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RESPONSE OF LEAF CHARACTERISTICS OF BT COTTON PLANTS TO RATIO OF NITROGEN, PHOSPHORUS, AND POTASSIUM

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Abstract

Application of nitrogen (N), phosphorous (P) and potassium (K) is an important practice for cotton (*Gossypium hirsutum* L.) production and is a useful tool to enhance cotton plant growth and physiological activities, especially during boll setting and boll development. This study investigated the effects of the ratio of N, P, and K on physiological parameters of leaves during boll development using *Bacillus thuringiensis* (Bt) cotton cultivars Siza 1 and Sikang 1. As compared with the treatment without P and K application, combined application of N, P and K significantly increased chlorophyll *a* content by 5.7% and 14.6%, chlorophyll *b* content by 22.6% and 3.8%, carotenoids content by 14.7% and 9.9%, soluble proteins by 0.6% and 17.5% as well as soluble sugar by 16.8% and 7.9% in the two growing seasons. Similarly, catalase was enhanced by 10.0% and 14.6%, peroxidase by 45.5% and 47.5%, superoxide dismutase contents by 4.9% and 42.2%, and seed cotton yield by 29.2% and 18.7%, respectively. Our study indicated that combined application of N, P, and K at appropriate ratios enhanced the activity of physiological parameters and yield and the ratio of 1:0.6:0.9 considerably performed best for Sikang 1 cultivar amongst all treatments.

Key words: Cotton, Genotypes, Physiology, Nutrients management.

Abbreviations: DAF: Day after flowering, SOD: Superoxide dismutase, CAT: Catalase, POD: Peroxidase, N: Nitrogen, P: Phosphorous; K: Potassium, chl: Chlorophyll.

Introduction

Efficient supply of macronutrients is necessary for cotton (*Gossypium hirsutum* L.) growth and production. Of all the nutrients needed by cotton plants, nitrogen (N), phosphorous (P), and potassium (K) are the three most important nutrients needed in higher quantity to sustain and coordinate vegetative and reproductive growth of cotton plants, and guarantee cotton productivity (Guo *et al*., 2010). The distribution pattern of macronutrients in cotton plants varies considerably at different stages of cotton plant growth and development. During the vegetative growth period, N, P, and K are used to meet the requirement of subtending leaf of cotton boll emergence, expansion, photosynthesis and stem growth (Egli & Bruening, 2001; Dong *et al*., 2012; Silvertooth *et al*., 2011). During the flowering and boll growth period, macronutrients are mainly used for accumulation of biomass and its transportation from leaves to reproductive organs for boll growth and development (Kavakli *et al*., 2000; Saleem *et al*., 2010).

As compared with P and K application, cotton growers pay higher attention to N application. With the wide extension of *Bacillus thuringiensis* (Bt) transgenic cotton in the past two decades, the role of N in cotton production has been given even more attention. It has been widely acknowledged that proper use of nitrogen can increase physiological activities and protein content of Bt cotton plants, especially during flowering and boll development (Lu *et al*., 2006). The application of P fertilizer is also acknowledged by cotton producers due to the interactive effects between N and P in enhancing cotton plant growth and increasing cotton yield. However, the importance of K on Bt cotton is less accepted by cotton producers as compared with N and P. Pettigrew *et al*., (2005) reported there had been remarkable improvements in cotton yield resulting from optimum K application. Johnston & Milford (2012) reported that sufficient K supply was necessary to manage efficiency of N as they are directly correlated to each other.

The plant available K (exchangeable K) status of soil has a substantial effect on the uptake of N by crops. When the exchangeable soil K content is less than a basic target level, the effect of N on increasing cotton yield is reduced. The interaction between the K and N applications on plant growth and yield attributes that are seen at agronomic level can be explained by their effects and the interactions on the growth process within the plants at the cell and tissue levels. K application and its relative proportion to N and P is the most important factor for crop productivity (Awais *et al*., 2017). Leaf net photosynthesis rate, stomatal conductance, and enzyme activity (Rubisco) were also reported to be significantly affected by K (Zhao *et al*., 2001; Wang *et al*., 2012). Therefore, by understanding how N, P and K work together, a superior methodology can be adopted for improving the physiological activity and cotton yield. Besides this, the understanding of how the combination of N, P, and K work together is extremely important to develop an appropriate strategy to improve cotton production. In previous research different studies has been conducted to investigate the individual effects of N, P, and K application on cotton. Additionally, there are also many previous studies about the interactive effects of N and P, and N and K. However, very few studies have been conducted to examine the combined effects of integrated application of N, P, and K at various ratios (Ahmad *et al*., 2019). The main objectives of this study were (i) to investigate the effects of different ratios of N, P, and K on physiological characteristics of Bt cotton leaves, biomass accumulation and yield attributes and (ii) to compare two cotton cultivars and determine the appropriate ratio of N, P and K for the enhancement of physiological activities of Bt cotton leaves and seed cotton yield.

Materials and Methods

A field experiment was conducted on the Experimental Farm of Yangzhou University, Jiangsu Province, China (32°30′ N, 11°925′ E) during two cotton growing seasons of 2016 and 2017. The field soil (0-20 cm layer) contained 1.27 mg kg^{-1} total N, 107.2 mg kg^{-1} mineral N (sum of $NO3^{-}$ and $NH4^{+}$-N), 15.1 mg kg^{-1} $Bray^{-1}$ P, and 77.9 mg kg^{-1} soil test K with pH 6.9 (1:1 in water). The determination of N in soil following the Kjeldhal method, available phosphorus (P) following Micro-Vanadate-Molybdate method and available potassium (K) following neutral ammonium acetate extract method was determined by flame photometer (Ibrahim *et al*., 2018). In both years, the study was arranged in a 2 × 4 factorial completely randomized design with three replicates, with cultivar and the ratio of N, P, and K as the two experimental factors. Siza 1 (a Bt transgenic hybrid, marked as A1 in the figures) and Sikang 1 (Bt transgenic parent of Sikang 1, marked as A2 in the figures) were used. The second experimental factor was the ratio of N, P, and K applied at four levels, including 1:0:0, 1:0.3:0.6, 1:0.6:0.9 and 1:0.9:1.2 (N: P_2O_5: K_2O) marked as B1, B2, B3, and B4 in the figures of this paper.

