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ORIGINAL ARTICLE

Effect of nitrogen levels and leaf position on carbohydrate and nitrogen metabolism in FCV tobacco (*Nicotiana tabacum* L.)

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Abstract Interplay of nitrogen and carbohydrate metabolism, which influenced by nitrogen nutrient management determines the quality and chemical composition of cured tobacco leaf. Tobacco variety Kanchan is grown in Northern light soils of Andhra Pradesh and is a high yielding variety showing response to higher levels of nitrogen. Effect of nitrogen (0, 40, 80, 120, 160 and 200 kg N ha^{-1}) on nitrate reductase, nitrate accumulation and starch content in different leaf position of variety Kanchan was studied in pot (150 kg) culture with Northern light soil of Andhra Pradesh. The starch content decreased significantly with increase in application of nitrogen in three growth stages. But the maximum decrease in starch content was observed with increase in application of nitrogen from 120 to 160 kg N ha^{-1} in all leaf positions except in the top position. The in vivo nitrate reductase (NR) activity increased significantly with increase in leaf position from bottom to top and decreased with age of the plant from 50 to 70 days. Nitrate–N content increased from 0.14 mg g^{-1} dry wt. in the bottom leaves of control plants to 8.615 mg g^{-1} dry wt in the leaves of plants treated with 200 kg N ha^{-1}. The nitrate content increased from bottom to top position in all treatments with increase in nitrogen application. Increased application of nitrogen increased the NR activity and accumulation of nitrate–N, thus a positive relationship was observed between NR activity and nitrate–N accumulation whereas starch content decreased with increased application of nitrogen. The negative relationship was observed between NR activity and starch accumulation and also between nitrate–N and starch. The recommendation of 120 kg N ha^{-1} for the variety Kanchan grown in northern light soils of Andhra Pradesh was found to be optimum as NR activity was at a par between 120 and 200 kg N ha^{-1} application at 60 and 70 days of growth. Thus NR activity may be taken as one of the indicators to the maturity of leaf and availability of nitrogen to the plants.

Keywords Tobacco · Nitrogen levels · NR activity · Starch · Nitrate nitrogen

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Flue-cured Virginia tobacco (*Nicotiana tabacum* L) is one of the important commercial crops grown in India and is a highly quality conscious crop. Balanced composition of carbohydrate and nitrogen components in the leaf is a predisposing factor for quality (Tso 1972). Nitrogen plays an important role on the yield and quality of tobacco. The first step in nitrate assimilatory pathway is the reduction of NO_3^- to NO_2^- catalyzed by the enzyme nitrate reductase, which is a rate-limiting step that regulates the inflow of inorganic nitrogen (NO^{-3}) to organic form in plants. Several studies indicated that the level of nitrate reductase in plant tissues is altered by exogenous application of nitrogen sources.

Though nitrogen is a key nutrient in tobacco fertilization, but tobacco is also sensitive to nitrogen fertilization. In tobacco, leaf being the economic product, inadequate or excess nitrogen show adverse effect on growth and chemistry of flue-cured tobacco. From the seedling stage to final harvest, the soil nitrogen regimes affect the process of plant development and chemistry of cured tobacco more than any other element. Weybrew et al. (1983) reported that influence of nitrogen nutrition on interaction of nitrogen and carbohydrate metabolism determined the quality and chemical composition of cured leaf. Inadequate levels of nitrogen causes premature yellowing of leaves, which when cured are generally pale in colour, close grained and thick bodied and their smoke is flat, insipid and imbalance of quality constituents. An increase in nitrogen generally produce higher yields of tobacco but leaf maturity is delayed and cured leaf will be dark brown to black in colour, dry and chaffy, and have a strong and pungent smoke (Court et al. 1984). The accumulation of N-nitrogen in tobacco leaf influences the health related smoke constituents (Shi et al. 2013). Tobacco is multi-level harvesting crop and leaves are harvested from the bottom when ever the leaves are matured. Nitrate concentration increases in the tobacco leaf as the nitrogen fertilizer dose increases (Chandrasekhararao et al. 2013). Accumulation of nitrate is one of the major problems encountered in most plants. Plants accumulate nitrate, when the uptake of this anion exceeds its metabolism (Ruiz et al. 1998). The inducible enzyme nitrate reductase (EC 1.6.6.1) can be used to differentiate between metabolically active and inactive nitrogen fractions, and provide a measure of plant nitrogen status and crop response to added nitrogen. In tobacco, leaf is the economically important product and more than 80 % of nitrate reduction occurs in the leaves. Tobacco variety Kanchan is grown in Northern light soils of Andhra Pradesh and is a high yielding variety showing response to higher levels of nitrogen. In the present paper, effect of different levels of nitrogen has been studied on leaf position and growth stages on accumulation of starch, nitrate–N and activity of nitrate reductase, as these are mainly involved in carbohydrate and nitrogen metabolism.

