Investigation of the Efficiency of the Total Antioxidants Assays in Silicon-Treated Lemon Fruit (*Citrus limon*)

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Abstract

The effect of postharvest silicon dips on the concentration of total antioxidants, total phenolics, and malondialdehyde, as well as the susceptibility to chilling injury of lemon fruit rind was studied. Fruit from two orchards were soaked into different silicon concentrations (0, 50, 150, and 250 mg/L K_2SiO_3) for 30 min and thereafter stored at -0.5 or 2°C for 28 days with weekly evaluation of chilling injury and total flavedo antioxidants. Total antioxidants were determined using three assays, the ferric reducing antioxidant power (FRAP), the 1,1-diphenyl-2-picrylhydrazyl (DPPH), and the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay. Silicon concentration in the flavedo was determined using Scanning Electron Microscope (SEM). Significant differences in endogenous silicon as well as chilling susceptibility between fruits from the two sources were observed. Fruit stored at -0.5°C was less chilling susceptible compared with fruit stored at 2°C. However, the total antioxidant capacity in the rind did not differ between the two storage temperatures. Postharvest silicon application had no effect on total phenolics, but an increase in the total antioxidants concentration and reduced malondialdehyde formation, a sign of membrane disintegration, was observed. High silicon concentrations were found to impair visual fruit quality. Chilling injury was reduced by 50 mg/L K_2SiO_3 and total antioxidants and total phenolics were significantly reduced at 150 and 250 mg/L K_2SiO_3 as determined by the FRAP, ABTS and DPPH assays. Fruit source impacted on total antioxidants, total phenolics and malondialdehyde. Fruits with high total antioxidants, total phenolics and low malondialdehyde were also found to have high silicon concentration. Therefore, silicon has the potential to reduce postharvest chilling injury; however, preharvest silicon application should be considered as high silicon concentration from postharvest drench application impairs visual quality although improving total antioxidants in the rind.

INTRODUCTION

The citrus industry is an important component of the South African economy. Internationally, South Africa is the second largest exporter of citrus, following Spain (Solomon, 2010). Approximately 61% is of the SA citrus production is exported comprising of Valencia (46%), navels (23%), grapefruit (14%), lemons (10%) and soft citrus (8%) (Solomon, 2010). Citrus fruit, in particular lemons, have limited postharvest life; therefore, chilling temperatures are commonly used to reduce respiration and to prolong the commodity's postharvest life. Furthermore, the presence of the fruit fly and the false codling moth requires cold sterilization as a quarantine treatment to export citrus fruit (Serry, 2010). However, such cold treatment can result in chilling injury.

Several techniques have been used to reduce chilling injury in order to extend fruit shelf life. However, health concerns regarding certain chemicals have been raised, hence, there has been a move towards less hazardous chemicals, such as silicon, which is able to

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reduce postharvest stress (Liang et al., 2007; Tesfay et al., 2011). The accumulation of phenolics in avocado (*Persea americana*) trees following silicon application has been reported (Bekker et al., 2007), as well as improvement in postharvest quality due to increased total antioxidant capacity (Tesfay et al., 2011).

Antioxidants alleviate the effect of free radicals which contribute to the development of chilling injury. Citrus flavedo has an array of antioxidant compounds that are responsible for scavenging reactive oxygen species (ROS), thereby alleviating stress (Abeysinghe et al., 2007; Mathaba et al., 2008; Cronje et al., 2011). Antioxidants have different scavenging mechanisms; it is therefore imperative to perform several antioxidant assays to gain a holistic view of mechanisms counteracting ROS effects (Wong et al., 2006). Previous studies have revealed that these assays either measure hydrophilic (ascorbic acid and phenolic groups) or lipophilic antioxidants (α-tocopherol, β-carotene and lycopene) (Wong et al., 2006; Pérez-Jiménez et al., 2008).

