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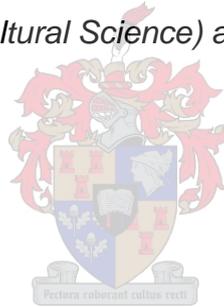
# A Study of Broken Stones in Japanese Plums (*Prunus salicina* Lindl.)

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by

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*Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Agriculture (Horticultural Science) at the University of Stellenbosch*



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**December 2015**

## DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: December 2015

## SUMMARY

### **A Study of Broken Stones in Japanese plums (*Prunus salicina* Lindl.)**

In order to export South African plums to overseas markets strict quality standards must be maintained. Among these quality classifications are specifications about the presence of cavities and pieces of broken stone/pit within the flesh of the fruit. If more than 10% of the fruit in a carton are affected by severely broken stones or large flesh cavities, the fruit have to be marketed as Class 2. A substantial amount of plums destined for export from South Africa is affected by broken stones and thus have to be marketed as Class 2. Lower prices are attained for Class 2 fruit, therefore, the presence of broken stones has a detrimental effect on the income generated from these fruit.

The main aim of this study was to gain a better understanding of broken stone development and compare the growth characteristics of Japanese plum cultivars differing in their susceptibility to broken stones.

Stone breakage in ‘Laetitia’ was observed as soon as stone hardening was initiated. At the start of stone hardening the parts of the stone that are still ‘soft’ are not strong enough to resist the pulling forces of the growing mesocarp and the stone is subsequently pulled apart. Regression analysis indicated that lengthwise growth of the fruit, fresh weight of the endo- and mesocarp, minimum orchard temperature and orchard night temperature, and relative humidity (RH) early in the growing season could possibly be used to predict the incidence of broken stones at harvest.

Differences in the incidence of broken stones were observed between ‘Laetitia’, ‘Sapphire’ and ‘Songold’ plums and between seasons. Furthermore, significant differences were observed in the density of the endocarp in different parts of the stone. For ‘Laetitia’ and ‘Songold’, stone breakage was observed when rapid increases in stone density coincided with rapid increases in fruit growth. The stones broke in positions where an interface exists between high and low density parts in the stone and when rapid radial growth takes place in the direction where the stone is least dense. In contrast, in ‘Sapphire’, stone breakage was observed before the stones had started to lignify, indicating that the endocarp was pulled apart by the expanding flesh because it was too soft to withstand the strong pulling forces created by the flesh. The incidence of broken stones was influenced by environmental factors, as higher temperatures during the stone development and hardening period could lead to more complete endocarp formation (more stone cells are formed under such conditions). Such fruit would thus have higher endocarp density, which, if coupled with rapid radial growth, could lead to a higher incidence of broken stones.

Foliar and/or root applications of calcium nitrate and potassium silicate were applied to ‘Laetitia’ plums to determine whether the incidence of broken stones could be reduced by increasing the strength of the

endocarp cell walls. However, no such effect was observed. Hence, neither calcium nor silicate treatments can be recommended for reducing broken stones in plums.

## OPSOMMING

### **‘n Studie van gebreekte pit in Japanese pruime (*Prunus salicina* Lindl.)**

Om Suid-Afrikaanse pruime na oorsese markte uit te voer moet vrugte aan streng kwaliteitstandaarde voldoen. Daar is ondermeer spesifikasies in verband met die teenwoordigheid van holtes en stukkie gebreekte pit binne die vrug. Indien meer as 10% van die vrugte in ‘n karton deur ernstige gebreekte pit of groot vrugholtes geaffekteer word, moet die vrugte as Klas 2 bemark word. Aangesien ‘n groot hoeveelheid van die pruime wat vir uitvoer bestem is, geaffekteer word deur gebreekte pit en gevolglik as Klas 2 bemark moet word, word aansienlike finansiële verliese gelei. Laer pryse word behaal vir Klas 2 vrugte, en dus het die teenwoordigheid van gebreekte pitte ‘n negatiewe effek op die wins wat deur die uitvoer van hierdie vrugte gegenerer kan word.

Die hoofdoel van hierdie studie was om die ontwikkeling van gebreekte pit in Japanese pruime te ondersoek en om die groei-patrone van kultivars wat geneig is tot gebreekte pit te vergelyk met ‘n nie-sensitiewe kultivar.

Gebreekte pit in ‘Laetitia’ is opgemerk sodra die pit begin verhard het. Met die aanvangs van pit-verharding is die gedeeltes van die pit wat nog nie volkome verhard het nie, nie sterk genoeg om die sterk trekkragte van die groeiende mesokarp te weerstaan nie en die pit word gevolglik uitmekaar-getrek. Regressie-analise het gewys dat lengtegroei van die vrugte, vars massa van die endo- en mesokarp, minimum boordtemperatuur en boord-nagtemperatuur, asook relatiewe humiditeit gedurende die vruggroei-periode moontlik gebruik kan word om die voorkoms van gebreekte pit by oes te voorspel.

Verskille in die voorkoms van gebreekte pit is opgemerk tussen ‘Laetitia’, ‘Sapphire’ en ‘Songold’ pruime, en ook tussen seisoene. Verder is beduidende verskille opgemerk in die digtheid van die endokarp in verskillende dele van die pit. By beide ‘Laetitia’ en ‘Songold’ is gebreekte pit opgemerk wanneer vinnige toename in pit-digtheid saamgeval het met ‘n vinnige toename in vruggroei. Die pitte breek veral in die oorgang tussen dele van die pit met hoë en lae digtheid en as dit gekombineer is met vinnige radiale vruggroei in die rigting waar die pit die minste dig is. In teenstelling hiermee is gebreekte pit in ‘Sapphire’ opgemerk selfs voordat die pitte begin verhard het. Dit dui daarop dat die endokarp uitmekaar-getrek is deur die vinnig groeiende mesokarp, omdat dit te sag was om die trekkragte van die groeiende vrugvles te weerstaan. Die voorkoms van gebreekte pit word ook deur weerstoestande beïnvloed, want hoër temperature gedurende die pit-ontwikkeling en verhardingsperiode, kan lei tot die ontwikkeling van endokarpe met meer steenselle. Hierdie vrugte sal dus ‘n hoër digtheid hê, en as dit saamval met vinnige radiale groei, kan dit lei tot ‘n groter voorkoms van gebreekte pit.

Blaar- en/of worteltoedienings van kalsiumnitraat en kaliumsilikaat is gemaak om te bepaal of die voorkoms van gebreekte pit in 'Laetitia' verminder kon word deur die versterking van die endokarp-selwande. Geen van hierdie behandelings het tot 'n vermindering in gebreekte pit gelei nie en nie kalsiumnitraat of kaliumsilikaat kan dus aanbeveel word om gebreekte pit in pruime te verminder nie.

## ACKNOWLEDGEMENTS

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To my extended family for their unconditional love, motivation and encouragement.

## **DEDICATION**

In memory of Hester Elizabeth Ackermann

The light I follow

**TABLE OF CONTENTS**

<b>DECLARATION</b> .....	<b>i</b>
<b>SUMMARY</b> .....	<b>ii</b>
<b>OPSOMMING</b> .....	<b>iv</b>
<b>ACKNOWLEDGEMENTS</b> .....	<b>vi</b>
<b>DEDICATION</b> .....	<b>vii</b>
<b>TABLE OF CONTENTS</b> .....	<b>viii</b>
<b>LIST OF FIGURES</b> .....	<b>ix</b>
<b>LIST OF TABLES</b> .....	<b>xi</b>
<b>NOTE</b> .....	<b>xiii</b>
<b>GENERAL INTRODUCTION AND OBJECTIVES</b> .....	<b>1</b>
<b>LITERATURE REVIEW: A review of broken and split stones in stone fruit</b> .....	<b>8</b>
<b>PAPER 1: The effect of climate and growing area on the incidence of broken stones in ‘Laetitia’ plums (<i>Prunus salicina</i> Lindl.)</b> .....	<b>33</b>
<b>PAPER 2: The use of computed tomography scans to evaluate broken stones in three Japanese plum cultivars (<i>Prunus salicina</i> Lindl.)</b> .....	<b>64</b>
<b>PAPER 3: Effect of foliar and root applications of calcium and silicon on broken stones and fruit quality in Japanese plum cv. ‘Laetitia’</b> .....	<b>92</b>
<b>GENERAL DISCUSSION AND CONCLUSION</b> .....	<b>118</b>

## LIST OF FIGURES

### GENERAL INTRODUCTION

Figure 1	Fruit classified as Class 1 according to internal and external fruit quality	6
Figure 2	The small cavity in the fruit flesh allowed under Class 1	6
Figure 3	Plum fruit with a cavity and a piece of broken stone at the stylar end, which is not allowed under Class 1 specifications and is thus marketed as Class 2	7

### LITERATURE REVIEW

Figure 1	Split pit in peach fruit, indicating how the stone has split along the suture	30
Figure 2	A broken stone in the Japanese plum cultivar ‘Laetitia’	30
Figure 3A	The dorsal bundle, which supplies the endocarp and lies in the groove opposite the suture	31
Figure 3B	The two ventral bundles, which supply the endocarp and lies in grooves on both sides of the suture	31
Figure 4	Distribution of the vascular bundles of a peach fruit in cross-section	31
Figure 5	Vertical section of a peach fruit, with the arrow pointing to the funicular bundle	32

### PAPER 1

Figure 1A-B	Incidence of broken stones	53
Figure 2A-B	Rate of change in fruit length per day	54
Figure 3A-B	Rate of change in fresh weight accumulation of the endocarp per day	55
Figure 4A-B	Average fresh weight of the endocarp	56
Figure 5A-B	Average minimum orchard temperature	57
Figure 6A-B	Average minimum orchard relative humidity	58
Figure 7A-B	Average orchard night temperature	59
Figure 8A-B	Average fruit length	60
Figure 9A-B	Average orchard night relative humidity	61
Figure 10A-B	Average fresh weight of the mesocarp	62
Figure 11A-B	Rate of change in fresh weight accumulation of the mesocarp per day	63

### Paper 2

Figure 1	Progression of stone hardening, as measured by change in relative stone density in ‘Laetitia’ and ‘Songold’ plums during the 2013/2014 season	84
Figure 2	Progression of stone hardening as measured by a change in relative stone density in ‘Sapphire’, ‘Laetitia’ and ‘Songold’ plums during the 2014/2015 season	85
Figure 3	Progression of radial growth in relation to days after full bloom of ‘Laetitia’ and ‘Songold’ plums in the 2013/2014 season	86
Figure 4	Progression of radial growth of ‘Laetitia’ and ‘Songold’ in relation to days after full bloom during the 2013/2014 season	86
Figure 5	Progression of radial growth of ‘Laetitia’, ‘Sapphire’ and ‘Songold’ plums in relation to days after full bloom and direction of growth during the 2014/2015 season	87
Figure 6	Split pit in peach fruit indicating how the stone has split along the suture	88
Figure 7	A broken stone in the Japanese plum cultivar ‘Laetitia’	88
Figure 8A	2D slice made along the suture of Japanese plum fruit used for three dimensional computed tomography scans	89
Figure 8B	Three dimensional computed tomography scan of a ‘Songold’ plum with a view of the suture	89
Figure 9	CT-scan showing the progression of endocarp lignification between ‘Laetitia’, ‘Songold’ and ‘Sapphire’	90
Figure 10	‘Laetitia’ plums exposed to Phloroglucinol-HCl solution, which stains tissue pink in the presence of lignin	91

### PAPER 3

Figure 1	Chart used to grade the severity of broken stones	117
Figure 2	Chart indicating the size of the cavity formed within the flesh	118

## LIST OF TABLES

### PAPER 1

Table 1	Summary of farms, rootstocks, orchard age, planting distance, training system, orchard layout and full bloom dates	49
Table 2	ANOVA indicating the differences in the incidence of broken stone at harvest in ‘Laetitia’ plums sampled in two production areas	50
Table 3	Summary of the 20 ‘best’ regression for the prediction of the percentage of broken stones on the optimum harvest date	51
Table 4	Regression table for the prediction of percentage broken stone on the optimum harvest date	52

### PAPER 2

Table 1	Summary of South African export standards and requirements for plums and prunes with regards to shape and broken stones	82
Table 2	Summary of temperature and rainfall data	83

### PAPER 3

Table 1	The effect of pre-harvest calcium and pre-harvest silicate treatments on the incidence and severity of broken stones as well as the size of the cavity formed in the flesh due to stone breakage for Trial 1 of the 2013/2014 season	107
Table 2	The effect of pre-harvest calcium and pre-harvest silicate treatments on the incidence and severity of broken stones as well as the size of the cavity formed in the flesh due to stone breakage for Trial 1 of the 2014/2015 season	108
Table 3	The effect of pre-harvest calcium and pre-harvest silicate treatments on the incidence and severity of broken stones as well as the size of the cavity formed in the flesh due to stone breakage for Trial 2 of the 2014/2015 season	108
Table 4	The effect of pre-harvest calcium and pre-harvest silicate treatments on the incidence and severity of broken stones as well as the size of the cavity formed in the flesh due to stone breakage over both seasons	109
Table 5	Maturity indexing and quality parameters of ‘Laetitia’ plums measured on the harvest date for fruit which received pre- and post-harvest calcium treatments as well as pre-harvest silicate treatments in the 2013/2014 season	110

Table 6	Maturity indexing and quality parameters of ‘Laetitia’ plums measured on the harvest date for fruit which received pre- and post-harvest calcium treatments as well as pre-harvest silicate treatments in the 2014/2015 season	111
Table 7	Maturity indexing and quality parameters for ‘Laetitia’ plums measured on the harvest date for fruit which received pre-harvest calcium and silicate sprays in the 2014/2015 season	112
Table 8	Maturity indexing and quality parameters for ‘Laetitia’ plums measured on the harvest date over both seasons	113
Table 9	Quality parameters measured after cold storage and after shelf life in the 2013/2014 season	114
Table 10	Quality parameters measured after cold storage and after shelf life in the 2014/2015 season	115
Table 11	Quality parameters measured after cold storage and after shelf life in the 2013/2014 season	116
Table 12	Quality parameters measured after cold storage and after shelf life on fruit which received pre-harvest foliar calcium and silicate applications respectively	117

## **NOTE**

This thesis is a compilation of chapters, starting with a literature review, followed by three research papers. Each paper is prepared as a scientific paper for submission to *Scientia Horticulturae*. Repetition or duplication between papers might therefore be necessary.

## GENERAL INTRODUCTION AND OBJECTIVES

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Approximately 5000 hectares of plums are planted across South Africa. The three most important cultivars are 'Laetitia', 'Songold' and 'Sapphire', contributing 12%, 11% and 7%, respectively, to the total planted plum area (HORTGRO, 2014). In the 2013/2014 season approximately 74054 tons of plums were produced in South Africa, of which 74% were exported, including 1881 125 cartons of 'Laetitia'. The main export markets for South African plums are the European Union, Russia, the United Kingdom, Middle and Far East, and Asia. Net realisation for the export of South African plums amounted to R15390 per ton in the 2013/2104 season.

However, in order to export fruit to overseas markets, strict quality standards must be maintained. According to the standards of the Organisation for Economic Co-operation and Development (OECD), plums destined for export are classified into classes according to internal and external quality (OECD, 2002). For Class1 fruit only very slight defects (in shape and development or colouring) are allowed, provided that these defects do not affect the general appearance or quality of the product (Fig. 1). Class 2 is indicated for fruit that do not qualify for the higher classes, but still satisfy the minimum quality requirements. Yet, there is a 10% tolerance limit in terms of these quality parameters, which means that fruit can still be classified as Class 1 even though 10% of the fruit only meet the requirements for Class 2 fruit.

Among these quality classifications are specifications about the presence of cavities and pieces of broken stone/pit within the flesh of the fruit. Under Class 1 classification a small cavity at the stem and/or stylar end of the stone is allowed, but only if the flesh is not discoloured (Fig. 2). Additionally, it is also permissible for the stone to be broken while still clinging to the flesh. However, if the size of the cavity is large or stone breakage more severe (Fig. 3), fruit have to be classified as Class 2, even though they adhere to all other Class1 specifications. Thus, if more than 10% of the fruit in a carton are affected by broken stones, the fruit have to be marketed as Class 2. The Laetitia plum cultivar is very prone to stone breakage, and as this cultivar is one of the main cultivars produced in South Africa, it is clear that stone breakage is a serious problem. A substantial volume of 'Laetitia' fruit destined for export is affected by broken stones and thus has to be marketed as Class 2. Lower prices are attained for Class 2 fruit, and therefore, the presence of broken stones has a detrimental effect on the income generated from these fruit.

Broken/split stones affect both peach and plum crops (Personal observations), but the exact causes of stone breakage are not yet fully understood and, to our knowledge, no information about this phenomenon is available for plums. In peaches, the occurrence of split stones is influenced by both

genetic and environmental factors (Claypool et al., 1972; Engin et al., 2010). The stone can break in almost any place and often in multiple places and as the fruit continues to grow, large cavities often form in the flesh in the areas where the stone had broken (Chatzitheodorou et al., 2004; Engin et al., 2010).

Stone breakage in peaches seems to generally occur during or just prior to stone hardening and is most often only detected when fruit are cut open, although it can be observed as malformed fruit or even fruit that are pulled apart at the stem end in severe cases (Ragland, 1934; Woodbridge, 1978). Affected fruit are commonly larger than normal, especially in cross diameter, and contain a higher than normal proportion of flesh (Davis, 1933; Evert et al., 1988). As fruit growers aim to produce larger fruit to meet the demands of consumers, cultivation practices can often exacerbate the extent of stone breakage (Nakano, 2006). Techniques such as fruit thinning and shoot pinching are used to promote fruit growth, but this may also promote the occurrence of broken stones.

Furthermore, peaches affected by broken or split stones have been found to ripen earlier than normal fruit, and as a consequence their shelf life are reduced (Crisosto et al., 1997; O'Malley and Proctor, 2002; Woodbridge, 1978). Split stones may sometimes cause tearing of the skin at the stem end of the fruit which makes it susceptible to insects and disease producing organisms (Chatzitheodorou et al., 2004; O'Malley and Proctor, 2002; Woodbridge, 1978). Fruit with broken stones may also contain stone residues that are unacceptable to the consumer and create difficulties in the canning process (Han et al., 1992).

Therefore, the main aim of this study was to gain a better understanding of broken stones in Japanese plums. In Paper 1 the effect of climate and growing area on the incidence of broken stones in 'Laetitia' (a cultivar susceptible to broken stones) was investigated in order to determine at which stage during fruit development stone breakage occurs and whether the occurrence was correlated to climatic conditions or fruit growth patterns. Hence, fruit were sampled over two seasons from farms in Stellenbosch and Robertson during the growing season, and orchard temperature and relative humidity, fruit length, diameter, along with the fresh weight of the endocarp and mesocarp were determined.

The literature indicates that the extent of stone splitting or breakage varies greatly among cultivars and between seasons in peaches (Sotiropoulos et al., 2010; Woodbridge, 1978). Thus, in Paper 2, computed tomography scans were used to determine how fruit growth patterns, with specific reference to the density of the stone, differ between cultivars that are susceptible to broken stones as opposed to a cultivar that is not susceptible to stone breakage.

While studies have been conducted on the effect of both calcium and silicon on the incidence of broken/split stones in peaches (Evert et al., 1988; Sotiropoulos et al., 2010), to our knowledge, no such studies have been conducted on plums. Knowing the roles of both calcium and silicon in strengthening

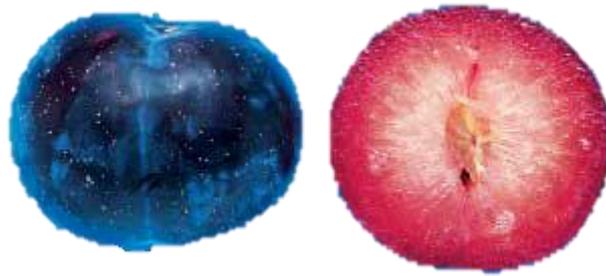
the cell wall, in Paper 3, we set out to determine whether foliar and root applications of these products could reduce the incidence and severity of broken stones in the Japanese plum cultivar Laetitia.

The results from these studies aimed to broaden the understanding of broken stones in Japanese plums and are the first steps in finding solutions for a problem that has a significant financial impact on the South African stone fruit industry.

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**Fig. 1.** Fruit classified as Class 1 according to internal and external fruit quality. For Class 1 fruit only very slight defects in shape, development, and colouring are allowed, provided that these defects do not affect the general appearance or quality of the product (Organisation for economic co-operation and development (OECD), 2002).



**Fig. 2.** The small cavity in the fruit flesh allowed under Class 1 (Organisation for economic co-operation and development (OECD), 2002)



**Fig. 3.** Plum fruit with a cavity and piece of broken stone at the styler end, which is not allowed under Class 1 specifications and is thus marketed as Class 2 (Organisation for economic co-operation and development (OECD), 2002).

## LITERATURE REVIEW

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### A review of broken and split stones in stone fruit

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#### 1. Introduction

Broken and split stones are physiological phenomena that affect plum and peach crops. Broken stones are observed in plums/prunes and peaches and is defined as a condition that occurs when a piece(s) of the stone has broken off during fruit development (Department of Agriculture Forestry and Fisheries, South Africa, 1990). However, they define instances where the stones have broken along its longitudinal axis (suture) as split stone/pit. Both broken and split stones are most often only detected when fruit are cut open. The exact causes of stone breakage or splitting are not yet fully understood.

In the case of broken stones, the stone can break at almost any position and often breaks at multiple locations (Chatzitheodorou et al., 2004; Engin et al., 2010). The extent of breakage also varies greatly among cultivars and between seasons (Sotiropoulos et al., 2010; Woodbridge, 1978). Stone splitting generally occurs during or just prior to stone hardening and can, in severe cases, be detected as malformed fruit or fruit that are pulled apart at the stem end (Ragland, 1934; Woodbridge, 1978). The occurrence of split stones in peaches is influenced by both genetic and environmental factors (Claypool et al., 1972; Engin et al., 2010). Cultivation practices that cause sudden or sharp increases in the fruit growth rate at the beginning of Stage II, such as thinning, N application, girdling, and irrigation, can also increase the incidence of split stones (Claypool et al., 1972; Engin et al., 2010; Nakano, 2006; Ragland, 1934; Sotiropoulos et al., 2010). Affected fruit are commonly larger than normal fruit, especially in cross diameter (Davis, 1933; Evert et al., 1988). As fruit growers aim to produce larger fruit to meet the demands of consumers, techniques such as fruit thinning are used to promote fruit growth, which could contribute towards incidence of split stones (Nakano, 2006). The presence of split stones leads to reduced quality, as fruit that contain broken or split stones have been found to ripen earlier than non-affected fruit and, as a consequence, their shelf life is reduced (Crisosto et al., 1997; O'Malley and Proctor, 2002; Woodbridge, 1978). Split stones in peaches may sometimes cause tearing of the peel near the stem which makes the fruit susceptible to insect damage and disease producing organisms (Chatzitheodorou et al., 2004; O'Malley and Proctor, 2002; Woodbridge, 1978). Fresh peaches with broken or split stones may contain stone residues that are unacceptable to the consumer and these pieces of stone also create difficulties in the canning process (Han et al., 1992).

To our knowledge no research has been done on broken stones to date, but research on split stones, especially in peaches, has been conducted (Claypool et al., 1972; Engin et al., 2010; Nakano, 2006; Ragland, 1934; Sotiropoulos et al., 2010). Hence, this literature review will focus on the occurrence of the phenomenon in peaches. While peach stones most often split along the suture/seam (Fig. 1)

(Chatzitheodorou et al., 2004; Engin et al., 2010), plum stones tend to break anywhere along the stone and often in multiple places (personal observation) (Fig. 2). This literature review will describe the growth and development of peach fruit and examine the lignification process of the stone and the genes involved during stone hardening. Broken or split stones will then be defined in more detail and the cultivation and environmental factors that influence their occurrence will be discussed.

## **2. Stone fruit growth and development**

Information on fruit growth of Japanese plums is lacking in literature and consequently, the growth of peaches, sour cherries and prunes will be discussed instead.

### **2.1 Double sigmoidal growth curve**

The pericarp of *Prunus* species can be divided into an endocarp (the stone), a fleshy mesocarp and the exocarp or peel (Ragland, 1934). The endocarp of a mature peach is composed of thick-walled, lignified, stone-like cells that are packed tightly with minimal intercellular spaces. Soon after full bloom (anthesis) there is only a slight distinction between the stone and the flesh, with the general outline of the stone only appearing at about 14 days after anthesis. The mesocarp, or flesh, consists of thin-walled parenchyma cells which are interwoven with numerous vascular strands. Most of the increase in the bulk of the flesh is due to cell enlargement, which does not occur at a uniform rate in all planes. Peach fruit do not have a distinctive exocarp – it consists of only an epidermis and a hypodermal layer that is between six and ten cell layers thick.