The seeds were sown on April 20 in 28 m^2 (8.0 m × 3.5 m) plots at a line spacing of 100 cm. At the second leaf age, the cotton plants were thinned to 30000 plants hm^{-2} at an approximate plant spacing of 33.3 cm. For all the plots, N was applied twice at an equal rate of 150 kg hm^{-2} after seed sowing and at initial flowering. Field management practices were performed in accordance with local recommendations.

Observations and measurements

Leaf physiological parameters: From July 22 to July 26 in both growing seasons, all the flowers of the plants of each plot were tagged and the flowering date was marked. At the 3^{rd}, 10^{th}, 17^{th}, 24^{th}, 31^{st}, 38^{th}, and 45^{th} day after flowering (DAF), ten leaves of each treatment were sampled and immersed in liquid nitrogen for 15 min and then stored at -80°C for determination of physiological parameters, including pigment content (chl *a*, chl *b*, and carotenoids), soluble protein, soluble sugar and enzymatic activity of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) (Ahmad *et al*., 2019) with minor modification.

Soluble protein: For the extraction and analysis of soluble proteins, approximately 0.5 g of leaf sample was homogenized at 4ºC in 5 ml Na-phosphate buffer (pH 7.2) and then centrifuged at 4ºC. The supernatant was placed on ice for further analysis. Soluble protein contents were determined by using the Coomassie blue dye-binding assay (Bradford, 1976). Absorbance readings were converted using bovine serum albumin (BSA) as the standard curve. Supernatants and dye were pipetted in spectrophotometer cuvettes and absorbance was determined with a spectrophotometer (Model 721, Shanghai Mapada Instruments Co. Ltd, Shanghai) at 595 nm.

Soluble sugar: 0.5 g sample was dried at 105°C in an oven for total soluble sugar determination. The content of total soluble sugar was determined in ethanol extract of cotton leaves by following the phenol-sulfuric acid method with minor modifications as described by (Cerning-Beroard, 1975).

Chlorophyll content: 0.2 g leaf sample on the same position of a leaf was selected for determination of leaf chl *a*, chl *b* and carotenoids. The leaf sample was cut into small pieces and put it into a 10-ml test tube with 96% ethanol solution and kept in dark. After preparation of all samples, all the test tubes were incubated for 3 h at 40ºC in a boiler. The samples were analyzed after the leaf color changed from green to white by using a spectrophotometer (Lamda 650, Perkin Elmer, USA) at the wavelengths of 470, 646, and 663 nm. The content of chlorophyll (*a*, *b*, and carotenoids) was calculated according to the procedure of (Lichtenthaler & Wellburn, 1983).

Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD): 0.5 g of leaf sample was collected for the extraction and analysis of SOD, POD, and CAT. The leaf sample was homogenized at 4ºC and centrifuged at 10,000 rpm for 15 minutes. Then the supernatant was stored on ice for further determination. The activities of CAT and POD were determined according to the method of (Xu & Ye, 1989) and SOD was measured according to the method of (Gupta *et al*., 1993).

Yield determination: The cotton bolls on 10 selected plants were handpicked every other week after the beginning of boll opening during two growing seasons and afterward sun dried, ginned and weighed to calculate the seed yield (kg hm^{-2}).

Statistical analysis

The study was arranged in a 2 × 4 factorial completely randomized design with three replicates. The data of each variable were subjected to the analysis of variance (ANOVA) according to MSTATC (Steel & Torrie, 1960). When F values were significant, means were separated by the LSD test at the 0.05 probability level.

Results

The content of chl *a* was affected by cultivar, ratio of N, P and K, year, and the interaction between cultivar fertilizer and year at 24, 31, and 38 days after flowering (DAF) (Table 1). Compared with Sikang 1, Siza 1 was 5.7% and 14.6% higher in chl *a* content at 31 and 38 DAF in 2016 and 16.6% and 2.6% higher at 24 and 38 DAF in 2017 (Fig. 1). At 31 DAF, the content of chl *a* was increased by 9.6%, 19.3%, and 19.3% with the ratios of 1:0.3:0.6, 1:0.6:0.9, and 1:0.9:1.2 as compared with the control of 1:0:0. At DAF 38, the ratios of 1:0.3:0.6 and 1:0.6:0.9 increased chl *a* content by 24.6% and 29.2%, but the ratio of 1:0.9:1.2 decreased chl *a* content by 1.5% in the first year. However, during the second year, the content of chl *a* was increased by 33.3%, 17.9%, 32.7%, and 48.6% with the ratio of 1:0.3:0.6, 1:0.6:0.9, and 1:0.9:1.2 at 24 DAF respectively. The chl *a* content was initially increased until 17 DAF, but decreased dramatically at 24 DAF, and increased at 31 and 38 DAF.

Table 1. ANOVA table for leaf chl *a*, chl *b*, carotenoids, CAT, POD, SOD, and soluble protein of two cotton cultivars, Siza 1 and Sikang 1 during 2016 and 2017 year.