As silicon is known to increase the antioxidant concentration and, subsequently, reduce stress (Gunes et al., 2007; Tesfay et al., 2011), the aim of this study was to determine the potential of Si to mitigate chilling injury through increasing the rind antioxidant concentration, and to further investigate which antioxidant assay is efficient in measuring a holistic antioxidant action. Principal component analysis (PCA) was used to identify the total variation in the antioxidant activities of the fruit by the methods used.

MATERIALS AND METHODS

Lemon fruit were harvested in July 2010 from the University of KwaZulu-Natal Research Farm, Ukulinga (29°40'00"S; 30°24'00"E) and as well as Ithala Farm (29°52'00"S; 30°16'00"E) located in the KwaZulu-Natal Midlands. Fruit were transported to laboratory, where it was selected according to good appearance and absence of blemishes. Prior to treatments fruit were dipped in Sporekill® solution. Fruit were treated with various concentrations (0, 50, 150, 250 mg/L) of potassium silicate (K_2SiO_3) dips for 30 min. The fruit were waxed (Avoshine®, (Citrashine) (Pty) Ltd.), weighed and subsequently stored at -0.5 or 2°C under 85-90% relative humidity (RH) for 7, 14, 21 or 28 days. After storage the fruit was evaluated for weight loss and kept at room temperature for five days before weight loss was recorded again and chilling injury evaluated. Thereafter, the flavedo of fruit was peeled, freeze-dried, milled and stored at -21°C for further analysis.

Chilling Injury Evaluation

After 7, 14, 21 and 28 days cold storage at -0.5 or 2°C plus 5 days shelf-life fruit were evaluated for appearance of chilling symptoms and chilling injury was expressed as percentage. Chilling injury (%) = (Number of fruit with chilling symptoms/total number of fruit evaluated)*100.

Analysis

Various antioxidant assays were used to determine total antioxidant concentration to gain a well-rounded view of mechanisms counteracting ROS effects. The total antioxidant concentrations were standardized to µmol Trolox equivalents per gram dry weight (DW)W to compare the assays. Total antioxidant capacity (TAC) was determined using the FRAP assay according to the method of Abeysinghe et al. (2007), with slight modifications. The ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) assay was performed according to Re et al. (1999) with some modifications. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was also used following the procedure of Wong et al. (2006).

The free phenolic content was determined according to Abeysinghe et al. (2007), with some modification according to Tesfay et al. (2011). Lipid peroxidation is measured by the accumulation of malondialdehyde (MDA) with high MDA accumulation signifying high lipid peroxidation. To determine the Si concentration all samples were scanned under a Scanning Electron Microscope equipped with EDX detector (Zeiss EVO

LS15,Oxford XMax detector, and INCA Energy EDX software). Solid particles were dispersed on a graphite adhesive tab placed on an aluminum stub. Data were subjected to analysis of variance (ANOVA) using GenStat, 12th edition. Means were separated using Duncan's test at $P \leq 0.05$ levels. Furthermore, data was subjected to principle component analysis (PCA) using Unscrambler (Ver.9.8).

RESULTS AND DISCUSSION

Antioxidants play an important role in stress reduction and have the ability to delay, reduce or prevent the destructive action of free radicals produced during stress (Halliwell, 1990; Salah et al., 1995). There are various many citrus fruit antioxidants including vitamin C, phenolics, flavonoids, that have been identified to play a role in reducing stress. Antioxidants in the rind/exocarp of the fruit are thought to be important in mitigating postharvest stresses such as chilling injury.

Silicon has been proven to induce stress resistance and to enhance antioxidant capacity in plants (Liang et al., 2008). Similarly, Si applied at 50 ml L^{-1} K_2SiO_3 reduced chilling injury (Fig. 1B). The difference in silicon concentration between Ithala and Ukulinga fruit seemed to be an important factor for the susceptibility of Ithala Farm fruit and resistant of Ukulinga Farm fruit to chilling injury (Fig. 1A and B). Postharvest treatment with 50 mg/L K_2SiO_3 reduced chilling injury symptoms; a finding in agreement with Agarie et al. (1998), Bekker et al. (2007), Liang et al. (2008), and Epstein (2009). These authors found Si to play an important role in inducing stress resistance. However, high Si concentrations proved to be disadvantageous to rind quality as chilling injury symptoms were increased by 150 and 250 mg/L K_2SiO_3 compared with control treatment (Fig. 1). Source of plant material can have an impact on total antioxidant capacity, as previously discovered on moringa (*Moringa oleifera*) leaves where production location has demonstrated a profound effect on total antioxidants (Iqbal and Bhanger, 2006). The difference in silicon concentration between the fruit source may explain the difference in total antioxidants as silicon increases the antioxidant capacity (Liang et al., 2007). Furthermore, the reduced lipid peroxidation in the fruit containing high endogenous silicon concentrations fruit source further proves relationship between region of harvest and chilling injury.

