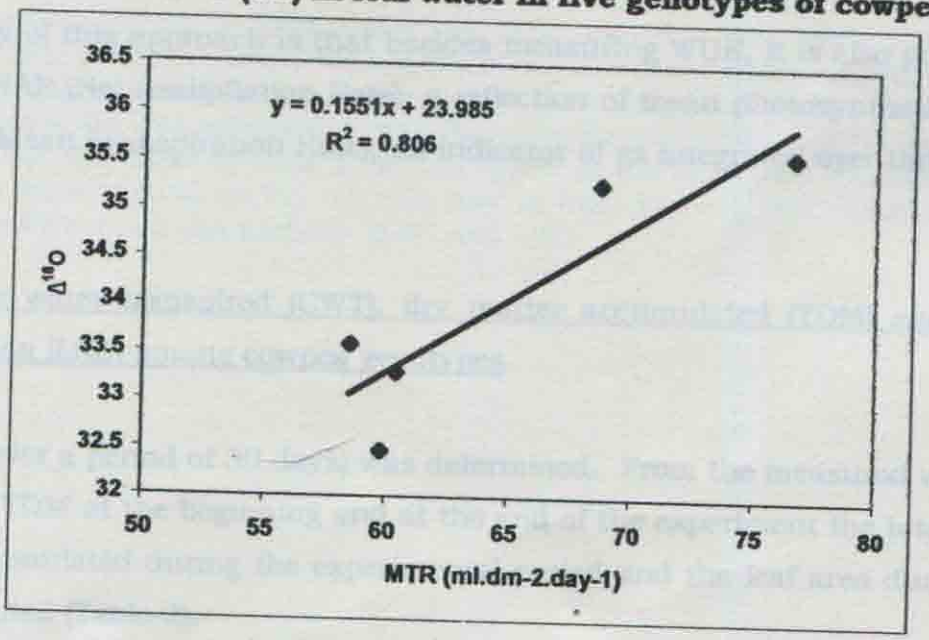


Fig. 10: Relationship between mean transpiration rate and $\Delta^{18}O$ (‰) in leaf water in five genotypes of cowpea



The g_s was determined using a gas exchange equipment on top fully expanded leaves (light intensity >1500µmol.m^{-2}.s^{-1}). MTR was determined gravimetrically based on amount of water transpired on a specific day (50th DAS). Leaf water was extracted from fully expanded leaves and analyzed for $\Delta^{18}O$

composition of leaf water, the ^{18}O composition of the CO_2 changes. Therefore, at high transpiration rate higher would be $H_2{}^{18}O$ in leaf water and hence $C^{18}O^{16}O/C^{16}O^{16}O$ ratio. Higher the leaf $C^{18}O^{16}O$ higher will be the assimilation of $C^{18}O^{16}O$ during photosynthesis and hence cellulose/biomass will be enriched with higher ^{18}O composition (Sternberg et al.,1986).

Therefore, the $\Delta^{18}O$ in leaf biomass would be a result of transpiration integrated over time. To examine this aspect, several experiments were conducted to assess the genetic variability in MTR, $\Delta^{18}O$ in biomass and associated physiological parameters in a few selected crop species.

I. Cowpea (field grown)

Gravimetric approach was followed to assess genetic variability in water use and WUE in cowpea genotypes.

The novelty of this approach is that besides measuring WUE, it is also possible to assess NAR (Net Assimilation Rate), a reflection of mean photosynthetic rate and MTR (Mean Transpiration Rate), an indicator of gs integrated over time at a given VPD.

Cumulative water transpired (CWT), dry matter accumulated (TDM) and leaf area duration (LAD) among cowpea genotypes.

The CWT (over a period of 30 days) was determined. From the measured values of LA_1 and TDM at the beginning and at the end of the experiment the total dry matter accumulated during the experimental period and the leaf area duration were computed (Table-2).

Statistically, significant differences in CWT, LAD and TDM over the experimental period were found among cowpea genotypes. Among these, APC-982 recorded the mean CWT of 27.23 kg, closely followed by APC-370 (27.12 kg), while APC-229 had the least CWT (19.11 kg), representing a variability of

Table-2: Genetic variability in physiological traits associated with WUE in field grown cowpea

Genotypes	CWT	LAD	MTR	TDM	WUE	$\Delta^{13}C$	$\Delta^{18}O$
APC-982	27.23	33.77	80.78	54.10	2.08	19.02	28.71
APC-478	20.62	34.05	66.36	68.14	3.29	18.51	27.04
APC-370	27.12	29.90	64.00	57.65	2.14	18.45	27.35
APC-580	19.72	32.48	68.82	69.20	3.49	17.88	29.18
APC-229	19.11	37.43	55.71	55.08	2.96	18.86	27.22
CD (p=0.05)	**2.11**	**1.33**	**5.90**	**4.36**	**0.82**	**0.92**	**1.01**

Index:
CWT – Cumulative Water Transpired (kg.plant^{-1})
LAD – Leaf Area Duration (cm^2 days)
MTR – Mean Transpiration Rate (ml.dm^{-2}.day^{-1})
TDM – Total Dry Matter (g.plant^{-1})
WUE – Water Use Efficiency (g.kg^{-1})
$\Delta^{13}C$ – Carbon Isotope Discrimination (per mil)
$\Delta^{18}O$ – Oxygen Isotope composition in leaf biomass (per mil)

WUE was quantified by gravimetric approach in a few selected genotypes under field lysimeter condition between 35 to 65 days after sowing (DAS). CWT was determined by weighing minilysimeters daily and the functional leaf area (LAD) was assessed during this experimental period. MTR was arrived on the basis of total water transpired to the functional leaf area during experimental period. On the day of final harvest, leaf samples were collected and analysed for $\Delta^{13}C$ and $\Delta^{18}O$.

42 %. Similarly APC580 yielded the highest TDM accumulation of 69.2 g.plant^{-1} and the lowest TDM was seen in APC-982 (54.1 g) representing around 28 per cent variability.

Carbon isotope discrimination (Δ^{13}C) and WUE

Though accurate, the quantification of WUE by gravimetry is cumbersome and cannot be adopted for large scale screening for the variability in WUE. With an advent of Δ^{13}C technique (ability of plants to actively discriminate against the heavy isotope of carbon), a phenomenal progress has been achieved in screening for genetic variability in WUE. The Δ^{13}C is widely being used as a surrogate estimate of WUE integrated over a period of time.

In the present experiment, an attempt was made to determine the genetic variability in Δ^{13}C among cowpea genotypes. The result presented in Table-2 indicates a significant genotypic variation in Δ^{13}C. While APC–580 had lowest discrimination (17.88 per mil), APC-229 and APC- 982 showed higher extents of discrimination against ^{13}C (18.86 and 19.02 per mil respectively). A strong inverse relationship between these two traits was observed (Fig. 11).

Mean transpiration rate and Δ^{18}O in leaf biomass

Significant genotypic variability in Δ^{18}O was observed. APC-580 showed a maximum Δ^{18}O of 29.18 per mil, followed by APC 982 (28.71 per mil) and lowest Δ^{18}O was recorded in APC-370 (27.36 per mil). MTR was positively correlated with Δ^{18}O (Fig. 12).

Grouping of genotypes using standardized normal Z-distribution plot

Genetic variability in WUE can be arrived based on carbon isotope discrimination technique. It is inadequate if genotypes possess only high WUE character since, crop growth rate (CGR) depends also on total water use which to large extent determined by transpiration rate at a given leaf area. One of the

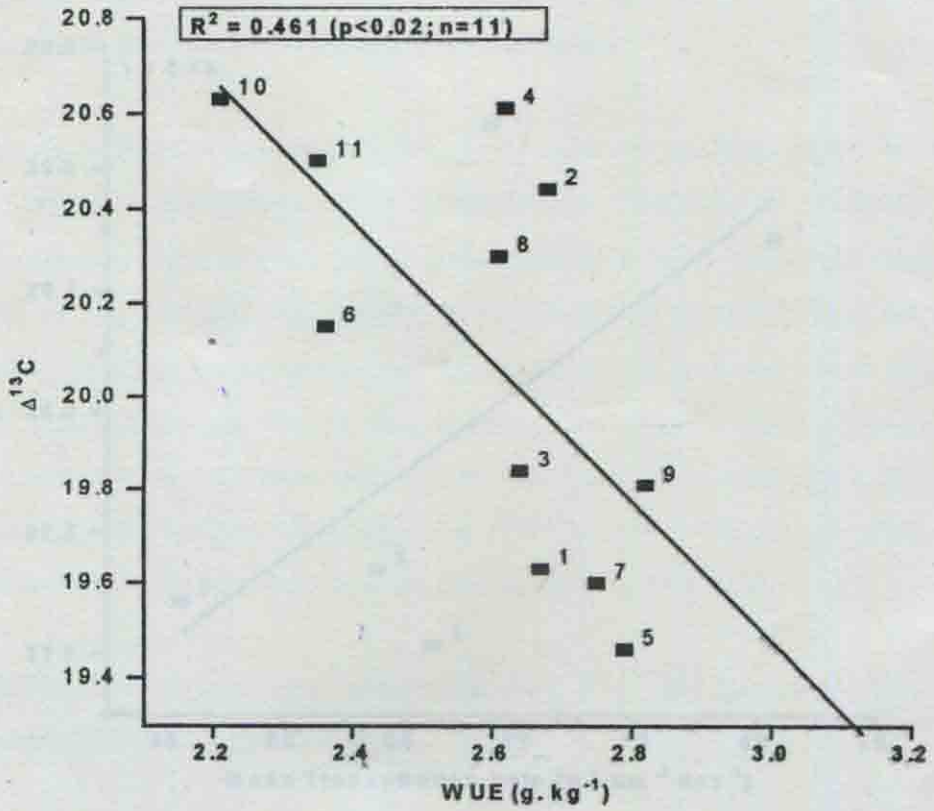


Fig. 11. Relationship between $\Delta^{13}C$ (‰) and WUE among 11 genotypes of Cowpea.

The genetic variability in WUE was gravimetrically determined in the eleven container grown cowpea genotypes (See flow chart A in material and methods for details on protocol). Leaves that developed during the experimental period were dried and the powder was used for the de termination of $\Delta^{13}C$ using an IRMS.

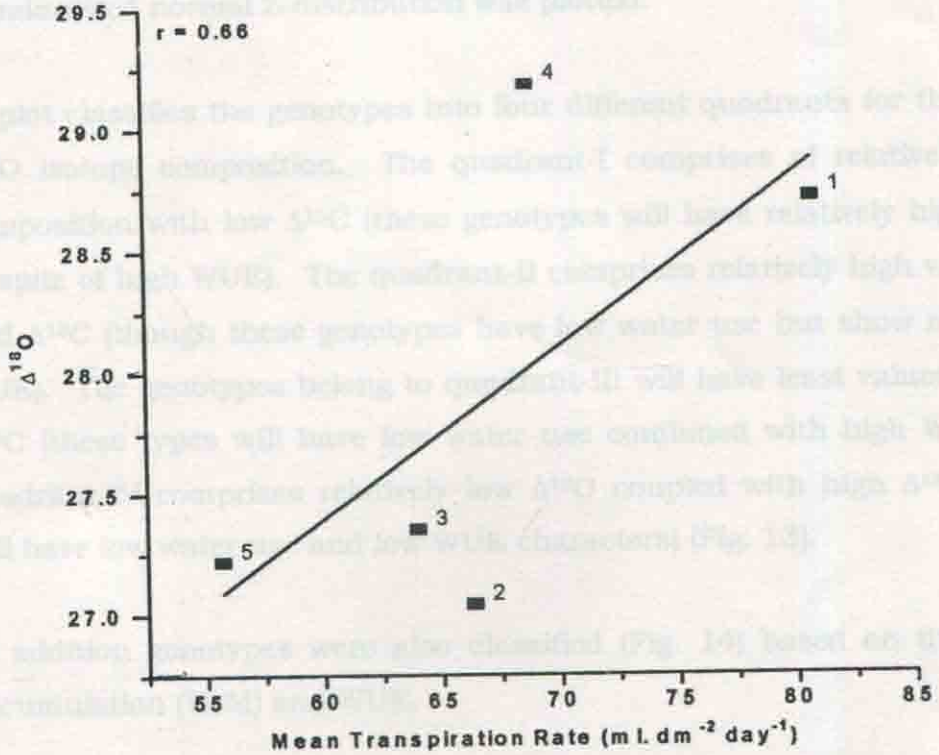


Fig. 12. Association between Mean transpiration rate and $\Delta^{18}O$ (‰) in leaf biomass in a few selected genotypes of Cowpea.

From the container experiment with 11 cowpea genotypes (see Fig 11), a few genotypes with contrasting differences in transpiration rate were identified. The dry leaf powder of these genotypes was analyzed for the $\Delta^{18}O$ by high temperature pyrolysis using an IRMS.

approaches to identify relatively high water use coupled with high WUE is by identifying types with low $\Delta^{13}C$ and high $\Delta^{18}O$. Based on these parameters standardized normal Z-distribution was plotted.

Z- plot classifies the genotypes into four different quadrants for their $\Delta^{13}C$ and $\Delta^{18}O$ isotope composition. The quadrant-I comprises of relatively high $\Delta^{18}O$ composition with low $\Delta^{13}C$ (these genotypes will have relatively high water use in spite of high WUE). The quadrant-II comprises relatively high values of $\Delta^{18}O$ and $\Delta^{13}C$ (though these genotypes have low water use but show relatively high WUE). The genotypes belong to quadrant-III will have least values of $\Delta^{18}O$ and $\Delta^{13}C$ (these types will have low water use combined with high WUE) and the quadrant-IV comprises relatively low $\Delta^{18}O$ coupled with high $\Delta^{13}C$ (genotypes will have low water use and low WUE characters) (Fig. 13).

In addition genotypes were also classified (Fig. 14) based on the dry matter accumulation (TDM) and WUE.

Quadrant-I: relatively high dry matter accumulation coupled with low WUE.
Quadrant-II: genotypes having high TDM with high WUE belongs to this quadrant.
Quadrant-III: genotypes in this quadrant will have low values of both TDM and WUE.
Quadrant-IV: comprises of genotypes having relatively low dry matter accumulation but high WUE.

Based on Z-plot for these two sets of characters ($\Delta^{18}O$ and $\Delta^{13}C$; TDM and WUE) contrasting cowpea genotypes were identified. For instance, APC-580 showed relatively high $\Delta^{18}O$ and low $\Delta^{13}C$ and this genotype also showed high dry matter accumulation coupled with high WUE (high water use character was also seen). Where as APC-229 showed relatively low $\Delta^{18}O$ in leaf biomass with high $\Delta^{13}C$. Therefore, it had low TDM coupled with low WUE.

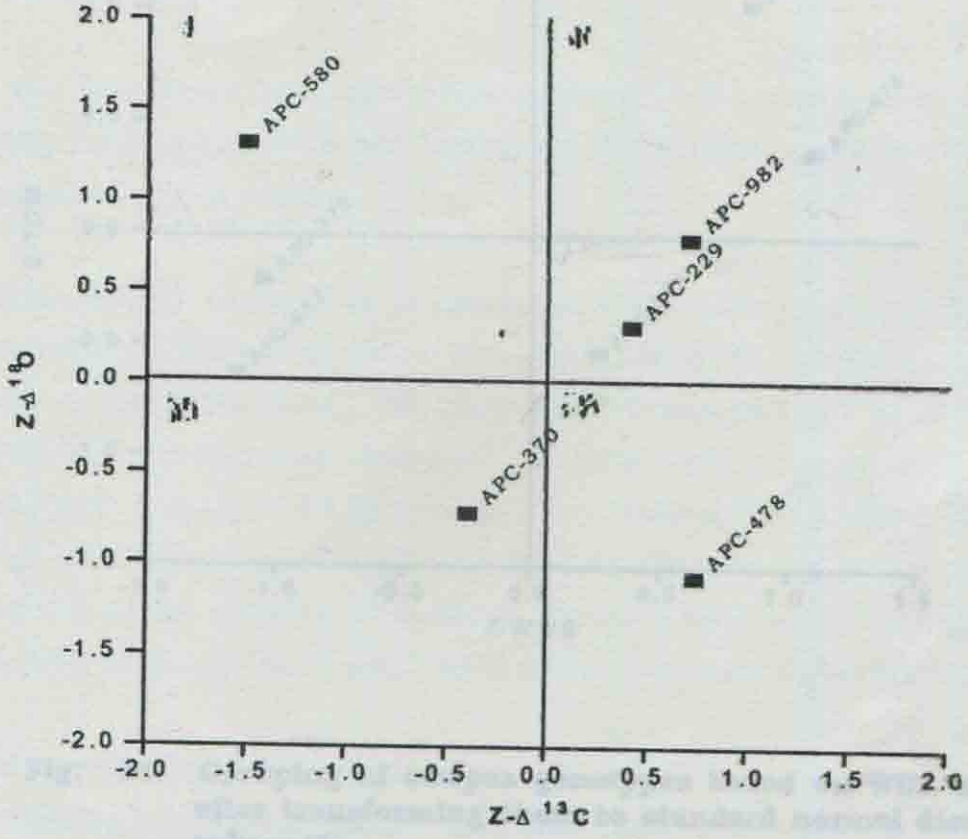


Fig. 13. Grouping of cowpea genotypes based on $\Delta^{13}C$ and $\Delta^{18}O$ in leaf biomass after transforming the values to obtain standard normal distribution (Z).

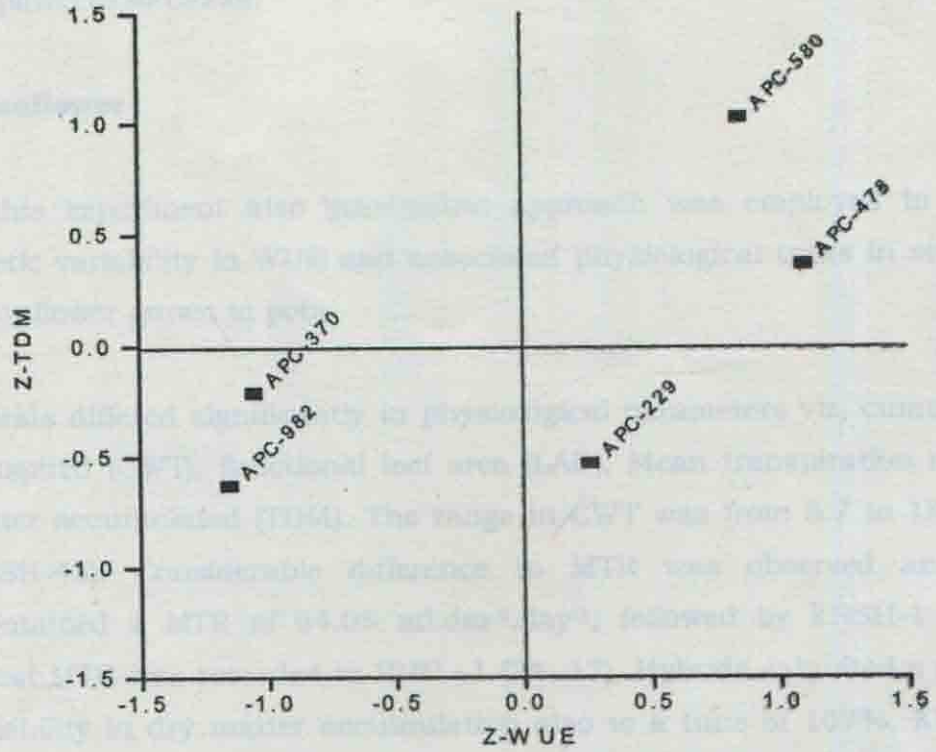


Fig. 14. Grouping of cowpea genotypes based on WUE and TDM after transforming them to standard normal distribution values (Z)

Results presented in histogram (Fig. 15) indicate that, APC-580 with $\Delta^{18}O$ coupled with low $\Delta^{13}C$ had other intrinsic desirable physiological traits compared to APC-229.

II Sunflower

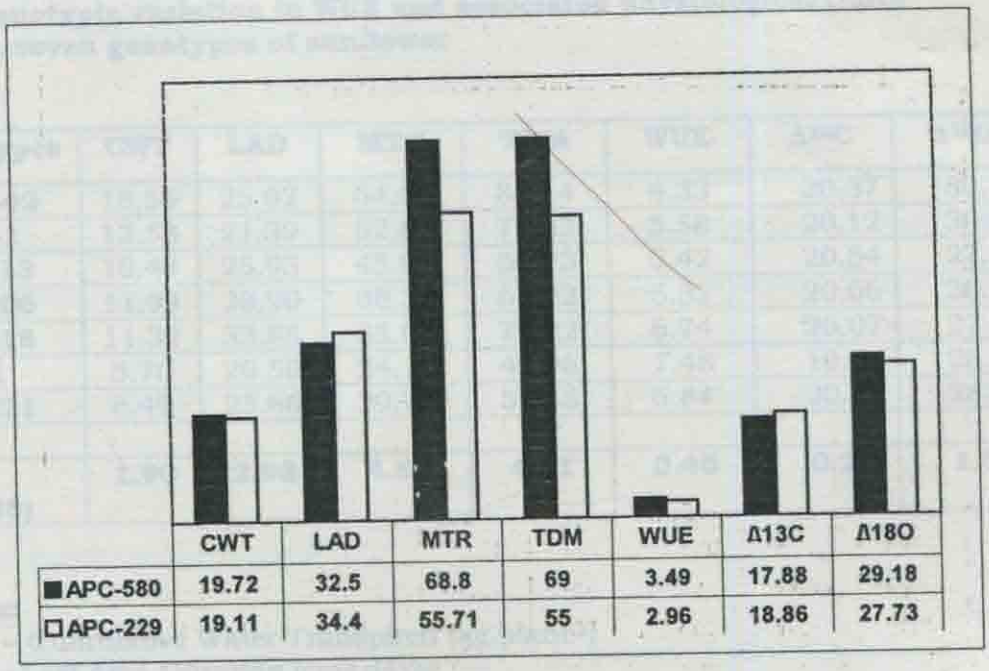
In this experiment also gravimetric approach was employed to assess the genetic variability in WUE and associated physiological traits in seven hybrids of sunflower grown in pots.