Source	*DAF	Chl *a*	Chl *b*	Carotenoids	CAT min^{-1}	POD	SOD	Soluble protein
		mg g^{-1}	mg g^{-1}	mg g^{-1}	µg min^{-1}	µg min^{-1}	µg min^{-1}	mg g^{-1}
C (cultivar)	3	5.5*	60.0**	3.2 ns	3.6 ns	21.7**	49.1**	4.1*
R (ratio of N, P and K)		0.2 ns	6.6**	5.3**	3.5*	8.1**	33.3**	8.4**
Y (year)		64.84**	522.4**	916.4**	4.8*	30.1**	32.9**	65.3**
C×R		0.6 ns	0.6 ns	2.0 ns	3.2*	0.3 ns	5.3**	1.4 ns
C×Y		1.2 ns	54.2**	0.3 ns	5.2*	3.0 ns	19.8**	4.5*
R×Y		1.1 ns	6.0**	0.5 ns	2.5 ns	16.1**	28.4**	7.8**
C×R×Y		0.5 ns	1.7 ns	3.6*	2.4 ns	0.7 ns	3.8*	1.7 ns
C (cultivar)	10	33.8**	37.0**	2.3 ns	0.1 ns	1.4 ns	8.4**	3.1 ns
R (ratio of N, P and K)		2.6 ns	6.6**	0.8 ns	3.5*	1.9 ns	2.7 ns	9.2**
Y (year)		100.8**	542.1**	494.7**	33.4**	27.0**	1639.8**	67.6**
C×R		1.5 ns	0.2 ns	1.6 ns	2.5 ns	0.6 ns	4.0**	0.3 ns
C×Y		15.3**	5.6*	2.1 ns	8.0**	5.5*	35.2**	3.3 ns
R×Y		2.9*	9.8**	8.6**	2.4 ns	1.0 ns	4.6**	8.6**
C×R×Y		0.7 ns	2.5 ns	3.2*	3.5*	0.7 ns	1.8 ns	0.4 ns
C (cultivar)	17	0.1 ns	13.6**	32.0**	13.6**	33.7**	54.6**	4.3*
R (ratio of N, P and K)		1.5 ns	6.0**	5.4**	16.7**	10.0**	4.0**	8.4**
Y (year)		0.1 ns	726.0**	256.2**	1640.6**	0.3 ns	1252.6**	38.1**
C×R		0.1 ns	0.1 ns	4.6**	14.7**	5.6**	0.9 ns	2.0 ns
C×Y		0.3 ns	6.2**	11.6**	12.9**	0.8 ns	27.3**	0.1 ns
R×Y		6.7**	11.8**	0.2 ns	10.9**	2.6 ns	19.3**	8.0**
C×R×Y		3.0*	2.2 ns	1.3 ns	13.1**	5.0**	3.9**	0.6 ns
C (cultivar)	24	18.1**	0.5 ns	4.9*	1.5 ns	21.2**	30.8**	349.7**
R (ratio of N, P and K)		7.8**	1.6 ns	2.6 ns	0.6 ns	36.0**	3.0*	157.3**
Y (year)		98.0**	278.0**	43.8**	0.3 ns	11.4**	3.7 ns	1177.1**
C×R		0.4 ns	2.5 ns	1.5 ns	0.3 ns	21.5**	4.1**	48.0**
C×Y		0.6 ns	0.1 ns	42.5**	0.2 ns	28.5**	3.7 ns	49.0**
R×Y		0.5 ns	0.5 ns	1.0 ns	0.2 ns	27.0**	3.0*	14.5**
C×R×Y		3.2*	0.9 ns	2.2 ns	0.2 ns	22.1**	1.1 ns	22.8**
C (cultivar)	31	5.3*	0.9 ns	0.5 ns	1.3 ns	0.2 ns	9.7**	12.8**
R (ratio of N, P and K)		11.3**	2.1 ns	2.3 ns	5.5**	10.5**	2.3 ns	65.3**
Y (year)		14.5**	101.3**	37.0**	2631.1**	9169.9**	0.1 ns	2.9 ns
C×R		3.3*	4.5**	0.9 ns	2.8*	24.0**	2.4 ns	12.4**
C×Y		11.8**	0.2 ns	2.8 ns	5.0*	0.1 ns	0.0 ns	12.8**
R×Y		2.7 ns	1.0 ns	1.2 ns	9.8**	14.4**	6.5**	49.2**
C×R×Y		4.5**	1.8 ns	0.2 ns	3.5*	20.2**	4.6**	12.5**
C (cultivar)	38	7.6**	1.1 ns	-	-	-	0.9 ns	53.0**
R (ratio of N, P and K)		18.4**	8.3**	-	-	-	6.6**	20.0**
Y (year)		113.0**	443.4**	-	-	-	101.2**	415.2**
C×R		3.8*	3.3*	-	-	-	2.4 ns	4.4**
C×Y		2.5 ns	0.8 ns	-	-	-	37.9**	33.7**
R×Y		8.4**	4.8**	-	-	-	2.8*	20.3**
C×R×Y		9.3**	1.8 ns	-	-	-	5.4**	6.0**

DAF represents days after flowering, *p<0.05, **p<0.01, - (no data)

