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Classification of 'Granny Smith' apples with different levels of superficial scald severity based on targeted metabolites and discriminant analysis

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Summary

To study the metabolic changes in 'Granny Smith' apples with different severities of superficial scald, fruit were stored in normal refrigerated air (0 °C, 95% RH) for 12 weeks followed by 7 d shelf-life under room conditions (20 °C, 65% RH). Fruit were graded to five groups based on scald severity and analysed for ethylene, α-farnesene and 6-methyl-5-hepten-2-one (MHO) levels. Reactive oxygen species (ROS) were measured by confocal laser-scanning microscopy on apple peel treated with fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate. Ethylene production rate, α-farnesene and MHO contents and ROS intensity increased with increasing scald severity but declined in severely scalded fruit. Malondialdehyde (MDA) concentration in fruit peel, a measure of membrane damage, increased linearly (R = 0.891) with increase in scald severity. Discriminant analysis was used to classify fruit by scald severity on the basis of metabolites accumulated. The stepwise model indicated that three attributes (ROS, ethylene production and MDA) contributed significantly ($R^2 \geq 0.5$) to the separation of the five scald severity indexes, with ROS having the highest contribution (partial $R^2 = 0.961$; $p < 0.0001$), followed by ethylene ($R^2 = 0.718$; $p < 0.0001$) and MDA ($R^2 = 0.578$; $p < 0.0001$).

Introduction

Superficial scald is the major physiological disorder that develops after long term cold storage of apples and is associated with the disruption of tissues immediately beneath the epidermis of the fruit, with tissue browning not extending to the mesocarp of pulp (Bain, 1956; Sabban-Amin et al., 2011; Lurie and Watkins, 2012). Array of factors such as cultivar, seasonality, maturity, and duration of cold storage significantly influence scald development (Rao et al., 1998; Watkins et al., 2000; Ahn et al., 2007). Some apple cultivars such as 'Granny Smith' and 'Law Rome' are more susceptible (Watkins et al., 1995; Pechous and Whitaker, 2004; Jemric et al., 2006; Sabban-Amin et al., 2011) whilst cultivars such as 'Golden Delicious' and 'Royal Gala' are resistant (Ingle, 2001; Zanella et al., 2008; Beuning et al., 2010).

In particular, fruit maturity plays an important role in scald susceptibility, with scald incidence declining with advancing fruit maturity (Erkan and Perkmezci, 2004). There are physiological changes preceding superficial scald development. Generation of reactive oxygen species or oxidative stress coupled with α-farnesene synthesis is one of the primary events leading to scald symptoms (Rudell et al., 2009). Moreover, conjugated trienols (CT) and 6-methyl-5-hepten-2-one (MHO), both oxidised products of α-farnesene, increase with scald incidence (Mir et al., 1999; Rowan et al., 2001; Moggia et al., 2010). Although concerted research efforts have been made to understand metabolic events leading to scald development, the relationship between scald severity and accumulation of metabolites linked to scald development remains unclear. The objective of this research work was to investigate the relationship between scald severity and metabolic changes in 'Granny Smith' apples.

Materials and methods

Fruit source and treatments

Seventeen-year old 'Granny Smith' apple trees grafted into M109 rootstock grown on a commercial orchard in Grabouw (34° 12'12" S, 19° 02'35" E) were used in this study. The tree spacing was 4 × 1.5 meters giving a total of 1667 trees per hectare. All the trees were pruned to a central leader and irrigated by micro sprinklers. Fruit were hand-picked at optimal maturity, and transported to the Research Laboratory at Agricultural Research Council, Stellenbosch and sorted to remove fruit with physical defects. The experiment was laid in a completely randomised design. Uniformly sized fruit with diameter of 70±2 mm and mass of 160±5 g were randomly selected to provide three replications of 100 fruit each. Fruit was placed in slotted and stacked high-density polyethylene plastic crates. Fruit were stored in regular atmosphere (RA) at 0 °C (95% RH) for 12 weeks followed by 7 days shelf-life at normal room conditions (20 °C, 65% RH). At the end of shelf life, fruit were individually assessed and rated for scald severity based on the percentage of the surface area affected and sorted into five groups based on as follows: 0 = no scald, 1 = 1-25% (slight), 2 = 26-50% (moderate), 3 = 51-75% (high), and 4 = 76-100% (very high) (Fig. 1). For each level of scald severity, six replicates with five fruit per replicate were used for analysis.

