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# Effect of pH and magnesium on colour development and anthocyanin accumulation in tuberose florets

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**Abstract** Anthocyanins are a group of plant pigments responsible for colors ranging from red to violet and blue. Anthocyanins are pigments that accumulate in the vacuoles of plant epidermal cells. Chroma and hue are dependent on conditions inside and outside the vacuoles. Also conditions within the vacuole that facilitate formation of complexes with several metal ions. The aim of this study was to examine changes in accumulation of anthocyanins and the resultant colour in tuberose (*Polianthes tuberosa* Linn.) after application of amendments to the soil. Treatments were applied six weeks after planting as a top dress. Magnesium was given as magnesium nitrate and nitrogen was given as calcium ammonium nitrate CAN was neutral and ammonium sulphate (AS) was acidic. Soil and plant tissues were analysed every two weeks for nitrogen, phosphorus, potassium, magnesium, and pH. Colour was determined using a photoelectric tri-stimulus colorimeter and expressed in CIE L*a*b* colour space coordinates. In addition, chlorophyll and anthocyanins in the florets were quantified. The amendments lowered the soil pH, especially for the AS and CAN. However, chroma and hue values as determined by the space coordinates, as well as the concentrations of anthocyanins in the floret, were not significantly linked to the soil pH following the amendments. Equally, the tissue elements and chlorophyll contents were similar between the amendments. The results of this study show that supplying magnesium through fertiliser application to the soil does not necessarily increase accumulation of Mg in tissues, and may ultimately not lead to accumulation of anthocyanins.

Key words: Chroma, hue, metal complexes, *Polianthes tuberosa*, plant pigments

## Introduction

Anthocyanins are a group of plant pigments responsible for colors ranging from red to violet and blue (van Tunen & Mol 1991). These pigments accumulate in the vacuoles of epidermal cell, and both their chroma and hue are dependent on external conditions, as well as on the pH in the vacuoles (Harborne & Grayer, 1988). Anthocyanins are able to accumulate in epidermal vacuoles and blend with the plastid pigments to give various hues that vary with light exposure and night and day temperatures (Sachray *et al.*, 2002). In most flowers, anthocyanin synthesis occurs with petals growth and is under developmental control. For example, in petunia it occurs during the corolla elongation ( Weiss & Halevy, 1989) while in *Lisianthus* colouration occurs prior to the unfurling of the petals after the buds have reached their final size (Oren-Shamir *et al.*, 1999).

Temperature is one of the main external factors affecting anthocyanin accumulation in plant tissues: low temperatures increase, and elevated temperatures decrease, anthocyanin concentration (Zhong & Yoshida, 1993; Oren-Shamir & Nissim-Levi, 1997; 1999; Zhang *et al.*, 1997). In petunia (Shvarts *et al*., 1997) temperature has been shown to have a significant effect on the expression of anthocyanin genes: low temperature conditions were accompanied by a several fold increase in transcript levels of genes whose products are either key enzymes in the general phenylpropanoid pathway such as phenylalanine ammonia lyase (PAL), or genes whose products catalyse reactions specific to flavonoid and anthocyanin biosynthesis, such as chalcone synthase (CHS), chalcone isomerase (CHI) and dihydroflavonol reductase (DFR). In addition, temperature may also affect the stability of anthocyanins. Therefore, the decrease in anthocyanin concentration at elevated temperatures may result from both a decrease in synthesis and an increase in degradation.

Several studies have examined the effect of different metals on anthocyanin stability and hue in solutions. Mazza & Miniati (1993) reported that tin, copper, and aluminium ions are capable of forming stable complexes with anthocyanins. Stable ternary complexes containing anthocyanin, an unidentified colourless compound and magnesium (or magnesium plus ferric ion or aluminium) have also been described (Takeda *et al*., 1990; 1994; Kondo *et al.*, 1992). The main known effect of metals on anthocyanins in flowers is a change in hue of the flower colour (Kondo *et al*,. 1992; Takeda *et al*., 1994).

