

## Comparative changes in the rheological properties and cell wall metabolism in rind of healthy and creased fruit of Washington Navel and Navelina sweet orange (*Citrus sinensis* [L.] Osbeck)

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### Abstract

Creasing is a physiological disorder in navel oranges and causes serious economic losses. In 2007, the comparative changes in rheological properties of the rind, levels of starch, phenolics, pectins and the activity of pectinesterase (PE) in albedo and flavedo tissues of the healthy and the creased fruit of 'Washington Navel' and 'Navelina' sweet orange at ripe stage were investigated. During 2009, dynamics of the activities of PE, exo and endo polygalacturonase (exo-PG, endo-PG), and endo-1, 4- $\beta$ -D-glucanase (EGase) enzymes in albedo and flavedo tissues of the healthy as well as the creased 'Washington Navel' sweet orange fruit at different maturation and ripening stages were researched. The rind hardness, stiffness and tensile force were substantially lower in the creased fruit than the healthy ones in 'Washington Navel' and 'Navelina'. The levels of starch, total phenolics, total pectins and water insoluble pectins decreased in the albedo and flavedo of the creased than the healthy fruit in both the cultivars. The levels of water soluble pectins increased in the albedo and flavedo of the creased fruit when compared to the healthy ones in both the cultivars. The activities of PE in albedo and flavedo tissues were higher in the creased fruit when compared to the healthy fruit of 'Washington Navel' and 'Navelina' in 2007. The activities of PE, exo and endo PG and Endo-1, 4- $\beta$ -D-glucanase were higher in the albedo tissue of creased fruit of 'Washington Navel' orange at different fruit maturation and ripening stages. In conclusion, the higher activities of pectinesterase, exo- polygalacturonase, endo- polygalacturonase, and endo-1, 4- $\beta$ -D-glucanase in the albedo of creased fruit at commercial harvest seem to be associated with the enhanced loss of pectins and starch in the cell walls of albedo tissue, leading to cell wall loosening and cracks formation consequently reducing hardness, stiffness and tensile force of the rind.

**Keywords:** Creasing; EGase; PE; pectins; PG; rind texture; sweet orange.

**Abbreviations:** CH\_commercial harvest; DACH\_days after commercial harvest; DBCH\_days before commercial harvest; FW\_fresh weight; EGase\_endo-1, 4- $\beta$ -D-glucanase.

### Introduction

Creasing in navel sweet oranges causes serious economic losses to the citrus growers. The creased fruit portion is characterized by multiple cracks in albedo (Bower, 2004), separation of cells in the albedo tissue resulting in channels in the rind (Storey and Treeby, 1994; Treeby et al., 1995), and separation of albedo from the flavedo causing formation of sunken areas on the peel (Agusti et al., 2001). Alquezar et al., (2010) reported that the disorder first appeared at the transition zone between flavedo and albedo, making the albedo cells flat and compact; later the affected cells extended progressively to outer albedo and inner flavedo. Various factors have been associated with creasing of sweet orange fruit such as lower levels of calcium in the albedo tissue (Treeby and Coote, 1997; Story et al., 2002), climatic changes (Zaragoza and Alonso, 1975; Agusti and Zaragoza, 2000), water stress (Zacarias et al., 2001), fruit position on the tree (Holtzhausen, 1981), crop load (Nagy et al., 1982), rootstocks (Treeby et al., 1995) and rind thickness (Koo and Reese, 1977; Holtzhausen, 1981; Ali et al., 2000). Earlier reports also indicate decreased pectin content in the cell wall

(Yang et al., 2008; Li et al., 2009), hemicellulose and cell wall polysaccharides (Jona, 1989) and higher activity of pectin methylesterase in the creased tissues leading to increased water-soluble pectins (Monselise et al., 1976) as well as uronic acid oxidase enzyme as a gelling factor (Bower, 2004). The albedo and flavedo comprise of polysaccharides including pectic substances like pectin, protopectin, pectic acid and pectinic acid (Be Miller, 1986; Yang et al. 2008; Barany et al. 2010), which are degraded by pectic enzymes like pectinesterases (PE), polygalacturonases (PG) (Kashyap et al., 2001; Yoo et al., 2003) and Endo-1, 4- $\beta$ -D-glucanase (EGase) (Brummell et al., 1997). The enzymatic peeling of citrus fruit exhibited that albedo is efficiently degraded by the activity of enzyme which act on polygalacturonic acid (Pretel et al., 2005). Galacturonic acid is the main constituent of pectins in albedo of citrus fruit (Schroder et al., 2004). Phenolics and starch have also been involved in fruit firmness, and cell wall integrity, whilst increased soluble phenolics enhance cell wall endurance (Diaz et al., 2001). The starch contributes to fruit firmness

being part of plant cell wall (Saltveit, 1999). No research work has been reported on the comparative changes in the levels of phenolics and starch in the albedo and flavedo tissues of the creased and the healthy sweet orange fruit. The rate of creasing in sweet orange fruit has been reported to be closely associated with changes in fruit firmness (Li, 2006). Later on, Li et al. (2009) reported that enhanced loss of pectins and cellulose in the cell walls of rind tissue of sweet orange has been associated with creasing. Additionally, Li et al (2009) also examined the comparative changes in cell wall components, activities of the cell wall degrading enzymes such as PE, PG and cellulase and expression of  $\alpha$ -expansin genes in the albedo of creased fruit only during fruit development in two cultivars varying in severity of creasing. We surmised that the estimation of comparative changes in these parameters of albedo and flavedo of the healthy and the creased fruit will improve understanding of this disorder. The putative physiology of albedo breakdown may be similar to fruit softening caused by cell wall disassembly (Brummel, 2006). It was hypothesised that creasing may be due to architectural changes in structures of cell wall components like pectins, phenolics and activities of various enzymes involved in cell wall degradation. The objective of this study was to examine the comparative changes in the rheological properties of the rind, levels of starch, pectins, phenolics and the activities of cell wall degrading enzymes such as PE, exo and endo-PG and EGase in the albedo and flavedo of the creased and the healthy fruit during maturation and ripening to underpin the role of cellular wall metabolism in development of creasing in sweet orange fruit.

