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Effects of timing of defoliation on nitrogen assimilation and associated changes in ethylene biosynthesis in mustard (*Brassica juncea*)

Pervez M. Lone, Rahat Nazar, Sarvajeet Singh & Nafees A. Khan*

Department of Botany, Aligarh Muslim University, Aligarh 202002, India; e-mail: naf9@lycos.com

Abstract: Mustard (*Brassica juncea* L.) is characterized by oblong-shaped leaves on the lower layers of axis. These leaves are poorly illuminated, remain below light photosynthetic compensation point and abscise at maturity. Earlier research has shown that the removal of these shaded leaves improves photosynthetic potential of the rest of the leaves, the overall growth and yield of the crop. Now we show that 50% removal of these leaves at pre-flowering stage, i.e. 40 days after sowing (DAS) enhances nitrogen assimilation in the leaves more substantially than defoliation at post-flowering stage, i.e. 60 DAS. Further, the changes in N assimilation were concurrent with the ethylene evolution. It is suggested that strategies that lead to the abscission of lower leaves at early stage of growth may be adopted for efficient N utilization, and ethylene may be considered as an important physiological tool.

Key words: defoliation; ethylene; mustard; nitrogen assimilation

Introduction

Partial or complete removal of leaves has been described as defoliation. Responses of plants to defoliation are of considerable economic importance (Mc Naughton 1979). Defoliation in mustard (*Brassica juncea* L.) is an age-old practice. Mustard leaves on lower layers contribute to the development of supra-optimal leaf area indices with accompanying self shading and shading by other leaves within the plant axis. These shaded leaves receive reduced irradiation and thus are less photosynthetically active (Khan 2002, 2003; Khan & Lone 2005). Earlier research has shown that removal of these shaded leaves improves assimilate balance, growth and photosynthetic potential of the rest of the leaves (Khan et al. 2002). The crop has high nitrogen (N) requirement for optimal growth and yield responses as the role of N in growth and photosynthesis is well established (Marschner 1995). Modifications in source/sink relations are considered as prominent factors in N accumulation (Bouchart et al. 1998). The reported study was conducted with the assumption that N assimilation in leaves is enhanced after defoliation and the N assimilation is linked with the ethylene biosynthesis as ethylene has vital influence in providing physiological adaptive signals (Abeles et al. 1992; Khan 2005, 2006). The objective of the reported research was to test the timing of defoliation on N assimilation and concurrent changes in ethylene biosynthesis.

Material and methods

Plant material and treatments

Mustard (*Brassica juncea* L. cv. Alankar) seeds were sown in 10 m^2 experimental plots of Aligarh Muslim University, Aligarh India. The available NO_3^-- N of the sandy loam experimental plot determined at a depth of 25 cm was at a concentration of 18 g N m^{-2} soil. Sufficient phosphorus (P) and potassium (K) at 2 g P and 3 g K m^{-2}, respectively were added to the soil at the time of sowing so as these nutrients were non-limiting. At seedling establishment, a uniform population of 12 plants m^{-2} was maintained.

Defoliation of 50% lower leaves on plant axis was done as described earlier (Khan et al. 2002) at pre-flowering, i.e. 40 days after sowing (DAS), or post-flowering, i.e. 60 DAS, and N assimilation and ethylene biosynthesis were measured at 80 DAS. The number of leaves at 40 and 60 DAS was 12 and 32, respectively. At 80 DAS, the number of leaves on non-defoliated control plants was 43, and was 49 and 46 on plants defoliated at 40 and 60 DAS, respectively (Khan & Lone 2005). At the two timing of defoliation (40 or 60 DAS), half of the total leaf number, 6 and 16, respectively present on lower half axis was removed. For the control set, all the leaves were left intact. The treatments were arranged in a randomized block design. Each treatment was replicated three times.

For estimation of N assimilation and ethylene biosynthesis, the uppermost three fully expanded leaves from four plants in each treatment were used.

