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Changes in Chloroplast Lipid Composition in Leaves of a *Brassica juncea* Variety as a Consequence of Infection by *Cuscuta reflexa*

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Abstract
Brassica juncea was infected with the angiosperm parasite, Cuscuta reflexa, on 24 DAS (days after sowing). A noteworthy reduction (44%) in carbon dioxide assimilation in leaves was found compared to normal plant, when both were assayed on 38 DAS. At this stage, the chloroplast lipid in infected leaves had decreased by 33%, with a reduction of 11, 39 and 53%, respectively, in neutral, glyco- and phospholipids. Among the glycolipids, the decrease in the content of MGDG, DGDG and SQDG was 23, 69 and 45%, respectively, with 2.4-fold increase in the MGDG/DGDG ratio. The chlorophyll content was reduced by 26%, while the carotenoid level increased by 35%. The free fatty acid content was enhanced by 45% which may be attributed to an enhancement in lipase activity. The contents of saturated fatty acids (capric, lauric, myristic, palmitic and stearic) increased while the contents of unsaturated fatty acids (palmitoleic, oleic, linoleic and linolenic) decreased. The ratio of unsaturated to saturated fatty acids declined over 50%. These findings may entail chloroplasts as reactive sites of the host-parasite interaction in case of the angiosperm parasite.

Key words: *Brassica juncea, carotenoid, chlorophyll, chloroplast, crucifereae, Cuscuta reflexa, leaf, lipid composition, parasitism*

Abbreviations. *DAS= day after sowin; DGDG= digalactosyl diglyceride; FAME= fatty acid methyl esters; FFA= free fatty acids; MGDG= monogalactosyl diglyceride; SQDG= sulfoquinovosyl diglyceride.*

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I. Introduction

Various stress conditions viz. nutritional stress [Sharma and Sanwal, 1992; Singh, 2016], light stress [Powles, 1984; Kumar and Khare, 2019] and biotic stress [Sreenivasulu et al., 1977; Singla and Krattinger, 2017] have been observed to affect the chloroplast biochemistry of higher plants. Studies on photosynthetic contribution of assimilates by different organs to the total lipid production of rape plant led to the conclusion that leaf was the main assimilatory organ during initial siliqua formation [Nalborczyk et al., 1987; Kirkegaard et al., 1997; Dreccer et al., 2000]. Earlier studies on seed oil quality as a consequence of biotic and abiotic stress have revealed drastic reduction in the quantity and quality of seed oil of various oil seed bearing plants (Mishra and Sanwal, 1992; Amir et al., 2005; Alqudah et al., 2010; Sabagh et al., 2019). Taking into the possibility of alteration in photosynthetic properties in leaves, and thus choloplast lipids upon biotic stress the present study has been undertaken to monitor the effect of infection by *Cuscuta reflexa* on leaf chloroplast lipid composition of *Brassica juncea.*

II.Materials And Methods

Indian mustard *(Brassica juncea* L. Cv. AHBJ-7044) belonging to the family of the *crucifereae* was chosen as the host for *Cuscuta reflexa* roxb. Plants were raised separately in flower pots under natural outdoor conditions of light and humidity from October to March. Infection by *C. reflexa* was carried out on the 24th DAS. Leaves were collected from the host plants on the 38th DAS in order to investigate the effect of *C. reflexa* on host plants during the preflowering growth phase. Growth patterns of the plants and mode of induction of parasitism was similar to that described by Mishra and Sanwal [1992].

Measurement of carbon dioxide assimilation during photosynthesis. Carbon dioxide assimilation during photosynthesis was measured in terms of co_2 uptake according to Long and Hallgren (1985). Co_2 uptake was measured in a closed system, Li-6000 IRGA (Li-Cor. Inc., USA). Normal leaf of 1 cm width was taken for the measurements. The flow rate was set at 6.0 ml s^{-1}. Observations were made in one cycle, with

intermissions of 10 sec. The data were stored in a computer and calculations for carbon dioxide assimilation were made later.

