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Ecological Implications Of The □^{13}c Values of Plant Species Growing in Natural Environment, Greenhouse and Plant Respired Carbon Dioxide (Captured as Carbonate)

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Abstract: *□^{13}C values have been used to differentiate the C_3 plant species from the C_4 species. Light isotope (^{12}C) is favoured against the heavier isotope (^{13}C) during the carbon fractionation in plant species. The □^{13}C values of terrestrial plant are useful in diverse applications in ecological, forensic, microbial diagnostic, biochemical and other scientific studies. There is variation of the □^{13}C values between the intraspecies grown in the greenhouse under controlled climatic conditions except respired carbon dioxide concentration. Also, □^{13}C values variation exist between interspecies, both grown in the greenhouse and field conditions. Isotopic composition of respired carbon dioxide (carbonate, CO_3^-) was different from that of plant carbon dioxide (carbonate, CO_3^-) and may be accounted due to respired carbon dioxide refixation. Further differences in the respired carbon exist between the C_3 and C_4plant species. Diffusion of carbon dioxide, interconversion of carbon dioxide and bicarbonate, assimilation of carbon dioxide by Ribulose bisphosphate carboxylase or Phosphoenol pyruvate carboxylase during carbon fractionation affect the final □^{13}C values. Different climatic factors and carboxylating enzymes explain the variation in the □^{13}C values within and amongst the C_3 and C_4 plant species. Furthermore, the variation in □^{13}C values may be caused by genetic differences in either leakiness of the bundle sheath cells due to light-use efficiency or by differences in the ratio of assimilation rate of stomatal conductance due to transpiration efficiency. Thus, both kinetics and thermodynamic modelling can be applied to explain the carbon fractionation process and the□^{13}C values.*

Keywords: *□^{13}C values, C_3 and C_4 plant species, respired carbon dioxide, climatic factors, Ribulose bisphosphate carboxylase, Phosphoenol pyruvate carboxylase, carbon fractionation.*

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I. Introduction

Carbon discrimination values (□^{13}C values) have been used to differentiate between the C_3 and C_4 species (Bender, 1971[1]; Epstein, Lauenroth, Burke and Coffin, 1997[2]; Sikolia, Beck, Kinyamario, Onyango andOuma, 2008[3]). Plants promotes isotopic fraction of carbon dioxide atmospheric source during photosynthesis, such that lighter isotope (^{12}C) is favoured than heavier isotope (^{13}C) [3]. This results to less ^{13}C isotope compared to higher atmospheric carbon dioxide in plants. Therefore, the isotopic ratio of ^{13}C to ^{12}C in plant tissue is less than the isotopic ratio of ^{13}C to ^{12}C in the atmosphere, implying that plants discriminates against ^{13}C during photosynthesis (Gregg and Zhang, 2000 [4]). Isotopic fractionation is caused by physical (slow diffusion of ^{13}C in plant tissues due to increased atomic weight) and biochemical (preference of ^{12}C by two enzymes- the Ribulose bisphosphate carboxylase/Oxygenase (RUBISCO) and Phosphoenol Pyruvate Carboxylase (PEPCase)) factors. Further, carbon discrimination of plants with the C_3pathway may be used to detect genetic differences in water-use efficiency of individual plants and of crops (Farguhar, Ehleringer and Hubick, 1989[5]).Baertschi (1953[6])observed an isotopic fractionation between carbon dioxide and fixed plant carbon is 1.026. There is measured differences between the $^{13}C/^{12}C$ ratios of atmospheric carbon dioxide and plant material in the controlled laboratory experiment [6]. Further, respired carbon dioxide has the same $^{13}C/^{12}C$ ratio as the plant [6]. Wickman (1952[7]) studied a variety of plant species from different ecological habitats for their $^{13}C/^{12}C$ ratios, analyzed and concluded that the variations of the ratios between different plant species were due to variations in the $^{13}C/^{12}C$ ratio of atmospheric carbon dioxide.The discrimination index varies between C_3 and C_4 species but also amongst the species of a given photosynthetic pathway [3] and among plants growing in different environments (Cernusak, Ubierna, Winter, Holtum, Marshall and Farguhar, 2013[9]) [7] and even between genotypes, for example in *Sorghum bicolor* Moench (Hubick, Hammer, Farguhar, Wade, Von Caemmerer and Henderson, 1990[10]). The C_4 species show less negative values compared to the C_3 species. There is evidence of interspecific differences in the carbon discrimination values among plants possessing the C_4 pathway but little variation within species [10]. However, analysis of isotope composition in one hundred

