Carbon isotope discrimination ($\Delta^{13}C$) as a physiological marker for shade tolerance in black pepper *(Piper nigrum* L.*)*

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Abstract: Light is the major limiting factor for intensive agriculture. Harnessing the light energy efficiently or developing the crop genotypes that yield better under low light is the need of the hour. However, due to non availability of easily quantifiable tool, the screening for shade tolerance has become complex. Hence, an attempt was made in this investigation to employ the Carbon Isotope Discrimination ($\Delta^{13}C$) as a physiological marker/tool in assessing the extent of shade tolerance in black pepper, as it has plant types differing in shade tolerance. On the basis of Photosynthetically Active Radiation (PAR), different shade levels of 50, 65 and 90 percent were created using shade nets. The growth and physiological parameters were measured periodically. Among the genotypes, the one which has relatively high adaptation to the shade has shown to utilize the light more efficiently thus maintaining low Ci, and hence the low $\Delta^{13}C$. It also maintained higher photosynthetic rate and lower transpiration rate. In this paper we discuss the physiological basis for this variation and currently we are contemplating to test the same hypothesis in other shade loving species too.

Keywords: Carbon Isotope Discrimination ($\Delta^{13}C$), Shade tolerance, Inter cellular CO_2 concentration (Ci), Black Pepper.

I Introduction

A whooping 55 percent of the total cropped area is occupied by plantation crops in Kerala, which succumbs to drastic vagaries of climate on a large scale. Over the years, area under plantation crops is increasing and owing to the limited land area availability in the state, intensive cultivation has become inevitable. Light becomes major limiting factor for productivity of under-story crops of the plantations. Though some plants have different habitat adaptation, on a larger scale shading has been a predominant constraint for overall productivity of these shrubs, which warrants breeding for shade tolerant plants.

Simple and easily quantifiable parameter which reflects shade tolerance is unavailable for breeding shade tolerant type/s. Plants adapt morphological, anatomical, physiological and biochemical changes to maximize the light absorption under shaded condition [1]. Any trait/parameter which congregates the effect of all the strategies that plants adapt, acts as a screening tool for selecting shade tolerant type.

In this context, carbon isotope discrimination ($\Delta^{13}C$) of the leaf tissue provides ample information on plant metabolism in general and photosynthesis in particular [2,3]. Carbon isotope discrimination is used extensively as a surrogate for water use efficiency in many crop plants [4,5,6]. Discrimination of heavy $^{13}CO_2$ during photosynthesis by the primary carboxylating enzyme RuBisCO depends on the internal CO_2 concentration (Ci). Low light results in less production of assimilatory power (ATP and NADPH) and therefore high Ci in the leaf. At a given level of low light intensity the Ci is largely depends on the ability of leaf to absorb and utilize light energy. Leaf with high efficiency to utilize low intensities of light maintains low Ci and results in low $\Delta^{13}C$. Therefore it is hypothesized that among the genotypes grown under low light intensity, genotype which shows least discrimination ($\Delta^{13}C$) value can utilize light most efficiently and therefore it is shade tolerant type.

Kennedy et al, 2006 [7] shown that shade tolerant forest species are identified using carbon isotope discrimination. But no such information is available among the crop plants growing under shade. In this paper we report on the use of $\Delta^{13}C$ as a signature for shade tolerance using black pepper genotypes differing in shade tolerance and demonstrate that the Karimunda, a shade tolerant type [8] has lower $\Delta^{13}C$ compared to

Panniyur-1, a shade susceptible type, under very low light intensity.

Materials and methods

Plant material establishment and modulation of light regimes: Photosynthetically Active Radiation (PAR) was measured using a quantum sensor. Based on the PAR values, 90, 65 and 50 percent shade levels were created using shade nets. Different cultivars of black pepper, viz., Panniyur-1, 2, 3, 4 & 5 and Karimunda procured from Pepper Research Station, Kerala Agricultural University, Panniyur were grown in pots as bush peppers. Rooted cuttings were transferred to 10 inch earthen pot and allowed to establish at 50 percent shade for 45 days. Established plants were shifted to 65 and 90 percent shade and one set was retained in 50 percent shade. Growth and physiological parameters were examined periodically in plants growing under three different shades and $\Delta^{13}C$ was analyzed 70 days after exposing the plants to each shade levels. All treatments include at least three replications.