A pot culture experiment was conducted for two seasons (2009–2010) in completely randomized block design with six nitrogen levels (0, 2.4, 4.8, 7.2, 9.6, 12 g N plant^{-1} corresponding to 0, 40, 80, 120, 160 and 200 kg N ha^{-1} respectively) with five replications. Cemented pots were filled with 150 kg light textured soil collected from CTRI research farm, Jeelugumilli, West Godavari district, Andhra Pradesh. The soil was slightly acidic, low in soluble salts, chlorides, available nitrogen and available potassium and medium in available phosphorous. Uniform seedlings grown in poly-trays (60 days) of flue-cured tobacco variety Kanchan were transplanted @ one seedling per pot. The plants were grown as per the recommended package of practices. Each pot received P_2O_5 and K_2O @ 3.6 and 7.2 g plant^{-1}, respectively. The leaves of the test plants were tagged as and when they formed to find out the exact leaf number. Leaf samples were taken uniformly from the same position on the stalk and the data were analyzed statistically (Gomez and Gomez 1984). The leaf number 3 and 4 (bottom), 10 and 11 (middle) and 17 and 18 (top) on the stalk were taken for the experiment.

In vivo nitrate reductase activity was estimated in fresh leaves (Ushasri et al. 1986) with modification. The leaf discs (100 mg) were incubated in 5 ml reaction mixture containing 2 ml of 0.5 M KNO_3, 1 ml of 0.05 % triton ×100 and 1 ml of 15 % propanol in potassium phosphate buffer (0.1 M, pH 6.0) for 30 min in dark after evacuating for 1 min. The enzyme activity was expressed as µmol of nitrite formed g^{-1} fresh weight min^{-1}. Nitrate–N was estimated by taking 200 mg of tobacco leaf powder in 150 ml Erlenmeyer flasks and 30 ml water added to it and kept shaking for 2 h and filtered through Whatman No. 1 filter paper (Cataldo et al. 1975). Aliquots of 0.2 ml of the extracts were taken into 25 ml volumetric flasks, and mixed thoroughly with 0.8 ml of 5 % (w/v) salicylic acid in concentrated H_2SO_4 (Sa-H_2SO_4). After 20 min of incubation at room temperature, 19 ml of 2 N NaOH was added slowly with a burette to raise the pH to 12. Samples were cooled to room temperature and absorbance was recorded at 410 nm. The nitrate–N content was estimated from standard curve prepared using potassium nitrate as standard. Starch content was estimated in the dried powder as per Gaines and Meudt (1968).

Starch content decreased with increase in position from bottom to top leaf on the stalk, whereas it increased with increase in age of the plant from 50 to 70 days. Plants with out nitrogen application showed higher starch content compared to all other nitrogen treatments at all leaf positions (Table 1). The starch content decreased significantly with increase in application of nitrogen in all three growth stages, highest decrease was observed from 120 to 160 kg N ha^{-1} at all the leaf positions, except at the top position. Bottom position leaves showed highest starch at all nitrogen levels, followed by middle and top. The starch content decreased by 31.4, 36.5 and 34 % in bottom, middle and top position leaves, respectively with the application of 160 kg N ha^{-1} over 120 kg N ha^{-1}.

With increase in age of the plant, the starch content increased significantly from 50 to 70 days in nitrogen applied plants, whereas the starch content increased from bottom to middle and decreased from middle to top in plants without nitrogen application. The starch content decreased by 97, 52.2 and 11 % at 50, 60 and 70 days, respectively with increase in nitrogen application from 120 to 160 kg N ha^{-1}. At 70 days, the starch content in the bottom position leaves was significantly higher than middle and top position and also over 50 and 60 days.