## Results

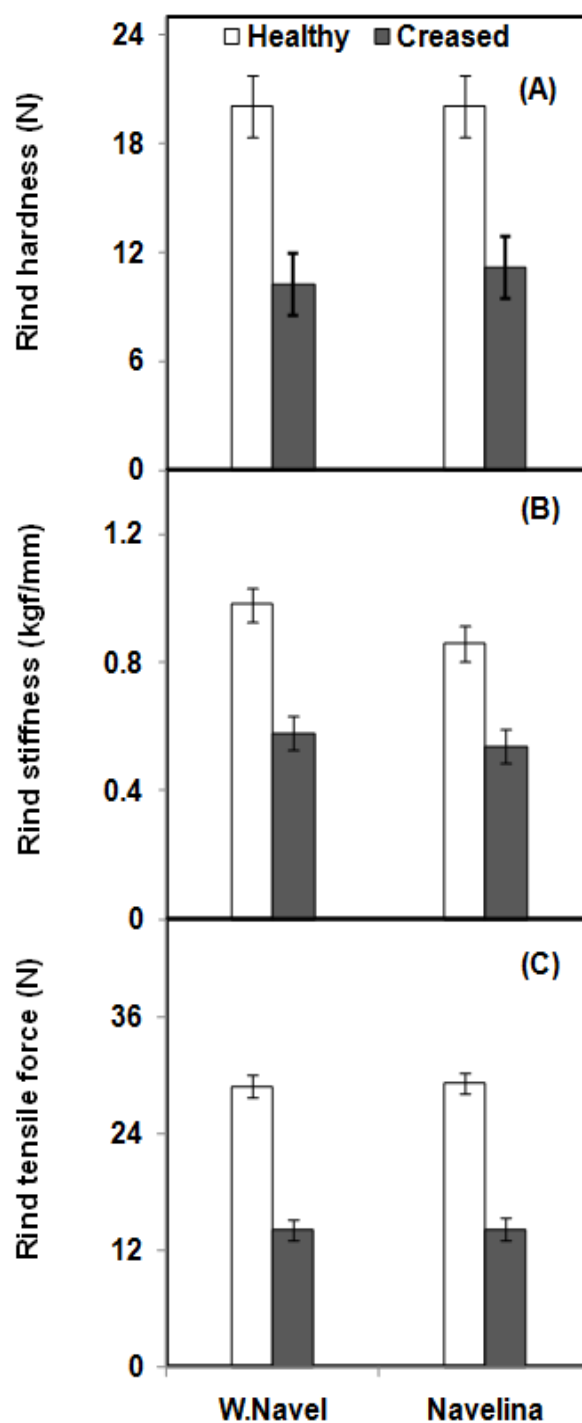
### *Effect of creasing on rheological properties of rind, levels of starch, phenolics, pectins and activity of pectinesterase (PE) in the albedo and flavedo of 'Washington Navel' and 'Navelina' fruit*

#### *Effect of creasing on rheological properties of rind*

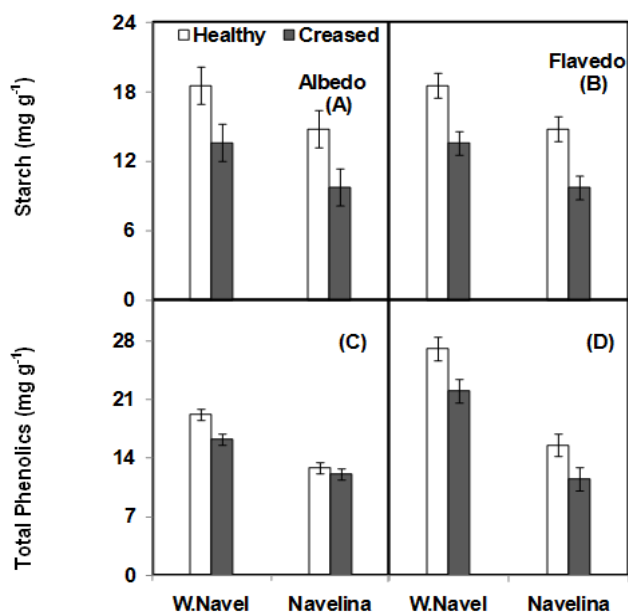
The rind of creased fruit exhibited significantly ( $P \leq 0.05$ ) lower hardness (1.9-fold and 1.8-fold) than the rind of healthy fruit of 'Washington Navel' and 'Navelina' sweet orange fruit respectively (Fig. 1A). The rind stiffness and tensile force of the creased fruit were also significantly ( $P \leq 0.05$ ) lower (1.7-fold, 1.6-fold, 2.0-fold and 2.1-fold) than the rind of healthy fruit of 'Washington Navel' and 'Navelina' respectively (Fig. 1B and C). The rind fracture force, springiness and cohesiveness did not vary significantly ( $P \leq 0.05$ ) between the creased and the healthy fruit in both cultivars (data not shown). When averaged over both cultivars, the rind of creased fruit showed significantly ( $P \leq 0.05$ ) lower hardness, stiffness and tensile force (10.69 N, 0.56 kgf mm<sup>-1</sup> and 14.11 N) than the rind of healthy fruit (20.12 N, 0.92 kgf mm<sup>-1</sup> and 28.97 N).

