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Effect of Vacuum Impregnation with Sucrose and Plant Growth Hormones to Mitigate the Chilling Injury in Spinach Leaves

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Abstract: Vacuum impregnation (VI) has been immensely used in modifying the physicochemical properties, nutritional values and sensory attributes of fruits and vegetables. However, the metabolic consequences of the plant tissue upon impregnation have not been profoundly explored although shelf life is strongly dependent on this factor. In this study, spinach leaves were impregnated with salicylic acid (SA), γ-aminobutyric acid (GABA) and sucrose to improve its quality and storage ability by reducing the chilling injury through the improvement of proline content. The spinach leaves were stored at 4 °C for 7 days and were analyzed at 12 h interval. Upon 1 day of impregnation, the proline content in GABA, sucrose and SA impregnated leaves was increased by 240%, 153% and 103%, respectively, while in non-impregnated leaves, the proline content was decreased by 23.8%. The chlorophyll content of GABA impregnated leaves exhibited the lowest reduction (49%) followed by sucrose (55%) and SA (57%); meanwhile, non-impregnated leaves reduced 80% of chlorophyll content at the end of storage. Sensory evaluation showed that GABA, sucrose and SA impregnated leaves respectively, obtained higher score in terms of freshness, color, texture and overall appearance as compared to non-impregnated leaves.

Keywords: physicochemical changes; metabolic consequences; proline; GABA; shelf life

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1. Introduction

Vacuum impregnation (VI) is a food processing technique used to introduce different substances to the porous matrices of plant tissue. It has been commonly used in the enrichment of vegetables and fruits with probiotics and micronutrients [1], texture enhancement [2], modification of the sensory attributes [3] and extension of shelf life by pH reduction [4]. The metabolic consequences of impregnating different substances in plant tissues have not been widely studied, although it is an important factor that can affect the product shelf life. It is important to understand that the introduction of other substances into the plant tissue might affect its metabolism and might change the quality of the vegetable and subsequently affect the shelf life. This could lead to the increase in food waste.

According to Dou and Toth [5], along with roots and tubers, fruits and vegetables have the highest wastage rates (1.3 billion tons per year) of any food products due to their perishable nature. One of the most common methods used to prolong the post-harvest shelf life of fruits and vegetables is by storing them at low temperatures. As for leafy vegetables like spinach, the storage at low temperatures might reduce the microbiological deterioration but the sensitive tissue might be vulnerable to chilling injury. The effect of chilling injury can be seen as browning, surface pitting, wilting and loss of flavor [6] and, thus, might affect the consumer acceptability and resulted in the increase of post-harvest loss.

Most research has been done to treat chilling injury and most research has been focusing on treating and improving the physical appearance of the leafy vegetables. However,

to the best of our knowledge, none of the research has explored the effectiveness of GABA, sucrose and SA compounds scientifically reported to mitigate the chilling injury effect.

Salicylic acid (SA), a type of phenolic compounds, is widely distributed in plants and is considered as a plant hormone because of its roles in plant growth and development, as well as in responses to environmentally stressful conditions [7–9]. In recent years, a few studies have reported the effects of SA on chilling injury in fruits and vegetables, such as increasing antioxidant enzyme activities in banana [10], enhancing total antioxidant activity and preserving bioactive compounds in orange fruit [11], improving proline content in banana [12], delaying activities of polyphenol oxidase (PPO) in banana [10] and reducing electrolyte leakage in banana and orange fruit [10,11].

γ-aminobutyric acid (GABA), a non-protein amino acid, is regarded as an endogenous signal molecule that plays a pivotal role in regulating the stress response and plant growth and development [13]. In plants, GABA content is typically low, but abiotic stresses such as chilling, heat, drought, UV irradiation and low level of oxygen can cause GABA to accumulate rapidly [14,15]. Recent studies have reported that GABA could be used as a post-harvest treatment to alleviate chilling injury in zucchini [16] and white clover [17] by enhancing proline accumulation and also delays senescence in cherry [18] and blueberry [19] by enhancing antioxidant system activity.

Sucrose represents the major transport form of photosynthetically assimilated carbohydrates and plays an important role in plants [20]. Sugars as sources of carbon skeleton are necessary to maintain energy supply and extend the post-harvest life of perishable fruits and vegetables [20]. It has been proven that exogenous sucrose supply can delay senescence in asparagus [21], reduce yellowing and enhance antioxidant capacity of broccoli [22] and reduce nitrate content in baby spinach leaves [23].

In this study, we suggest that exogenous administration of SA, GABA and sucrose using VI reduce the chilling injury and maintain a good quality of spinach leaves during cold storage by improving the proline content, slowing the chlorophyll degradation and showing evidence that impregnated leaves directly influence the physicochemical changes of spinach leaves.

2. Materials and Methods

2.1. Plant Material

Spinach leaves (*Spinacia oleracea* cv. Amaranthus) were purchased fresh from Pasar Borong Seri Kembangan, Selangor, Malaysia. The leaves were placed in a plastic bag before being transferred to a laboratory in UPM within 20 min. In the laboratory, the spinach leaves were stored at 4 °C. Only spinach leaves with a blade of 8.0 ± 0.5 cm length and 7.0 ± 0.5 cm width and petiole of 1.0 ± 0.1 cm length were selected for experiments. The leaves were subjected to VI treatment within 3 h after purchasing.

2.2. Impregnating Solutions

Salicylic acid (SA) solution of 2 mM (pH 3.11) and γ-aminobutyric acid (GABA) solution of 5 mM (pH 5.94) were prepared based on the most commonly used concentrations for the treatment of fruits and vegetables [10,13]. An isotonic sucrose solution of 0.3 M (pH 6.43) in equilibrium with spinach leaves was designed with respect to the cell sap. The isotonic solution concentration was determined by immersing three spinach leaves in a series of solutions with different concentrations ranging from 0.2 M to 0.6 M [24]. The variation of tissue weight was recorded every hour until equilibrium.