Accumulation of higher levels of starch in leaves in nitrogen limited plants was a consequence of decrease in demand of carbon-skeleton for synthesis of amino acids,

Table 1 Effect of nitrogen, leaf position and growth stages on starch (% dry wt.) content in FCV tobacco variety Kanchan

Treatment kg N ha^{-1}	Days after planting											
	Bottom leaves				Middle leaves				Top leaves			
	50	60	70	Mean	50	60	70	Mean	50	60	70	Mean
Control	9.28	19.40	15.50	14.73	8.31	15.92	13.53	12.58	6.98	13.34	11.69	10.76
40	7.40	10.61	13.42	10.47	5.21	10.01	11.10	8.77	3.45	6.95	7.46	5.95
80	4.04	7.71	12.47	8.07	2.52	6.41	10.96	6.63	1.01	5.38	8.90	5.09
120	4.31	7.57	9.80	7.23	2.10	5.79	9.40	5.76	0.95	5.22	6.80	4.32
160	1.01	4.33	9.43	4.92	0.75	2.56	7.67	3.66	0.53	2.00	6.01	2.85
200	0.80	1.60	5.72	2.71	0.31	1.03	2.73	1.36	0.18	0.85	1.92	0.98
Mean	4.47	8.53	11.05	8.02	3.20	6.95	9.23	6.46	2.18	5.62	7.17	4.99

	SEM±	CD ($P = 0.05$)		SEM±	CD ($P = 0.05$)
Nitrogen	0.12	0.32	Position × age	0.14	0.40
Position	0.08	0.23	N × P × age	0.35	0.97
Age	0.09	0.26	Nitrogen × position	0.20	0.56
			Nitrogen × age	0.18	0.54

Table 2 Effect of nitrogen, leaf position and age on in vivo NR activity (μmol g^{-1} fr. wt. min^{-1}) in FCV tobacco variety Kanchan

Treatment kg N ha^{-1}	Days after planting												Treatment means
	Bottom leaves				Middle leaves				Top leaves				
	50	50	50	50	50	50	50	50	50	50	50	50	
Control	5.36	4.56	2.9	4.3	13.3	8.93	6.9	9.7	23.73	12.36	8.5	14.86	9.9
40	13.86	7.7	8.6	10.06	25.86	15.56	10.23	17.2	40.73	31.6	19.4	30.56	19.26
80	23.6	19.43	16.3	19.76	28.36	22.2	22.1	24.2	44.2	36.5	26.7	35.8	26.6
120	30.93	23.2	17.6	23.9	38.46	29.06	22.7	30	65.4	42.13	27.8	45.3	33.1
160	28.9	25.6	22.5	25.66	31.16	31.16	28.3	30.2	69.13	69.13	29.8	56	37.3
200	35.7	29.96	19.73	28.46	61.66	36.6	24.3	40.9	62.5	62.66	31.6	52.26	42.03
Mean	23.06	18.4	14.6	18.7	33.13	23.93	19.1	25.36	50.93	42.5	23.9	39.13	

	SEM±	CD ($P = 0.05$))		SEM±	CD ($P = 0.05$)
Nitrogen	0.59	1.6	Position × age	0.72	2.01
Position	0.45	1.22	N × P × age	1.77	4.93
Age	0.42	0.16	Nitrogen × position	1.06	2.94
			Nitrogen × age	1.02	2.84

proteins and other nitrogenous compounds under N-deficient conditions. Carbohydrate metabolism of sink leaves was altered in nitrogen-limited plants, as starch and sucrose levels were elevated relative to nitrogen supplied, even as growth was being restricted (Thomas et al. 1988). Accumulation of carbohydrates, especially starch, mannitol, sucrose and glucose has been observed in nitrogen-deficient leaves of olive cultivars (Boussadia et al. 2010). The starch content of the plants grown under the high-nitrogen condition at the heading stage was lower than that under a low-nitrogen condition (Hirano et al. 2005). Decrease in starch accumulation with increase in nitrogen application in burley and FCV tobacco has been reported by Sims and Atkinson (1971) and Chandrasekhararao et al. (2013).