The red colour in many fruits and flowers has been found to be due to anthocyanins with a common aglycone (cyanidin) bound to different sugars, thereby producing different cyanidin glycosides (Montefiori *et al*., 2005). Huang *et al*. (2002) investigated the environmental effects on flower anthocyanin pigmentation using reddish-purple tuberose, and established that the primary component of the anthocyanin pigment in tuberose is cyanidin.

The cut flower value of tuberose can be improved by increasing the reddish pink colouration attributed to anthocyanins. To identify the soil related factors associated with the formation of this colour in tuberose the study was carried out. The combined effect of low pH

and increased Mg concentrations in the soil on the colour and accumulation of anthocyanins in tuberose flowers was investigated.

## Materials and methods

Tuberose bulbs were planted in March 2007 at a spacing of 20cm between plants and 20cm between the rows. Flower bud initiation was detected in June 2007 and plants continued to flower during the course of the experiment. The tuberose flower spike emerges with a flower head enclosed in bract leaves. Flowers were staged to determine the appropriate time for anthocyanin extraction based on morphology. Table 1 gives a description of the stages and Plate 1 shows a photograph of each stage. The appropriate extraction stage was 9.

The field experiment was carried out at the Kenya Agricultural Research Institute -National Horticultural Research Centre. The Centre is in Thika District, Central Province and is situated at 0° 59' South and 37° 04' East at an altitude 1548 metres above sea level.

Three chemical fertilisers, Calcium ammonium nitrate (CAN), Ammonium sulphate (AS) and Magnesium nitrate ($Mg(NO_3)_2$) were used as soil amendments. AS well as different chemical compositions, these fertilisers also have different pHs. Each fertiliser was applied at a dose of 13 g N per $2m^2$ plot.

Soil analysis was carried out before planting, after planting, before adding the fertilisers, and the every 2 weeks thereafter until the 18th week.

Foliar chlorophyll was obtained using a hand held Minolta SPAD502 -chlorophyll meter. Newly expanded leaves were used for determination of chlorophyll. Three measurements were taken along the midrib, averaged and recorded. These same leaves were detached for plant tissue analysis.

Petals from each flower spike representing the color shade and intensity were detached to increase the surface area of the petal used for colour determination using the 8-mm diameter head of a portable tristimulus color analyser (Chromameter II; Minolta.). Flower colour was expressed in Commission Internationale d'Eclairage (CIE) L*, a*, b* color-space coordinates. The meter was calibrated using the manufacturer's standard white tile. CIELAB coordinates Scale measured relate to:

L*(Lightness) Dark-Bright (0=black, 100=white)
a* Green-Red (negative value=more green, positive value=more red)
b* Blue-Yellow (negative value=more blue, positive value=more yellow)
h (Hue angle) Degree of brownness (the higher the more brown)
C* (Chroma) Color intensity (the higher the more intense)

Table 1. Description of floret stages in tuberose flower spike development.

| Stage | Name | Description |
|---|---|---|
| 1 | Tight bud | A bud that is tightly enclosed by the bract leaves terminating in a pointed end |
| 2 | Swollen | A bud with expanded florets under tight bracts giving it a more elliptical shape (blunt end) |
| 3 | Cabbage head | A bud with expanded florets and loosened bracts giving a flat head resembling a cabbage |
| 4 | Bud break | The bracts completely loosened exposing green florets |
| 5 | Elongation | The spike is elongated and individual florets increase in size with visible internodes. 1st internode clearly visible |
| 6 | Colouration | More than two internode clearly visible pinkish tinge appearing on florets |
| 7 | Spike elongation | More than three (3) internodes visible |
| 8 | Floret expansion | Swollen basal florets |
| 9 | Harvest for export | The flower spike is fully elongated basal florets enlarged and white, partially open |
| 10 | Harvest for local | The basal floret on flower spike is in bloom |