Antioxidant capacity was not significantly influenced by storage temperature (Fig. 2A-C). Previous studies on apple (*Malus* × *domestica*) fruit revealed that cold storage temperature does not influence antioxidant concentration nor activity (Van Der Sluis et al., 2001). The fruit in this experiment could have been pre-conditioned in the orchard, hence storage temperature did not affect antioxidant concentration. The ability of silicon to maintain or increase the antioxidant capacity, and subsequently reduce the risk of abiotic stress, has been reported in previous studies (Agarie et al., 1998; Tesfay et al., 2011). In this study, silicon enhanced the antioxidant capacity (DPPH) which increased with the silicon concentration applied (Fig. 5A-B). However, 50 mg/L K_2SiO_3 was seen as the best treatment as it also reduced chilling injury, unlike other treatments. Total phenolics were not influenced by postharvest silicon dips, a finding in contrast with the increased phenolics in the avocado exocarp stored at 5.5°C (Tesfay et al., 2011). The reduced lipid peroxidation, as determined by malondialdehyde accumulation (MDA) following silicon treatment is in agreement with the reduction in MDA in salt-treated barley leaves (Liang, 1999).

Our data has shown that the endogenous silicon concentration is correlated to the antioxidant capacity as measured by the DPPH and ABTS assay thereby reducing lipid peroxidation and ultimately reducing chilling injury (Fig. 2D-E). The rind of Ukulinga fruit had a higher silicon concentration than that of Ithala fruit, the probable reason for the chilling resistance of Ukulinga fruit. Moreover, in as much as postharvest silicon dips have shown to improve total antioxidants, and reduce MDA, high silicon concentration (150 and 250 mg/L K_2SiO_3) have shown to impair visual fruit quality as whitish deposits are often observed on the fruit surface.

Total antioxidant assays have been proven to differ substantially in determining

the antioxidant concentration due to the complexity of antioxidants. Wong et al. (2006) using principal component analysis found a strong correlation between total antioxidant capacity values of sweet potato (*Ipomea batatas* C.) obtained for DPPH and FRAP assay. In this study, principal component analysis (Fig. 4) showed a correlation between ABTS and DPPH, with DPPH probably estimating both, lipophilic and hydrophilic antioxidants, sufficiently accurate (Wong et al., 2006). Moreover, Awika et al. (2003) found a correlation between ABTS and DPPH in sorghum. All three assays rank the strength of antioxidants in order of: vitamin C>phenolics>flavonoids>hesperidin>naringin (Fig. 3). The ABTS and DPPH assay had similar antioxidants except that DPPH detected a higher antioxidant strength than ABTS. Furthermore, rind antioxidants, as determined by the DPPH assay had slightly higher values than determined with other antioxidant assay.

In conclusion, orchards factors i.e., soil and climate impact on the antioxidants present in the flavedo, on lipid peroxidation and on chilling injury. The analysis of antioxidants with different antioxidant assays (FRAP, ABTS, DPPH) seems important to obtain a holistic estimate of the total antioxidant activity of lemon flavedo. Postharvest silicon soaks increase the total antioxidant capacity; however, as the visual fruit quality is partly impaired by postharvest silicon dips, preharvest silicon applications should be considered as the preferred method to reduce postharvest chilling injury. The FRAP, ABTS and DPPH assays gave comparable results for the antioxidant strength of lemon flavedo with ABTS and DPPH showing high correlation. Therefore, these assays constitute the best techniques for measuring the total antioxidants of lemon flavedo with major contributors to the total antioxidant capacity of lemon flavedo being Vitamin C, phenolics and flavonoids.