Ethylene production

Ethylene production was measured as described by Oz (2011) with slight modifications. Briefly, each fruit was weighed using a Mettler Toledo digital balance (±0.01g), and thereafter enclosed in 1 L airtight jar for 1 h at 20 °C. Infrared ethylene analyser (ICA56 ppm) was used for measurement and the results were expressed as $\mu L kg^{-1} h^{-1}$.

Headspace volatile analysis

Fresh peel (5 g) was weighed into a 20 mL solid phase microextraction (SMPE) glass vials. 10 µL of 3-octanol internal standard was added, and the vials were sealed. Vial headspace was analysed according to Mayuoni-Kirshinbaum et al. (2012) and Caleb et al. (2013). The vials were equilibrated for 10 min at 50 °C in the CTC autosampler incubator. After equilibration, a 50/30 µm divinylbenzene/-carboxen/-polydimethylsiloxane coated fibre was

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Fig. 1: 'Granny Smith' apples with different superficial scald severity: 0, no scald; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%.

then exposed to the sample headspace for 20 min at 50 °C. After extraction, the trapped volatile compounds from the fibre coating were desorbed for 2 minutes in the injection port of the gas chromatograph operated in a splitless mode. The temperature was maintained at 250 °C for the injection. The fibre was cleaned after each sample heating for 10 min in the fibre conditioned station maintained at 270 °C. Chromatographic separation of the extracted volatiles was performed on a Agilent 6890N (Agilent, Palo Alto, CA) connected through a transfer line to a Agilent 5975B MS (Agilent, Palo Alto, CA) mass spectrometer detector. The GC-MS system was equipped with a polar DB-FFAP column from J&W (part number 122-3263) with the following dimensions: (60 m length; 250 μm internal diameter; and 0.5 μm film thickness). Helium was used as carrier gas at a flow rate of 1.3 mL min^{-1}. The oven temperature program was as follows: initial temperature of 40 °C for 5 minutes; then ramped at 5 °C min^{-1} up to a final temperature of 230 °C with a final hold time of 6 minutes. The ion source and quadropole were maintained at 240 °C and 150 °C, respectively. The transfer line temperature was maintained at 280 °C. Alpha-farnesene and MHO were identified by a library search and quantified using abundance characteristic ion 93 and 108, respectively. Generally, α-farnesene gave a single peak at 22.2 min while MHO gave a peak at 16.6 min. A reading of 10^4 in abundance was defined as one unit and expressed as U g^{-1} (Ju and Curry, 2000).