2.3. Vacuum Impregnation

Ten leaves were submerged in a beaker containing the solutions of interest and were immediately introduced to VI process at temperature 25 °C ± 2 °C, which was carried out in a desiccator connected to a vacuum controller (VACUUBRAND GMBH + CO KG, Wertheim, Germany) and a vacuum pump, as described by [24]. Based on preliminary experiments, to establish maximum weight gain and avoid tissue damage, a protocol with

a minimum absolute pressure of 150 mbar is chosen. During the first phase of VI, the pressure was gradually decreased from 1000 mbar to 150 mbar in 16 min and was kept at 150 mbar for 2 min. During the second phase, the vacuum was released and the pressure progressively increased to atmospheric pressure for 30 min and was kept at atmospheric pressure for 15 min. The total treatment time was 63 min and this cycle was repeated twice. After VI process, the excess solution on the surface of the spinach leaves was removed with tissue paper and the weight gain (50% ± 1.5) of each leaf was recorded.

2.4. Sample Preparation

Non-impregnated and impregnated leaves were placed in a closed polypropylene container (10 leaves per container) with saturated humidity and left in darkness at 4 °C ± 0.3 °C for 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 and 7 days. For the analysis of proline and chlorophyll, freeze-drying was carried out using a laboratory freeze-dryer (FreeZone, Labconco, Kansas City, MO, USA) for 3 days. After drying, the leaves were ground to a fine powder with mortar and pestle.

2.4.1. Proline

The proline was extracted from non-impregnated and impregnated spinach leaves powder in 30 g/L sulfosalicylic acid at 100 °C for 10 min with shaking. Then, the extract was centrifuged at 10,000 rpm for 15 min and the supernatant was collected and stored at 4 °C for proline determination. Of supernatant, 2 mL was mixed with 2 mL glacial acetic and 3 mL acid ninhydrin reagent and boiled for 30 min. After 5 min of cooling, the reaction mixture was extracted by 4 mL of toluene and the absorbance of the organic phase was recorded at 520 nm using spectrophotometer (GENESYS 30, Thermo Scientific, Waltham, MA, USA). The proline content was expressed as mg proline per 100 g dry mass. The experiment was done in triplicates for each non-impregnated and impregnated spinach leaves [25].

2.4.2. Chlorophyll

Dried samples of non-impregnated and impregnated spinach leaves were weighed at 0.5 g and homogenized in tissue homogenizer with 10 mL of acetone and centrifuged for 10,000 rpm for 15 min at 4 °C. Of supernatant, 0.5 mL was mixed with 4.5 mL acetone and was analyzed for chlorophyll content using a spectrophotometer (GENESYS 30, Thermo Scientific, Waltham, MA, USA). The absorbance of the solution mixture was taken at 663 nm for chlorophyll a and 645 nm for chlorophyll b [26].

2.4.3. Weight Loss

The weight of non-impregnated and impregnated leaves was recorded before and after storage at 4 °C for every 12 h until 7 days. The weight loss of the spinach leaves was expressed as the percentage (%) [27].

2.4.4. Total Soluble Solids

Non-impregnated and impregnated spinach leaves were blended and 1 mL of the juice was used to determine the total soluble solids using a digital refractometer (PR-201α, ATAGO, Tokyo, Japan). The total soluble solids were expressed as Brix [28].

2.4.5. Titratable Acidity

Non-impregnated and impregnated spinach leaves were blended and 5 mL of the juice was used to determine the titratable acidity by titration with 0.1 M sodium hydroxide. The volume of sodium hydroxide used to reach the endpoint was recorded for the calculation of titratable acidity. The titratable acidity was expressed as the percentage (%) of oxalic acid [28].

2.4.6. Color

Color measurements were performed using a chroma meter (CR-410, Konica Minolta, Chiyoda City, Tokyo, Japan). The illumination area and observer of ϕ50 mm/ϕ53 mm and 2° closely matches CIE 1931 Standard Observer (illuminant D65) were used, respectively. The L*, a*, b* values of the spinach leaves were recorded for non-impregnated and all impregnated leaves every 12 h for 7 days.

2.4.7. Overall Visual Quality

The visual observation of non-impregnated and all impregnated spinach leaves was taken with a camera (Apple iPhone 6 Plus, Apple Inc., Hessen, Germany) for every 12 h for 7 days.

2.4.8. Sensory Evaluation

A total of 60 untrained panelists aged from 22 to 35 corresponding to 25 men and 35 women) were involved in the sensory evaluation. They were required to evaluate the non-impregnated and impregnated spinach leaves of 0, 1, 3, 5 and 7 days in terms of freshness, color, texture, smell and overall appearance. For each category, they were given a 5-scale score. Note that 5 is the highest score, whereas 1 as the lowest. Total score (%) for each spinach were calculated as follow [29]:

$$\frac{\sum(\text{scale} \times \text{no. of panellist})}{5 \times \text{total panellist}} \times 100 \quad (1)$$

2.5. Statistical Analysis

The statistical significance ($p < 0.05$) of the treatments was tested by means of two-way analysis of variance (ANOVA) using Minitab Statistical Software (Minitab 19, LLC, Dayton, OH, USA). The Tukey–Kramer multiple comparison test was used to evaluate true differences in treatment means.

3. Results

3.1. Long Term Metabolic Response: Effects of Different Impregnating Solutions on Proline Content of Spinach Leaves

Our findings (Figure 1a) show that as soon as it reaches 0.5 days after impregnation, GABA impregnated leaves showed a sharp improvement of proline content by 310% (865.6 ± 30.9 mg/100 g), but decreasing steadily afterward and starting to decrease significantly on the 4th day (236.1 ± 5.8 mg/100 g) of storage. Sucrose impregnated leaves recorded an increment of proline content by 97% (409.5 ± 28.1 mg/100 g) on 0.5 days and keep increasing by 214% (652.4 ± 40.0 mg/100 g) on the 2nd day before started to decrease throughout the storage. The increment of proline content was also observed in SA impregnated leaves on 0.5 days after impregnation by 60% (354.2 ± 16.7 mg/100 g) and the highest increment was observed on 1st day of storage by 103% (453.2 ± 49.0 mg/100 g) but started to decrease afterward until the end of storage. The proline content in both GABA and sucrose impregnated leaves were seen to return to its initial value on day 4.5 of storage, whereas in SA impregnated leaves, the proline content gets back to its initial value on day 2 of storage time.

On the other hand, non-impregnated leaves decrease up to 94% of proline content on the 7th day of storage (12.8 ± 2.4 mg/100 g). Statistical analysis shows that the proline content of GABA and sucrose impregnated leaves for all days were significantly different from non-impregnated leaves, but there were no significant differences observed between non-impregnated and SA impregnated leaves.