#### *Comparative levels of starch and total phenolics in albedo and flavedo of creased and healthy fruit*

The albedo of the creased fruit exhibited lower levels of starch (1.4-fold and 1.5-fold) than the albedo of healthy fruit in both cultivars respectively (Fig. 2A). Similarly, in both cultivars, the levels of starch were lower (1.2-fold and 1.6-fold) in the flavedo of the creased fruit of 'Washington Navel' and 'Navelina' than the healthy ones respectively (Fig. 2B). Averaged over both cultivars, the mean



**Fig 1.** Comparative changes in rind hardness (A), stiffness (B) and tensile force (C) of healthy and creased fruit of 'Washington Navel' and 'Navelina' sweet orange.  $n = 30$  (three replicates, ten fruit per replication). Vertical bars represent S.E. of means. (A) Rind hardness LSD ( $P \leq 0.05$ ) for healthy vs. creased = 3.94, cultivars = 3.94, healthy vs. creased x cultivars = 5.58. (B) Rind stiffness LSD ( $P \leq 0.05$ ) for healthy vs. creased = 0.12, cultivars = 0.12, healthy vs. creased x cultivars = 0.17. (C) Rind tensile force LSD ( $P \leq 0.05$ ), healthy vs. creased = 2.62, cultivars = 2.62, healthy vs. creased x cultivars = 3.70.



**Fig 2.** Comparative levels of starch in albedo (A) and flavedo (B), total phenolics in albedo (C) and flavedo (D) of creased and healthy fruit of ‘Washington Navel’ and ‘Navelina’ sweet orange.  $n = 15$  (three replicates, 5 fruit per replication); Vertical bars represent S.E. of means. LSD ( $P \leq 0.05$ ) starch albedo (A), healthy vs. creased = 3.71, cultivars = 3.71, healthy vs. creased  $\times$  cultivars = 5.25; LSD ( $P \leq 0.05$ ) starch flavedo (B), healthy vs. creased = 2.36, cultivars = 2.36, healthy vs. creased  $\times$  cultivars = 3.34; LSD ( $P \leq 0.05$ ) total phenolics albedo (C), healthy vs. creased = 3.20, cultivars = 3.20, healthy vs. creased  $\times$  cultivars = 4.53; LSD ( $P \leq 0.05$ ) total phenolics flavedo (D), healthy vs. creased = 2.77, cultivars = 2.77, healthy vs. creased  $\times$  cultivars = 3.92

concentrations of starch in both albedo and flavedo of the creased fruit were 1.4-fold lower than the albedo and flavedo of the healthy fruit. The concentration of total phenolics in the albedo of the creased fruit was significantly ( $P \leq 0.05$ ) lower than in the healthy fruit of ‘Washington Navel’ only, but these differences were not significant in cultivar ‘Navelina’ (Fig. 2C). The flavedo of creased fruit showed 1.2-fold and 1.4-fold lower concentrations of total phenolics than the healthy one in cultivar ‘Washington Navel’ and ‘Navelina’ respectively (Fig. 2D). Averaged over both cultivars, the mean concentrations of total phenolics in both albedo and flavedo of the creased fruit were 1.1-fold and 1.3-fold lower than the albedo and flavedo of the healthy fruit.

#### **Comparative levels of total, water insoluble and water soluble pectins in albedo and flavedo of creased and healthy fruit**

The albedo of the creased fruit of both cultivars exhibited decreased levels of total pectins (1.2-fold and 1.2-fold) and water insoluble pectins (1.3-fold and 1.3-fold) than the albedo of healthy fruit respectively (Fig. 3A and C). The concentrations of total pectins and water insoluble pectins in the flavedo of the creased ‘Washington Navel’ and ‘Navelina’ fruit were lower (1.2-fold and 1.3-fold; and 1.3-

fold and 1.5-fold) as compared to the flavedo of healthy fruit of both cultivars respectively (Fig. 3B and D). When averaged over both cultivars, the mean concentrations of total

pectins and water insoluble pectins in both albedo and flavedo of the creased fruit were lower (1.2-fold and 1.2-fold) and (1.3-fold and 1.4-fold) than albedo and flavedo of the healthy fruit respectively. The concentrations of water soluble pectins in the albedo and flavedo of the creased ‘Washington Navel’ and ‘Navelina’ fruit were higher (1.1-fold and 1.5 fold) and (1.3-fold and 1.5-fold) as compared to the albedo and flavedo of the healthy fruit of both cultivars respectively (Fig. 3E and F). Averaged over both cultivars, the mean concentrations of water soluble pectins in both albedo and flavedo of the creased fruit were 1.2-fold and 1.4-fold higher than the albedo and flavedo of the healthy fruit.

#### **Comparative PE activity in albedo and flavedo of the creased and the healthy fruit**

The albedo tissue of the creased fruit in both cultivars exhibited 1.4-fold and 1.4-fold higher activity of PE as compared to the albedo tissue of the healthy fruit of ‘Washington Navel’ and ‘Navelina’ respectively in 2007 (Fig. 4A). The flavedo tissue of the creased fruit exhibited 1.4-fold and 2.2-fold higher activity of PE as compared to the albedo tissue of the healthy fruit of both cultivars in 2007 (Fig. 4B). Averaged over cultivars, the mean activity of PE was 1.4-fold and 1.8-fold higher in the albedo and flavedo tissue of the creased fruit than in the healthy ones respectively.

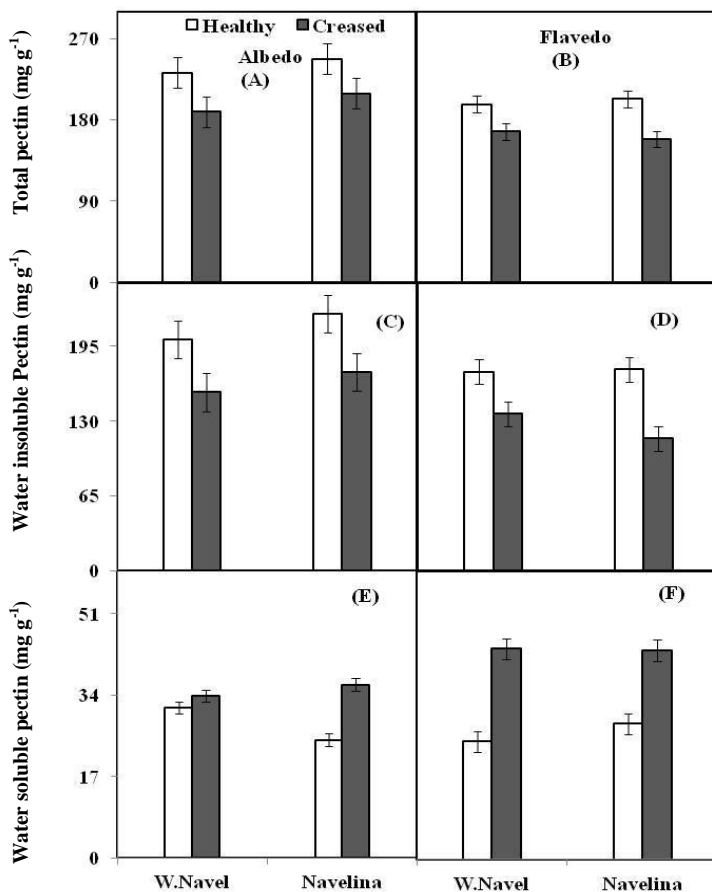
#### **Effect of creasing on the activities of PE, exo and endo polygalacturonase (exo-PG, endo-PG), and endo-1, 4-β-D-glucanase (EGase) in the albedo and flavedo tissues of ‘Washington Navel’ fruit at different maturation and ripening stages**