and twenty genotypes of *Zea mays* failed to show any significant variation (O'Leary, 1981[11]).Thedifferences in the discrimination against ^{13}C during photosynthesis has been attributed to various factors, including stomatal conductance limitations, photosynthetic capacity leakiness and enzymatic processes [4] and is highly correlated to water use efficiency [9]. Knowledge of the □^{13}C values is mainly derived from studies of the monocots. However, the degree of carbon isotopic fractionation in the dicot species require more □^{13}C value data from diverse climatic conditions for comprehensive and ecological comparison [8]. Stable carbon isotopes ratios (□^{13}C) of terrestrial plants are employed across a diverse range of applications in environmental and plant sciences; however, the kind of information that is desired from the □^{13}C signals often differs [9]. Further, □^{13}C values have been used to solve scientific problems, for example, to trace the flow of carbon as differential dietary inputs (Hobson, 1999[12]), follow the transport of carbon across ecosystems (Conte and Weber, 2002[13]; Ehleringer, Cerling and Dearing, 2002 [14]), address forensic problems such as determining the origin of illicit drugs (Carter, Titterton, Murray and Sleeman 2002[15]), trace the origin of infectious microbial source (Kreuzer-Martin, Lott, Dorigan and Ehleringer 2003[16]), Kreuzer-Martin, Chesson, Lott, Dorigan and Ehleringer2004[17]), fingerprints of biological agents as forensic tool (Horita and Vass, 2003[18]) and differentiating between C_3 and C_4 dicot species of the Centrospermeae families and understanding climatic factors affecting their distribution along altitudinal gradient in Kenya [3][8]. Thus, at the extremes, the □^{13}C value applications ranges between diverse and dynamic field of studies in the society and not only pure environmental and biological criteria [9].

II. Materials And Methods

2.1 Isotopic analysis

The isotopic analysis was done at the University of Bayreuth, Germany. It involved two sets of plants. One set was for those plant leaf samples collected from natural environment (field collected plant materials) in Kenya while the second set involved plants which were raised under controlled conditions at the University of Bayreuth.

2.1.1 □^{13}C values of plants collected from natural environment (field plant material collections)

Leaves from plants of different ecotypes varying in climatic conditions vis-á-visaltitudes, latitude, temperature, potential rates of evaporation, rainfall, relative humidity and radiation (Sikolia, 2016[19]) and dried under natural conditions at 25℃- 30℃, until there was no change in weight of the leaf organs. The leaves were safely preserved in well labelled paper bags. The label on each bag included the conditions of the ecotypic study area of the species. The dried leaves were used for □^{13}C value analysis. Each leafy material was milled into a powder using isotopic ball mill at 90 shaking speed per minute. The resultant fine powder was put in an enclosed 10cm^{3}bottle. Sample weights of between 60µg-80µg were transferred into aluminum cups, which were tightly enclosed for isotopic □^{13}C values measurements. Two replicate measurements were made to ascertain the reliability of the results.

2.1.2 □^{13}C values for greenhouse plants

Three species of the Centrospermeae were studied for □^{13}C value under controlled environmental conditions. The results were compared with those of the species sampled from natural environments. Ten seeds were germinated in the glass chamber under controlled germination conditions for two weeks. The seedlings were transplanted in half-litre pots containing optimum mineral nutrients. They were irrigated at an interval of 24 hours. After two months of growth the leaves were expanded. The experiments were replicated two times.
Fully expanded fresh leaves (3-4) were dried in the oven at 65℃-80℃ for 3-5 days until no change in weight was achieved. Dried leaves were milled as described by [3]. The powder was placed in a desiccator for 2-3 days to be fully desiccated. The powder was then weighed at between 60µg-80µg and then transferred into aluminum cups and tightly enclosed for □^{13}C isotopic analysis. Two replicate measurements were made to ascertain the reliability of the results.

