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Effects of Rhizobia Inoculation, Phosphorus and Potassium on Chlorophyll Concentration of Soybean Grown under Maize Intercropping System

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Authors' contributions

This work was carried out in collaboration between both authors. Authors DN and PAN designed the study. Author DN managed literature searches, conducted field experiment, managed the laboratory analyses of the study, performed the statistical analysis and wrote the first draft of the manuscript. Author PAN reviewed the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: The study was conducted to assess the effects of Rhizobia inoculation, supplemented with phosphorus (P) and potassium (K) under intercropping system on soybean chlorophyll content.
Study Design: The design of the experiment was split-split plot with three factors factorial and replicated thrice.
Place and Duration of Study: The experiment was carried out for two consecutive years 2015 and 2016 at the Tanzania Coffee Research Institute farm in Northern Tanzania.
Methodology: Two inoculation treatments, four intercropping systems and seven fertilizer levels (kg ha^{-1}): Control, 20, 40 K, 26, 52 P, 26 P + 20 K and 52 P + 40 K. Chlorophyll concentrations were extracted using dimethyl sulphoxide (DMSO). Spectrophotometer was used to read the absorbance values at 645 nm (Chlorophyll b) and 663 nm (Chlorophyll a).
Results: Rhizobia inoculation significantly (p=.05) increased total soybean leaf chlorophyll content from 4.25±0.30 to 5.32±0.34 and 7.20±0.27 to 7.88±0.29 in 2015 and 2016 cropping seasons respectively. P and K fertilization also significantly (p=.05) increased soybean total leaf chlorophyll

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content from 1.69±0.23 to 7.17±0.51 and 4.62±0.33 to 9.87±0.48 in 2015 and 2016 cropping seasons respectively. The combined fertilizers had higher mean values of chlorophyll concentration over all other treatments in both 2015 and 2016 cropping seasons.

Conclusion: Therefore, for improved chlorophyll concentration, P and K should be applied in combination at low rate of 20 K + 26 P (kg ha^{-1}). Doubling of these fertilizers may be costly and will not significantly change the leaf chlorophyll content.

Keywords: Intercropping systems; BNF; plant pigments; photosynthesis; soybean; plant nutrition.

1. INTRODUCTION

Photosynthesis is a process by which green plants and other photosynthetic organisms use the energy from sunlight and convert it to produce useful chemical energy in presence of water, carbon dioxide and chlorophyll [1]. Chlorophyll may be referred to as a green pigments found in photosynthetic organisms such as plants, algae, and photosynthetic bacteria. For the purpose of this article, we will be referring to Soybean chlorophyll. The molecule plays the central function in photosynthesis [2]. Therefore, decreased chlorophyll concentration may inhibit photosynthesis [3], and hence reduce production of food in crops.

The general equation for the photosynthesis process is shown bellow

$$6CO_2 + 6H_2O \rightarrow \frac{\text{Chlorophyll}}{\text{Sun light}} \rightarrow C_6H_{12}O_6 + 6O_2$$

Where:

CO_2 = carbon dioxide
H_2O = water } Input
$C_6H_{12}O_6$ = glucose
O_2 = oxygen } Output

Since the chlorophyll is necessary for the photosynthesis process [4], which is vital for the life of nearly all organisms, it is important to enhance it in the cropping systems to allow production of enough food to feed the sky-rocketing human population. One way of enhancing chlorophyll concentration in the cropping systems is to improve nutrition and adequate exposure of plants to sunlight.

Several researches have been done to assess photosynthetic activities of plant and their responses under different factors. For example, studies have shown that plant beneficial microorganisms (Rhizobia) have enhanced photosynthesis because they improve plant nutrition hence increased leaf area that reflects photosynthesis [5]. In another study done by Nyoki and Ndakidemi [6], it was reported that total leaf chlorophyll content of cowpea was significantly increased following inoculation of *Bradyrhizobium japonicum*. The same results were found in another study by Bambara and Ndakidemi [7] which showed that *P. vulgaris* L. inoculated with Rhizobia had increased leaf chlorophyll content compared with that of control plants. However, much of these studies have focused on inoculation of legumes grown as monocrop. There is little information on chlorophyll content of inoculated legumes grown under intercropping systems. Therefore, there was a need to conduct a study assessing the chlorophyll content of inoculated soybean and un-inoculated soybean grown under maize intercropping systems.