The growth of stone fruit exhibit a double-sigmoidal growth curve with three stages (I, II and III) (Ragland, 1934; Tukey, 1936). Stage I consists of a period of rapid development of the pericarp (mesocarp, endocarp and exocarp), beginning at the time of anthesis and lasting for about 7 weeks. During this time the fruit rapidly increases in volume, and while the pericarp increases in volume the nucellus and integuments also attain their maximum size, but the embryo remains in a state of arrested development. Stage I is followed by Stage II, a period of slow mesocarp growth between 7 and 11 weeks after anthesis, during which the embryo grows and the stone hardens (Chalmers and Van den Ende, 1975). By the end of Stage II the endosperm tissue will have been completely replaced by the embryo. The stone is, therefore, the first part of the fruit to mature and it reaches its maximum size long before the rest of the fruit does (Ragland, 1934). Stone hardening starts at the styler end of the stone and progresses towards the stem end. The ventral suture (seam) and inner walls harden more rapidly than the rest of the stone. Although hardening does not begin at the same time each year, the process of hardening stays relatively constant from year to year. Finally, during Stage III, the mesocarp develops rapidly prior to fruit ripening between 12 and 18 weeks after anthesis. This stage is often referred to as "final swell" (Tani et al., 2007; Tukey, 1936).

The different growth stages can further be broken down into fresh and dry weight accumulation (Chalmers and Van den Ende, 1975). Although both show a double sigmoidal growth curve, their times of onset and cessation are slightly different for the various growth stages. During Stage I dry and fresh weight accumulation starts simultaneously. The rate of fresh weight accumulation increases rapidly during Stage I, and when this rate starts to decline Stage II of fruit growth starts. However, the first stage of dry weight accumulation continues to increase until nearly the middle of Stage II. This can most likely be explained by lignification of the endocarp during this time. During Stage II the rate of fresh weight accumulation declines, with commencement of Stage III occurring when fresh weight accumulation starts to increase again. The second stage of dry weight accumulation starts during the middle of Stage II, and the rate of accumulation declines until the middle of Stage III. Therefore, the final phase of dry weight accumulation starts in the middle of Stage III and continues for about a week or less after fresh weight accumulation for this stage has ceased.

Some hypotheses to explain the regulation of the different fruit growth stages include competition for carbohydrates, either between different fruit parts, or between fruit and vegetative activity of the tree (Chalmers and Van den Ende, 1977, 1975; Lott, 1932). Chalmers and Van den Ende (1975) hypothesised that competition between the growing flesh and embryo could explain the need for the growth stages. They observed that the transition from Stage I to Stage II is characterized by an almost complete change from flesh growth to stone growth. There appears to be strong competition between the stone and flesh for the supply of available substrates to support growth. Furthermore, they found a drop in the levels of organic acids during this time, which could indicate activation of the pentose-phosphate pathway. This pathway produces precursors for phenols required for synthesis of lignin – which is required for stone hardening. They also found that the first stage of dry weight accumulation ended when the growth rate of the stone started to decline rapidly (i.e. in the middle of Stage II). However, no compensating increase in flesh growth rate occurred and a rapid increase in soluble sugars was observed. This, in turn, initiated rapid growth of the embryo. Thus, Chalmers and van den Ende (1975) concluded that if the peach embryo requires higher substrate levels than other parts of the fruit in order to grow, it would be necessary to 'turn off' the growth of other parts of the fruit to ensure sufficient supply of substrate to the growing embryo. It is also possible that the cells of the flesh and stone deplete these substrates before they can reach the embryo, because the vascular tissues that transport the substrates cross the flesh and stone before reaching the embryo. This would also require the growth of flesh and stone cells to be 'turned off' to allow substrate to pass on to the seed. Although growth of the embryo may initially depend upon the material in the endosperm, this state cannot persist for long. Chalmers and Van den Ende (1977) found that the final weight of the embryo was almost ten times greater than the final weight of the seed coat plus endosperm. Thus, it is likely that most of the growth requirements of the embryo are met from current assimilates.

Another theory regarding the necessity for different growth stages is the competition between vegetative and reproductive growth. Reproductive growth seems to dominate vegetative growth in the fruit, with the stone and seed representing reproductive tissues and the flesh the vegetative tissue (Lott, 1932). While the seed is strictly the only reproductive tissue of the fruit, the stone is more intimately associated with the seed than the flesh, since the stone protects the seed. Lott (1932) further found that, after it reached its maximum dry weight, the stone was the source of supplies for seed development. He also argued that supplies were translocated from the flesh to the seed after the flesh matured and stated that the anatomy of the peach fruit is conducive to transport of supplies from the flesh and stone to the seed (most likely via the vascular bundles embedded within the stone). Thus, the growth rate of the fruit must be low during the period of stone hardening, because the stone and seed are dominant over the flesh during this period. Therefore, this period of slow growth rate of the fruit would occur regardless of crop load.

The length of the different growth stages varies with cultivar, species and season (Tukey, 1936). Early maturing cultivars undergo a short period of development, whereas late maturing cultivars show a longer Stage II. Due to the shorter Stage II in early cultivars, Stage III growth begins before the embryo has reached its full size and these cultivars are consequently prone to non-viable or aborted embryos. In studies that simulated embryo abortion by damaging the embryos at different times during the growth phase, destruction of the embryo caused an increase in the ripening process parallel with/following the growth curve. In early maturing cultivars, the growth curve is nearly linear because of the short Stage II, which can be as short as 5 days (Ognjanov et al., 1995). Drought and high temperatures can alter the double-sigmoid curve of fruit growth towards a linear pattern, indicating that such conditions reduce Stage II. In a study on sweet cherries, a close similarity was reported between the growth and development of late and early maturing cultivars during Stage I (Tukey, 1933). The first difference between the two cultivars was noticed during Stage II. Early maturing cultivars had a shorter Stage II compared to those of late maturing cultivars. Thus, Stage III of growth started during the period when the embryo and endosperm were still developing in the early maturing cultivar. In the late maturing cultivar Stage III started about 6 days after the rate of embryo growth started to decrease. Late maturing cultivars consequently had a longer development period than early maturing cultivars. In early maturing cultivars the embryo often aborts, but it is unclear whether rapid growth of the pericarp causes embryo abortion or whether embryo abortion causes rapid pericarp growth.

## **2.2 Cell division and enlargement**

Cell division and enlargement determine final fruit size and these processes start even before full bloom (Scorzal et al., 1991). Large-fruited cultivars already have higher rates of cell division in their ovaries during the pre-bloom stages and pre-bloom cell divisions play an important role in determining ultimate

fruit size. Four weeks after full bloom the final cell number in the mesocarp and endocarp of peach appears to be fixed (Masia et al., 1992; Ragland, 1934), and further growth of the fruit is mainly due to cell enlargement in the mesocarp, which is dependent on processes of cell wall loosening, cell separation and the formation of intercellular spaces (Ragland, 1934; Zanchin et al., 1994). The rate of cell division and enlargement declines during Stage II when the embryo grows and the stone begins to harden (Zanchin et al., 1994). The rate of cell enlargement then increases again during Stage III, indicating that, in *Prunus*, cell division is only responsible for the early stages of fruit development and thereafter, growth is mostly due to cell enlargement.

During Stage I the rate of increase in cell size is similar in all planes. In Stage II the longitudinal and tangential diameters of cells increase very little while the radial diameters continue to enlarge at the same rate as during Stage I (Ragland, 1934). The radial increase in cell diameter during Stage II causes internal stresses within the fruit. According to Ragland (1934) these internal stresses are most prominent in the inner third of the flesh and leads to a change in the shape of the cells.

### **2.3 Hormone activity during the double sigmoidal growth curve**

Indole-3-Acetic acid (IAA) is the principal natural auxin in plants and promotes cell elongation, both by increasing the extensibility and acidification of the cell walls, which, in turn, allows expansin proteins to loosen the cell walls, allowing the cells to expand in response to turgor pressure (Taiz and Zeiger, 2010). Levels of free IAA in the pericarp are high during Stage I of fruit growth, which induces rapid cell enlargement (Masia et al., 1992; Miller et al., 1987). As cell division ceases and the fruit enters Stage II of growth, the levels of IAA decline in both the endo- and mesocarp. Miller et al. (1987) found that, when IAA levels were at their highest in the seed, levels were, contrary to what was previously believed, at their lowest in the mesocarp. During Stage III IAA levels in the mesocarp increase again. It was concluded that IAA may serve as a significant growth promoter during Stages I and III, supporting cell enlargement (Masia et al., 1992; Miller et al., 1987).

Tonutti et al. (1991) found that ethylene evolution in peach fruit decreased from the beginning towards the end of Stage I, but whether or not the high concentrations of ethylene detected at the beginning of Stage I played a role in growth, was not established. They suggested that the modulation of ethylene biosynthesis was regulated by auxin at both the beginning of Stage I and at the transition between Stage II and Stage III. This correlates with the IAA levels observed by Masia et al. (1992) during the different growth stages. In contrast, during late Stage III, ethylene biosynthesis appeared to be regulated by ripening-associated events during the climacteric (Tonutti et al., 1991). Furthermore, ethylene production was highest in the epicarp (peel) and it was found that this tissue was the most efficient at converting ACC (1-aminocyclopropane-1-carboxylic acid, the precursor to ethylene) to ethylene. The

peach epicarp is very sensitive to wounding and it may be adapted to react to physical injuries by increasing ethylene biosynthesis.

In two different studies on both peaches and apricots levels of gibberellins (GAs) increased after anthesis and, in apricots, reached a maximum about 20 days after anthesis (Jackson, 1968; Jackson and Coombe, 1966). Initially, the seed contained the highest levels of GA activity and the endocarp contained the lowest levels of GA activity. During subsequent growth (from 20 days after anthesis) the levels of GA decreased in all fruit tissues – most rapidly in the endocarp and seed, and slowest in the mesocarp. GA concentration was closely correlated with the rate of cell expansion in the different fruit tissues, but not with the rate of cell division. GA activity in the endocarp declined concurrent with stone hardening, which was between 30 to 45 days after anthesis for apricots, after which the GA levels remained low (Jackson, 1968). GA activity in the apricot seeds remained high from anthesis until 35 days after full bloom (dafb) and started to decline between 35 to 60 dafb. During this time, the growth rate of the seed also declined. At 60 dafb it was only the mesocarp which still grew and this tissue, therefore, contained the highest levels of GA during this period.

## **2.4 From ovary to fruit**

The peach flower has a uni-carpellate (one carpel) ovary that contains two ovules, with one usually degenerating later (Lilien-Kipnis and Lavee, 1971). Sterling (1953) found that the ovarian tissues of ‘Imperial’ and ‘French’ prunes were meristematically active and observed that different tissue layers showed different rates and directions of cell division from full bloom onwards. Divisions in the inner epidermis were mainly anticlinal, but periclinal divisions also occurred. The inner epidermis was surrounded by a layer or two of cells that were larger than those of the former. Division activity in these layers was the same as within the inner epidermis (anticlinal). External to this layer was a zone consisting of two to four layers of cells in which cell divisions occurred in all directions. These cells formed the outer part of the tissues that would later form the stone of the fruit. These layers were further surrounded by 10 to 14 cell layers which enclosed most of the vascular strands in the fruit. The cells in this outer layer divided in all planes and eventually developed into the flesh of the mature fruit. Finally, the cell layers of the outer layer were followed by two layers of hypodermal cells in which mostly anticlinal divisions took place. Similar tissue layers (inner epidermis, outer epidermis, stone and flesh) were observed in sour cherries (Tukey and Young, 1939).

During bloom the originally single layered inner epidermis begins to divide actively by periclinal and anticlinal divisions (Sterling, 1953; Tukey and Young, 1939). The cells expand little after they have divided and are quite small in the transverse and longitudinal section. The inner epidermis continues to divide and cell sizes are reduced as the layer expands to between four to six tangential rows. Rapid development of the nucellus starts near the end of bloom following the rapid increase in ovule size

(Ognjanov et al., 1995). The time of ovule, and consequently nucellus, growth cessation closely coincides with the beginning of Stage II of growth. Disintegration of the nucellus occurs when rapid endosperm and embryo development commences. In mid and late maturing cultivars the endosperm completely disintegrates during embryo growth, while early maturing cultivars retain endosperm that accounts for 9% to 18% of the seed volume at the time of fruit maturity. Embryo development starts with the first division of the zygote 3 to 5 days after the endosperm nucleus starts to divide. Development of the embryo is very slow in the early stages of growth. In early maturing cultivars the period of rapid embryo development starts 8 weeks after anthesis, and in mid and late maturing cultivars it starts approx. 9 weeks after anthesis. The embryos of early ripening cultivars rarely grow large enough to fill the ovule, even though the embryo is morphologically complete. Peach fruit exhibit a fast development of the nucellus and endosperm, followed by a slow development of the embryo in the first phase of embryogenesis.

Within 2 weeks after anthesis the rates of cell division in the three layers that will form the stone increases rapidly (Sterling, 1953; Tukey and Young, 1939). The cells that surround the inner epidermis continue to divide periclinally to form four to six cell layers. Due to continuing transverse divisions the vertical diameter of the cells in this zone stays relatively small. The cells in the outer epidermis continue to divide in all planes leading to the formation of eight to ten cell layers with isodiametric cells within 2 weeks after anthesis. These patterns of development and division continue for several weeks after anthesis. Ragland (1934) reported that in peaches, the cells of the layers that form the stone are orientated with their longest diameters perpendicular to the surface of the stone at the margins of the two halves of the stone, but the cells are parallel to each other in the halves of the stone where they merge to form the suture (seam). Thus, a union does not form between the cells along the ventral suture of the stone, and, as a consequence, a weak area exists along the suture. The suture is thus a site of potential cleavage if the stone is exposed to external stresses.

In the layers of cells that surround the stone continuing divisions in all planes leads to the formation of the total number of cells that will form the flesh of the mature fruit (Sterling, 1953). These cells are about 43 layers thick in 'French' prunes and about 48 layers in 'Imperial' prunes. Ragland (1934) found that in peaches cell division continues longest in the epidermal and hypodermal layers of cells. This accounts for the increase in the circumference of the fruit during the early stages of growth. In contrast to cell division, which does not continue long after full bloom, cell enlargement proceeds continually from the time of flowering (Sterling, 1953).

### **3. Vasculature**

Within the peach fruit different vascular bundles supply nutrients to the different fruit parts (Jun et al., 2009). The main vascular bundles supply nutrients to the embryo, endocarp and mesocarp, respectively. The strands of the vascular cylinder that stretches from the pedicel to within the fruit terminate at the

base of the carpel (Ragland, 1934). From here the vascular cylinder branches off to form the different groups of bundles in the fruit and stone. The dorsal bundle and two ventral bundles are initiated first and supply nutrients to the endocarp. These bundles are not embedded within the “stony” portion of the stone at any point during development. The dorsal bundle lies in a shallow groove on the dorsal side of the stone (Zhang et al., 2009) (Fig. 3A). After following the grooves of the stone for two-thirds of its length, the dorsal bundle reaches the apex of the fruit (stylar end) via a more direct route. The two ventral bundles are in closer contact with the stone as they lie in two deep grooves on each side of the suture over the entire length of the stone (Fig. 3B). The ventral bundles branch very little along the basal half of the fruit, but they branch freely in the upper half. The dorsal and ventral bundles converge near the stylar end of the ovary from where they move into the style. Besides the dorsal and two ventral bundles in peaches, which lie in grooves along the stone, there are also bundles that lie partly or completely within the stone. These bundles are called ‘pit bundles’. Peaches also contain funicular bundles that lie within the stone and which feed the embryo.

The pit bundles are only initiated after the dorsal and ventral bundles have formed (Ragland, 1934). These bundles form a ring of distinct bundles that are about the same distance from the ovarian cavity as the ventral bundles, and they are embedded within the stone for various lengths (Fig. 4). Most of the vascular system of the flesh is supplied by the pit bundles. They branch at all levels and the grooved surface of the peach stone represents the points of exit of the pit bundles from the stone into the flesh (Ragland, 1934). There are usually about 10 or 12 pit bundles, but this number is subject to variation. Following the initiation of the pit bundles the funicular bundles start to form. They lie between the ventral bundles, but are slightly closer to the ovarian cavity than the former. The funicular bundles lead to the embryo and they are often so fused that they resemble a single, large bundle (Fig. 5). The funicular bundle reaches and passes into the funiculus and down to the chalazal region (the region opposite the micropyle, where the integuments fuse with the funiculus (Raghavan, 1997) of the ovule (Sterling, 1953). The same observations regarding vascular bundles were made in sour cherries, with the exception that, with cherries, the pit bundles are not embedded in the stone (Tukey and Young, 1939). Initially the growth of the embryo is supported by nutrients in the endosperm, but these materials are soon depleted (Chalmers and Van den Ende, 1975). Further needs of the embryo are then met from current assimilate transported through the vascular bundles.

#### **4. Lignification**

The endocarp of stone fruit is hardened through the process of lignification (Hatfield and Vermerris, 2001). Lignin is formed from the phenylpropanoid pathway, the end products of which are coniferyl and sinapyl alcohols. These alcohols serve as the basis for lignification whereby lignin is formed via oxidative processes which involves peroxidase and laccase enzymes. Numerous genes within the phenylpropanoid and lignin pathways are induced in peach stones concurrent with the onset of

lignification (Dardick et al., 2010). Although endocarp lignification occurs during Stage II, the cells are recognizable from early Stage I, being smaller and synthesizing a large amount of precursors of phenolic compounds (Masia et al., 1992). Because phenols are absent in the larger adjacent cells of the mesocarp their synthesis seems to be specific to the endocarp. These phenolic compounds disappear during lignification, which suggests that they might be related to the formation of lignin, as phenols are lignin precursors.

Four weeks after anthesis wall-thickening and lignification begins at the distal end of the area that will eventually form the stone (Dardick et al., 2010; Hayama et al., 2006). Lignification proceeds very slowly from the distal end towards the base of the fruit during a period of 4 weeks (Lilien-Kipnis and Lavee, 1971; Sterling, 1953). The cell walls start to thicken and contain numerous simple pits (Sterling, 1953). Lignification of the individual cells begins in the middle lamella and then continues towards the cell walls. Lignin is first deposited along the ventral suture until it reaches two thirds the length of the stone (Ryugo, 1961). Subsequently, the smooth inner lining of the ventral side starts to harden, after which the dorsal side of the stone begins to harden. As soon as the dorsal side of the stone has hardened, lignification continues radially from the inside of the stone towards the margins. At 8 weeks after anthesis the cells within the stone area reach their final size and wall thickening and lignification is visible throughout the entire area (Dardick et al., 2010; Hayama et al., 2006; Sterling, 1953).

Dardick et al. (2010) found that, when the lignin pathway was active in the peach endocarp during lignification, the flavonoid pathway was concurrently active in the mesocarp and exocarp. These pathways are competitive, because they use the same precursors which are generated by the phenylpropanoid pathway. The flavonoid pathway is involved in the synthesis of anthocyanins that give fruit their red colour, but this early induction of the pathway (during Stage II) is limited to genes that encode the enzymes involved only in the initial steps of flavonoid biosynthesis and proanthocyanidin production. Even though lignin and flavonoid biosynthesis genes are activated at the same time, the expression of the flavonoid biosynthesis genes diminishes before the endocarp hardens substantially. It is suggested that the energy resources in the fruit are partitioned so that flavonoids can accumulate before stone hardening depletes the energy and metabolic resources needed for their synthesis. The elevated activity of the lignification and flavonoid pathways could serve as another explanation for the slowdown in mesocarp growth during Stage II, as substantial energy resources are required for these processes to occur.

## **5. Gene expression during stone hardening**

Peach trees and the model plant *Arabidopsis* both belong to the clade of Rosids and share some anatomical and physiological similarities between their flowers and fruits (Tani et al., 2009). This enables application of the knowledge obtained from *Arabidopsis* to peaches. Both peach fruit and *Arabidopsis* pods originate from the carpel of the ovary and can be divided into an exocarp, mesocarp,

endocarp and embryo (Dardick et al., 2010). Differentiation of peach endocarp tissues may be regulated similarly to endocarp lignification in *Arabidopsis* as these processes share similar transcription factors.

In *Arabidopsis* the two valves of the silique (seedpod) are lignified during fruit development and breaks open at maturity to release the seeds (Roeder and Yanofsky, 2006). The valves of the silique break away/separate from each other at the valve margins due to internal forces generated when the layers of the endocarp dry out and become lignified (Tani et al., 2007). Lignification of the endocarp layers generates internal tension which causes the valves to shatter and release the seeds. Tani et al. (2007) suggest that lignification in the dehiscence zone (a layer within the valve margins) contributes to the weakening of the suture where the two valves come together. A somewhat similar process occurs in peaches during lignification of the stone. During hardening and lignification the stone gradually loses flexibility and becomes very rigid while the flesh is still tightly attached to it. Then, in the final stage of fruit growth, the continuing growth of the mesocarp generates forces that pull on the stone. If these forces are large enough, they can pull the stone apart in its weakest parts, which is generally along the suture.

Multiple studies have been conducted to identify the genes involved in endocarp lignification of both peaches and *Arabidopsis* (Dardick et al., 2010; Tani et al., 2009, 2007). In *Arabidopsis* dehiscence (splitting along a weakened margin to release seeds) is controlled by the activities of INDEHISCENT (IND), SHATTERPROOF (SHP) and FRUITFUL (FUL) genes (Seymour et al., 2008). The SHATTERPROOF-genes are MADS-box genes that are involved in the differentiation of the valve margin along which the silique breaks open (Tani et al., 2007). If the SHP genes are inactivated, the silique cannot break open and the seeds cannot be dispersed. FUL genes control the formation of the dehiscence zone (a layer within the valve margin) via its repression of SHP, which means that if FUL genes are expressed, lignification of the valve margins is prevented and as a result the silique cannot break open to release the seeds (Tani et al., 2007). Sequences that are related to FUL and SHP of *Arabidopsis* and that are involved in lignification have been identified in peach (Tani et al., 2009). These MADS-genes were PPERFFRUITFUL (PPERFUL) and PPERSHATTERPOOF (PPERSHP).

SHP genes are endocarp specific and their expression steadily declines from the earliest stages of fruit growth, while FUL expression is consistently lower in the endocarp than in the mesocarp or exocarp (Dardick et al., 2010). FUL expression does not increase in the endocarp as SHP declines. SHP genes are not actively regulated by dynamic FUL levels in the endocarp; rather, it is probably the relative ratio of FUL that enables SHP to promote endocarp differentiation. SHP gene expression declines just prior to the onset of lignification.

## 6. Split pit/ broken stones

### 6.1 Definition

The exact causes of stone breakage are not yet fully understood, but its occurrence is influenced by both genetic and environmental factors (Claypool et al., 1972; Engin et al., 2010). With peaches the stone usually splits or breaks along the suture. However, multiple breaks can also occur, and as the fruit continue to grow cavities often form in the flesh in the areas where the stone had split (these cavities sometimes contain a gummy substance) (Chatzitheodorou et al., 2004; Engin et al., 2010). The extent of splitting or breakage varies greatly among cultivars and between seasons (Sotiropoulos et al., 2010; Woodbridge, 1978).

The phenomenon seems to generally occur during or just prior to stone hardening and is most often only detected when fruit are cut open, although it can be observed as malformed fruit or even fruit that are pulled apart at the stem end in severe cases (Ragland, 1934; Woodbridge, 1978). Affected fruit are commonly larger than normal fruit, especially in cross diameter and contain a higher than normal proportion of flesh (Davis, 1933; Evert et al., 1988). There is a close relationship between the size or growth rate of fruits and the occurrence of splitting: there is a negative relationship between fruit diameter and the ability of the stone to resist external stresses – with fruit diameter over 45 mm (for peaches) during Stage II being especially vulnerable to splitting (Nakano, 2006).