2.1.3 Experiments for □^{13}C values of the greenhouse circulating air, respired C_3 plant species carbon dioxide and respired C_4 plant species carbon dioxide collected as carbonate (CO_3^-) precipitate

The C_3 and C_4 plant species were randomly selected and used for respired carbonate (CO_3^-) precipitate collections. The plant species included: *Phytolaca americana, Amaranthus lividus, Portulaca grandiflora, Mirabilis jalaba, Trianthema triquetra, Amaranthus retroflexus, Dianthus barbatus, Portulaca oleracea, Chenopodium ambroisoides, Amaranthus patulus, Fagopyrum tataricum, Rumex triqulivalis, Gysophila paniculata, Rumex rugosus, Lychnis coronaria, Amaranthus hybridus, Silene vulgaris, Kochia scoparia, Silene dioica, Chenopodium capitatum, Rumex acetosa, Pleuropetalum darwinii, Rumex rugosus, Chenopodium album, Amaranthus dubius, Polygonum setulosum, Vaccaria pyramidata, Minuartia juniperiana.* Ten to twelve seeds of each of the twenty-eight, were germinated in 0.5 liter pots on vermiculite in the growth chamber under

controlled conditions (automatic irrigation system, 20℃ – 30℃ temperature and normal bulb light system). The seedlings were ready for transplanting 2-3 weeks after germination. An 18-hour monitoring duration was carried out on a daily basis. Two to three week old seedlings were transplanted into 1 litre pots in normal sterilized nutrient rich soil for continual growth. The plant species were irrigated on a daily interval until they were seven weeks old when the respired carbon dioxide samples of the C_3 and C_4 were collected as carbonate (CO_3^-) precipitate.Three set of closed photosynthetic chambers for the circulation of the greenhouse through the empty-chamber (greenhouse air without plant species), chamber with C_3 plant species and C_4 plant species were constructed. A six-week old plant in a pot was enclosed in a gas-tight cover using Vaseline just before the experiment to prevent gas exchange with the soil. It was put in an air tight, 19liter glass chamber at a controlled temperature of 20℃. Air circulation was allowed to enter through airtight channel to reach the enclosed plant. The existing respired air (carbon dioxide gas plus other gases) was passed through calcium hydroxide solution in airtight flat-bottom flask forming the required carbonate precipitate. The carbon dioxide of the greenhouse (normal air), respired carbon dioxide of the C_3 plant species and respired carbon dioxide of the C_4 species, were collected as carbonate (CO_3^-) precipitate. The precipitate was warm-dried at normal temperatures (20℃ – 30℃) in semi-enclosed jar and grinded using isotopic ball mill at a 95 shaking speed per minute. Dried fine powder of the carbonate precipitate was weighed using electronic balance to the required weight and put in an enclosed 10cm^3 bottle. Sample weights of between 60µg-80µg were transferred into aluminum cups, which were tightly enclosed for isotopic □^{13}C values measurements. Two replicate measurements were made to ascertain the reliability of the results.

III. Results

3.1 Isotopic □^{13}C values for the greenhouse species and field species

There was observable differences in the□^{13}C values in different species collected at different altitudes (Table 4) for the twenty-eight species studied. The standard deviation show that the variation in □^{13}C values for given species were not very significant and within the normal range. The minimal variation can be attributed to ecotypic plasticity and climatic factors during carbon fractionation processes in plant species, especially in the C_3 species as compared to the C_4species. The differences in the □^{13}C values was much noticeable in the C_3 plant species between the field species and greenhouse experiments as shown in *Gysophila paniculata* which thrives at 2675m a.s.l. in the semi-arid habitats of Kenya. For instance, experimental data –I, data-II and field data shows -28.83‰, -31.73‰ and -26.82‰, respectively. Similar phenomenon was observed in the *Phytolaca americana,Mirabilis jalaba and Dianthus barbatus* growing at least 2350m a.s.l. and above. These observations were the contrast in the *Chenopodium ambroisoides*, *Fagopyrumtataricum, Polygonum setulosum* that thrive at 1965m, 2115m, 2500m a.s.l., respectively.