Growth Parameters: Leaf area and dry weight of root, shoot, and leaf were recorded before shifting the plants to shade, 45 days and 90 days after shifting to different shade levels. On the basis of dry weight and leaf area, different parameters were calculated as per Dhopte and Patil (2002)[9].

Leaf Area Ratio (LAR): The ratio of leaf area to plant dry weight which reflects the photosynthetic surface area to fix carbon to produce biomass was measured in all the genotypes.

Physiological parameters: Stomatal frequency and chlorophyll content were measured in fully opened leaf which was formed under each shade level. Chlorophyll content was measured invasively using SPAD chlorophyll meter (SPAD 502, Konika-Minolta, Japan) and expressed as SPAD Chlorophyll Meter Reading (SCMR).

Carbon Isotope discrimination: Newly formed leaf under particular shade was harvested and dried in hot air oven (80^0C for 3 days). Leaf samples were powdered and analyzed for carbon isotopes at National facility for stable isotope studies in biological sciences, Department of Crop Physiology, UAS, Bangalore, India. $\Delta^{13}C$ was measured using continuous flow Isotope Ratio Mass Spectrometer (IRMS). The extent of $\Delta^{13}C$ was computed as per Farquhar et al (1989) [3] and expressed in per mil (‰, parts per thousand) notation.

Chlorophyll fluorescence and electron transport rate (ETR): Chlorophyll fluorescence parameters were recorded using pulse amplitude modulation Fluorometer (PAM-200) (Make Heinz Walz Gmbh, Germany). Fully mature leaf (2nd or 3rd leaf from the top) was used to take all the measurements. Fv/Fm (Maximum quantum yield of PSII) and electron Transport Rates were measured as per Maxwell and Johnson (2000) [10]. The definition of electron transport rate (ETR) is ETR = Yield X PAR X 0.5 X Absorptivity, wherein, Yield = Fv/Fm and PAR is the actinic light intensity. Leaves were irradiated with a series of default actinic light intensities (0, 1, 21, 56, 111, 186, 281, 336, 396, 461, 531, 611, 701 lmol m-2 s-1) for 10 s each which finishes with a saturating pulse after each step. Simultaneously, ETR values were determined and plotted against irradiance automatically using the given software.

Gas exchange parameters: Nondestructive measurements of the photosynthetic and transpiration rate and stomatal conductance obtained with the Portable Photosynthesis System (ADC, BioScientific Ltd., England) at 10:00 a.m. on healthy, completely expanded young leaves. All the measurements were conducted on sunny days in the Net house, 70 days after shifting of the plants to their respective light regimes.

Results and Discussion

Black pepper genotypes established in the pots as bush peppers were transferred to very low light intensity. Seventy days after exposure, $\Delta^{13}C$ determined in the leaf that was formed under particular shade condition. $\Delta^{13}C$ was increased with decrease in light intensity in all the genotypes (Table-I).

The mean value of $\Delta^{13}C$ at 50 % shade was 20.93‰ whereas for 90 % shade it was 23.341‰. Low light intensity leads to increased $\Delta^{13}C$ in black pepper. Similar observation was also made by Flanagan *et al* (1997)[11] in plants of hanging garden (Ribbon garden) communities in Southern Utah, USA, where plants were shaded throughout the day. They attributed this high $\Delta^{13}C$ to high internal CO_2 concentration (Ci). It is interesting to note a considerable variation in $\Delta^{13}C$ (21.184 to 25.370) among the genotypes at lowest light intensity. It is well established that the $\Delta^{13}C$ depends on Ci (Farquhar et al, 1989)[3] and the variation in Ci depends on stomatal as well as mesophyll factors [12]. In order to dissect this, we examined the effect of the shade on stomatal index/frequency.

On a whole, stomatal frequency was reduced with decrease in light intensity except in Panniyur-1 and panniyur-4 (Table-II). Maintaining less number of stomata under low light is beneficial to the plant to conserve moisture [13]. Plant is unable to fix carbon due to low available Photosynthetic Photon Flux Density (PPFD). If leaf maintains more number of stomata under such a situation, it is likely to loose water without much carbon gain. Stomatal conductance is not a limiting factor under shade for photosynthesis. Therefore, we can argue that the observed variation in $\Delta^{13}C$ at 90 % shade was probably due to the variation in the mesophyll associated factors.