Embryos of peaches with split-pit tend to abort in a high percentage of cases (Ragland, 1934). Nevertheless, the flesh can still develop to maturity as in unaffected fruits. Ragland (1934) hypothesized that the position of the vascular bundles in the stone might explain why the fruit are able to still develop normally when the embryo aborted relatively early during development. The funicular bundles, which lie close to the line of cleavage along the ventral suture, are especially vulnerable to breakage if the stone splits. As the funicular bundles feed the ovules, it makes sense that a high percentage of embryo abortions are observed in fruit with split stones, because the source of nutrients to the embryo would be cut off if the funicular bundles were damaged. On the other hand, 10 to 12 pit bundles enter the stone at points well removed from the suture, along which splitting occurs, and are, therefore, in relatively little danger of being damaged when splitting occurs. Since the pit bundles branch profusely to all parts of the flesh, growth of this portion of the fruit can proceed normally even though the stone has split. In plums, stone breakage is observed in all parts of the stone and not necessarily along the suture. If the pit bundles are embedded within the stone, as with peaches, stone breakage in plums would likely cause considerable damage to the pit bundles located in that part of the stone. The pit bundles supply the flesh with nutrients, and, therefore, it seems likely that damage to these bundles would have a negative effect on the development of the mesocarp. However, broken stones generally do not affect the development of the mesocarp and therefore, the vascular bundles of

plums might rather resemble those of sour cherries. Tukey and Young (1939) observed that, with cherries, the pit bundles are not imbedded within the stone as is the case with peaches. Split-pit of peaches could be related to the morphology of the stone (Han et al., 2015). The two ventral bundles lie in two deep grooves on each side of the suture, over the entire length of the stone, and it was found that in fruit affected by splitting these grooves were broader and deeper compared to those of intact stones. Thus, these deeper grooves rendered the suture of the stone thinner and more fragile, which led to splitting in this part.

## 6.2 Occurrence

It has been suggested that the occurrence of splitting is associated with environmental conditions or cultivation practices that cause an increased growth rate of the fruit (Davis, 1933). Environmental conditions such as low temperatures and/or freeze damage during flowering and early fruit development can increase the incidence of splitting (Claypool et al., 1972), while favourable temperatures and moisture levels as well as initial crop set also affect the occurrence of split pit (Nakano, 2006). High temperatures during the final growth phase of early ripening cultivars resulted in a higher ratio of split and shattered stones (Ryugo, 1961), because higher temperatures shorten the duration of fruit development, creating a strong pulling force by the fruit flesh on the stone (Engin et al., 2010). Furthermore, cultural practices that enhance fruit growth at the beginning of Stage II (such as thinning, nitrogen application, girdling, and excessive irrigation) also tend to increase the incidence of split stones (Ragland, 1934; Claypool et al., 1972; Nakano, 2006; Engin et al., 2010; Sotiropoulos et al., 2010).

Traditionally early maturing cultivars are more prone to split stones than later maturing cultivars (Claypool et al., 1972; Chatzitheodorou et al., 2004; Tani et al., 2007). This is because the stone hardening and final swell phases of fruit growth occur relatively close together due to the short Stage II of early cultivars. The ventral edge of the stone closes only during the late part of stone hardening, explaining why in early maturing cultivars, where the endocarp has often not completed development before the final swell begins, stones split along the suture are more prevalent (Brady, 1993). During the hardening and lignification processes of Stage II the stone starts to lose flexibility and becomes very rigid, with the flesh still tightly attached (Tani et al., 2007). In early ripening cultivars Stage III of growth commences before the attachment between the stone and the flesh has weakened. The expansion of the fruit cells during Stage III then creates internal forces that pull on the stone. If these forces are sufficiently strong they can pull the stone apart along its weakest parts, because during this stage the stone is not strong enough to withstand such external forces (Ragland, 1934; Tani et al., 2007). In contrast to this theory Han et al. (2015) argue that stone splitting is caused by an excessively large seed that exerts an outward force on the stone, rather than the pulling force of the expanding flesh, as proposed by Ragland (1934) and Tani et al. (2007). Han et al. (2015) suggest that seed development is

controlled by the embryo vascular bundle and excessive moisture and nutrient transport into the seed leads to increased turgor inside the endocarp. If this is coupled by enlarged ventral bundles, which leads to the formation of deep grooves on either side of the stone suture, fruit are more prone to splitting as the enlarging seed pushes against the stone and breaks it apart in its weakest areas, i.e. along the suture.

Water content in the stone decreases rapidly during stone hardening, and the water content decreases more in stones of unaffected- than in fruit with broken/split stones (Evert et al., 1988). As a result of the slower rate of water loss during stone hardening of affected fruit, these fruit remain vulnerable to splitting for a longer period of time. In affected fruit the failure of the water content to decrease normally during stone hardening, together with the larger than normal amount of flesh usually observed in affected fruit at the start of stone hardening, and the continued growth of the flesh during hardening would increase tension on the stone. Split-pit would occur if the stone could not withstand this tension.

### **6.3 Genetic factors**

On a genetic basis Tani et al. (2007) suggest that early formation and lignification of the dehiscence zone (the zone within the valve margin along which Arabidopsis pods break open to release the seeds) may contribute to suture weakening, and that temporal regulation of PPERFUL and PERSHP expression may have effects on the splitting process in peaches. They further reported that the extent of lignin formation differed between peach cultivars at the same stage of development. Cultivars also differ in their susceptibility to the formation of split stones. Further studies suggested that MADS-box gene regulation of fruit development and lignification could be responsible for the formation of split stones in susceptible cultivars. According to Tani et al. (2009), the genes PPERFUL and PERSHP show temporal expression patterns that leads to earlier formation of the dehiscence zone in cultivars that are susceptible to split stones compared to cultivars that are non-susceptible. Susceptible cultivars furthermore showed a larger decrease in FUL expression and a lower suppression of SHP expression compared to non-susceptible cultivars. These patterns of gene expression could lead to fast formation and lignification of the dehiscence zone along the suture, allowing separation of the two halves of the stone if there are strong pulling forces created by the growing fruit (Tani et al., 2007).

Anatomical and physiological comparisons indicate that the peach pericarp is analogous to Arabidopsis valves, because both originate from the carpel of the ovary (Tani et al., 2007). When the expression patterns of PPERFUL and PERSHP were compared in split stones susceptible versus non-susceptible cultivars, a pattern that might be relevant to split-pit formation was observed. Initially, PPERFUL in both cultivars was expressed at high levels, followed by a decrease during stone hardening, and then an increase in expression during the final stage of fruit growth. The differences between the split-pit susceptible cultivar and the non-susceptible cultivar were initially small when PPERFUL expression was high. During Stage II, when stone hardening took place and PERSHP expression decreased, the

non-susceptible cultivar had significantly lower PPERSHIP expression than the susceptible cultivar. During the final stage of fruit growth PPERFUL expression increased and was higher in the susceptible cultivar than in the non-susceptible cultivar. These findings indicate that a change in the ratio of PPERFUL/PPERSHIP expression during Stage II of growth could be an important factor that affects susceptibility to split stone formation in peaches. Tani et al. (2007) argued that an understanding of the genetic factors that influence susceptibility to splitting could provide the means for breeding resistant cultivars and for identifying molecular markers that could help improve cultivation practices that minimize the occurrence of split-pit formation in peaches.

## 7. Cultivation practices

Fruit growers aim to produce larger fruit to meet the demands of consumers, and, therefore, techniques such as fruit thinning and shoot pinching are used to promote fruit growth (Claypool et al., 1972; Davis, 1933; Engin et al, 2010; Nakano, 2006). However, such practices may also promote the occurrence of split stones, leading to reduced fruit quality. It would, therefore, be advantageous to develop new cultivars or cultivation practices to produce larger fruit without increasing the incidence of splitting.

Claypool et al. (1972) found that high soil moisture was positively correlated with splitting of peaches and suggested that this was due to higher turgidity within fruit cells under these conditions. The forces acting on the stone when splitting occurs are stronger under conditions of high turgidity. During the period when the stone is least resistant to external forces acting on it (i.e. prior to stone hardening), the timing of irrigation may be a factor in the amount of splitting that occurs. It is unlikely that splitting can be controlled effectively by controlling the timing of irrigation only, because climate and crop load also influence the occurrence of splitting, but a reduction of irrigation volumes during the sensitive period for splitting (Stage II) may contribute towards reducing the incidence of splitting. Choi et al. (2002) supported this, as they reduced the incidence of split stones in peaches with soil water management. Covering the soil with polyethylene film or applying mulch reduced the incidence of spitting from 20% in controls (no film) to 10% in treatments.

Lopez and DeJong (2007) tested the hypothesis that early spring weather conditions have a significant effect on peach fruit size and that fruit size is generally smaller when early spring temperatures are high. Low temperatures seemed to limit fruit growth potential initially, while higher temperatures during this 4-week period led to high rates of fruit development. Bergh (1990) reported that temperatures during the period of 7 to 21 dafb significantly influenced apple fruit growth. Higher temperatures were found to enhance the rate of apple fruit growth and increase the cell numbers of different regions of the fruit, because temperature affects the rate of cell division (Bergh, 1990; Greybe et al., 1988; Stanley et al., 2000). In contrast with the effect of higher day temperatures, high night temperatures were found to have a negative effect on apple growth. This could be due to greater depletion of carbohydrate substrate

overnight, which would then require a longer time the following day before a positive growth increment could be achieved (Stanley et al., 2000).

The length of the fruit development period for peaches seems to be related to early spring temperatures (Weinberger, 1948). Growing degree hour (GDH) accumulation during the first 30 dafb affects the length of the peach fruit development period (Ben Mimoun and DeJong, 1998). Fruit growth is dependent on the amount of resources available to support growth and the competition among the different plant organs for these resources (Grossman and DeJong, 1994). High heat unit accumulation during early spring increases the rate of fruit development (Lopez and DeJong, 2007). This increased rate of development leads to increased fruit growth potential, not necessarily to increased carbohydrate supply to support fruit growth. A negative correlation was found between the number of dafb until 10 days after stone hardening (defined as reference date) and heat unit accumulation for the first 30 dafb. Fruit size on the reference date was also correlated with the number of dafb until the reference date, which indicates that heat accumulation during the first 30 dafb drives fruit growth and development. High temperatures lead to high fruit development rates, but trees are not necessarily always capable of supplying enough resources at such a fast rate to support the growing fruit and maintain the potential fruit growth rate (either due to lack of resources or due to transport limitations or environmental conditions). Therefore, fruit size on the reference date was smaller in years when heat accumulation was very high, because actual fruit growth rates could not keep up with the potential growth rate. According to Lopez and DeJong (2007), stone hardening was substantially earlier when temperatures during the first 30 dafb were very high and heat units substantially more than 6000 were accumulated, compared to seasons with less than 6000 heat units. Furthermore, high temperatures during early spring decreased peach fruit size.

High nitrogen (N) levels tend to increase the levels of splitting (Claypool et al., 1972). It possibly delays stone hardening which would make the stone more vulnerable to the pulling forces of the growing pericarp and could lead to increased incidence of splitting. Saenz et al. (1997) found that N fertilization extended the fruit maturation period of 'O'Henry' peaches. They hypothesized that, since peaches accumulate most of their final dry mass during the last few weeks of growth, and if fruit maturation is delayed, the longer the period of rapid fresh and dry mass accumulation would be. This seemed to be the major reason why fruit on N-fertilized trees exhibited a higher final fruit size potential than fruit from non-fertilized tree (Saenz et al., 1997) – and larger, fast growing fruit tend to be more prone to split stones. Another theory to explain the relationship between high N levels and stone splitting is that N can increase the turgor pressure inside the fruit and promote the development of the vascular bundles (Han et al., 2015). Over-developed ventral vascular bundles lead to the formation of very deep grooves in the stone, which makes it weak and fragile. This, coupled with the increased turgor in the seed (due

to an over-developed embryo vascular bundle) causes the stones to split along the suture due to the outward force exerted on it by the enlarged seed.

Claypool et al. (1972) found that between N levels, soil moisture and crop load, the latter had the most significant effect on the occurrence of splitting. When fruit set is very low or heavy thinning is carried out, fruit size tends to be larger. As a consequence stone splitting or breakage may be higher. Early thinning increased the chance of splitting. The timing of fruit thinning is important, as fruit thinned too early tend to be large and this leads to an increased incidence of splitting (Claypool et al., 1972; Nakano and Nakamura, 2002). Subsequent studies corroborated the findings of Claypool et al. (1972). The percentage of fruit with split stones increased by 58.2% in heavily-thinned (fruit 20 cm apart) compared to moderately- (10 cm apart) or lightly-thinned (5 cm apart) trees (Drogoudi et al., 2009; Nakano, 2006). The higher incidence of split stones in fruit from heavily-thinned trees could be due to increased photosynthetic carbon-partitioning to the fruit, as there were fewer fruits present after thinning. Early thinning (15 days before stone hardening) also lead to a 22.9% increase in the occurrence of splitting compared to later thinning. This was probably because the stones were at a more sensitive stage of development, increasing vulnerability to splitting (Drogoudi et al., 2009). Due to the reduction in fruit yield caused by late thinning and the increased incidence of splitting due to early thinning, Drogoudi et al. (2009) recommended that the optimum time to thin 'Andross' peaches was during stone hardening.

The marked increase in fruit size after thinning is partly due to the removal of competition for carbohydrate supply to the developing flesh during the final swell period (Stage III) (Lott, 1932). It has been proposed that early thinning should be more effective than late thinning, because of the removal of competition between fruit for the carbohydrates that are needed by the stone during hardening. Early thinning would thus conserve carbohydrates that could be used for increased growth of the fruit that remain on the tree.

## **8. Conclusion**

The phenomenon of broken and split stones is a complex problem and a multitude of factors influence its occurrence. We cannot assume that information regarding the occurrence and causes of split stones in peaches is directly applicable to the phenomenon of broken stones in Japanese plums. Therefore, to better understand the causes of broken stones in plums, investigation to determine differences in susceptibility between early and late maturing cultivars is necessary. If there are also differences in susceptibility between early maturing plum cultivars, it will be important to determine whether these cultivars have different growth patterns, and whether differences in the direction of the pulling forces on the stone influences susceptibility to stone breakage.

In plums, where the stone usually does not split along the suture, but breaks in any part of the stone and often right at the base of the stone (personal observation), it seems improbable that the pit bundles

are embedded within the stone as in peaches. If so, and the stone breaks near the base, it will be difficult for the fruit to continue to grow, as the nutrient supply would be cut off. Therefore, in plums, as in cherries, the pit bundles might not traverse the stone. It is unclear where these pit bundles start to branch after entering the fruit, but it seems that they are not damaged when the stone breaks. If this were the case, the flesh supplied by these bundles would likely show altered growth due to lack of nutrient supply, similar to the embryo abortion observed after stone splitting in peaches (due to damage to the funicular bundles). Consequently, it might be useful to investigate the vasculature of Japanese plums.

## 9. References

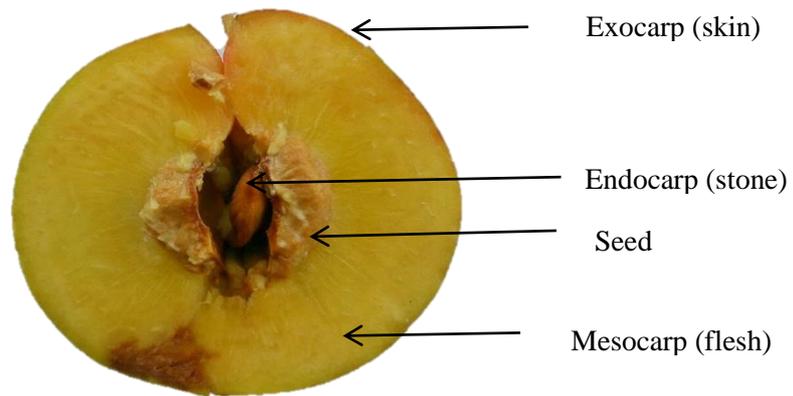
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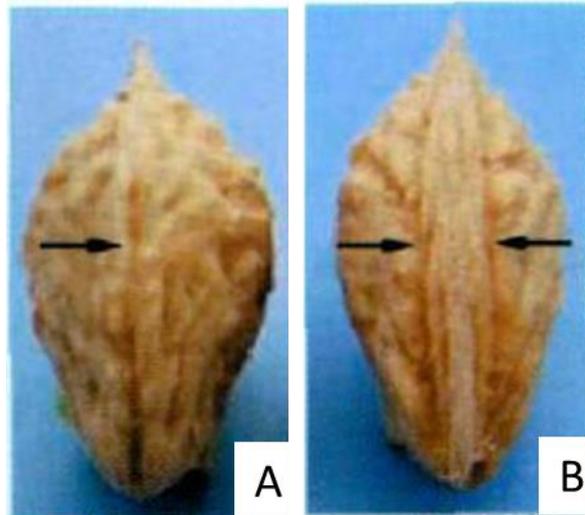
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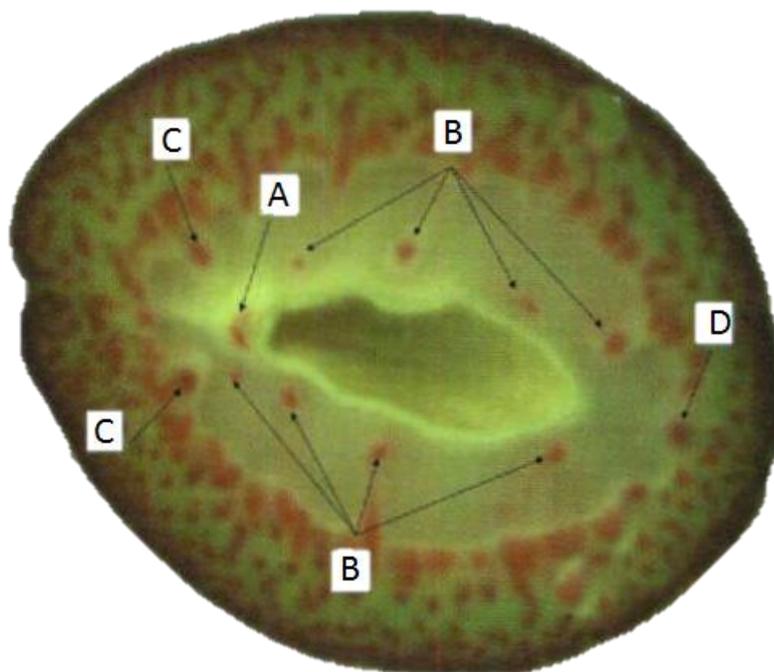
**Fig. 1.** Split pit in peach fruit, indicating how the stone has split along the suture.



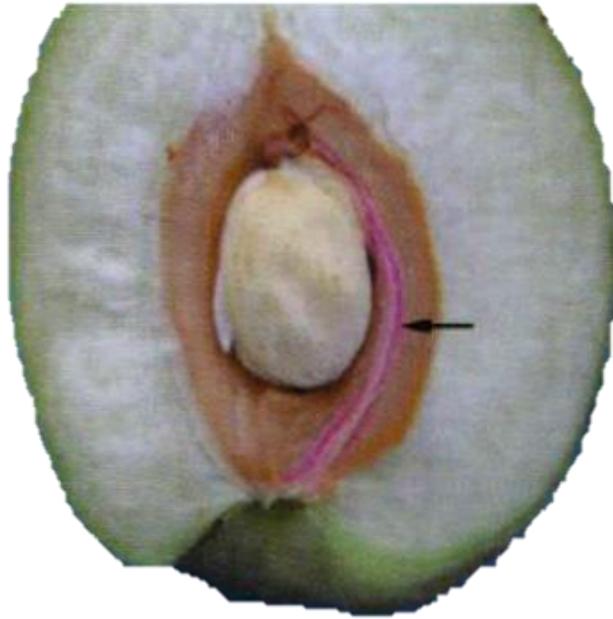
**Fig. 2.** A broken stone in the Japanese plum cultivar Laetitia – unlike what is observed with peaches, the stone did not split along the suture, but is broken near the stem end of the fruit (personal observation).



**Fig. 3A.** The dorsal bundle, which supplies the endocarp and lies in the groove opposite the suture. Fig 3B. The two ventral bundles, which supply the endocarp and lies in grooves on both sides of the suture (Zhang et al., 2009).



**Fig. 4.** Distribution of the vascular bundles of a peach fruit in cross-sections (66 dafb). A. Funicular bundle; B. Pit bundles; C Ventral bundles; D. Dorsal bundle (Zhang et al., 2009).



**Fig. 5.** Vertical section of a peach fruit, with the arrow pointing to the funicular bundle (Zhang et al., 2009).

## PAPER 1

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# The effect of climate and growing area on the incidence of broken stones in 'Laetitia' plums (*Prunus salicina* Lindl.)

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### ABSTRACT

Stone breakage affects both peaches and plums and leads to significant financial losses for the agricultural industry as affected fruit have to be marketed as Class 2, thus obtaining lower prices on international markets. As no information exists on the development of broken stones in Japanese plums the aim of this study was to determine whether climatic differences between growing areas would affect the incidence of broken stones and whether certain parameters, measured throughout the growing season, could give an indication of the incidence of stone breakage to be expected at harvest. This would enable producers to determine their marketing strategies early in the season. Stone breakage was observed as soon as stone hardening was initiated. At the start of stone hardening the parts of the stone that are still 'soft' are not strong enough to resist pulling forces of the growing mesocarp on it and the stone is subsequently pulled apart. Regression analysis indicated that lengthwise growth of the fruit, fresh weight of the endo- and mesocarp, minimum orchard temperature and orchard night temperature, and relative humidity early in the growing season (especially between 42 and 52 days after full bloom) could possibly be used to predict the incidence of broken stones at harvest. However, data from more seasons should be included in order to make the model more robust.

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### **Keywords:**

Broken stones; Fruit growth; 'Laetitia'; Plums; Temperature

## 1. Introduction

Broken stones is a phenomenon that affects peaches and plums and can lead to significant financial losses as affected fruit have to be marketed as Class 2 (Organisation for Economic Co-operation and Development, 2002). In plums, we have observed that the endocarp can break apart anywhere and often in multiple areas. Environmental conditions, such as temperature, as well as cultivation practices that result in increased fruit growth rates, are associated with split stones in peaches (Claypool et al., 1972; Davis, 1933; Engin et al., 2010).

The growth of stone fruit follow a three-stage (I, II and III), double-sigmoidal growth curve (Ragland, 1934; Tukey, 1936). Stage I consists of a period of rapid fruit development. Stage II comprises a period of slow mesocarp growth during which the embryo grows and the stone (endocarp) hardens (Chalmers and Van den Ende, 1975). Stage III, often referred to as “final swell”, is characterized by rapid mesocarp development until fruit ripening (Tukey, 1936; Tani et al., 2007). Furthermore, the length of the different growth stages varies between cultivars, species and seasons (Tukey, 1936). Early maturing cultivars have a shorter Stage II, and they continue to enlarge during this stage, which is in contrast to the longer Stage II observed for late maturing cultivars during which fruit growth slows down. Consequently, early maturing peach cultivars are usually more prone to split stones than later maturing cultivars (Claypool et al., 1972; Chatzitheodorou et al., 2004; Tani et al., 2007). Stage II and Stage III of fruit growth, therefore, occur relatively close together in early maturing cultivars, and if the stone is not yet hard enough to resist the forces exerted on it by the mesocarp, it can be pulled apart (Ragland, 1934; Tani et al., 2007).

During Stage I fruit increases in size due to cell division, and by 28 days after full bloom (dafb) the final cell numbers in the mesocarp and endocarp of peaches are fixed (Masia et al., 1992; Ragland, 1934). Subsequent fruit growth is mainly due to cell enlargement in the mesocarp (Ragland, 1934; Zanchin et al., 1994). Temperature affects the rate of cell division during Stage I of fruit growth (Stanley et al., 2000). Since cell division and enlargement determine final fruit size (Scorzal et al., 1991), temperature thus has a significant effect on fruit size. Furthermore, it is known that larger peach fruit (especially in cross diameter) are more prone to split stones compared to smaller fruit (Davis, 1933; Evert et al., 1988; Nakano, 2006).

Hence, our hypothesis was that relatively high (favourable) temperatures during Stage II of fruit growth (when the stone is supposed to harden), cause mesocarp growth to continue, resulting in the hardening stone being pulled apart by the fast growing flesh. The aim of our study was thus to establish the effect of the climate and/or temperature differences between growing areas on the occurrence of broken stones in Japanese plums, via its influence on the rate of fruit growth.

## 2. Materials and Methods

Two climatically different growing areas, namely Stellenbosch and Robertson, were used to sample ‘Laetitia’ plums. ‘Laetitia’ plums are highly susceptible to broken stones. In the 2013/2014 season fruit were sampled from three farms per area. In Robertson sampling occurred at Lucerne (S33°51.180’ E19°58.035’), Goedemoed (S33°50.895’ E19°57.966’) and St Kilda (S33°51.635’ E19°58.840’), and in Stellenbosch, from farms in the Devonvalley area, namely Waterkloof (S33°54.955’ E18°47.713’), Dwars-in-die-Weg (S33°55.627’ E18°48.668’), and The Firs (S33°54.637’ E18°49.186’).

In the 2014/2015 season two additional farms per area were included to make the model more robust. In the Robertson/Bonnievale area these were Edendale (S33°56.812’ E20°7.063’) and Concordia (S33°55.063’ E19°59.494’). In Stellenbosch, the two additional farms were Welgevallen (S33°56.83’ E18°52.277’) and Bon Esperance (S33°53.202’ E18°50.979’).