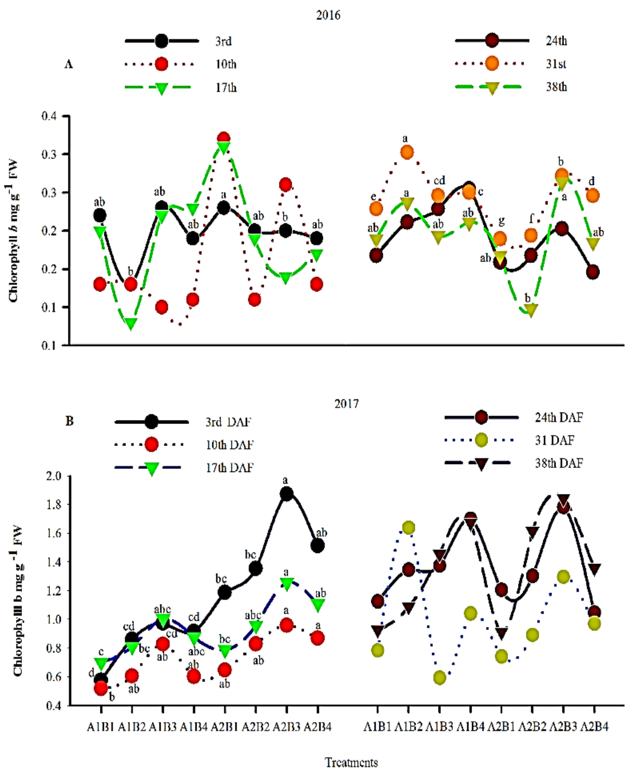

Fig. 1. Effects of ratio of N, P, and K on leaf chlorophyll *a* content of Siza 1 and Sikang 1 cotton plants at different days after flowering in 2016 and 2017 years. A and B represent the 3rd, 10th, 17th, 24th, 31st, and 38th day after flowering respectively. Different letters on the bars in each figure are statistically different at the 0.05 probability level by an ANOVA-protected test. Bars with no letters are not significantly different in each figure.

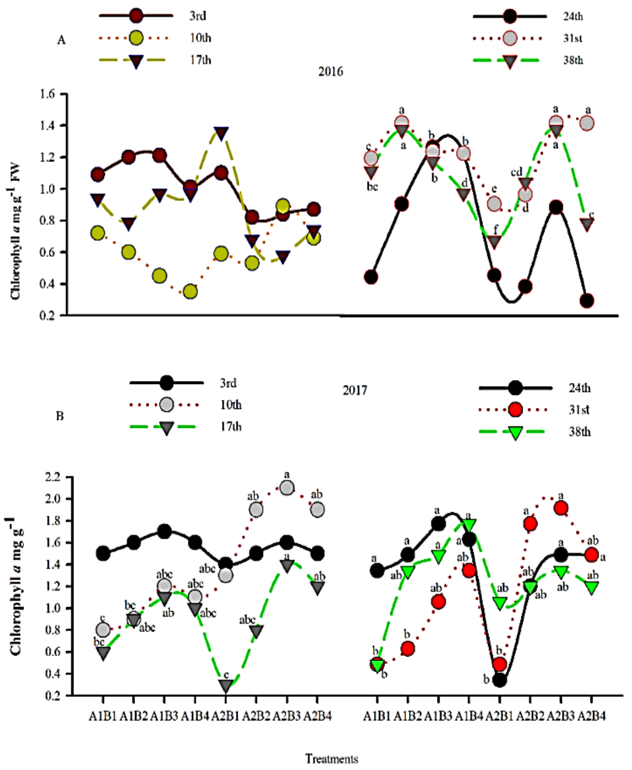

Fig. 2. Effects of ratio of N, P, and K on leaf chlorophyll *b* content of Siza 1 and Sikang 1 cotton plants at different days after flowering during two growing seasons. A and B represent the 3rd, 10th, 17th, 24th, 31st, and 38th day after flowering respectively. Different letters on the bars in each figure are statistically different at the 0.05 probability level by an ANOVA-protected test. Bars with no letters are not significantly different in each figure.

Similar to chl *a*, chl *b* content was affected by cultivar, fertilizer and year and the interaction between fertilizer and year at 3, 10, 17, and 38 DAF (Table 1). Siza 1 was 22.6% and 3.8% higher in chl *b* content at 31 and 38 DAF in during the first year compared to Sikang 1 (Fig. 2A). In 2016, at 31 DAF the content of chl *b* was increased by 37.0%, 44.4% and 37.0% while increased by 45.0%, 60.0% and 25.0% at 38 DAF with the ratios of 1:0.3:0.6, 1:0.6:0.9 and 1:0.9:1.2 as compared to the control. At DAF 17 the ratios of 1:0.3:0.6, 1:0.6:0.9 and 1:0.9:1.2 significantly reduced chl *b* content by 50.0%, 30.0% and 23.0%. However, in 2017 at 17 DAF the content of chl *b* was increased by 20.0%, 50.7% and 46.7% with the ratios of 1:0.3:0.6, 1:0.6:0.9 and 1:0.9:1.2, respectively. Similar to chl *a*, chl *b* was also increased at the initial stage until 17 DAF, decreased at 24 DAF, and increased again at 31 and 38 DAF.

The carotenoid content was affected by year and the interaction between cultivar fertilizer and year at 3 and 10 DAF (Table 1). Siza 1 was 14.6% and 9.9% higher in carotenoid content at 24 and 31 DAF during the first year and 19.0% higher at 38 DAF during the second year as compared to Sikang 1 (Fig. 3A and B). In 2016 at 24 DAF the carotenoid content was increased by 21.7%, 25.7% and 18.3% with the ratios of 1:0.3:0.6, 1:0.6:0.9 and 1:0.9:1.2 compared to the control (1:0:0). However, in 2017 At 31 DAF the ratios of 1:0.3:0.6 and 1:0.6:0.9 increased the content by 23.7% and 29.8% but the ratio of 1:0.9:1.2 decreased the content by 9.6% respectively.

The content of soluble protein was affected by cultivar, fertilizer, and the interaction between cultivar fertilizer and year at 24, 31 and 38 DAF (Table 1). Compared with Sikang 1, Siza 1 was 0.6% and 17.5% higher in soluble protein content at 10 and 24 DAF in 2016-2017 (Table 2). At 24 DAF, the content was increased by 27.6-41.8%, 46.7-44.1% and 33.2-54.5% with the ratios of 1:0.3:0.6, 1:0.6:0.9 and 1:0.9:1.2 compared to the control (1:0:0) during two growing seasons, respectively. Moreover, during the first year soluble protein content was decreased by 0.5% and 0.4% with the ratios of 1:0.3:0.6 and 1:0.6:0.9 at 3 and 31 DAF. The content of soluble protein was increased until 10 DAF decreased from 17 DAF and increased again at 38 DAF.