N assimilation

For estimation of N assimilation, the activities of nitrate reductase (NR), nitrite reductase (N_iR) and glutamine syn-

* Corresponding author

Table 1. Effect of defoliation of 50% of leaves from lower layers of plant axis of mustard (*Brassica juncea*) at pre-flowering, i.e. 40 days after sowing (DAS) or post-flowering, i.e. 60 DAS on the activities of nitrate reductase (NR; mmol NO_2 g^{-1} FW h^{-1}), nitrite reductase (NiR; μmol NO_2 g^{-1} FW h^{-1}) and glutamine synthetase (GS; μmol γ-glutamyl hydroxamate g^{-1} FW h^{-1}) determined at 80 DAS (Mean ± SE).

Treatment	NR	NiR	GS
Control	7.47 ± 0.36c	18.26 ± 0.80c	34.51 ± 1.22c
Defoliation at 40 DAS	8.39 ± 0.42a	21.18 ± 0.94a	38.24 ± 1.36a
Defoliation at 60 DAS	8.08 ± 0.38b	19.16 ± 0.82b	35.43 ± 1.42b
No defoliation control at 40 DAS	4.28	15.31	18.15
No defoliation control at 60 DAS	6.12	17.22	22.40

Data followed by the same letter within a column are significantly not different at $P < 0.05$.

thetase (GS) were assayed. Root and leaf N concentrations were determined in acid-peroxide digested leaf sample according to Lindner (1944).

Activity of NR in leaves was estimated by the method of Jaworski (1971). Fresh 200 mg of leaf tissue segments was incubated for 2 h at 30 ℃ in a reaction mixture containing 2.5 mL of 0.1 M phosphate buffer (pH 7.5), 0.5 mL of 0.2 M potassium nitrate solution and 2.5 mL of 5% isopropanol. After incubation the reaction was stopped by adding 0.3 mL each of 1% sulphanilamide and 0.02% NED-HCl to 0.4 mL reaction mixture, and NO_2^- released was measured spectrophotometrically at 540 nm (SL-164 UV-VIS, Elico, Hyderabad, India).

Nitrite reductase activity was determined using methyl viologen as the reductant (Lillo 1984). Enzyme extract was prepared by homogenizing 5 g of leaf tissue with 50 mL of 0.5 M Tris-HCl buffer (pH 7.5) in a blender. The homogenate was passed through multilayered cheesecloth and used as enzyme extract. To 0.3 mL enzyme extract, a reaction mixture consisting of 6.25 mL of 0.5 M Tris-HCl buffer (pH 7.5), 2 mL of 3 mM sodium nitrite, 2 mL of 0.75 mM methyl viologen were added. Freshly prepared $Na_2S_2O_4$ (0.2 mL 17 mg /mL in 0.29 M $NaHCO_3$) was added to start the reaction. After incubation at 30 ℃ for 15 min, 14.75 mL of distilled water was added, and air was led into the mixture to ensure that oxidation of methyl viologen was complete. To 20 μL aliquot, 1.0 mL each of 1% sulphanilamide and 0.02% NED-HCl was added and nitrite content was measured spectrophotometrically at 540 nm.

The enzyme extract for GS assayed by the method of Farnden & Robertson (1980) was prepared by homogenizing 1.0 g leaf material in 5 mL of 50 mM imidazole-acetate buffer (pH 7.8) containing 0.5 mM EDTA, 1 mM dithiothreitol, 2 mM $MnCl_2$ and 20% glycerol. The homogenate was centrifuged at 10 000 *g* at 4 ℃ (CPR 24 Remi, New Delhi) for 3 min and supernatant was used as enzyme extract. To 0.2 mL of enzyme extract, 2.0 mL of 0.2 M L-glutamine, 0.5 mL of 20 mM sodium arsenate and 0.3 mL of 2 mM $MnCl_2$ were added followed by the addition of 0.5 mL of 1 mM ADP and 50 mM hydroxylamine and incubated at 37 ℃ for 30 min. The final pH was 8.0. The reaction was stopped by adding 1.0 mL of 2.5% $FeCl_2$ and 5% trichloroacetic acid in 1.5 mM HCl to the reaction mixture, and absorbance was read at 540 nm.