### 2.1 Site description

See Table 1 for a description of rootstocks, orchard age, planting distance, training system, orchard layout and full bloom dates.

### 2.2 Experimental layout

A randomised design was used with the farms in each growing area serving as replicates. In the 2013/2014 season 60 fruit of uniform size were sampled from 10 trees, selected at random (30 fruit from each side of the tree) on each sampling date, from each farm. This was done twice weekly from 28 dafb until the end of stone hardening (approximately 40 dafb), after which sampling was done once a week until the commercial harvest date.

In the 2014/2015 season 30 fruit per farm were sampled from 10 trees, selected at random (15 fruit from each side of the tree) on each sampling date. Fruit were sampled twice a week from 21 dafb until 52 dafb, which was when stone hardening was complete (the stones could not be cut in half anymore). All farms followed standard cultivation practices.

### 2.3 Measurements

On each sampling date the fruit were stored at 4 °C until the following morning. The diameter (mm) and length (mm) of each fruit was measured using a digital calliper (Mitutoyo, Japan). Fruit were then cut open and examined for the presence of broken stones. Of the 60 fruit sampled per farm in the 2013/2014 season, 12 were dissected into their individual endo- and mesocarps. Each endo- and

mesocarp was weighed to determine their fresh weight (g). In the 2014/2015 season, six of the 30 sampled fruit were used for dissection.

Rate of change in the diameter, length and fresh weight of the individual endo- and mesocarps were calculated by dividing the difference in measured growth between sampling dates by the number of days between sampling dates to obtain rate of change day<sup>-1</sup>.

## 2.4 Temperature calculations

‘Tiny Tag’(TGP-4500) (Gemini Data Loggers Ltd, Chichester, West Sussex, UK) temperature and relative humidity (RH) loggers, placed inside logger shields, were installed in each orchard to measure RH and temperature for the duration of the season. Measurements were logged every hour. The minimum and maximum temperature and RH were calculated for each day. In addition, average night temperature and RH (measured between 7 pm and 6 am) and average day temperature and RH (measured between 7 am and 6 pm) were calculated. Growing degree days (GDD) were calculated for each farm using 5, 7.5 and 10 °C as base temperatures. Rumayor- Rodríguez (1995) used 5 °C as a base temperature for the growth of Japanese plums, while a base temperature of 7 °C is often used for peaches (Day et al., 2007; Marra et al., 2002) and 10°C is used for apples (Stanley et al., 2000). GDD for each day was calculated using the following formula:

$$GDD = \frac{(Minimum\ temperature + Maximum\ temperature)}{2} - Base\ temperature$$

## 2.5 Statistical Analysis

To determine whether areas and/or seasons differed from one another in terms of the incidence of broken stones at harvest, a one-way fixed effect analysis of variance was performed. Since neither seasons nor areas differed from each other and in order to build an accurate and robust prediction model the data of the two seasons and the two areas were pooled. ‘Best’-subset regressions were performed in order to determine whether the incidence of broken stones at harvest could be predicted by the different variables measured between 21 and 52 dafb. Separate regressions were performed for 21, 24, 28, 31, 35, 38, 42, 45, 49 and 52 dafb. The variables included in the regressions were: average fruit diameter (mm), average fruit length (mm), average fresh weight (g) of the endocarp and mesocarp, respectively, rate of fruit diametric growth (mm day<sup>-1</sup>), rate of fruit length extension (mm day<sup>-1</sup>), and rate of fresh weight change of the endocarp and mesocarp (g day<sup>-1</sup>), respectively. ‘Leave one out’ cross validation was then performed to test the ability of the models obtained for each sampling date to predict

the incidence of broken stones at harvest. In addition, a one-way analysis of variance was performed for each of the variables that appeared in the models obtained from the regressions. The analyses were performed using Statsoft STATISTICA version 12 (Statsoft, Inc., 2011).

### 3. Results

#### 3.1 Incidence of broken stones

In both seasons the incidence of broken stones increased significantly over time (Fig. 1). The first signs of stone breakage were observed at 31 dafb in the 2013/2104 season, while it was only observed at 35 dafb in the 2014/2015 season. This coincided with the onset of stone hardening, which was when the stones started to show resistance when cut open. Stone breakage reached a maximum between 42 and 45 dafb, which was when the stones were very close to completion of hardening. There were no significant differences in the incidence of broken stones at harvest between seasons or growing areas (Table 2).

#### 3.2 Regression analysis

Table 3 shows a summary of the best regressions for each sampling date and the 'leave one out' cross validation  $R^2$ , which indicates the accuracy with which the model could predict the percentage of broken stones on the optimum harvest date. Seasonal and orchard data were pooled and the analysis was performed on the parameters measured between 21 and 52 dafb (when stone hardening was completed). The 'leave one out' cross validation  $R^2$  values were very low (except for 42 dafb), indicating that none of the models could accurately predict the incidence of stone breakage. This is due to the small data set and it is clear that more seasons and/or more farms need to be included in the study in order to build a more accurate and robust model. However, from Table 3 it is evident that data obtained closer to the completion of stone hardening (from 42 until 52 dafb) constantly gave  $R^2$  values higher than 65% and could most likely be used in future to predict the percentage broken stones at harvest. Consequently, only parameters that occurred most frequently in the 20 'best' regressions for each sampling date between 42 and 52 dafb were analysed further in this study to determine if they could give an indication as to why broken stones occurred and if there were value in using them as predictors of the percentage broken stones at harvest. Separate ANOVA's were performed for each of these parameters. Table 4 gives a summary of the parameters that made a statistically significant contribution to the explanation of the variation in the data and that were included in the 20 'best' regressions for each of the sampling dates from 42 to 52 dafb.

### 3.3 Rate of change in fruit length (mm day<sup>-1</sup>)

For the 42 dafb measurement, the rate of change in fruit length was only determined in 2014/2015 at 42 dafb and the parameter appeared 11 times in the 20 ‘best’ regressions (Table 4). The regression results at 45 and 52 dafb represent data from the 2013/2014 and 2014/2015 seasons. Although two seasons’ data were used, the rate of change in fruit length appeared only three times in the 20 ‘best’ regressions performed at 45 dafb, compared to 20 times at 52 dafb.

In the 2013/2014 season, daily lengthwise growth of the fruit decreased significantly from 31 dafb until 35 dafb and thereafter grew at a constant rate of approx. 0.5 mm day<sup>-1</sup> (Fig. 2A). In contrast with the 2013/2014 season, the rate of lengthwise fruit growth was very slow in the period between 24 and 52 dafb, and did not differ significantly between the sampling dates in the 2014/2015 season (Fig. 2B). Together with the relatively slow rate of lengthwise growth in the 2014/2015 season, the average rate also varied greatly between fruit on each sampling date. Figure 2 shows that the rate of lengthwise fruit growth at 42, 45 and 52 dafb did not differ significantly from each other or from the dates preceding them in the respective seasons. Therefore, there is no physiological explanation why this variable was selected by the 20 ‘best’ regression models to predict the percentage broken stones on the optimum harvest date. This result strengthens the finding of the ‘leave one out’ cross validation that more seasons and/or more farms need to be included in the study in order to build a more accurate and robust prediction model.

### 3.4 Rate of change in the fresh weight accumulation of the endocarp (g day<sup>-1</sup>):

The regression analysis indicated that the rate of change in the fresh weight of the endocarp determined at 42 dafb could be used to predict the incidence of broken stones at harvest (Table 4). However, the values obtained at 42 dafb represent only data from the 2014/2015 season, as sampling was not performed on this date in the 2013/2014 season. Upon further investigation the ANOVA results for the two seasons individually indicated that there were no significant changes in the rate of fresh weight accumulation of the endocarp from 31 to 52 dafb (Fig. 3A and B). However, the average fresh weight of the endocarps increased steadily and sometimes significantly between 28 to 52 dafb for both seasons (Fig. 4A and B). In the 2013/2014 season there was a lag in fresh weight accumulation of the endocarp between 35 to 45 dafb (Fig. 4A), and in the 2014/2015 season this lag was more pronounced and happened slightly later, i.e. between 42 to 49 dafb (Fig. 4B). In both seasons the lag in endocarp fresh weight accumulation happened when the percentage broken stone manifestation also reached a maximum (Fig. 1).

### 3.5 Minimum orchard temperature (°C)

The regression analysis indicated that minimum orchard temperature at 42 dafb could be used to predict the incidence of broken stones at harvest (Table 4). However, the values obtained at 42 dafb represent only data from the 2014/2015 season, as sampling was not performed on this date in the 2013/2014 season. In both seasons the average minimum orchard temperature increased with an increase in time, but this increase was not statistically significant in the 2013/2014 season (Fig. 5A). There were significant differences in minimum temperatures between the sampling dates in the 2014/2015 season (Fig. 5B); however, at 42 dafb the minimum temperature did not differ significantly from the dates just prior to or after it. Consequently, there is no physiological reason at this stage why the average minimum orchard temperature at or just before 42 dafb could predict the percentage broken stones at harvest.

### 3.6 Minimum orchard relative humidity (%)

The minimum average orchard RH appeared 11 times in the 20 ‘best’ regressions at 45 dafb (Table 4). In the 2013/2014 season there were no significant differences in minimum RH between sampling dates (Fig. 6A). Nevertheless, a rapid increase in RH was observed between 38 and 45 dafb. In the 2014/2015 season differences in RH were observed between sampling dates, but differences were not always significant (Fig. 6B). It cannot be explained why minimum RH would be included in the regression model, because the values for the two seasons are contradictory and differ substantially on the sampling date (45 dafb) in which it was included in the model (48.71% in the 2013/2014 season versus 37.74% in the 2014/2015 season). Together with the low ‘leave one out’ cross validation  $R^2$  value it is clear that data from more seasons are needed in order to increase the robustness of the model.

### 3.7 Average orchard night temperature (°C)

At 45 dafb the regression analysis indicated that average night temperature appeared 19 times in the 20 ‘best’ regressions (Table 4). For both seasons there was a gradual increase in the average orchard night temperatures over time, but this difference was not always significant between sampling dates (Fig. 7A and B).

### 3.8 Average fruit length (mm)

The regression analysis indicated that average fruit length at 49 dafb could predict stone breakage at harvest (Table 4). It is important to note that these values represent only data from the 2014/2015 season, as sampling was not performed at 49 dafb in the 2013/2014 season. Since the significance level ( $p =$

0.056) for average fruit length was not very high together with the fact that this parameter only appeared 5 times in the 20 ‘best’ regressions, it is questionable if this parameter can be used to accurately predict stone breakage at harvest. In both seasons average fruit length increased over time (although not statistically significantly between all the dates) (Fig. 8A and B). However, there were distinct differences in the growth curves between the two seasons. In the 2014/2015 season growth followed a clear single sigmoidal growth curve in the period when measurements took place (Fig. 8B). In contrast, the growth curve of the 2013/2014 season showed no lag phase in growth, but this might be because sampling in the 2013/2014 season only started at 28 dafb (Fig. 8A). Also, fruit were much longer (by approx. 8 mm) at 28 dafb in the 2013/2014 season compared to the 2014/2015 season. At 49 dafb, when regression analysis indicated that fruit length could be used to predict the incidence of broken stones at harvest, fruit length were similar for both seasons and did not differ significantly from the dates preceding or following it (Fig. 8A and B).

### **3.9 Average orchard night relative humidity (%)**

At 49 dafb, average night RH formed part of the 20 ‘best’ regressions to predict the incidence of broken stones at harvest (Table 4). This only represents data from the 2014/2015 season, as sampling was not done on this date in the 2013/2014 season. Furthermore, this variable only appeared 7 times in the 20 ‘best’ regressions. In both seasons average night RH generally followed a decreasing trend over the sampling period (Fig. 9A and B). However, no significant differences were found in the average night RH between sampling dates in the 2014/2015 season (Fig. 9B) and, therefore, it cannot be explained why this variable would be able to predict the incidence of broken stones at harvest. Data from more seasons will have to be included in order to validate these results.

### **3.10 Average fresh weight of the mesocarp (g)**

At 52 dafb a regression analysis indicated that the average fresh weight of the mesocarp could be used to predict the incidence of broken stones at harvest (Table 4). However, the parameter only appeared twice in the 20 ‘best’ regressions. In both seasons the average fresh weight of the mesocarp increased over time, however, not always significantly between dates (Fig. 10A and B). Similar to fresh weight accumulation of the endocarp (Fig. 4), fresh weight accumulation of the mesocarp also experienced a lag phase between 35 to 38 dafb in the 2013/2014 season and between 42 and 49 dafb in the 2014/2015 season (Fig. 10A and B).

### 3.11 Rate of change in the fresh weight accumulation of the mesocarp ( $\text{g day}^{-1}$ )

At 52 dafb, a regression analysis indicated that the rate of change in the fresh weight accumulation of the mesocarp could be used to predict the incidence of broken stones at harvest (Table 4). However, the parameter only appeared 4 times in the 20 ‘best’ regressions. In both seasons there were no significant differences in the rate of change in the fresh weight accumulation of the mesocarp over time (Fig. 11A and B). Therefore, there is no physiological reason that can explain why this variable occurred in the regression models at 52 dafb.

## 4. Discussion

### 4.1 Incidence of broken stones

The increase observed in the incidence of broken stones over time (Fig. 1A and B) was most likely due to a combination of continued fruit growth (Fig. 8A and B) during the onset of stone hardening. In peaches, stone splitting generally occurs during or just before stone hardening (Ragland, 1934; Woodbridge, 1978). Ragland (1934) and Tani et al. (2007) found that the hardening peach stones are not able to resist the forces of the rapidly expanding flesh that pulls on it and are subsequently pulled apart at its weakest parts. Our results indicate that the same holds true for Japanese plums, as the onset of stone breakage in our study coincided with the start of stone hardening (Fig. 1A and B).

### 4.2 Average fruit length and rate of change in in fruit length per day

Peaches affected by split stones are commonly larger than unaffected fruit (Davis, 1933; Evert et al., 1988). Fruit that show a high increase in growth during the second growth phase are more vulnerable to splitting, because during this time the stone is not yet able to resist the external forces of the rapidly expanding mesocarp and can be pulled apart (Claypool et al., 1972; Nakano, 2006; Tani et al., 2007). We observed that the stones generally broke near the stem end of the plum fruit. In peaches stone lignification begins at the styler end of the fruit by 4 weeks after anthesis and proceeds very slowly towards the stem end over a 4 week period (Dardick et al., 2010; Hayama et al., 2006; Lilien-Kipnis and Lavee, 1971; Sterling, 1953). This was corroborated for Japanese plums in Paper 2. Therefore, by 31 dafb (in the 2013/2014 season) and 35 dafb (in the 2014/2015 season), when the first broken stones were observed, lignification of the stone had just started. It is therefore possible that rapid lengthwise extension at 45 dafb, as observed in this study (Fig. 8), pulled the stone apart at the interface between the parts of the stone that have started to lignify and the parts that were still ‘soft’. However, since lengthwise growth per day at 42 dafb in both seasons did not differ from the dates preceding or following it (Fig. 2), it could not provide an explanation as to why it was chosen by the regression to predict broken stones at harvest. The rate of change in fruit length  $\text{day}^{-1}$  declined in the 2013/2014

season and remained constant in the 2014/2015 season, but average length in both seasons increased. This may be due to the rate of growth which did not differ between sampling dates, but average length increased with the same increment on each sampling date. Hence, more seasons are needed to establish if this parameter can accurately predict broken stones at harvest.

#### **4.3 Fresh weight of the endo- and mesocarp**

Lignin forms chemical bonds with the hemicellulose components of the wall during the deposition of lignin in the cell walls, and water is gradually eliminated from the cells (Boerjan et al., 2003). Water content of the peach stone decreases rapidly during stone hardening, and decreases more in stones of unaffected fruit than in fruit with broken/split stones (Evert et al., 1988). The slower rate of water loss during stone hardening of fruit with broken/split stones makes these fruit vulnerable to splitting for a longer period of time. In affected fruit the failure of the water content to decrease normally during stone hardening, together with the larger than normal amount of flesh usually observed in affected fruit, and the continued growth of the flesh during stone hardening, would increase tension on the stone. Split-pit or stone breakage would occur if the stone could not withstand this tension. Furthermore, a study on apricot stones found that when the stones had a high moisture content less force was required to break the stones (Vursavus and Özgüven, 2004).

Our results indicated that the fresh weight of both the endocarp and mesocarp were at a maximum at 52 dafb, which was when these variables were included in the 20 'best' regressions (Fig. 4 and Fig. 10). One would expect that the rate of fresh weight accumulation of the endocarp would slow down during the lignification process, however, this was not the case in the 2014/2015 season and it slowed down only slightly in the 2013/2014 season. Thus, our results are in agreement with the aforementioned observations that stones with higher moisture content tend to be more susceptible to breaks, especially if this is coupled with increased growth of the mesocarp, which was observed in terms of lengthwise fruit growth (Fig. 8A and B). Therefore, it is suggested that data from more seasons needs to be included in the study to verify these relationships

#### **4.4 Minimum average orchard temperature and average orchard night temperature**

In this study stone breakage was observed as soon as stone hardening was initiated (Fig. 1A and B). Lopez and DeJong (2007) found a negative correlation between temperatures during the first 30 dafb and the time between full bloom and stone hardening of peaches, i.e. lower temperatures during this period lengthened the time needed for the stone to harden completely. Our results seem to be in agreement with their findings as stone hardening started approx. 4 days later in the 2014/2015 season

(which experienced cooler minimum orchard temperatures and night temperatures) compared to the 2013/2014 season (Fig. 5 and 7).

Furthermore, it was found that high temperatures during early spring lead to smaller fruit size (Lopez and DeJong, 2007). Smaller fruit size under high temperature conditions is most likely due to increased respiratory demands by the growing organs, coupled with competition between vegetative organs and fruit (Grossman and DeJong, 1995; 1994). Peaches affected by split stones are commonly larger in cross diameter compared to unaffected fruit (Davis, 1933; Evert et al., 1988). Fruit that have increased fruit growth during the onset of stone hardening are more vulnerable to splitting, because during this time the stone has not yet hardened completely and is not able to resist the external forces of the rapidly expanding mesocarp and can thus be pulled apart (Claypool et al., 1972; Nakano, 2006; Tani et al., 2007). If the same holds true for plums, lower temperatures during early spring may contribute to a higher incidence of stone breakage, because the lower temperatures will cause less competition between the growing plant organs and will aid in the development of larger fruit. However, at the end of the sampling period (52 dafb) there were no clear differences in fruit size (expressed as average length) between the two seasons (Fig. 8).

However, in this study a higher, though not statistically significant, percentage of broken stones was observed in the 2013/2014 season which experienced warmer temperatures during stone hardening. Therefore, the current plum data do not support the peach data that less broken stones could be expected after a warmer spring. However, these results agree with our results from Paper 2, which also showed a higher incidence of broken stones in the 2013/2014 season. A study comparing a 'Stoneless' prune cultivar (a cultivar that forms an incomplete endocarp) to a 'normal' cultivar indicated that the 'Stoneless' cultivar had decreased and abnormal endocarp formation (significantly lower endocarp dry weight compared to the 'normal' cultivar) (Callahan et al., 2009). Endocarp development differed between seasons – in years with higher spring temperatures, the fruit tended to contain more complete stones (Dardick and Callahan, 2014). Hence, higher spring temperatures may also lead to the formation of more complete endocarps (more endocarp cells) in plum cultivars susceptible to broken stones. A higher number of endocarp cells would certainly increase the density (and hence, rigidity) of the stone, and if this was coupled with rapid radial growth, stone breakage could occur. Since the minimum average orchard temperature and the average orchard night temperature in the 2013/2014 season were higher than that of the 2014/2015 season (Fig. 5 and Fig. 7) it is suggested that 'Laetitia' was able to form a more complete stone, and hence, more stone cells which lignified to form denser, more rigid stones. Such a scenario would create a more pronounced interface between already hardened and less hardened stone parts during Stage II of fruit growth, which, in combination with rapid increases in radial fruit growth during Stage II, might serve as an explanation as to why 'Laetitia' had a higher percentage of broken stones in the 2013/2014 season compared to the 2014/2015 season.

#### 4.5 Minimum relative humidity and average night relative humidity of the orchard

Claypool et al. (1972) observed a higher incidence of stone splitting in peaches under conditions that led to high turgidity within the fruit cells. They suggested that the forces acting on the stone when splitting occurs would be greater under conditions of high turgidity. Under conditions conducive to high cell turgor (e.g. high RH), there is increased tension in the cell walls (Konstankiewicz and Zdunek, 2001; Oey et al., 2007). The expanding flesh exerts strong pulling forces on the stone and can pull the stone apart if it has not hardened completely (Claypool et al., 1972; Nakano, 2006; Tani et al., 2007). Consequently, when the stone is not completely hard, conditions that induce high cell turgor, such as high RH and high soil moisture, could lead to a higher incidence of stone breakage or splitting. However, the results from this study (Fig. 6 and Fig. 9) are contradictory, since, except for the average minimum orchard RH in the 2013/2014 season (Fig. 6A), the RH in the orchard was relatively low at 45 dafb (Fig. 6B) and at 49 DAFB (Fig. 9A and B) and, therefore, data from more seasons will have to be included to verify these observations.

#### 5. Conclusions

In this study we set out to determine whether climatic differences between growing areas would affect the incidence of broken stones and whether certain parameters, measured throughout the growing season, were related to the incidence of stone breakage at harvest.

The same pattern in the incidence of broken stones was observed in both seasons and in both growing areas – stone breakage was observed as soon as stone hardening was initiated. At the start of stone hardening the parts of the stone that were still “soft” were not strong enough to resist pulling forces of the growing mesocarp on it and the stone is subsequently pulled apart. We found that the stones generally broke near the apical end of the fruit, which is the last part of the stone to harden (lignification proceeds from the distal end towards the apical end of the stone). However, no significant differences were observed in the incidence of broken stones between areas or seasons. Regression analysis indicated that lengthwise growth of the fruit, fresh weight of the endo- and mesocarp, minimum orchard temperature and orchard night temperature, and RH early in the growing season (especially between 42 and 52 dafb) could possibly be used to predict the incidence of broken stones at harvest. However, data from more seasons must be added (to add more seasonal variation in the data) and more farms or areas must be included in the study. This will make these models more robust. Consequently no predictions or recommendations can be made at this stage. It would be beneficial for producers to use easily measurable parameters (such as fruit length and orchard temperature) early in the season to predict the incidence of broken stones that might be expected at harvest in order to better plan their marketing strategies in seasons when a high percentage of broken stones (and thus more Class 2 fruit) are expected.

Hopefully, repeating this trial over more seasons will produce an accurate model to enable producers to do so.

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**Table 1**

Summary of farms, rootstocks, orchard age, planting distance, training system, orchard layout and full bloom dates.

Area	Farm	Rootstock	Plant year	Planting distance	Training system	Cross-pollinator	Full bloom date
Robertson	Concordia	'Marianna'	1998	4m x 2m	Palmette	1 row 'Laetitia' and 1 row 'Songold'	15 Sept. 2014
	Edendale	'Viking'	2010	4m x 2m	Palmette	Inter-planted with 'Songold'	28 Sept. 2014
	Goedemoed	'GF3'	2010	5m x 1.5m	V-Haag	1 row 'Laetitia' and 1 row 'Larry Anne'	22 Sept. 2013 14 Sept. 2015
	Lucerne	'Marianna'	1992	4m x 2m	Palmette	1 row 'Laetitia' and 2 rows 'Songold'	30 Sept. 2013 14 Sept. 2015
Stellenbosch	Bon Esperance	'Marianna'	2003	4m x 1.5m	Palmette	1 row 'Laetitia' and 1 row 'Songold'	9 Sept. 2014
	Dwars-in-die-weg	'Marianna'	2003	4m x 2m	Palmette	Inter-planted with 'Songold'	3 Oct. 2013 9 Sept. 2014
	The Firs	'FG967'	1994	4m x 1.5m	Palmette	1 row 'Laetitia' and 2 rows 'Songold'	3 Oct. 2013 9 Sept. 2014
	Waterkloof	Peach seedling	-	4m x 1.5m	Palmette	1 row 'Laetitia' and 1 row 'Songold'	4 Oct. 2013 9 Sept. 2014
	Welgevallen	'Marianna'	1998	4m x 1.25m	Palmette	1 row 'Laetitia' and 1 row 'Songold'	9 Sept. 2014

**Table 2**

ANOVA table indicating the differences in the incidence of broken stones at harvest in 'Laetitia' plums sampled in two production areas, Stellenbosch and Robertson, in the 2013/2014 and 2014/2015 seasons.