Table 1: □^{13}Cvalues for greenhouse grown plants (Experiment I and II) and field collected plants (field experiments). Greenhouse experiments were carried out at Bayreuth University, Germany.

Sample	□^{13}C values (‰) for different species				Standard Deviation of Mean
	Exp. data I	Exp. data II	Field Exp. data	Altitude(m) a.s.l.	

Phytolaca americana, C_4 species	-25.65	-26.37	-28.37	2850	1.3
Amaranthus lividus, C_4 species	-14.66	-14.34	-12.96	850	1.1
Portulaca grandiflora, C_4 species	-15.15	-13.96	-12.33	1365	1.2
Mirabilis jalaba, C_3 species	-27.87	-30.34	-30.26	3250	1.1
Trianthema triquetra, C_4 species	-14.18	-14.31	-13.25	1355	0.5
Amaranthus retroflexus, C_4 species	-13.54	-13.49	-13.48	1450	0.02
Dianthus barbatus, C_3 species	-26.70	-29.49	-29.53	2365	1.3
Portulaca oleracea, C_4 species	-14.83	-14.98	-14.88	975	0.1
Chenopodium ambroisoides	-29.54	-29.44	- 29.49	1965	0.04
Amaranthus patulus, C_4 species	-16.13	-15.73	-13.12	1200	1.3
Fagopyrum tataricum, C_3 species	-26.55	-26.88	-26.73	2115	0.2
Rumex triqulivalis, C_3 species	-29.26	-27.76	-27.86	2565	0.7
Gysophila paniculata, C_3 species	-28.83	-31.73	-26.82	2675	2.0
Rumex rugosus, C_3 species	-28.14	-27.37	-27.96	1875	0.3
Lychnis coronaria, C_3 species	-27.86	-27.69	-25.58	2348	1.0
Amaranthus hybridus, C_4 species	-14.71	-13.12	-14.70	1095	0.8
Silene vulgaris, C_3 species	-28.20	-30.27	-27.27	2765	1.3
Kochia scoparia, C_4 species	-14.91	-13.61	-13.55	1350	0.6
Silene dioica, C_3 species	-27.46	-25.76	-25.70	2975	0.8
Chenopodium capitatum,C_3 species	-30.31	-28.92	-29.13	1695	0.6
Rumex acetosa, C_3 species	-28.19	-30.15	-30.25	1550	0.9
Pleuropetalum darwinii, C_3 species	-28.05	-27.25	-27.64	2300	0.3
Rumex rugosus, C_3 species	-28.25	-27.97	-28.15	1765	0.1
Chenopodium album, C_3 species	-28.91	-29.10	-26.70	2540	1.1
Amaranthus dubius, C_4 species	-15.07	-14.94	-15.14	990	0.1
Polygonum setulosum, C_3 species	-27.74	-27.66	-27.18	2500	0.2
Vaccaria pyramidata, C_3 species	-30.60	-28.15	-28.75	1750	1.0
Minuartia juniperiana, C_3 species	-30.24	-27.69	-27.57	2110	1.2

Generally, the C_4 plant species showed a high stability in the □^{13}C values for greenhouse grown plants and field collected plants with standard deviation of mean not greater than 1.2 for each species.

The paired t-test statistical tests were performed for the experimental data collected as follows:

I) paired t-test between the experimental data-I of the greenhouse species and experimental data-II of the greenhouse species. The results revealed, t-value of 0.394485 and p value of 0.69632.

II) unpaired t-test was carried out between the:

a) experimental data-I of the greenhouse species and experimental data-II of the collected field species. The results revealed, t-value of 0.34168 and p value of 0.7848, with 54 degrees of freedom;

b) experimental data-I of the greenhouse species and experimental data-II of the collected field species. The results revealed t-test value of 0.2745 and p value of 0.7848 with 54 degrees of freedom.

The statistical values show that there is some significant differences between the □^{13}Cvalues for greenhouse grown plants. Further, there was slight differences between the□^{13}Cvalues for greenhouse grown plants and field collected plant species.