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Figures

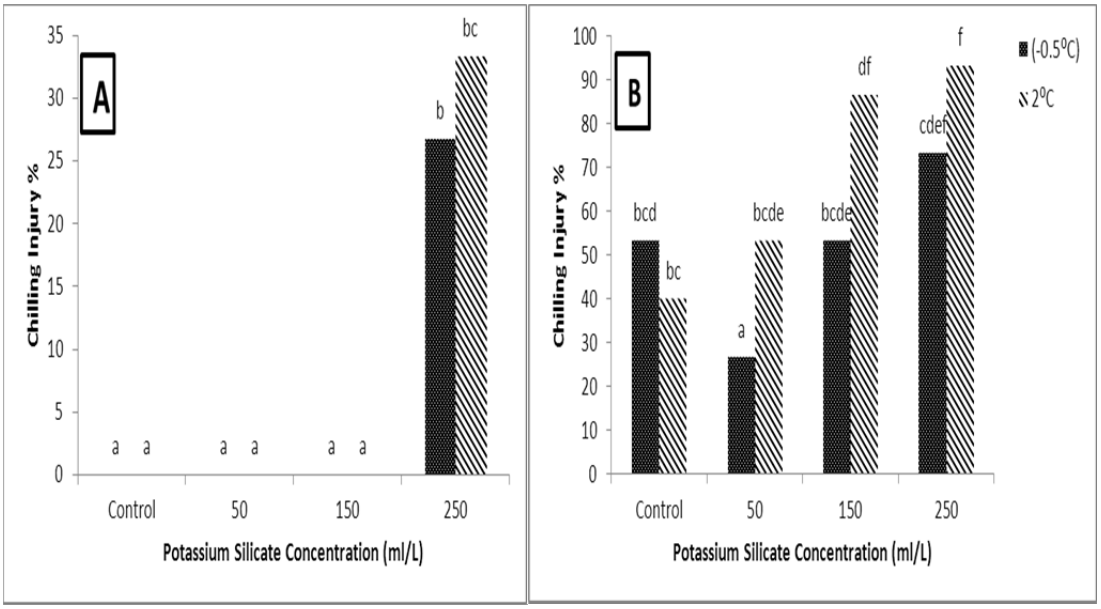

Fig. 1. The effect of K_2SiO_3 concentration and storage temperature on chilling injury percentage of lemon fruit sourced from Ithala farm at 21 days (A) and 28 days (B) storage time.