A supply of different mineral elements is another factor which is reported to enhance chlorophyll concentration and photosynthesis in general. Potassium and phosphorus are particularly important in plant chlorophyll concentration and photosynthesis. Hossain et al. [8] and Longstreth and Nobel [9] pointed out that the limited supply of these elements impaired plant growth in terms of cell division and expansion, and photosynthesis. Wu et al. [10] reported an increase in chlorophyll content following application of phosphorus on the seedlings of *Larix olgensis*. Furthermore, Onanuga et al. [11] observed that the plants treated with relatively high levels of P and K improved chlorophyll a, b and a/b production in cotton leaves. Study by Zhao et al. [12] showed that K deficient was associated with low chlorophyll content in cotton leaves. In another study, Lamrani et al. [13], who investigated the influence of nitrogen, phosphorus, and potassium on pigment concentration in cucumber leaves, observed that K nutrition promoted formation of both chlorophyll a and b.

Another factor that may affect chlorophyll content and photosynthesis is by growing crops of different height in the mixture (intercropping). This practice has been reported to improve yield over sole crop by many researchers [14-18]. Though, this may result in the suppression of one crop in the mixture by preventing the sunlight from reaching the crop. It was previously reported that Mungbean suffered a shading stress when it was intercropped with sorghum at different growth stages [19]. The grain filling stage is very much light sensitive. Therefore, if one has to improve and maximize yield, grain filling stage needs to be given special attention in intercropping systems [19].

It is evident from different literature cited that *Rhizobium* inoculation and mineral elements supplemtation increases the chlorophyll content of leaves, and hence improves plant biomass production. However, these treatments need to be studied under cereal-legume intercropping systems to assess their effects on leaf chlorophyll content of legumes. Therefore, the objective of this study was to assess the effects of Rhizobia inoculation, supplemented with phosphorus (P) and potassium (K) under intercropping system on chlorophyll synthesis in soybean.

2. MATERIALS AND METHODS

2.1 Experimental Design and Treatments

The experiment was carried out at Tanzania Coffee Research Institute (TaCRI) for two consecutive cropping seasons (April – September 2015 and April – September 2016). The experiment was laid out in Split-split plot with three factors factorial and replicated thrice. The plot size was 3 x 3 m, with main plot comprised two inoculation treatments: i) With rhizobia inoculation and ii) Without rhizobia inoculation. The subplots was assigned with cropping systems as follows: maize (sole crop) at a spacing of 75 x 60 cm; soybean (sole crop) at a spacing of 75 x 20 cm; maize/soybean (intercropping system) at a spacing of 75 x 60 cm and 75 x 20 cm, maize and soybean respectively; and the last cropping system was Maize/ soybean (intercropping system) at a spacing of 75 x 60 cm and 75 x 40 cm, maize and soybean respectively. The following fertilizer levels (kg ha^{-1}) were assigned to the sub-subplots. (i) Control (Without fertilizer). (ii) 20 K. (iii) 40 K. (iv) 26 P. (v) 52 P. (vi) 26 P + 20 K. (vii) 52 P + 40 K.

2.2 Chlorophyll Extraction and Determination

Chlorophyll concentrations were extracted using dimethyl sulphoxide (DMSO) as it was previously described in Hiscox and Israelstam, [20]. In this method, a third leaf from the top of the plant for each treatment was collected for chlorophyll extraction. From the sampled leaves, a hundred (100) mg of the middle portion of fresh leaf slices was placed in a 15 mL vial containing 7 mL DMSO and incubated at 4℃ for 72 h. After the incubation, the extract was diluted to 10 mL with DMSO. This technique helps to extract chlorophyll from shoot tissue without grinding or maceration [20]. From the chlorophyll extract, 3 mL sample was transferred into curvets for absorbance determination. A spectrophotometer (UV/Visible Spectrophotometer, Pharmacia LKB Ultrospec II E) was used to determine absorbance values at 645 (Chlorophyll b) and 663 nm (Chlorophyll a), which were then be used in the equation proposed by Arnon [21] to determine total leaf chlorophyll contents against DMSO blank, expressed as mg L^{-1} as follows:

$$\text{Chlorophyll total } (\text{Chlt} = 20.2\text{D}_{645} + 8.02\text{D}_{663})$$

Where “D” is the density at the respective wavelengths which was obtained from spectrophotometer.