<b>Source of variation</b>		<b>Broken stones (%)</b>
Year	2013/2014	22.78
Year	2014/2015	19.00
<b>Pr &gt; F</b>		<b>0.593</b>
Area	Robertson	27.87
Area	Stellenbosch	13.91
<b>Pr &gt; F</b>		<b>0.065</b>

**Table 3**

Summary of the 20 ‘best’ regressions for the prediction of the percentage of broken stones on the optimum harvest date, for each sampling date respectively. The ‘leave one out’ cross validation  $R^2$  value indicates the accuracy with which the model could predict the percentage of broken stones determined at harvest. Seasonal (2013/2014 and 2014/2015 seasons) and orchard ( $\geq$  three orchards each in Robertson and Stellenbosch per season) data were pooled for the respective dates.

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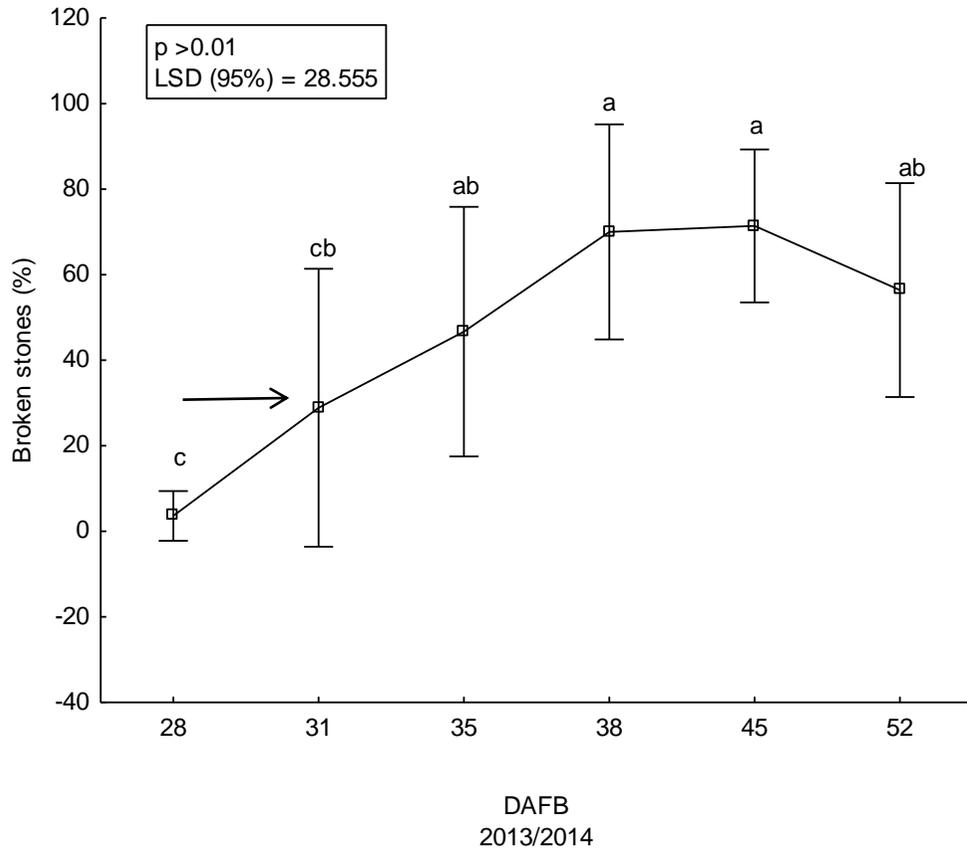
<b>DAFB</b>	<b><math>R^2</math></b>	<b>Cross-validation <math>R^2</math></b>
21	0.46	0.11
24	0.78	0.43
28	0.65	0.20
31	0.24	0.04
35	0.61	0.41
38	0.24	0.06
42	0.92	0.85
45	0.75	0.44
49	0.67	0.46
52	0.81	0.58

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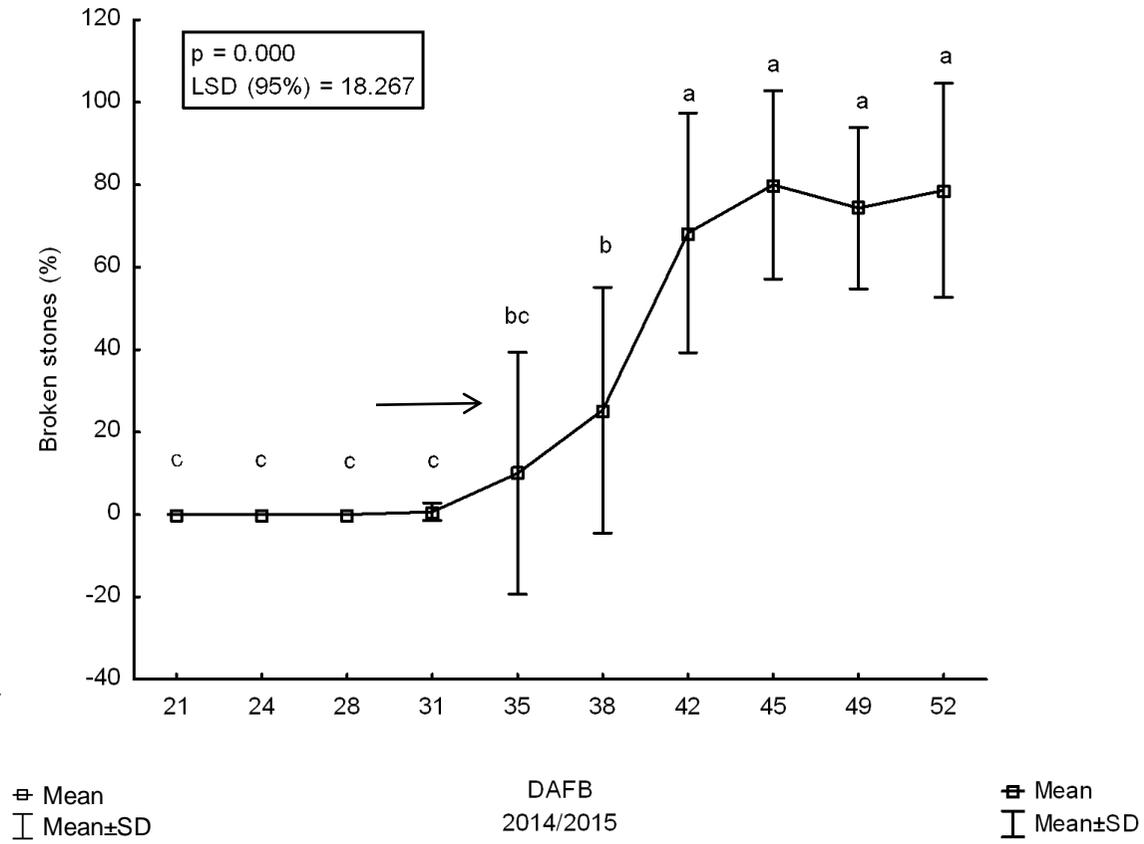
**Table 4**

Regression table for the prediction of percentage broken stones on the optimum harvest date. The ‘leave one out’ cross validation  $R^2$  value indicates the accuracy with which the regressions could predict the percentage of broken stones determined at harvest. Seasonal (2013/2014 and 2014/2015 seasons) and orchard ( $\geq$  three orchards each in Robertson and Stellenbosch per season) data were pooled for the respective dates. Only the dates with  $R^2$  values above 0.65 are shown.

<b>42 days after full bloom: <math>R = 0.960</math>; <math>R^2 = 0.921</math>; ‘leave one out’ cross validation <math>R^2 = 0.85</math></b>		
Variable	p-value	Number of times in the 20 ‘best’ regressions
Rate of change in fruit length (mm day <sup>-1</sup> )	0.0186	11
Rate of change in fresh weight of endocarp (g day <sup>-1</sup> )	0.0010	15
Average minimum orchard temperature (°C)	0.0004	8
<b>45 days after full bloom. <math>R = 0.868</math>; <math>R^2 = 0.754</math>; ‘leave one out’ cross validation <math>R^2 = 0.44</math></b>		
Variable	p-value	Number of times in the 20 ‘best’ regressions
Rate of change in fruit length (mm day <sup>-1</sup> )	0.0226	3
Average minimum orchard RH (%)	0.0061	11
Average orchard night temperature (°C)	0.0008	19
<b>49 days after full bloom: <math>R = 0.8176</math>; <math>R^2 = 0.6685</math>; ‘leave one out’ cross validation <math>R^2 = 0.46</math></b>		
Variable	p-value	Number of times in the 20 ‘best’ regressions
Average fruit length (mm)	0.0558	5
Average orchard night RH (%)	0.0219	7
<b>52 days after full bloom: <math>R = 0.9012</math>; <math>R^2 = 0.8121</math>; ‘leave one out’ cross validation <math>R^2 = 0.58</math></b>		
Variable	p-value	Number of times in the 20 ‘best’ regressions
Fresh weight of mesocarp (g)	0.0240	2
Rate of change in fruit length (mm day <sup>-1</sup> )	0.0012	20
Rate of change in fresh weight of mesocarp (g day <sup>-1</sup> )	0.0125	4

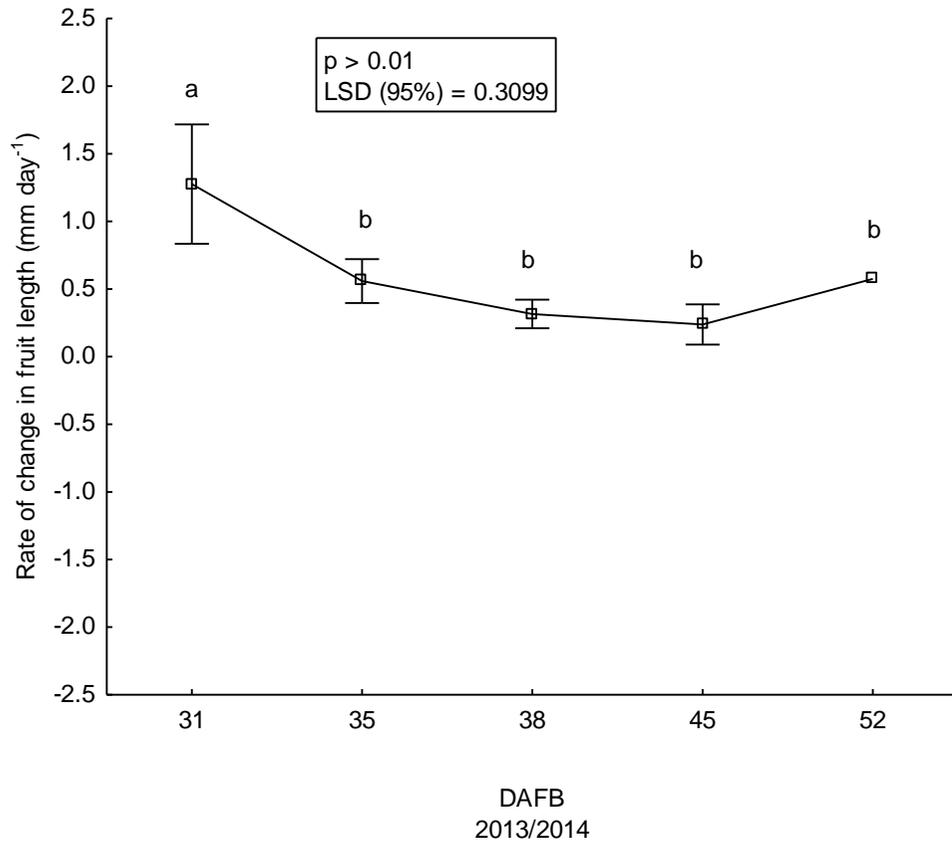


(A)

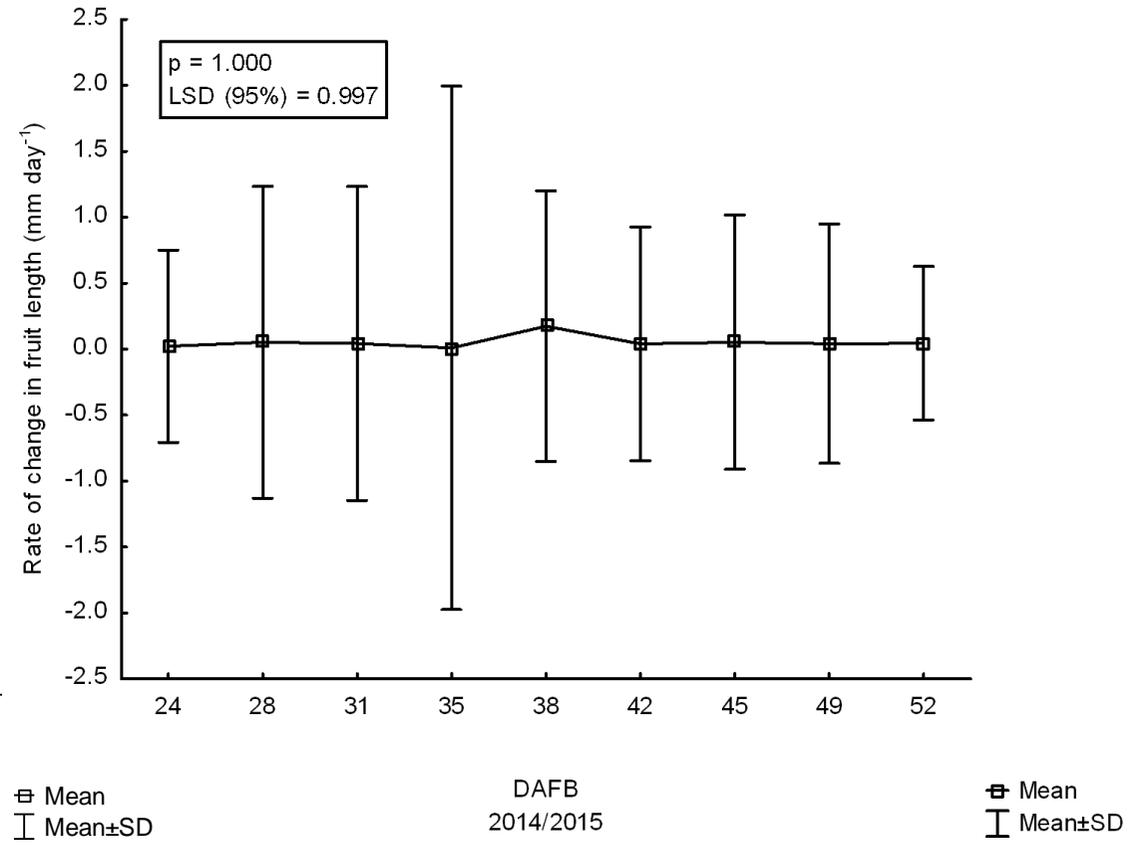


(B)

**Fig. 1.** Incidence of broken stones in the 2013/2014 (A) and 2014/2015 (B) season, respectively. Significant differences are indicated as lower case letters. Whiskers depict standard deviation. Arrows on the graphs indicate when the first signs of stone breakage (which coincided with the onset of stone hardening) occurred.

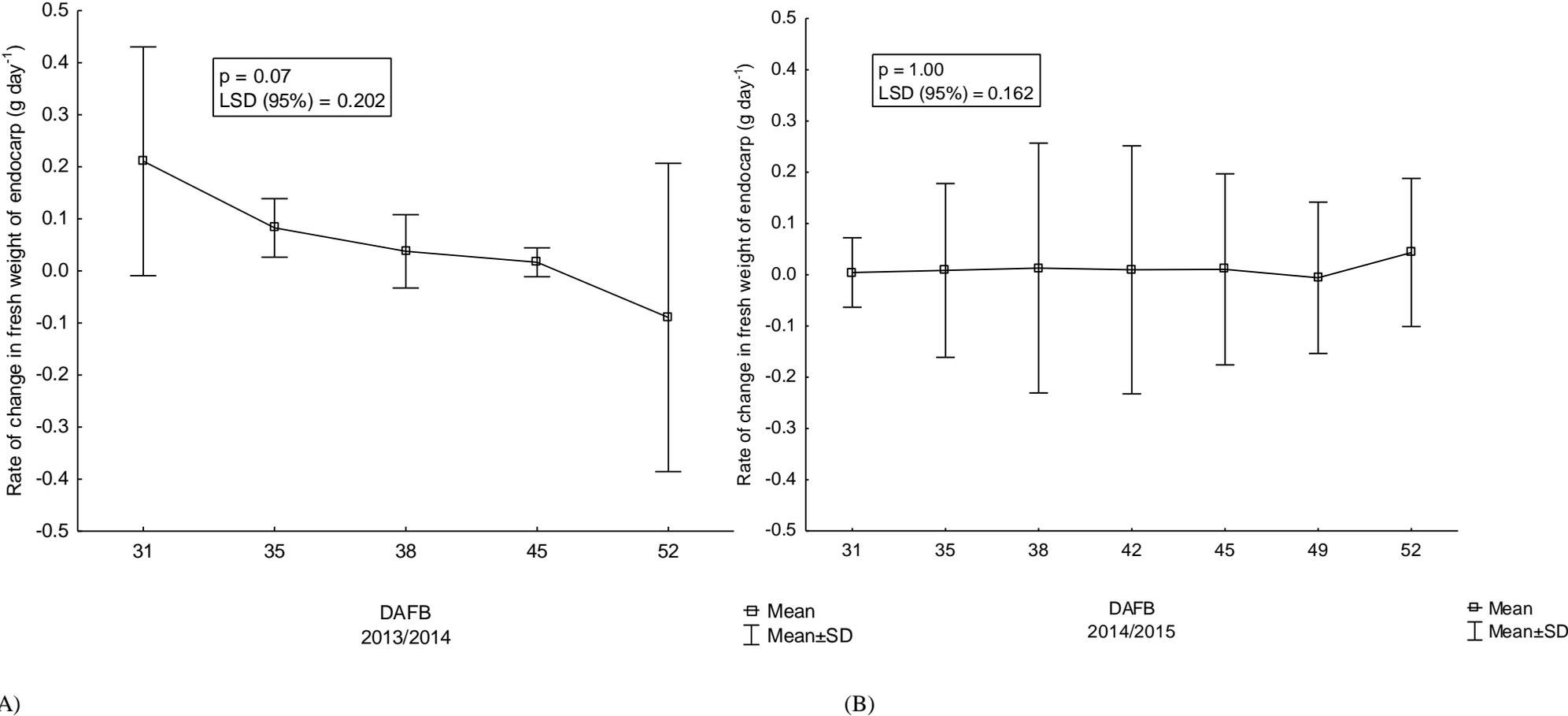


(A)

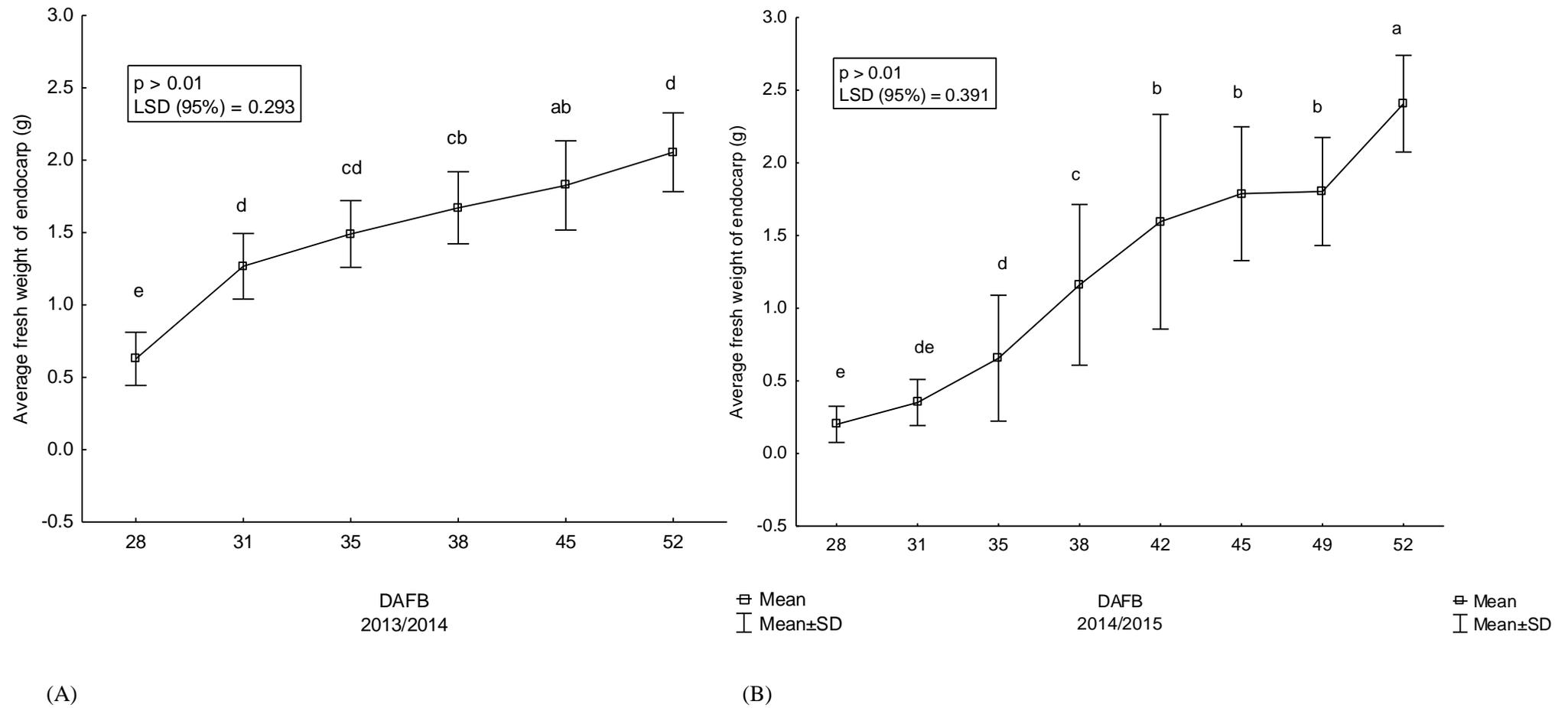


(B)

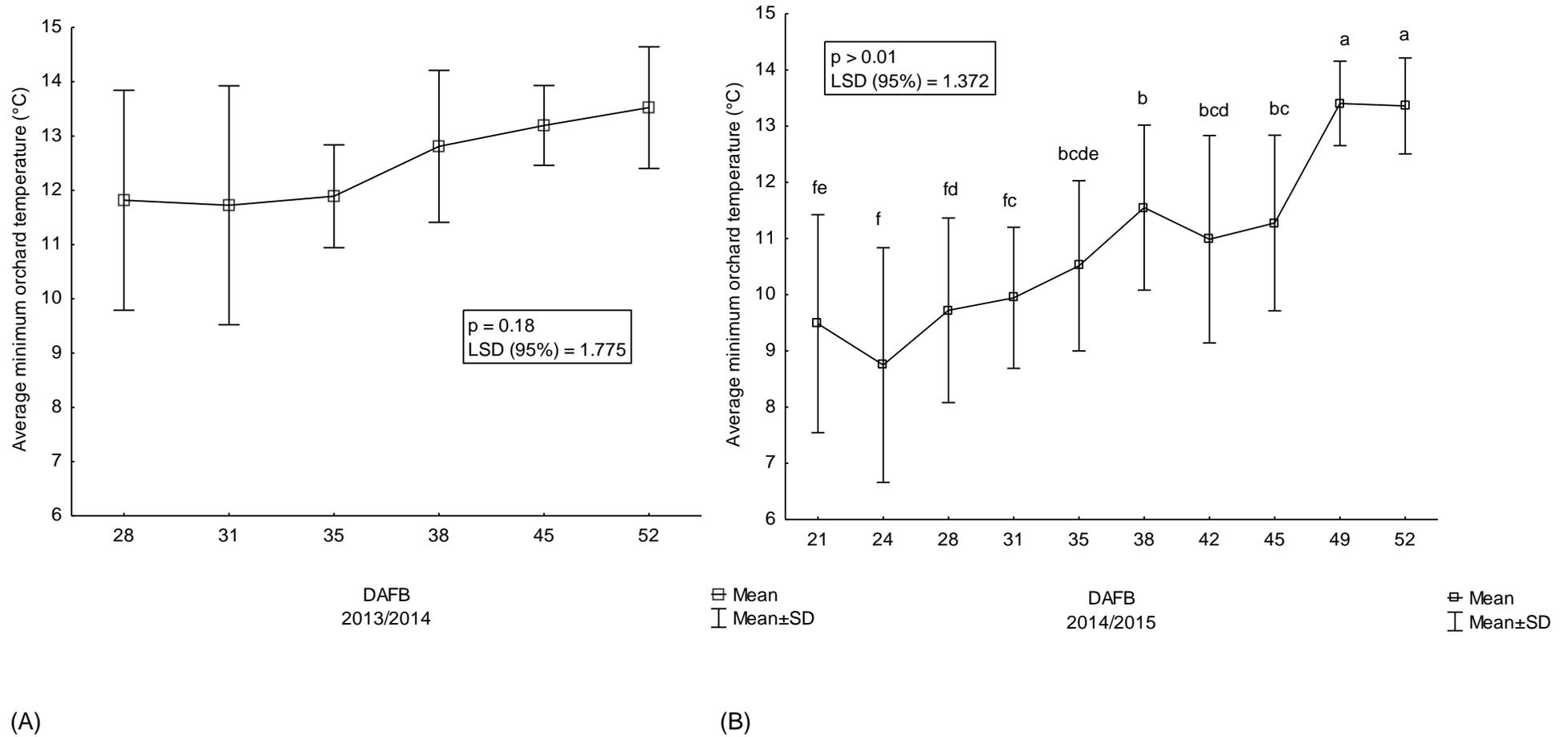
**Fig. 2.** Rate of change in fruit length per day in the 2013/2014 (A) and 2014/2015 (B) season, respectively. Significant differences are indicated as lower case letters. Whiskers depict standard deviation.



