Pattern of phenolic content, antioxidant activity and senescence-related enzymes in granulated vs non-granulated juice-sacs of ‘Kinnow’ mandarin (*Citrus nobilis* x *C. deliciosa*)

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Abstract ‘Kinnow’ is a hybrid mandarin, developed at California (USA) but could not become successful there. However, it revolutionized citrus industry in India, Pakistan and Bangladesh. Recent reports indicate that like other citrus fruits, it also suffers from juice-sac granulation but exact cause of this malady is not known. Fully-mature ‘Kinnow’ fruits were harvested and observations on some physical and biochemical attributes were recorded and their relationship was established with occurrence of granulation. About 12.8 % ‘Kinnow’ fruits were affected by juice-sac granulation. Granulated fruits had higher average weight (178 ± 2.26 g), peel thickness (3.72 ± 0.23 mm), and less soluble solids concentrates (7.4 ± 0.21 %) than non-granulated fruits. Granulated fruits exhibited lower concentrations of total phenolics compounds (4.3 ± 0.56 mg 100^{-1} g gallic acid equivalent fresh weight) and antioxidants activity (1.78 ± 0.29 μ mol Trolox g^{-1} FW) but produced higher rates of carbon dioxide and ethylene, and exhibited higher activities of senescent-related enzymes such as lipoxygenase (LOX) (1.3 ± 0.31 μ moles $min^{-1} g^{-1}$ FW) and pectin methylesterase (PME) (0.52 ± 0.12 μ mol of NaOH g^{-1} FW min^{-1}) and had strong relationships with the occurrence of granulation. From this study, it can be concluded that total phenolics compounds, antioxidants and PAL enzyme activity have strongly negative co-relation; whereas, senescent-related enzymes such as LOX, and PME and rates of respiration or ethylene evolution have

strongly positive relationships with the occurrence of granulation in ‘Kinnow’ mandarin.

Keywords Granulation · Antioxidant activity · Phenyl ammonia lyase · Lipoxygenase · Pectin methylesterase

Introduction

Citrus is considered as the second most important group of fruits in the World, which includes fruits such as oranges, mandarins, grapefruits, pummelos, tangerines, tangors, citranges etc. (FAOSTAT 2014). Under each sub-group of citrus fruits, several varieties and/or hybrids have been developed in the world. Under mandarin group, a tight-skinned hybrid, ‘Kinnow’ was developed by Dr. H.B. Frost at California, the USA in 1935 by crossing ‘King’ (*Citrus nobilis*) and Willow Leaf (*C. deliciosa*) mandarins. ‘Kinnow’ mandarin could not become very successful in the USA. However, it was introduced by different citrus growing countries of the World and it revolutionized citrus industry in India, Pakistan and Bangladesh (Sharma and Saxena 2004; Sharma et al. 2006; Jhalegar et al. 2015). Now it occupies a place of eminence and contributes as a major share in the area and production of citrus grown in India. It has replaced sweet orange in several parts of India and had become a major citrus fruit because of its better adaption under semi-arid climatic conditions, where other citrus varieties have failed. Besides, its also have attractive orange coloured fruits, high juice content and better fruit quality than other citrus fruits (Ladanyia 2008; Singh 2001).

Citrus fruits suffer from several physiological disorders, of which juice-sac granulation is of serious nature. In this disorder juice sacs become hard, dry, get enlarged, and assume a grayish colour (Ladanyia 2008; Singh 2001). It is known by

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different names in different countries such as vesicle drying, *kaosan* and crystallisation, but the term granulation is commonly used throughout the World (Ladanyia 2008; Sharma and Saxena 2004; Singh 2001). Almost all citrus types suffer from the problem of granulation of juice-sacs, but sweet oranges and mandarins are worst-affected (Ladanyia 2008; Sharma and Saxena 2004; Singh 2001). Although, most reports have indicated that ‘Kinnow’ mandarin is free from juice-sac granulation, but Singh (1977) and later Sharma and Saxena (2004) and Sharma et al. (2006) have reported that like other several mandarins, ‘Kinnow’ is also affected by this disorder to variable extent.