Visual assessment of plant color was done in a scale of 1 – 5. This assessment was based on previous studies by Xu et al. [22] Maher et al. [23] and Ndakidemi and Makoi [24]. In this study, the scale of 1 was assigned to plots which were observed to be more dark green and 5 was assigned to plots with yellowish color. This scale enabled the researcher to quantify the color intensity of plants in different treatments.

2.3 Statistical Analysis

The statistical analysis was performed using the 3-way analysis of variance (ANOVA) in factorial arrangement. The computation was performed with the software program STATISTICA. The fisher’s least significance difference (L.S.D.) was used to compare treatment means at p = 0.05 level of significance [25].

3. RESULTS

3.1 Soil Results

The results of selected chemical properties of the soil from the study area before the start of experiment are presented in Table 1.

Table 1. The selected chemical properties of soil

pH 1:2.5		Total N %	Avail. P, Bra-I mg/kg	K meq/100 g
H_2O	KCl			
6.43	6.14	0.183	5.21	0.93

3.2 Effect of Cropping Systems on Chlorophyll Content of Soybean

The results presented in Table 2, indicated that for the year 2015, the cropping systems had no significant effect on the chlorophyll a, b and total of the soybean leaves. The chlorophyll concentrations were almost the same in sole soybean and maize-soybean intercropped at different spacing. In the second season (2016 cropping season), cropping systems did not show significant differences in chlorophyll a, b and total concentration. However, soybean intercropped with maize at a spacing of 75 x 20 cm, and 75 x 60 cm soybean and maize respectively numerically had lower chlorophyll a, b and total concentration when compared with the soybean in monocrop (Table 3).

3.3 Effects of Rhizobium Inoculation (*Bradyrhizobium japonicum*) on Chlorophyll Content in Soybean

In both cropping seasons, i.e. 2015 and 2016, Rhizobia (*Bradyrhizobium japonicum*) inoculation had a positive effect and significantly (P = .05) increased the chlorophyll a, b and total concentration over the control (Table 2 and 3). In 2015 cropping season (Table 2), the concentration of chlorophyll a, b and total were increased by 27, 23 and 25% respectively in the inoculated plots over the control (un-inoculated plots). In 2016 cropping season (Table 3), Rhizobia inoculation significantly improved chlorophyll a, b and total relative to un-inoculated plots. Inoculation significantly increased chlorophyll a, b and total by 8.40, 10.70 and 9.35% respectively.

3.4 Effects P and K Fertilization on Chlorophyll Content in Soybean

Different levels of K and P significantly affected soybean leaf chlorophyll concentrations. In both cropping seasons (2015 and 2016), the higher rate of potassium fertilizer (40 kg ha^{-1}) significantly increased chlorophyll a, b, and total compared with the lower rate (20 kg ha^{-1}) and the control. Furthermore, when compared with the control, the lower rate of potassium (20 kg ha^{-1}) significantly increased the concentration of chlorophyll a, b and total (Table 2 and 3). Following potassium fertilization, the concentration of chlorophyll in both 2015 and 2016 cropping seasons followed a trend of control <20 <40 (kg ha^{-1}). The data presented in Table 2 (2015 cropping season), showed that the higher rate of potassium (40 kg ha^{-1}) increased chlorophyll a, b, and total by 137, 133 and 135% respectively over the control. Likewise, in Table 3 (2016 cropping season), the higher rate of potassium (40 kg ha^{-1}) significantly increased chlorophyll a, b, and total by 58, 41 and 50% respectively relative to the control.

Referring to the Table 2 and 3, phosphorus fertilization significantly increased chlorophyll a, b and total. The concentration levels of chlorophyll in phosphorus fertilized plots followed the same trend (control <26 <52 (kg ha^{-1}) as those in potassium treated plots. For both cropping seasons, doubled treatment of phosphorus (52 kg ha^{-1}) significantly increased chlorophyll a, b and total chlorophyll over the lower rate (26 kg ha^{-1}) and the control. In 2015 cropping season, application of phosphorus at the level of 52 kg ha^{-1} significantly increased chlorophyll a, b and total by 18, 19 and 18% respectively over 26 kg P ha^{-1} treated plots and by 251, 243 and 245% respectively over the control. For the 2016 season, application of phosphorus at the level of 52 kg ha^{-1} significantly increased chlorophyll a, b and total by 10, 1 and 6% respectively over the 26 kg ha^{-1} treated plots and by 83, 67 and 76% respectively over the control.