##### **PE activity in the albedo and flavedo of the creased and the healthy fruit at various maturation and ripening stages**

The activities of PE in the albedo of the creased fruit at 15 DBCH, CH and 35 DACH were significantly ( $P \leq 0.05$ ) higher (1.4-fold, 1.5-fold and 2.2-fold) as compared to the healthy fruit at these stages of maturation and ripening respectively (Fig 5A). When averaged over maturation and ripening stages, the mean PE activity in the albedo of creased fruit was significantly higher (1.6-fold) than the healthy ones. The activities of PE showed different trend in the flavedo tissue as it was significantly higher (1.5-fold and 1.3-fold) in the flavedo of healthy fruit at 15 DBCH and 35 DACH than the creased fruit flavedo (Fig 5B).

##### **Comparative exo-PG and endo-PG activities in the albedo and the flavedo of the creased and the healthy fruit at various maturation and ripening stages**

The activities of exo-PG in the albedo of creased fruit at 15 DBCH, CH and 35 DACH were significantly higher (1.2-fold, 1.3-fold and 1.2-fold) as compared to the healthy ‘Washington Navel’ sweet orange fruit at these stages of maturation and ripening respectively (Fig. 6A). Averaged over maturation and ripening stages, the mean exo-PG activity in the albedo of creased fruit was significantly higher (1.5-fold) than the healthy ones. Whilst, the exo-PG activities in the flavedo of creased fruit at 15 DBCH, CH and 35 ACH showed reverse trend than its activity in the albedo at



**Fig 3.** Comparative levels of total pectins in albedo (A) and flavedo (B), water insoluble pectin in albedo (C) and flavedo (D) and water soluble pectin in albedo (E) and flavedo (F) of creased and healthy fruit of 'Washington Navel' and 'Navelina' sweet orange. Vertical bars represent S.E. of means.  $n = 30$  (three replicates, 10 fruit per replication) LSD ( $P \leq 0.05$ ) total pectins in albedo (A), healthy vs. creased = 38.72, cultivars = 38.72, healthy vs. creased  $\times$  cultivars = 54.75, LSD ( $P \leq 0.05$ ) total pectins in flavedo (B), healthy vs. creased = 21.06, cultivars = 21.06, healthy vs. creased  $\times$  cultivars = 29.79; LSD ( $P \leq 0.05$ ) water insoluble pectins in albedo (C) healthy vs. creased = 37.67, cultivars = 37.67, healthy vs. creased  $\times$  cultivars = 53.28, LSD ( $P \leq 0.05$ ) water insoluble pectins in flavedo (D) healthy vs. creased = 24.67, cultivars = 24.67, healthy vs. creased  $\times$  cultivars = 34.88; ; LSD ( $P \leq 0.05$ ) water soluble pectins in albedo (E) healthy vs. creased = 2.90, cultivars = 2.90, healthy vs. creased  $\times$  cultivars = 4.10, LSD ( $P \leq 0.05$ ) water soluble pectins in flavedo (F) healthy vs. creased = 5.12, cultivars = 5.12, healthy vs. creased  $\times$  varieties = 7.24

these stages (Fig. 6B). The albedo of creased fruit at 15 DBCH, CH and 35 DACH showed significantly higher endo-PG activity (3.0-fold, 2.3-fold and 1.5-fold) than in the albedo of healthy fruit at these stages (Fig. 6C) respectively. Averaged over maturation and ripening stages, the mean endo-PG activity in the albedo of the creased fruit was significantly higher (2.2-fold) than in the albedo of the healthy fruit. The endo-PG activity in the flavedo of the creased fruit as compared to the healthy ones at various maturation and ripening stages did not differ significantly (Fig 6D).

### EGase activities in the albedo and flavedo of the creased and the healthy fruit at various maturation and ripening stages

The albedo of creased fruit at 15 DBCH, CH and 35 DACH showed significantly higher EGase activity (9.0-fold, 3-fold and 2.8-fold) than in the albedo of healthy fruit at these stages (Fig. 7A) respectively. Averaged over maturation and ripening stages, the mean EGase activity in the albedo of creased fruit was significantly 3.9-fold higher than in the albedo of healthy 'Washington Navel' sweet orange fruit. The EGase activity in the flavedo of creased fruit than in the flavedo of healthy ones at 15 DBCH, CH and 35 DACH did not differ significantly (Fig 7B).