Thickness of the leaf measured in terms of Specific Leaf Weight (SLW), which is increased under low light in all the genotypes of black pepper (Table-II). Number of leaf per plant decreased considerably under low light and leaves became thin under low light. Similar observation was made in groundnut genotypes [14, 15] under different growing conditions.

The rate at which carbon is getting fixed by the leaves was measured based on Net Assimilation Rate (NAR), another estimate of photosynthetic rate. An apparent decline in NAR was observed with decrease in light intensity, which is expected as light is the driving force for photosynthesis and associated physiological process [16]. However, genotypes did respond differentially with different light regimes. Among the genotypes, Karimunda and panniyur-4 showed high NAR of 2.85 $gm^{-2}day^{-1}$ and 2.12 $gm^{-2}day^{-1}$ respectively at 90 % shade and Panniyur-1 and Panniyur-5 showed low NAR (Table-III).

Leaf Area Ratio was highest in Panniyur-5 among the genotypes in all the shade treatments (Table-3). Karimunda and Panniyur-4 maintained relatively high LAR under 90 % shade. Together with high NAR, these two genotypes found to be relatively shade tolerant. On the other hand, Panniyur-1 showed low LAR coupled with low NAR, hence can be categorized as a shade sensitive type. Similar evidence in the case of coffee cultivars have been reported earlier [17]. Among the genotypes, Karimunda and Panniyur-1 found to be contrasting in their physiological parameters under very low light intensity. It is interesting to note that these two types also have contrasting $\Delta^{13}C$. Karimunda a shade tolerant type, showed lower $\Delta^{13}C$ where as Panniyur-1 showed higher $\Delta^{13}C$ (Table-I and III), substantiating the hypothesis that $\Delta^{13}C$ reflects the efficiency of CO_2 fixation by the leaf under low light conditions.

This study highlights the use of carbon isotope discrimination technique in screening the black pepper genotypes for photosynthetic carbon assimilation under varying shade conditions. In these two genotypes adapted to low light photosynthetic efficiency and gas exchange parameters were analyzed.

Table-I: Effect of shade on Carbon Isotope Discrimination

Variety/ Hybrids	**$\Delta^{13}C$ (‰)**		
	50 % shade	**65 % shade**	**90 % shade**
Karimunda	20.415	21.730	22.719
Panniyur-1	21.736	21.116	24.830
Panniyur-2	19.984	20.894	21.184
Panniyur-4	20.386	21.650	22.602
Panniyur-5	22.130	21.440	25.370
Mean	**20.930**	**21.366**	**23.341**

Table-II: Effect of shade on growth and physiological parameters of black pepper (60 days after the shade treatment)

Genotypes/Shade level	50 %			65 %			90 %		
	No. of Leaves	SLW cm^2g^{-1}	Stomatal $No.mm^{-2}$	No. of Leaves	SLW cm^2g^{-1}	Stomatal $No.mm^{-2}$	No. of Leaves	SLW cm^2g^{-1}	Stomatal $No.mm^{-2}$
Karimunda	25.0	116	23.25	28.3	146	20.93	16.0	215	15.12
Panniyur-1	9.0	114	22.09	9.3	114	20.93	5.0	146	25.58
Panniyur-2	17.7	139	22.09	14.7	120	22.03	10.7	160	19.77
Panniyur-3	9.7	119	19.77	14.7	119	19.77	8.7	147	13.95
Panniyur-4	19.7	104	22.09	16.7	130	16.28	11.0	155	19.77
Panniyur-5	11.4	105	22.09	10.0	132	21.51	5.4	158	23.25
Mean	**15.4**	**116**	**21.7**	**15.6**	**127**	**20.2**	**9.46**	**164**	**19.6**

CD (Critical Difference) at 0.05 probability for No. of leaves = 2.35; SLW=23.7; Stomatal number = 0.51