Ethylene biosynthesis

Activity of 1-aminocyclopropane carboxylic acid synthase (ACS) was measured according to Avni et al. (1994) and Woeste et al. (1999). Leaf tissue was ground in 100 mM N-2 hydroxyethyl piperazine-N-2 ethanesulfonic acid buffer (pH 8.0) containing 4 mM dithiothreitol, 2.5mM pyridoxal phosphate, and 2.5% polyvinylpyrrolidone. After thorough homogenization, the preparation was centrifuged at 12 000 *g* for 15 min. One mL of the supernatant was placed into a 30 mL tube and 0.1 mL of 5 mM S-adenosyl methionine (AdoMet) was added. This was incubated for 1 h at 22 ℃. The 1-aminocyclopropane carboxylic acid (ACC) formed was converted to ethylene by the addition of 0.1 mL of 20 mM $HgCl_2$, followed by 0.1 mL of 1:1 mixture of saturated NaOH/NaOCl. The tubes were capped immediately after addition of NaOH/NaOCl and incubated on ice for 10 min. For control set, AdoMet was not added. A 5 mL of gas phase was removed with a syringe and ethylene was measured on a gas chromatograph GLC 5700 (Nucon, New Delhi) equipped with 1.8 m Porapack N (80/100mesh) column, a flame ionization detector, and an integrator. Nitrogen gas was used as a carrier. The flow rates of nitrogen, hydrogen and oxygen were 0.5, 0.5 and 5 mL s^{-1}, respectively. The oven temperature was 100 ℃ and the detector was at 150 ℃. Ethylene identification was based on the retention time and quantified comparing with the peaks from standard ethylene concentration.

Data analysis

Data were analyzed statistically using SPSS (10.0 for Windows). Standard error was calculated and ANOVA was performed on the data to determine least significant difference (LSD) at $P < 0.05$. The treatment mean was separated by Duncan's multiple range test. Different letters indicate significant difference at $P < 0.05$.

Results and discussion

Defoliation of lower leaves significantly affected N assimilation and ethylene biosynthesis in new leaves (Tables 1–3). The activities of nitrate reductase, nitrite reductase and glutamine synthetase and leaf N concentration were higher in plants defoliated at 40 DAS than the control or plants defoliated at 60 DAS. The activities of NR, N_iR and GS were enhanced by 12.3, 16.0 and 10.8% with defoliation at 40 DAS over control, and 8.2, 5.0 and 2.7% with defoliation at 60 DAS compared to the control. Root N and leaf N concentrations were increased by 17.1 and 7.9% in plants defoliated at 40 DAS and 9.7 and 5.3% in plants defoliated at 60 DAS compared to the control.

The activity of ACS and ethylene evolution showed significant increase in plants following defoliation, the greatest increase was found with defoliation treatment at 40 DAS. The activity of ACS was increased by 12.5% when defoliation was done at 40 DAS, and by 7.5% when defoliated at 60 DAS compared to the control.

Table 2. Effect of defoliation of 50% leaves from lower layers of plant axis of mustard (*Brassica juncea*) at pre-flowering, i.e. 40 days after sowing (DAS) or post-flowering, i.e. 60 DAS on root and leaf N concentration (mg g^{-1} dry mass) determined at 80 DAS (Mean ± SE).

Treatment	Root N	Leaf N
Control	16.4 ± 0.52c	26.5 ± 0.62c
Defoliation at 40 DAS	19.2 ± 0.62a	28.6 ± 0.74a
Defoliation at 60 DAS	18.0 ± 0.56b	27.9 ± 0.68b
No defoliation control at 40 DAS	11.2	18.6
No defoliation control at 60 DAS	14.6	22.4

Data followed by the same letter within a column are significantly not different at $P < 0.05$.

Table 3. Effect of defoliation of 50% leaves from lower layers of plant axis of mustard (*Brassica juncea*) at pre-flowering, i.e. 40 days after sowing (DAS) or post-flowering, i.e. 60 DAS, on 1-aminocyclopropane carboxylic acid synthase (ACS) activity (nmol ACC g^{-1} leaf FW h^{-1}) and ethylene evolution (nL g^{-1} leaf FW h^{-1}) in the leaves of plants analyzed at 80 DAS (Mean ± SE).