Hybrids differed significantly in physiological parameters viz, cumulative water transpired (CWT), functional leaf area (LAD), Mean transpiration rate and dry matter accumulated (TDM). The range in CWT was from 5.7 to 18.55 $Kg.pot^{-1}$ (KBSH-42). Considerable difference in MTR was observed and KBSH-42 maintained a MTR of 64.05 $ml.dm^{-2}.day^{-1}$, followed by KBSH-1 (52.85) and lowest MTR was recorded in RHC –1 (24 .17). Hybrids exhibited a considerable variability in dry matter accumulation also to a tune of 107%, KBSH 42 had high TDM of 85.14 g and least TDM of 42.96 g was recorded in RHC-1.

WUE in sunflower hybrids varied from 4.33 in KBSH-42 to 7.48 g/kg in RHC-1 representing a significant genetic variability in this trait (Table-3).

$\Delta^{13}C$ and WUE

Sunflower genotypes showed a considerable genetic variability in $\Delta^{13}C$ (19.75 to 20.66). Among hydrids, RHC-1 discriminated less (19.75per mil) followed by IHM-318 (20.09per mil) and highest $\Delta^{13}C$ was recorded in IHM-306 (20.66per mil). The variability in $\Delta^{13}C$ to an extent of 4-5 % was noticed across the hybrids. $\Delta^{13}C$ showed a strong inverse relationship with WUE (Fig. 16).



	CWT	LAD	MTR	TDM	WUE	Δ13C	Δ18O
■APC-580	19.72	32.5	68.8	69	3.49	17.88	29.18
□APC-229	19.11	34.4	55.71	55	2.96	18.86	27.73

Fig. 15: Differences in physiological traits associated with WUE in two contrasting field grown cowpea genotypes

Two contrasting genotypes selected based on their relative dual isotope composition ($\Delta^{13}C$ and $\Delta^{18}O$) were compared for their associated physiological traits.

Table-3 : Genotypic variation in WUE and associated physiological traits in seven genotypes of sunflower

Genotypes	CWT	LAD	MTR	TDM	WUE	$\Delta^{13}C$	$\Delta^{18}O$
KBSH-42	18.55	25.62	64.05	85.14	4.33	20.37	30.15
KBSH-1	13.58	21.39	52.85	75.63	5.58	20.12	30.85
IHM-313	10.48	25.93	45.05	56.73	5.42	20.54	27.63
IHM-306	11.99	28.90	38.30	63.92	5.32	20.66	30.17
IHM-318	11.32	33.88	35.04	76.22	6.74	20.07	27.86
RHC-1	5.70	20.50	24.17	42.96	7.48	19.75	28.24
IHM-321	8.49	25.66	29.20	57.65	6.84	20.13	28.48
CD (p=0.05)	**1.90**	**2.93**	**4.52**	**4.01**	**0.45**	**0.26**	**1.12**

Index:

CWT – Cumulative Water Transpired ($kg.plant^{-1}$)
LAD – Leaf Area Duration (cm^2 days)
MTR – Mean Transpiration Rate ($ml.dm^{-2}.day^{-1}$)
TDM – Total Dry Matter ($g.plant^{-1}$)
WUE – Water Use Efficiency ($g.kg^{-1}$)
$\Delta^{13}C$ – Carbon Isotope Discrimination (per mil)
$\Delta^{18}O$ – Oxygen Isotope composition in leaf biomass (per mil)

Gravimetric approach was employed to assess the genotypic variability in WUE and associated physiological traits in pot grown sunflower hybrids between 30 to 55 days after sowing (DAS). CWT was determined by weighing the pots daily. MTR was computed on the basis of total water transpired to the functional leaf area during experimental period. Leaves developed during the experimental period were collected at final harvest and carbon and oxygen isotope composition was determined.

MTR and $\Delta^{18}O$ in leaf biomass

As a more integrated approach to assess genetic variability in transpiration rate, $\Delta^{18}O$ in all seven genotypes was determined. Significant difference across sunflower genotypes in $\Delta^{18}O$ was noticed. Among them, IHM-306 showed $\Delta^{18}O$ of 30.17 per mil, least by IHM-313 (27.63 per mil). The variability in $\Delta^{18}O$ was around 10-12 %. The $\Delta^{18}O$ had a strong positive linearity with MTR (Fig. 17), suggesting the possibility of using this trait as a surrogate for g_s/T integrated over time.

Grouping of sunflower genotypes

Sunflower genotypes were grouped by adopting normal Z distribution plot to identify contrasting genotypes for the characters of high $\Delta^{18}O$ and low $\Delta^{13}C$ composition in leaf biomass and also for relatively high TDM and high WUE traits.

The emphasis here is that, genotype having high $\Delta^{18}O$ and low $\Delta^{13}C$ values would also have high water use coupled with high WUE (Quadrant-I) when compared to genotypes belonging to quadrant-IV (low $\Delta^{18}O$ and high $\Delta^{13}C$ value, low water use combined with low WUE).

Z-analysis plot (Fig. 18 and 19) suggests the existence of two such hybrids having contrasting features of both stable isotope and WUE traits. KBSH-1 (quadrant-I) had relatively high $\Delta^{18}O$ and low $\Delta^{13}C$ coupled with high TDM and WUE. In contrast, IHM-313 had low biomass $\delta^{18}O$ and high $\Delta^{13}C$, also showed relatively low TDM and WUE characters. Results presented in histogram (Fig. 20) further reiterate that other physiological traits associated with these two hybrids also showed considerable variability.

Fig. 16: Relationship between WUE and $\Delta^{13}C$ (‰) in a few genotypes of sunflower

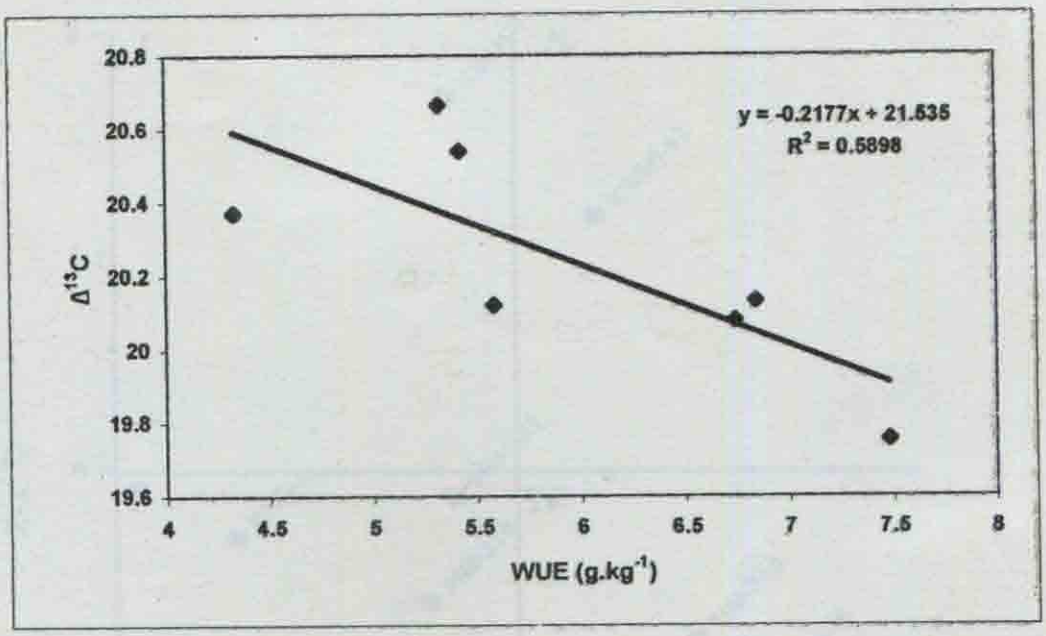
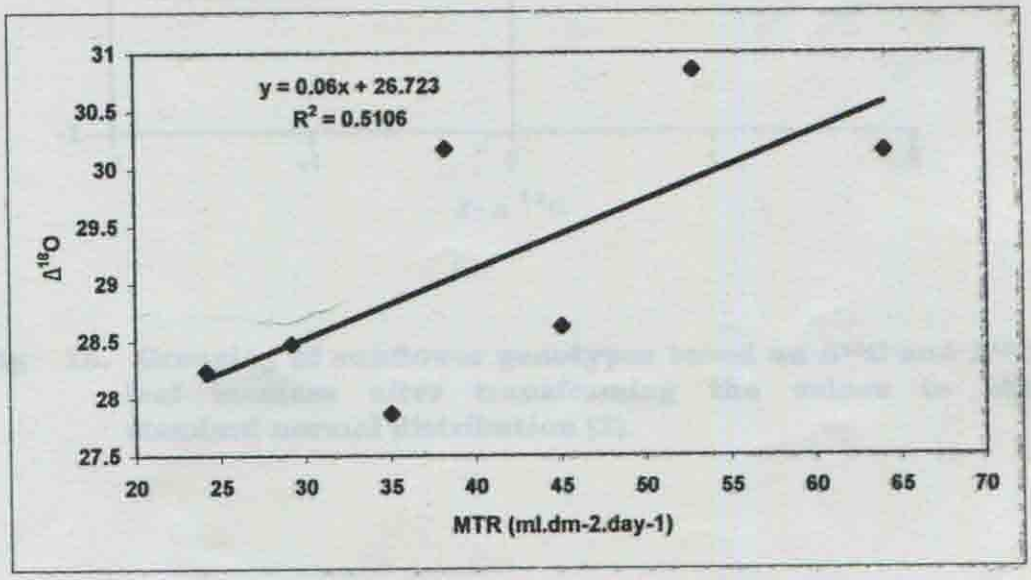


Fig. 17: Relationship between MTR and $\Delta^{18}O$ (‰) in a few genotypes of sunflower



WUE was determined gravimetrically and the leaves developed during the experimental period were analyzed for both $\Delta^{13}C$ and $\Delta^{18}O$ in leaf biomass.

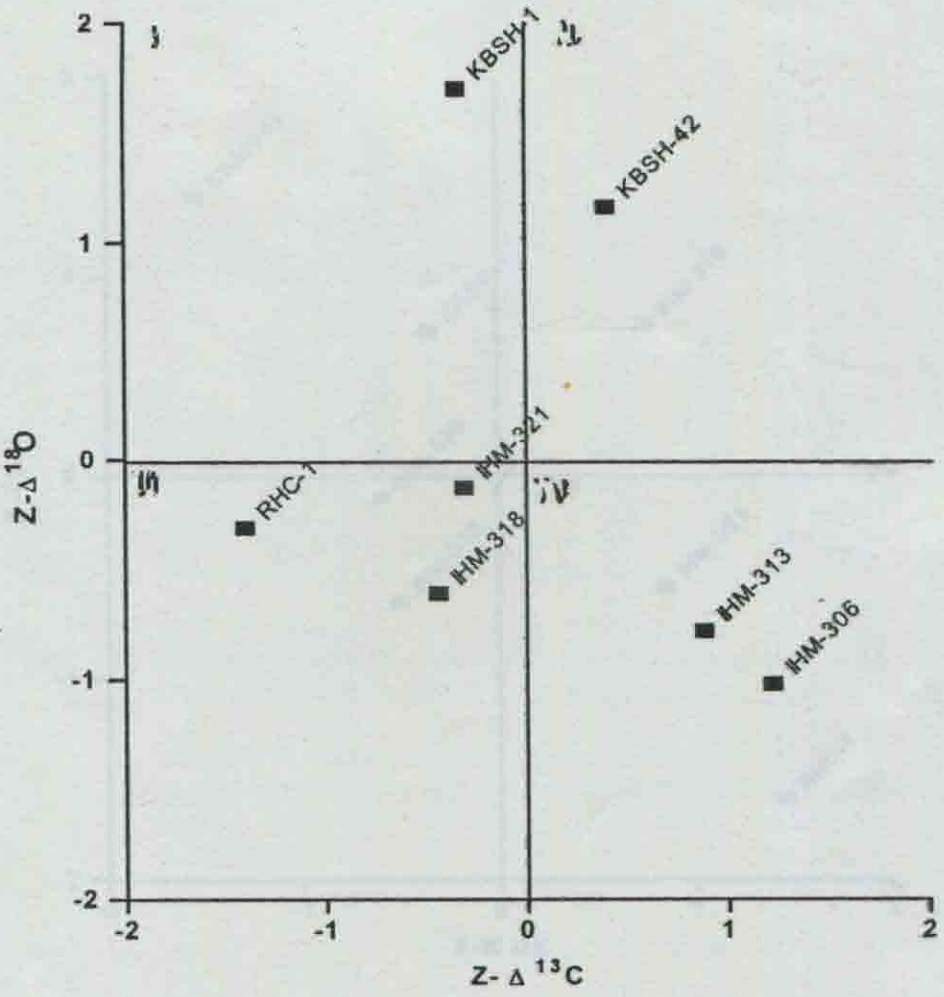


Fig. 18. Grouping of sunflower genotypes based on $\Delta^{13}C$ and $\Delta^{18}O$ in leaf biomass after transforming the values to obtain standard normal distribution (Z).

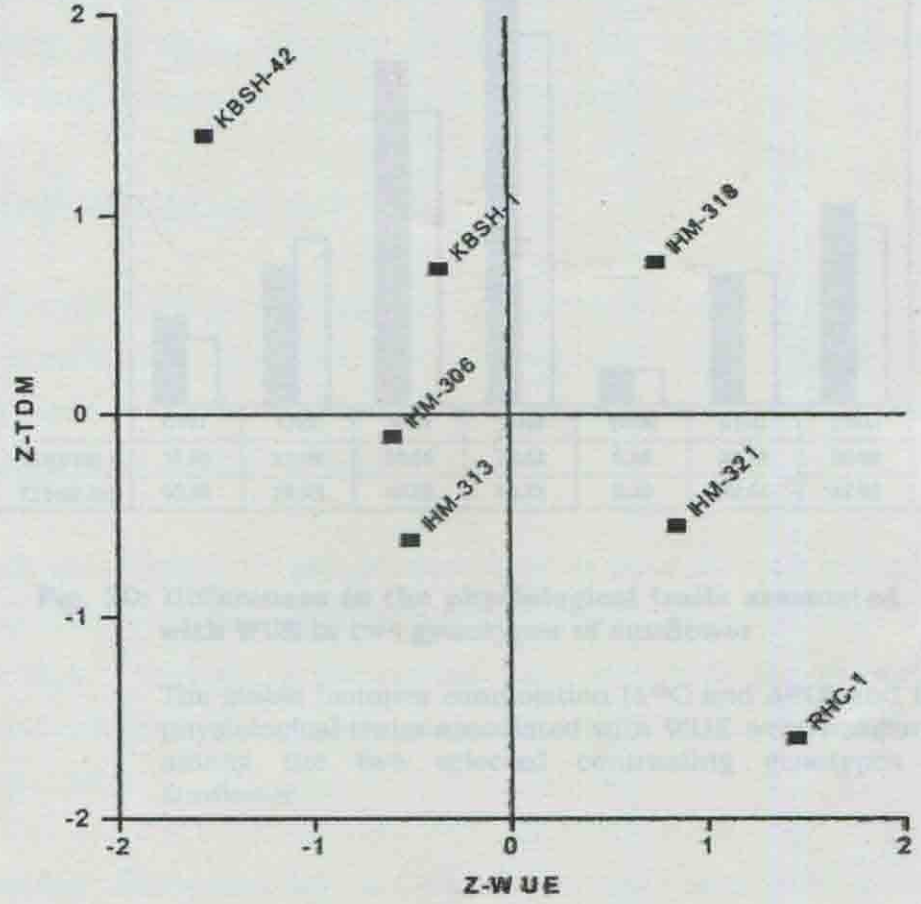
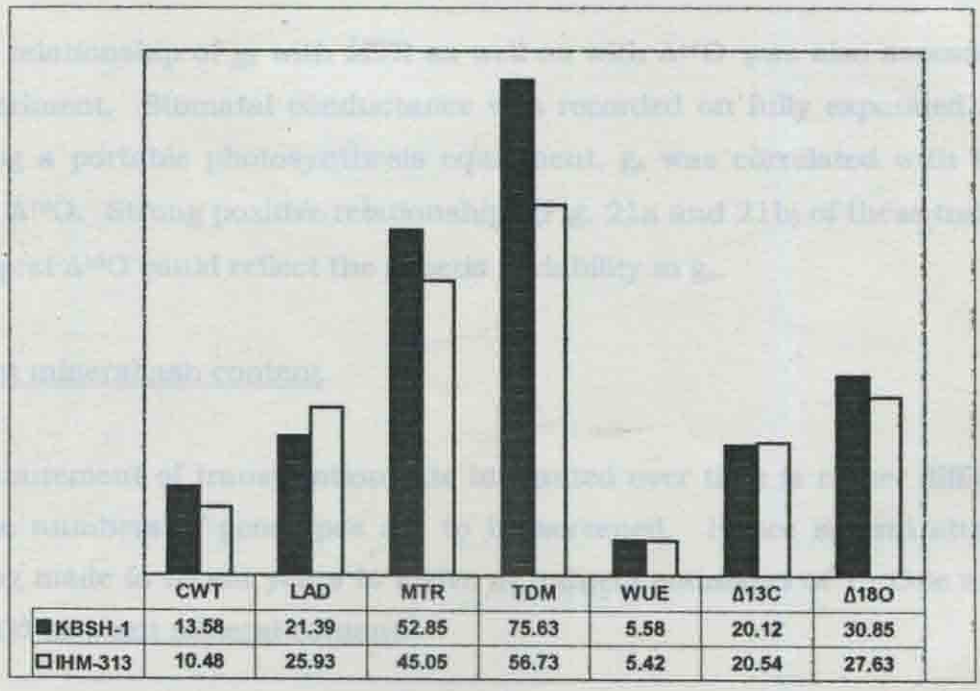


Fig. 19. Grouping of sunflower genotypes based on WUE and TDM after transforming them to standard normal distribution values (Z).



	CWT	LAD	MTR	TDM	WUE	Δ13C	Δ18O
■KBSH-1	13.58	21.39	52.85	75.63	5.58	20.12	30.85
□IHM-313	10.48	25.93	45.05	56.73	5.42	20.54	27.63

Fig. 20: Differences in the physiological traits associated with WUE in two genotypes of sunflower

The stable isotopes composition ($\Delta^{13}C$ and $\Delta^{18}O$) and the physiological traits associated with WUE were compared among the two selected contrasting genotypes of sunflower.

Stomatal conductance, MTR and $\Delta^{18}O$

The relationship of g_s with MTR as well as with $\Delta^{18}O$ was also assessed in this experiment. Stomatal conductance was recorded on fully expanded 3rd leaves using a portable photosynthesis equipment. g_s was correlated with both MTR and $\Delta^{18}O$. Strong positive relationships (Fig. 21a and 21b) of these traits with g_s suggest $\Delta^{18}O$ could reflect the genetic variability in g_s.

Plant mineral ash content

Measurement of transpiration rate integrated over time is rather difficult when large numbers of genotypes are to be screened. Hence several attempts are being made in recent years to arrive at indirect estimates of T. One such traits would be plant mineral content.

During the process of transpiration there is a continuous uptake of water by the roots from the soil. Several ions (nutrients) also get an entry along with the mass flow. Recently a few reports have suggested the possibility of using mineral ash content as a reflection of transpiration rate (Masle, et al., 1992; Brown, 1997).

To explore this possibility, an experiment was conducted to determine the mineral ash content in all the seven hybrids by subjecting the dried leaf samples for ashing. Mineral ash content showed a significant genetic variability ranging from 126.0 to 193.0 $g.Kg^{-1}$.

MTR and mineral ash content

A strong positive relationship was observed between ash content and MTR (Fig. 22), signifying mineral ash content could be an initial indicator of genetic variability in transpiration rate in sunflower.