The mean NR activity increased significantly from bottom to top leaf position and the activity in the top position leaves was 2.1 times higher than the bottom leaves and decreased with age of the plant from 50 to 70 days (Table 2). In the control plants the NR activity increased from 4.3 in bottom position leaves to 14.86 μmol g^{-1} fr. wt. min^{-1} in top position leaves, whereas at 160 kg N ha^{-1} treatment, the NR activity increased from 25.66 in bottom

Table 3 Effect of nitrogen, leaf position and growth stages on nitrate nitrogen (mg g^{-1} dry wt.) content in FCV tobacco variety Kanchan

Treatment kg N ha^{-1}	Days after planting												Treatment means
	Bottom leaves				Middle leaves				Top leaves				
	50	50	50	50	50	50	50	50	50	50	50	50	
Control	0.14	0.280	0.316	0.25	0.179	0.345	0.384	0.30	0.229	0.345	0.435	0.33	0.29
40	0.163	0.641	0.483	0.43	0.858	0.745	0.503	0.70	0.894	0.745	0.818	0.92	0.69
80	0.787	1.059	0.513	0.79	0.965	1.184	0.595	0.91	1.164	1.184	0.827	1.15	0.95
120	2.051	0.975	1.007	1.34	4.365	1.392	1.019	2.26	6.28	1.392	1.128	2.97	2.19
160	3.395	1.735	1.102	2.08	7.275	2.905	1.086	3.76	8.295	2.905	1.125	4.26	3.37
200	6.430	2.919	1.118	3.49	8.308	5.760	2.513	5.53	8.615	5.760	3.544	6.22	5.08
Mean	2.16	1.26	0.75		3.65	2.05	1.02		4.23	2.37	3.544		

	SEM±	CD ($P = 0.05$)		SEM±	CD ($P = 0.05$)
Nitrogen	0.07	0.08	Position × age	0.08	0.22
Position	0.05	0.13	N × P × age	0.20	0.55
Age	0.07	0.18	Nitrogen × position	0.11	0.32
			Nitrogen × age	0.12	0.36

position leaves to 56 µmol g^{-1} fr. wt. min^{-1} in top position leaves. Highest NR activity was observed in top position leaves at 70 days of age in 160 kg N ha^{-1} treatment.

The NR activity at 120 kg N ha^{-1} was at a par with 160 kg N ha^{-1} in bottom and middle position leaves, whereas it significantly increased in top position leaves. The increase in NR activity with increase in nitrogen application from 120 to 200 kg N ha^{-1} was 18.6, 36 and 13.2 % in bottom, middle and top position leaves, respectively (Table 2). The interaction effect between age, leaf position and nitrogen application was significant. NR activity was at par among the 120, 160 and 200 kg N ha^{-1} treatments at 60 and 70 days age in bottom leaves, at 70 days of age in middle leaves and at 50 and 70 days age in top position leaves. NR activity was at par between the nitrogen treatments 120 and 160 kg N ha^{-1} at 50 days in bottom, and at 50 and 60 days in middle position leaves.

The NR activity increased with increase in application of nitrogen from 40 to 200 kg N ha^{-1}, with the exception from 120 to 160 kg N ha^{-1} at 50 days of growth where the NR activity decreased. The interaction effect between the age and nitrogen on NR activity was at par between the N treatments of 120 and 160 kg N ha^{-1} at 50 days. NR activity was at par between 160 and 200 kg N ha^{-1} applications at 60 and 70 days of age. The interaction between leaf position and age of the plants was significant. NR activity increased significantly at each age with increase in leaf position from bottom to top.

Shangguan (2007) repotted a significant linear correlation between NR activity and nitrate content at tillering and jointing stages of winter wheat. The NR activity began to decline at about 70 DAP and this served as a useful indicator of when to apply nitrogen fertilizer for its best utilization. Santamaria et al. (2002) reported the accumulation of nitrate–N increased with increase in nitrogen supply in rocket salad.

Nitrate–N content in the bottom leaves of plants without nitrogen application increased from 0.14 to 8.615 mg g^{-1} dry wt. in the leaves of plants treated with 200 kg N ha^{-1} (Table 3). The nitrate content increased from bottom to top position in all treatments with increase in nitrogen application. The mean nitrate–N content increased significantly with increase in leaf position from bottom to top. In the control plants, the nitrate–N content increased non-significantly with the increase in leaf position from bottom to top, whereas accumulation of nitrate–N increased significantly with increase in nitrogen from 80 to 200 kg N ha^{-1} (Table 3). The nitrate–N content increased by 55.2, 66.4 and 42.5 % in bottom, middle and top positions, respectively with increase in nitrogen from 120 to 160 kg N ha^{-1}.