Confocal microscopic analyses of ROS production

Reactive oxygen species were determined following a method described by Macarisin et al. (2007) and Sabban-Amin et al. (2011). The fluorescent probe 2,7-dichlorodihydrofluorescein diacetate in which dichlorodihydrofluorescein (DCF) fluorescence measurement quantifies general oxidative stress was used. 2,7-dichlorodihydrofluorescein enters cells in the diacetate form (H_2DCF-DA), and the acetate form (H_2DCF) is hydrolyzed by intracellular esterases and then reacts with oxidants, resulting in the highly fluorescent DCF. Acetate detects a broad range of oxidizing molecules rather than a single ROS form, and it is efficient in localizing ROS within plant cells (Joo et al., 2005). Immediately before microscopic analysis, slices of apple peel were cut from fruit and immediately immersed in a small Petri dish containing 10 mL of 10.0 μM H_2DCF-DA in loading buffer (50 mM MES buffer, pH 6.5). The H_2DCF-DA was freshly prepared from a 20 mM stock solution in dimethyl sulfoxide (DMSO). To prevent light-inducible oxidation, the slices were kept in the dark for 10 min and were thereafter transferred to a new Petri dish containing loading buffer to wash off excess dye. Model IX 81 inverted confocal laser-scanning microscope (FLUOVIEW 500, Olympus, Japan) equipped with a 488 nm argonion laser was used for sample examination and image acquisition. The fluorescent probe was excited with a 488 nm laser beam and the emission was collected through a BA 515-525 filter. For autofluorescence, a BA 660 IF emission filter was used. Magnification was increased by focusing the scanning laser beam onto a smaller area of the tissue. The transmitted-light images were obtained with Nomarski differential interference contrast (DIC) optics. The relative intensity of the fluorescence signal was estimated by calculating average pixel intensity from each successive focal plane of the apple peel slice, in 5 μm steps, with MICA software (Multi-Image Analysis, CytoView, Israel). The value of fluorescence intensity presented is the mean (± standard error).

Lipid peroxidation

MDA was measured by the method described by Dhindsa et al. (1981) and Siboza et al. (2013) with slight modifications. Freeze dried and pulverised apple peel (0.1 g) was homogenised with 10 mL of ice cold 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10000 rpm for 15 min at 4 °C to precipitate particulates. A 1 mL aliquot of the supernatant was thoroughly mixed with 4 mL of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was incubated at 95 °C for 30 min and thereafter quickly cooled in an ice bath. After centrifugation at 10000 for 15 min at 4 °C, the absorbance of the supernatant was read at 532 nm and corrected for nonspecific absorbance at 600 nm using UV-Visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). The concentration of MDA was calculated using an extinction coefficient (∈) of 155 mM^{-1} cm^{-1}.

Statistical analysis

Data was subjected to Statistica 11 (StatSoft Inc. Oklahoma, USA) for analysis of variance (ANOVA) according to Duncan's multiple range test. Graphical data presentations were performed using GraphPad Prism software, ver. 6 (GraphPad Software, Inc. San Diego, USA). Discriminant analysis (DA) was performed using XLStat, ver. 7.5.2 (Addinsoft, New York, USA).

Results and discussion

Ethylene

Ethylene production has an influence on physiological changes associated with superficial scald (Ingle, 2001). Several reports have shown that ethylene is involved in regulating α-farnesene, a key volatile involved in the induction of superficial scald in apples (Mir et al., 1999; Ju and Curry, 2000; Pechous et al., 2005). In this study, ethylene production increased gradually as scald severity increased from none (0) to slight (2), and declined as scald severity progressed (Fig. 2). Reduced ethylene production with increasing scald severity could be an indication of its completed role in scald etiology. This observation is in agreement with previous findings by Mir and Beaudry (1999), who demonstrated that ethylene production increases with scald-development but reduces in severely scalded fruit. A similar trend has also been reported for 'Bartlett' pears stored at -1 °C for 24 weeks (Ekman et al., 2004; Whitaker et al., 2009).

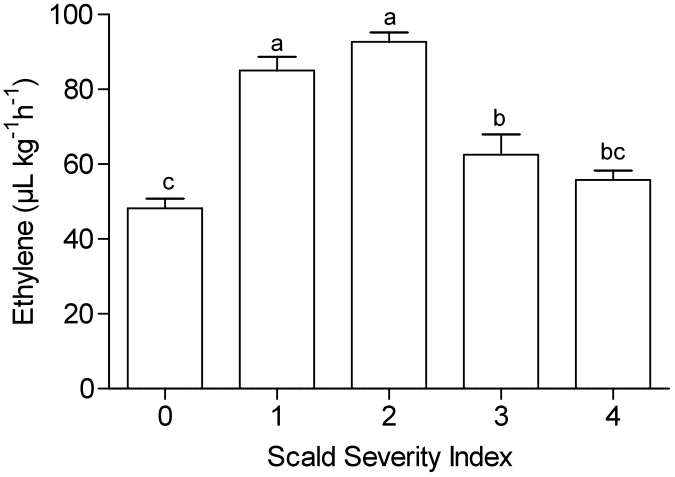

Fig. 2: Ethylene production in 'Granny Smith' apples with different scald severity. The data are the means ± standard error (SE) of six replicates with five fruits per replicate. Different letter(s) on the bars mean statistical differences according to Duncan's multiple range test ($p<0.05$).