### Discussion

The creased fruit exhibited lower rind hardness, stiffness and tensile force than the healthy fruit of 'Washington Navel' and 'Navelina' (Fig. 1A-C). It may be ascribed to weaker rind tissue caused by cellular disassembly, and a reduction in cellular wall adhesion following dissolution of the pectinaceous middle lamella (Wakabayashi, 2000; Harker et al., 1997, Li et al. 2009). The symptoms of albedo breakdown include multiple cracks and separation of cells in the albedo tissue resulting in channels in the rind and formation of sunken areas on the peel confirms weakening the rind tissue (Storey and Treeby, 1994; Treeby et al., 1995; Bower, 2004; Agusti et al., 2001; Alquezar et al., 2010). The healthy rind tissue was more compact and firm than the creased one. Similarly, cell wall loosening in the rind due to extended storage period resulted in reduced puncture force and cutting energy of 'Nagpur' mandarin fruit (Singh and Reddy 2006). Whilst, 'Valencia' sweet orange fruit sprayed with GA<sub>3</sub> before, at, and after fruit colour break resulted in 10% to 20% greater tensile strength than control possibly due to delayed over ripening of the fruit (Fidelibus et al., 2002). The reduction in starch concentration in the albedo and flavedo of creased fruit compared to the albedo and flavedo of the healthy ones in both cultivars (Fig. 2A and B) may be attributed to higher enzymatic activities of cellular wall-degrading metabolism. The reduction in the levels of total phenolics in the albedo and flavedo of creased fruit as compared to the albedo and flavedo of healthy fruit in 'Washington Navel' and 'Navelina' (Fig. 2C and D) may possibly be attributed to lower activity of phenol biosynthesizing enzyme such as phenylalanine ammonia lyase (PAL) and higher activities of phenol catabolising enzymes such as polyphenol oxidase and peroxidase which warrants to be investigated. The phenolic metabolism has been claimed to be required for building protecting barriers that would help 'Navelate' sweet orange fruit to reduce non-chilling peel pitting (Cajuste and Lafuente, 2007). The phenolics have also been involved in fruit firmness, and cell wall integrity, whilst increased soluble phenolics enhance cell wall endurance (Diaz et al., 2001). The phenolic compounds have also been reported to be involved in many interactions of plants with their biotic and abiotic factors. These substances accumulate in different plant tissues and cells during ontogenesis and under the influence of various environmental stimuli, respectively (Hutzler, 1998). The levels of total and water insoluble pectins were substantially reduced in the albedo and flavedo of creased fruit than the healthy ones in both the cultivars (Fig. 3A-D). The levels of water soluble pectins were substantially increased in the albedo and flavedo of creased fruit than the healthy one in both cultivars (Fig. 3E and F). The increased levels of water soluble pectins in the albedo and flavedo of creased fruit may

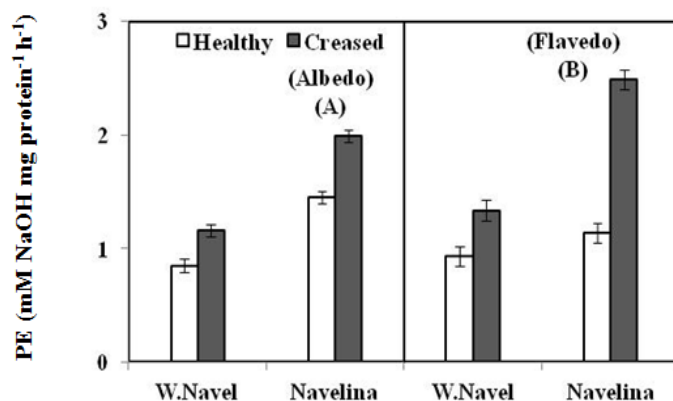
be ascribed to the higher activities of PE in the albedo and flavedo of creased fruit than in these tissues of the healthy ripe fruit in both cultivars (Fig. 4A, B and 5A, B). The PE is known to catalyze the hydrolysis of methylester groups of cell wall pectins (Christensen et al., 1998; Kashyap et al., 2001). In plants, PE plays an important role in cell wall metabolism through changes in the cell wall composition (Yang et al., 2008) with solubilization of pectins (Kesta et al., 1999) and considered the basic reasons of fruit softening during fruit ripening. Similarly, the increased levels of water-soluble pectins in the rind of creased sweet orange fruit have also been reported previously by Monselise et al., (1976) and Li et al., (2009). The albedo tissue of creased fruit exhibited significantly higher activities of PE, exo-PG, endo-PG, and EGase as compared to the albedo tissue of healthy fruit at commercial harvest in 'Washington Navel' and 'Navelina' and 'Washington Navel' in 2009 (Fig. 4-7). The activities of PE in the flavedo of creased fruit were also significantly higher than in the flavedo of healthy fruit in 'Washington Navel' and 'Navelina' (Fig. 4-5). Earlier, the PE activity showed gradual increase in the albedo tissue from creased fruit during maturation and involved in pectins disassembly, which may be causing creasing in sweet orange. The PG has been reported to release galactose from pectins and against other galactose containing hemicellulosic polymers consequently influence cell wall structure or physical properties (Ross et al., 1993; Brummell et al, 1997; Li et al, 2009; Micheli, 2001). The EGase is known to catalyze the hydrolysis of cellulose, which is a main carbohydrate that forms the structure of the cell wall. The higher activities of PE, PG and EGase may possibly have led to loss of pectins and cellulose in the cellular wall of albedo of sweet orange leading to separation of cells in the albedo tissue resulting in channels development (cracks) which are typical symptoms of creasing. Moreover, the EGase activity and gene expression have also been reported to increase and correlate with events in fruit abscission zone in 'Valencia' sweet orange and 'Tahiti' lime (Kazokas and Burns, 1998). The PG activity has also been reported to increase during abscission of 'Shamouti' leaf (Riov, 1974). Similarly, Li et al., (2009) reported a linear correlation of creasing fruit incidence in sweet orange with the activities of PG and cellulase in cultivar 'Hong Jiang' and 'An Liu' sweet orange.