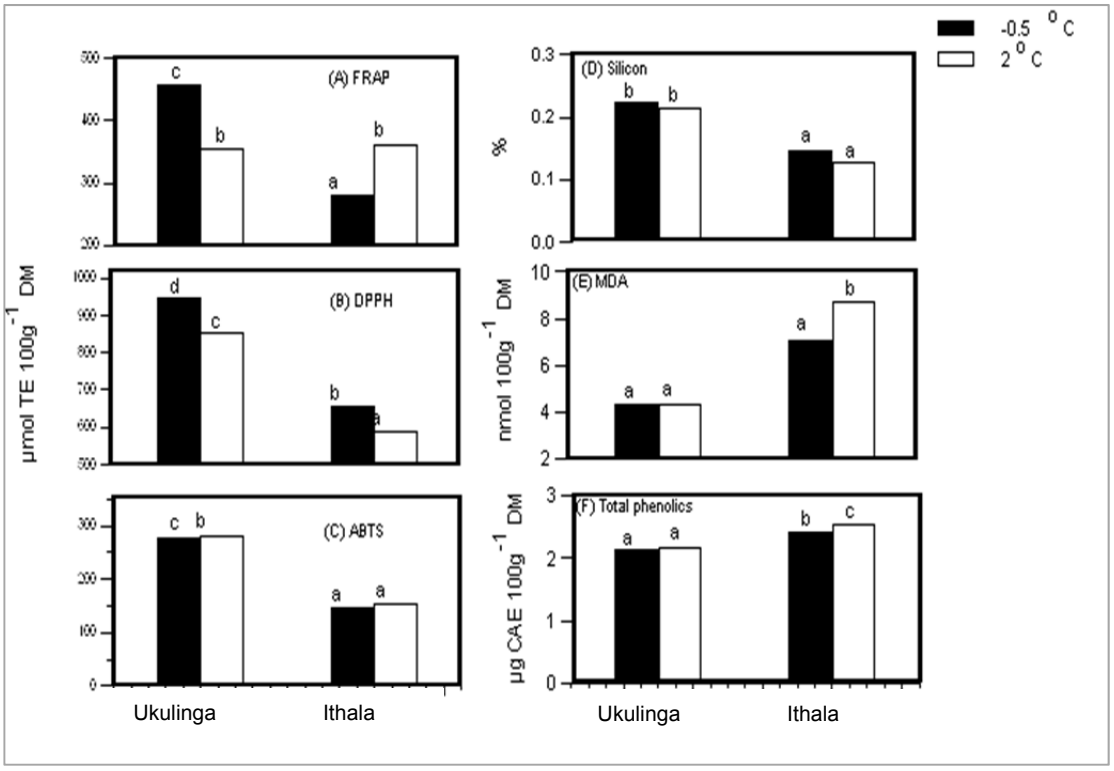

Fig. 2. Effect of fruit source on total antioxidants, FRAP (A), DPPH (B), ABTS (C), and silicon (D), lipid peroxidation (MDA) (E) and total phenolics (F).