With increase in age from 50 to 70 DAS, the mean nitrate–N content decreased significantly. The nitrate–N content increased non-significantly in the plants with out nitrogen application with growth of the plants from 50 to 70 days, whereas in 40 and 80 kg N ha^{-1} treatments, the nitrate–N content was at par at the three growth stages (Table 3). But in other N treatments, the nitrate–N content decreased significantly from 50 to 70 days of age. The nitrate–N content increased by 49.4, 106.9 and 4.76 % at 50, 60, and 70 days of age, respectively by increase in application in nitrogen from 120 to 160 kg N ha^{-1}. The interaction between the leaf position and age of the plant was significant. The nitrate–N content increased significantly with increase in leaf position from bottom to top at each stage of growth and decreased with age of growth in each position.

Accumulation of nitrate–N with increased levels of nitrogen supply has been reported in lettuce (Mars and Osvald 2002). The accumulation of nitrate in plants depends on their genetic characteristics as well as on many environmental factors, such as nitrogen supply or methods of application, light intensity, photoperiod, temperature or water supply. All factors influencing the nitrate uptake, translocation and assimilation processes may affect nitrate concentration in plant tissue. The increased nitrate content in plants may be the result of intensified nitrate uptake and/or decreased activity of NR. Variation in nitrate content in different genotypes of FCV tobacco varieties has been reported (Padmavathi 2008). Raja Rao and Suryanarayana (1988) reported that accumulation of higher levels of nitrate–N in air-cured burley tobacco genotypes, which was considered to be a genetic factor. Chandrasekhararao et al. (2013) reported that nitrate nitrogen content increased significantly with increased level of nitrogen application from 40 to 160 kg N ha^{-1} and decreased at 200 kg N ha^{-1} in cured tobacco and also reported increase in nitrate–N content with increase in leaf position. Burton and Walton (1989) have reported a wide variation in nitrate content of cured tobacco depending on cultural practices.

In the present study increased application of nitrogen increased the NR activity and accumulation of nitrate–N, thus a positive relationship was observed between NR and nitrate–N accumulation. NR is an inducible enzyme and its activity will be increased with increase in availability of its substrate nitrate. The starch content decreased with increase in application of nitrogen and with increase in NR activity both in leaf positions and growth stages. Thus a negative relationship was observed between NR activity and starch accumulation and also between nitrate–N and starch. It indicated that till the NR activity is very active due to availability of nitrogen in the form of nitrate in growth medium, starch accumulation was not taking place. In FCV tobacco, leaf matures generally about 70 days after transplanting, it means that when leaves are nearing maturity, the nitrate metabolism especially uptake of nitrate should be low for maximum accumulation of starch so that more sugars will be formed during the curing. This is one of the reasons to recommend that application of nitrogen fertilizer should be completed after 40 days of transplanting so as to reduce the availability of nitrogen to the plant when it comes to maturity to allow the maximum accumulation of starch. Accumulation of higher levels of nitrate was positively correlated with tobacco specific nitrosamines (TSNA), which are considered to be associated with tobacco related health problems. Shi et al. (2013) reported that as the nitrate–N increased in cured burley tobacco, TSNA formation increased significantly during leaf storage at high temperature. Addition of nitrate onto flue-cured tobacco to the level equivalent to burley tobacco followed by high-temperature treatment increased the TSNA concentration comparable to burley tobacco.

The results showed that higher levels of nitrogen application has negative effect on the starch accumulation, whereas positive effect on NR activity and accumulation of nitrate–N. At recommended dose of nitrogen, i.e., 120 kg N ha^{-1} for the variety Kanchan, the NR activity was at a par between 120 and 160 kg N ha^{-1} indicating that the substrate concentration, i.e., applied nitrogen was sufficient for the maximum induction of NR activity for utilizing the applied nitrogen fertilizer. Time of application for the last dose of nitrogen fertilizer to the FCV tobacco is very important so that the NR activity and accumulation of nitrate nitrogen should come to lowest by the time of flower bud initiation stage, which leads to the rapid accumulation of starch in the lower leaves as the demand for photosynthate in reduction process decreases and leads to maturity. Thus NR activity may be taken as one of the indicators to the maturity of leaf and availability of nitrogen in the plants.

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