#### Material and methods

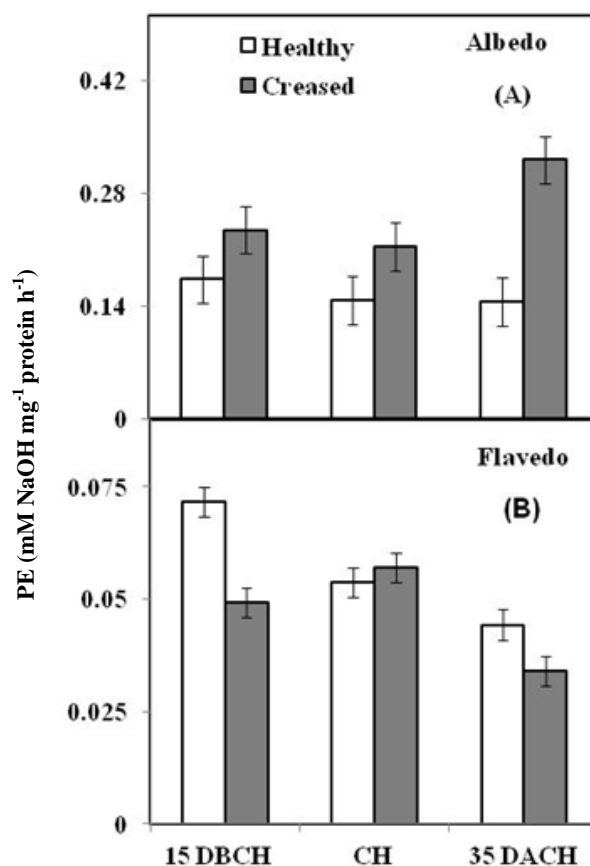
Two independent experiments were conducted in 2007 and 2009 to compare rind texture, biochemical changes and activities of cell wall degrading enzymes in albedo and flavedo of the creased and healthy fruit. In experiment 1 during 2007, we investigated the comparative changes in rheological properties of the rind, levels of starch, phenolics, pectins and the activity of PE in albedo and flavedo tissues of the healthy and the creased ripe fruit of 'Washington Navel' and 'Navelina' sweet orange. In the experiment 2 during 2009, we investigated dynamics of activities of PE, exo-PG, endo-PG, and EGase enzymes in albedo, flavedo tissues of the healthy and the creased 'Washington Navel' sweet orange fruit during various maturation and ripening stages.

#### Plant materials

These investigations were carried out during 2007 and 2009 on fruit of 'Washington Navel' and 'Navelina' sweet orange

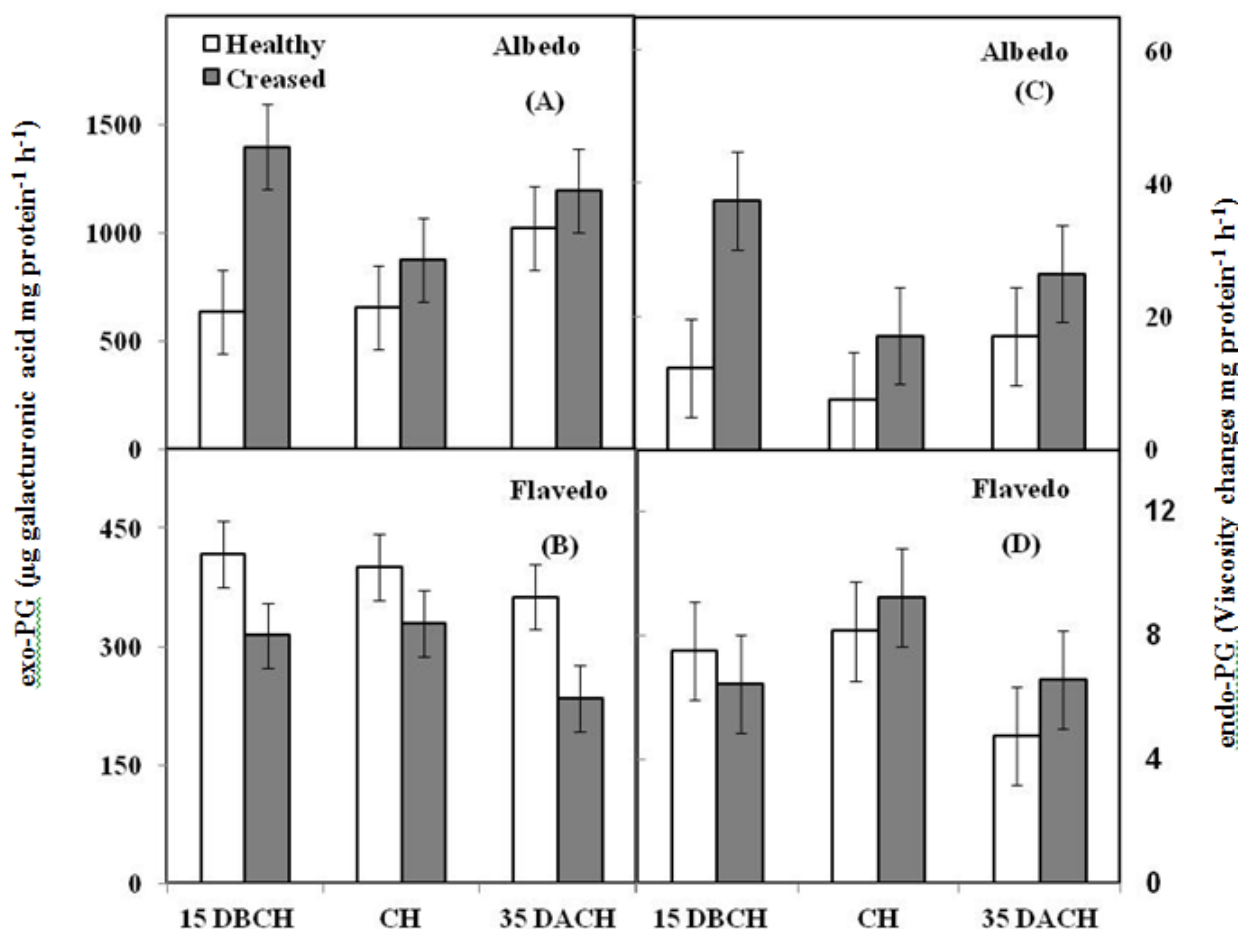


**Fig 4.** Comparative activity of PE in the albedo (A) and flavedo (B) of the creased and healthy 'Washington Navel' and 'Navelina' sweet orange fruit. n = 15 (three replicates, 5 fruit per replication). Vertical bars represent S.E. of means. LSD ( $P \leq 0.05$ ) for PE activity in the albedo, healthy vs. creased = 0.13, cultivars = 0.13, healthy vs. creased  $\times$  cultivars = 0.19, PE activity in the flavedo, healthy vs. creased = 0.20, cultivars = 0.20, healthy vs. creased  $\times$  cultivars = 0.28