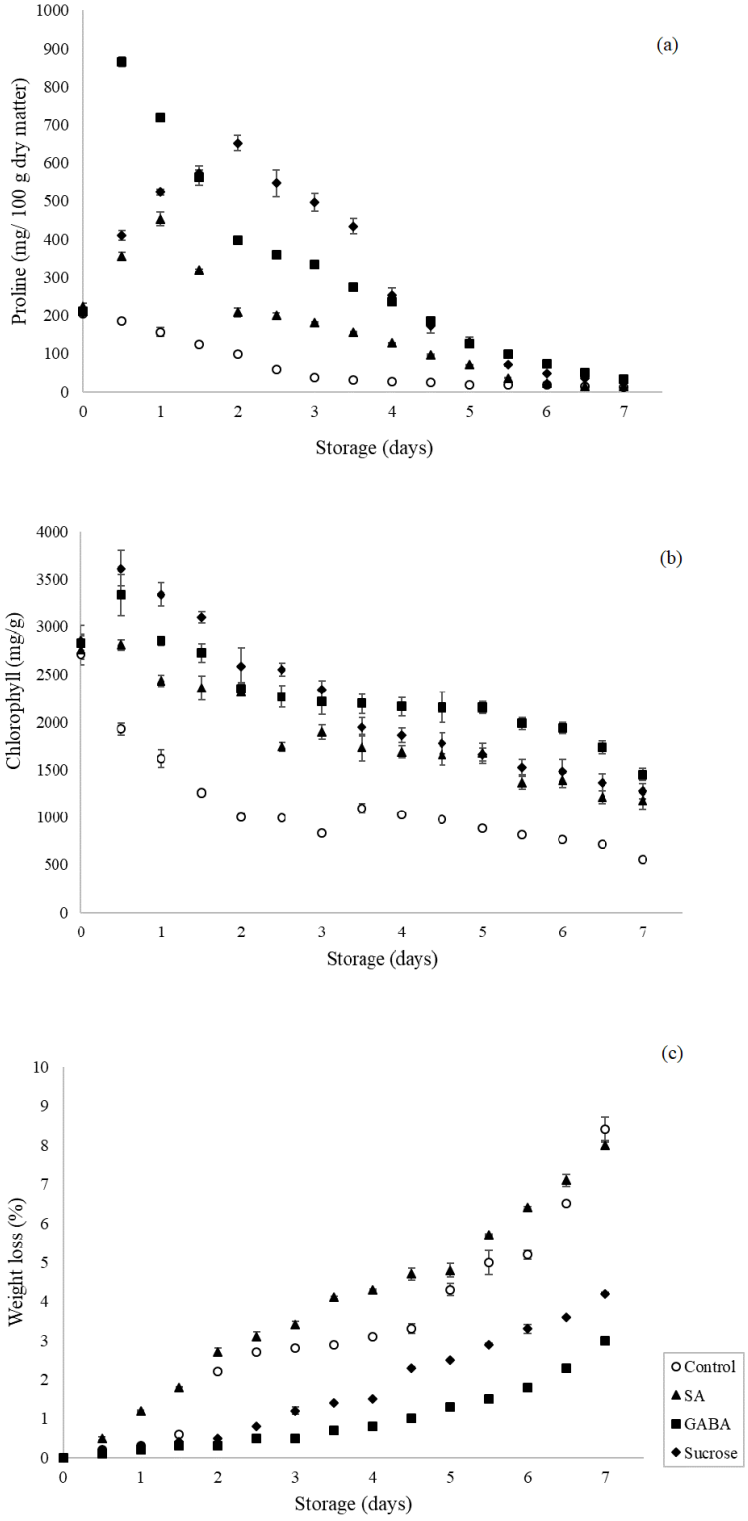

Figure 1. (**a**) Proline content in non-impregnated leaves (○) and leaves vacuum impregnated with 2 mM SA (▲), 5 mM GABA (■) and 0.3 M sucrose (♦) during 7 days at 4 °C. The first point of the impregnated leaves represent the proline content 20 min after VI. (**b**) Chlorophyll content in non-impregnated leaves (○) and leaves vacuum impregnated with 2 mM SA (▲), 5 mM GABA (■) and 0.3 M sucrose (♦) during 7 days at 4 °C. The first point of the impregnated leaves represent the chlorophyll content 20 min after VI. (**c**) Weight loss in non-impregnated leaves (○) and leaves vacuum impregnated with 2 mM SA (▲), 5 mM GABA (■) and 0.3 M sucrose (♦) during 7 days at 4 °C. The first point of the impregnated leaves represent the weight of spinach right after VI. Bars represent ± S.E.

3.2. Long Term Metabolic Response: Effects of Different Iimpregnating Solutions on Chlorophyll Degradation in Spinach Leaves

Impregnation with all three solutions resulted in an increasing in chlorophyll content as compared to non-impregnated leaves. The chlorophyll content of all impregnated leaves for all storage days was significantly different with non-impregnated leaves. Figure 1b shows that an increase of chlorophyll content was observed in leaves impregnated with SA by 2% (2814 ± 200.6 mg/g), GABA by 18% (3332 ± 432.7) and sucrose by 26% (3613 ± 380.8) after 0.5 days of storage and started to reduce gradually throughout 7 days of storage with a significant decrease on the 2nd day. It was observed that the chlorophyll content of SA, GABA and sucrose impregnated leaves started to get back to its initial value on day 1, day 1.5 and day 2, respectively.

At the end of storage, much lower chlorophyll reduction was observed in SA, GABA and sucrose impregnated leaves, which were 57% (1180 ± 155.0 mg/g), 49% (1451 ± 117.0 mg/g) and 55% (1278 ± 148.6 mg/g), respectively, as compared to non-impregnated leaves, which recorded up to 80% (551 ± 23.3 mg/g) loss of chlorophyll on the 7th day of storage.

3.3. Physicochemical Changes: Weight Loss

Figure 1c shows that, after 7 days of storage, non-impregnated leaves showed a 8.4% loss of weight which, was slightly higher (not significantly different) as compared to 8% weight loss in SA impregnated leaves. Meanwhile, both GABA (3%) and sucrose (4.2%) impregnated leaves showed noticeably lower weight loss throughout 7 days of storage time as compared to non-impregnated leaves. Both non-impregnated leaves and all impregnated leaves showed significant weight loss after 3.5 days of storage.