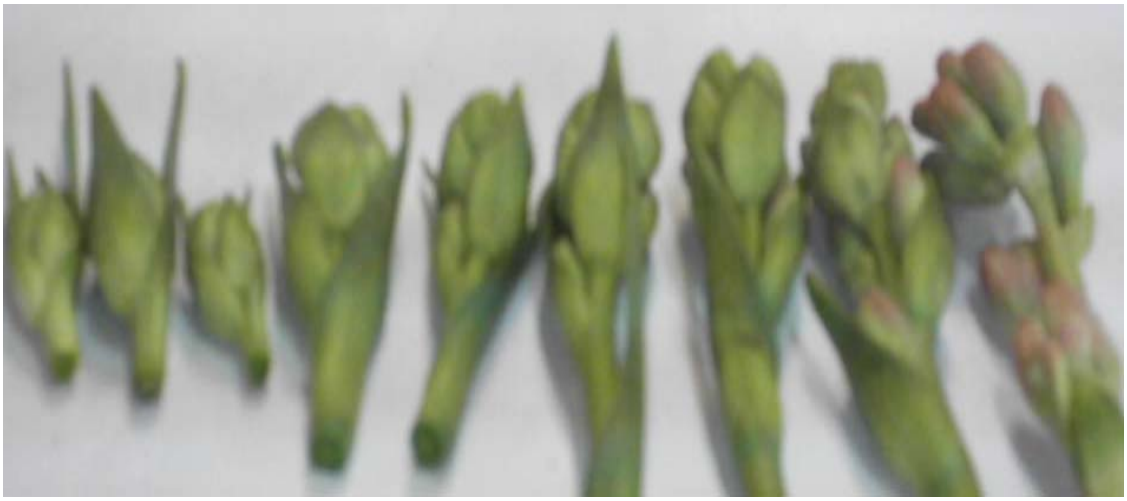

Plate 1. Various tuberose floret stages of tuberose plants grown at KARI-Thika in 2007.

Pre-chilled petals (0.5 to 1.0 g) were ground with a pestle and mortar and mixed with chilled 80% acetone. The residue was re-extracted with 5 mL aliquots of 80% acetone until the acetone remained clear. The combined extracts were adjusted to 20 mL with 80% acetone and centrifuged at 5000 x *g* for l0 min. Absorbance was measured at 645 and 663 nm for chlorophyll a and b, respectively, and at 480 nm for carotenoids. Chlorophyll and carotenoid content was calculated from the data using the equations of Ross (1974).

To determine total anthocyanin content, acetone extracts of outer flower petals were prepared. The extracts were filtered through Whatman filter paper and the filtrates were made up to a final volume of 5 mL with distilled water. The total anthocyanin content of the acetone extract was measured using a pH differential method. A UV mini 1240 Shimadzu spectrophotometer was used to measure absorbance at 510 and 700 nm in buffers at pH 1.0 and 4.5. Absorbance readings were converted to total mg of cyanidin 3-glucoside per 100 g fresh weight of tuberose petals using the molar extinction coefficient of 26,900 and absorbance of A = [(A510 _ A700)pH1.0 _ (A510 _ A700)pH4.5]. Data are reported as means ± SD for three replications.

Florets (2.5 g) were dried at 70°C for 48 h. 0.25 g of dry material was added to 10 mL phosphoric acid at 140°C. Magnesium concentration in the solutions was determined using the wet ashing technique for phosphorus, potassium, calcium and *magnesium analysis* in *plant tissue (Okalebo*, 1985).

A one-way analysis of variance (ANOVA) test was used to determine whether differences between soil nutrient content, plant tissue mineral content, SPAD values, colour and anthocyanin content of samples were statistically significant. The differences between means were determined using the Tukey's multiple comparison test. To assess the relationship between the activities and the phenolic content, Pearson's correlation coefficients were calculated with 95% confidence. The Statistical Package SAS 9.1 was used to analyse the data.

## Results

Temperature, a key determinant of anthocyanin accumulation, was generally low during the production period and therefore favourable for anthocyanin accumulation (Fig. 1). The monthly average maximum and minimum temperature ranged from 23 to 28°C, and 13 to 16 °C, respectively. Daily temperature ranged from 17 to 29°C during the day and as low as 10°C at night to 22°C (data not shown); rainfall was heavy during establishment but minimal during top dressing; solar radiation was low between June and September (2007) and relative humidity was low during the month of March and June.