**Fig 5.** Comparative activity of PE at different fruit maturity stages in the albedo (A) and flavedo (B) of the creased and healthy 'Washington Navel' fruit at different maturity stages. n = 24 (three replicates, eight fruit per replication). Vertical bars represent S.E. of means. LSD ( $P \leq 0.05$ ) activity of PE in albedo (A) healthy vs. creased = 0.05, maturity stage = 0.06, albedo  $\times$  maturity stage = 0.09, flavedo (B) healthy vs. creased = 0.006, maturity stage = 0.007, flavedo  $\times$  maturity stage = 0.01.



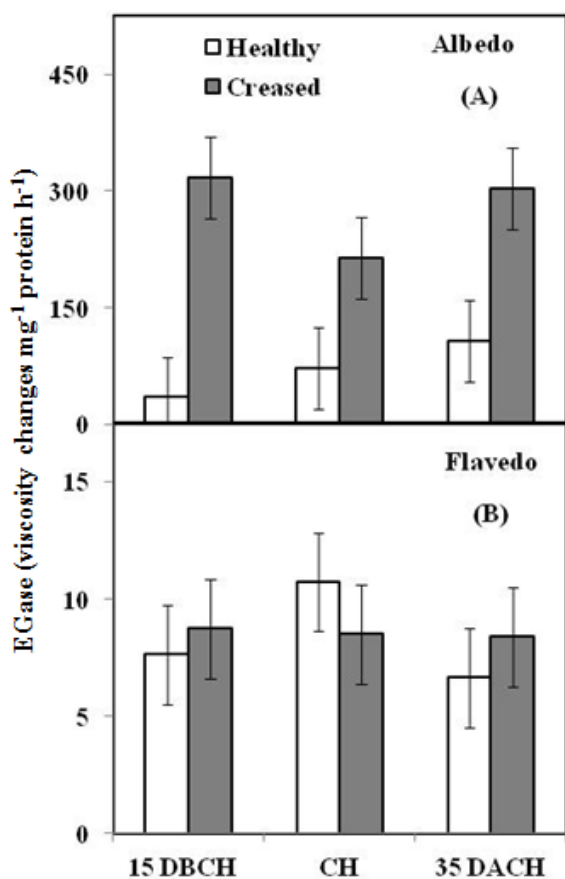


**Fig 6.** Comparative activity of exo PG at different fruit maturity stages in the albedo (A) and flavedo (B) and endo PG in albedo (C) and in flavedo (D) of the creased and healthy 'Washington Navel' fruit.  $n = 15$  (three replicates, five fruit per replication). Vertical bars represent S.E. of means. LSD ( $P \leq 0.05$ ) for exo-PG albedo (A) healthy vs. creased = 344.30, harvest dates = 421.60, albedo  $\times$  maturity stage = 596.30, exo-PG flavedo (B) healthy vs. creased = 74.2, maturity stage = 90.90, flavedo  $\times$  maturity stage = 128.60, for endo-PG, albedo (C) healthy vs. creased = 13.01, maturity stage = 15.93, albedo  $\times$  harvest dates = 22.53, flavedo (D) healthy vs. creased = 2.83, maturity stage = 3.47, flavedo  $\times$  maturity stage = 4.90

trees [*Citrus sinensis* (L.) Osbeck] grafted on [*Poncirus trifoliata* (L.) Raf.] rootstock, grown at commercial orchards at Gingin (longitude 115°55' E, latitude 31°21'S), Western Australia. Experiments 1 and 2 were conducted on 23- and 25-year old trees respectively. The trees were spaced 7.5 m between rows and 2.70 m within rows with row direction of north – south. All the experimental trees of both cultivars received similar cultural practices including irrigation, nutrition, plant protection and weed control during the period of investigations. The orchard soil is a sandy loam. The climate is described as a winter dominant with wet winters and hot, dry summers.

In first experiment, the creased and healthy 'Washington Navel' and 'Navelina' sweet orange fruit were collected on 10<sup>th</sup> July 2007 at the time of CH from thirty trees. The healthy fruits were free from visual symptoms of any disease and albedo breakdown. Fruit maturity was assessed based upon total soluble and acid ratio in the fruit juice. At least two fruit (one healthy and one creased) were harvested from each tree. Two samples of peel per fruit were used for rind texture analysis. Albedo and flavedo from the healthy and the creased fruit were separated from rind and allowed to dry separately at  $60 \pm 2$  °C to constant dry weight. The powder of dried albedo and flavedo of creased and healthy fruit was used for estimation of starch and pectins. The concentrations of total phenolics and activity of PE were determined from

the fresh albedo and flavedo tissues of the creased and healthy fruit. The experiment used a completely randomised design; including the creased and the healthy fruit as one factor and cultivar as a second one. Ten fruit were treated as an experimental unit and replicated three times. In experiment 2 in 2009, healthy and creased fruit of Washington Navel sweet orange were collected. The healthy and creased fruit were randomly harvested from tree canopy of thirty trees at various fruit maturation and ripe stages viz. 15 DBCH (on 16<sup>th</sup> June), CH (on 3<sup>rd</sup> July) and 35 DACH (on 7<sup>th</sup> August 2009. At least two fruit (one healthy and one creased) were harvested from each tree. After sampling, albedo and flavedo tissues were separated from the healthy and the creased fruit and packed in aluminum foil prior to dipping in liquid nitrogen for a few minutes, and later kept at  $-80^{\circ}\text{C}$  for determining the activities of PE, exo-PG, endo-PG, and EGase. The experiment was laid out by following a completely randomised design; including the creased and healthy fruit as one factor and the fruit maturity stage as a second factor. Ten fruit were used as an experimental unit and replicated three times.