3.4. Physicochemical Changes: Total Soluble Solid

Figure 2a shows that the initial value of total soluble solid recorded by non-impregnated leaves was 8.0 °Brix. Throughout storage time, total soluble solid content in non-impregnated and all impregnated leaves were decreasing gradually, with a significant decrease observed on day 2.5. At the end of storage, sucrose (5.7 °Brix) impregnated leaves showed the highest total soluble solid content followed by non-impregnated (3.7 °Brix) leaves, GABA (3.3 °Brix) and SA (2.7 °Brix) impregnated leaves. Statistical analysis shows that the total soluble solids content of all three impregnated leaves for all storage days are significantly different from non-impregnated leaves.

3.5. Physicochemical Changes: Titratable Acidity

Figure 2b shows that the initial oxalic acid percentage in non-impregnated leaves was 0.09%. After 0.5 days, the non-impregnated and SA impregnated leaves recorded an increment in oxalic acid by 67% and 120%, respectively, while in GABA and sucrose impregnated leaves, the oxalic acid percentage reduced steadily with time. Based on the statistical analysis, there is no significant difference in oxalic acid (%) between storage days. However, the percentage of oxalic acid for all three impregnated leaves is significantly different from non-impregnated leaves.

3.6. Physicochemical Changes: pH Value

Our findings in Figure 2c shows that the same trend of TSS/TA and pH were observed in non-impregnated and all impregnated leaves. Throughout 7 days of storage time, the pH values of non-impregnated and SA impregnated leaves were decreased but increased gradually in GABA and sucrose impregnated leaves. However, there is no significant difference in pH value throughout 7 days of storage of all non-impregnated and impregnated leaves.

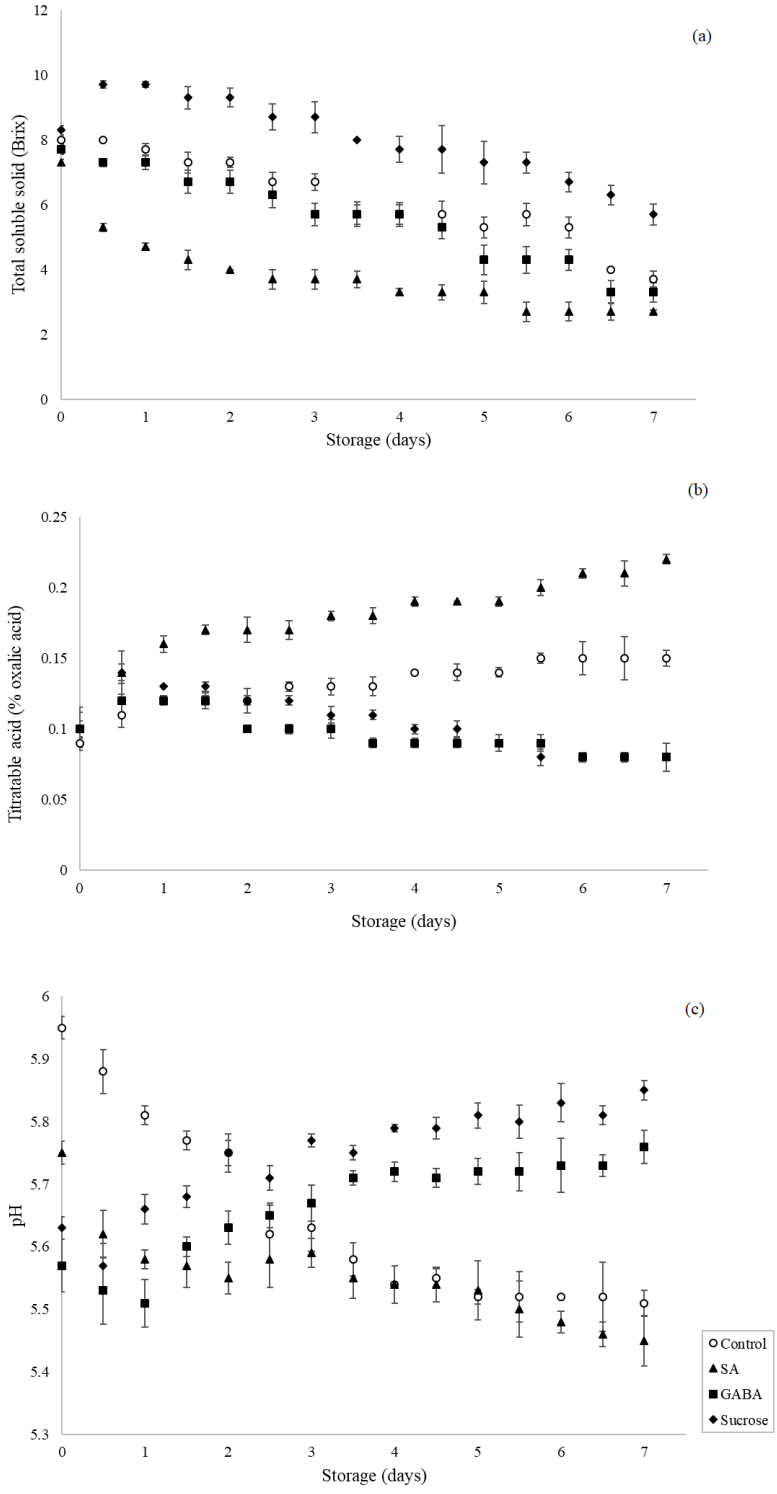

Figure 2. (**a**) Total soluble solid content in non-impregnated leaves (○) and leaves vacuum impregnated with 2 mM SA (▲), 5 mM GABA (■) and 0.3 M sucrose (♦) during 7 days at 4 °C. The first point of the impregnated leaves represent the total soluble solid content 20 min after VI. (**b**) Titratable acid content in non-impregnated leaves (○) and leaves vacuum impregnated with 2 mM SA (▲), 5 mM GABA (■) and 0.3 M sucrose (♦) during 7 days at 4 °C. The first point of the impregnated leaves represent the titratable acid content 20 min after VI. (**c**) pH value of non-impregnated leaves (○) and leaves vacuum impregnated with 2 mM SA (▲), 5 mM GABA (■) and 0.3 M sucrose (♦) during 7 days at 4 °C. The first point of the impregnated leaves represent the pH value of leaves 20 min after VI. Bars represent ± S.E.