The fertilisers AS and $Mg(NO_3)_2$ had low pH compared to CAN (Table 2). The soils were generally of a weak acidic pH ranging from 5.9 to6.6 before the fertiliser additions (Table 3). After application of fertilisers, the soil pH was significantly lower in soils treated with CAN and AS (Table 4).

Leaf growth was similar between the fertiliser types in terms of leaf area and leaf dry weight (Fig. 2). SPAD values did not show significant differences between the fertilisers applied except at 70 days after application when the $Mg(NO_3)_2$ treatment had significantly higher values, with values reaching higher levels ranging from 61 to 64 between 56 and 98 days. By 126 days, the values had declined to 51-55 (Fig. 3).

The type of fertiliser applied had no significant effect on the L values. However, L values in plants where $NHSO_4$ was applied increased gently up to the 16th week before

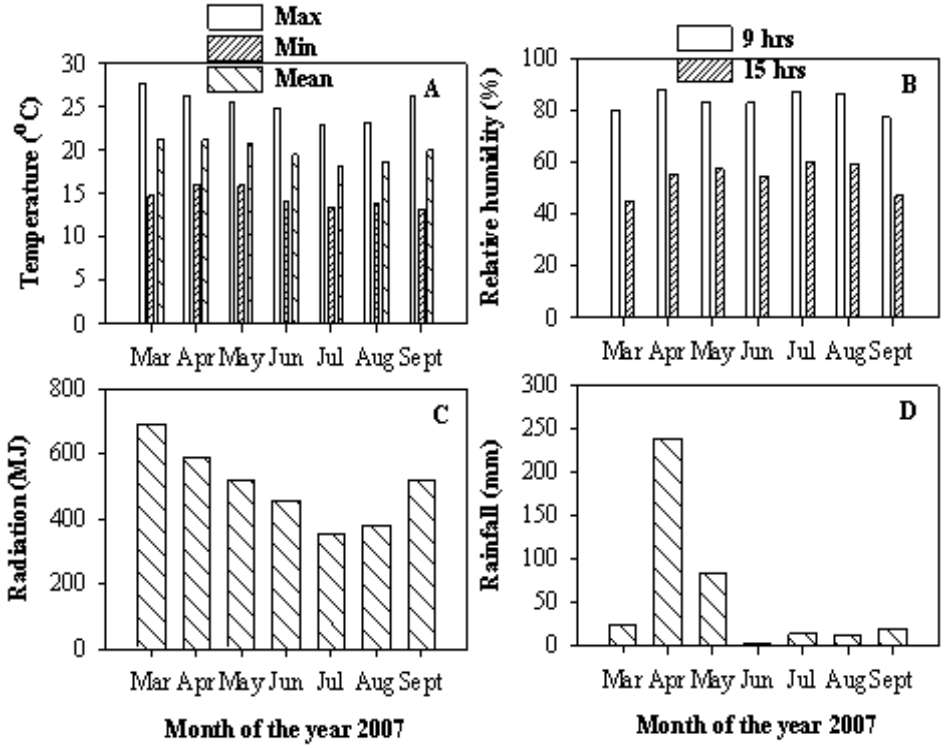


Figure 1. Pattern of key weather elements during the experimental period.

Table 2. Chemical and physical characteristics of the applied amendments.

| Treatment | pH (measured) | Nutrient content (commercial information) | Weight of fertilizer (g) required to give 13g of N per $2m^2$ plot |
|---|---|---|---|
| Calcium Ammonium Nitrate (CAN) (farmer's practice –control) | 5.3 | 26% N26.5% CaO | 50 |
| Ammonium Sulphate(AS) | 2.7 | 21% N24% S | 62 |
| Magnesium Nitrate ($MgNO_3$) | 3.7 | 15% Mg11% N | 118 |

Table 3. Chemical and physical characteristics of the soil in the experimental field.