Fig. 21 a: Relationship between g_s and MTR in a few genotypes of sunflower

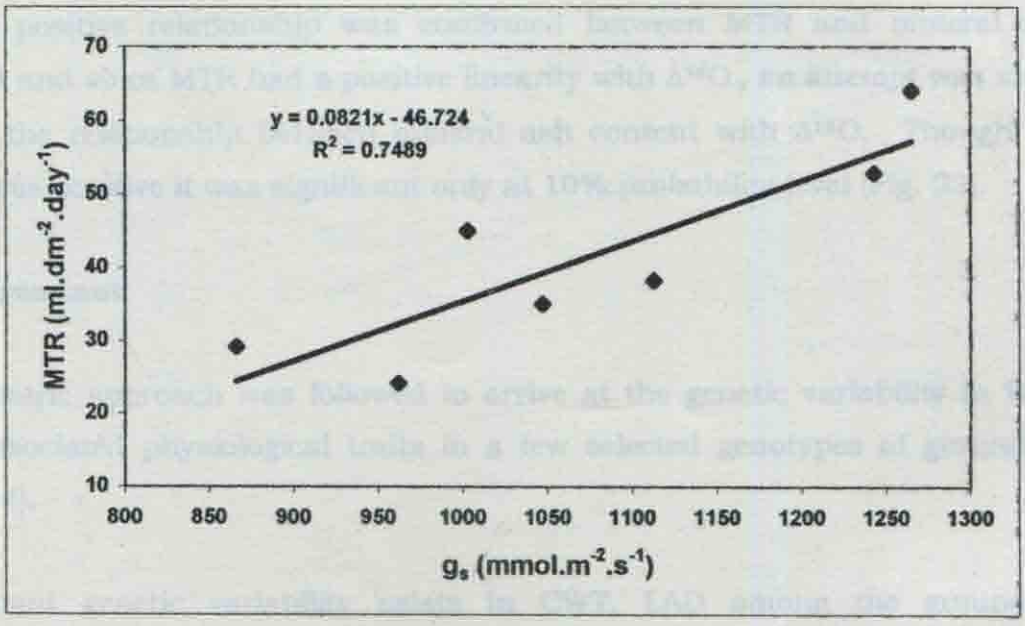
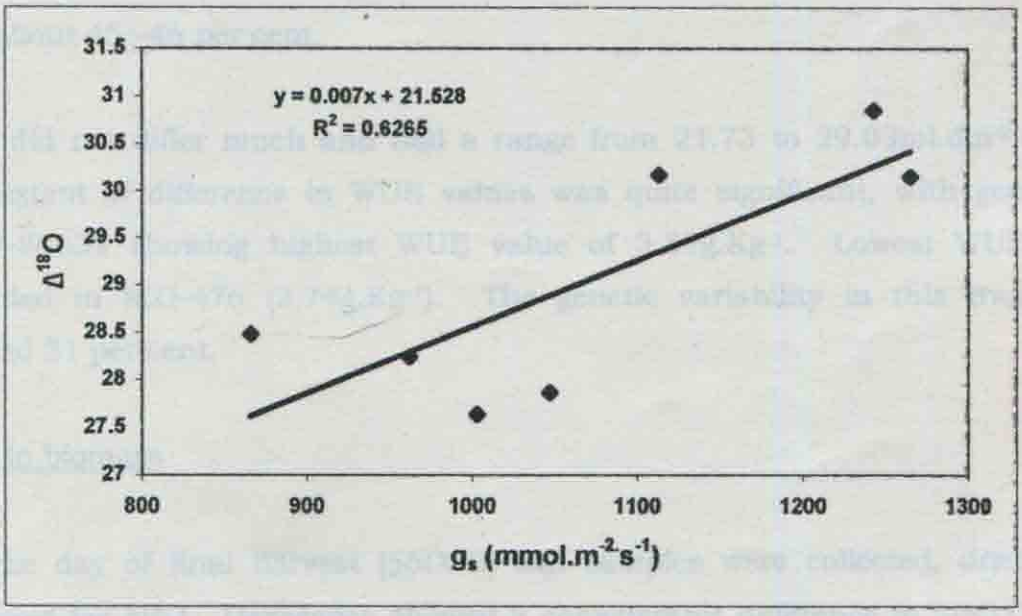


Fig. 21 b: Relationship between g_s and $\Delta^{18}O$ (‰) in a few genotypes of sunflower



Stomatal conductance was recorded using a portable photosynthesis equipment and the MTR was determined gravimetrically. The leaves that developed during the experimental period were analyzed for $\Delta^{18}O$.

Mineral ash content and $\Delta^{18}O$

Strong positive relationship was confirmed between MTR and mineral ash content and since MTR had a positive linearity with $\Delta^{18}O$, an attempt was made to see the relationship between mineral ash content with $\Delta^{18}O$. Though the trend was positive it was significant only at 10% probability level (Fig. 23).

III: Groundnut

Gravimetric approach was followed to arrive at the genetic variability in WUE and associated physiological traits in a few selected genotypes of groundnut (Table-4).

Significant genetic variability exists in CWT, LAD among the groundnut genotypes. The groundnut genotypes showed a marked difference in dry matter accumulation. Among them, TMV-2 produced maximum biomass of 44.57g.plant^{-1} and ICGS 44 stood second in the order (41.8g), ICG 476 had accumulated a minimum biomass of 28.59 g.plant^{-1}. The variability in TDM was about 45 –46 per cent.

MTR did not differ much and had a range from 21.73 to 29.03ml.dm^{-2}.day^{-1}. The extent of difference in WUE values was quite significant, with genotype ICGV-86031 showing highest WUE value of 3.59g.Kg^{-1}. Lowest WUE was recorded in ICG-476 (2.74g.Kg^{-1}). The genetic variability in this trait was around 31 per cent.

$\Delta^{18}O$ in biomass

On the day of final harvest (55DAS) leaf samples were collected, dried and analyzed for $\Delta^{18}O$. Genotypes showed a considerable difference in $\Delta^{18}O$ (Table-4). ICGS-44 showed higher enrichment of 27.33per mil followed by ICGS-86031 (26.70 per mil), $\Delta^{18}O$ was least in TMV-2 (26.42 per mil).

Fig. 22: Relationship between MTR and mineral ash content in sunflower

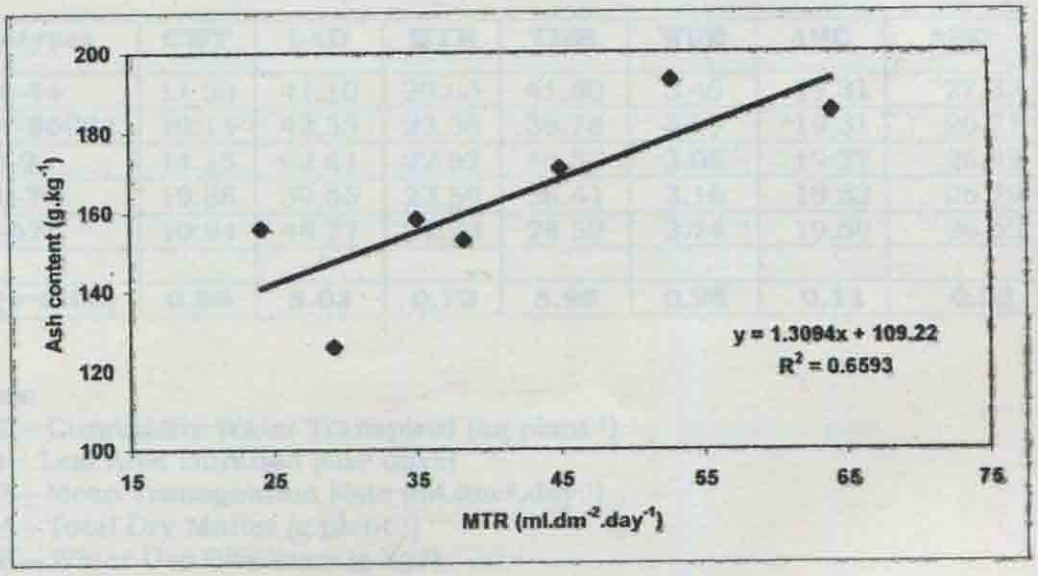
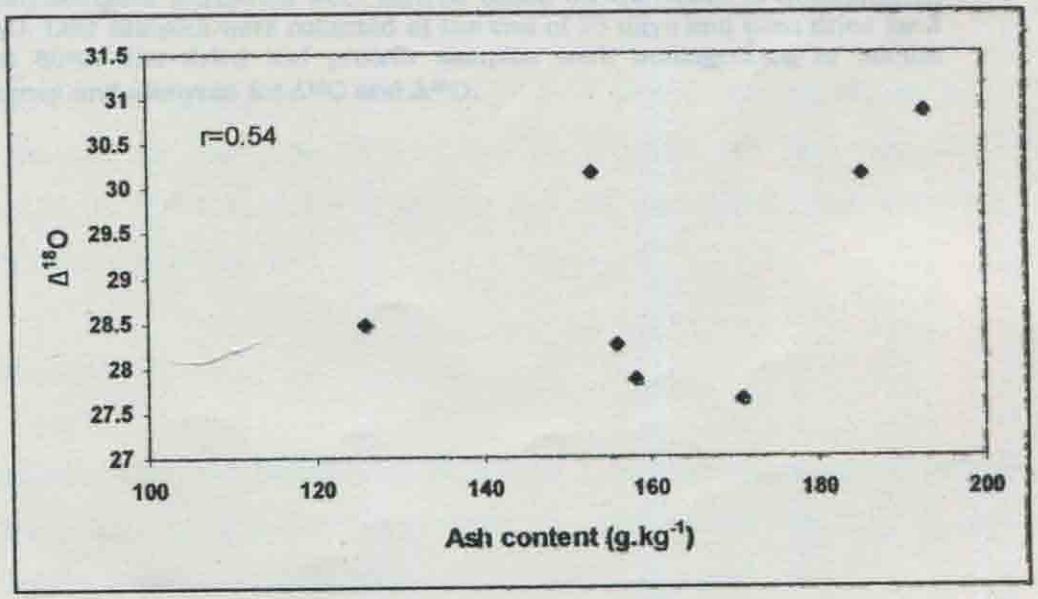


Fig. 23: Association between mineral ash content and $\Delta^{18}O$ (‰) in biomass in sunflower



MTR was quantified gravimetrically in seven pot grown genotypes of sunflower. The leaves that were developed during the experimental period were analyzed for both $\Delta^{18}O$ and mineral ash content

Table-4: Variation in WUE and associated physiological attributes among the genotypes of groundnut

Genotypes	CWT	LAD	MTR	TDM	WUE	$\Delta^{13}C$	$\Delta^{18}O$
ICGS-44	11.50	41.10	29.03	41.80	3.46	19.31	27.33
ICGV-86031	10.11	42.35	23.36	35.78	3.59	19.31	26.71
TMV-2	14.15	62.41	22.67	44.57	3.08	19.27	26.42
ICGS-76	10.88	39.65	23.59	36.41	3.16	19.52	26.79
ICG-476	10.94	48.77	21.73	28.59	2.74	19.69	26.65
CD (p=0.05)	**0.86**	**5.03**	**0.72**	**5.98**	**0.95**	**0.11**	**0.22**

Index:
CWT – Cumulative Water Transpired (kg.plant^{-1})
LAD – Leaf Area Duration (cm^2 days)
MTR – Mean Transpiration Rate (ml.dm^{-2}.day^{-1})
TDM – Total Dry Matter (g.plant^{-1})
WUE – Water Use Efficiency (g.kg^{-1})
$\Delta^{13}C$ – Carbon Isotope Discrimination (per mil)
$\Delta^{18}O$ – Oxygen Isotope composition in leaf biomass (per mil)

The potted plants were weighed once daily to assess the transpirational losses over a period of 20 days (35-55DAS) and MTR was computed based on this value (also considered the functional leaf area developed in this period). WUE and other physiological attributes were arrived based on the value of transpiration and LAD. Leaf samples were collected at the end of 55 days and oven dried for 3 days at 80°C. The dried leaf protein samples were homogenized to talcum consistency and analyzed for $\Delta^{13}C$ and $\Delta^{18}O$.

The relationship presented in Table below illustrates the inter-dependency between WUE and associated physiological traits. The variability in WUE was brought mainly by the differences in net carbon gain, as evident by strong linearity between NAR and WUE (r=0.55). WUE and MTR did not show any relationship, signifying that groundnut is a capacity type. The positive correlation between WUE and dry matter accumulation further reiterates this fact.

Relationship of WUE with other physiological traits in groundnut

Parameter	Relationship (r-value)
WUE v/s NAR	Positive (0.55)
WUE v/s MTR	Non significant (-0.08)
WUE v/s TDM	Positive (0.47)

$\Delta^{13}C$ and WUE

Significant genetic variability in both WUE and $\Delta^{13}C$ exists. Among the selected genotypes, though the variation in $\Delta^{13}C$ (19.31 – 19.69 per mil) was small compared to WUE (2.74 to 3.59) a strong inverse correlation was observed between these two traits (Fig. 24).

MTR, g_s and $\Delta^{18}O$

In this experiment also, relationship of MTR with $\Delta^{18}O$ was assessed. A strong positive relationship was found between these two parameters (Fig. 25). g_s also showed a positive correlation with MTR and $\Delta^{18}O$ (Fig. 26a and 26b).

Grouping of genotypes

As explained earlier standardized normal Z-distribution was employed to group the genotypes for physiological traits. Similarly one more classification was

Fig. 24: Relationship between WUE and $\Delta^{13}C$ (‰) in a few genotypes of groundnut

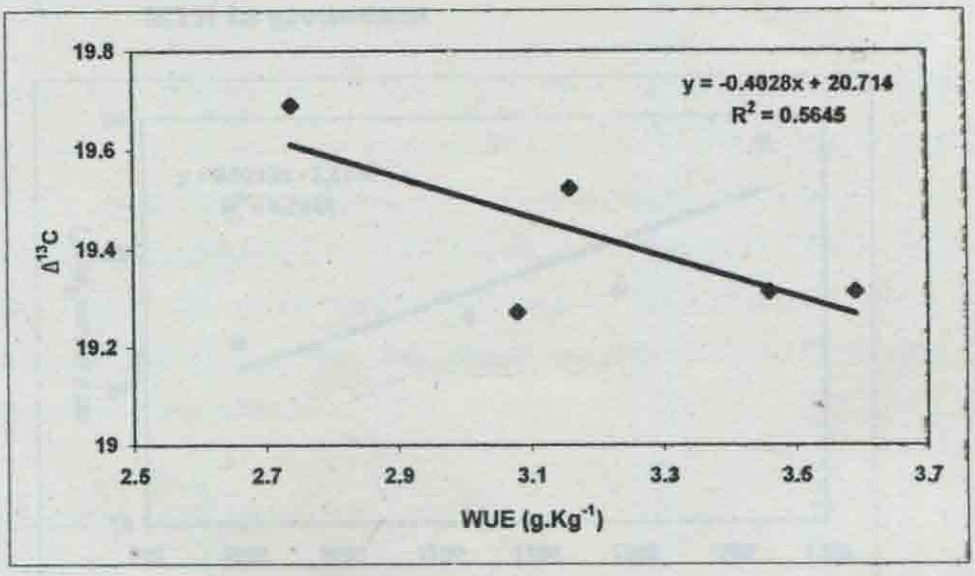
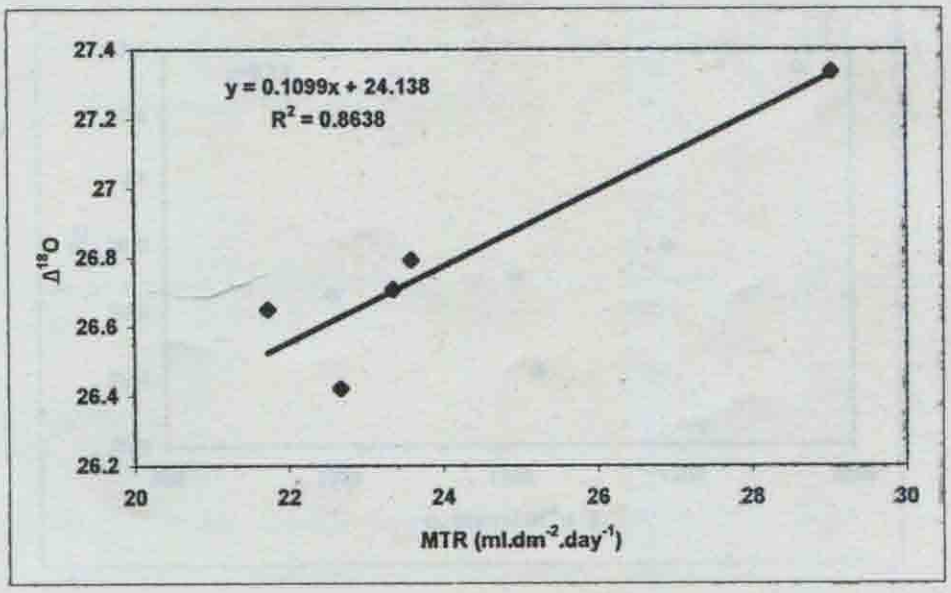


Fig. 25: Relationship between mean transpiration rate and $\Delta^{18}O$ (‰) in leaf biomass in groundnut genotypes



WUE and MTR were gravimetrically determined in pot grown genotypes of groundnut. The leaves that developed during the experimental period were analyzed for $\Delta^{13}C$ and $\Delta^{18}O$.

Fig. 26 a: Relationship between stomatal conductance and MTR in groundnut

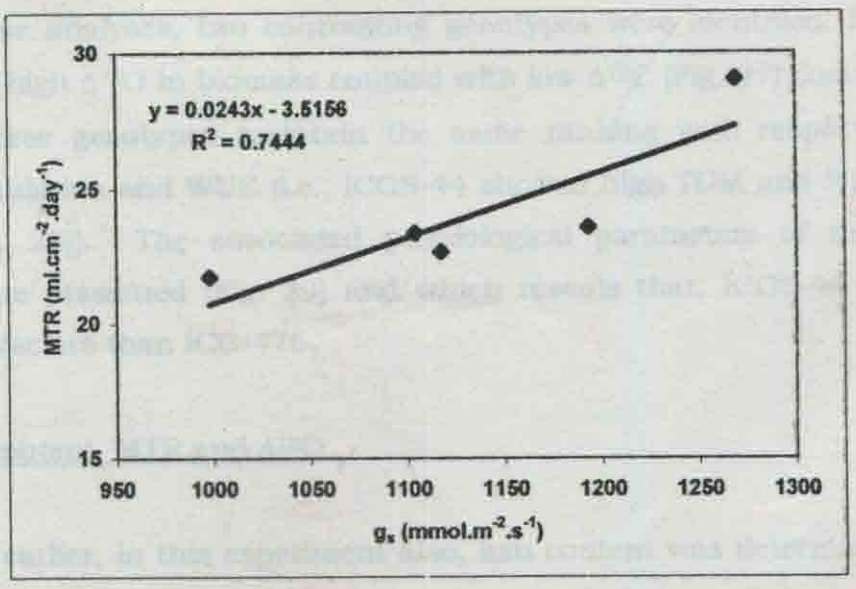
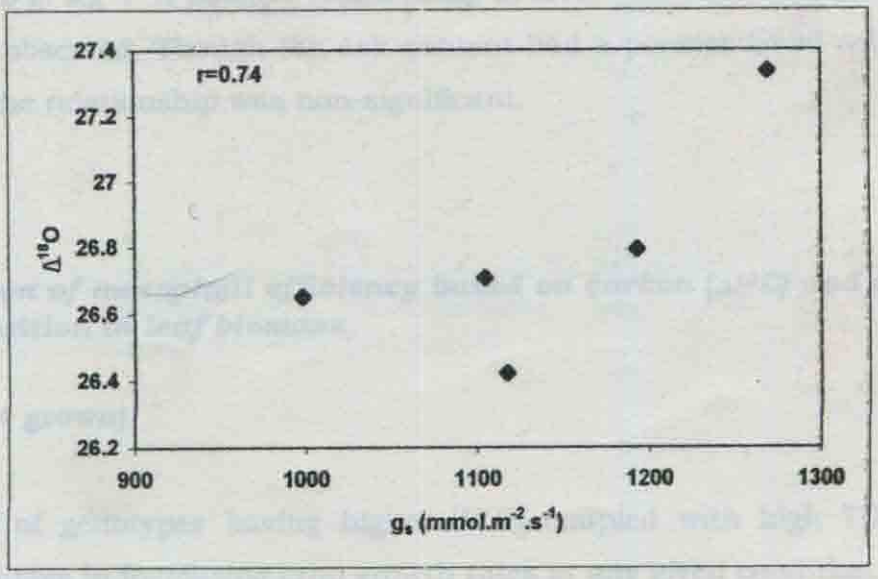


Fig. 26 b: Relationship between stomatal conductance and $\Delta^{18}O$ in leaf biomass in groundnut



Stomatal conductance was recorded by using a portable photosynthetic system (LCA-4) and MTR was determined gravimetrically in genotypes of groundnut grown in pots. The leaves that were developed during the experimental period were analyzed for $\Delta^{18}O$.

done between dry matter accumulation and WUE. The genotype belongs to Quadrant-II was selected (having high WUE coupled with high TDM, also has relatively high $\Delta^{18}O$ but low $\Delta^{13}C$).

Based on these analyses, two contrasting genotypes were identified. ICGS-44 had relatively high $\Delta^{18}O$ in biomass coupled with low $\Delta^{13}C$ (Fig. 27) compared to ICG-476. These genotypes maintain the same ranking with respect to dry matter accumulation and WUE {i.e., ICGS-44 showed high TDM and WUE than ICG-476 (Fig. 28)}. The associated physiological parameters of these two genotypes were presented (Fig. 29) and which reveals that, ICGS-44 has the desirable characters than ICG-476.