Similar to soluble protein, soluble sugar was significantly affected by cultivar, ratio of N, P and K and their combination at all the sampling dates except 3 DAF (Table 3). Compared with Sikang 1, Siza 1 was 16.8% and 7.9% higher in soluble sugar content at 10 and 24 DAF. The soluble sugar content was decreased by 23.2%, 23.9% and 26.8% with the ratios of 1:0.3:0.6, 1:0.6:0.9 and 1:0.9:1.2 at 17 DAF as compared to control, respectively. Soluble sugar content was decreased until 31 DAF and increased up to 45 DAF.

Table 4. Effects of the ratio of nitrogen, phosphorous and potassium on leaf SOD activity of Siza 1 and Sikang 1 during 2016 and 2017.

*Cotton plants were sampled at 3rd, 10th, 17th, 24th, 31st, and 38th day after flowering. Different letters in the same column within the same year in the table are statistically different at the 0.05 probability level by an ANOVA-protected test.

The activity of SOD was affected by cultivar, ratio of N, P, and K, year, and the interaction between cultivar and year and between the fertilizer and year at 3 and 17 DAF (Table 1). Siza 1 was 4.9% and 42.2% lowered in SOD activity at 3 and 17 DAF during the two growing seasons compared to Sikang 1 (Table 4). The SOD activity was increased by 4.9% with the ratio of 1:0.3:0.6 at 24 DAF and increased by 20.5% and 4.7% with the ratios of 1:0.6:0.9 and 1:0.9:1.2 at 38 DAF during the first year compared to the control (1:0:0). Moreover, at 10 DAF it was increased by 4.6%, 15.4% and 8.2% with the ratios of 1:0.3:0.6, 1:0.6:0.9 and 1:0.9:1.2 during the second year, respectively. The content of SOD was increased until 31 DAF and decreased at 38 DAF.

The content of CAT (catalase) was significantly affected by the ratio of NPK, year, and the interaction between cultivar and year at 3, 10, 17, and 31 DAF (Table 1). Compared with Sikang 1, Siza 1 was 10.0% and 14.6% lowered in CAT activity at 17 DAF during 2016 and 2017 (Fig. 4A and B). However, the activity of CAT was increased by 17.5-4.5%, 23.9-15.4% and 22.1-8.2% with the ratios of 1:0.3:0.6, 1:0.6:0.9 and 1:0.9:1.2 at 17 DAF during the two year growing season as compared with control, respectively. The activity of CAT was abruptly increased at 24 DAF.

The POD (peroxidase) content was affected by cultivar, fertilizer, and the interaction between cultivar and fertilizer and between the cultivar fertilizer and year at 17 and 24 DAF (Table 1). Compared with Sikang 1, Siza 1 was 47.5% and 45.0% lowered in POD activity at 17 DAF during both years (Fig. 5A and B). At 17 DAF, the POD activity was increased 179.0-22.1%, 203.0-20.5% and 12.3-24.5% with the ratios of 1:0.3:0.6, 1:0.6:0.9 and 1:0.9:1.2 during the two years as compared to the control (1:0:0), respectively. However, the activity was decreased by 24.0% and 44.6% with the ratios of 1:0.3:0.6 and 1:0.9:1.2 at 3 DAF and decreased by 46.0% with the ratio of 1:0.6:0.9 during 2016. The POD activity was increased until 24 DAF and decreased at 31 and 38 DAF.

Different letters in the same column within the table are statistically different at the 0.05 probability level by an ANOVA-protected test.

The seed yield was affected by fertilizer and year (Table 1). Siza 1 was 0.7% and 2.6% lower in seed yield as compared to Sikang 1 during the two growing seasons (Table 5). The seed yield was increased by 7.7% and 6.1% with the ratio of 1:0.3:0.6, increased by 29.2% and 18.7% with the ratios of 1:0.6:0.9, and increased by 7.5% and 10.0% with the ratio of 1:0.9:1.2 during 2016 and 2017, respectively.

Table 2. Effects of the ratio of nitrogen, phosphorous and potassium on leaf soluble protein content of Siza 1 and Sikang 1 during 2016 and 2017.

Years	Cultivar	Ratio of N, P, and K	Days after flowering (DAF)						
			*3	10	17	24	31	38	45
2016	Siza1	1:0:0	27.01 b	35.4 b	26.1 a	14.8 fg	22.6 ab	24.6 a	23.5 ab
		1:0.3:0.6	26.82 c	35.3 bcd	26.8 a	15.6 e	22.5 ab	24.9 a	23.6 a
		1:0.6:0.9	27.30 a	35.6 a	25.2 a	18.4 d	23.09 ab	25.1 a	23.3 cd
		1:0.9:1.2	26.79 c	35.4 bc	26.4 a	15.3 ef	22.4 ab	25.0 a	23.5 ab
	Sikang1	1:0:0	27.01 b	35.2 d	26.5 a	14.3 g	22.6 ab	25.1 a	23.2 d
		1:0.3:0.6	26.88 c	35.3 cd	26.1 a	21.6 c	22.4 b	25.4 a	23.3 cd
		1:0.6:0.9	27.06 b	35.6 a	27.2 a	24.6 a	23.2 a	24.6 a	23.4 bc
		1:0.9:1.2	27.10 b	35.4 b	27.1 a	23.6 b	22.5 ab	25.5 a	23.3 cd
2017	Siza1	1:0:0	28.7 bc	25.7 b	26.4 ab	7.9 d	19.5 e	18.1 c	-
		1:0.3:0.6	29.6 abc	27.7 ab	27.6 ab	12.4 bc	22.9 bc	21.9 b	-
		1:0.6:0.9	32.6 ab	31.1 ab	29.6 ab	12.2 bc	25.3 a	22.0 b	-
		1:0.9:1.2	34.4 a	34.3 ab	28.8 ab	10.5 bcd	23.8 ab	19.9 bc	-
	Sikang1	1:0:0	25.8 c	29.4 ab	26.0 b	9.7 cd	20.5 de	21.6 b	-
		1:0.3:0.6	30.1 abc	29.8 ab	27.9 ab	12.6 bc	21.3 cde	22.0 b	-
		1:0.6:0.9	32.1 ab	31.7 ab	30.9 a	13.1 b	22.4 bcd	24.8 a	-
		1:0.9:1.2	30.3 abc	36.1 a	30.6 ab	16.6 a	23.8 ab	22.4 ab	-