| Prior to planting (fallow 2 years) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| pH | % N | % OM | P % | K Meq | Ca Meq | % Mg | Zn (ppm) | Fe (ppm) | Mn (ppm) | Cu (ppm) |
| 5.9 | 0.4 | 11.0 | 0.0046 | 8.1 | 3.3 | 4.2 | 1.6 | 27.2 | 208.4 | 2.1 |
| After planting (before top dressing) | | | | | | | | | | |
| 6.6 | 0.058 | | 0.094 | 5.6 | 0.82 | 4.06 | 14.6 | 76.6 | 563.6 | 1.7 |

Table 4. Soil pH during the experimental period.

| | Weeks after amendment | | | |
|---|---|---|---|---|
| Amendment | 0 | 12 | 16 | 18 |
| CAN | 6.8 | 5.1a | 5.3a | 5.3a |
| AS | 6.8 | 5.1a | 4.8a | 4.6a |
| MgSO4 | 6.8 | 6.5b | 6.5b | 6.8b |
| LSD | - | 0.5 | 0.5 | 0.6 |

Means followed by the same letter down the column are not significantly different at 5 % significance level.

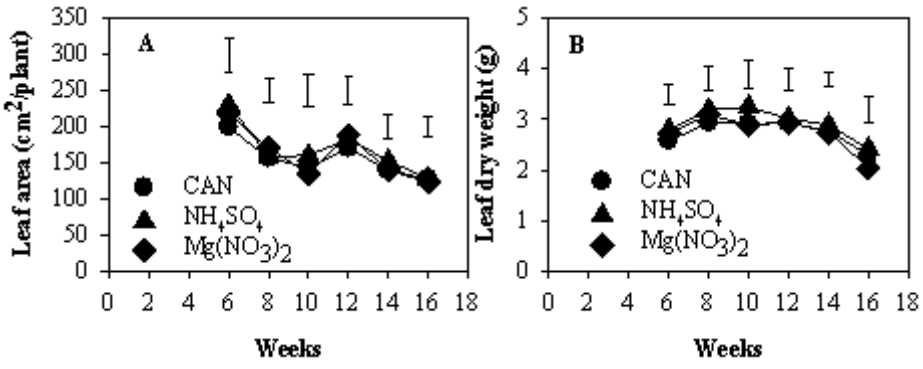


Figure 2. Leaf area and dry weight of tuberose plants. Vertical bars show $LSD_{0.05}$.

declining (Fig. 4a). L values were generally in the range 55-70. The a values were significantly higher in plants supplied with CAN at 16 weeks, but the other sampling dates showed no clear trends (Fig. 4b). Fertiliser types had no significant effect on b values, but generally plants supplied with CAN and $Mg(NO_3)_2$ saw a decline in b values over time. In contrast, plant supplied with $NH_4SO_4$ showed a general increase in b values before a decline at week 16 (Fig. 4c).

Chroma was significantly higher in petals of plants supplied with CAN at 16 weeks (Fig. 5a). However, there were no clear trends in chroma levels on the other sampling dates. The values generally ranged from 53 to 60. Fertiliser types had no significant effect on hue values, but generally plants supplied with CAN and $Mg(NO_3)_2$ saw declines in hue values over time, while plant supplied with AS had a general increase until week 16 before a decline at week 18

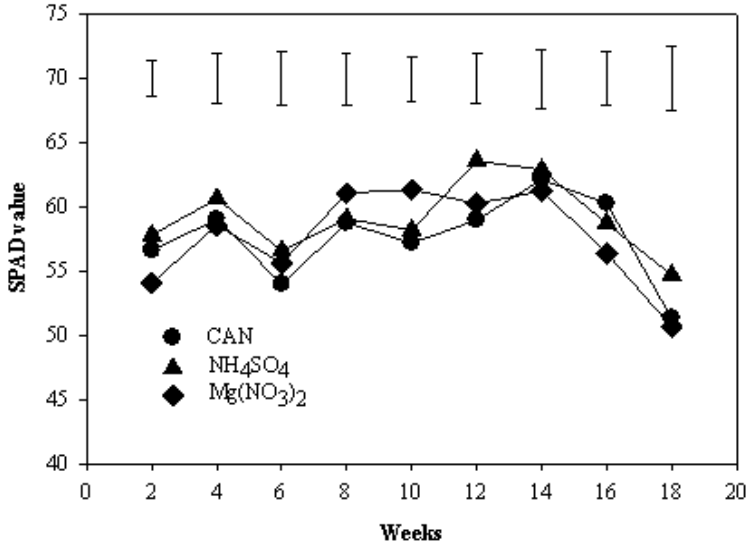


Figure 3. Foliar SPAD values of tuberose plants. Vertical bars show $LSD_{0.05}$.