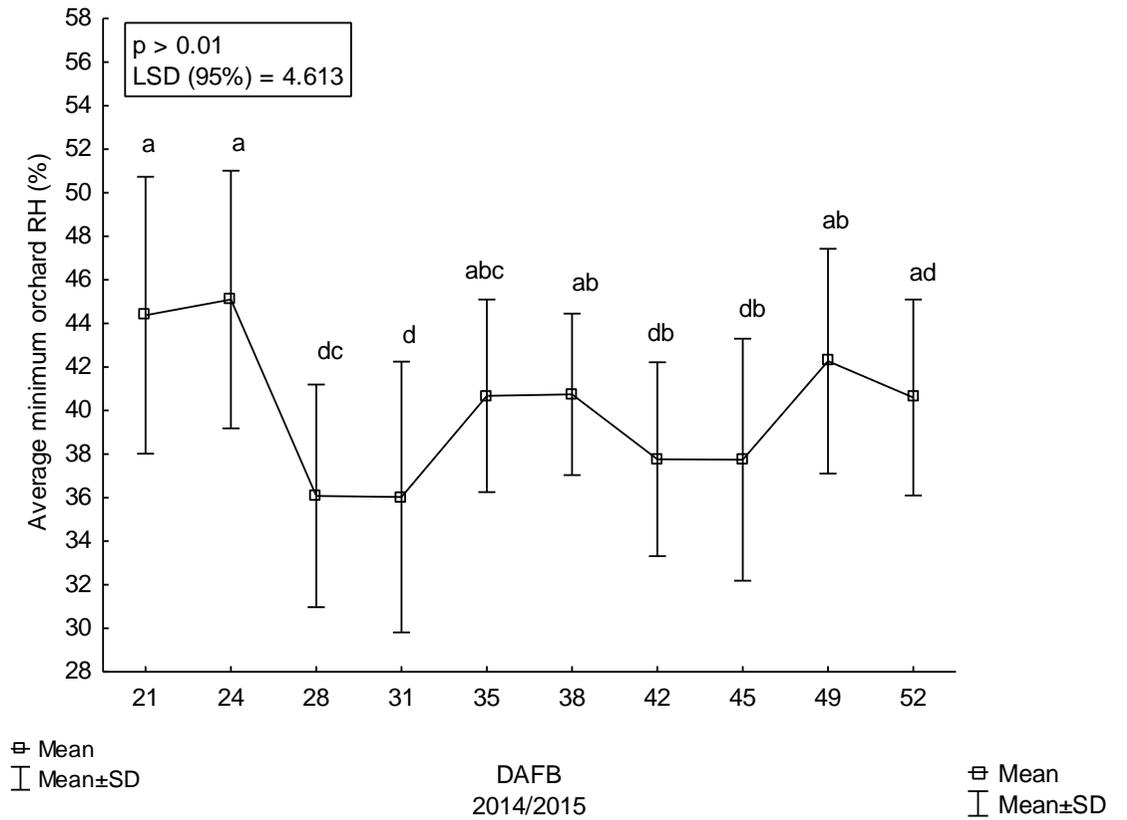
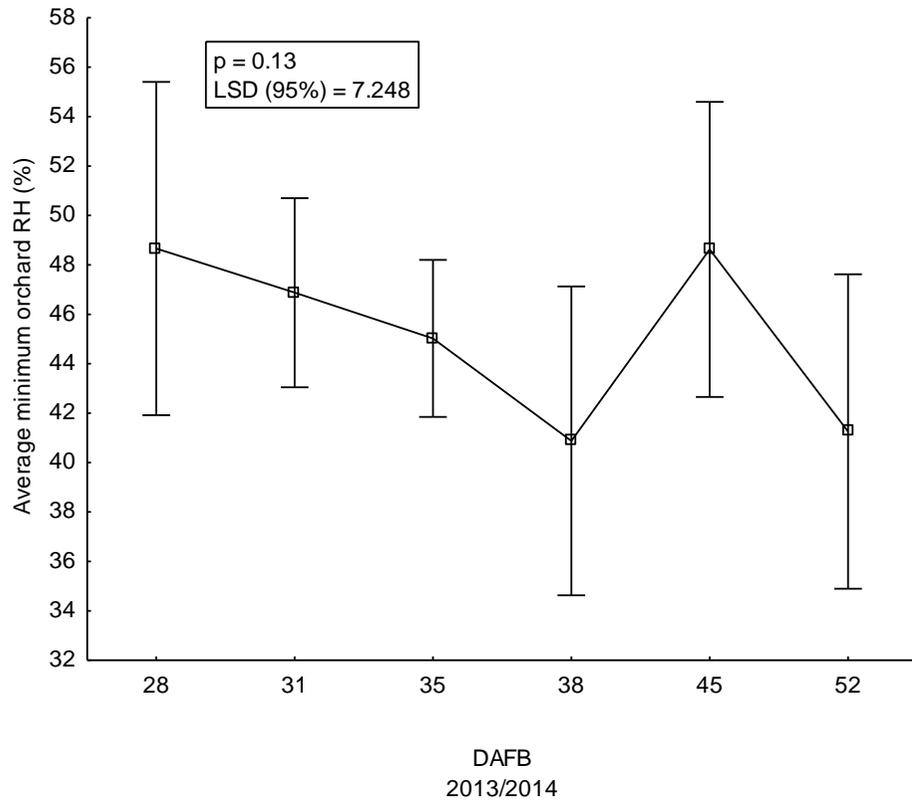
**Fig. 3.** Rate of change in fresh weight accumulation of the endocarp per day in the 2013/2014 (A) and 2014/2015 (B) season, respectively. Whiskers depict standard deviation.



**Fig. 4.** Average fresh weight of the endocarp in the 2013/2014 (A) and 2014/2015 (B) season, respectively. Significant differences are indicated in lower case letters. Whiskers depict standard deviation.



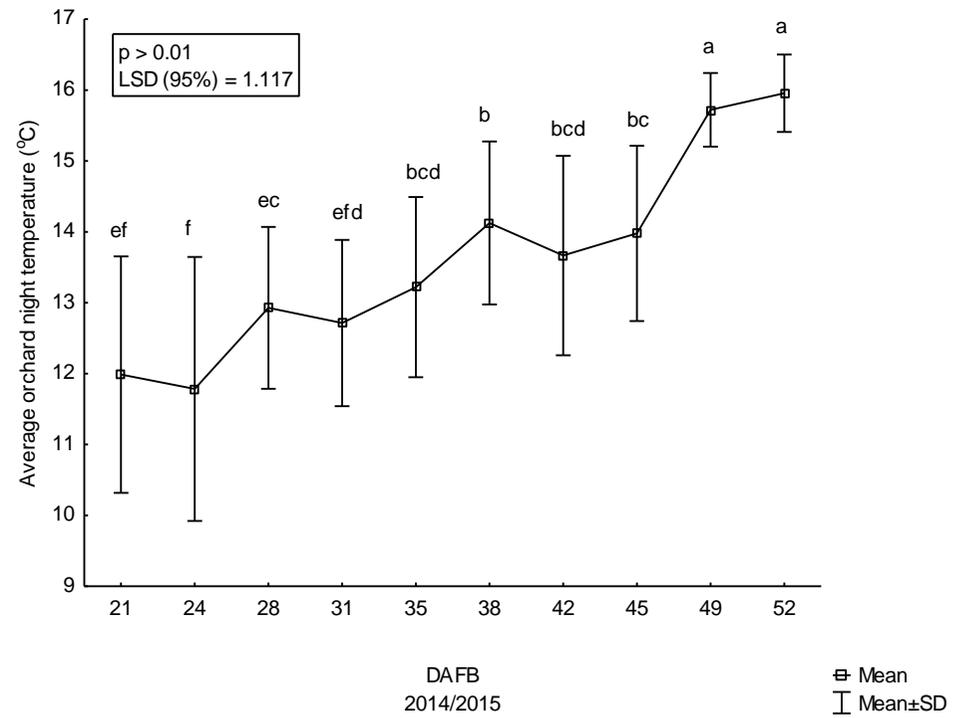
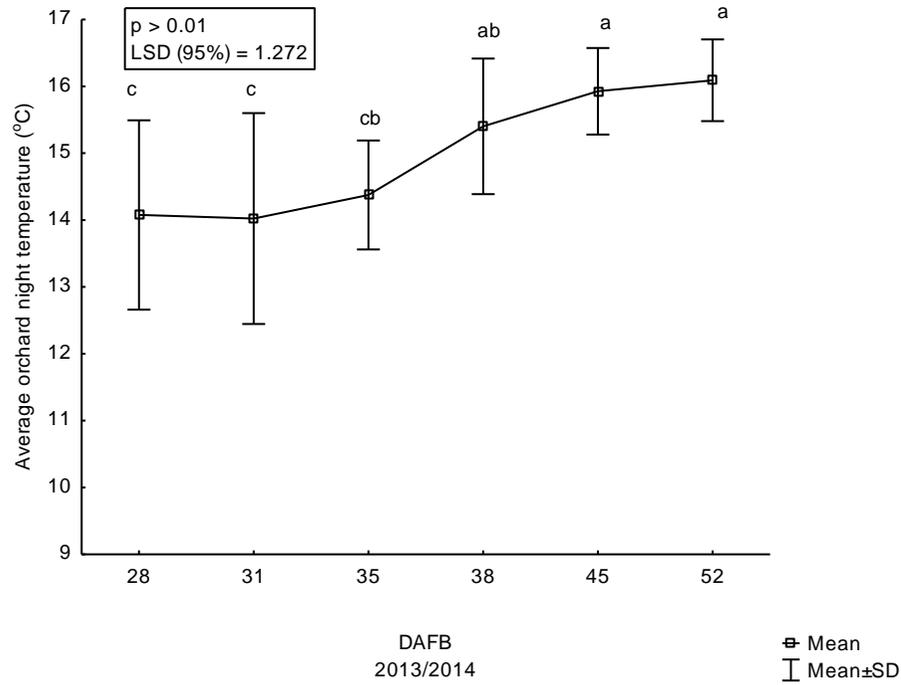
**Fig. 5.** Average minimum orchard temperature in the 2013/2014 (A) and 2014/2015 (B) season, respectively. Significant differences are indicated in lower case letters. Whiskers depict standard deviation.



(A)

(B)

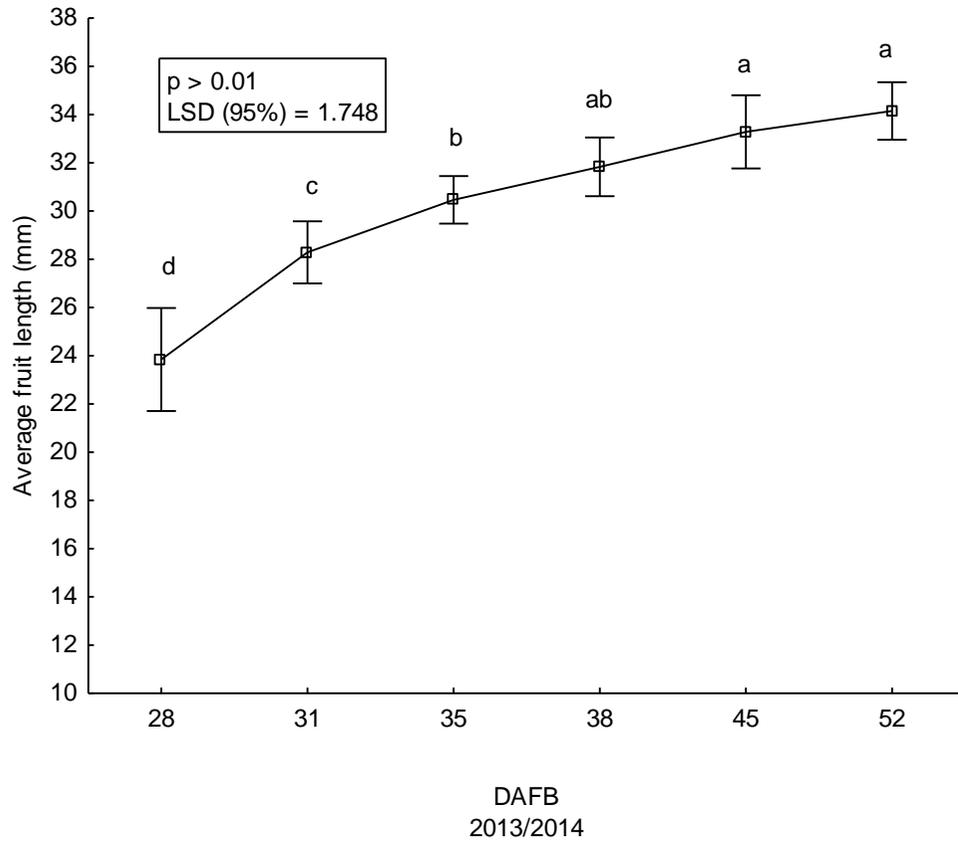
**Fig. 6.** Average minimum orchard relative humidity in the 2013/2014 (A) and 2014/2015 (B) season, respectively. Significant differences are indicated in lower case letters. Whiskers depict standard deviation.



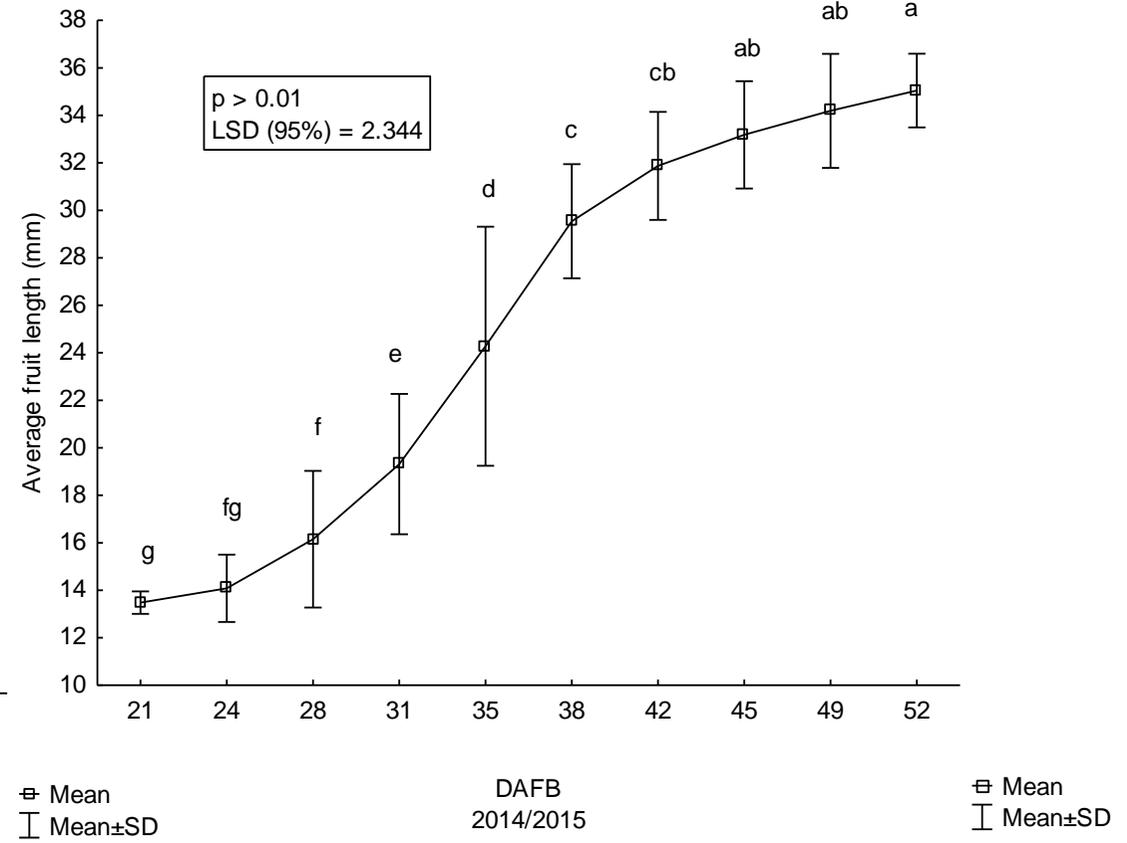
(A)

(B)

**Fig. 7.** Average orchard night temperature in the 2013/2014 (A) and 2014/2015 (B) season, respectively. Significant differences are indicated in lower case letters. Whiskers depict standard deviation.

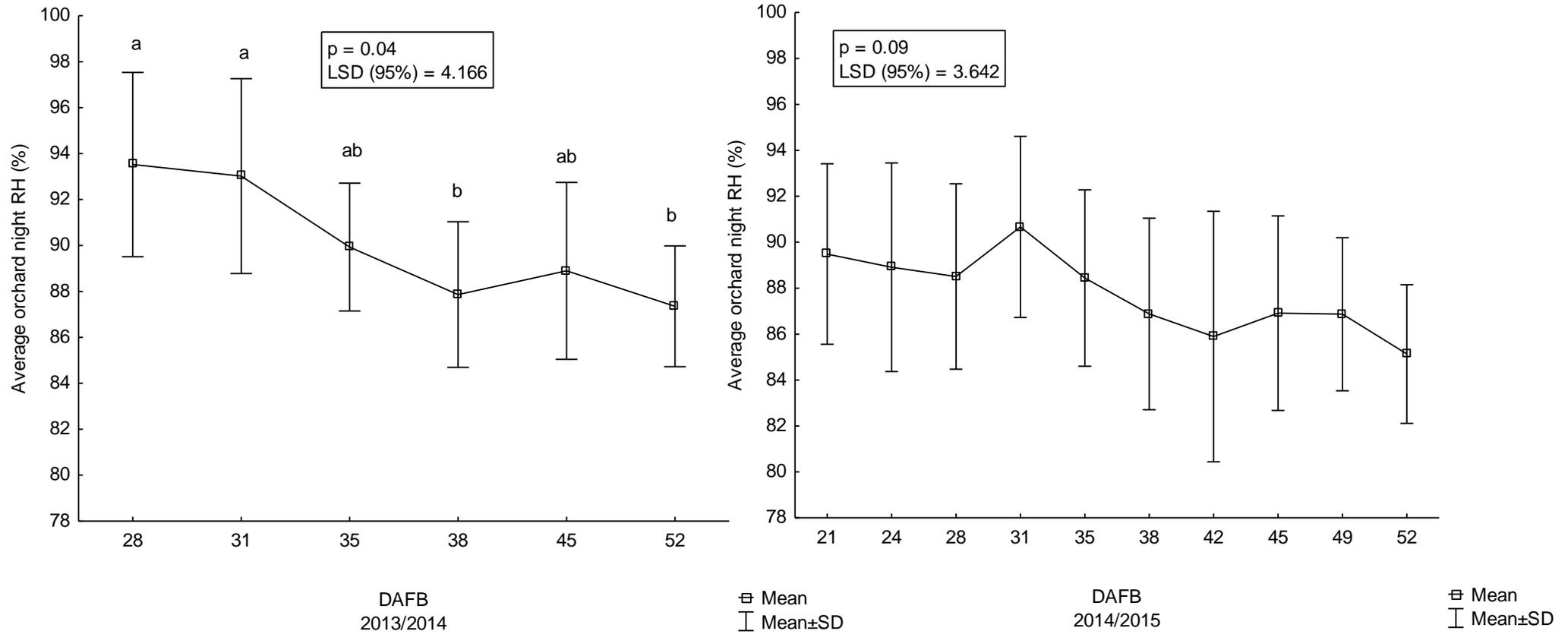


(A)



(B)

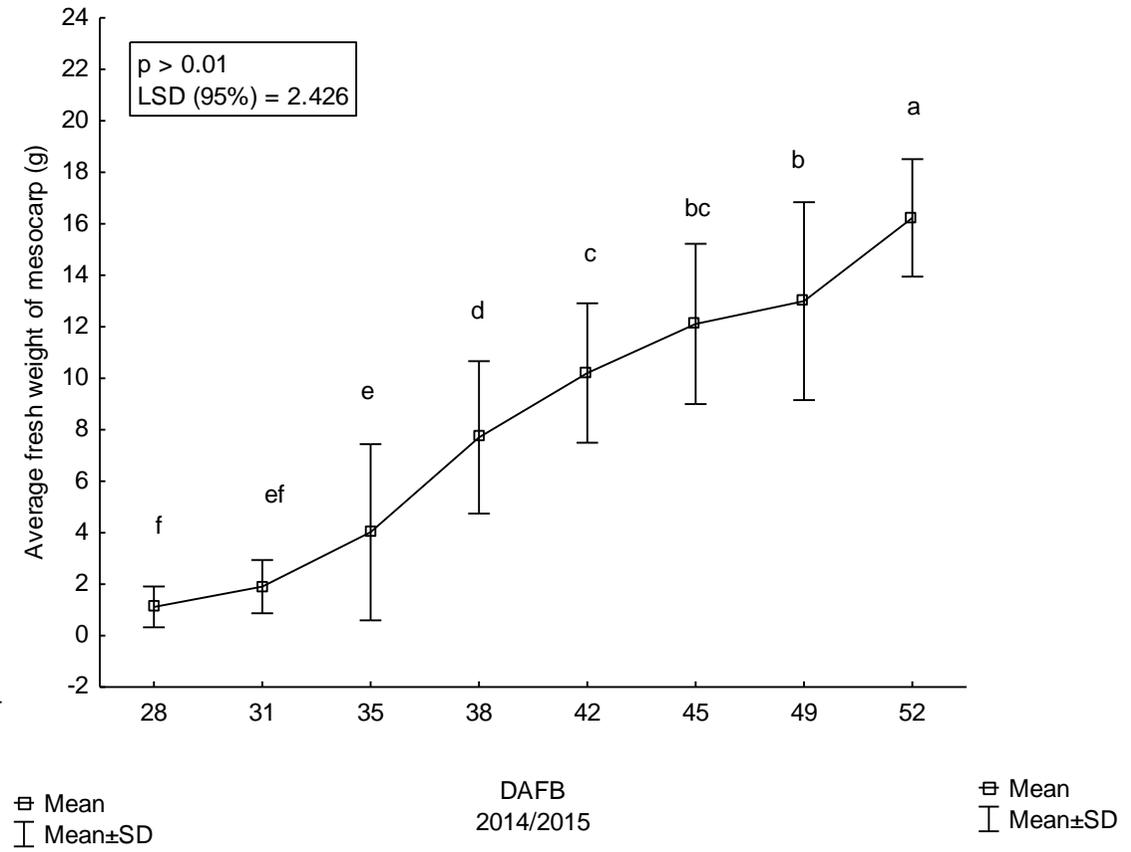
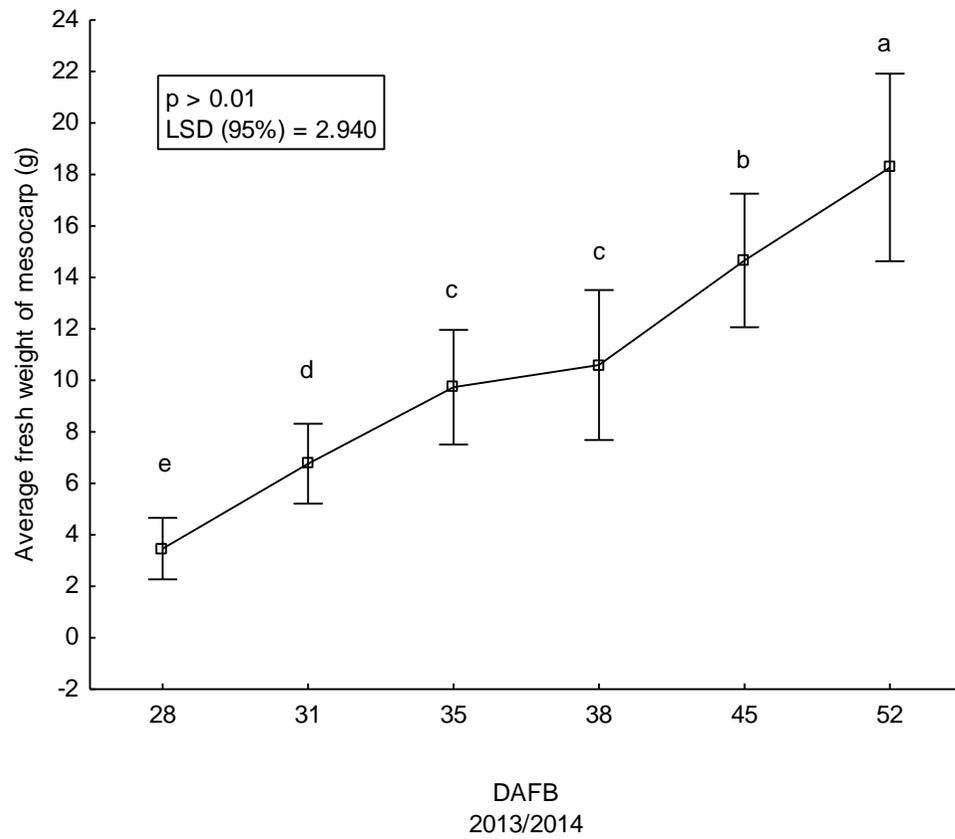
**Fig. 8.** Average fruit length in the 2013/2014 (A) and 2014/2015 (B) season, respectively. Significant differences are indicated as lower case letters. Whiskers depict standard deviation.