Mineral ash content, MTR and $\Delta^{18}O$

As explained earlier, in this experiment also, ash content was determine in few genotypes of groundnut.

Mineral ash content showed a considerable genetic variability ranging from 124.0 to 144.0 g. Kg^{-1}. A positive relationship of MTR with mineral ash content (Fig. 30) was observed. Though the ash content had a positive trend with $\Delta^{18}O$ (Fig. 31) but the relationship was non-significant.

Section-III

Quantification of mesophyll efficiency based on carbon ($\Delta^{13}C$) and oxygen ($\Delta^{18}O$) composition in leaf biomass.

COWPEA (pot grown)

Identification of genotypes having higher WUE coupled with high TDM has specific advantage in increasing crop growth rates at any given condition.

Since WUE and T directly influence the crop growth rates, interdependency between these traits is not desirable. This necessitates the identification of

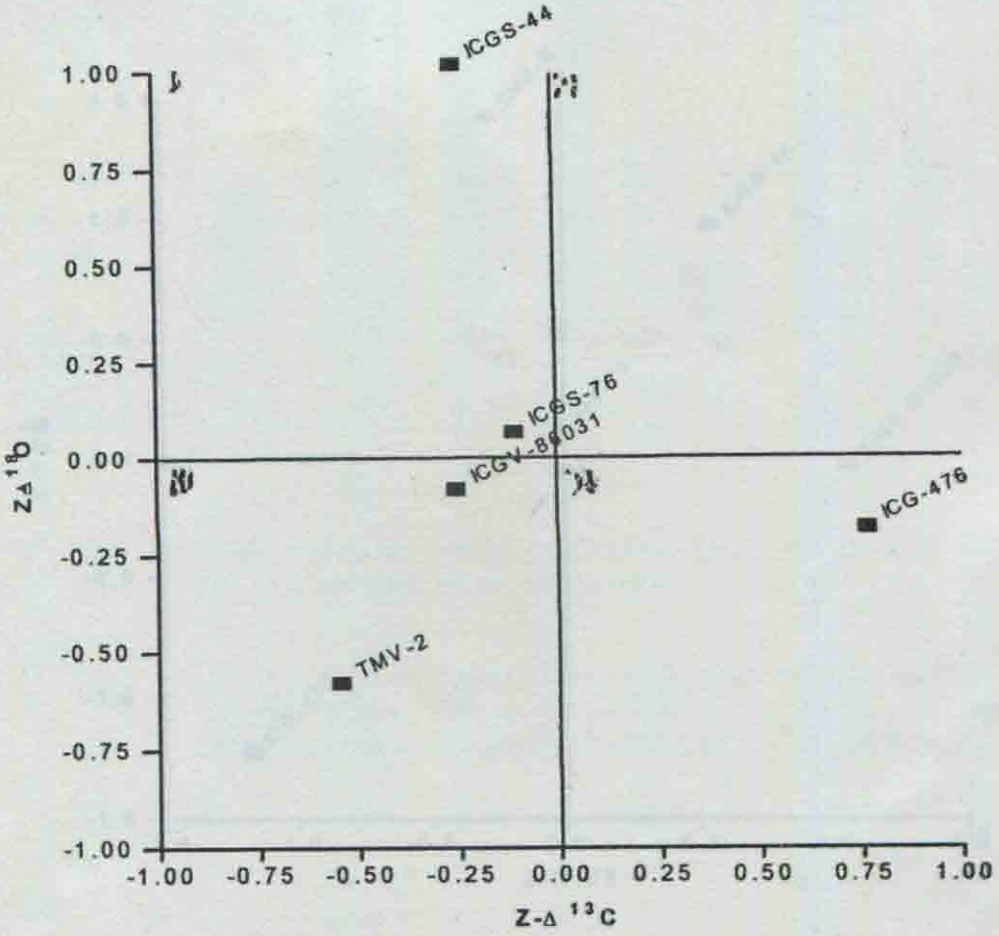


Fig. 27. Grouping of groundnut genotypes based on $\Delta^{13}C$ and $\Delta^{18}O$ in leaf biomass after transforming the values to obtain standard normal distribution (Z).

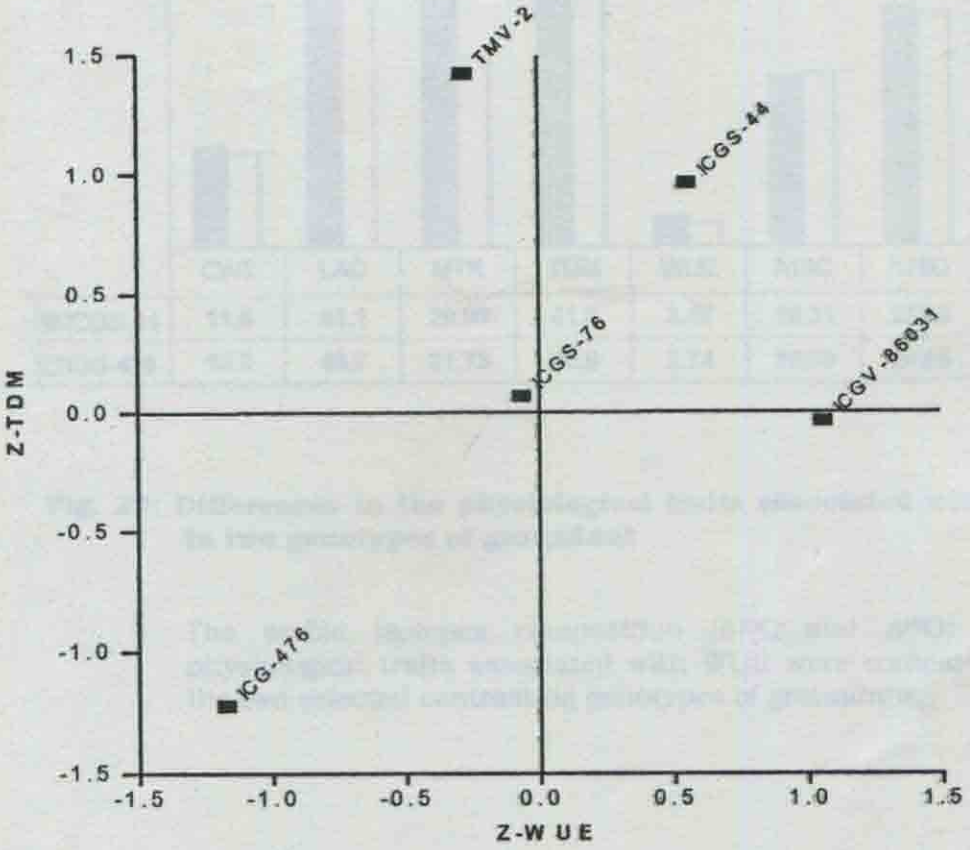
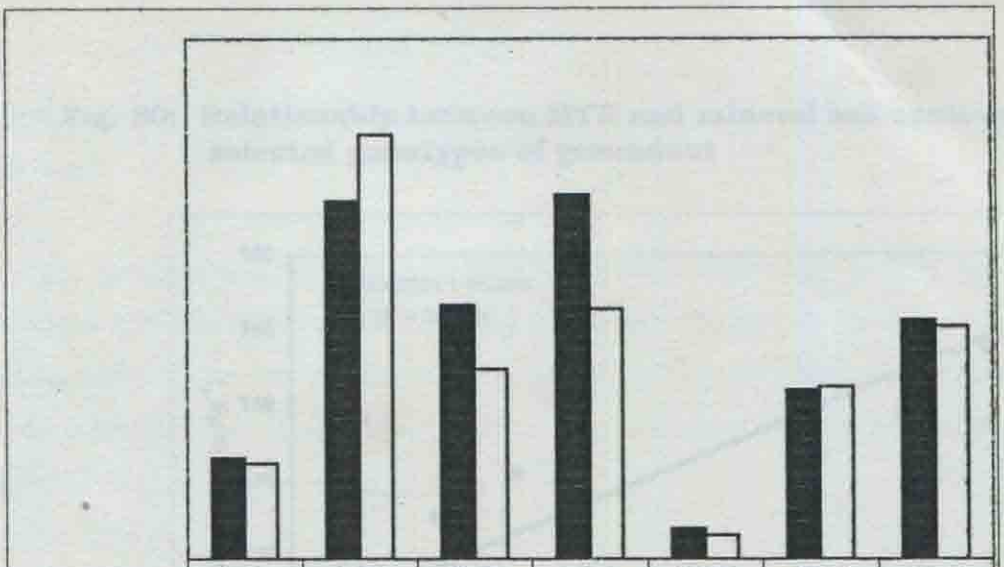


Fig. 28. Grouping of groundnut genotypes based on WUE and TDM after transforming them to standard normal distribution values (Z).



	CWT	LAD	MTR	TDM	WUE	Δ13C	Δ18O
■ICGS-44	11.5	41.1	29.03	41.8	3.46	19.31	27.33
□ICG-476	10.9	48.7	21.73	28.6	2.74	19.69	26.65

Fig. 29: Differences in the physiological traits associated with WUE in two genotypes of groundnut

The stable isotopes composition ($\Delta^{13}C$ and $\Delta^{18}O$) and the physiological traits associated with WUE were compared among the two selected contrasting genotypes of groundnut.

Fig. 30: Relationship between MTR and mineral ash content in selected genotypes of groundnut

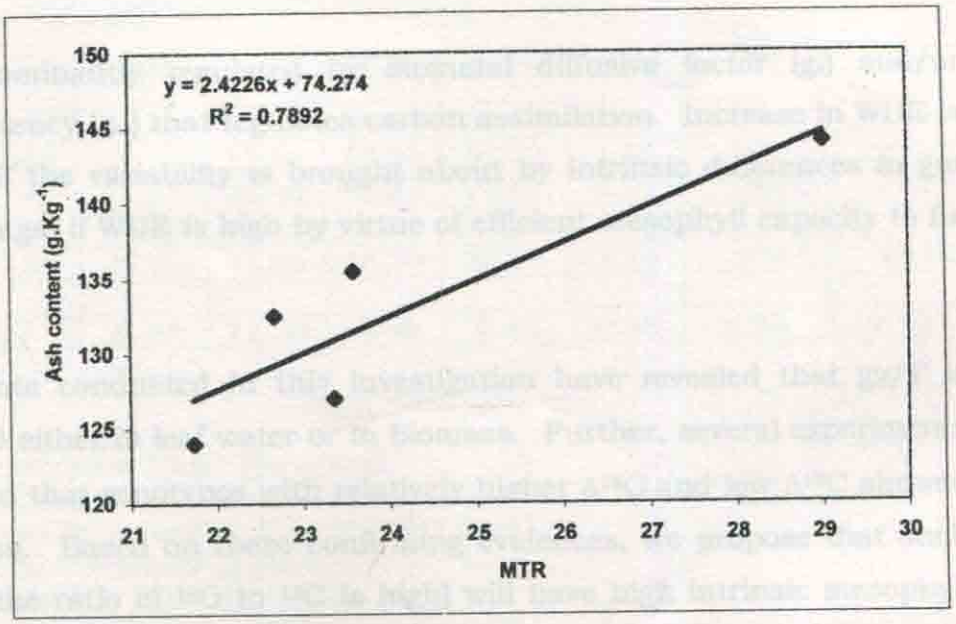
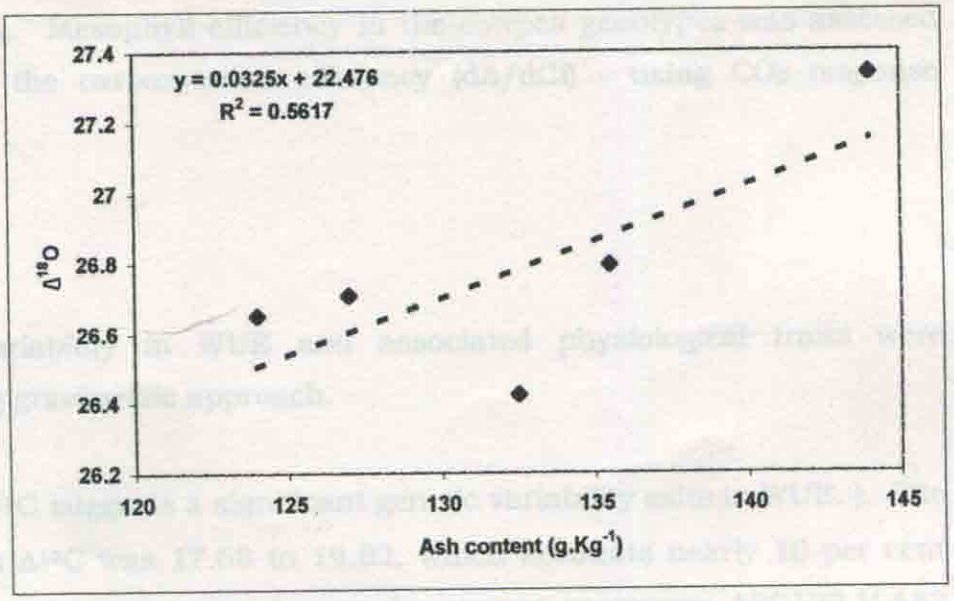


Fig. 31: Association between mineral ash content and $\Delta^{18}O$ (‰) in leaf biomass in groundnut genotypes



Mean transpiration rate in a few groundnut genotypes was determined gravimetrically. The leaves that were developed during the experimental period were analyzed for both mineral ash content and $\Delta^{18}O$. The regression line (dotted), though not significant, depicts the direction of the trend of the association between mineral ash and $\Delta^{18}O$ in Fig. 31.

genotypes where this inter-relationship is weak (i.e., high WUE despite high water use).

WUE is predominantly regulated by stomatal diffusive factor (g_s) and/or mesophyll efficiency (g_m) that regulates carbon assimilation. Increase in WUE is relevant only if the variability is brought about by intrinsic differences in gm (i.e., at a given gs, if WUE is high by virtue of efficient mesophyll capacity to fix carbon).

The experiments conducted in this investigation have revealed that gs/T is related to $\Delta^{18}O$ either in leaf water or in biomass. Further, several experiments have confirmed that genotypes with relatively higher $\Delta^{18}O$ and low $\Delta^{13}C$ showed higher biomass. Based on these confirming evidences, we propose that such types (where the ratio of ^{18}O to ^{13}C is high) will have high intrinsic mesophyll efficiency. To examine these aspects a comprehensive experiment was conducted in cowpea, which is generally a conductance type (i.e., stomatal diffusive factors influence the genetic variability in WUE traits). The aim of this experiment was proposed to identify type/s which will have higher WUE by virtue of high g_m. Mesophyll efficiency in the cowpea genotypes was assessed by determining the carboxylation efficiency (dA/dCi) - using CO_2 response curves.

WUE and $\Delta^{13}C$

The genetic variability in WUE and associated physiological traits were quantified using gravimetric approach.

The result on $\Delta^{13}C$ suggests a significant genetic variability exits in WUE.). The overall range in $\Delta^{13}C$ was 17.68 to 19.82, which accounts nearly 10 per cent variability across the genotypes. Among the cowpea genotypes, APC123 V-683 had highest discrimination value of 19.82 per mil and APC-4125 had least value of 17.68 (Table-5). A strong inverse relationship between $\Delta^{13}C$ and WUE was observed (Fig. 32).

Table-5: Variations in physiological traits associated with WUE in five genotypes of cowpea grown in pots

Genotypes	CWT	LAD	MTR	TDM	WUE	$\Delta^{13}C$	$\Delta^{18}O$
APC-40 GC-20	41.42	54.02	76.64	57.17	1.38	19.66	25.89
APC-123 V-683	32.37	55.04	58.52	57.30	1.78	19.82	24.23
V-585370	37.87	63.30	59.82	78.78	2.09	18.78	24.25
APC-4125	30.28	50.12	60.42	68.38	2.51	17.68	24.94
APC-121 P-132	49.19	71.60	68.70	89.54	1.82	18.88	25.59
CD (p=0.05)	**6.32**	**11.52**	**3.48**	**5.43**	**0.95**	**0.88**	**0.33**

Index:
CWT – Cumulative Water Transpired
LAD – Leaf Area Duration (cm^2 days)
MTR – Mean Transpiration Rate ($ml.dm^{-2}.day^{-1}$)
TDM – Total Dry Matter ($g.plant^{-1}$)
WUE – Water Use Efficiency ($g.kg^{-1}$)
$\Delta^{13}C$ – Carbon Isotope Discrimination (per mil)
$\Delta^{18}O$ – Oxygen Isotope composition in leaf biomass (per mil)

WUE was quantified by gravimetric technique in a few selected genotypes grown in pot between 35 to 60 days after sowing (DAS). CWT was determined by weighing the pots daily and the functional leaf area (LAD) was assessed during this experimental period. MTR was arrived on the basis of total water transpired to the functional leaf area during experimental period. The leaves that developed during this treatment period were collected and analyzed for $\Delta^{13}C$ and $\Delta^{18}O$.

$\Delta^{18}O$ in leaf biomass was also determined along with $\Delta^{13}C$. The results on $\Delta^{18}O$ indicated a considerable genetic variability. The $\Delta^{18}O$ had a range from 24.25 to 25.89 per mil. Among the genotypes, APC-40-GC-20 had maximum ^{18}O composition (25.89 per mil), closely followed by APC 121-P-132 (25.52 per mil) and APC 4125 (24.92 per mil) and minimum $\Delta^{18}O$ of 24.25 per mil was observed in V-585. Variability of about eight per cent was noticed in $\Delta^{18}O$ across the genotypes.

The interrelationship between the physiological traits associated with WUE both at whole plant level and at single leaf level was also examined in the present experiment.

At whole plant level

1. CWT and TDM

At any given situation, total biomass is directly linked to the total transpiration of the plants, similar relationship did exit in the present study also. A strong positive correlation (r=0.61) was observed between total water use during the experimental period and dry matter accumulation.

2. WUE and CWT

An inverse relationship (r=-0.54) was observed between WUE and CWT suggesting, in cowpea, genotypes with less transpiration showed higher WUE.

3. MTR and WUE

Significant negative correlation (r=-0.72) was seen, signifying stomatal regulation of WUE in cowpea genotypes.

To assess the genetic variability in A/g_s, gas exchange parameters were recorded on 55th DAS.

From the primary values such as Assimilation rate ('A'), stomatal conductance (g_s) and inter-cellular CO_2 concentration (Ci), A/g_s, Ci/g_s (intrinsic mesophyll efficiency) and A/Ci were computed.

The result suggests that a considerable genotypic variation in the mentioned gas exchange traits (Table-6). In addition, the relationship of gas exchange traits with integrated growth traits was also examined.

Table-6: Relationship across gas exchange traits in selected cowpea genotypes

Parameters	Relationship(r value)
A v/s g_s	+ve (0.91)
A v/s Ci	-ve (-0.80)
g_s v/s MTR	+ve (0.63)
g_s v/s WUE	-ve (0.53)
A/g_s and WUE	+ve (0.40)
$\Delta^{13}C$ v/s A/g_s	-ve (0.70)

The following inferences can be drawn:

A. The variability in WUE in cowpea is predominantly governed by stomatal diffusive factors. WUE was inversely related with stomatal conductance and MTR was directly related to g_s (r=0.63).

B. The relationship between intrinsic WUE (A/g_s) and WUE measured gravimetrically was poor, suggesting, instantaneous measure of WUE, though dynamic but not stable, hence difficult to depend on this trait.

$\Delta^{18}O$ and its interaction with a few physiological traits

From previous experiments it is evident that $\Delta^{18}O$ in biomass could be a reflection of T. In this experiment also, $\Delta^{18}O$ was analyzed and its relationships with MTR and stomatal conductance were examined.

MTR and $\Delta^{18}O$

The result in Table-6 indicates a significant genotypic variability in $\Delta^{18}O$ in cowpea (LSD=0.33). Among cowpea genotypes, the $\Delta^{18}O$ in APC-40-G-20 was relatively high (25.89per mil) closely followed by APC 121-P-132 (25.52per mil). Low $\Delta^{18}O$ was recorded in V-585 (24.25per mil). Though prominent difference was not observed in absolute values, still variability of around 8 per cent was seen across the genotypes. Strong positive relationship between MTR and $\Delta^{18}O$ (Fig-33) further signifies the fact that $\Delta^{18}O$ is a reflection of transpiration rate averaged over time.

Grouping of genotypes based on their physiological characters

The emphasis was to identify genotypes where in WUE is governed by efficient mesophyll characters rather than by stomatal characters using stable isotopes of carbon and oxygen. In those types WUE will be more despite high water use and hence will have high crop growth rates. Keeping this in view, cowpea genotypes were grouped using a standard Z analysis.

Z-distribution plot (Fig. 34) between $\Delta^{18}O$ and $\Delta^{13}C$ reveals that among selected genotypes, APC 4125 and APC 121-P-132 represent the I-Quadrant (which comprises high $\Delta^{18}O$ and low $\Delta^{13}C$ isotope composition). Apart from this, these genotypes had relatively high TDM coupled with high WUE {confirmed from Z-analysis between TDM and WUE traits (Quadrant-I in Fig. 35)}. Both these genotypes had higher water use and WUE characters.