3.2 □^{13}C Values for greenhouse circulating air, respired c_3 plant species carbon dioxide and respired c_4 plant species carbon dioxide collected as carbonate (CO_3^-) precipitate

The □^{13}C values for the carbon dioxide of the greenhouse, respired carbon dioxide of the C_3 plant species and respired carbon dioxide of the C_4 plant species collected as carbonate (CO_3^-) precipitate were measured and analyzed. The resultant □^{13}C value data was tabulated for each sample of the carbonate (CO_3^-) precipitate collected (Table 2).

Table 2: Mean □^{13}C values for greenhouse carbon dioxide, C_3species respired carbon dioxide and C_4 species respired carbon dioxide collected as carbonate precipitate (CO_3^-).

Sample Source-Precipitate	□^{13}C values
Greenhouse carbon dioxide CO_3^- -I	-23.25‰
Greenhouse carbon dioxide CO_3^- -II	-24.25‰
C_3 species respired carbon dioxide CO_3^- -I	-24.57‰
C_3 species respired carbon dioxide CO_3^- -II	-24.88‰
C_4 species respired carbon dioxide CO_3^- -I	-25.25‰
C_4 species respired carbon dioxide CO_3^- -I	-25.09‰

Table 2 show that there is some significant difference in the □^{13}C values between the sample source replicate of the greenhouse carbon dioxide CO_3^- -I and greenhouse carbon dioxide CO_3^- -II, C_3species respired carbon dioxide CO_3^- -Iand C_3 species respired carbon dioxide CO_3^- -II and C_4 species respired carbon dioxide CO_3^- -I and C_4 species respired carbon dioxide CO_3^- -II. Further, Table 2 shows there was significant variation between the C_3species respired carbon dioxide and C_4species respired carbon dioxide. This phenomenon was even more significant between greenhouse carbon dioxide CO_3^- and C_4 species respired carbon dioxide CO_3^- but lesser between greenhouse carbon dioxide CO_3^- and C_3speciesrespired carbon dioxide CO_3^-.

IV. Discussion

4.1 Carbon isotope discrimination value of the greenhouse and field plant species

Studies have shown that the isotope fractionation of carbon formed during photosynthesis in a given plant species differs significantly based on the nature and pattern of photosynthesis of the species [3] [5] [8] [11] [19]. Plants promote the isotopic fractionation of carbon dioxide atmospheric source during photosynthesis, such that the lighter isotope (^{12}C) is favoured than heavier isotope (^{13}C). This results into less ^{13}C isotope compared to higher atmospheric carbon dioxide in plants. The extent of discrimination varies between the C_3 and C_4 species but also amongst the species of a specific photosynthetic pathway. This is because carbon fixed by carbon dioxide assimilating enzymes Ribulose Bisphosphate Carboxylase (RUBISCO) in the C_3 plant species and Phosphoenol Pyruvate (PEP) carboxylase in the C_4 and CAM species show different carbon discrimination values (□^{13}C values) since the former enzyme discriminates -27‰ to -30‰ values whereas the latter only 0‰ to 2‰. Therefore, the □^{13}C values for the C_4 plant species utilizing the PEP carboxylase range from -7.0‰ to -17.0‰ while the C_3 plant species that use RUBISCO show an interval range of -20.0‰ to -32.0‰ [5] [3].
The variation of the □^{13}C values amongst the C_3 and C_4 dicot species may be influenced by climatic factors especially the temperature, rainfall, altitude and light-use efficiency (light energy), water-use efficiency, genetic differences either in leakiness of the bundle-sheath cells or by differences in the ratio of assimilation rate of stomatal conductance. The difference in the □^{13}C values of C_4 species was slightly significant in *Kochia scoparia (-14.91*‰, -13.61‰, -13.55 *with man standard deviation ofwith mean standard deviation of 0.6), Amaranthus patulus (-16.13*‰, *-15.73*‰, *-13.12*‰ *with mean standard deviation of 1.3*), *Amaranthus lividus* (-14.66‰,-14.34‰, -12.96‰ *with man standard deviation of 1.1)* and*Portulaca grandiflora (-15.15*‰,*-13.69*‰, *-12.13*‰ *with man standard deviation of 1.2).*The mechanism of discrimination in plants possessing C_4 syndrome is such that these plants discriminate less than do those with the C_3 syndrome (Bender, 1968[20]; Smith and Epstein, 1971[21]). The leakiness factor measure the reuse by PEPCase compared to RUBISCO, and therefore ofextra light energy required for carbon fixation. This suggest the differences in □^{13}C values among the C_4 pathway and therein C_4 species reflect differences in the leakiness and light-use efficiency (Farguhar, 1983 [22]). This leakiness effect is modified by the ratio of mesophyll intercellular and atmospheric partial pressures of carbon dioxide (p_i/p_a). The difference in □^{13}C values amongst C_4 species at the leaf level could be affected by the light efficiency and /or transpiration efficiency index. Since pi/$p_a$is affected by stomatal conductance and assimilation capacity [5], it has direct bearing on the carbon fixed per unit of water utilized, or transpiration efficiency, and this association has been investigated in the C_3 species. These factors can be influenced by temperature, carbon dioxide concentration, rainfall and other modifying climatic variables.