(A)

(B)

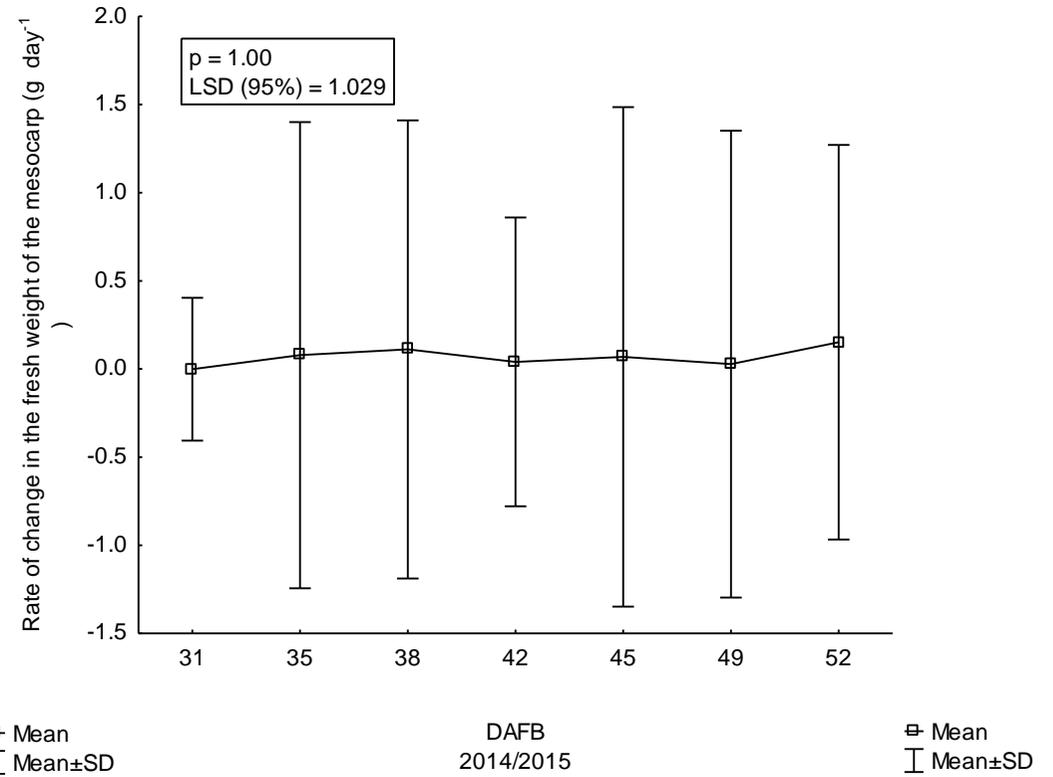
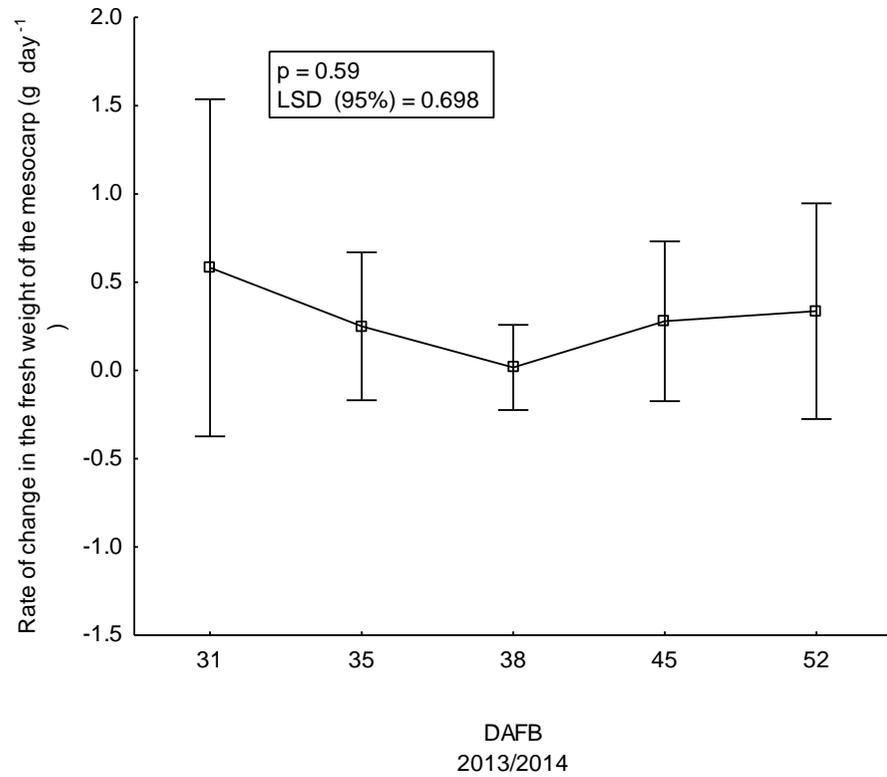
**Fig. 9.** Average orchard night relative humidity in the 2013/2014 (A) and 2014/2015 (B) season, respectively. Significant differences are indicated in lower case letters. Whiskers depict standard deviation.



(A)

(B)

**Fig. 10.** Average fresh weight of the mesocarp in the 2013/2014 (A) and 2014/2015 (B) season respectively. Significant differences are indicated in lower case letters. Whiskers depict standard deviation.



(A)

(B)

**Fig. 11.** Rate of change in fresh weight accumulation of the mesocarp per day in the 2013/2014 and 2014/2015 season, respectively. Whiskers depict standard deviation.

## PAPER 2

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# The use of computed tomography scans to evaluate broken stones in three Japanese plum cultivars (*Prunus salicina* Lindl.)

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### ABSTRACT

Stone breakage affects both peaches and plums and leads to significant financial losses for the agricultural industry as affected fruit have to be marketed as Class 2, thus obtaining lower prices on international markets. Since no information exists on the development of broken stones in Japanese plums, the aim of this study was to determine when and why the defect develops during fruit development by comparing susceptible and less susceptible cultivars. Information on differences in growth patterns between susceptible and less susceptible cultivars and indications on effects thereof on the incidence of broken stones may help to forecast severity of the defect in some seasons, assist in marketing of the fruit and/or could support the South African plum breeding program to select cultivars not susceptible to the defect. Computed tomography was used to compare the growth characteristics and endocarp density of 'Laetitia' and 'Sapphire' (which are prone to stone breakage) and 'Songold' (not susceptible to stone breakage) plums during the period of stone hardening. Lignification started at the styler end of the fruit and progressed towards the stem end, between 28 to 63 days after full bloom. This was accompanied by an increase in endocarp density. The inner lining of the stone started to harden first, after which lignification continued radially from the inside of the stone towards the margins. Clear differences in the incidence of broken stones were observed between cultivars and seasons. Broken stone manifestation coincided with rapid radial fruit growth in one or more directions before the stones were completely hardened. It seems that the stones are not able to resist the strong pulling forces of the growing mesocarp when they are not completely hardened and this leads to the stones being pulled apart.

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### **Keywords:**

Broken stones; Density; Endocarp; Lignification; Plums

## 1. Introduction

Stone breakage and/or splitting is a phenomenon that affects peaches and plums and can lead to significant financial losses as affected fruit have to be marketed as Class 2 (Organisation for Economic Co-operation and Development, 2002). There are usually no external indications of stone splitting or breakage and it is only detected when fruit are cut open (Ogawa et al., 2003; Ragland, 1934; Woodbridge, 1978). However, in severe cases the fruit may be malformed or even pulled apart at the stem end and these fruit cannot be exported as Class 1 (see Table 1 for a summary of South African export standards pertaining to broken stones in plum/prunes). Stone residues in peaches cause difficulties during the canning process, and in fresh fruit, pieces of broken stone in the flesh are unacceptable to the consumer (Han et al., 1992). In peaches, the stone usually splits open along the suture/ seam of the endocarp (Fig. 6) (Department of Agriculture Forestry and Fisheries, South Africa, 1990). However, with stone breakage in Japanese plums, the endocarp can disintegrate (break apart) anywhere, and often in multiple areas (Personal observation) (Fig.7).

It is known that the occurrence of split and broken stones in peaches is associated with environmental conditions during fruit development on the tree, cultivation practices that cause an increased growth rate of the fruit, as well as genetic factors (Claypool et al., 1972; Engin et al., 2010 Tani et al., 2007; Ragland, 1934). Fruit with broken or split stones are often larger than fruit with intact stones (Davis, 1933; Evert et al., 1988; Nakano, 2006) and early maturing cultivars are more prone to stone breakage than later maturing cultivars (Chatzitheodorou et al., 2004; Claypool et al., 1972). Stone fruit have three growth stages during fruit development, with the first (I) and third (III) stages being fast and the second stage, being slower to allow the stone to harden (Chalmers and Van den Ende, 1975). However, in early maturing cultivars, under certain environmental conditions, or as a result of cultivation practices that promote fruit growth, fruit enlargement often does not slow down during Stage II of fruit development (Claypool et al., 1972; Ognjanov et al., 1995; Scorzal et al., 1991; Tukey, 1936). Consequently, the stone is not allowed to harden properly to resist the growing forces of the rapidly expanding fruit flesh, and is pulled apart, resulting in split or broken stones (Ragland, 1934; Tani et al., 2007). Although numerous studies have been conducted on the split stone phenomenon in peaches and nectarines, no research has been carried out on the broken stone phenomenon in Japanese plums.

Since the occurrence of broken stones in Japanese plums has a pronounced economic impact on the South African plum export industry, it is of the utmost importance to obtain a better understanding of the reasons why the defect only develops in certain cultivars in order to develop protocols to reduce the incidence of the defect. We hypothesized that susceptible and non-susceptible cultivars differ in fruit growth rates during Stage II and that susceptible cultivars grow more prominently in length, while non-susceptible cultivars tend to grow more rapidly in diameter at the end of Stage II. Thus, we compared the growth characteristics of 'Laetitia' and 'Sapphire' plum cultivars (which are prone to stone breakage) to 'Songold' (which is not susceptible to stone breakage). Computed tomography (CT) scans were used to visualise the occurrence of broken stones and monitor fruit growth patterns non-destructively. In

recent years, there has been an increase in the use of non-destructive methodology to determine the internal quality of fresh produce (Barcelon et al., 1999a). Methods such as nuclear magnetic resonance, infrared radiation, X-rays and CT scans have been applied successfully to numerous fruit types to quantify internal quality (Barcelon et al., 1999a, 1999b; Han et al., 1992; Ogawa et al., 2003). Computed tomography specifically has been used to evaluate the moisture content, acidity, soluble solids and density in mangoes and peaches (Barcelon et al., 1999a, 1999b). Maturity of tomatoes, the presence of water core in apples and internal insect damage to fruit are further examples of the application of CT scans to evaluate fruit quality (Brecht et al., 1991; Ogawa et al., 2003). Furthermore, it has also been employed to visualise the presence of split or broken stones non-destructively in peaches (Han et al., 1992; Ogawa et al., 2003).

## **2. Materials and Methods**

### **2.1 Site description**

#### **2.1.1 2013/2014 season**

The trial was conducted at the Stellenbosch University experimental farm, Welgevallen (33°56'49.8"S 18°52'16.6"E), Stellenbosch. Two cultivars, namely 'Laetitia' (highly susceptible to broken stones) and 'Songold' (low susceptibility to broken stones) on 'Marianna' rootstock, were used. The 'Laetitia' orchard was planted in 1992, while the 'Songold' orchard was planted in 1998. Both cultivars were trained according to a palmette system at a spacing of 4.5 x 1.25 m. 'Songold' was used as a pollinator in the 'Laetitia' orchard, while 'Laetitia' was used as a pollinator in the 'Songold' orchard.

#### **2.1.2 2014/2015 season**

The same trial site was used during two consecutive seasons (2013/2014 and 2014/2015). 'Sapphire', an early season cultivar which is also highly susceptible to broken stones, was included in the 2014/2015 season. The 'Sapphire' trial was carried out at Morgenzon farm in the Helshoogte area of Stellenbosch (33°55'29.33"S 18°55'48.446"E). Trees were planted in 2002 on 'Marianna' rootstock at a spacing of 4 x 1.25 m, with 'Harry Pickstone' as cross-pollinator. The trees were trained according to a palmette system.

In both seasons, and for all the cultivars, a complete randomised design was used. Temperature and rainfall data were obtained from Rustenberg weather station in Stellenbosch (33°53S 18°54E).

## **2.2 Fruit sampling**

### **2.2.1 2013/2014 season**

#### **2.2.1.1 Fruit sampling to determine the incidence of broken stones during fruit development**

Sampling started 28 days after full bloom (dafb) and continued until the first commercial harvest date (28 January 2014). Full bloom dates were 30 September 2013 for ‘Laetitia’ and 2 October 2013 for ‘Songold’. Two hundred fruit per cultivar, chosen at random from various positions in the trees, were sampled once a week from 20 trees per cultivar (10 fruit per tree) to determine the incidence of broken stones.

#### **2.2.1.2 Fruit sampling for computed tomography scans**

On each sampling date an additional six fruit per cultivar, chosen at random from various positions and from randomly selected trees in the orchard, were used for 3D CT scans. Initially, the fruit intended for the CT scans were sampled twice a week until the onset of stone hardening, which was observed at approximately 30 and 37 dafb for ‘Songold’ and ‘Laetitia’, respectively. The onset of stone hardening was defined as the first date where the stone started to show resistance when cut open along the fruit suture. After the onset of stone hardening, fruit were sampled once a week, until the first commercial harvest date (28 January 2014).

#### **2.2.1.3 Fruit sampling on commercial harvest date to determine final incidence of stone breakage**

On the commercial harvest date, 20 trees per cultivar were strip picked. All fruit were individually cut along the suture (in the length) to determine the incidence of broken stones.

### **2.2.2 2014/2015 season**

#### **2.2.2.1 Fruit sampling to determine the incidence of broken stones during fruit development**

Full bloom dates were 25 August 2014 for ‘Sapphire’, 9 September 2014 for ‘Laetitia’ and 16 September 2014 for ‘Songold’. Sampling started 21 dafb and continued until stone hardening was complete (about 63 dafb, when the stones could not be cut in half anymore). Fruit were sampled once a week during this period. The onset of stone hardening for ‘Songold’ was at 28 dafb, for ‘Laetitia’ at 35 dafb, and for ‘Sapphire’ at 42 dafb. Once a week, 15 fruit per cultivar chosen at random from various positions in the trees, were sampled from randomly selected trees, to determine the incidence of broken stones.

#### **2.2.2.2 Fruit sampling for computed tomography scans**

On each sampling date, an additional three fruit per cultivar, chosen at random from various positions in the trees, were harvested from randomly selected trees and used to perform the CT scans.

### **2.2.2.3 Fruit sampling on commercial harvest date to determine final incidence of stone breakage**

On the commercial harvest date of each cultivar, 100 fruit chosen at random from various positions in the trees, were sampled from randomly selected trees to determine the incidence of broken stones.

## **2.3 Computed tomography analysis**

Fruit were scanned either on the day of sampling, or the morning after sampling, at the CT scanner facility at Stellenbosch University, South Africa. X-ray micro computed tomography was used to create images of the scanned fruit in order to measure the relative density of the stones as well as fruit growth at different angles, non-destructively. A General Electric Phoenix V|Tome|X L240 / NF180 was used and the X-ray settings were 160 kV and 120 microA. Two-thousand images were acquired in a full rotation at an image acquisition time of 500 ms per image, with no averaging and no skipping of images. Detector shift was activated to minimize ring artefacts. Background calibration was performed and the scan time was approximately 20 min per fruit. Reconstruction was performed with system-supplied Datos reconstruction software and analysis was performed with Volume Graphics VGStudio Max 2.1 commercial 3D analysis software.

After completion of the 3D scans, a 2D, lengthwise slice was made through the suture of the fruit in order to measure radial growth in different directions, as well as the relative density of the stone in different parts of the endocarp (Fig. 8A and 8B). Since it was not possible to measure the actual density of the stone, relative density was expressed as a ratio of the grey-value of a piece of plastic included in every scan, to the grey-value determined in different parts of the stone (plastic/stone grey value ratio) (Fig. 8B). The grey-value indicates how much of the X-ray beam was attenuated or “blocked” by the tissue. Therefore, darker shades on a CT image represent areas that blocked less X-rays directed at it (e.g. air), while lighter tissues represent areas where the X-ray beams were strongly attenuated. Hence, as the endocarp lignified, we expected the ratio between the plastic and the stone to increase as the stone became harder and denser. The density of the stones was measured at 0° (stem end of the fruit or the top of the 2D slice), 180° (stylar end of the fruit or the bottom of the 2D slice) and along the middle/diameter of the fruit (90° and 270° combined) (Fig. 8A). Furthermore, the radial growth at different angles (from 0° to 330° at 30° intervals) was recorded, but for the sake of clarity, some of these measurements were combined to divide the fruit into 4 different quadrants namely, stem end, stylar end, left side and right side (Fig. 8A). The latter measurements were performed from the middle towards the outer edge of the fruit.

## **2.4 Lignin staining**

In the 2014/2015 season, the endocarps of five fruit per cultivar per sampling date were stained according to the method of Callahan et al. (2009) to determine when and where in the endocarp

lignification started and how it spread throughout the endocarp, and if it corroborated with the information obtained from the CT scans. Each fruit was cut in half through the suture/ seam and placed in a phloroglucinol-HCl solution [1% (w/v) phloroglucinol (Sigma-Aldrich, South Africa Product number: 79330 ALDRICH), 12% HCl (v/v), and 85% ethanol (v/v)] for 1 h. The stained tissue turned pink where lignin was present. Subsequently the fruit halves were rinsed with a 96% ethanol solution and photographed.

## **2.5 Statistical analysis**

To determine the effect of cultivar on the percentage of broken stones, a one-way analysis of variance (ANOVA) was performed per season. The effect of cultivar, direction of growth and the number of dafb on the growth of the fruit and density of the endocarp (as obtained from the CT scan data) was determined by performing a three-factorial, one-way ANOVA per season. ANOVA-generated P-values and the significant differences between means were determined using Fisher's least significant differences (LSD) test with a 95% confidence interval. Analyses were performed on the data from each season separately.

It must be noted that the trial was conducted in only one orchard per cultivar and, therefore, results may be applicable to either real cultivar differences or to the specific orchard. However, the 'Laetitia' and 'Songold' trees were planted in the same orchard with the same elevation, soil type, management practices and climatic conditions and thus it can be assumed that the differences observed were due to cultivar differences.

Statistical analysis was performed using STATISTICA, version 12 (Statsoft, Inc., 2011).

## **3. Results**

### **3.1 Broken stones**

#### **3.1.1 2013/2014 season**

At harvest, 'Laetitia' showed a broken stone incidence of 32.69%, which differed significantly ( $p < 0.01$ ) from the incidence of 7.23% observed in 'Songold'.

#### **3.1.1 2014/2015 season**

At harvest there were no significant differences ( $p = 0.167$ ) between the incidence of broken stones for 'Laetitia' (7%), 'Songold' (15%) and 'Sapphire' (14%).

## 3.2 Density of the endocarp

### 3.2.1 2013/2014 season

There was a significant interaction between dafb and direction of growth on the density of the endocarp (Fig. 1). For both ‘Laetitia’ and ‘Songold’ the density of the stone in all the measured positions (stem end, middle and stylar end) increased with an increase in number of dafb. Density in the middle of the stone started off higher than both the stem and stylar ends and did not increase significantly until 42 dafb. From 35 dafb and onwards, relative density at the stylar end was significantly higher than in the other two positions (except at 63 dafb when it did not differ significantly from density in the middle of the stone). Density at the stem end of the stone was the lowest compared to the other positions on all sampling dates.

### 3.2.2 2014/2015 season

There was a significant interaction between dafb and cultivar on the density of the endocarp (Fig. 2). ‘Laetitia’ and ‘Songold’ had a similar pattern of stone hardening, namely a slight decrease from 21 dafb until 35 dafb (‘Songold’) and 42 dafb (‘Laetitia’), followed by a sharp increase in stone density. There was no significant differences in the density of ‘Songold’ and ‘Laetitia’ stones between 21 and 35 dafb. Stone hardening in ‘Laetitia’ started one week later compared to ‘Songold’. This was corroborated by the phloroglucinol-HCl stains, where pink staining near the stylar end of the stone was observed at 35 dafb for ‘Songold’, while the first signs of staining (also at the stylar end) for ‘Laetitia’ were only observed at 42 dafb (data not shown). Staining progressed from the stylar end of the stone towards the stem end, as well as outward from the inner edges of the stone, in both cultivars (Fig.10). The intensity of the pink stain increased thereafter in both cultivars, suggesting an increase in the lignin content of the endocarp. In ‘Laetitia’ stone hardening seemed to stop after 56 dafb. In contrast, ‘Songold’ showed a decrease in stone hardening after 49 dafb, followed with a second sharp increase in stone density between 56 and 63 dafb (Fig. 2). This second stage of stone hardening for the ‘Songold’ stones could be ascribed to sampling error due to the relatively small sample size (three fruit per sampling date). The density of ‘Sapphire’ stones did not differ significantly from the other cultivars at 21 dafb. Thereafter density showed a zig-zag (increase-decrease) pattern until 42 dafb, which was probably due to high variation between samples again due to the relatively small sample. In contrast to ‘Laetitia’ and ‘Songold’, the density of the ‘Sapphire’ stones remained constant and significantly lower between 42 and 56 dafb. ‘Sapphire’ fruit treated with the phloroglucinol-HCl-solution started to show slight/light pink staining only after 42 dafb, but the intensity of the stain increased substantially by 49 dafb (data not shown). Subsequently, the ‘Sapphire’ stones hardened at a rapid rate between 56 and 63 dafb. Notwithstanding this rapid increase, the density of the ‘Sapphire’ stones at 63 dafb was significantly lower compared to the final densities of the ‘Laetitia’ and ‘Songold’ plums.

‘Laetitia’ stone breakage was first observed at the onset of the rapid increase in endocarp density at 42 dafb. ‘Sapphire’ stone breakage was first observed at 49 dafb, which was immediately prior to the rapid increase in stone density. However, with ‘Songold’ stone breakage was only observed at harvest. The fact that breakage was not observed during the sampling period for ‘Songold’ plums may be ascribed to the relatively small sample sizes.

### **3.3 Radial growth**

#### **3.3.1 2013/2014 season**

There was a significant interaction between dafb and cultivar for radial growth (Fig. 3). For both cultivars, radial growth increased over time. Except for 42 dafb, the radial growth of ‘Songold’ was significantly higher than that of ‘Laetitia’. Although ‘Laetitia’ showed a slower rate of radial growth than ‘Songold’, broken stones were observed (mostly at the stem end of the fruit) from 35 dafb onwards, while broken stones (mostly observed at the stylar end of the fruit) were observed later, from 42 dafb onwards in ‘Songold’.