*Cotton plants were sampled at 3rd, 10th, 17th, 24th, 31st, 38th, and 45th day after flowering. Different letters in the same column within the same year in the table are statistically different at the 0.05 probability level by an ANOVA-protected test

Table 3. Effects of the ratio of nitrogen, phosphorous and potassium on leaf soluble sugar content of Siza 1 and Sikang during 2016.

Cultivar	Ratio of N, P, and K	Days after flowering (DAF)						
		*3	10	17	24	31	38	45
Siza 1	1:0:0	47.44 a	34.1 b	24.0 b	20.1 bc	51.7 a	49.9 c	51.6 bc
	1:0.3:0.6	47.34 a	26.7 d	21.7 c	21.2 b	50.0 de	50.5 bc	51.5 c
	1:0.6:0.9	42.15 a	28.7 c	16.9 f	19.6 c	49.9 de	50.9 ab	52.4 a
	1:0.9:1.2	52.92 a	36.1 a	18.4 e	14.3 e	50.6 c	50.3 bc	50.5 de
Sikang 1	1:0:0	53.27 a	32.5 b	29.8 a	23.4 a	50.3 cde	51.7 a	51.2 c
	1:0.3:0.6	57.92 a	20.1 e	19.6 d	16.3 d	51.1 b	50.1 bc	50.4 e
	1:0.6:0.9	53.15 a	29.0 c	20.8 c	14.8 e	50.3 cd	50.1 bc	51.1 cd
	1:0.9:1.2	47.39 a	25.8 d	21.0 c	15.3 e	49.8 e	50.5 bc	52.3 ab

*Cotton plants were sampled at 3rd, 10th, 17th, 24th, 31st, 38th, and 45th day after flowering. Different letters in the same column within in the table are statistically different at the 0.05 probability level by an ANOVA-protected test

Table 4. Effects of the ratio of nitrogen, phosphorous and potassium on leaf SOD activity of Siza 1 and Sikang 1 during 2016 and 2017.

Years	Cultivar	Ratio of N, P, and K	Days after flowering (DAF)					
			*3	10	17	24	31	38
2016	Siza1	1:0:0	10.0 ab	14.8 a	15.1 a	14.5 b	18.4 a	10.0 ab
		1:0.3:0.6	8.0 c	15.6 a	15.6 a	15.0 ab	17.8 ab	8.3 b
		1:0.6:0.9	9.9 ab	15.3 a	14.4 a	14.7 b	17.0 b	12.4 a
		1:0.9:1.2	10.2 ab	16.3 a	16.7 a	16.4 ab	18.3 a	11.7 ab
	Sikang1	1:0:0	9.7 ab	16.2 a	16.3 a	16.9 ab	18.4 a	11.8 ab
		1:0.3:0.6	9.2 bc	16.8 a	16.9 a	17.9 a	17.8 ab	13.2 a
		1:0.6:0.9	11.0 a	15.5 a	15.1 a	16.4 ab	18.4 a	13.9 a
		1:0.9:1.2	10.2 ab	15.7 a	15.5 a	16.5 ab	18.1 ab	11.4 ab
2017	Siza1	1:0:0	5.5 d	9.0 abc	5.2 c	13.6 a	17.5 a	15.1 a
		1:0.3:0.6	8.4 d	9.4 ab	6.3 c	15.0 a	17.7 a	15.4 a
		1:0.6:0.9	8.4 b	9.9 a	8.7 b	16.2 a	18.2 a	15.7 a
		1:0.9:1.2	8.0 bc	10.1 a	6.0 c	15.7 a	18.2 a	14.1 a
	Sikang1	1:0:0	6.0 cd	7. 6c	8.3 b	15.8 a	18.0 a	13.6 a
		1:0.3:0.6	10.0 ab	7.9 bc	8.5 b	15.9 a	18.2 a	13.5 a
		1:0.6:0.9	11.3 a	9.2 abc	11.0 a	16.4 a	18.3 a	14.2 a
		1:0.9:1.2	11.7 a	7.8 bc	9.5 ab	15.9 a	18.3 a	13.3 a

*Cotton plants were sampled at 3rd, 10th, 17th, 24th, 31st, and 38th day after flowering. Different letters in the same column within the same year in the table are statistically different at the 0.05 probability level by an ANOVA-protected test