The application of the combined P and K fertilizers significantly increased chlorophyll a b and total over all the treatments in the two (2015 and 2016) cropping seasons. However, the doubling of combined P and K did not show any significant difference between the lower rate (20 K + 26 P (kg ha^{-1}) and the doubled rate (40 K + 52 P (kg ha^{-1}) (Tables 2 and 3).

3.5 Interactive Effects of Rhizobia, Cropping Systems and Fertilizer Levels

The results from this study did not show any significant interactions of the main plots and subplots.

Table 2. Effect of cropping systems, Rhizobia inoculation, P and K fertilization on concentration of soy bean leaf chlorophyll a, b and total in 2015 cropping season

Treatments	Chl a 2015	Chl b 2015	Chl T 2015
Cropping system			
SB	2.08±0.16b	2.11±0.19b	4.19±0.35b
M+B (A)	2.24±0.22a	2.71±0.25a	4.95±0.47a
M+B (B)	2.31±0.18a	2.54±0.20a	4.85±0.37a
Rhizobia			
With out	1.95±0.14b	2.31±0.16b	4.25±0.30b
With	2.47±0.17a	2.85±0.18a	5.32±0.34a
Fertilizer levels (kg ha^{-1})			
Control	0.76±0.12e	0.93±0.13e	1.69±0.23f
20 K	1.60±0.26d	1.66±0.16d	3.26±0.38e
40 K	1.80±0.15cd	2.17±0.17cd	3.97±0.32de
26 P	2.26±0.17bc	2.69±0.21bc	4.95±0.39cd
52 P	2.67±0.22ab	3.19±0.28ab	5.86±0.49bc
20 K + 26 P	3.31±0.26a	3.86±0.26a	7.17±0.51a
40 K + 52 P	3.06±0.29a	3.53±0.35a	6.59±0.63ab
3-Way ANOVA F-statistics			
CroSyt	0.59*	0.52*	0.43*
Rhiz	9.17**	8.47**	9.29**
Fert	15.22***	18.40***	17.71***
CroSyt*Rhiz	0.71 ns	1.40 ns	1.02 ns
CroSyt*Fert	0.55 ns	0.39 ns	0.45 ns
Rhiz*Fert	0.53 ns	0.57 ns	0.56 ns
CroSyt*Rhiz*Fert	0.30 ns	0.15 ns	0.18 ns

*CroSyt: Cropping Systems; Fert: Fertilizers; Rhiz: Rhizobium; Chl: Chlorophyll; M+B (A): Maize/soybean intercropped at a spacing of 75 x 60 cm and 75 x 20 cm, maize and soybean respectively; M+B (B): Maize/soybean intercropped at a spacing of 75 x 60 cm and 75 x 40 cm, maize and soybean respectively; Values presented are means ± SE; **, ***: significant at p ≤ 0.01, p ≤ 0.001 respectively, ns = not significant, SE = standard error. Means followed by dissimilar letter(s) in a column are significantly different from each other at p = 0.05 according to Fischer least significance difference (LSD)*

3.6 Visual Assessment of Crop Pigmentation

Dark green colour is an indication of healthy plants. Dark green colour is also an indication that active growth and active photosynthesis is taking place. Visual assessment of plant greenness showed that for the two cropping seasons (2015 and 2016) Rhizobia inoculation and fertilizer (P and K) application significantly increased plant greenness over the control. The cropping systems did not show any significant difference in plant greenness for the 2015 season. However, cropping systems significantly affected the crop greenness in the second season (2016) whereby soybean planted as monocrop were greener compared with those in intercropped plots (Table 4). It is clearly seen in the Image (3) that soybean intercropped with maize without Rhizobia inoculation suffered both effects of shading and nitrogen deficiency compared with monocropped soybean which suffered only nitrogen deficiency (Image 1). Rhizobia inoculated soybean under intercropping system did not suffer shading effect from its companion crop (Image 4). The greenness of rhizobial inoculated soybean under monocropping was not different from that of Rhizobia inoculated soybean under intercropping systems (Images 4 and 2).