3.7. Physicochemical Changes: Colour

Figure 3a and shows the L* (lightness) values for non-impregnated and all impregnated leaves with a significant decrease on day 3 and onwards. However, comparing to non-impregnated leaves, there is no significant difference in L* values for all days of sucrose impregnated leaves but significant difference was observed in GABA and SA impregnated leaves.

For −a* values (Figure 3b) which represent the green color, showed a continuous reduction in non-impregnated and all impregnated leaves throughout storage, particularly in non-impregnated and SA impregnated leaves. A significant decrease was observed starting at day 1 and onward for both non-impregnated leaves and all impregnated leaves. Statistical analysis shows that −a* values of all three impregnated leaves are significantly different with non-impregnated leaves.

Figure 3c shows the b* (yellow) values for non-impregnated and all impregnated leaves showed a continuous increase throughout 7 days of storage time with the most significant increase observed in non-impregnated leaves on day 3.5 and onwards. Statistical comparisons show that all impregnated leaves except for SA impregnated leaves show significant difference in b* values of all storage days with non-impregnated leaves.

3.8. Overall Visual Quality

Figure 4 shows the visual observation of non-impregnated leaves and SA, GABA and sucrose impregnated leaves throughout 7 days of storage in chilling condition. It can be seen that non-impregnated leaves started to show chilling injury symptoms as early as 5 days of storage. However, all impregnated leaves were still in good condition even on the 7th day of storage.

3.9. Sensory Evaluation

The sensory evaluation showed that the freshness, color, texture, odor and overall appearance scores of non-impregnated leaves on day 0 which were the freshly bought spinach leaves were 62.7%, 71.7%, 53%, 69.3% and 62.7%, respectively.

Based on Table 1, the non-impregnated leaves, SA, GABA and sucrose impregnated leaves were significantly different in the category of texture. The non-impregnated leaves were significantly different from GABA and sucrose impregnated leaves in freshness and overall appearance categories whereas, in the category of color, only GABA impregnated leaves showed significant differences with non-impregnated leaves. However, for the odor parameter none of the impregnated leaves recorded significant difference with non-impregnated leaves.

Throughout 7 days of storage time, there was no significant difference in the category of freshness, texture, odor and overall appearance for non-impregnated and all impregnated leaves. The significant difference was observed only in color category between day 0 and day 7.

Nevertheless, on the 7th day of storage time, non-impregnated leaves showed the lowest score for freshness (42%), color (58%), texture (44%) and overall appearance (51%) as compared to the impregnated leaves. Among the impregnated leaves, GABA impregnated leaves showed the highest score for all categories, which are freshness (67%), color (71%), texture (69%), odor (68%) and overall appearance (69%) followed by sucrose and SA impregnated leaves.

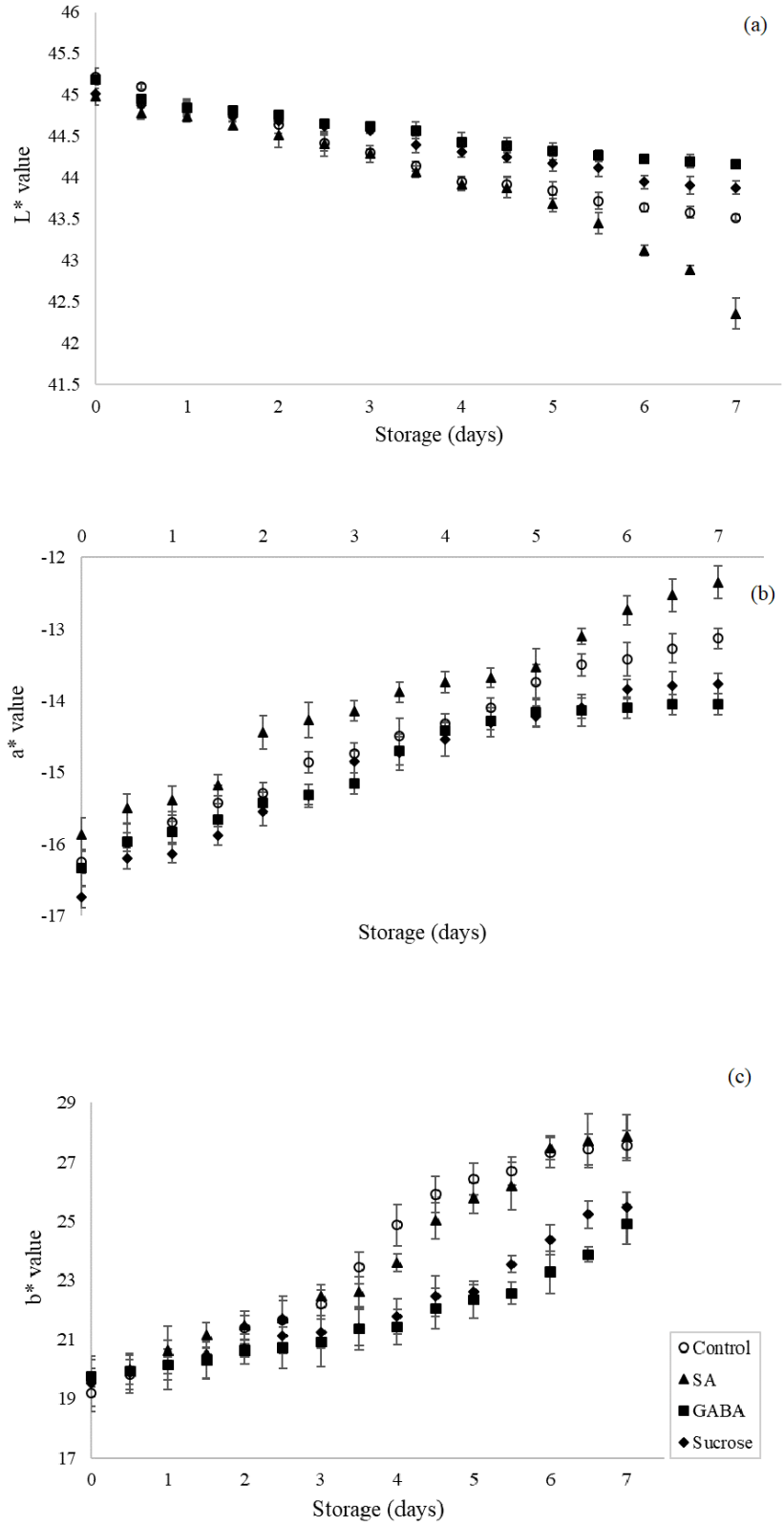

Figure 3. (**a**–**c**) Effect of different impregnating solutions on color changes during storage at 4 °C. Spinach leaves were either non-impregnated (○) or impregnated with 2 mM SA (▲), 5 mM GABA (■) and 0.3 M sucrose (♦) solutions. The leaves were stored for 7 days. Color parameters: (**a**) L* (from 0 black to 100 white), (**b**) a* (from −a* green to +a* red), (**c**) b* (from −b* blue to +b* yellow). Bars represent ± S.E.