Troughton (1972[23]) reported that □^{13}C values become slightly negative (by up to 2%) with increasing temperature in several C_3 and C_4 plants. Smith, Oliver and McMillan (1976 [24]), Smith, Herath and Chase (1973[25]) and Bender and Berge (1979 [26])observed similar results in different plant species. These observations were not consistent in other investigations (Troughton, Card and Bjorkman, 1974 [27]; Pardue, Scalan, Van Baalen and Parker, 1976[28]). The effect of temperature affect the enzymatic kinetics and rate of carbon dioxide diffusion through the stomata into the intercellular space where the carbon dioxide concentration becomes the critical parameter. Higher intercellular carbon dioxide concentration establish positive gradient that enhance CO_2 entry into the cellular protoplasm via the mesophyll and bundle sheath shuttle system. This gradient would be favourable in the C_3 plant species but the C_4 plant species would not loose muchin the process. Low carbon dioxide concentration in the intercellular space in the leaf organ cause low differential

gradient that would benefit the C_4 shuttle mechanism against that in the C_3 plant species. Water availability ensure efficiency in photolysis providing hydrogen ion for reduction of carbon dioxide during the dark stage of photosynthesis. Less concentration of the hydrogen ions limit the rate at which the reduction process occurs. These effects of temperature, water availability and carbon dioxide concentration may have caused the differences in the □^{13}C values between the greenhouse C_3 plant species and field C_3 plant species, and the greenhouse C_4 plant species and field C_4 plant species in the present study.

Interspecific and intraspecific variation of the□^{13}C values were observed in the plant species. The main and critical factor that affected the isotopic carbon discrimination must have been due to the presence of the C_3 and C_4 photosynthetic mechanisms in operational in the plant species and modified by climatic factors. Specific and individual differential capacity of RUBISCO metabolic activity during carbon dioxide carboxylation in different plant species cause differential carbon fractionation that lead to varied □^{13}C values in the C_3 plant species. C_4 species initiate different sub-metabolic carbon dioxide carboxylation pathway that lead to the observed different □^{13}C values in different plant species. This differential capacities during carbon fractionation in different species led to variation in the □^{13}C values. Interestingly, Lowden (1969[29]) found no significant differences in □^{13}C values for several strains of *Zea mays*. But, studies showed carbon isotope discrimination varied among the genotypes in the leaves of *Sorghum bicolor* Moench C_4 species [10]. The variation in carbon isotope discrimination may be caused by genetic differences in either leakiness of the bundle-sheath cells or by differences in the ratio of assimilation rate of stomatal conductance [10]. At the leaf level, the former should be related to light-use efficiency of carbon fixation and the latter should be related to transpiration efficiency. All these factors are based mainly on the nature and specific photosynthetic metabolism functioning in a given species and/or genotype.