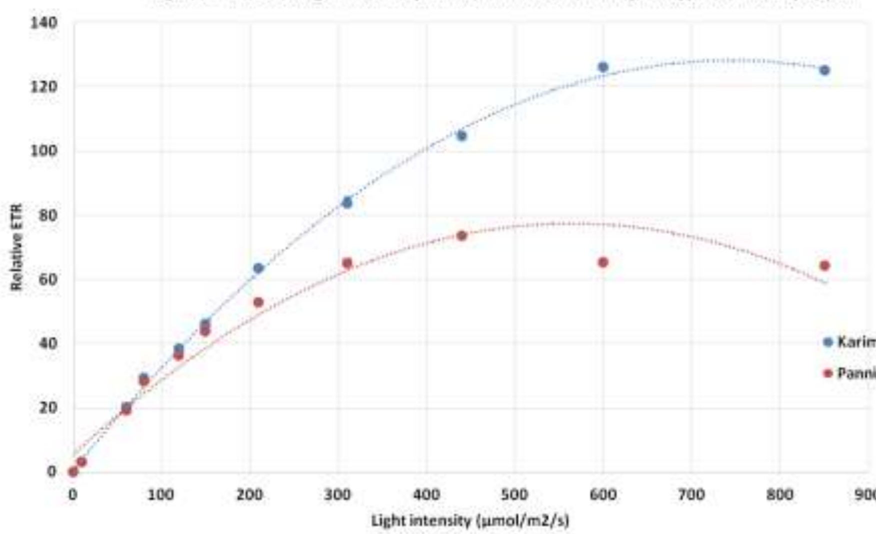
Table-III: Effect of shade on physiological parameters of black pepper

Genotypes/Shade level	50 %			65 %			90 %		
	NAR	LAR	SCMR	NAR	LAR	SCMR	NAR	LAR	SCMR
Karimunda	3.55	58.40	56.32	3.17	70.52	61.00	2.85	61.43	69.40
Panniyur-1	2.98	47.26	52.79	3.18	46.29	60.00	0.98	47.58	69.57
Panniyur-2	2.82	44.15	55.37	3.02	40.64	59.72	1.87	48.97	66.56
Panniyur-3	3.30	54.10	51.98	3.55	55.70	50.44	1.83	65.09	64.56
Panniyur-4	4.08	54.33	52.5	2.93	62.04	51.34	2.12	64.44	65.42
Panniyur-5	1.854	76.70	52.84	2.03	75.32	59.10	0.83	88.42	70.23
Mean	**3.09**	**55.8**	**53.63**	**2.98**	**58.41**	**56.90**	**1.74**	**62.65**	**67.62**

NAR = Net Assimilation Rate ($gm^{-2}day^{-1}$), LAR = Leaf Area Ratio (cm^2g^{-1}), SCMR= SPAD Chlorophyll Meter Reading. CD at 0.05 probability for NAR = 0.31; LAR=5.1; SCMR = 2.8

Relative electron transport rate (ETR) is an indication of photosynthetic efficiency. ETR was measured in a non invasive way using Chlorophyll fluorescence meter. The relative electron transport rate was measured in two contrasting genotypes Karimunda and Panniyur-1 adapted to low light intensity.

Figure-1: Effect of light intensity on Fv/Fm ratios of two genotypes of black pepper



It was found that Karimunda is having higher electron transport rate than Panniyur-1 at low light intensity (Fig-1). Therefore Karimunda is efficient in absorbing light than panniyur-1 under low light intensity. Gas exchange parameters were measured in Karimunda and Panniyur-1 grown under two different light intensities. Under low light Karimunda registered higher photosynthetic rate than Panniyur-1. Interestingly the internal carbon dioxide concentration was much lower compared to Panniyur-1 under shade. Lower carbon isotope discrimination noticed earlier in Karimunda may be due to its ability to maintain lower Ci under low light. Similar conclusions were also drawn by Carelli et al 1999 [18] in *Coffea* species. In both the cultivars of black pepper decrease in transpiration is observed under shade. But Ci is relatively low in Karimunda compared to Panniyur-1 under low light. Low Ci in Karimunda indicates its ability to fix carbon at low light intensity. This is further strengthen by high instantaneous photosynthetic rate.

Table-IV: Photosynthetic rate and gas exchange parameters in black pepper exposed to different light intensities

Variety	Treatment	Light (µmole.$m^{-2}.s^{-1}$)	Ci (ppm) (Internal CO_2 concentration)	Phtotosynthetic rate (µmole.$m^{-2}.s^{-1}$)	Transpiration (µmole.$m^{-2}.s^{-1}$)
Karimunda	Light	602	271	4.98	2.10
	Shade	182	276	3.73	1.53
Panniyur1	Light	612	253	6.23	1.97
	Shade	181	294	2.53	0.90
CD at 5%		2.58	5.40	0.33	0.100

The experiment on mechanism of light interception at low light by Karimunda is underway to provide better clarity on the observed differences among the genotypes. Further, we are also contemplating to examine the $\Delta^{13}C$ as screening tool in other shade loving species too.

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