Headspace volatile analysis

Scald development has been strongly associated with α-farnesene accumulation in apple peel (Pesis et al., 2009). In this study, headspace levels of α-farnesene increased with scald severity (Tab. 1) but declined thereafter when fruit became highly scalded. This result is in agreement with previous findings which demonstrated that α-farnesene increased with scald severity in 'Law Rome' apples (Watkins et al., 2000) and 'Granny Smith' apples (Shaham et al., 2003; Zubini et al., 2007) stored at 0 °C for up to 26 weeks. As noted in this study, Zubini et al. (2007) also reported a decline in α-farnesene levels in severely scalded fruit.

Interestingly, Gapper et al. (2006) reported a similar trend for 'd' Anjou' pears and concluded that *AFS1* transcript is mediated by ethylene.

MHO (a product of α-farnesene oxidation) was significantly lower in fruit with no scald and increased with the onset of scalding and up moderate severity however, after severity level 2 (26-50 %), there was a decline in MHO content (Tab. 1). Our findings are corroborated by those reported in 'Granny Smith' apples by several researchers (Fan et al., 1999; Mir et al., 1999; Wang and Dilley, 2000) who demonstrated an initial increase in MHO content with onset of scald and a subsequent decline as scald incidence and severity increased. It is worth noting that the reduction in both α-farnesene and MHO levels coincided with a decline in ethylene production. These results suggest that both the production and oxidation of α-farnesene may require ethylene action. In fact, previous studies focused on reducing superficial scald in apples reported a reduced α-farnesene and MHO production after 1-MCP treatment, and consequently low scald incidence (Ghahramani and Scott, 1998; Fan and Mattheis, 1999; Shaham et al., 2003; Gapper et al., 2006; Jung and Watkins, 2008). Recently, Gao et al. (2015) has also demonstrated that 1-MCP reduces superficial scald development in 'Wujiuxiang' pears by retarding α-farnesene and CTols accumulation. Mir et al. (1999) indicated that the temporary relationship between scald severity and MHO may indicate that MHO is not directly involved in scald development. Contrary to the prevailing hypothesis linking MHO and superficial scald, Rupasinghe et al. (2000) reported that methyl heptenol (MHOL) in 'Delicious' apples stored at 0 °C for 17 weeks was 60 % and 20 % higher in scald-developing and severely scalded tissues, respectively. Other research findings have concluded that MHO production rather than its presence, is the important aspect involved in scald appearance (Ju and Curry, 2002; Lurie and Watkins, 2012).

Tab. 1: Headspace accumulation of α-farnesene and MHO in 'Granny Smith' apples with different levels of scald severity. The data are the means ± SE of three replicates with five fruits per replicate. Means in each column followed by different letter(s) differ significantly according to Duncan's multiple range test ($p<0.05$).

Superficial scald severity	α-farnesene (U g^{-1})	MHO (U g^{-1})
0	6612.09±577.18 c	56.18±6.58 c
1	11216.05±588.63 b	90.17±4.04 b
2	15286.12±355.12 a	132.04±6.38 a
3	11716.10±462.87 ab	110.01±4.99 b
4	8940.15±304.31 bc	84.13±1.46 bc