Treatment	ACS	Ethylene
Control	3.75 ± 0.12c	10.52 ± 0.42c
Defoliation at 40 DAS	4.22 ± 0.16a	12.45 ± 0.58a
Defoliation at 60 DAS	4.03 ± 0.14b	11.07 ± 0.44b
No defoliation control at 40 DAS	2.34	6.94
No defoliation control at 60 DAS	2.86	8.88

Data followed by the same letter within a column are significantly not different at $P < 0.05$.

Ethylene evolution was18.3 and 5.2% higher with defoliation treatment at 40 and 60 DAS, respectively compared to the control.

Nitrogen assimilation is an important aspect in N acquisition for overall growth, physiology and development of plant (Marschner 1995). An understanding of the process that governs N assimilation in crops is of major importance with respect to both environmental concerns and the quality of crops (Gastal & Lemaire 2002). Plants after defoliation required large amount of N to compensate growth and maintenance of new leaves emerging on the upper axis. Earlier, it has been reported that defoliation at 40 DAS enhanced emergence of new leaves on the upper axis with higher photosynthetic capacity (Khan et al. 2002; Khan & Lone 2005). Marriott & Haystead (1990) reported that defoliation increased the rates of leaf emergence and the development of young leaves to maturity.

The emergence of new leaves has been shown to have greater efficiency for CO_2 assimilation (Alderfer & Eagles 1976; Caemmere & Farquhar 1984). The new leaves which emerged on the upper axis following defoliation require more N for the maintenance of growth and photosynthesis. The plants subjected to defoliation showed increased N concentration in root and were able to meet the increased N demand of leaf as shown by higher concentration of leaf N (Tables 1–2). The maximum increase in N concentration in root and leaf occurred in plants subjected to defoliation at 40 DAS. It has been reported that about 40–60% of the total N in roots was remobilized to meet the N demand of shoots during early leaf growth (Lefevre et al. 1991; Hendershot & Volenec 1993). The adaptation to defoliation, partial or complete, in many grass species involved a capacity of remobilization of N stored in roots allowing N to be supplied to the growing zones (Thornthon et al. 1993; Volenec et al. 1996; Goulas et al. 2002). The increase in the enzyme activities of N assimilation and leaf N content following defoliation in the present study show that the plants defoliated at 40 DAS assimilated more N than the plants defoliated at 60 DAS (Table 1). Crafts-Brandner et al. (1983) reported enhanced nitrate assimilation in soybean after the removal of vegetative shoot apex. The significant increase in total plant nitrate reductase activity was found to highly correlate with shoot regrowth. Boucaud & Bigot (1989) reported an increased glutamine synthetase activity in regrowing tissue of ryegrass.

The concurrent changes in ethylene biosynthesis with N assimilation after defoliation suggest involvement of the hormone in this physiological phenomenon. The higher activity of ethylene biosynthesis in plants subjected to defoliation at 40 DAS compared to those subjected to defoliation at 60 DAS is attributed to the emergence of more new leaves of higher assimilatory capacity at early stage of growth, i.e. 40 DAS as the younger leaves produce more ethylene than older leaves because of their higher auxin concentration (Abeles et al. 1992; Khan et al. 2002). Moreover, higher N assimilation by plants defoliated at 40 DAS led to higher ethylene evolution in comparison with plants defoliated at 60 DAS. The interaction of nitrogen with ethylene evolution has been reported. Tari & Szen (1995) and Lynch & Brown (1997) reported that nitrogen availability affected ethylene synthesis. N-use efficiency of wheat and barley has been increased with the application of ethephon, an ethylene-releasing compound (van Sanford et al. 1989; Bulman & Smith 1993). Young leaves following defoliation at 40 DAS had higher ethylene level and irradiance, which increased nitrate reductase activity (Streit & Feller 1982), and the activities of nitrite reductase and glutamine synthetase and leaf N content. Moreover, ethylene has been reported to enhance glutamine synthetase activity (Pujade-Renaud et al., 1994). Thus, the pre-flowering stage (40 DAS) is a more active stage of metabolism than 60 DAS. It was, therefore, found to be superior in N assimilation, which is linked with ethylene biosynthesis.

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