Several researchers have worked on causes of this malady in many citrus growing countries of the World, but its causes have not yet been fully understood. However, several factors, e.g., frequent irrigation, higher application rates of nitrogenous fertilizers, luxuriant growth following heavy pruning or fertilization, delayed harvesting, etc., have been reported to be associated with this malady (Ladanyia 2008; Sharma and Saxena 2004; Singh 1977, 2001). Similarly, some climatic factors, rootstock, fruit size, tree age, etc., are also known to accentuate the problem of granulation (Al-Hosni et al. 2011; Sharma and Saxena 2004; Singh 2001). Furthermore, some investigators have also reported the association of enzymes such as peroxidase (Awasthi and Nauriyal 1972a, b) and superoxide dismutase (SOD) with citrus granulation (Chen et al. 1994). However, attempts have not been made to establish relationships between granulation and senescence-related enzymes. Thus, the primary aim of this study was to determine phenolic content pattern and activities of senescence-related enzymes such as lipoxigenase, pectin methylesterase or phenyl ammonia lyase and to derive their relationships with the occurrence of juice-sac granulation in ‘Kinnow’ mandarin.

Material and methods

Experimental site and fruit material

The research was carried out at the Division of Food Science and Postharvest Technology, Indian Agricultural Research Institute (I.A.R.I.), New Delhi 110 012, India in the fruiting season of 2013–14. Two-hundred fully mature ‘Kinnow’ fruits were randomly harvested from uniformly maintained 6-year-old trees budded on *Jatti Khatti* (*Citrus jambhiri*) rootstock, from the orchard of Division of Fruits and Horticultural Technology, I.A.R.I., New Delhi-12. The harvested fruits were washed with tap water and dried under fan in a laboratory, divided into four lots of 50-each and then stored under ambient conditions in the laboratory.

From each lot, ten fruits were randomly selected for taking observations on the fruit weight, peel thickness and respiration rate and ethylene evolution rate and fruit quality attributes

such as soluble solids concentrates (SSC), ascorbic acid contents (ASC), total phenolic contents (TPC), antioxidant (AOX) activity and enzymes such as phenyl ammonia lyase (PAL), lipoxigenase (LOX) and pectin methyl esterase (PME) were determined.

Incidence and degree of granulation

The incidence of granulation was expressed as the percentage of the fruit affected by the disorder. The degree of granulation was based on the estimate of the portion of the fruit affected, which was classified as zero, below 10, 10–25, 25–50, 50–75 and 75–100 % (Singh 1977). All the fruits of each lot (50) in each replication were used to study the extent and degree of granulation. For this, the fruits were first peeled and their finger removed. Then, individual finger was hand-peeled for observing the percentage of portion affected by granulation (Sharma and Saxena 2004; Sharma et al. 2006).

Determination of the rates of respiration and ethylene evolution

The rates of ethylene production and respiration were measured using the static headspace technique (Sharma et al. 2012a) using a gas analyser (Checkmate 9900 O₂/CO₂; PBI Dansensor, Ringsted, Denmark) and was expressed in ml CO₂ kg⁻¹ FW fruit h⁻¹. To determine ethylene contents, 1.0 ml of headspace gas was withdrawn using a gas-tight syringe and injected into a gas chromatograph (HP 5890; Hewlett Packard, Avondale, PA, USA), which was calibrated using standard ethylene gas (Laser Gases, New Delhi, India) expressed as µl C₂H₄ kg⁻¹ FW fruit h⁻¹.

Determination of phenyl ammonia lyase (PAL), lipoxigenase (LOX) and pectin methylesterase (PME) activity

The phenyl ammonia lyase (PAL) activity was estimated by the method of Khan and Vaidyanathan (1986) and expressed as µmoles of cinnamic acid produced min⁻¹ g⁻¹ FW. The lipoxigenase (LOX) activity was determined by the procedure adopted by Sharma et al. (2012b) for apple with slight modifications (Jhalegar et al. 2012) and expressed as µmoles min⁻¹ g⁻¹ FW. The pectin methylesterase (PME) activity was measured by following the method of Sharma et al. (2012c) with minor modifications and expressed as µmol of NaOH g⁻¹ FW min⁻¹.

Determination of total phenolic contents (TPC), antioxidant (AOX) activity and fruit quality attributes

The total phenolic content (TPC) of fruits were determined by the Singleton and Rossi method (1965) with some

modifications, and expressed in mg of gallic acid equivalents (GAE) mg per gram FW (Sharma et al. 2014). The antioxidant (AOX) activity was determined by following CUPRAC method (Apak et al. 2004) with slight modifications (Sharma et al. 2014), and expressed as $\mu\text{mol Trolox g}^{-1}$. The soluble solids concentrate (SSC) in fruits were estimated using Fisher hand-hold Refractometer (scale 0–50) and values were expressed in °Brix at 20°C. The ascorbic acid contents (AAC) were determined according to Ranganna (2000) and expressed in mg ascorbic acid 100 mg per 100 g FW.