Table 5. The chlorophyll, anthocyanin and Mg contents of tuberose petals at week 18 after soil applied amendments.

| Amendment | Chlorophyll (µg/g) | Anthocyanin (%w/w) | Mg (%) |
|---|---|---|---|
| CAN | 2.36 | 0.88 | 0.46 |
| AS | 1.69 | 2.64 | 0.49 |
| MgSO4 | 1.67 | 2.61 | 0.47 |

(Fig. 5b). The hue values were generally in the range of 27 to 30.

Generally, the tissues had similar levels of Mg, N, P and K irrespective of the type of fertiliser applied (Fig. 6). Magnesium content increased up to the 4th week and peaked at levels of 2.5 to 2.8 %. Nitrogen showed a general decline from high levels of 6 to about 3 % in the 10th week. P increased to peak levels at 4-6 weeks ranging from 0.16 to 0.23 % before declining to 0.07-to 0.13 % by the 10th week. L was generally in the range of 2.13 to 3.43 %.

The chlorophyll content and anthocyanin content were closely linked. CAN, which gave the highest chlorophyll content, gave the lowest anthocyanin content as compared to AS and $Mg(NO_3)_2$ (Table 4). Mg content was marginally higher for AS and $Mg(NO_3)_2$ treatments, which also had the highest anthocyanin and the lowest chlorophyll contents (Table 5).

## Discussion

Temperatures during the period of the trial were favourable for colour development. It was expected that application of fertilisers AS and $Mg(NO_3)_2$ would lower the soil pH more than CAN because of their lower pH. The soil pH declined over time after the fertilisers were applied and by the 18th week, AS treatments had the lowest soil pH as expected. However, $Mg(NO_3)_2$-treated soils had a higher

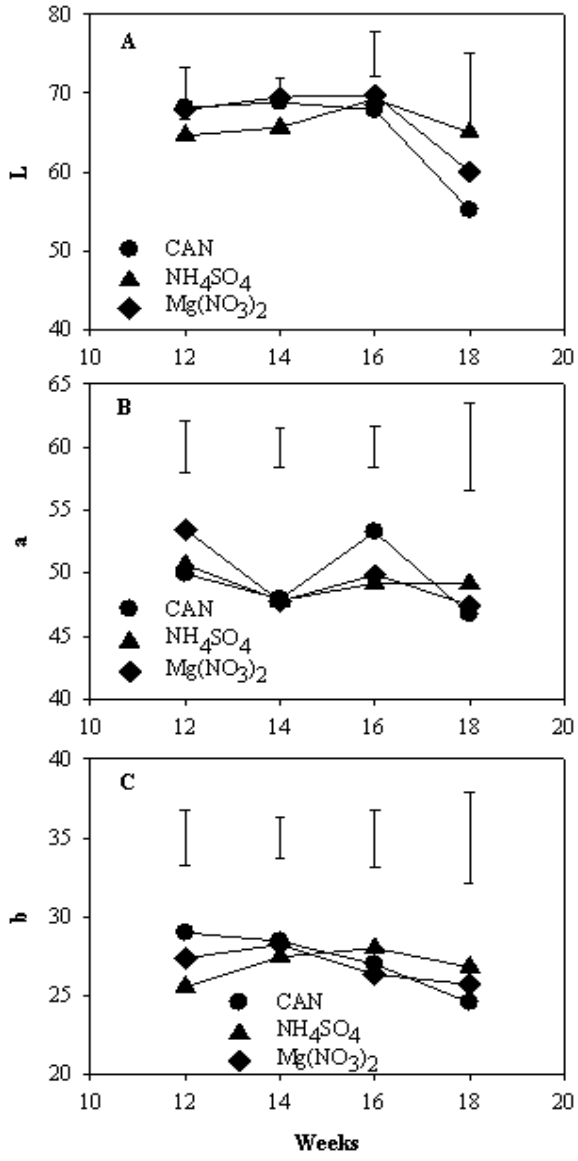


Figure 4. The L, a and b values of tuberose petals. Vertical bars show $LSD_{0.05}$.