4.2 Respired Carbon

Respired carbon dioxide was captured as carbonate and analyzed for □^{13}C values. Also, greenhouse carbon dioxide was captured for □^{13}C values analysis. It should be pointed out that not all carbon dioxide as a result of respiration and other carbon dioxide forming processes within the plant species was released to the environment because some were refixed. This refixation can fractionate carbon isotopes and therefore the measured □^{13}C values for the respired carbon may differ from that formed by respiratory processes. The carbon isotope discrimination values varied between -23.25‰ to -24.25‰, -24.57‰ to -24.88‰, -25.09‰ to -25.25‰ for the greenhouse carbon dioxide CO_3^-, C_3 species respired carbon dioxide CO_3^-, C_4 species respired carbon dioxide CO_3^-, respectively. The variations are within range carbon fractionation of the photosynthetic grouping of the C_3 and C_4 plant species.

The isotopic composition of a plant is controlled by the isotopic composition of the carbon dioxide source, the isotope fractionation following carbon dioxide assimilation, and isotopic fractionation and the amount of carbon dioxide lost through respiratory processes (O'Leary, 1981 [11]). This study follows number of measurements of □^{13}C values for respired carbon by trapping the carbon dioxide released by a plant in a carbon dioxide-free atmosphere and measuring its isotopic composition. It has been shown that isotopic composition of CO_2 released from plants in the dark differs only slightly from that of the whole plant. Thus, CO_2 released by dark respiration in tomato was 2‰ to 5‰ more positive than the whole plant (Park and Epstein, 1961[30]) but in *Triticum aestivum* the released carbon was 5 more positive than the leaf (Troughton, Card and Hendy, 1974 [31]). Further, greater difference in □^{13}C values between whole leaves and carbon released in the light, for example, *Gossypium hirsutum* and *Triticum aestivum* (both C_3 plants) the released carbon was 10‰ and 12‰, respectively, more positive than the leaf [31]. This differences may be due to the occurrence of partial refixation of respired carbon dioxide, leading to a discrimination against ^{13}C and the release of relatively positive carbon to the atmosphere. *Paspalum dilatatum* and *Zea mays* (both C_4 plants) showed 3‰ and 6‰ more negative than the leaf for the released respired carbon, respectively [31]). Thus, variation of the intraspecific species □^{13}C values could have been due to refixation of respired carbon dioxide within the greenhouse when the greenhouse carbon dioxide CO_3^- replicates I and II are compared. The extent of refixation will also be affected because there is no atmospheric carbon dioxide to compete with respired carbon dioxide for ribulose bisphosphate carboxylase. Therefore, there is no certainty that isotopic composition of the respired carbon dioxide in a free environment is the same as would be obtained in a normal atmosphere. Infact, Sternberg, Mulkey and Wright (1989[32]) found that respired carbon dioxide can have an important effect on the carbon isotope ratios of leaf material in the understorey. Thus, it may be of practical significance to avoid isotope analysis of carbon dioxide from the air near study plants (i.e. avoiding respired carbon dioxide effect)in order to use □^{13}C values as indicator of water-use efficiency.

Carbon isotope discrimination value differences of the C_3 plant species may not have been influenced by photorespiration. This is because there was no change in oxygen concentration (%) observed during the experimental process unlike the case observed in the*Atriplex patula*(a C_3 species) where oxygen varied from 20% to 4% such that the whole plant □^{13}C value became more positive by 2‰ - 4‰ (Berry, Troughton and Bjorkman, 1972 [33]). However, no oxygen effect was recorded in the *Atriplex rosea* (C_4 species)[33]. Further, the source of respired carbon dioxide (carbohydrate or lipid in nature) has been reported to be the cause for variation in the carbon isotope discrimination. Plant lipids are significantly more negative than other components (Jacobson, Smith, Epstein and Laties, 1970 [34]). This observations have been confirmed in other plant studies (Park and Epstein, 1960[35]) [31]. The difference between whole leaf and lipid is often near 5‰, but may be large as 10% (Ziegler, 1979 [36]). This depletion may be caused by the isotope fractionation associated with decarboxylation of pyruvic acid (DeNairo and Epstein, 1977 [37]). Thus, there are small environmental and intrinsic variations in the □^{13}C values. These account for the present and observed variations in the □^{13}C values of the plant species.