Fig. 32: Relationship between WUE and $\Delta^{13}C$ (‰) in selected genotypes of cowpea

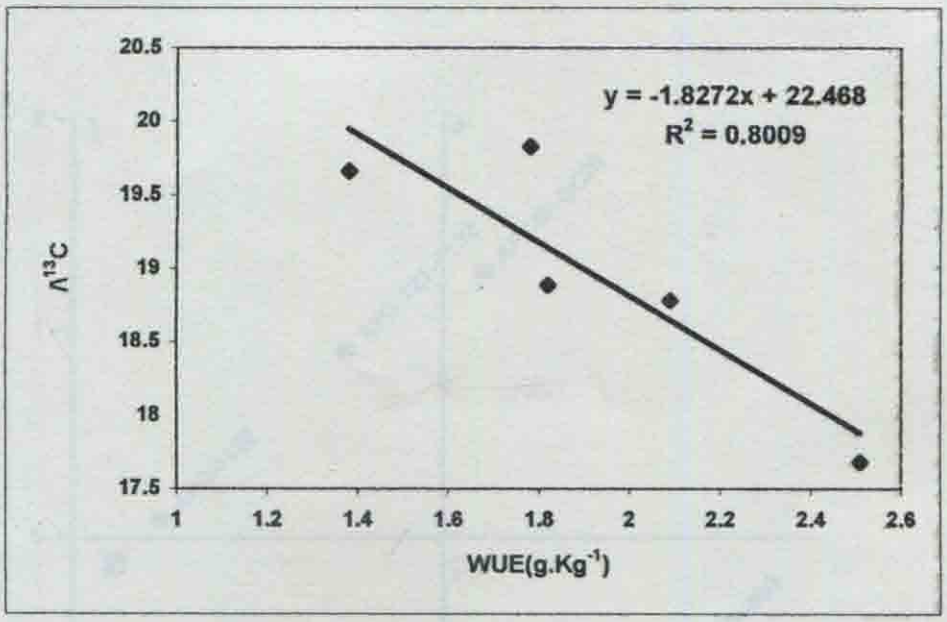
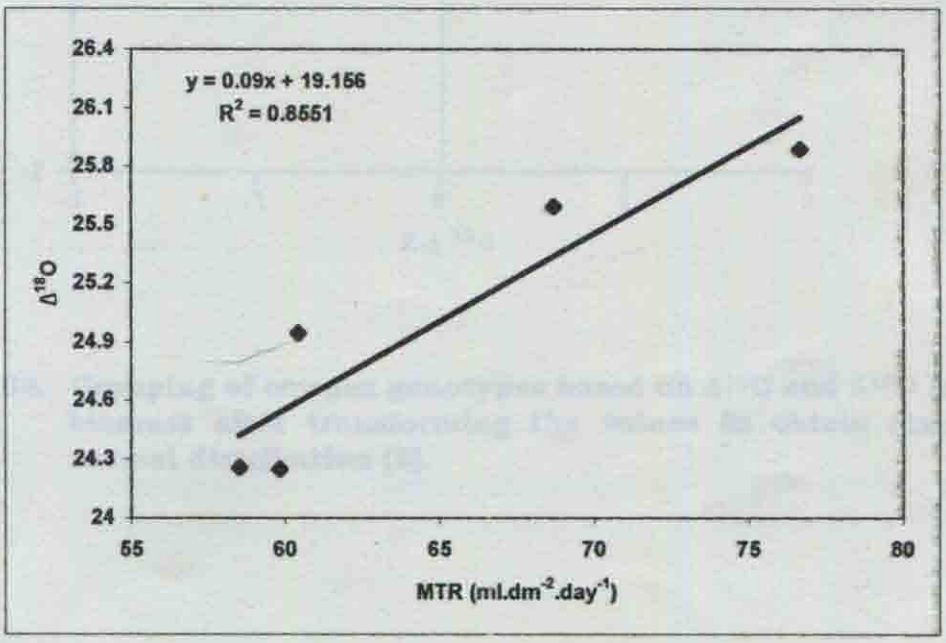


Fig. 33: Relationship between MTR and $\Delta^{18}O$ (‰) in leaf biomass in cowpea



WUE and MTR between 35-50 DAS were assessed gravimetrically in a few genotypes of cowpea grown in pots. The leaves that developed during the experimental period were analyzed for $\Delta^{13}C$ and $\Delta^{18}O$.

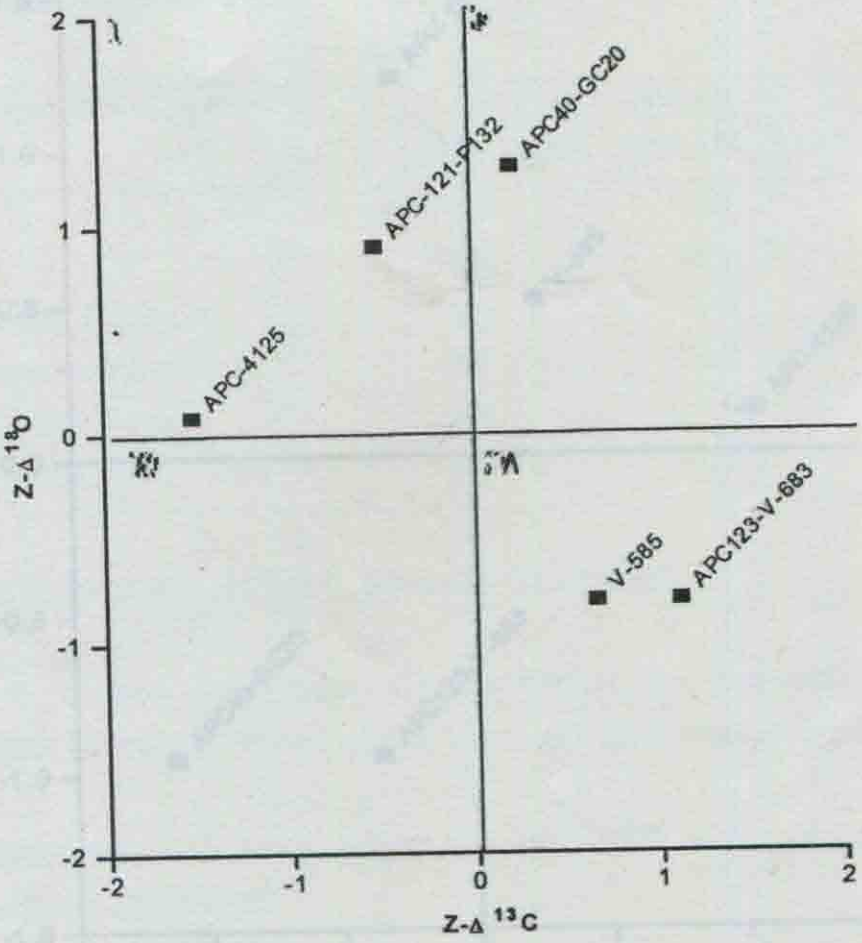


Fig. 34. Grouping of cowpea genotypes based on $\Delta^{13}C$ and $\Delta^{18}O$ in leaf biomass after transforming the values to obtain standard normal distribution (Z).

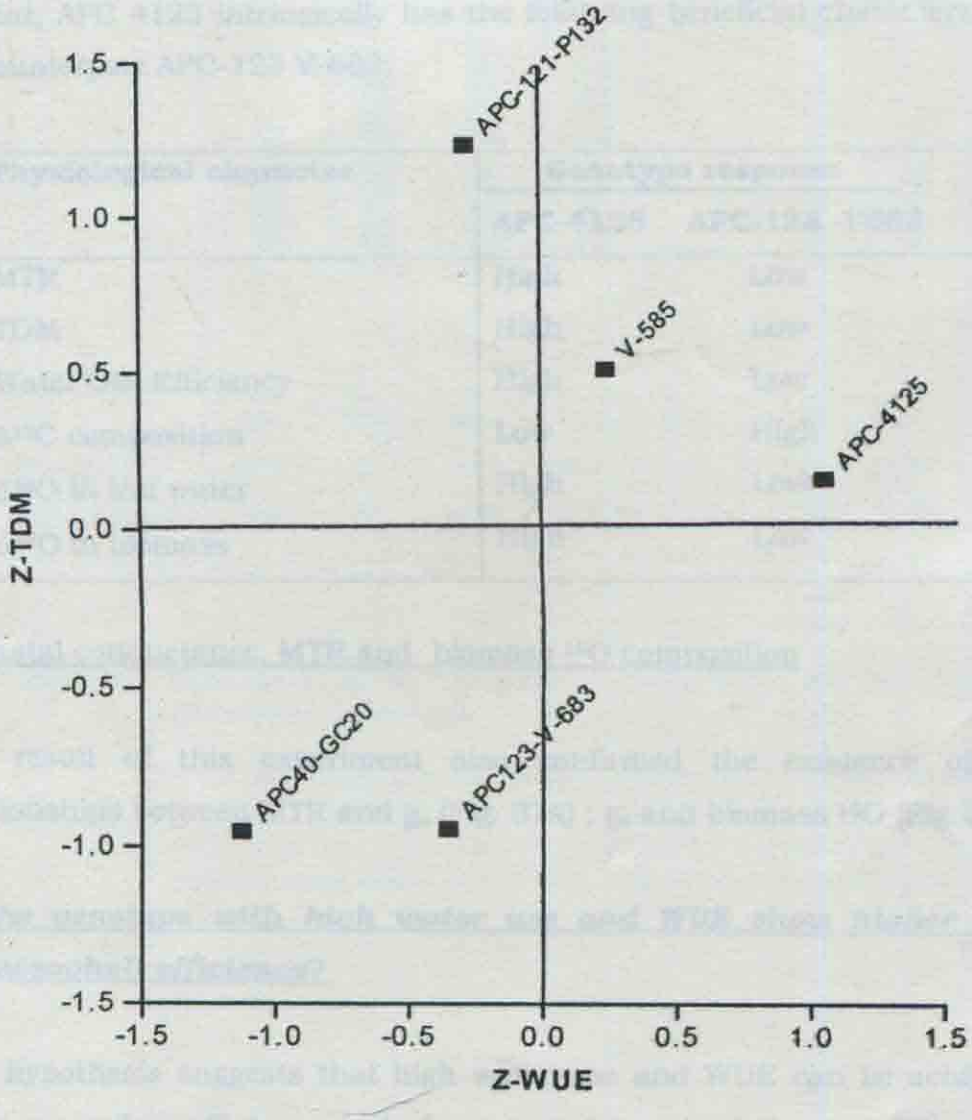


Fig. 35. Grouping of cowpea genotypes based on WUE and TDM after transforming them to standard normal distribution values (Z).

The genotype, APC-4125 in Quadrant-I with high $\Delta^{18}O$ and low $\Delta^{13}C$ showed a characteristic feature of mesophyll capacity type when rest of physiological traits were compared with APC-123 V-683. The histogram (Fig. 36) reveals that, APC 4125 intrinsically has the following beneficial characters than its counterpart APC-123 V-683.

Physiological character	**Genotype response**	
	APC-4125	**APC-123 -V683**
MTR	High	Low
TDM	High	Low
Water Use Efficiency	High	Low
$\Delta^{13}C$ composition	Low	High
$\Delta^{18}O$ in leaf water	High	Low
$\Delta^{18}O$ in biomass	High	Low

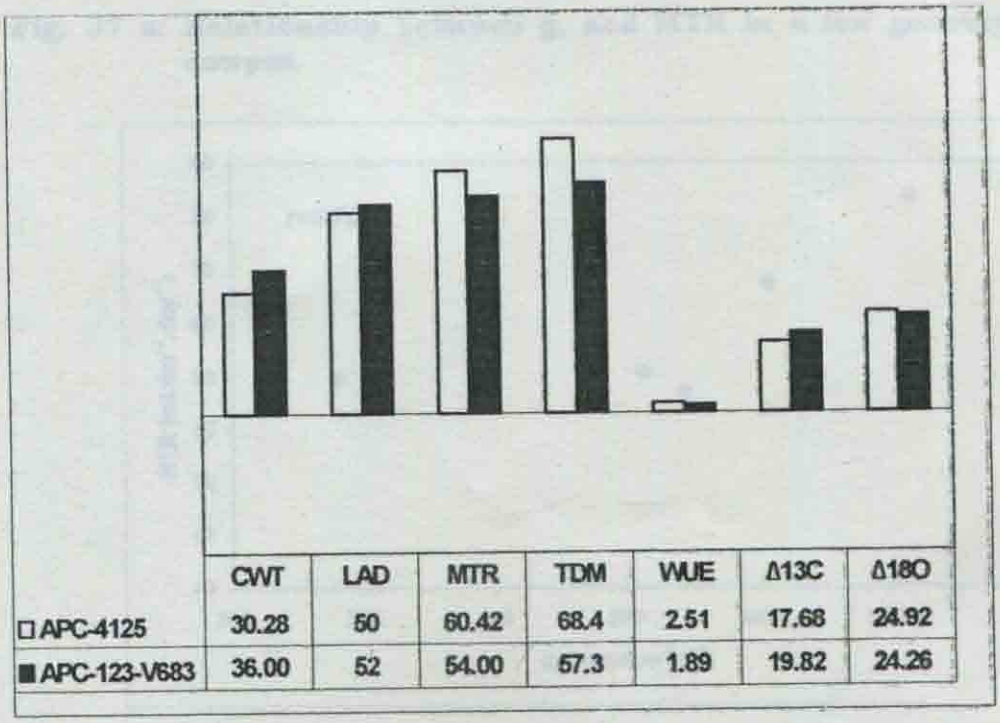
Stomatal conductance, MTR and biomass ^{18}O composition

The result of this experiment also confirmed the existence of positive relationships between MTR and g_s (Fig. 37a) ; g_s and biomass ^{18}O (Fig. 37b).

Is the genotype with high water use and WUE show higher intrinsic mesophyll efficiency?

Our hypothesis suggests that high water use and WUE can be achieved only when mesophyll efficiency (g_m) characters determined the variability in WUE. To examine this aspect, CO_2 response curves were developed using gas exchange approach (to arrive at dA/dCi) in contrasting genotypes of cowpea.

Initial slope of CO_2 response curve (dA/dCi, a reflection of initial carboxylation efficiency during photosynthesis) showed a considerable variation across genotypes (Table-7). APC 4125 had high initial carboxylation efficiency of 30.53 closely followed by APC 121-P-132 (19.32) and APC 123-V-683 (18.66). The least dA/dCi of 13.54 was recorded in V-585 (Fig. 38a, 38b, 38c and 38d). A



	CWT	LAD	MTR	TDM	WUE	Δ13C	Δ18O
□ APC-4125	30.28	50	60.42	68.4	2.51	17.68	24.92
■ APC-123-V683	36.00	52	54.00	57.3	1.89	19.82	24.26

Fig. 36: Variability in a few physiological traits in two contrasting genotypes of cowpea

Two contrasting genotypes were identified based on Z-analysis for their stable isotopes ($\Delta^{13}C$ and $\Delta^{18}O$) composition. In these genotypes, variation in few physiological traits associated with WUE was compared.

Fig. 37 a: Relationship between g_s and MTR in a few genotypes of cowpea

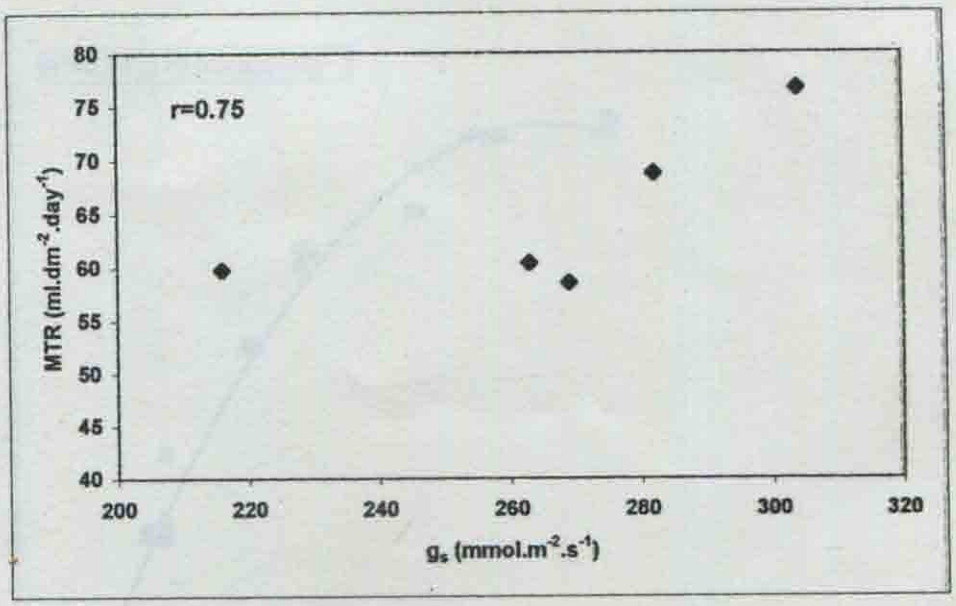
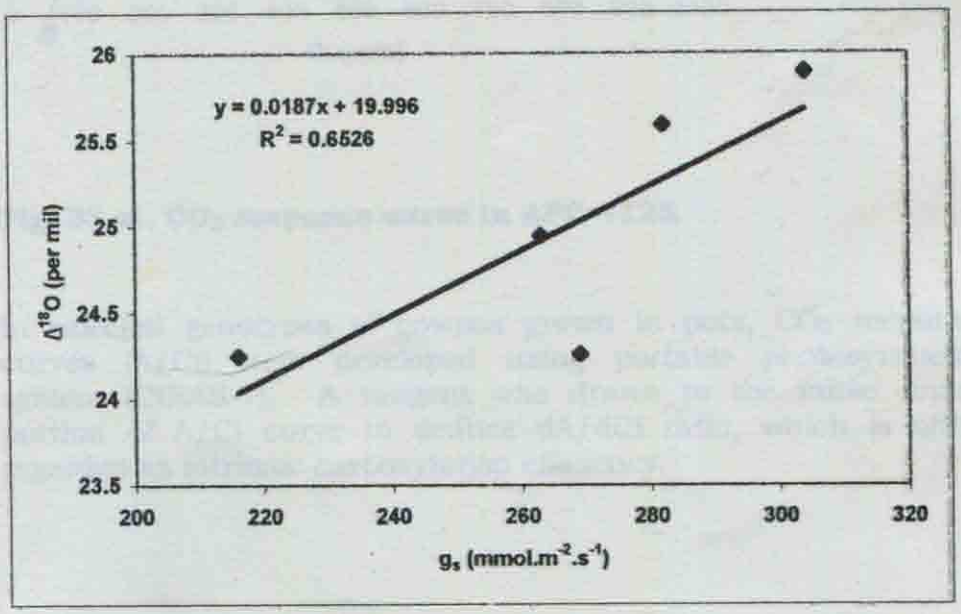


Fig. 37 b: Relationship between g_s and $\Delta^{18}O$ (‰) in leaf biomass in a few genotypes of cowpea



Stomatal conductance and MTR were determined by employing gas exchange and gravimetric approaches respectively. $\Delta^{18}O$ was analyzed in the leaf samples collected at the end of the experiment.

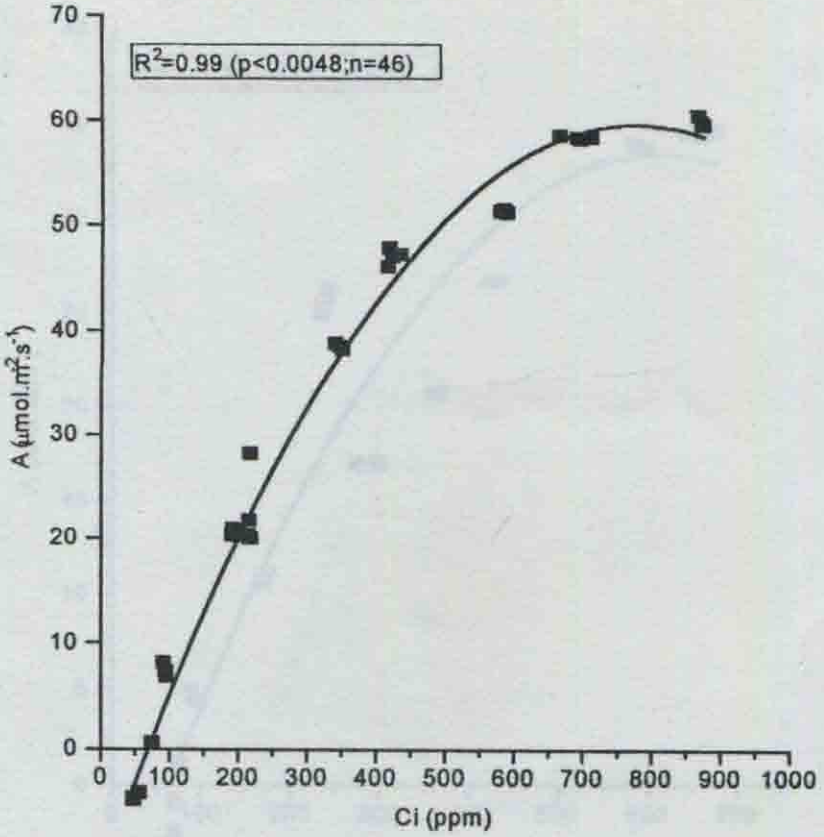


Fig. 38 a: CO_2 response curve in APC-4125

In selected genotypes of cowpea grown in pots, CO_2 response curves (A/Ci) were developed using portable photosynthesis system (CIRAS-1). A tangent was drawn to the initial linear portion of A/Ci curve to deduce dA/dCi ratio, which is often regarded as intrinsic carboxylation efficiency.