ROS detection and quantification

Low storage temperatures trigger plant tissues to produce reactive oxygen species (ROS) which are the by-products of electron flow disruption in the mitochondria (Purvis et al., 1995; Pinhero et al., 1997) resulting in physiological disorders such as chilling injury (Lyons, 1973; Sala, 1998) and superficial scald (Watkins, 1995; Sabban-Amin et al., 2011). Physiologically, ROS cause the oxidative stress that consequently results in imbalances in metabolism, high respiration rate, reduced ability of biological systems to detoxify toxic metabolites (Lyons, 1973). The fluorescence appearing during cold storage and shelf-life was quantified as fluorescence units related to ROS levels (Sabban-Amin et al., 2011; Pesis et al., 2012). In the current study, fluorescence intensity significantly increased with scald severity (Fig. 4A-E). Similarly, ROS levels increased with scald severity (Fig. 3); however, low fluorescence and ROS levels were detected in severely scalded fruit (Fig. 4E).

These results have demonstrated that ROS play a role in superficial scald development in 'Granny Smith' apples. This observation is consistent with previous findings that ROS are involved in scald etiology. For instance, Rao et al. (1998) found scald incidence to be related to ROS levels in hybrid 'White Angel × Rome Beauty' apple stored for 16 weeks at 0.5 °C. Moreover, hydrogen peroxide (H_2O_2) was reported by Zubini et al. (2007) to increase with scald incidence and severity in 'Granny Smith' apples. Recently, Lu et al. (2014) also reported scald severity to be highly dependent on the

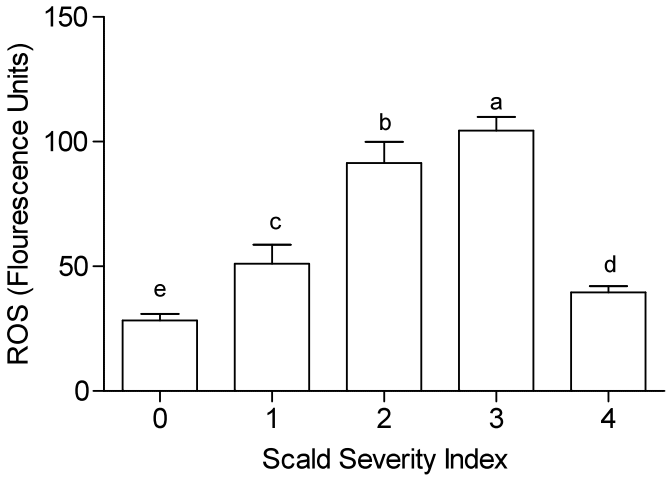

Fig. 3: Production of reactive oxygen species (ROS), as quantified by relative intensity of dichlorodihydrofluorescein diacetate (DCF) fluorescence in peel slices of 'Granny Smith' apples with different scald severity. The data are the means ± standard error (SE) of six replicates with five fruits per replicate. Different letter(s) on the bars mean statistical differences according to Duncan's multiple range test ($p<0.05$).

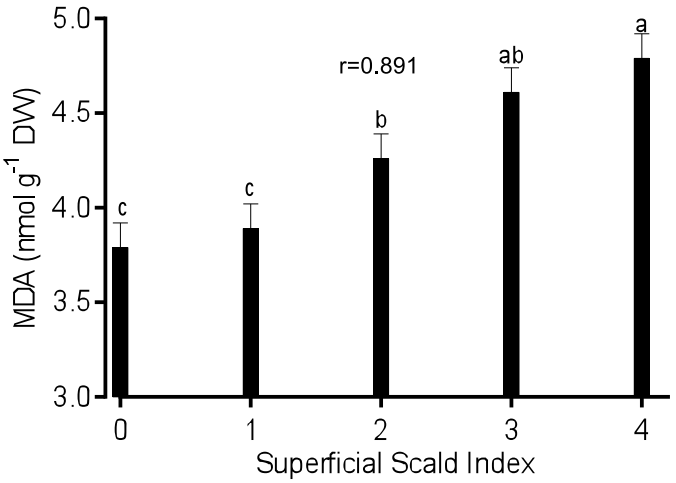

Fig. 5: Changes in malondialdehyde (MDA) concentration of 'Granny Smith' apple peel slices as influenced by scald severity. The data are the means ± standard error (SE) of six replicates with five fruits per replicate. Different letter(s) on the bars mean statistical differences according to Duncan's multiple range test ($p<0.05$).

accumulation of H_2O_2 concentration in 'Fuji' apples stored at 0 °C for 28 weeks. Our study shows that the accumulation of ROS is linked to scald severity, and the low ROS levels in high to severely scalded fruit could be linked to reduced reactivity of oxygen species, and hence the corresponding reductions in metabolites implicated in scald development and severity such as α-farnesene and MHO.