Chloroplast isolation. Chloroplasts were isolated from leaves according to Douce et al. (1973). Leaves (10g) from *Brassica* plants were chopped, mixed with 50 ml of chilled grinding medium A containing 0.33 m sucrose in 0.15 M sodium phosphate buffer (pH 7.3) and homogenized in a waring blender for 3 sec. The leaf debris, removed through squeezing, was rewashed twice with 25 ml medium A and squeezed again. The filtrate was centrifuged at 2500x g for 2 min in a refrigerated centrifuge at 4^0C and the crude chloroplast pellet suspended in 0.8 ml medium A. The suspension was loaded on the top of a density gradient consisting of 6 ml 46% and 9 ml 50% sucrose in 0.15 m phosphate buffer (pH 7.3) and centrifuged at 1000x g for 30 min in a swing out rotor. A discrete green band appeared just above the junction of two gradients. The band was removed, suspended in 2 vol of medium A and centrifuged in a swing out rotor at 2000x g for 5 min, which sedimented intact chloroplasts as a dark green pellet at the bottom of the tube.

To assess the extent of functional chloroplasts present in the preparation, intactness was determined polarographically using a Hansatech oxygen electrode DW2. A chloroplast suspension equivalent to 50 mg chl ml^{-1}, suspended in medium b containing Tricine (50 mM, pH 7.6), KCl (5 mM), $MgCl_2$ (mM) and NH_4Cl (5 mM), was placed in the electrode well and stirred magnetically for 2 min to ensure complete rupture of chloroplasts. Ferricyanide dependent 2 evolution was assayed by adding 3 mm of K-ferricyanide. The same procedure was repeated but with 0.33 m sucrose added to the medium. The intactness of isolated chloroplasts was determined according to Mishra and Sanwal (1994).

The assessment of freedom of contamination by mitochondria was made by assaying the mitochondrial marker enzyme cytochrome c oxidase in the preparation. Cytochrome c oxidase activity was determined according to Potter (1959).

Determination of total chlorophyll, chlorophyll a, b and carotenoid content.Total chlorophyll, chlorophyll a, b and carotenoids were determined according protocol adopted by Mishra and Sanwal (1994).

Lipid extraction and analysis. The chloroplast lipid was extracted according to the method of Roughan et a1. (1978) using a chloroform — methanol (2:1, v/v) mixture and purification of lipid fraction performed according to Folch et al. (1957). The purified lipid fraction in chloroform was stored under no at -20 ^{0}c till further use. Dry weight of lipid was determined according to Mishra and Sanwal (1992).

Lipids were fractionated into neutral, glyco- and phospholipids by chromatography on silicic acid column with the solvent sequence — chloroform, acetone, methanol (Rouser et al., 1976). In addition,

Two-dimensional TLC with chloroform/ methanol/ 28% aqueous ammonia (65/ 35/ 5, v/v) followed by chloroform/ acetone/ methanol/ acetic acid/ water (5/ 2/ 1/ 1/ 0.5, v/v) was employed to check the purity of each fraction. Chloroform eluate contained MGDG, DGDG and SQDG (plus diphosphatidyl glycerol and phosphatidic acid in traces), while methanol eluate contained mostly phospholipids with traces of glycolipids. Each lipid class was quantified on the basis of dry weight as well as by chemical estimation. The acetone fraction, containing glycolipids, was subjected to thin layer chromatography on silica gel G employing the developing solvent : chloroform-acetone-methanol-acetic acid-water (5:2:1:1:0.5, v/v). Identification of individual glycolipids was carried out by running authentic reference standards simultaneously and also by staining with α-naphthol reagent (Siakotes and Rouser, 1965). Water spray (Gardner, 1968) was used for analytical purposes. The glycolipids separated chromatographically were estimated quantitatively by determining the amount of sugar after hydrolysis employing the method of Roughan and Batt (1968).

FFA content was determined according to Lowry and Tinsley (1976). The total fatty acid composition was determined as described by Mishra and Sanwal (1992). FAME were separated and detected by gas liquid chromatography. The identification of fame was carried out using Heptadecanoic fatty acyl ester as internal standard. The values for each fatty acid are given as percent by weight of total fatty acids.