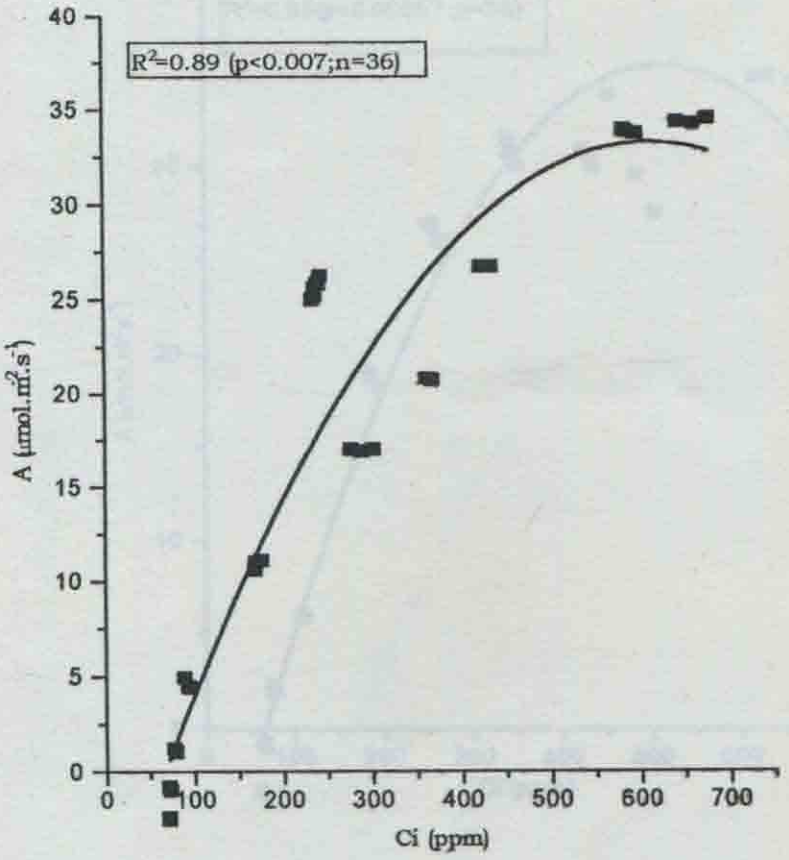


Fig. 38 b: CO_2 response curve in V-585

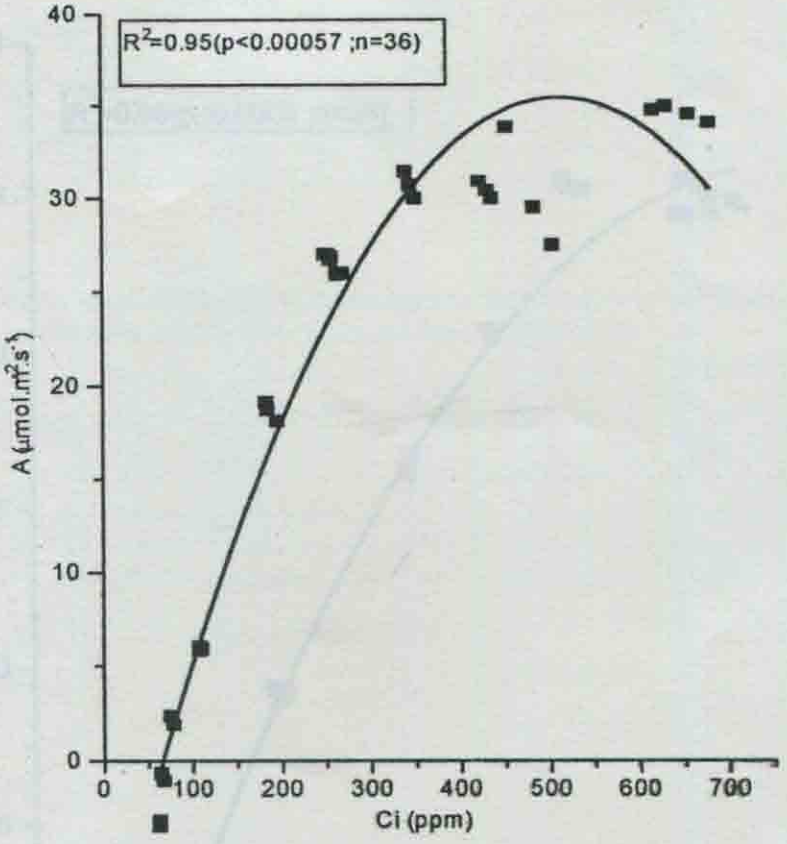


Fig. 38 c: CO_2 response curve in APC 123-V-683

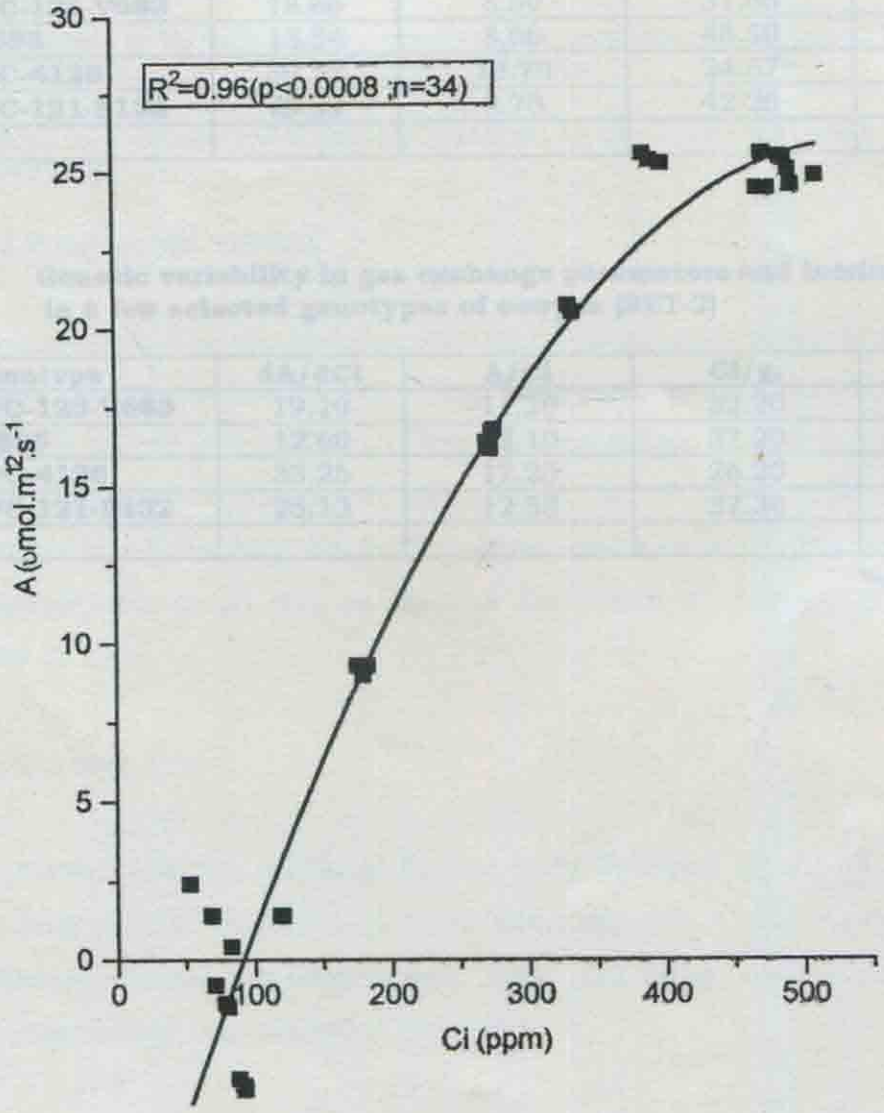


Fig. 38 d: CO_2 response curve in ACP 121-P-132

Table-7: Genetic variability in gas exchange parameters and intrinsic WUE in a few selected genotypes of cowpea (SET-1)

Genotype	dA/dCi	A/Ci	Ci/g_s	A/g_s
APC-123-V683	18.66	8.50	31.66	2.50
V-585	13.54	8.60	48.50	2.20
APC-4125	30.53	13.70	24.57	5.20
APC-121-P132	19.32	9.70	42.26	5.10

Genetic variability in gas exchange parameters and intrinsic WUE in a few selected genotypes of cowpea (SET-2)

Genotype	dA/dCi	A/Ci	Ci/g_s	A/g_s
APC-123-V683	19.10	11.20	32.20	3.01
V-585	12.60	13.10	37.20	2.50
APC-4125	33.25	17.25	26.20	4.65
APC-121-P132	25.13	12.58	37.30	4.32

strong negative correlation between dA/dCi and Ci/g_s was also observed among the genotypes (Fig. 39). We conclude that:

- Variations in dA/dCi exits in cowpea. The genotype, which had high water use and WUE also, showed high dA/dCi character.
- There is a considerable genetic variation in gas exchange traits (A/g_s, Ci/g_s, A/Ci etc).

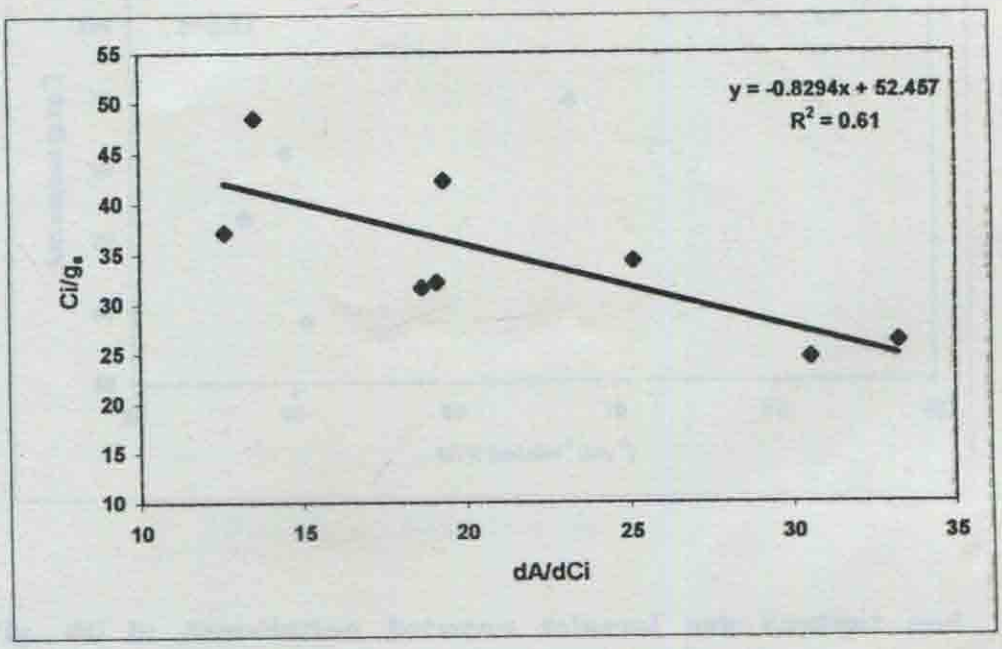
MTR and mineral ash content

In this experiment also an attempt was made to quantify mineral ash content. Significant genetic variability exits among cowpea genotypes, APC 40-GC-20 accumulated higher ash content (158g.Kg^{-1}) compared to other genotypes and relatively lower ash content was determined in APC-121-P132 (89g.Kg^{-1}). A strong positive relationship between ash content and MTR (Fig. 40a) signifies that mineral ash could act as one of the traits in assessing the genetic variability in T.

Ash content and $\Delta^{18}O$

Since a strong positive relationship was seen between MTR and mineral ash content and also MTR had a positive linearity with $\Delta^{18}O$, we examined the association of mineral ash content with $\Delta^{18}O$. Though a positive trend was seen but the relationship was scattered (Fig. 40b).

Fig. 39: Relationship between intrinsic carboxylation and mesophyll efficiency in selected genotypes of cowpea



Using the gas exchange equipment (CIRAS-1), CO_2 response curves were developed to deduce dA/dCi. Apart from this other gas exchange parameters like, A, g_s and Ci were also measured and the ratio of Ci/g_s was computed.

Fig. 40 a: Relationship between transpiration rate and mineral ash content in selected genotypes of cowpea

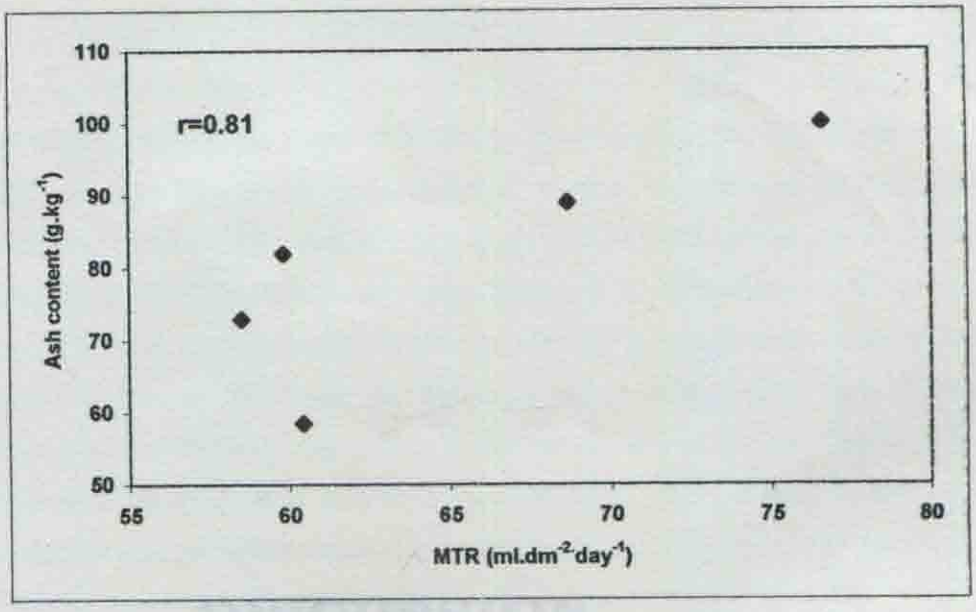
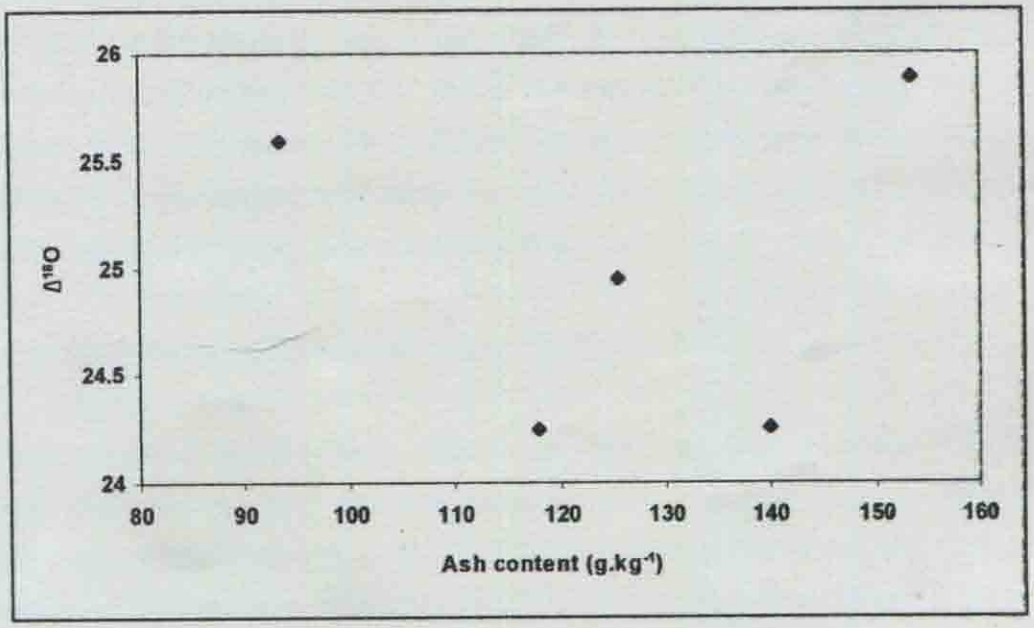


Fig. 40 b: Association between mineral ash content and $\Delta^{18}O$(‰) in biomass in cowpea



MTR was determined gravimetrically. The leaves that developed during experimental period were collected and analyzed for ash content and $\Delta^{18}O$.

DISCUSSION

DISCUSSION

Maximizing the efficiency of biomass production per unit amount of water transpired (WUE) has been a major research focus of plant scientists, especially under tropical conditions (Passioura, 1986). Increase in WUE is often achieved through a reduction in water loss. Though this is an important water conserving strategy evolved by plants, due to a strong relationship between total water loss and biomass production, selecting for high WUE often resulted in low biomass production (Matus, *et al.,* 1995; Udaya Kumar, *et al.,* 1998a). Therefore, the significant genetic variability in WUE among several crop species has not been successfully exploited. To increase breeding success for high WUE one needs to adequately analyze the significance of physiological traits that regulate WUE.

The two important traits that can be attributed for the variability in WUE are photosynthetic rate (A), responsible for the dry matter production and the transpiration rate (T). While the stomatal factors determine T at a given VPD, the carbon assimilation rate is determined by both stomatal diffusive characteristics (supply function) and the intrinsic mesophyll efficiency to fix carbon (capacity function).

Inter-dependence of Transpiration and WUE:

Since stomatal conductance determines the exchange of water vapour and CO_2 between the ambient environment and the plant, a strong interdependence between T and WUE often exists. In order to enhance breeding success for improved WUE, it is essential to identify plant genotypes in which this interdependence is weaker (Udaya Kumar *et al.,* 1998a). This necessitates the determination of genetic differences in both T and g_s and WUE.

Carbon isotope discrimination during photosynthesis ($\Delta^{13}C$) has been well established, as a time averaged surrogate for WUE (Farquhar and Richards, 1984). Several others have successfully screened large number of genotypes for variations in WUE by this approach (Hubick, *et al.,* 1994: Hall, *et al.,* 1996; Wright *et al.,* 1983; Rao, *et al.,* 1995, Ashok, *et al.,* 1999).

Transpiration rate on the other hand is a highly dynamic physiological process strongly influenced diurnally and seasonally by VPD, light intensities, temperature etc. Further the ontogenic changes make the time-integrated estimate of T and g_s rather difficult. However, gas exchange approach is often adopted to determine the differences in T and g_s. Being time instantaneous measures, the gas exchange parameters do not sufficiently explain the diurnal and seasonal variations in T and g_s. Although gravimetric method can be employed for time-integrated measurement of T, this procedure is laborious and cannot be adopted for large-scale screening.

Recent studies indicate that the enrichment of the heavy isotope of oxygen (^{18}O) in leaf water and further in the leaf biomass could hold the potential as a rapid surrogate for the determination of transpiration rate and g_s. The major objective of this study was to assess the relevance of ^{18}O enrichment as a potential approach to determine T/ g_s and examine the feasibility of a dual isotope technique (^{13}C and ^{18}O) to identify the desirable genotype where the interdependence between T and WUE is weak.

Series of experiments were conducted to examine the relationship of oxygen isotope enrichment with both g_s and transpiration. The ^{18}O enrichment in leaf water was determined in plants growing under different VPD, by altering the g_s and in selected genotypes intrinsically differing in g_s. ^{18}O enriched water in the leaf makes an entry into intermediates of metabolic pathway leading to synthesis of

cellulose/biomass (Sternberg, *et al.*, 1986). Hence, in this investigation, we examined the composition of ^{18}O in leaf biomass to assess the variability in transpiration rate across the species/genotypes.

Besides the stable isotope ratios, gas exchange and gravimetric approaches were also employed for the determination of WUE and the physiological traits associated with it (to quantify intrinsic WUE (A/g_s), and mesophyll efficiency (dA/dCi) among the selected genotypes). In addition, plant mineral ash content was also quantified as an indirect measure of transpiration integrated over experimental period.

The results obtained in these experiments are discussed in this chapter.

VPD induced changes in transpiration rate (T) altered the $\Delta^{18}O$ in leaf water

In this investigation, the ^{18}O enrichment in leaf water in a few crop species (both C_3 and C_4) grown under different VPD was examined.

Transpiration rate increased in all the species at higher VPD, though the species differed in the extent of response to VPD. The $\Delta^{18}O$ of the leaf water closely followed the increase in VPD (Figs. 3a, 3b and 3c).

In drier environments, the differences in transpiration rate are predominantly determined by the evaporative demand. Since transpiration rate is known to increase with VPD at any given g_s, the higher $\Delta^{18}O$ of leaf water at high VPD could be expected to match the changes in transpiration rate. To prove that evaporation of water results in ^{18}O enrichment, the oxygen isotope ratios of water taken in metallic cups and exposed to different VPD was analyzed. The extent of evaporation closely matched the $\Delta^{18}O$.