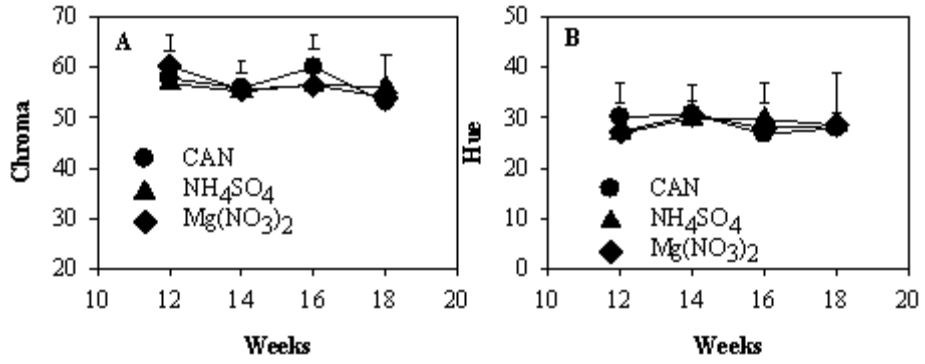


Figure 5. Chroma and hue values of tuberose petals. Vertical bars show $LSD_{0.05}$.

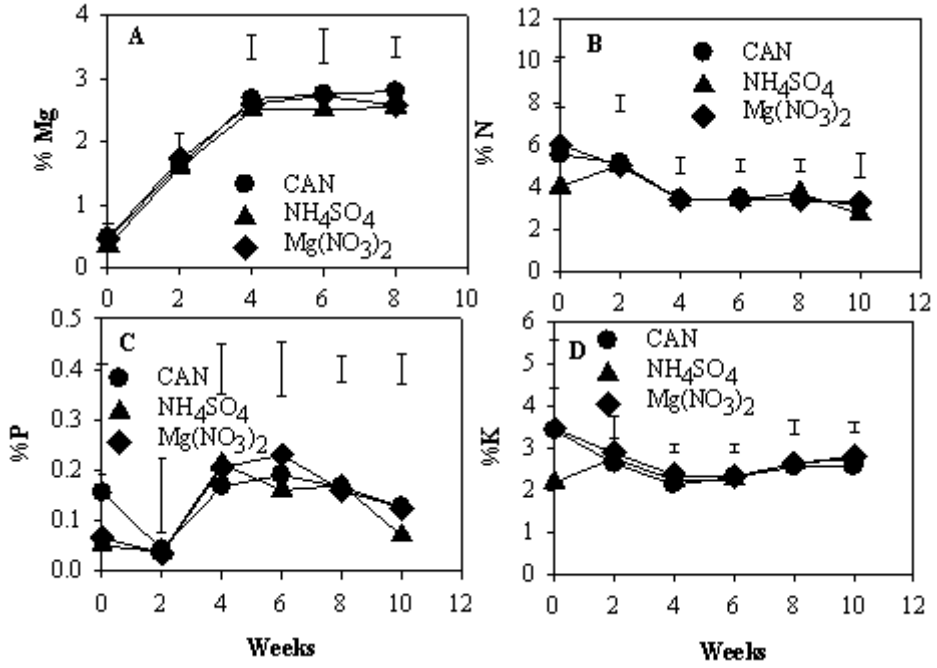


Figure 6. Levels of chemical elements in tuberose plants. Vertical bars show $LSD_{0.05}$.

pH than would have been expected. Thus, plants treated with AS experienced a much lower soil pH, followed by plants in the CAN treatment, while those in $Mg(NO_3)_2$ had more or less neutral pH.