Lipase activity. Lipase activity was measured spectrophotometrically by following the protocol of Schmidt et al. (1974). Leaf homogenate was prepared by grinding 10 g leaves in 500 ml of homogenizing medium Consisting of 0.6 m sucrose, 1 mm EDTA, 10 mm kCl, 1 mm MgCl2, 2 mm DTT, and 0.15 m phosphate buffer, pH 7.5.

III. Results

Intactness and purity of isdldted chloroplasts. According to authors' computation 40-45% of the isolated chloroplasts appeared to be intact. There was no difference in intactness between chloroplasts isolated from the control and infected plants. The mitochondrial contamination to the chloroplast preparation was negligible as revealed by nondetectable activity of cytochrome c oxidase.

Effect of Cuscuta infection on carbon dioxide assimilation during photosynthesis. Data expressed as (pmols CO_2 assimilated m^{-2} s^{-1} show (Table 1) that on about two weeks after initiation of infection, *Cuscuta* was found to cause a 44% reduction in carbon dioxide assimilation in normal leaves of AHBJ-7044

Table 1. Effect of *Cuscuta reflexa* on Carbon dioxide assimilation during photosynthesis in leaves of *Brassica juncea.* Results are expressed as pmols CO_2 assimilated $m^{-2}S$-1 mean ±SD of three sets of experiments with triplicates in each set.

Control	Infected	Decrease(%)
34±4	19±3*	44

*Differences significant at P< 0.001

Alterations in contents of total lipid, various lipid classes and glycolipid components. *Cuscuta* infestation led to a significant decrease in total chloroplast lipid in AHBJ-7044 leaves. There was a 33% reduction in total chloroplast lipid (Table 2). *Cuscuta* infection caused reduction to different extent in contents of various lipid classes; the reduction being 11, 39 and 53% in neutral, glyco- and phospholipid, respectively. A highly significant alteration was observed amongst the glycolipid components of leaf chloroplasts. Upon infection, the decrease in MGDG, DGDG and SQDG contents was 23, 69 and 45, respectively (Table 3). The ratio of MGDG/DGDGin leaf chloroplasts increased from 1.8 to 4.4 upon infection by *Cuscuta.*

Table 2. Effect of by *Cuscuta* infection on the contents of total chloroplast lipid and various lipid classes of AHBJ-7044 leaves. Results are mean ±SD of three sets of experiments with triplicates in each set.

	Lipid content µg- (100 gFW leaf)$^{-1}$	
	Control	Infected
Total lipid	6450 ± 111	4318 ± 104[a]
Neutral lipids	1105 ± 28	988 ± 23[b]
Glycolipids	4364 ± 87	2659 ± 65[a]
Phospholipids	720±28	336 ± 23[a]

[a]Difference significant at $P < 0.001$; [b]Difference significant at $P < 0.05$.

Table 3. Alterations in galacto- and sulpholipids of leaf chloroplasts of AHBJ-7044 upon infection by *Cuscuta reflexa.* Results are mean ±SD of four sets of experiments with duplicates in each set and differences between control and infected sets are significant at $P < 0.001$

	Lipid content µg- (100 gFW leaf)$^{-1}$	
	Control	Infected
MGDG	2292 ± 62	1761 ± 24
DGDG	1292 ± 45	402 ± 14
SQDG	392 ± 23	216 ± 11
MGDG	1.8	4.4
DGDG		

Alterations in total chlorophyll, chlorophyll a, b and carotenoid contents of* Brassica *leaves upon infection by Cuscuta. Reduction in total chlorophyll content of *Brassica* leaves observed on 38 DAS was highly significant (26%) when infection was initiated on 24 DAS (Table 4). A significant decrease was also found in chlorophyll a and b. The reduction in chlorophyll a and b was 29% and 33%, respectively. In contrast, total carotenoid content showed a significant enhancement (35%) as a consequence of infection by C. *refiexa.*

Changes in FFA content of leaf chloroplasts of Brassica juncea. *Cuscuta* infestation resulted in a significant increase in FFA content of mustard leaf chloroplasts (Table 5). Expressed as µg per 100g fresh weight of leaves, the FFA content increased by 45% in infected plants compared to the control plants.