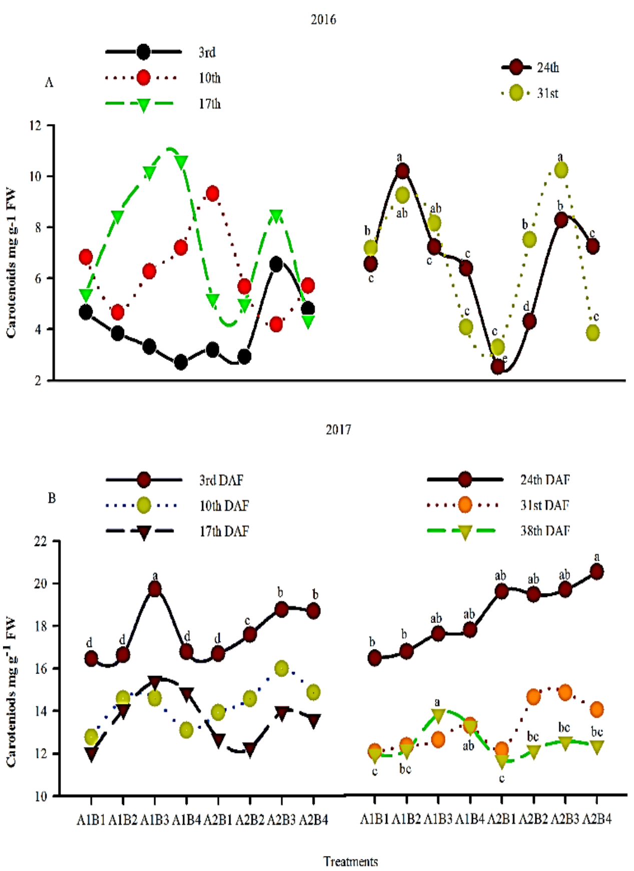

Fig. 3. Effects of ratio of N, P, and K on leaf carotenoid contents of Siza 1 and Sikang 1 cotton plants at different days after flowering during two years. A represent the 3rd, 10th, 17th, 24th and 31st day after flowering, and B represent the 3rd, 10th, 17th, 24th, 31st, and 38th day after flowering respectively. Different letters on the bars in each figure are statistically different at the 0.05 probability level by an ANOVA-protected test.

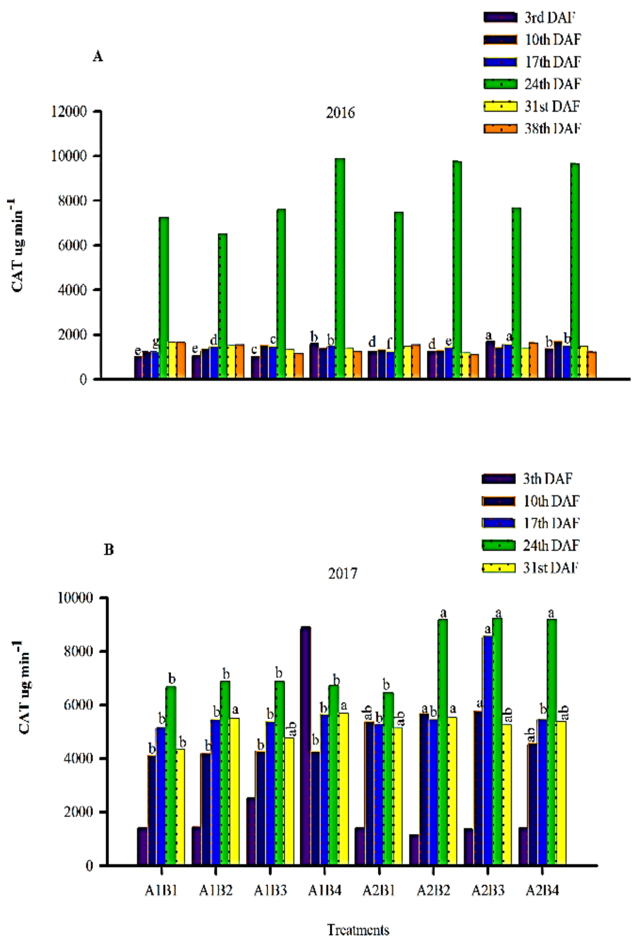

Fig. 4. Effects of ratio of N, P, and K on leaf CAT activity of Siza 1 and Sikang 1 plants at different weeks after flowering during two years. A and B represent 1-6 weeks, respectively. Bars with no letters are not significantly different in each figure.

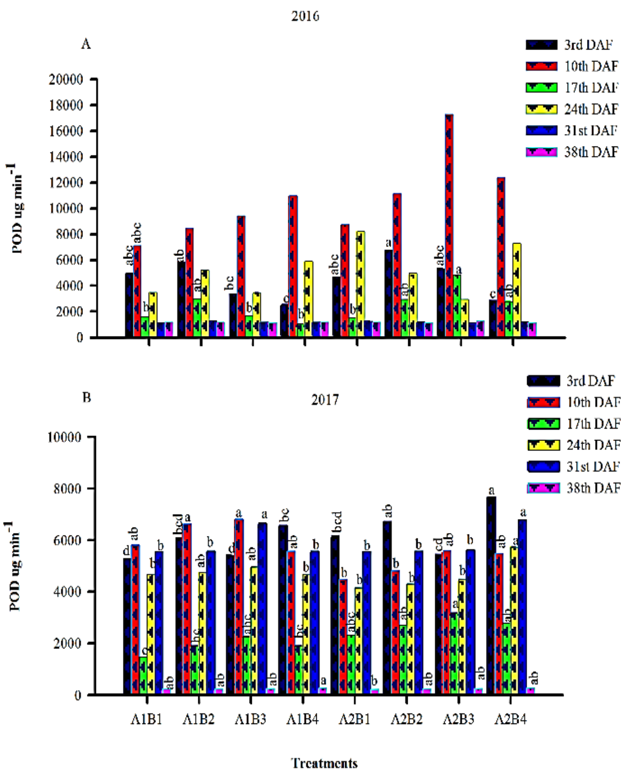

Fig. 5. Effects of ratio of N, P, and K on leaf POD activity of Siza 1 and Sikang 1 plants at different weeks after flowering in 2016 and 2017 years. The POD activity at the 3rd, 10th, 17th, 24th, 31st, and 38th day after flowering is represented in Fig. A and B. Different letters on the bars in each figure are statistically different at the 0.05 probability level by an ANOVA-protected test. Bars with no letters are not significantly different in each figure.