4. DISCUSSION

Chlorophyll concentration of the plants is generally affected by the treatments received by the respective plants. The current study examined the effects of cropping systems, Rhizobia inoculation and P and K fertilizers on chlorophyll concentration in soybean leaves. Form this study; it was generally observed that cropping systems had no significant effect on chlorophyll concentration in leaves of soybean.

Table 3. Effect of cropping systems, Rhizobia inoculation, P and K fertilization on concentration of soy bean leaf chlorophyll a, b and total 2016 cropping season

Treatments	Chl a 2016	Chl b 2016	Chl T 2016
Cropping system			
SB	2.27±0.18b	2.64±0.16b	4.91±0.33b
M+B (A)	4.05±0.17a	3.36±0.17a	7.41±0.33a
M+B (B)	3.97±0.20a	3.32±0.19a	7.30±0.38a
Rhizobia			
With out	3.93±0.14b	3.27±0.13b	7.20±0.27b
With	4.26±0.16a	3.62±0.15a	7.88±0.29a
Fertilizer levels (kg ha^{-1})			
Control	2.46±0.18e	2.17±0.17e	4.62±0.33e
20 K	3.28±0.14d	2.71±0.12d	5.99±0.24d
40 K	3.88±0.14c	3.06±0.16cd	6.94±0.28cd
26 P	4.11±0.14bc	3.59±0.14bc	7.69±0.25bc
52 P	4.51±0.17b	3.63±0.15b	8.14±0.29b
20 K + 26 P	5.13±0.22a	4.40±0.23a	9.53±0.44a
40 K + 52 P	5.33±0.22a	4.54±0.28a	9.87±0.48a
3-Way ANOVA F-statistics			
CroSyt	1.75*	2.00*	2.09*
Rhiz	5.79*	6.14*	6.67*
Fert	31.39***	21.08***	28.78***
CroSyt*Rhiz	0.87 ns	0.99 ns	0.88 ns
CroSyt*Fert	0.54 ns	0.82 ns	0.65 ns
Rhiz*Fert	1.04 ns	0.24 ns	0.60 ns
CroSyt*Rhiz*Fert	0.49 ns	0.77 ns	0.60 ns

*CroSyt: Cropping Systems; Fert: Fertilizers; Rhiz: Rhizobium; Chl: Chlorophyll; M+B (A): Maize/soybean intercropped at a spacing of 75 x 60 cm and 75 x 20 cm, maize and soybean respectively; M+B (B): Maize/soybean intercropped at a spacing of 75 x 60 cm and 75 x 40 cm, maize and soybean respectively; 3Values presented are means ± SE; *, ***: significant at p≤ 0.5, p ≤ 0.001 respectively, ns = not significant, SE = standard error. Means followed by dissimilar letter(s) in a column are significantly different from each other at p = 0.05 according to Fischer least significance difference (LSD)*

Table 4. Visual assessment of plant greenness scored in a scale of 1 – 5

Treatments	Greenness	
Cropping system	**2015 Cropping season**	**2016 Cropping season**
SB	1.56±0.22b	2.00±0.19b
M+B (A)	2.48±0.25a	2.54±0.19a
M+B (B)	2.79±0.25a	2.39±0.18a
Rhizobia		
With out	3.92±0.11a	3.13±0.14a
With	1.29±0.09b	1.35±0.06b
Fertilizer levels (kg ha^{-1})		
Control	3.33±0.35a	3.06±0.26a
20 K	2.53±0.39ab	2.50±0.35b
40 K	2.56±0.36ab	2.44±0.30b
26 P	2.56±0.37ab	1.83±0.23c
52 P	2.61±0.37ab	1.72±0.23c
20 K + 26 P	2.03±0.31b	2.11±0.27bc
40 K + 52 P	2.04±0.42b	2.03±0.31bc
3-Way ANOVA F-statistics		
CroSyt	1.86 *	4.12*
Rhiz	375.21***	176.39***
Fert	1.37 *	6.69***
CroSyt*Rhiz	0.25 ns	0.59ns
CroSyt*Fert	1.12 ns	0.77ns
Rhiz*Fert	2.38ns	0.85ns
CroSyt*Rhiz*Fert	1.08 ns	1.05ns