Studies have shown that respired carbon dioxide is responsible for 31 and 37% of the variation in isotope composition in leaves of two species of herbaceous bamboo grown in a well-ventilated sun treatment and in the forest understorey [32]. Further, respired carbon dioxide accounts for 45-70% of the difference in □^{13}C values between understorey and canopy leaves for three tree species growing inlarge-scale irrigation and control treatments (Mulkey, 1986 [38]). Studies indicated that understorey leaves of these species show □^{13}C values consistent with higher ratios of intercellular to ambient carbon dioxide in irrigated relative to control treatments. This observations corroborate the present

phenomenon where respired carbon dioxide contributed to the varied carbon isotope discrimination values between the intraspecies and interspecies grown in the greenhouse compared to the field species. This phenomenon can be replicated in the canopy-understorey environment as a function of the respired carbon dioxide concentration gradient in the forest and/or greenhouse set up. It does imply that there is need to adjust the effect of the carbon isotope discrimination of respired carbon dioxide when estimating water-use efficiency from leaf carbon isotope content in closed canopy forests during ecological studies, especially equatorial and tropical forests near the equator.

The present findings implies the effect of respired carbon dioxide concentration gradient maybe observed in the stratification of photosynthetic species in the forest canopy towards the understorey. Ehleringer, Field, Lin and Kuo, 1986 [39] showed that carbon isotope ratios of plant tissue in the forest understorey are frequently lower compared to the tissue in the canopy. This partly due to high respired carbon dioxide concentration in the understorey (Medina and Minchin, 1980[40]; Schleser and Jayasekera, 1985[41]; Medina, Montes, Cuevas and Rokczandic, 1986[42]).Further, respired carbon dioxide comes from the soil bacteria acting on the pant detritus during decomposition process. This respiration output and thereby input of respired carbon dioxide result into □^{13}C values between -25‰ and -28‰ (Peterson and Fry, 1987[43]), otherwise the forest air would be approximately -7.8‰ (Evans, Sharkey, Berry and Farguhar, 1986[44]).Thus, the stratification of understorey-canopy carbon dioxide gradient varies as a function of diurnal gas exchange between the boundary layer above the forest and the free troposphere, and is more pronounced near the ground where air mixing is slow (Wofsy, Harriss and Kaplan, 1988 [45]) [32]. This canalso influence partition of the different photosynthetic species to assume the anticipated paradigm shift of C_3 monocots > C_3 dicots>C_4 monocots>C_4 dicots diffuse arrangement in the canopy–understorey in a dense forest ecosystem. The NAD-ME. NADP-ME and PEPCK subtypes of the C_4 pathway may show slight preferential stratification especially in well-established and undisturbed forests. However, these observations require further investigations for confirm the paradigm shift of the C_3 and C_4 photosynthetic pathways and their C_4 subtypes.

V. Conclusion

The research data on thecarbon isotope discrimination values has been discussed based on integrated kinetics and thermodynamics during carbon fractionation process under different climatic conditions. The climatic factors and the metabolic enzymatic activity act synergistically at the same time within varying timescale. The dominant factor plays major determinant role in the measured□^{13}C value of the plant organ of the plant species. Here, the leaf organ was used in the investigations to measure □^{13}C values of different plant species. The □^{13}C values varied between the greenhouse species and the field species. The variation of the □^{13}C values was small between the intraspecies grown in the greenhouse and may have been caused by refixation of the respired carbon dioxide. Further, variation of the□^{13}C values between interspecific species may have been due to climatic factors including carbon dioxide concentration, altitude and the carboxylation enzymatic component in a given plant species. The differential capacity of the either RUBISCO in the C_3 plant species or PEPCase in the C_4 plant species significantlydetermines the resultant and varied □^{13}C values amongst the plant species. Thus, the abiotic factors modified by internal factors in the plant species affect carbon fractionation process that results into the analyzed □^{13}C values. It is suggested that climatic factors and RUBISCO/or PEPCase enzymatic factor along the latitudinal and altitudinal gradients be investigated under controlled laboratory and environmental conditions for explicit data in an attempt to explain□^{13}C value variations between intraspecies and interspecies of the C_3 and C_4plant species and between their different plant organs.

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