Physical characteristics

Standard procedures were followed for measuring fruit weight (g), peel thickness (mm), and extractable juice (%).

Statistical design and analysis of data

The experiment was laid out in a completely randomised factorial design with four lots, each lot consisting of 50 fruits with four replications, for measuring the incidence and degree of granulation, and 10 fruit per lot with four replicates for all other biochemical observations. The data were then analysed using the SAS statistical package (SAS Institute Inc., Cary, NC, USA). The effects were compared from ANOVA by calculating the least square difference (LSD) at the 5 % level of significance ($P \leq 0.05$). Correlations (r^2) between total phenolics compound vs granulation, antioxidant activity vs granulation, LOX activity vs granulation, PME activity vs granulation, PAL enzyme vs granulation, rates of respiration and ethylene evolution vs granulation were determined. These correlations were calculated on the basis of 40 fruits ($n = 10$, with four replicates).

Results and discussion

Incidence and degree of granulation

About 12.8 % of ‘Kinnow’ fruits were observed to be affected by granulation with about 4.3 % degree of granulation in affected fruits. In earlier studies, Sharma and Saxena (2004) and Sharma et al. (2006) have also reported occurrence of granulation in ‘Kinnow’ under Indian conditions, although variable results have been reported, which might be due to yearly variations.

Fruit weight, peel thickness, juice content and soluble solids concentrates in granulated vs non-granulated fruits

The average weight of granulated fruit was significantly higher (178.6 ± 2.26 g) than non-granulated fruits (170.3 ± 2.21 g) (Fig. 1a). Granulated fruits produced thicker

Fig. 1 Fruit weight (a), peel thickness (b), juice content (c) and soluble solids concentrates (d) in granulated and non-granulated juice-sacs of ‘Kinnow’ mandarin. The data are means of 10 samples (fruits) with four replications. The bars represent the mean $\pm$ standard error

peel (3.72 ± 0.23 mm) than non-granulated fruits (3.25 ± 0.40 mm) (Fig. 1b). Similarly, granulated fruits produced significantly lesser juice (45.3 ± 2.16 %) than non-granulated fruits (55.4 ± 2.33 %) (Fig. 1c) and had low SSC (7.4 ± 0.21 %) than non-granulated fruits (10.1 ± 0.26 %) (Fig. 1d). Granulated fruits have been reported to have large volume because of puffiness, and juice in bound form, hence their weight is more, and such fruits produce less juice and have low SSC (Awasthi and Nauriyal 1972a; Sharma and Saxena 2004; Sharma et al. 2006; Singh 1977).

Rates of respiration rate and ethylene evolution in granulated vs non-granulated fruits

Like other citrus fruits, ‘Kinnow’ is a non-climacteric fruit, it liberates very little amount of ethylene and does not show respiration peak. We observed that the respiration rate of the

Fig. 2 Respiration rate (a) and ethylene evaluation (b) in granulated and non-granulated juice-sacs of 'Kinnow' mandarin. The data are means of 10 samples (fruits) with four replications. The bars represent the mean $\pm$ standard error

Fig. 3 Total phenolic contents (a), ascorbic acid contents (b) and antioxidant activity (c) in granulated and non-granulated juice-sacs of 'Kinnow' mandarin. The data are means of 10 samples (fruits) with four replications. The bars represent the mean $\pm$ standard error

granulated fruits ($34.45 \pm 2.19 \text{ mL CO}_2 \text{ kg}^{-1} \text{ FW h}^{-1}$) was much higher than non-granulated fruits (Fig. 2a) and such fruits also produced higher amounts of ethylene ($0.22 \pm 0.01 \mu\text{L C}_2\text{H}_4 \text{ produced kg}^{-1} \text{ FW h}^{-1}$) (Fig. 2b). It indicated that granulated fruit were more senescent than non-granulated fruits. Senescent apples (Sharma et al. 2012a), kiwifruits (Jhalegar et al. 2012; Sharma et al. 2012b) and plums (Sharma et al. 2012c) have been reported to liberate higher amount of CO_2 and C_2H_4 .