***Lipase activity in leaves of AHBJ-7044 plants upon inyection by C.* reflexa**. Lipase activity in leaves of infected plants was 46'% higher compared to the control plants (Table 5). The increase was significant.

Table 4. Effect of *Cuscuta reflexa* on total chlorophyll, chlorophyll a, b and carotenoid contents of *Brassica juncea* leaves. Results are mean ±SD of three sets of experiments with triplicates in each set and differences are significant at $P < 0.001$

	Pigments µg (100 gFW leaf)$^{-1}$		% Increase (+) Decrease (—)
	Control	Infected	
Chlorophyll	890 ± 24	662 ± 21	—26
Chlorophyll a	638 ± 17	456 ± 14	—29
Chlorophyll b	246 ± 16	166 ± 13	—33
Carotenoids	166 ± 4	224 ± 9	+35

Table 5. Changes in FFA content of leaf chloroplasts of *Brassica juncea* upon infection by *Cuscuta* reflexa and lipase activity of leaves. Results are mean ± SD of three sets of experiments with duplicates in each set and difference between control and infected sets is significant at $P < 0.05$

Plant sets	FFA µg (100 gFW leaf)$^{-1}$	**Lipase activity** pmoles FA released h^{-1}(gFW leaf)$^{-1}$
Control	49 ± 3	48 ± 2
Infected	71 ± 3	70 ± 2

Effect of Cuscuta infection on total fatty acid composition of leaf chloroplasts. Upon infection, there was increase in saturated fatty acids and decrease in unsaturated fatty acids of rape chloroplasts (Table 6). The saturated fatty acids, capric, lauric, myristic, palmitic and stearic, enhanced by 100, 91, 67, 75 and 23%, respectively. While there was a highly significant decrease in unsaturated fatty acids. The decrease in palmitoleic, oleic, linoleic and linolenic acid was 55, 38, 47 and 26%, respectively.
The ratio of unsaturated to saturated fatty acids reduced over 50%.

IV.Discussion

The infestation of *Brassica* plant by *Cuscuta* led to a decrease of leaf chloroplast lipid. This could be explained as a consequence of the enhancement in lipase activity of *Brassica* leaves by over 45% compared to the control plant. This is substantiated by the examination that FFA content of *Brassica juncea* leaf chloroplast increased in response to infection by *Cuscuta reflexa.* It has been observed that the carbon dioxide assimilation during photosynthesis in *Brassica* leaves is reduced by 44% as a result of infection. This is expected to decrease the synthesis of sucrose and other photosynthates and thus may lead in reduction in the amount of pyruvate in chloroplasts. Pyruvate is known to be converted to acetyl- CoA in pea [Williams and Randall, 1979; Alban et al., 1994] and spinach (Liedvogel, 1985; Lemmark and gardestrdm**,** 1994) chloroplasts by the action of pyruvate dehydrogenase. Acetyl-CoA is used as a substrate for acetyl-CoA carboxylase with the product being malonyl-CoA in leaf mesophyll cells. This enzyme has been localized with in the chloroplast (Nikolau et al., 1984; Elborough et al., 1994).

Table 6. Effect of *Cuscuta* infection on total **fatty** acid composition of leaf chloroplasts of *Brassica juncea* plants. Results are expressed as % of total fatty acids and are mean ±SD of three sets of experiments with duplicates in each set and differences are significant at $P < 0.001$.