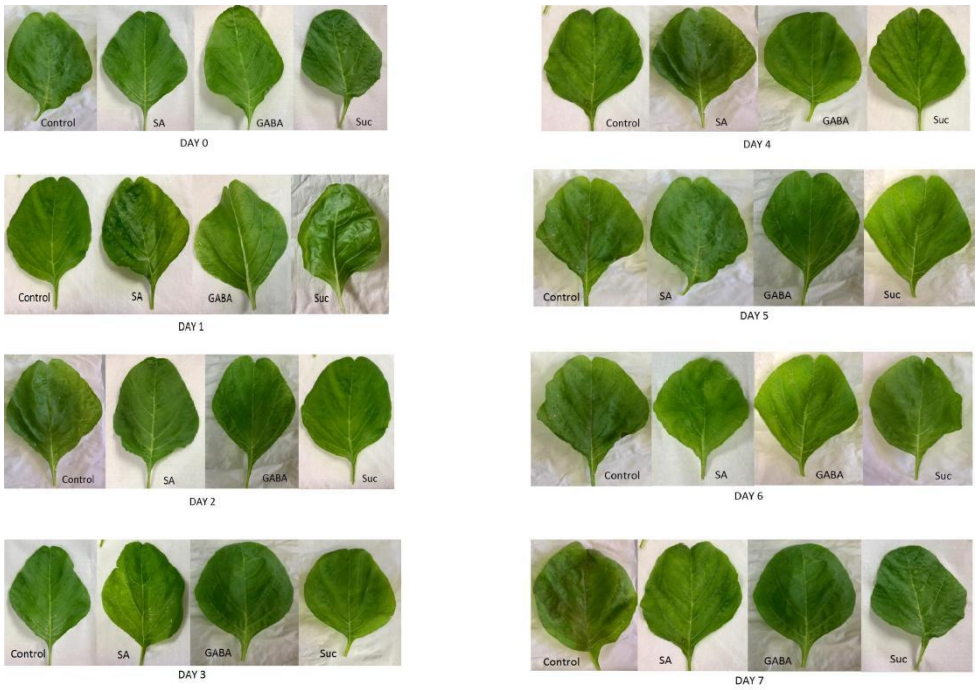

Figure 4. Overall visual quality of non-impregnated leaves and vacuum impregnated leaves with 2 mM SA, 5 mM GABA and 0.3 M sucrose during 7 days at 4 °C. The picture of the impregnated leaves on Day 0 shows the picture of leaves 20 min after impregnation with the solutions.

Table 1. Effect of different impregnating solutions on freshness, color, texture, smell and overall appearance of spinach leaves during storage at 4 °C for 7 days. Spinach leaves were either non-impregnated or impregnated with 2 mM SA, 5 mM GABA and 0.3 M sucrose solutions. The scores were given by 60 panelists. Capital letters represent the significant difference between impregnated compounds and small letters represent the significant difference between storage days.

Days	Leaves	Freshness (%)	Colour (%)	Texture (%)	Odour (%)	Overall Appearance (%)
0	Non-impregnated	62.7 ^{Ba}	71.7 ^{Aa}	53 ^{Aa}	69.3 ^{Aa}	62.7 ^{Aa}
	SA-impregnated	64.7 ^{Aa}	70.7 ^{ABa}	53 ^{Ac}	64.7 ^{Bb}	63 ^{Ab}
	GABA-impregnated	63.3 ^{Ad}	70 ^{Bd}	51.7 ^{Be}	64.7 ^{Bb}	62.3 ^{Ad}
	Sucrose-impregnated	61.7 ^{Bb}	71 ^{Ab}	51.7 ^{Bd}	64.7 ^{Ba}	63.3 ^{Ac}
1	Non-impregnated	57.7 ^{Db}	68 ^{Bb}	54.3 ^{Ca}	68 ^{Aab}	61.7 ^{Ca}
	SA-impregnated	58.7 ^{Cc}	69.7 ^{Aab}	57.3 ^{Bb}	65.3 ^{Bb}	65.3 ^{Ba}
	GABA-impregnated	63.7 ^{Ad}	70 ^{Ad}	66 ^{Ad}	65.3 ^{Bb}	70 ^{Ab}
	Sucrose-impregnated	62 ^{Bb}	70 ^{Ac}	66.3 ^{Ab}	65 ^{Ba}	69.7 ^{Aa}
3	Non-impregnated	53.3 ^{Dc}	65.7 ^{Dc}	51 ^{Db}	66 ^{Bbc}	61.7 ^{Da}
	SA-impregnated	60 ^{Cb}	69.3 ^{Cb}	69.3 ^{Ba}	67.3 ^{Aa}	64.7 ^{Ca}
	GABA-impregnated	64.7 ^{Bc}	73.7 ^{Ab}	68 ^{Cc}	68.3 ^{Aa}	71 ^{Aa}
	Sucrose-impregnated	69.3 ^{Aa}	72.7 ^{Ba}	70 ^{Aa}	65 ^{Ba}	70 ^{Ba}
5	Non-impregnated	53 ^{Dc}	66 ^{Cc}	47 ^{Bc}	66.7 ^{Bc}	58 ^{Db}
	SA-impregnated	59.7 ^{Cb}	70.3 ^{Bab}	68.7 ^{Aa}	68.3 ^{Aa}	64.3 ^{Ca}
	GABA-impregnated	68 ^{Aa}	75.3 ^{Aa}	70 ^{Aa}	68.3 ^{Aa}	71.3 ^{Aa}
	Sucrose-impregnated	62 ^{Bb}	66.7 ^{Cd}	69.7 ^{Aa}	62.3 ^{Bb}	65.7 ^{Bb}
7	Non-impregnated	42 ^{Dd}	58.3 ^{Cd}	44.3 ^{Cd}	64.3 ^{Bd}	51 ^{Dc}
	SA-impregnated	53.3 ^{Cd}	63 ^{Bc}	57.7 ^{Bb}	65.7 ^{Bb}	56.7 ^{Cc}
	GABA-impregnated	67 ^{Ab}	71 ^{Ac}	68.7 ^{Ab}	68.3 ^{Aa}	68.7 ^{Ac}
	Sucrose-impregnated	57.7 ^{Bc}	62 ^{Be}	58.3 ^{Bc}	62.7 ^{Cb}	61 ^{Bd}