$\Delta^{18}O$ in leaf water was related to g_s induced changes in T

Transpiration rate is determined both by the intrinsic g_s and the existing VPD. To study the effect of g_s on ^{18}O enrichment we altered the g_s by several approaches viz., *partial defoliation, partial shading, ABA induced reduction in g_s and transgenic tobacco plants over producing cytokinins*

Altered stomatal conductance

The partial defoliation resulted in increased g_s, whereas partial shading led to a reduction in g_s. Leaf water ^{18}O enrichment was high in first case and was low in the latter case. The transgenic tobacco plants recorded high g_s compared to wild (control) type and also showed high leaf water ^{18}O enrichment than wild plants.

In all the experiments, a strong positive relationship between g_s and $\Delta^{18}O$ was observed (Fig. 6) suggesting the role of g_s in determining $\Delta^{18}O$ in leaf water.

To prove this point further, differential stomatal closure was achieved by dipping the petioles of excised leaves in different concentrations of abscissic acid (ABA). The leaf kept in the highest concentration of ABA (10^{-4} M) recorded the lowest T (Fig. 5). Since the leaves of all ABA treatments were kept in the same environment (light intensity 1300 µmol. m^{-2} s^{-1}; RH=60% and air temperature of 28°C), the only factor determining the variability in transpiration rate was the ABA induced differences in stomatal conductance (g_s). Therefore, the changes in the ^{18}O composition of leaf water should be a function of g_s.

Genotypes intrinsically differing in g_s

In a few selected genotypes of cowpea intrinsically differing in growth rates, the transpiration rate on a specific day (49th and 50th DAS) was

analyzed gravimetrically. A strong positive relationship (R^2=0.83) exits between transpiration rate and $\Delta^{18}O$ of the leaf water. If the T of a specific day is considered as a time-averaged estimate of g_s, the result indicated that the observed $\Delta^{18}O$ in leaf water is determined by the intrinsic stomatal conductance.

The bulk leaf water ^{18}O composition was seen to increase in proportion with transpiration rate and stomatal conductance (g_s). A strong positive correlation between $\Delta^{18}O$ in leaf water and the T suggests that the $\Delta^{18}O$ is indeed a function of leaf transpiration rate and g_s. These experiments clearly suggest that both transpiration rate and stomatal conductance determine the $\Delta^{18}O$ of the bulk water. Under natural conditions with diurnally varying VPD, the intrinsic g_s and the existing VPD of a given day would determine the T of a plant. A positive correlation between $\Delta^{18}O$ and T indicates that differences in T or g_s can be estimated by $\Delta^{18}O$.

Quite a bit of disagreement and confusion exist in the literature on the understanding of the ^{18}O enrichment concept in plants. The existing environmental conditions such as VPD, light intensity and air as well as leaf leaf temperature have been shown to differently influence the $\Delta^{18}O$ of leaf water (Dongmann, *et al.*, 1974 and Flanagan, *et al.*, 1993). Since environmental parameters affect the $^{18}O/^{16}O$ ratios, one immediate application of the $\Delta^{18}O$ has been to determine the VPD of the atmosphere in which the vegetation grew.

The $^{18}O/^{16}O$ ratios of the bulk leaf water and chloroplast water depend primarily on the leaf to air VPD. The larger the gradient the higher the ratio of $^{18}O/^{16}O$ in leaf water (Flanagan *et al.*, 1993, 1994). Earlier Walker *et al.*, (1989) studied the pattern of ^{18}O enrichment in leaf water as influenced by atmospheric turbulence, resulting an alteration in boundary layer conductance characteristics, isotopic composition of source water (feed water) as well as the atmospheric water vapour. Walker and Lance (1991) observed a specific diurnal pattern of change in

$\Delta^{18}O$ of leaf water in association with RH of air and transpiration rates. I recomputed same data from this work and observed a strong positive correlation between $\Delta^{18}O$ in leaf water and transpiration rate (R^2=0.84). This suggests that the leaf water ^{18}O could indeed be a reflection of the transpiration rate. When transpiration was high, Sternberg, *et al.*, (1984) have reported, the lighter isotope of oxygen having high diffusion gradient coupled with greater vapour pressure escapes early out of leaf surface.

Farquhar and Lloyd (1993) opined that application of the Craig and Gordon model evaporative enrichment model for predicting transpiration might not be straightforward. The explanation being, $H_2{}^{18}O$ molecules in the leaf would likely to diffuse away from sites of evaporational enrichment to other tissues of the leaf. The convection stream of the unfractionated stem (or xylem) water would oppose this diffusion effect resulting overtime in the bulk leaf water, ^{18}O composition is to tend more or less towards the stem water (Farquhar, *et al.*, 1998). Hence a negative relationship would be expected between leaf/chloroplast water (at evaporative enrichment site i.e., sub-stomatal cavity) ^{18}O and transpiration rate influenced by leaf temperature and gs when plants were grown under controlled condition in glass house (Farquhar, *et al.*, 2000; Barbour, *et al.*, 2000a). They proposed a simple alternative model to describe the evaporational enrichment model of leaf water as influenced by transpiration rate.

Although the Craig and Gordon model and the subsequent models developed by others predict an increase in ^{18}O enrichment compared to the source water during transpiration, no unequivocal proof has been documented yet. We provide strong experimental evidences that a positive relationship exists between $\Delta^{18}O$, T and g_s in a number of experiments.

We discuss here, the present understanding of the ^{18}O enrichment during transpiration and how far the accepted theory still supports our findings.

Processes determining $^{18}O/^{16}O$ in leaf water

Fractionation associated with evaporation

- $H_2{}^{18}O$ has lower vapour pressure than $H_2{}^{16}O$, which is denoted by e^* (equilibrium fractionation) the proportional depression of isotopic equilibrium vapour pressure by the heavier isotope of oxygen
- $H_2{}^{18}O$ diffuses slowly: $H_2{}^{16}O$ diffusion is 1.028 times faster that of $H_2{}^{18}O$. This is referred to as kinetic fractionation (e_k). It is 28‰ for diffusion in air or through stomata and 19‰ through boundary layer

$$\Delta_E = [R_E/R_S - 1]$$

Δ_E – isotopic composition at the site of evaporation
R_E – $^{18}O/^{16}O$ ratio of water at the site of evaporation
R_s - $^{18}O/^{16}O$ ratio of source (stem) water

Based on this original evaporational enrichment model of Craig and Gordon, Farquhar and Lloyd (1993) simplified to derive a simple and straightforward model to predict the enrichment of ^{18}O of water at the evaporating site (Δ_E)

$$\Delta_E = \varepsilon^* + \varepsilon_k + (\Delta_v - \varepsilon_k)\, ei/ea$$

ε^* - Equilibrium fractionation
ε_k – Kinetic frationation
Δ_v – $^{18}O/^{16}O$ ratio of atmospheric water vapour compared to SMOW
ea and ei are water vapour pressures of atmospheric water and intercellular spaces

ε^* - Temperature dependent fractionation, which is 9.2‰ at 20°C and 9.6 ‰ at 25°C (Bottinga and Craig, 1969).

ε_k – through the stomata and boundary layer at any given stomatal resistance is calculated as follows:

$$\varepsilon_k = \frac{28.5\, r_s + (28.5)^{1/3} r_b}{r_s + r_b}$$

assuming the fractionation through the boundary layer is one-third function of the stomatal resistance.

Although this model predicts a higher enrichment of $H_2{}^{18}O$ at the evaporative site (sub-stomatal cavity), a large discrepancy between the enrichment at the site of evaporation and bulk leaf water was often noticed (Flanagan, *et al.*, 1991). Farquhar and Lloyd (1993) explained this discrepancy was caused by the **Péclet effect** ($\wp$), which is defined as,

$\wp$ = **EL/CD**

where E – evaporation/transpiration rate expressed in $mol.m^{-2}s^{-1}$

L- effective path length (l) of water flow in mesophyll tissue, which is 10^2 to 10^3 times the actual length

C- density of water ($5.55x10^4 mol.m^{-3}$)

D- diffusivity of $H_2{}^{18}O$ in water ($2.66x10^{-9} m^{-2}.s^{-1}$).

The model assumes at transpiration rate equals to flow rate of un-fractionated stem water (E). Thus the equation for predicting the ^{18}O enrichment in the bulk leaf water becomes:

$$\Delta^{18}O_L = \frac{\Delta^{18}O\,(1-e^{-\wp})}{\wp}$$

The péclet effect is more sensitive to evaporation (or transpiration) as other parameters in the equation are physical constants. Although the effective length through which water diffuses through the leaf is a complex parameter, it can also be assumed as a constant for a given leaf. Therefore, the model predicts lower ^{18}O enrichment in the bulk leaf water as E (or T) increases. Barbour *et al.*, (2000a) have provided experimental

evidences for the existence of péclet effect in castor bean plants grown under controlled environmental conditions. Thus, the model predicts that the bulk leaf water $\Delta^{18}O$ tend towards the $\Delta^{18}O$ of source water (Farquhar and Barbour, 1998).

The theory of Craig and Gordon (1965) predicts that ^{18}O gets enriched in water as evaporation increases. This enrichment heavily depends on the temperature at the evaporative site. Similarly, Farquhar and Lloyd's revised model predicts an increase in ^{18}O composition of water at the evaporating site as a function of leaf temperature. Under steady state conditions, any increase in leaf temperature results in an increase in the vapour pressure of water at the leaf inter-cellular spaces. This enhances the evaporation or transpiration leading to an isotopic enrichment (Gillon and Yakir, 2000). Barbour *et al.,* (2000a) later showed that the leaf temperature increases under conditions when the stomata are closed. Thus they noticed a strong inverse relationship between g_s and $\Delta^{18}O$.

Further Barbour *et al.,* (2000b) provided experimental evidences for the existence of a Péclet effect which is responsible for the increased discrepancy between the oxygen isotope ratio predicted by the theory and the observed ratio of bulk leaf water. These results in a way contradict our present findings.

The Craig-Gordon theory as well as the one developed subsequently to explain the leaf water evaporative enrichment assumes an isotopic steady state (ISS). Under an ISS, the ^{18}O signatures of the transpired water stream will be equal to that of the xylem or source water. Though such an equilibrium state can be reached with in about 2-3 hours (Flanagan, *et al.,* 1993), no such steady state can be attained under natural conditions (Harwood, *et al.,* 1998). Recently Roden and Ehleringer (1999) demonstrated that an ISS might not be mandatory for explaining the ^{18}O enrichment in leaf water.

An ISS can be attained in a precisely controlled environmental condition. But under diurnally varying VPD, the attainment of ISS is usually delayed till late afternoon (Harwood, *et al.,* 1998). Under natural conditions, plant transpiration rate is controlled b the intrinsic stomatal conductance during the cooler morning hours, while the control of transpiration would be predominantly by the VPD during the afternoons.

However, under natural conditions, with the diurnal changes in a number of environmental parameters, ISS would not be reached until late in the afternoon (Harwood *et al.,* 1998). Dynamic responses of the plant physiological processes to the changing environmental variables have been well documented. A reduction in the stomatal conductance towards afternoon is normally associated with an increase in the VPD. This physiological adjustment to the diurnally changing environmental conditions could delay the development of an ISS. In such diurnally changing environments, differences in T are brought about by g_s during the cooler hours of the morning and a larger vapor pressure gradient during the warmer afternoons. In other words, T can still be high despite a stomatal closure at high VPD. Therefore the positive relationship between T and $\Delta^{18}O$ in our studies is imminent under natural conditions (Bindu Madhava *et al.,* 1999).

Biomass ^{18}O composition – Its relevance

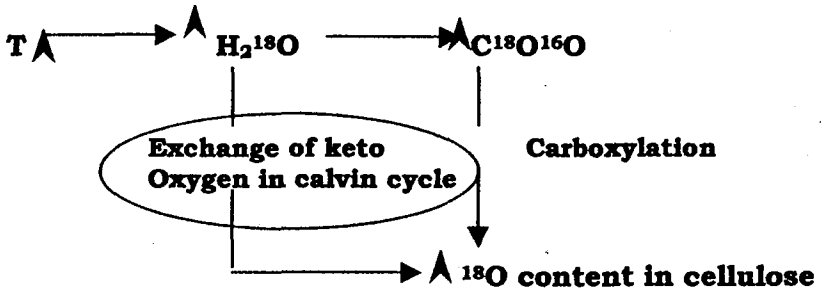
The experiments discussed above clearly indicate that $\Delta^{18}O$ has a strong positive association with g_s and T. The enrichment of leaf water with the heavy isotope of oxygen depends on the variation in g_s and T. These physiological parameters are strongly regulated by the existing environmental condition such as VPD that constantly change both on diurnal and seasonal scales. Therefore, though $\Delta^{18}O$ in leaf water is related to g_s, this parameter is time instantaneous hence, cannot be applied to transpiration rate occurred over a period of time. For

identifying genotypes that differ in T and g_s, we used a more time-averaged estimate for these two parameters.

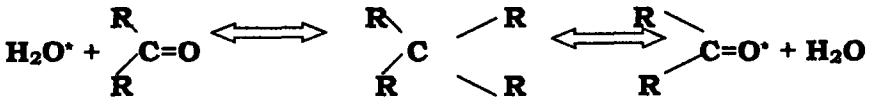
The ^{18}O signatures of leaf water find their path into the biomass through the metabolic cycles of photosynthesis and biomass formation. Thus it can be expected that the $\Delta^{18}O$ in leaf water would determine the $\Delta^{18}O$ of leaf biomass.

The theory

Oxygen isotope ratio of plant cellulose/biomass is determined by isotopic exchange occurring during hydration of carbonyl groups of the intermediates of cellulose synthesis (Sternberg, *et al.*, 1986).



Chemically,



(Carbonyl oxygen exchange with water)

Oxygen in other functional groups, such as hydroxyl, carboxyl and phosphate are not exchangeable under temperature and pH ranges found in plants (Sternberg *et al.*, 1986). The fractionation factor of carbonyl oxygen exchange with water has been measured in acetone to be 1.028 at 25°C (Sternburg and DeNiro, 1983) and which is constant in all molecules having carbonyl oxygen.

Berry *et al.*, (1978) fed labeled oxygen to leaves and observed that its presence in 3-PGA indicated that it probably enters the photosynthetic carbon reduction cycle. DeNiro and Epstein (1979) confirmed that it is the water in the plant that determines the ^{18}O signature of cellulose,

suggested that there could be exchange between the oxygen of glceraldehade phosphate and the oxygen of water. Sternberg and DeNiro (1983) suggested that the carbonyl oxygens of di-hydro acetone phosphate (DHAP) in the Photosynthetic Carbon Reduction cycle exchange with water. Any or all of these mechanisms could operate to transfer the leaf water ^{18}O signature to biomass. However, about 45% of the ^{18}O signal from sugars is found to be lost before it reaches cellulose, presumably exchanged with water or lost by respiratory metabolism (Sternberge, *et al.,* 1986). Farquhar and Barbour (1998) developed a theoretical model to predict the fractionation of ^{18}O during cellulose biosynthesis.

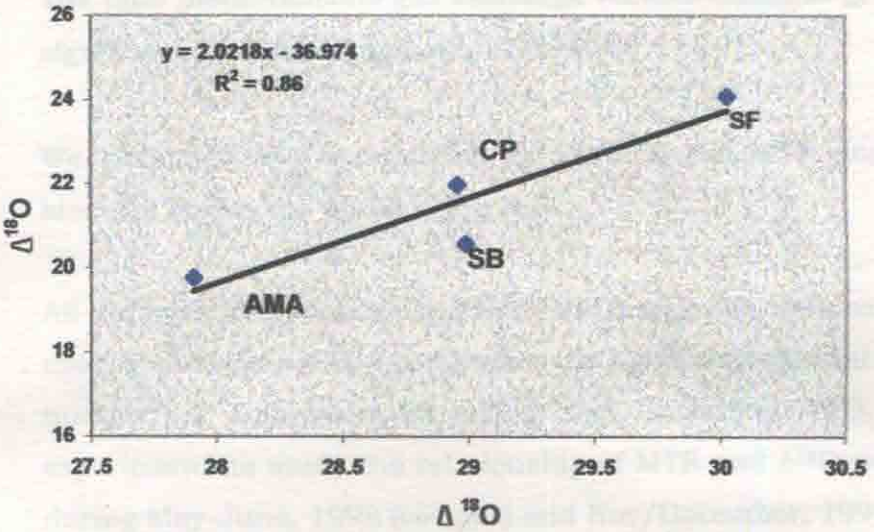
However $\Delta^{18}O$ in leaf water should be related to $\Delta^{18}O$ in the leaf biomass. We examined this relation by pooling the data of several experiments where both $\Delta^{18}O$ in leaf water and leaf biomass was determined. A positive relationship suggests that inspite of losses of ^{18}O during respiration, high ^{18}O enrichment in leaf water results in $\Delta^{18}O$ in leaf biomass (Fig. 41).

Since, biomass formation is a process that occurs over a longer period of time, the $\Delta^{18}O$ signatures of the biomass gets integrated over that period of time. We measured the oxygen isotope ratios of the leaf biomass by direct pyrolysis.

$\Delta^{18}O$ in leaf biomass is related to mean transpiration rate

Determination of the mean transpiration rate (MTR) by gravimetric approach in our investigation integrates diurnal variations in transpiration caused by differences in g_s and VPD. Thus, the MTR is a more time-averaged estimate of stomatal conductance. Across the genotypes of cowpea, sunflower and groundnut, a strong positive association of MTR with $\Delta^{18}O$ of leaf biomass indicates that the differences in the MTR can be accurately predicted by $\Delta^{18}O$ of biomass.

Fig. 41: Relationship between $\Delta^{18}O$ (‰) in leaf water and leaf biomss across the crop species



Plants of all the four species were maintained in pots for 35-40 days. Daily water loss was quantified gravimetrically. In one set of plants the leaf water was extracted and analyzed for $\Delta^{18}O$. In another set of plants, the leaves developed during the experimental period were harvested at the end of the experiment, and analyzed for $\Delta^{18}O$.

The time instantaneous gas exchange measurement of g_s also showed a significant positive relationship with $\Delta^{18}O$.

We examined this association by plotting the MTR and $\Delta^{18}O$ in leaf biomass across the species (Fig. 42).

All the experiments described in this investigation were conducted under natural environmental conditions with significant diurnal variations in a number of environmental parameters including VPD. Two major experiments to study the relationship of MTR and $\Delta^{18}O$ were carried out during May-June, 1998 (cowpea) and Nov/December, 1999 (Groundnut). During the experimental period the VPD increased from 10 mbar to ~30 mbar for cowpea and from 8mbar to 20 mbar for groundnut. Under such conditions, the transpiration rates are modulated by the intrinsic stomatal conductance during the cooler morning hours and by the high VPD during the later part of the day. Therefore, a specific diurnal pattern in the changes in T and gs can be expected on any given day. In most plant species, stomatal factors are more sensitive to changes in VPD than the carbon assimilation rate, especially in cowpea and groundnut. Despite the disagreement that exists in the literature, we found a strong positive correlation between T and $\Delta^{18}O$.

Since stomatal factors regulate both carbon exchange and transpiration rate and the water enriched with ^{18}O during transpiration transfers their signals to the biomass, hence, the ^{18}O signals of the biomass could be expected to reflect the differences in g_s as well as transpiration.