Fatty acid		Control	Infected	% Increase (+) Decrease (-)
Capric	(10:0)	1.0 ± 0.21	2.0 ± 0.20	+100
Laurie	(12:0)	1.1 ± 0.20	2.1 ± 0.21	+91
Myristic	(14:0)	1.2 ± 0.20	2.0 ± 0.20	+67
Palmitic	(16:0)	16.0 ± 1.24	28.0 ±2.40	+75
Palmitoleic	(16:1)	1.1 ± 0.05	0.5 ± 0.04	—55
Stearic	(18:0)	17.1 ± 0.24	21.0 ±0.21	+23
Oleic	(18:1)	1.6 ± 0.22	1.0 ± 0.21	-38
Linoleic	(18:2)	7.5 ± 1.10	4.0 ± 1.00	—47
Linolenic	(18:3)	53.4 ± 2.80	39.4 ± 2.40	—26

Both acetyl- CoA and malonyl- CoA are used for fatty acid biosynthesis. Fatty acids are lastly incorporated in a variety of classes of lipids. Therefore reduction in photosynthetic assimilation of CO_2 as a consequence of infestation by the parasite would eventually result in the reduced lipid synthesis in leaf chloroplasts. The decreased galactolipid in *Brassica* leaves can also be traced to reduced synthesis of sucrose as a result of decrease in carbon dioxide assimilation during photosynthesis and also channeling of sucrose to the parasite, *Cuscuta*.

Certain studies on phloem unloading [Wolswinkel, 1975, 1987; Ellis et al., 1992; Baluska et al., 2001) suggested parasitizing *Cuscuta* as a foremost sink of host with a potential to ovemile host sinks that makes it a tremendous sink. Thus during infection of the *Brassica* plant by *Cuscuta,* sucrose is channeled to the parasite rather than synthesis of lipid in host leaves. Diacylglycerol is synthesized from sucrose through a variety of enzymatic steps and is incorporated into the biosynthesis of phospholipids [Moore, 1982; Bates et al., 2012; Bates, 2016]. Therefore, a decline in sucrose synthesis of the host plant would lead to declined synthesis of diacylglycerol and consequently phospholipids. It was also pragmatic that MGDG/DGDG ratio of the host plant was augmented significantly from 1.8 to 4.4 upon *Cuscuta* infestation, which may lead to deterioration of the membrane bilayer. Badawi et a1. (2004) observed an enhancement in the MGDG/DGDG ratio in salt tolerant tobacco leaves. Because of the different arrangements of MGDG and DGDG within the thylakoid membrane, an alteration in their proportion is likely to be associated with an alteration in the physical properties of thylakoid membranes.

The infestation of *Brassica* plant by dodder resulted in decrease of the content of chlorophyll (a and b) in leaves. Baker et al. (1997) reported the transfer of glutamic acid and citrulline from host *(alnus glutinosa)* to the haloparasite *(Lathraea clandestina).* It is also relevant to consider that Christensen et al. (2003) confirmed extensive transfer of amino acids from host plant to *Cuscuta.* Any decrease in the content of glutamic acid, the precursor of the chlorophyll biosynthesis, of the *Brassica* plant upon its transport to the parasite *Cuscuta* can elucidate reduced synthesis of chlorophyll in the host plant. In addition, it is appealing to suppose that upon infection the increased activity of chlorophyllase might lead to enhanced degradation of chlorophyll molecule into chlorophyllide and phytol, eventually resulting into lowered level of chlorophyll pigments. *Cuscuta* infestation resulted in a lower chlorophyll content. Carotenoids appear to gather in chromoplasts, which develop from chloroplasts. It can be speculated that partial transformation of chloroplasts into chromoplasts takes place as a result of infection of the rape plant by *Cuscuta.*

The variation in fatty acid profile of rape leaves as a result of infection by *Cuscuta* was highly significant. The saturated fatty acids increased with significant decrease in unsaturated fatty acids upon infection. The prominent decrease was observed in linoleic and linolenic acid. Linolenic acid (18:3) is a product of a sequential desaturation involving 18:1 —› 18:2 —+ 18:3. Major repositories of 18:3 are the galactolipids (mgdg and dgdg) of the chloroplast. Hence a reduction in galactolipid contents in Leaves of the rape plant consequent to infection by the parasite could be the possible explanation for decrease in 18:3 fatty acids.

The reduction in MGDG, DGDG, linoleic and linolenic acid of *Brassica* leaves upon parasitization by *Cuscuta reflexa* indicates chloroplast as reactive sites of host- parasite interaction in the case of an angiosperm parasite.

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