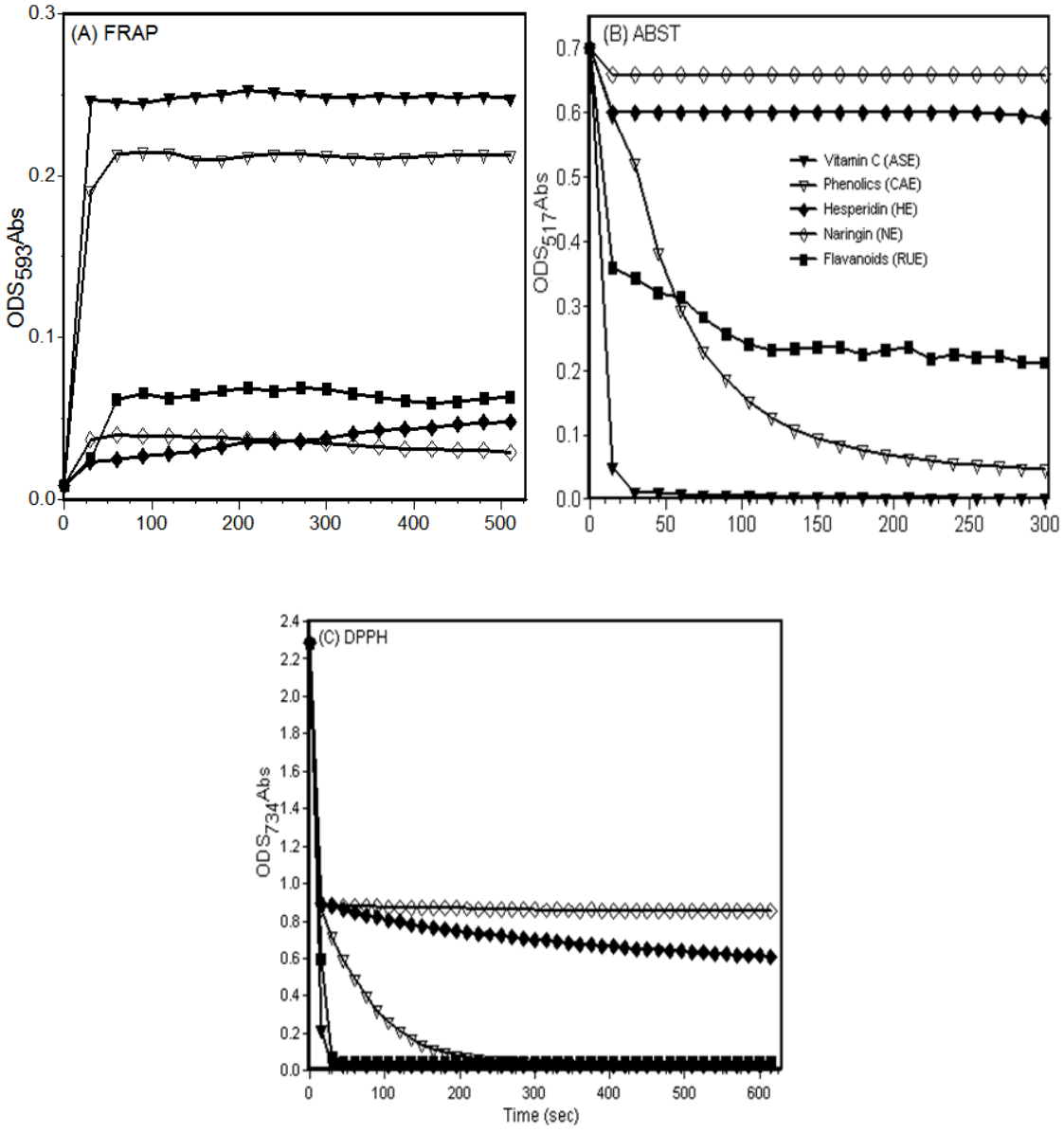

Fig. 3. Evaluation of antioxidant capacity over different trolox equivalent using FRAP (A), ABTS (B) and DPPH (C) assays.