However, a number of research documents from Graham Farquhar's lab (Farquhar *et al.*, 1998; Farquhar and Barbour, 2000; Barbour, *et al.*, 2000a, 2000b) largely indicated a negative relationship between g_s and $\Delta^{18}O$. The steady-state model for the $\Delta^{18}O$ in leaf water strongly depends on the vapour pressure of water in the inter-cellular spaces, which is primarily determined by the leaf temperature. Further, their model also

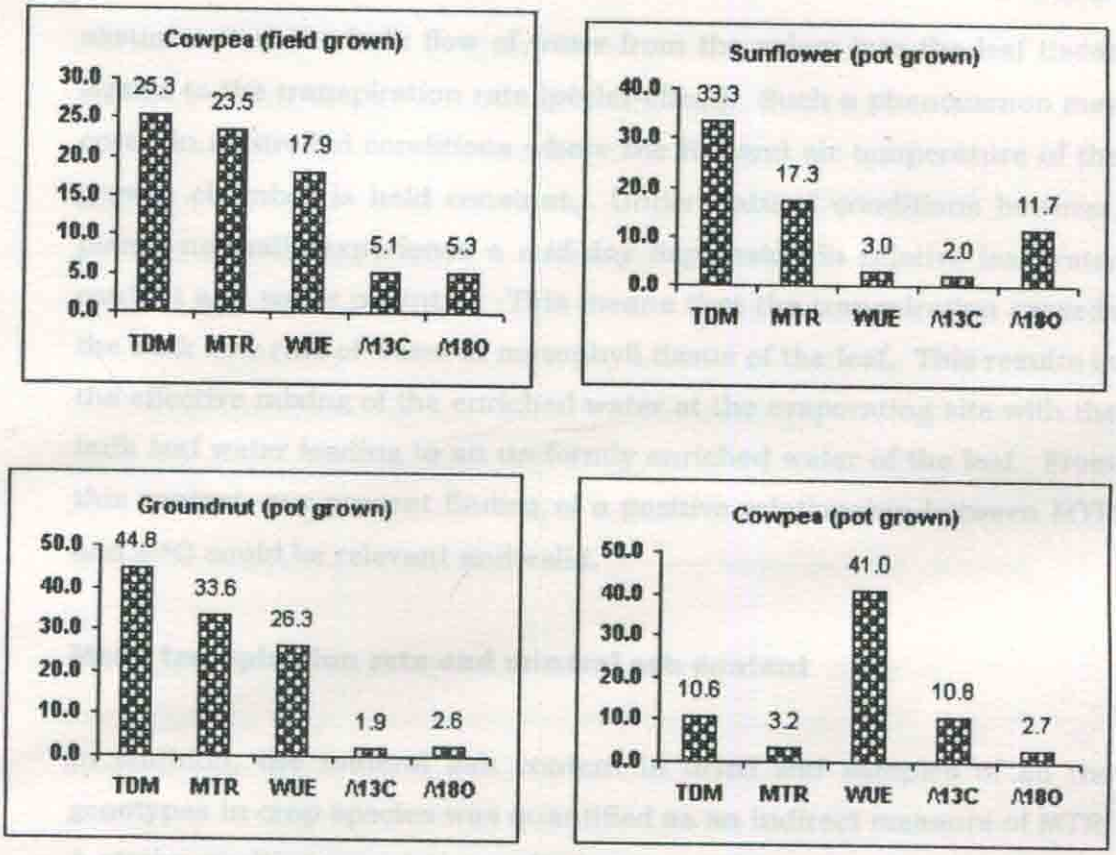


Fig. 42: Percent increase TDM, MTR and WUE in genotypes with high ^{13}C and ^{18}O composition compared to genotypes with low composition.

assumes that the bulk flow of water from the xylem into the leaf tissue equals to the transpiration rate (péclet-effect). Such a phenomenon may occur in controlled conditions where the RH and air temperature of the growth chamber is held constant. Under natural conditions however, plants normally experience a mid-day depression in relative leaf water content and water potential. This means that the transpiration exceeds the bulk flow rate of water in mesophyll tissue of the leaf. This results in the effective mixing of the enriched water at the evaporating site with the bulk leaf water leading to an uniformly enriched water of the leaf. From this context, our present finding of a positive relationship between MTR and $\delta^{18}O$ could be relevant and valid.

Mean transpiration rate and mineral ash content

In addition, the mineral ash content in dried leaf samples of all the genotypes in crop species was quantified as an indirect measure of MTR. A strong positive association exits between MTR and ash content (Figs. 22, 30), suggesting the possibility of employing mineral ash content as an initial indicator of transpiration. Similar observations were recorded by Masle *et al.,* (1992) in wheat, barley and tobacco and by Brown *et al.,* (1997) in peanut and pearl millet.

Identification of genotypes with high WUE and relatively high transpiration based on dual isotope approach

Among a number of adoptive strategies evolved by plants, WUE in most relevant especially under rainfed agro-ecosystems. Realizing the importance of WUE in crop improvement, several attempts were made to assess the genetic variability in this trait by adopting gravimetric or gas exchange approaches and more recently using the CID as a surrogate for WUE (Farquher and Richards, 1984; Hubick, *et al.,* 1985; Wright, *et al.,* 1988; Roy Stephan, 1995; Hubick and Farquhar, 1989; Ayman, 1999; Anil Koushik, 1999; Ashok *et al.,* 1999).

The significant genetic variability in WUE (Ashok *et al.*, 1999; Rao *et al.*, 1995) with high broad sense heritability (Hall and Ismail, 1986, 1990) and a low G x E interaction renders WUE a potential physiological trait amenable for crop improvement. From the agronomic point of view any trait will be worthwhile for crop improvement only if it is associated with superior growth rates (Uday Kumar *et al.*, 1998a; Passioura *et al.*, 1986). However, there has been a limited success in considering WUE trait for crop improvement program, though significant genetic variability was reported. A concomitant reduction in the total biomass while selecting for high WUE was perhaps the major reason for the lack of enthusiasm among breeders to include WUE in their crop improvement program.

A strong interdependence between TDM and total water use normally exists among crop plants. Hence any reduction in water use, though may increase WUE, could result in an unavoidable reduction in biomass production. From this context, it is important to identify genotypes where the interdependence between WUE and T is either weak or non- existent. Therefore, simultaneous determination of WUE as well as T is important.

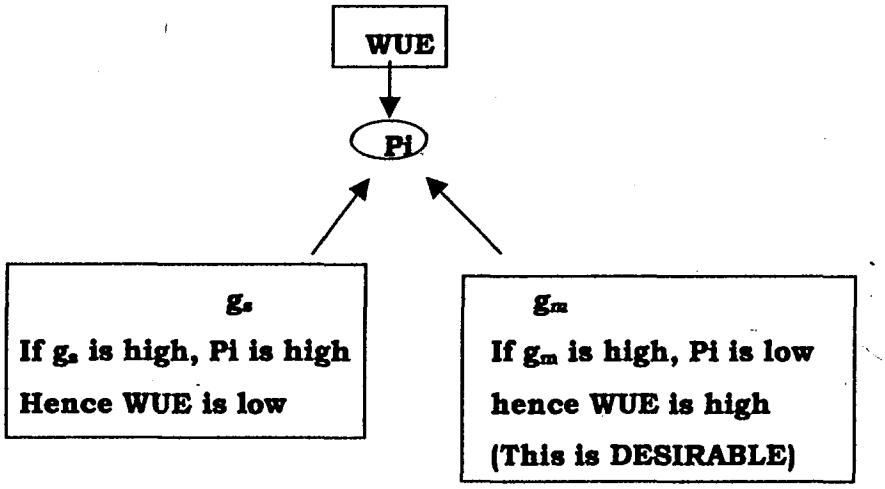
In this investigation experimental evidences were provided to show that the $\Delta^{18}O$ of leaf biomass could be used as a potential surrogate for the estimation of T and genotypes. In addition, the feasibility of an approach based on the composition of both the isotopes ($\Delta^{13}C$ and $\Delta^{18}O$) was examined to identify the desirable genotypes where the interdependence between T and WUE is weaker. To achieve this, we analyzed the standardized normal distribution plot between $\Delta^{13}C$ and $\Delta^{18}O$ (Z-transformation) in sunflower, groundnut and cowpea (Figs.18, 27, 34)

Based on the distribution of points, genotypes having high $\Delta^{18}O$ (high T) and low $\Delta^{13}C$ (high WUE) were selected. Several growth and physiological parameters were compared with the genotypes that had low $\Delta^{18}O$ and low $\Delta^{13}C$. We hypothesized that high $\Delta^{18}O$ types coupled with low $\Delta^{13}C$ would have an advantage of high TDM and high WUE.

We computed the **percent increase in** TDM, MTR, NAR and WUE in such group of genotypes **compared to the ones** with high $\Delta^{13}C$ and low $\Delta^{18}O$ in biomass (Fig. 42). In **all the species** (cowpea, sunflower and groundnut), the genotype which **had low $\Delta^{13}C$ and high** $\delta^{18}O$ showed a higher percent increase in **all associated physiological** parameters than the genotypes which had **high** $\Delta^{13}C$ **coupled with low** $\Delta^{18}O$ in leaf biomass. This amply illustrated the potential that the dual isotope discrimination helps in identifying the desirable genotypes for further crop improvement.

Importance of mesophyll capacity types

For a genotype to have high WUE despite relatively high T such types should be complemented with a superior capacity of the mesophyll to fix carbon. i.e.,



Determination of the mesophyll efficiency (g_m) is rather difficult. However, several indirect approaches such as the initial slope of CO_2 response curve (Caemmerer and Farquhar, 1981), the ratio of the A to Ci, function associated with RuBisCO (Henderson *et al.*, 1998; Mote *et al.*, 1998) are often used. Recently the ratio of Ci to g_s was also suggested as a good indication of mesophyll efficiency (Sheshshayee *et al.*, 1996; Krishnaprasad *et al.*, 1996). In several contrasting genotypes of cowpea

significantly differing in $\Delta^{18}O$, T, WUE, we measured the dA/dCi and Ci/g_s to determine the mesophyll efficiency.

Genotypes differed significantly in the mesophyll capacity characters. The genotype APC 4125 that had highest $\Delta^{18}O$ content among the cowpea genotypes showed high g_m (Fig. 43). APC 4125 also had relatively low $\Delta^{13}C$ (high WUE).

On the whole, from the results obtained in this investigation the following broad inference can be drawn:

- *$\Delta^{18}O$ is positively related to MTR and g_s*
- *Genotypes with high $\Delta^{18}O$ and low $\Delta^{13}C$ recorded high percentage increase in TDM and other physiological traits*
- *In genotypes with high $\Delta^{18}O$ and low $\Delta^{13}C$, the inter-relationship between WUE and T was weak, and such genotypes had high mesophyll capacity*
- *We hypothesized and provided experimental evidences, for the first time, that the dual isotope ($\Delta^{13}C$ and $\Delta^{18}O$) composition is indeed a powerful surrogate for identifying the desirable mesophyll capacity types among crop species*

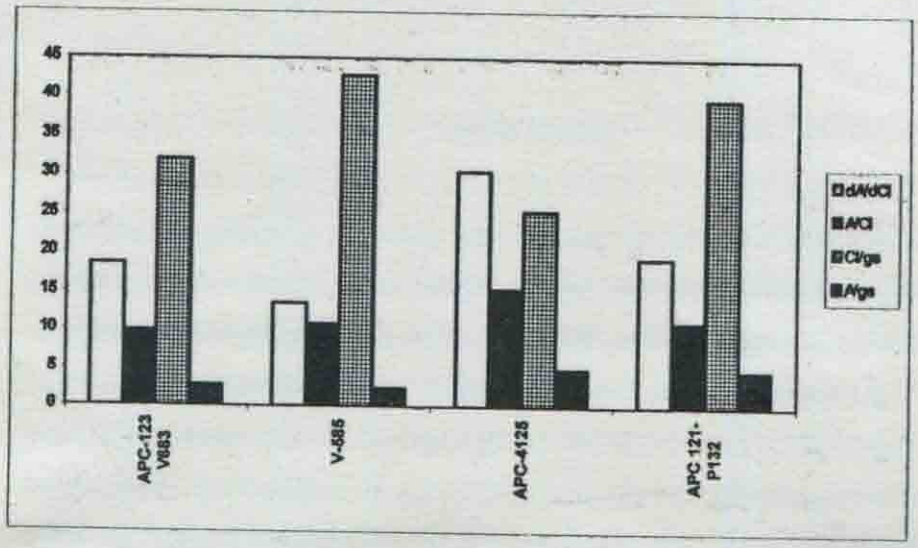


Fig. 43: Comparison of gas exchange characteristics among the selected cowpea genotypes

The gas exchange data obtained from the four different genotypes of cowpea were compared along with dA/dCi value.

SUMMARY

SUMMARY

Water availability is a major constraint for crop productivity especially under tropics. Among the naturally evolved adoptive strategies superior water harnessing characters associated with root system and the efficient use of water for biomass production are most noteworthy. Normally plants increase the WUE through a reduction in T. Although this is an important water conserving mechanism, due to a strong interdependence between total transpiration and TDM production, any increase in WUE is often associated with reduced dry matter. From the agronomical point of view this is not desirable.

To increase the success of breeding program through improving WUE it is essential to identify genotypes where the interdependence between T and WUE is weak. This necessitates simultaneous determination of T and WUE.

Carbon isotope discrimination ($\Delta^{13}C$) that occurs during photosynthesis has been well established as a potential surrogate to identify high WUE types (Farquhar and Richards, 1984; Farquhar et al., 1989). Significant progress in assessing genetic difference in WUE was possible with advent of this rapid technique. However, no such rapid techniques were available to determine the genetic difference in T. Though gravimetric and gas exchange approaches are often adopted for the determination of T and stomatal conductance (g_s) these methods are either cumbersome or time instantaneous. In this context, it is therefore essential to evolve a suitable technique for rapid and time integrated estimation of T and g_s.

Because of the slower diffusivity and lower vapor pressure of $H_2{}^{18}O$ compared to $H_2{}^{16}O$, water molecule with heavier isotope of oxygen hasbeen shown to get enriched in water during evaporation (Craig and Gordon, 1965; Flanagan and Farquhar, 1993). Transpiration being an

evaporative process, it is possible that ^{18}O enrichment in the leaf water could be associated with the difference in T and g_s at a given VPD.

Therefore, the major objective of this investigation was to study the relevance of ^{18}O enrichment as an estimate of T and g_s and to quantify the physiological traits associated with WUE using a novel approach based on the composition of carbon and oxygen isotopes.

Standardization of leaf extraction protocols and determination of $\Delta^{18}O$.

Initially, extraction of leaf water and the determination of ^{18}O signatures were standardized. Briefly, 50 leaf punches were frozen to liquid nitrogen temperature and thawed on a hot water bath to disrupt the cell membranes. On centrifugation the leaf water that collected at the bottom of the tapering plastic tube was drawn using a syringe and stored in vaccutainer tubes. A CO_2 water equilibration method developed by Scrimgrover (1995) was adopted with modifications to estimate ^{18}O signature in water. A known volume of CO_2 gas was introduced into the vaccutainers and equilibrated for 15 h at 28°C. The oxygen isotopic composition of CO_2 gas after equilibration was determined and expressed against vSMOW. The headspace CO_2 gas after equilibration was drawn through a helium carrier gas by a gas autosampler and introduced into the IRMS after scrubbing for water vapour. The signals for 44 and 46 mass ratios were amplified to determine the $^{18}O/^{16}O$ ratio of the water.

After standardizing the protocol for the determination of ^{18}O in water sample, a series of experiments were conducted to assess the enrichment of ^{18}O in leaf water. The salient findings of these experiments are summarized in this chapter.

^{18}O enrichment during transpiration

Transpiration rate and g_s were altered either by changing the VPD or altering the g_s by ABA. Further genotypes significantly differing in T were studied.

Transpiration rate increased with an increase in VPD in all the species. The oxygen isotope signatures in leaf water showed a progressive increase with increase in VPD. Alterations of g_s in sunflower leaves by dipping the leaf petioles in different concentrations of ABA closely matched the changes in the ^{18}O composition of leaf water. The leaf dipped in highest concentration of ABA had the lowest g_s and $\Delta^{18}O$. A strong positive correlation between g_s and ^{18}O was noticed.

A few genotypes of cowpea significantly differing in transpiration were grown in containers and T was estimated by gravimetric approach. The T measured on a specific day among these genotype showed a strong positive association with $\Delta^{18}O$ in leaf water.

These results indicated that the $\Delta^{18}O$ in the leaf water is dependent on T and g_s.

^{18}O signatures in leaf water and leaf biomass are related

Evaporative enrichment of leaf water is passed on to organic material due to exchange of carbonyl oxygen with water. The most important exchange occurs in trios phosphate as 2 of the 3 oxygen are in carbonyl group and the half time to equilibration is known to be rapid (Sternberg et al., 1986, 1989; Farquhar et al., 1998). Therefore we expect that the leaf water $\Delta^{18}O$ values would get imprinted into biomass and hence the biomass ^{18}O signatures would be a time-integrated reflection of leaf water $\Delta^{18}O$.

The leaf water ^{18}O composition was determined in contrasting genotypes of several species such as Amaranthus, Sunflower, Cowpea and Soybean. The dried leaf samples from the genotypes and species were analyzed for $\Delta^{18}O$ biomass at the labs of Dr. Dan Yakir, Israel, Dr. Mathias Saurer, Switzerland, Dr. Graham Farquhar, Australia and also at the Finnigan MAT research lab, Germany.

Among the species studied in this present investigation $\Delta^{18}O$ leaf water was found to have a strong association with $\Delta^{18}O$ of the biomass, reiterating the fact that the ^{18}O signatures of the water is progressively transferred to the biomass. Since biomass accumulation occurs over longer periods of time, $\Delta^{18}O$ in leaf biomass is a more time-integrated estimate of $\Delta^{18}O$ of leaf water.

On the relationship of MTR with $\Delta^{18}O$ of leaf biomass

We have demonstrated earlier that the $\Delta^{18}O$ in leaf water is related to g_s. On a time-averaged basis transpiration rate is best quantified by gravimetric approach. MTR over an extended period of time was determined in contrasting species viz., cowpea, sunflower and groundnut with an objective to examine its relationship with $\Delta^{18}O$ in leaf biomass.

In all the species the genetic difference in $\Delta^{18}O$ in biomass closely reflected the difference in MTR. These results clearly describe the potential of $\Delta^{18}O$ in leaf biomass to identify the differences in mean transpiration rate.

Mineral ash content in the leaf is related to MTR

The mineral ash content of the leaf is often determined as a measure of T (Masle, 1992; Brown et al., 1997). The leaf ash content has been shown to have a positive relationship with T in several crop species. We

determined the ash content in several of our studies in this investigation. A positive relationship between MTR and ash content was noticed. Accordingly, a positive trend, though not significant was observed between $\Delta^{18}O$ in leaf biomass and mineral ash content, indicating a possibility of initially identifying high T types based on leaf mineral ash content also.

Desirable genotypes with high WUE and high T can be identified by $\Delta^{13}C$ and $\Delta^{18}O$

Enhanced breeding success to improve WUE can be achieved only if the constituent physiological traits determining the observed variability in WUE are evaluated.

Based on the relative contributions of g_s or g_m determining the variations in WUE, the species or genotypes could be classified into Capacity or Conductance types. In capacity types with high g_m, WUE and T will be less dependent on each other. Such genotypes are desirable, as they possess relatively high WUE despite relatively high T. As per the well accepted model of Passioura (1986), both T and WUE conceptually contribute to the differences in TDM. Therefore, such types will have high WUE and TDM.

From our results, we confirm that $\Delta^{13}C$ is related to WUE and adequate experimental evidences were provided in this study to establish the relationship between T and $\Delta^{18}O$.

In view of this, we evaluated genotypes based on $\Delta^{18}O$ and $\Delta^{13}C$ to identify types with high WUE and TDM.

The genetic differences in WUE in a few crop species (cowpea, sunflower and groundnut) were determined by gravimetric technique. The $\Delta^{13}C$ values were also assessed in these species and genotypes. A strong inverse relationship between $\Delta^{13}C$ and WUE was observed as expected,

indicating that the $\Delta^{13}C$ is a surrogate estimate for WUE. A significant genetic and species variability in WUE was noticed by both these approaches.

The causal relationship of $\Delta^{13}C$ with Pi and Pi with WUE renders $\Delta^{13}C$ a powerful estimate of WUE. However, the difference in Pi is caused both because of stomatal diffusive characters (g_s) and mesophyll efficiency (g_m). We have provided experimental evidences to suggest that the $\Delta^{18}O$ is a surrogate for g_s and since Ci/g_s is an indication of g_m, the isotope compositions of carbon and oxygen could be used as a potential approach to identify the desirable mesophyll capacity types.

Since g_m types are desirable, it is essential to delineate the effect of g_s and g_m on Pi and hence $\Delta^{13}C$. To achieve this a standard normal distribution values of $\Delta^{13}C$ and $\Delta^{18}O$ were plotted. Genotypes with low $\Delta^{13}C$ coupled with high $\Delta^{18}O$ showed high WUE and biomass.

Genotypes with these desirable combinations of stable isotopes were identified and several physiological traits of these genotypes were compared. The increase in biomass in these types was as high as 20-30%. The identified capacity types recorded significantly higher MTR, WUE as well as TDM.

Theoretically, these genotypes should have an intrinsically superior g_m characters. We examined the mesophyll efficiency in these genotypes.

Characteristics of the mesophyll capacity types

Unlike the g_s, quantification of g_m is rather difficult. However, indirect estimates such as the initial slope (dA/dCi) of CO_2 response curve (Caemmerer and Farquhar, 1981) and the ratio of Ci to g_s (Sheshshayee, et al., 1996) are often adopted as a measure of g_m. We developed CO_2 response curves in the selected contrasting genotypes of cowpea.

The dA/dCi values were significantly higher in the genotype APC-4125 that had a desirable combination of low $\Delta^{13}C$ and high $\Delta^{18}O$. This genotype also recorded relatively higher TDM.

The Ci and g_s ratio (Ci/g_s) was also significantly lower, clearly indicating that this genotype has higher mesophyll capacity to fix carbon and such types will have high WUE despite high g_s.

This investigation reveals that the $d^{18}O$ has the potential to be used as a surrogate for g_s and T. The technique based on the dual isotope compositions could be effectively employed for the genetic analysis of physiological traits contributing for differences in WUE besides identifying the mesophyll capacity types from germplasm.

<u>The salient findings of this investigation are</u>

- *Evaporation of water during transpiration results in enrichment of the heavy isotope of oxygen ($H_2{}^{18}O$) in the leaf.*
- *The ^{18}O signatures of the leaf water are transferred to the biomass.*
- *The MTR measured over an extended period and biomass ^{18}O were positively related indicating the potentiality of ^{18}O as surrogate measure of both T and g_s integrated over time.*
- *Genotypes with low $\Delta^{13}C$ and high $\Delta^{18}O$ in biomass were associated with high WUE and high T.*
- *Such genotypes had superior mesophyll efficiency as evidenced by high dA/dCi and low Ci/g_s ratios.*

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