Lipid peroxidation

Malondialdehyde (MDA) is regarded to be a suitable biomarker for lipid peroxidation caused by ROS which is the major cause of membrane damage in plant tissues (KATSUHARA et al., 2005; LU et al., 2014). Unless metabolised, ROS cause lipid peroxidation and eventual symptoms of damage in plant tissues. Peroxidation of membrane lipids is an indication of membrane damage and electrolyte leakage under cold stress (KATSUHARA et al., 2005). Lipid peroxidation leads to membrane damage, and consequently chilling injury symptoms (LYONS, 1973). Membrane damage is the primary metabolism disorder preceding superficial scald in apples (RAO et al., 1998). In this study, lipid peroxidation expressed as MDA concentration (Fig. 5) had a significant effect on scald severity. The MDA was significantly lower in fruit with scald severity index of 0 and 1. However, lipid peroxidation gradually increased with scald severity. This result is in agreement with previous findings showing the accumulation of MDA in scalded apples. For instance, RAO et al. (1998) reported that lipid peroxidation increases with storage time and consequently scald severity in 'White Angel × Rome Beauty' apple. LU et al. (2014) also noted that MDA content increases with scald incidence and severity in 'Fuji' apples stored at 0 °C for 28 weeks. MOGGIA et al. (2010) reported increased membrane integrity in severely scalded 'Granny Smith' apples for 6 months at 0 °C. THOMAI et al. (1998) also demonstrated that membrane damage increases with scald incidence and severity. The low lipid peroxidation in fruit with no scald proves the relationship between scald incidence and membrane damage. Moreover, the continuous increase in lipid peroxidation indicates that superficial scald is not only a change in symptoms but also an accumulative damage (LU et al., 2011; GAO et al., 2015).

Discriminant analysis

Outcome of discriminant analysis of scald severity index and metabolic attributes is presented in Fig. 6. Confusion matrix showing the correct and incorrect predictions made by the model are presented in Tab. 2. The confusion matrix indicated 96.76% accuracy in classifying the five scald severity classes. Four indexes were particularly well discriminated with 100% accuracy; however, 16.67% confusion appeared in severely scalded fruit. The confusion between

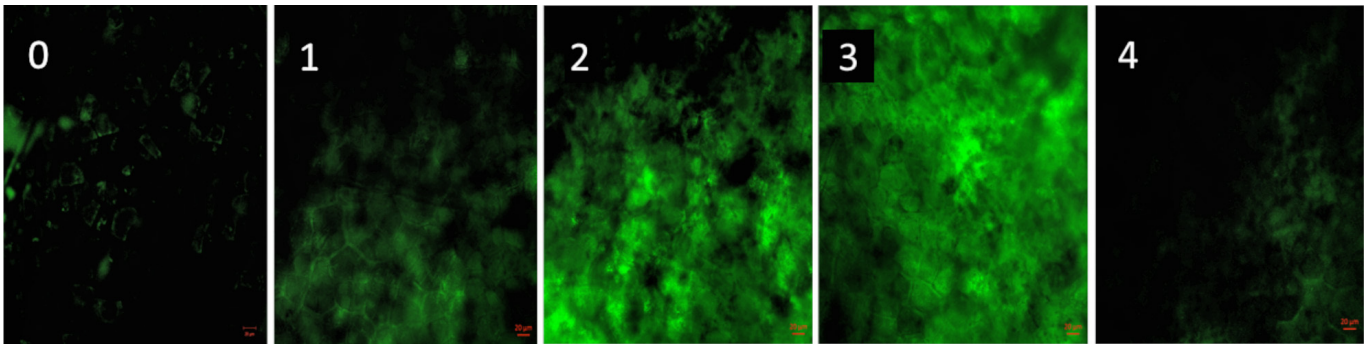

Fig. 4: Confocal laser-scanning fluorescence images of 'Granny Smith' apple peel slices. Superficial scald index 0, no scald; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%.