**Fig 7.** Comparative activity of EGase at different fruit maturity stages in the albedo (A) and flavedo (B) of the creased and healthy 'Washington Navel' fruit at different maturity stages.  $n = 24$  (three replicates, eight fruit per replication). Vertical bars represent S.E. of means. LSD ( $P \leq 0.05$ ) for EGase in albedo (A) healthy vs. creased = 0.05, maturity stage = 0.06, healthy vs. creased  $\times$  maturity stage = 0.09, for flavedo (B) healthy vs. creased = 0.006, maturity stage = 0.007, healthy vs. creased  $\times$  maturity stage = 0.01.

#### Rheological properties of rind

The effect of albedo breakdown on textural properties of the rind was determined by puncture and tensile strength properties using a texture analyzer (TA Plus, AMETEK Lloyd instruments Ltd., Hampshire, UK). A personal computer with Nexygen® software was interfaced to the texture analyzer.

##### Rind puncture test

Small rind sections (2.5 cm) having 6 mm thickness were prepared from the creased and the healthy fruit using a slicer (Zyliss Easy slice, folding Mandolin slicer, Swiss). The speed of the crosshead was 50 mm min<sup>-1</sup> with 70 % of rind compression with 4 mm probe attached to the load cell. The rind hardness, stiffness, cohesiveness, springiness and fracture force were estimated.

##### Rind tensile strength test

The rind sections (2.5 x 5 cm area having 6 mm thickness) from the creased and the healthy fruit were inserted

longitudinally into the clamps of the texture analyzer. One clamp was fixed to the base and other attached to the moveable load cell. The rind sample was subjected to axial tensile loading until rind deflection of 10.0 mm at the crosshead speed of 100 mm min<sup>-1</sup> and a preload of 10 N. The rind tensile strength force was calculated at the maximum load and limit points where the rind deflection occurred.

#### Determination of starch

The starch was determined following the method previously detailed by Singh and Dhillon (1993) with some modifications. One gram dried powdered tissues of albedo/flavedo was extracted in 70 % ethanol (3X) and the residue was oven dried at  $70 \pm 2^\circ \text{C}$ . The dried residue was transferred into 50 mL centrifuge tube along with 20 mL distilled water and 10 mL HClO<sub>4</sub> (52 %). The mixture was centrifuged at  $4628.5 \times g$  for 20 min at 4° C and the supernatant was transferred into a 100 mL flask. The residue was re-extracted following the same procedure and the supernatant was pooled in the same flask and volume was made to 100 mL. Following 25 times dilution of the above solution, three mL of the solution was taken into a test tube and six mL anthrone reagent (0.2 %) and heated in boiling water for eight min. Then it was allowed to cool at room temperature and the absorbance was recorded at 630 nm using an UV/Vis spectrophotometer (Model 6405; Jenway Limited, Gransmore Green, Felsted, Dunmow, Essex, UK). The concentrations of starch were expressed as glucose equivalent mg g<sup>-1</sup>.

#### Determination of total phenolics

The concentrations of total phenolics were determined following the method previously described by Chen et al. (2006) with some modifications. Fresh tissues of albedo/flavedo (0.5 g) were extracted in 50 mL of 2 % HCl in methanol (v/v) for 24 hours in dark at ambient temperature. The extract was centrifuged at  $12,000 \times g$  for 20 min at 4° C and the supernatant (200  $\mu\text{L}$ ) was transferred into 5 mL test tube. The Folin-Ciocalteu's reagent (1 mL) was added into the supernatant and the contents were mixed using a vortex. After three min, 0.8 mL of an aqueous solution containing 7.5 % sodium carbonate was added into this solution. The reaction mixture was incubated for 30 min at room temperature, and the absorbance was recorded at 765 nm using an UV/Vis spectrophotometer (Model 6405; Jenway Limited, Gransmore Green, Felsted, Dunmow, Essex, UK). The concentration of total phenolics was expressed as gallic acid equivalent in mg g<sup>-1</sup> fresh weight of the sample.

#### Determination of pectins

Total and water soluble pectins from albedo and flavedo tissues were determined by following the method of Wang et al. (2008) using an UV/Vis spectrophotometer (Model 6405; Jenway Limited, Gransmore Green, Felsted, Dunmow, Essex, UK). The water insoluble pectins were calculated by deduction of water soluble pectins from total pectins. The concentrations of pectins were expressed as galacturonic acid equivalent in mg g<sup>-1</sup>.

#### Protein determination

Protein contents from fruit albedo or flavedo tissues of fruit rind were estimated using the method of Bradford (1976) and were expressed as mg g<sup>-1</sup> FW.

### **Determination of activities of PE, exo-PG, endo-PG, and EGase**

The activities of cell wall degrading enzymes such as PE, exo and endo-PG and EGase in the albedo and flavedo of the creased and the healthy fruit were determined by following the modified method reported earlier by Khan and Singh (2007). The activity of PE was expressed as mM NaOH mg protein<sup>-1</sup> h<sup>-1</sup>, exo-PG activity as µg galacturonic acid mg protein<sup>-1</sup> h<sup>-1</sup> while endo-PG and EGase activities were expressed as viscosity changes mg protein<sup>-1</sup> h<sup>-1</sup>.

### **Statistical analysis**

The data were subjected to two-way analysis of variance (ANOVA), using Genstat 13 release (Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK). The effects of various treatments and their interactions were assessed within ANOVA and least significance differences (Fisher's LSD) were calculated following significant ( $P \leq 0.05$ ) F test. All the assumptions of analysis were checked to ensure validity of statistical analysis.

### **Conclusion**

In conclusion, the elevated activities of pectinesterase, exopolygalacturonase, endo-polygalacturonase, and Endo-1, 4-β-D-glucanase in albedo and flavedo of creased fruit than healthy ones at harvest appear to be associated with the enhanced loss of pectins, starch in the cell walls of albedo, consequently reducing hardness, stiffness and tensile force of the rind possibly leading to cell wall loosening and cracks formation in 'Washington Navel' and 'Navelina'.

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