*CroSyt: Cropping Systems; Fert: Fertilizers; Rhiz: Rhizobium; Chl: Chlorophyll; M+B (A): Maize/soybean intercropped at a spacing of 75 x 60 cm and 75 x 20 cm, maize and soybean respectively; M+B (B): Maize/soybean intercropped at a spacing of 75 x 60 cm and 75 x 40 cm, maize and soybean respectively; Values presented are means ± SE; *, ***: significant at p≤ 0.5, p ≤ 0.001 respectively, ns = not significant, SE = standard error. Means followed by dissimilar letter(s) in a column are significantly different from each other at p = 0.05 according to Fischer least significance difference (LSD)*

Image 1. Soybean monocrop without rhizobia inoculation

Image 2. Soybean monocrop with rhizobia inoculation

Image 3. Soybean maize intercropping without rhizobia inoculation

Image 4. Soybean maize intercropping with rhizobia inoculation

Rhizobia inoculation in the two cropping seasons significantly affected the concentration of chlorophyll a, b and total when compared with un-inoculated treatments. These findings are in line with the previous report [6,7,26-28] which showed that Rhizobia strain significantly increased chlorophyll concentration in the crops. Since Rhizobial inoculation increases chlorophyll contents and bearing in mind that chlorophyll in necessary in the photosynthesis, it is also ideal to say Rhizobia is necessary for the increased leaf photosynthesis [29]. The relationship between Rhizobia inoculation and chlorophyll content is found in the biological nitrogen fixation, a process by which plants convert atmospheric nitrogen into a usable form by plant. The fixed nitrogen is responsible for the increases greenness of the plant leaves [30] and the greenness of plant leaves is an indicator of the improved chlorophyll content of the plant leaves [31]. Since nitrogen is a structural element of chlorophyll [32], hence its availability to plants results in increased chlorophyll content [33].

P and K fertilization also improved chlorophyll concentration of soybean leaves. From the current study, increasing the level of fertilizers had positive effects on chlorophyll content of soybean leaves. Interestingly, in the two cropping seasons, the lower (26 kg ha^{-1}) and higher (52 kg ha^{-1}) rate of phosphorus fertilizer had higher mean values of chlorophyll relative to the potassium fertilized plots and the unfertilized plots. The related findings were previously reported that phosphorus increased leaf chlorophyll content [6,33]. However, contrary to our results in which higher rate of phosphorus increased chlorophyll content, [33] reported that the higher P rate significantly decreased chlorophyll content in their 1st year of experiment. Furthermore, when compared with the control, potassium fertilization increased chlorophyll content of soybean leaves. The findings of the current study agree with the Zhao et al. [12] who reported that potassium deficient was associated with the low chlorophyll content in cotton leaves. Doubling potassium rate from 20 to 40 (kg ha^{-1}) significantly increased the leaf chlorophyll content in the two cropping seasons. The combined fertilizer treatments at their lower rates (20 K + 26 P (kg ha^{-1}) resulted in higher mean values of chlorophyll content compared with the different fertilizer levels when applied singly. However, doubling of combined fertilizers (40 K + 52 P (kg ha^{-1}) did not significantly change the chlorophyll content of the soybean leaves. From this study, we learn that P and K deficiency reduced leaf chlorophyll content of soybean. This observation agrees with Watanabe and Yoshida, [34] who stated that deficiency phosphorus and potassium causes changes in the structure of chloroplasts and may affect the biochemical activity of chloroplast resulting to low leaf chlorophyll content.

5. CONCLUSION

The results from current study indicated the importance of mineral elements in chlorophyll formation in soybean leaves. It can be generalised that N, P and K are equally necessary for the formation of chlorophyll in crops thereby improving final yields. We have tested these elements, P and K from mineral fertilizers and N from BNF and found that both of them significantly increased soybean leaf chlorophyll content. The combined P and K at the lower rate resulted in higher mean values of chlorophyll content. From this observation it is recommended that for improved chlorophyll concentration, P and K should be applied in combination at low rate of 20 kg K ha^{-1}+26 kg P ha^{-1}. Doubling of these fertilizers may be costly and will not significantly change the leaf chlorophyll content.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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