Leaf growth in terms of leaf area and dry weight was similar between the different fertilisers applied. This can be attributed to the fact that the same amount of nitrogen was supplied in all the treatments. Despite the differences in soil pH, plants were able to uptake nitrogen and other nutrients and show similar amounts of growth. In the same way, SPAD values were generally similar irrespective of the fertiliser type. No differences in the greenness of the leaf could be detected, again possibly due to the plants having access to similar amounts of nitrogen from the different fertilisers. The amount of the elements N, K, P and Mg in the plant tissue gives further evidence that their uptake was relatively similar between the fertiliser types.

L values, which provide a measure of lightness, did not change significantly in response to the fertiliser types. However, the L values were higher in plants treated with AS at the 18th week, when the soil pH was the lowest in that treatment. The general decline in L value from about 68 to 70 at 16th week to 55 to 65 at the 18th week could be attributed to the change in colour of the florets. When colour changes occur during fruit ripening in cherry, the L values decline (Gonclaves *et al*., 2007). In contrast, no pronounced declines in chroma and hue were observed. Gonclaves *et al*. (2007) and Byamukama *et al.* (2006) have shown a negative correlation between chroma and hue with anthocyanin content in cherries and *Hippeastrum*, respectively, but this was not the case in our study. In Eustoma flowers (*Eustoma grandiflorum* Gries), total anthocyanin increased as L decreased L, but increased as chroma increased (Uddin *et al.*, 2004). Tuberose L values indicated a relatively lighter colour. In red *Hippeastrum* flowers, Byamukama *et al.* (2006) reported lower L values ranging 33-38,with chroma and hue values ranging 40-63, and 22-35, respectively. In Eustoma, most purple flowered cultivars had L, chroma and hue values in the ranges of 21-42, 45-76, and 319-342, respectively, reddish purple had ranges of 55-58, 40-50, and 326-341, respectively, pink flowered 49-84, 3-49, and 349-357, respectively and white flowered 85-88, 1-11, and 17-343, respectively. Therefore

the L and chroma values for tuberose in our study are similar to the ranges for reddish purple and purple flowered Eustoma, respectively (Uddin *et al*., 2004).

In this study, the anthocyanin content of tuberose did not show a strong link with tissue Mg content. It was expected that plants supplied with Mg $(NO_3)_2$ would have higher amounts of Mg in their tissues. However, this was not the case as Mg content was more or less similar between amendments. In aster flowers, Sachray *et al*. (2002) have shown that application of Mg salts to the flowers or whole plants increased the metal levels in the petals, and that anthocyanin production was increased at elevated temperatures. Colour was not different with Mg and pH. Anthocyanin content varied with Mg and pH.

The amendments were soil applied and the reaction between the fertiliser and the soil affects the uptake of Mg and therefore overall rate of anthocyanin synthesis in plants. It has been shown that anthocyanin synthesis occurs concurrently with petal growth, and decreases with unfurling of the petals (Weiss & Halevy, 1989; Martin & Gerats, 1993; Oren-Shamir *et al*., 1999). This suggests that PAL activity during flower development is under a different control than enzymes solely committed to flavonoid and anthocyanin biosynthesis. Anthocyanin synthesis is often initiated or increased by stress conditions. Nitrogen deficiency (Bongue-Bartelsman & Phillips, 1995), low temperature (Christie *et al*., 1994) and application of growth retardants (Avihai & Dougall, 1992) have been correlated with increased anthocyanin synthesis in plant tissues. Magnesium becomes limited at elevated temperatures, thus inhibiting the activity of these enzymes. Anthocyanin-magnesium complexes, if formed, may increase the half-life time of the pigments, and/or inhibit their catabolism. Over-expressing the vacuolar Mg/H exchanger (Shaul *et al*., 1999) may be a future way for improving flower pigmentation at elevated temperatures. Low absorbance recorded for anthocyanins could be an artifact due to interference from chlorophyll and phenolics (Cheng & Breen, 1991).

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