Total phenolic compounds, ascorbic acid contents and antioxidant activity in granulated vs non-granulated fruits

Granulated fruits of 'Kinnow' mandarin contained very low concentrations of phenolic compounds ($4.3 \pm 0.56 \text{ mg 100 ml}^{-1} \text{ GAE FW}$) (Fig. 3a), ascorbic acid contents ($16.2 \pm 1.63 \text{ mg 100 ml}^{-1}$) (Fig. 3b) and exhibited very low antioxidant activity ($1.78 \pm 0.29 \mu\text{mol Trolox g}^{-1} \text{ FW}$) than non-granulated 'Kinnow' fruits (Fig. 3c). Low antioxidant activity of granulated fruits may be due to lower

Fig. 4 Activities of phenyl ammonia lyase (a), pectin methyl esterase (b) and lipoxigenase (c) enzymes in granulated and non-granulated juice-sacs of 'Kinnow' mandarin. The data are means of 10 samples (fruits) with four replications. The bars represent the mean $\pm$ standard error

concentrations of ascorbic acid and total phenolic compounds. Both ascorbic acid and total phenolic compounds contribute significantly to antioxidant activity of any fruit or vegetable (Jhalegar et al. 2012; Sharma et al. 2012b; Sharma et al. 2012c). Higher the content of these attributes in any fruit, higher is the antioxidant activity, which is nowadays considered as an important attribute for health. Thus, it appears that granulated ‘Kinnow’ fruits were significantly less healthy than non-granulated fruits.

Activities of phenyl ammonia lyase, pectin methylesterase and lipoxygenase enzymes in granulated vs non-granulated fruits

The phenyl ammonia lyase (PAL) activity was lower in granulated fruits of ‘Kinnow’ mandarin (0.37 ± 0.10 nano moles cinnamic acid produced g^{-1} FW h^{-1}) than non-granulated fruits (Fig. 4a). PAL plays different roles in plant systems but its role in synthesis of phenolic compounds is considered

as most significant (Jones 2004), which is also evident from this study as granulated ‘Kinnow’ fruits produced very less concentrations of phenolic compound than non-granulated fruits. Granulated fruits exhibited higher activity of PME enzyme ($0.52 \pm 0.12 \mu\text{mol}$ of NaOH g^{-1} FW min^{-1}) than non-granulated fruits ($0.37 \pm 0.09 \mu\text{mol}$ of NaOH g^{-1} FW min^{-1}) (Fig. 4b). It is established that PME enzyme catalyses the de-esterification of pectin during fruit ripening (Micheli 2001), which is responsible for senescence of fruits. Thus, higher PME activity in granulated fruits indicated that there must be more de-esterification of pectin in such fruits as a result of which they become more senescent than non-granulated fruits (Goto 1989). Furthermore, the LOX activity of the granulated fruits was significantly higher ($1.3 \pm 0.31 \mu\text{moles min}^{-1} \text{g}^{-1}$ FW) than normal fruits ($0.92 \pm 0.18 \mu\text{moles min}^{-1} \text{g}^{-1}$ FW) (Fig. 4c). Like, the PME activity, the lipoxygenase (LOX) is an enzyme that is found in many fruits and vegetables, which catalyses the oxygenation of polyunsaturated fatty acids, which is greatly

Fig. 5 Correlation between granulation and total phenolics contents (a), granulation and antioxidant activity (b), granulation and PAL enzyme activity (c), granulation and PME enzyme activity (d), granulation and LOX enzyme activity (e), granulation and respiration rate (f), and granulation and ethylene evolution rate (g)

related to fruit senescence (Baysal and Demirdöven, 2007). Thus, higher LOX activity in granulated fruits indicated that there must be higher rate of oxygenation of polyunsaturated fatty acids in granulated fruits of Kinnow, as a result, such fruits become more senescent than non-granulated fruits. Therefore, such fruits would likely to stay for a short time in the market that non-granulated fruits, which exhibited lower activities of PME and LOX.

Relationships between granulation and antioxidant activity and senescence-related enzymes

The correlation between total phenolics compound vs granulation ($R^2 = -0.80$), antioxidant activity vs granulation ($R^2 = -0.78$), and phenyl ammonia lyase enzyme vs granulation ($R^2 = -0.81$) were negative (Fig. 5a,b, and c) and for pectin methylesterase enzyme activity vs granulation ($R^2 = +0.97$), lipoxygenase enzyme activity vs granulation ($R^2 = +0.95$), rates of respiration ($R^2 = +0.83$) (Fig. 5d,e, and f) and ethylene evolution rate vs granulation ($R^2 = +0.88$) were strongly positive (Fig. 5g). Similar relationships have also been established between granulation and active oxygen metabolism in pummelo by She et al. (2009) and mineral contents with granulation in *Citrus grandis* by Wang et al. (2014).

Conclusions

From these studies, it can be concluded that total phenolics compounds, antioxidants and PAL enzyme activity have strongly negative co-relation; whereas, senescent-related enzymes such as LOX, and PME and rates of respiration or ethylene evolution have strongly positive relationships with the occurrence of granulation in 'Kinnow' mandarin.

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