4. Discussion

4.1. Effect of VI on Proline Content of Spinach Leaves

Our results clearly show that impregnation of GABA, sucrose and SA into spinach tissue have resulted in the increment of proline by 240%, 153% and 103% respectively on day 1 of storage in chilling temperature (Figure 1a). Amino acid proline is known as one of the major organic osmolytes that accumulate in a variety of plants in response to environmental stresses such as temperatures, drought, salinity, UV radiation and heavy metals [30]. Luo et al. [31] stated that proline defends plants by functioning as a cellular osmotic regulator between cytoplasm and vacuole and by detoxifying of reactive oxygen species (ROS), thus protecting membrane integrity and stabilizing antioxidant enzymes. Therefore, higher proline accumulation can help in reducing the damages resulted from low temperature abuse during cold storage of spinach leaves.

In plants, the precursors for proline biosynthesis are L-glutamic acid and ornithine. Two enzymes involved in the proline biosynthetic pathway (Figure 5) through L-glutamic acid are *pyrroline-5-carboxylate synthetase* (P5CS) and *pyrroline-5-carboxylate reductase* (P5CR). Meanwhile, *ornithine δ-amino-transferase* (OAT) takes part through the ornithine pathway [32].

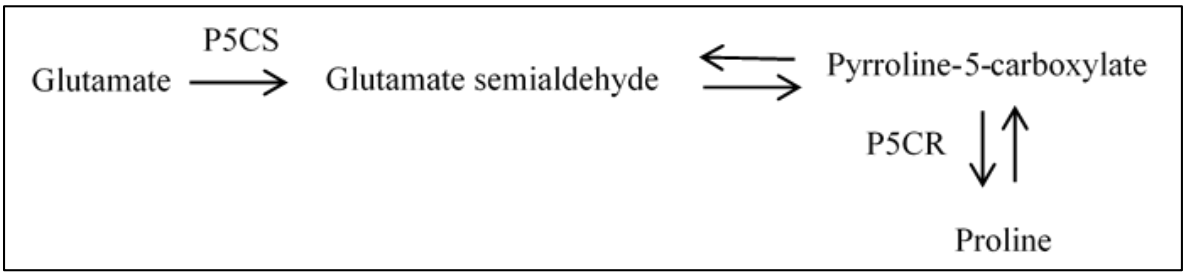

Figure 5. Proline biosynthesis from glutamate [30].

Based on our results, the highest proline content was observed in GABA impregnated leaves, followed by sucrose and SA impregnated leaves. In contrast, the proline content in non-impregnated leaves was decreasing by as much as 94% throughout 7 days of storage. The results were supported by previous research of Shang et al. [32] regarding the effect of exogenous GABA treatment on chilling injury in peach after long term cold storage. It has been reported that immersion of peaches in GABA for 10 min could reduce chilling injury by enhancing the accumulation of proline and also endogenous GABA. The study also mentioned that proline accumulation depends on its degradation, which catalyzed by *proline dehydrogenase* (PDH). Shang et al. [32] also revealed that treatment with GABA increased the activities of P5CS and OAT but decreased PDH activity in peaches under chilling stress. The increase of P5CS and OAT activities could enhance the proline biosynthesis, while the decrease of PDH activity would contribute to the lower degradation of proline in the GABA-treated peaches.

The increment of proline content in sucrose impregnated leaves was supported by previous research of Cao et al. [33]. In this study, it was found that the effect of exogenous sucrose feeding to cucumber seedlings on chilling tolerance and proline content has resulted in higher proline. Ashraf and Foolad [30] explained that proline helps in chilling injury by providing sufficient reducing agents upon relief of stress, which later supports mitochondrial oxidative phosphorylation and generation of ATP for recovery from stress and repairing of stress-induced damages. In addition to proline's role in chilling tolerance, endogenous sucrose also contributes to the mitigation of chilling injury by activating the antioxidant enzymes [33].

Previous researches [9–11] reported that the application of exogenous SA could improve proline accumulation in fruits and vegetables, thus alleviating damages from chilling temperature storage. This was also found in our results where vacuum impregnation

of SA in spinach tissue can increase proline accumulation by 103% as compared to untreated leaves. It is believed that exogenous SA can activate P5CS which triggers proline accumulation [34].

4.2. Effect of VI on Chlorophyll Content of Spinach Leaves

Chlorophyll degradation can cause loss of green color or yellowing in spinach leaves and subsequently decreasing the market value of spinach [35]. Our findings showed that GABA, sucrose and SA impregnated leaves were able to reduce chlorophyll degradation as much as 57%, 55% and 49%, respectively, as compared to the non-impregnated leaves which lost to 80% chlorophyll on 7th day of storage (Figure 1b). Similar findings were also reported by Rezaei-chiyaneh et al. [36], Huang et al. [37] and Xu et al. [22] on the effect of GABA, SA and sucrose, respectively, in chlorophyll degradation. Huang et al. [37] explained that plants suffering from chilling stress can cause membrane damage, ROS generation and toxic compound accumulation, which can lead to a reduction of chlorophyll content, the disintegration of chloroplast membranes, disruption of photosystem biochemical reactions and the reduction of photosynthetic activity. Meanwhile, Xu et al. [22] mentioned that delaying chlorophyll degradation in post-harvest treatment with sucrose might be related to the inhibition of enzyme activities and expression of genes associated with chlorophyll degradation.