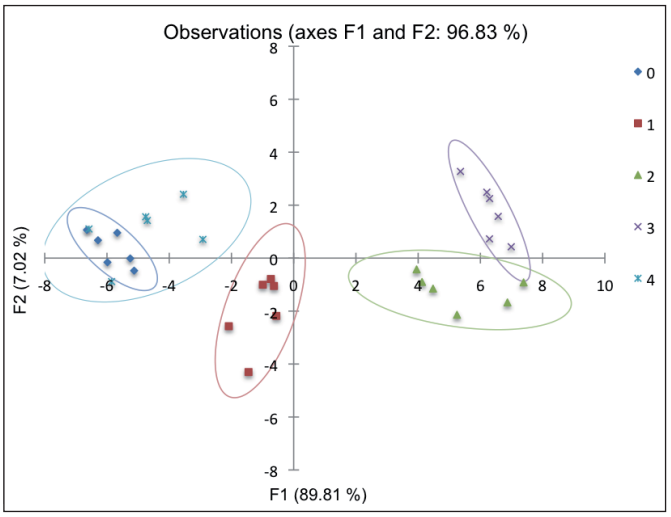

Fig. 6: Discriminant analysis (DA) observations chart of 'Granny Smith' apples with different scald severity: 0, no scald; 1, 1-25 %; 2, 26-50 %; 3, 51-75 %; 4, 76-100 %.

Tab. 2: Confusion matrix of 'Granny Smith' apples with different levels of superficial scald severity

Superficial Scald Index	0	1	2	3	4	Total	% correct
0	6	0	0	0	0	6	100.00
1	0	6	0	0	0	6	100.00
2	0	0	6	0	0	6	100.00
3	0	0	0	6	0	6	100.00
4	1	0	0	0	5	6	83.33
Total	7	6	6	6	5	30	96.67%

scald index 0 and 4 could be explained by the fact that the tissue in severely scalded fruit was almost dead, and this presumably reduced metabolism hence the similarity in accumulated metabolites between these two groups. The stepwise model indicated that three attributes, namely; ROS, ethylene production and MDA contributed significantly ($R^2 \geq 0.5$) to the separation of the five scald severity indexes (Tab. 3). Amongst the contributors, ROS had the highest significant ($p < 0.0001$) contribution with $R^2 = 0.961$, suggesting that ROS could indeed be strongly linked to scald severity in 'Granny Smith' apples. This result is in agreement with Rao et al. (1998), who indicated that although scald development mechanism is yet to be fully understood, the contribution of ROS maybe related to the disorder.

Conclusion

This study has demonstrated that scald severity is not directly related to some scald-associated metabolites in 'Granny Smith' apples. While increases in ethylene production, α-farnesene and MHO corresponded with the onset and progression of scald severity, this relationship did not hold in high to severely affected fruit. However, the accumulation of ROS leading to loss of membrane integrity corresponded strongly to the level of scald severity with the exception of severely scalded fruit which showed lower ROS levels.

Tab. 3: Summary of variable selection using stepwise analysis showing attributes that contribute most to superficial scald severity of 'Granny Smith' apples

Variable	Status	Partial R^2	F statistic	Pr > F
ROS	IN	0.961	153.506	<0.0001
Ethylene	IN	0.718	15.306	<0.0001
MDA	IN	0.578	7.887	<0.0001

ROS, reactive oxygen species; MDA, malondialdehyde.
Partial R^2 – determination coefficient; F statistic – F ratio test; Pr > F – p value at significance level of 0.05.

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