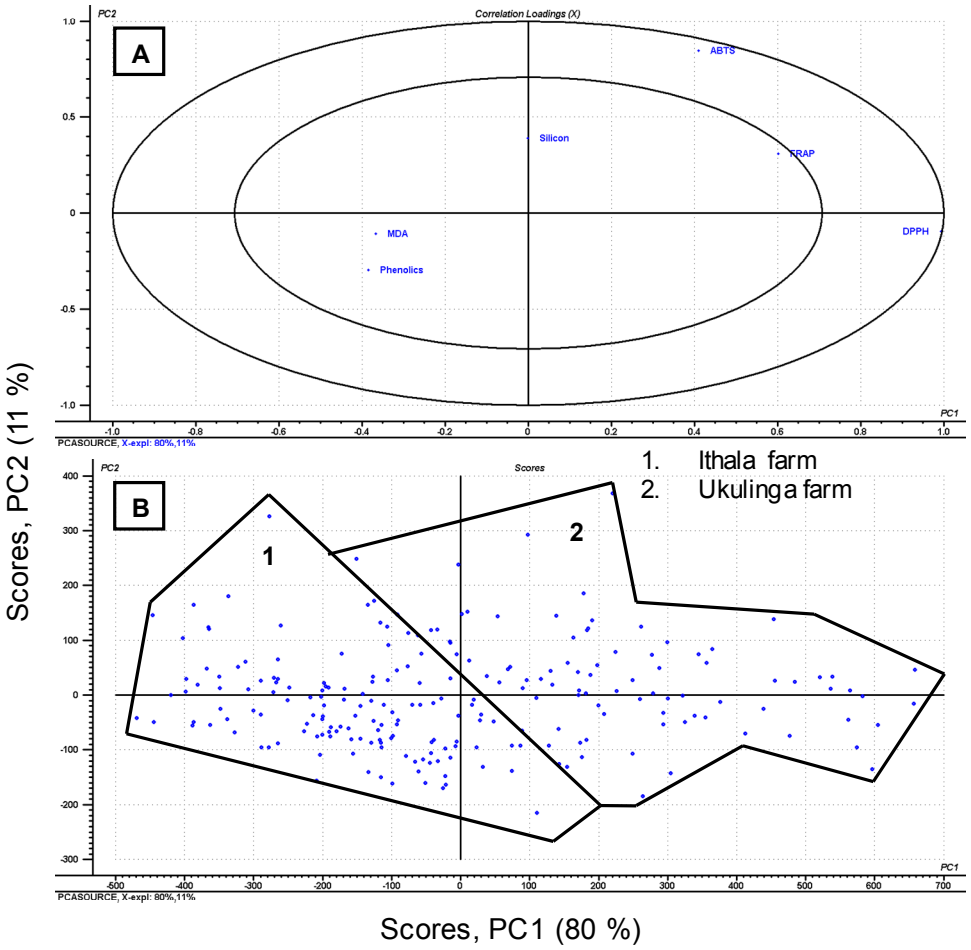

Fig. 4. Principal component analysis (PCA) showing correlation loadings (A). Score plot lemon total antioxidants (FRAP, DPPH, ABTS), MDA, Silicon, and total phenolics (B). Principal component analysis (PCA) led to variation of 90% with principal component 1 (PC1) explaining majority of variation (80%) and principal component 2 (PC2) explaining 11% of total variation.

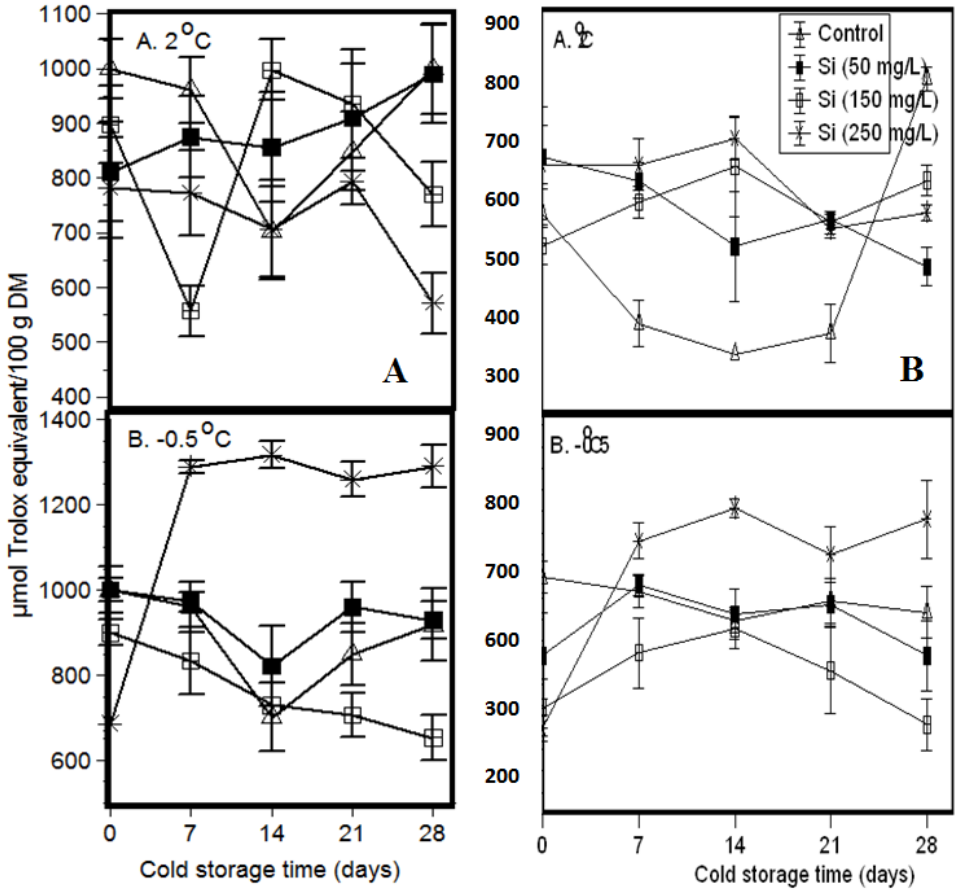

Fig. 5. The effect of silicon concentration, storage time, and storage temperature on total antioxidants under analysis of DPPH assay on lemon flavedo of Ukulinga Farm fruit (A) and Ithala Farm fruit (B).