Our results from chroma meter show that L* values (Figure 3a) for non-impregnated, GABA and sucrose impregnated leaves were increasing throughout 7 days of storage time. The exemption was made to SA impregnated leaves that shows a decreasing pattern on L* values. Meanwhile, for the $-$a* values (Figure 3b) which indicate the green color, recorded a decreasing trend for non-impregnated and all three impregnated leaves. These values were in line with the chlorophyll content that also decreasing throughout storage. However, a significant decrease was observed in non-impregnated leaves and SA impregnated leaves throughout the storage days. For b* values (Figure 3c) which signify yellow color, non-impregnated and SA impregnated leaves showed higher value than GABA and sucrose impregnated leaves at the end of storage time. These values explained that yellowing of spinach leaves can be delayed by GABA and sucrose treatments as they can also reduce chlorophyll degradation by 49% and 55%, respectively, as compared to 80% reduction in non-impregnated leaves at the end of storage days.

4.3. Effect of VI on Physicochemical Changes of Spinach Leaves: Weight Loss, Total Soluble Solids, Titratable Acidity and pH

Physicochemical changes of the non-impregnated and impregnated spinach leaves were observed as they could be the indicator or symptoms of chilling injury. Weight loss in fruits and vegetables is mainly due to the loss of water caused by transpiration and respiration processes [38]. Based on our results, GABA impregnated leaves recorded the lowest percentage of weight loss after 7 days of storage time with 3%, followed by sucrose (4.2%) and SA (8%) impregnated leaves while higher weight loss was observed in non-impregnated (8.4%) leaves (Figure 1c). Higher loss of water in SA impregnated leaves can be explained by the low solubility of SA in water as compared to GABA and sucrose. According to Nordstrom and Rasmuson [39], the hydroxyl group in SA is hydrogen bonded intramolecularly to the carbonyl oxygen and, thus, reduced intermolecular hydrogen bonding which explains its low solubility in water. Therefore, more non-bonded water is available in SA impregnated leaves as compared to GABA and sucrose impregnated leaves which leads to a higher loss of unbound water.

According to Cavalcanti et al. [40], total soluble solids reflects the sugar content in spinach leaves. Our results show that sucrose impregnated leaves have significantly highest total soluble solid followed by non-impregnated leaves, GABA and SA impregnated leaves (Figure 2a). The high soluble solid content in sucrose impregnated leaves (8 °Brix $\pm$ 1.2) was due to the exogenous sucrose feeding which result in higher sucrose content in the spinach leaves [33]. On the other hand, the average value of titratable acidity of GABA impregnated leaves (0.09% $\pm$ 0.01) recorded the lowest followed by sucrose impregnated leaves (0.1% $\pm$ 0.02), non-impregnated leaves (0.13% $\pm$ 0.02) and SA impregnated leaves

(0.18% ± 0.03) (Figure 2b). The results of TSS/TA are in line with the pH values of the leaves where on the 7th day of storage, sucrose (pH 5.85) recorded the highest pH or least acidic followed by GABA (pH 5.76), non-impregnated (pH 5.51) and SA (pH 5.45) impregnated leaves (Figure 2c). The changes in pH and TA in our findings were due to the administration of SA solution of pH 3.11, GABA solution of pH 5.94 and sucrose solution of pH 6.43. Throughout the storage, SA impregnated leaves and non-impregnated leaves show an increasing pattern in TA while GABA and sucrose impregnated leaves show a decreasing trend. Therefore, the TSS/TA ratio was increased for SA and non-impregnated leaves and was decreased for GABA and sucrose impregnated leaves. The pattern of changes in pH is in line with the changes in TSS/TA ratio. This result is in agreement with Taghipour et al. [41] findings where the pomegranate recorded the same trends in changes of pH and TSS/TA ratio as the result of intermittent warming.

4.4. Sensory Evaluation

Sensory evaluation of spinach leaves with and without impregnation of GABA, SA and sucrose was conducted in order to evaluate the chilling injury of the spinach and to acquire the consumers' preferences. The scores given by the 60 panelists were categorized by freshness, color, texture, odor and overall appearance. For freshness, there is no significant decrease in scores of GABA impregnated leaves stored from day 0 to day 7 at 4 °C. This indicates that spinach leaves impregnated with GABA can maintain their freshness even until 7 days of storage. On the other hand, non-impregnated leaves were given lower scores as compared to sucrose and SA impregnated leaves signifying that sucrose and SA impregnated leaves could improve the freshness of spinach leaves during storage in chilling temperature. For color and texture, spinach leaves impregnated with all three compounds showed higher scores as compared to non-impregnated leaves. These results are in line with both chlorophyll (Figure 1b) and proline contents (Figure 1a) as the green color of spinach leaves reflects the chlorophyll content and lower chlorophyll degradation with the increase of proline content. Thus, the green color is maintained. Meanwhile, for the odor, there were no significant differences in the scores for non-impregnated and impregnated spinach leaves throughout the storage time, as the original form of the impregnated substances did not exhibit any trace of odor upon treatment of the spinach leaves. From this result, it can be proven that leaves impregnated with these three compounds did not give any unpleasant smell of chemicals and was found not significantly different with the non-impregnated leaves. The score for the overall appearance revealed that at the end of storage, GABA impregnated leaves (68.7%) scored the highest followed by sucrose impregnated leaves (61%), SA impregnated leaves (56.7%) and non-impregnated leaves (51%). Through the organoleptic evaluation result, it can be concluded that all compounds used are able to reduce the chilling injury in spinach leaves with GABA being the most effective followed by sucrose and SA.

5. Conclusions

This study explores the metabolic responses of spinach tissue that follows the application of VI with SA, GABA and sucrose for the impregnation of fruit and vegetables. The following are the main results:

1. All of these substances are able to increase the proline content in spinach leaves with increment of 240%, 153% and 103% in GABA, SA and sucrose impregnated leaves respectively. At the same time, these substances are proven to mitigate the chilling injury in spinach leaves based on the results from sensory evaluation which all of the impregnated leaves score better result as compared to non-impregnated leaves.
2. The impregnation of these substances is also able to improve the chlorophyll content, subsequently lowering the chlorophyll degradation in all impregnated leaves as compared to the non-impregnated leaves. Thus, minimizing the chlorophyll degradation has delayed the yellowing of the spinach leaves.

3. Spinach leaves impregnated with GABA and sucrose recorded lower percentage of weight loss which indicates that GABA and sucrose are able to maintain its textural integrity by preventing loss of water from transpiration and respiration in spinach throughout 7 days of storage time.
4. The changes in pH value for all three impregnated leaves were not significantly different as compared to the non-impregnated leaves.
5. The organoleptic evaluation revealed that all compounds used are able to reduce the chilling injury in spinach leaves, with GABA being the most effective followed by sucrose and SA.

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