Table 5. Effects of the ratio of nitrogen, phosphorous and potassium on cotton seed yield (kg hm^{-2}) of Siza 1 and Sikang 1 during 2016 and 2017.

Cultivar	Ratio of N, P, and K	2016	2017
Siza 1	1:0:0	2241.9 b	2528.8 c
	1:0.3:0.6	2398.2 ab	2649.2 bc
	1:0.6:0.9	2867.4 ab	2980.9 ab
	1:0.9:1.2	2677.8 ab	2904.3 abc
Sikang 1	1:0:0	2358.2 b	2626.8 bc
	1:0.3:0.6	2556.2 ab	2819.8 abc
	1:0.6:0.9	3076.5 a	3136.2 a
	1:0.9:1.2	2646.2 b	2952.0 abc

Discussion

Macronutrient application, particularly the combined application of N, P, and K, plays an important role in cotton plant growth and productivity and are involved in different biological processes (Deng *et al*., 2014). The balanced ratio of N, P, and K is more critical than their separate value for better crop physiological activities, development and production (Usherwood & Segars, 2001; Yang *et al*., 2012; Suminarti *et al*., 2016). In the present study, we illustrated responses of combined nutrient application on leaf characteristics of two cotton cultivars using different ratios of N, P, and K. This study added new information on the importance of applying combined fertilizer on cotton. Our research indicated that combined nutrient application enhanced plant growth, including leaf physiological activities, and seed cotton yield of two cotton cultivars during two growing seasons.

In the present study, we noticed that all the higher ratios of N, P, and K increased the contents of chl *a* (Fig. 1A and B), chl *b* (Fig. 2A and B), and carotenoids (Fig. 3A and B) as compared with treatment with only N application. Our results are in agreement with the study of (Dheeba *et al*., 2014), who identified that plants treated with balanced N, P, and K fertilizer showed significant increase in chlorophyll pigments as compared to the control in maize. It was)also reported that appropriate combined application of N, P, and K stimulated the formation of active photosynthetic pigments, which enhanced plant productivity and physiological activity Li *et al*., (2010).

In this research, higher fertilizer ratios of N, P, and K enhanced the contents of soluble protein (Table 2) and soluble sugar of both cultivars (Table 3). Siza 1 had lower content of soluble protein as compared with Sikang 1. This observation was different from the results of Chen *et al*., (2005), who reported that Bt hybrid Kemian 1 had a higher content of soluble protein than its female parent Yumian 1, which is a non-Bt cultivar. The difference between the two studies might lie in the genetic backgrounds of cultivars. Yumian 1 is a non-Bt cultivar and Kemian 1 is a Bt hybrid with Yumian 1 as the female parent. Here in our study, Siza 1 is a Bt hybrid with Sikang 1 (a Bt conventional cultivar) as the parent. Generally, Bt transgenic cotton requires a higher rate of N, P, and K for producing more proteins for the composition of Bt insecticidal protein (Wang *et al*., 1998).

Antioxidant enzymes like SOD, CAT, and POD play a vital role during abiotic stress conditions in plants (Mittler, 2002). SOD is a major scavenger of O_2^- and its enzymatic action results in the production of H_2O_2 and O_2. Then H_2O_2 is scavenged by POD (Dionisio-Sese & Tobita, 1998). The H_2O_2 can also be directly broken into water and oxygen by CAT (Wang *et al*., 2008; Dazy *et al*., 2009). In our study combined application of N, P, and K significantly increased the activities of CAT (Fig. 4A and B), SOD (Table 4) and POD in leaves (Fig. 5A, B). Our results are in agreement with the findings of (Shabbir *et al*., 2016), who reported that the activities of CAT, and SOD could be enhanced by combined application of N, P, and K.

In the current work, the activity of CAT was suddenly increased during the first year (Fig. 4A). This might be the reason for the applied fertilizers or the effect of different environmental stresses. Our results are in line with Hammad & Ali (2014), who reported that during stress plants release different kinds of antioxidant enzymes for reducing oxidative damage to detoxify ROS (reactive oxygen species) and ROS scavengers are those antioxidant enzymes which include SOD, CAT and POD. The contents of chl *a, b* and carotenoids (Fig. 1A, 2A and 3A) were increased until 17 DAF, decreased at 24 DAF, and increased again at 31 and 38 DAF during the first year. It might depend on the applied fertilizer rates at different developmental stages. Our results agreed with the finding of Gormus *et al*., (2016), who reported that the requirements of fertilizers for cotton plants were different at various growth stages.

The higher fertilizer ratios of N, P and K significantly increased seed cotton yield as compared to control (Table 5). The relative increase in combined N, P, and K application as compared to N may change the carbon metabolism and finally increase seed cotton yield. In the present study, seed cotton yield was increased in 2016 and 2017 under the higher ratios of combined N, P, and K. The seed cotton yield was slightly lower during the first year as compared to the second year, due to heat stress.

Conclusion

Combined application of N, P, and K significantly influenced leaf physiological activities in both growing seasons. The ratio of 1:0.6:0.9 performed best across two years in which all treatments increased chl *a, b,* carotenoid contents, soluble protein, superoxide dismutase, catalase and peroxidase activity by 19.3%, 45.0%, 25.7%, 47.6%, 20.5%, 21.0% and 203%. Sikang 1 showed remarkably higher activity of SOD, POD and CAT during two growing seasons (2016-2017) and showed best results as compared to Siza 1. Results indicated that combined application of N, P, and K at appropriate ratios enhanced the activity of physiological parameters, yield and the ratio of 1:0.6:0.9 considerably performed best amongst all treatments for Sikang 1 variety during two growing seasons.

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