There was also a significant interaction between dafb and the direction of radial growth for the two cultivars (Fig. 4). All directions of growth (towards the stem end, diametric, and towards the stylar end of the fruit) increased over time. However, growth towards the stem end of the fruit generally progressed at a faster rate compared to the other directions (stylar and diametric). Although diametric and stylar growth proceeded at a slower rate than growth towards the stem end, diametric growth proceeded at an even slower rate than growth towards the stylar end of the fruit (although not always statistically significant) until 49 dafb, after which it overtook the stylar growth rate until sampling stopped at 63 dafb.

#### **3.3.2 2014/2015 season**

There was a significant interaction between dafb, cultivar and direction of growth for radial growth (Fig. 5A, B and C). When sampling started 21 dafb, the radial distance from the centre of the stone towards the stem end was significantly higher compared to the radial distance towards the stylar end and the left and right sides of the ‘Laetitia’ fruit (Fig. 5A). ‘Laetitia’ fruit generally continued to grow towards the stem and stylar ends of the fruit, while there was virtually no increase in fruit diameter until the final sampling date at 63 dafb, giving the fruit an oblong shape. Between 28 and 35 dafb, there was a sharp increase in fruit growth towards the stem end. This was followed by a lag period in growth between 35 and 49 dafb, and even a decrease in growth towards the stem end between 49 and 56 dafb. Growth towards the stem-end proceeded at a significantly faster rate between 56 and 63 dafb (Fig. 5A). Fruit growth towards the stylar end of the fruit occurred at a fast rate from 21 dafb until 42 dafb, where after it decreased significantly and remained constant until 63 dafb. Hence, fruit growth towards the stylar-end of the fruit continued at a relatively fast rate, while diametric growth did not occur and growth

towards the stem end ceased between 35 and 42 dafb. Stone breakage was observed from 42 dafb onwards and occurred mostly near the stem end of the fruit.

For 'Sapphire', the radial distance between the centre of the stone to the stem-end was significantly higher on the first sampling date (21 dafb), with the left and right sides of the fruit showing the shortest radial distance (Fig. 5B). As with 'Laetitia', this caused the 'Sapphire' fruit to have an oblong shape at the start of sampling throughout the sampling period. The distance from the centre of the stone to the stem end of the 'Sapphire' fruit was generally more compared to 'Laetitia', while the distance between the stone centre and the styler-ends were similar between the two cultivars. Radial growth towards the stem- and styler ends of the 'Sapphire' fruit more or less followed the same pattern during the sampling period, with an initial lag in growth followed by a steep increase after 35 dafb. Growth towards the stem-end ceased after 42 dafb. However, fruit growth towards the styler end continued at a fast rate until 49 dafb after which it ceased. At 63 dafb there was no significant difference in the distance between the centre of the stone and the stem- and styler ends of the fruit. In contrast to 'Laetitia', diametric growth increased significantly from 21 dafb until 35 dafb, thereafter it ceased until 49 dafb, but increased again between 49 and 63 dafb. However, the distance between the right and left sides of the fruit and the centre of the stone was always significantly less compared to the distance to the styler- and stem-ends of the fruit. Stone breakage (most often at the sides of the stone) was observed from 49 dafb when growth towards the styler- and stem-ends ceased, but diametric growth started to increase again (data not shown).

For 'Songold', growth towards the styler and stem end of the fruit followed a completely different pattern compared to 'Laetitia' and 'Sapphire'. When sampling started 21 dafb, the distance between the centre of the stone and the styler end of the fruit was the highest (sign.), followed by the left and right sides of the fruit, with the stem end having the shortest distance (sign.) from the stone centre (Fig. 5C). This gave the 'Songold' fruit a round shape compared to a more oblong shape of the other two cultivars. The 'Songold' fruit maintained this round shape throughout the sampling period. There was a small increase in the rate of growth towards the styler end of the 'Songold' fruit between 21 and 28 dafb, followed by a sharp decrease between 28 and 35 dafb. Subsequently, fruit growth towards the styler end increased again and continued at a relatively fast rate between 35 and 56 dafb, where after growth towards the styler end ceased between 56 and 63 dafb. Diametric growth of 'Songold' fruit at first showed a lag period between 21 and 28 dafb, followed by a steady, but not always statistically significant increase in growth between 35 and 63 dafb. Fruit growth towards the stem end first showed a lag period between 21 and 35 dafb, followed by a significant increase in growth between 28 and 35 dafb and a subsequent lag period between 35 and 42 dafb. Thereafter, fruit growth towards the stem-end of the fruit proceeded at a fast rate between 42 and 49 dafb as well as between 56 and 63 dafb. Stone breakage (mostly near the styler end of the fruit) was only observed on the optimum harvest date and not in the sampling period between 21 and 63 dafb (data not shown).

#### 4. Discussion

The endocarp of stone fruit is hardened through the process of lignification (Hatfield and Vermerris, 2001). Although endocarp lignification only occurs during Stage II of stone fruit growth, the cells are recognizable from early Stage I, being smaller and synthesizing a large amount of lignin precursors (Masia et al., 1992). By approx. 28 dafb, wall-thickening and lignification begins at the stylar end of the area that will eventually form the peach stone (Dardick et al., 2010; Hayama et al., 2006) and proceeds towards the stem end of the fruit over a period of four weeks (Lilien-Kipnis and Lavee, 1971; Sterling, 1953). Around 56 dafb, cells within the stone area of peach fruit reach their final size and wall thickening and lignification can be seen throughout the entire area (Dardick et al., 2010; Hayama et al., 2006; Sterling, 1953). This agrees with observations in the cultivars investigated in this study (Fig. 9 and 10).

Fruit samples that were stained with the phloroglucinol-HCl solution, showed that for all three cultivars in this study, the tip of the stone at the stylar end stained first (Fig. 10). Staining then progressed upwards, but was confined to the inner edges of the stone. As soon as a light stain was observed throughout the area forming the stone, it seems as if, for the remainder of the lignification process, increasing amounts of lignin were synthesized – indicated by the increase in the intensity of the pink colour caused by the stain. Images obtained from the CT scans clearly showed the same trend (Fig. 9). After the entire area of the stone had become slightly more dense (seen as light area on the CT images), density further increased as the light parts of the images became even lighter. Ryugo (1961) observed that lignification of the individual cells begins in the middle lamella and then continues to the cell walls in peach fruit. The inner lining of the stone hardens first, after which lignification continues radially from the inside of the stone towards the margins. Our observations confirmed this.

Clear differences in the incidence of broken stones were observed between cultivars and seasons. This was expected as it is known that split/broken stones in peaches varies greatly among cultivars, seasons, and as a result of cultivation practices and environmental conditions (Davis, 1933; Sotiropoulos et al., 2010; Woodbridge, 1978). Since ‘Laetitia’ and ‘Songold’ were sampled from the same orchard during both seasons (under the same cultivation practices), it is suggested that the differences observed between these two cultivars were not influenced by cultivation practices.

For ‘Laetitia’, the cultivar susceptible to stone breakage, there was a clear difference in the incidence of broken stones between seasons, as fewer broken stones were observed in the 2014/2015 season (7% versus 32.69% in the 2013/2014 season). In contrast, broken stone incidence was much lower in the 2013/2014 season (7.23%) compared to the 2014/2015 season (15%) for ‘Songold’.

In the 2013/2014 season, when a high percentage of broken stones were observed, both the ‘Laetitia’ and ‘Songold’ endocarps were much denser throughout the sampling period compared to the 2014/2015 season. At 28 dafb, ‘Laetitia’ endocarps had a 0.965 plastic/stone grey-value-ratio in the 2013/2014 season versus a 0.645 plastic/stone grey-value-ratio in the 2014/2015 season and at 63 dafb, in the 2013/2014 season, the plastic/stone grey-value-ratio was 1.096 versus 0.724 in the 2014/2015 season. For ‘Songold’ a similar trend was observed: at 28 dafb endocarps had a relative density of 0.965 (plastic/stone grey-value-ratio) in the 2013/2014 season versus a 0.629 plastic/stone grey-value-ratio in the 2014/2015 season and at 63 dafb this ratio was 1.076 in the 2013/2104 season versus a 0.745 plastic/stone grey-value-ratio in the 2014/2015 season. Compared to ‘Laetitia’, the density of the ‘Songold’ stones at the end of the sampling period (63 dafb) was lower in the 2013/2014 season (plastic/stone grey-value-ratio of 1.096 versus 1.076 for ‘Laetitia’ and ‘Songold’ respectively).

The stem end of the stone had the lowest average density (for all sampling dates combined) in both ‘Laetitia’ and ‘Songold’ fruit in the 2013/2014 season (data not shown). For ‘Laetitia’ the relative density at the stem end was 1.001 (plastic/stone grey-value-ratio), compared to a 1.019 plastic/stone grey-value-ratio in the middle and a 1.046 plastic/stone grey-value-ratio at the stylar end. For ‘Songold’ it was a 1.003 plastic/stone grey-value-ratio at the stem end, 1.017 plastic/stone grey-value-ratio in the middle and a 1.033 plastic/stone grey-value-ratio at the stylar end. In the 2014/2015 season, when a lower percentage of broken stones were observed in ‘Laetitia’, the stem end of the stone had the lowest density again (plastic/stone grey-value-ratio of 0.661) compared to middle (0.672 plastic/stone grey-value-ratio) and stylar end (0.668 plastic/stone grey-value-ratio). In contrast, the incidence of stone breakage in ‘Songold’ was twice as high as during the 2014/2015 season when the relative density was lowest at the stylar end (0.672 plastic/stone grey-value-ratio) compared to the stem end (0.678 plastic/stone grey-value-ratio) and the middle of the stone (0.680 plastic/stone grey-value-ratio).

It was interesting to note that stone breaks were mostly observed at the stem end of ‘Laetitia’ fruit, while they were more often noticed at the stylar end of the ‘Songold’ stones. It seems that the stones tend to break at the interface between high and low density areas, which explains why ‘Laetitia’ stones tend to break mostly at the stem end, while ‘Songold’ stones broke at the stylar ends. For ‘Laetitia’ these breaks were observed as soon as the stones started to lignify (35 dafb in 2013/2014 and 42 dafb in 2014/2015). This agrees with studies on split pit in peaches, where splitting occurs during or just prior to the onset of stone hardening (Ragland, 1934; Woodbridge, 1978). During lignification, the stone starts to lose flexibility and becomes rigid while the flesh is tightly attached to it (Tani et al., 2007). In early ripening peach cultivars, Stage III of fruit growth commences before the stone has hardened completely. The rapid mesocarp growth, which is characteristic of Stage III, then creates strong internal forces that pull on the incompletely hardened stone (Ragland, 1934; Tani et al., 2007). If these forces are large enough, they can pull the stone apart along its weakest parts.

As the density of the endocarp started to increase from 28 dafb in 'Laetitia' in the 2013/2014 season, there was also a rapid increase in radial growth, especially towards the stem end of the fruit (Fig. 4). Even though fewer broken stones were observed in the 2014/2015 season in 'Laetitia', a similar stone hardening pattern was observed compared to the 2013/2014 season. In the 2014/2015 season the first broken stones were observed at 42 dafb, which coincided with a rapid increase in stone density (Fig. 2), and subsequent to a rapid increase in fruit growth towards the stylar end of the fruit (Fig. 5A). Likewise, with 'Songold', density increased rapidly from 35 to 49 dafb in the 2014/2015 season and this was coupled with a significant increase in radial growth towards the stylar and stem ends of the fruit. For 'Sapphire', that is also highly susceptible to stone breakage, a rapid increase in stone density was only observed after 56 dafb (Fig. 2), which was much later than was observed for 'Laetitia' and 'Songold'. Similar to 'Laetitia', the lowest endocarp density was measured at the stem end of the 'Sapphire' fruit (0.633 plastic/stone grey-value-ratio at the stem end, 0.636 plastic/stone grey-value-ratio in the middle and 0.640 plastic/stone grey-value-ratio at the stylar end). The first signs of stone breakage were observed at 49 dafb – a week before stone hardening commenced (Fig. 2). Hence, stone breakage in 'Sapphire' commenced just after a sudden, although not statistically significant, increase in radial fruit growth towards the stylar end of the fruit (Fig. 5C), while diametric growth and growth towards the stem end were dormant. Stone breakage in 'Sapphire' was mostly observed at the sides of the stone. It seems that a semi-rigid stone combined with rapid radial fruit growth in the direction where the stone is least dense, is the cause of stone breakage in both 'Laetitia' and 'Songold'. However, for 'Sapphire' the first signs of stone breakage were observed before stone hardening commenced, which indicates that the non-lignified stone was probably not able to resist the strong pulling forces created by the expanding mesocarp and was subsequently pulled apart. This corroborates with observation in early maturing peach cultivars where split pit is often observed, because fruit enlargement does not slow down during Stage II of fruit development to allow the stone to harden properly and consequently it cannot resist the pulling forces of the expanding fruit flesh and is pulled apart (Claypool et al., 1972; Ognajov et al., 1995; Ragland, 1934; Scorzal et al., 1991; Tani et al., 2007).

Since clear differences were seen in the incidence of broken stones, as well as endocarp density between seasons, it seems that environmental conditions also contribute to the incidence of stone breakage. Loquat fruit are known to increase in firmness after harvest, and this has been shown to be due to lignification in the fruit flesh (Jincheng et al., 2006). Loquat fruit stored at 4 °C showed a higher degree of lignin accumulation compared to fruit kept at 12 °C. At the lower temperatures the activity of the enzymes involved in lignification was higher than at 12 °C. Mangosteen fruit are also known to increase in firmness after harvest due to increased lignification (Dangcham et al., 2008). Fruit firmness started to increase after 12 days of storage and this increase was higher in fruit stored at 6 °C compared to fruit stored at 12 °C. The increase in fruit firmness was due, in part, to an increase in fruit lignin content and increased activity of the enzymes involved in lignification. Similarly, Hausman et al. (2000) found that poplar cuttings kept at 10 °C accumulated more lignin compared to those kept at higher temperatures.

Our results indicate that stone density was much higher in the 2013/2014 season compared to the 2014/2015 season (Fig. 1 and 2). However, the 2013/2014 season experienced higher average temperatures during the stone development and hardening period compared to the 2014/2015 season (Table 2). Hence, according to the data on the loquat and mangosteen fruit as well as the poplar cuttings, the higher stone densities observed in the 2013/2014 season could not be due to a higher degree of lignin accumulation.

A study comparing a 'Stoneless' prune cultivar (a cultivar that forms an incomplete endocarp) to a 'normal' cultivar suggested that the lack of a hard stone could either be due to ineffective lignification, causing parts of the stone to not harden sufficiently, or to incomplete endocarp formation (Callahan et al., 2009). They found, however, that the lignification pathways were similar in both cultivars, but that the 'Stoneless' cultivar displayed decreased and abnormal endocarp formation (significantly lower endocarp dry weight compared to the 'normal' cultivar). Furthermore, endocarp development differed between seasons, suggesting that it might also be environmentally regulated. In years when higher spring temperatures were observed, fruit tended to contain more complete stones (Dardick and Callahan, 2014). It is possible that cultivars such as 'Laetitia' and 'Sapphire', which are susceptible to stone breakage, might also show abnormal endocarp formation during cooler springs. Hence, higher spring temperatures may also lead to the formation of more complete endocarps (more endocarp cells) in plum cultivars susceptible to broken stones. Higher temperatures during early fruit growth stages enhance the rate of apple fruit growth and increase the cell numbers of different regions of the fruit, because temperature affects the rate of cell division (Bergh, 1980; Greybe et al., 1988; Stanley et al., 2000). A higher number of endocarp cells would certainly increase the density (and hence, rigidity) of the stone, and if this is coupled with rapid radial growth in a specific direction before the stone has hardened completely, stone breakage could occur at the interface between high and low density areas in the stone. Since the average temperature during the stone development period (0-63 dafb) in the 2013/2014 season was higher than that of the 2014/2015 season (Table 2), it is suggested that 'Laetitia' was able to form a more complete stone, and hence, more stone cells which lignified to form denser, more rigid stones. Such a scenario would create a more pronounced interface between already hardened and less hardened stone parts during Stage II of fruit growth, which, in combination with rapid increases in radial fruit growth during Stage II, might serve as an explanation as to why 'Laetitia' had a higher percentage of broken stones in the 2013/2014 season compared to the 2014/2015 season. It is furthermore suggested that 'Songold' (less susceptible to broken stones) forms a complete endocarp irrespective of spring temperatures, but that the enzymes responsible for lignification are influenced by spring temperatures. Hence, the cultivar has a more flexible stone, resisting stone breakage, in a warmer spring when lignification is less pronounced and forms a much more rigid stone in a cooler spring when lignification is upregulated as suggested by the results of the loquat, mangosteen and poplar cutting research (Dangcham et al., 2008; Hausman et al., 2000; Jincheng et al., 2006). A more rigid stone would form a more pronounced interface between already hardened and less hardened stone parts during Stage II of fruit growth, and in

combination with rapid fruit growth during stone hardening, result in stone breakage. Data from more seasons will be required in order to validate these suggestions.

Higher rainfall levels were recorded in the 2013/2014 season compared to the 2014/2015 season, while the former also had a significantly higher incidence of broken stones (Table 2). This is in agreement with Claypool et al. (1972) who found that high soil moisture was positively correlated with splitting of peaches and suggested that this was due to higher turgidity within fruit cells under these conditions. The forces acting on the stone when splitting occurs are stronger under conditions of high turgidity.

## 5. Conclusions

The onset of stone hardening in Japanese plums varied slightly between cultivars and seasons, but was generally at about 30 dafb. Stone hardening started at the stylar end of the endocarp and progressed towards the stem end until about 63 dafb. The inner lining of the stone started to harden first, after which lignification continued radially from the inside of the stone towards the margins. Clear differences in the incidence of broken stones were observed between cultivars and seasons, which corroborates with studies on split-pit in peaches. Significant differences were also observed in relative density of the endocarp in different parts of the stone and between the two seasons. For ‘Laetitia’ and ‘Songold’, stone breakage was observed when rapid increases in stone density coincided with rapid increases in fruit growth. The stones had a tendency to break in positions where an interface exist between high and low density parts in the stone. When this was coupled with rapid radial growth in the direction where the stone is least dense, breaks occurred. In contrast, for ‘Sapphire’, stone breakage was observed even before the stones had started to lignify, indicating that the endocarp was pulled apart by the expanding flesh because it was too soft to withstand the strong pulling forces created by the flesh. Our results suggest that higher temperatures during the stone development period (0-63 dafb) may lead to higher endocarp density/rigidity since more stone cells are formed under such conditions and fruit growth is accelerated, thus leading to a higher incidence of broken stones in susceptible plum cultivars. Even though this study was only repeated over two seasons for ‘Laetitia’ and ‘Songold’ and one season for ‘Sapphire’, our results suggest that endocarp development in cultivars that are susceptible to stone breakage is abnormal and that less endocarp cells are present compared to cultivars which are less susceptible to stone breakage. This condition is influenced by environmental factors, as higher temperatures during the stone development and hardening period can lead to more complete endocarp formation. Such fruit would thus have higher endocarp density, which, if coupled with rapid radial growth, could lead to a higher incidence of broken stones. Our results also suggest that cultivars that are less prone to stone breakage will probably have a higher percentage of broken stones after cooler springs due to the upregulation of lignification under such conditions, creating more rigid stones which are more prone to break.

Spring rainfall might also have an influence on the incidence of stone breakage, as higher rainfall levels were recorded in the season where a higher percentage of stone breakage was observed. Higher soil moisture levels lead to higher turgidity of the fruit cells, which increased the pulling forces on the stone and in turn, lead to a higher incidence of stone breakage. It is evident that stone breakage in Japanese plums is controlled not only by genetic differences between cultivars, but also by environmental factors. A better understanding of this phenomenon will be gained if this study is repeated over multiple seasons.

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**Table 1**

Summary of South African export standards and requirements for plums and prunes with regards to shape and broken stones. At least two per cent of the containers in a consignment must be selected at random for inspection purposes. Broken stones, excluding split stones, are determined by taking a sample of 10 plums, which are, in the opinion of the inspector, the most likely to contain broken stones, from the inspection sample of 50 fruit per container. Fruit are cut open along the suture and the number of fruit containing broken stones is calculated as a percentage of the total number of fruit in the inspection sample. The average percentage of broken stones is then calculated for all the inspection samples (Department of Agriculture Forestry and Fisheries, South Africa, 1990 and 2013).

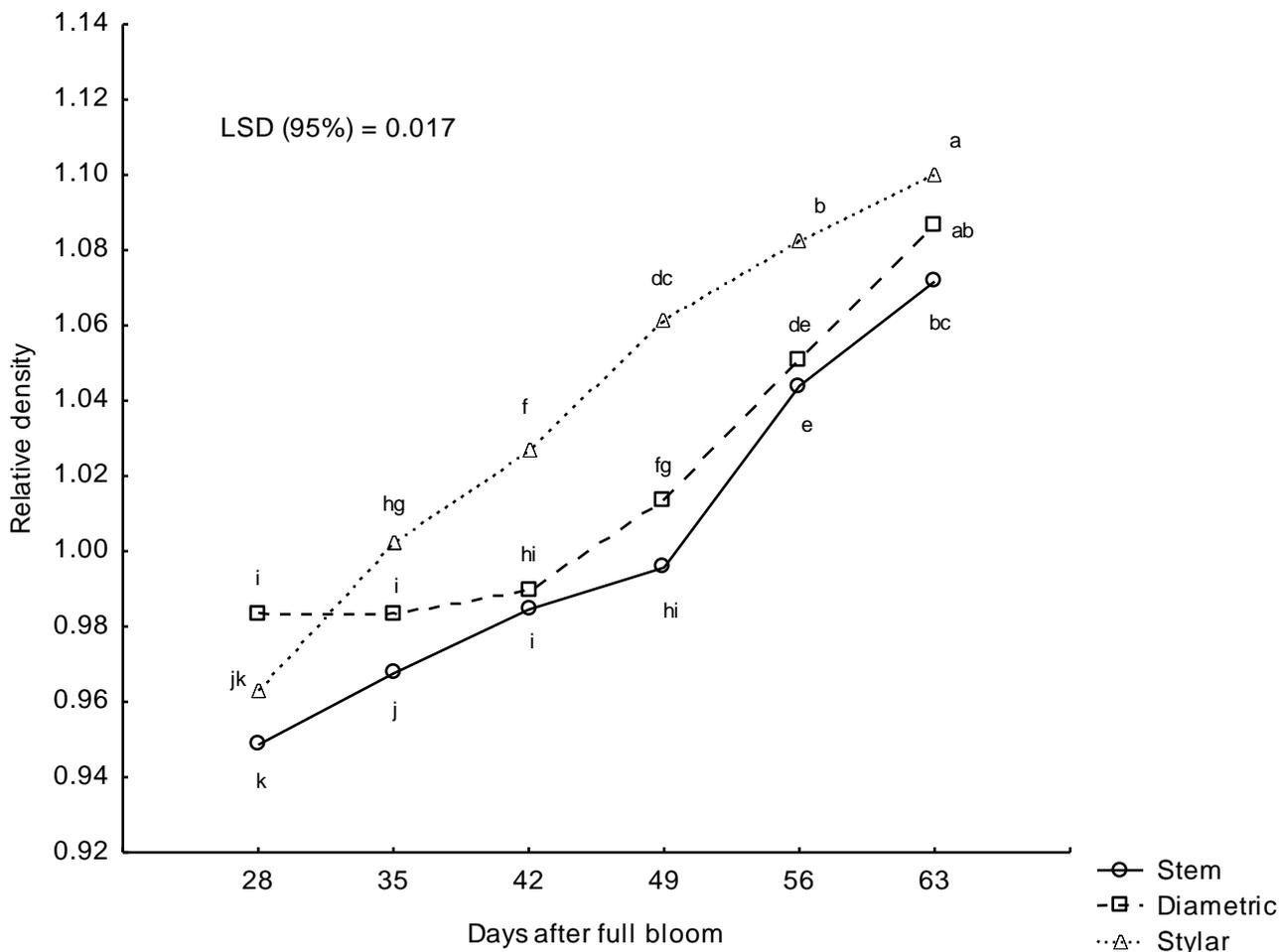
Quality factor	Extra Class	Class 1	Class 2
Shape	Characteristic of the cultivar/variety concerned	Characteristic of the cultivar concerned: Provided that a slight defect in shape, that does not affect the general appearance of the plums/prunes, is allowable	Characteristic of the cultivar concerned: Provided that a defect in shape, that does not seriously affect the general appearance of the plums/prunes, is allowable
Cavities in the flesh and around the stone	Shall not occur	A cavity around the stone is allowable if no decay, discoloration, gum pockets or aperture that externally exposes the cavity, is visible	A cavity around the stone is allowable if no decay, discoloration, gum pockets or aperture that externally exposes the cavity, is visible
Visible split stones, bruises or unspecified progressive defects	2%	5%	6%
Broken stones	3%	10%	-
Malformation	2%	5%	10%

**Table 2**

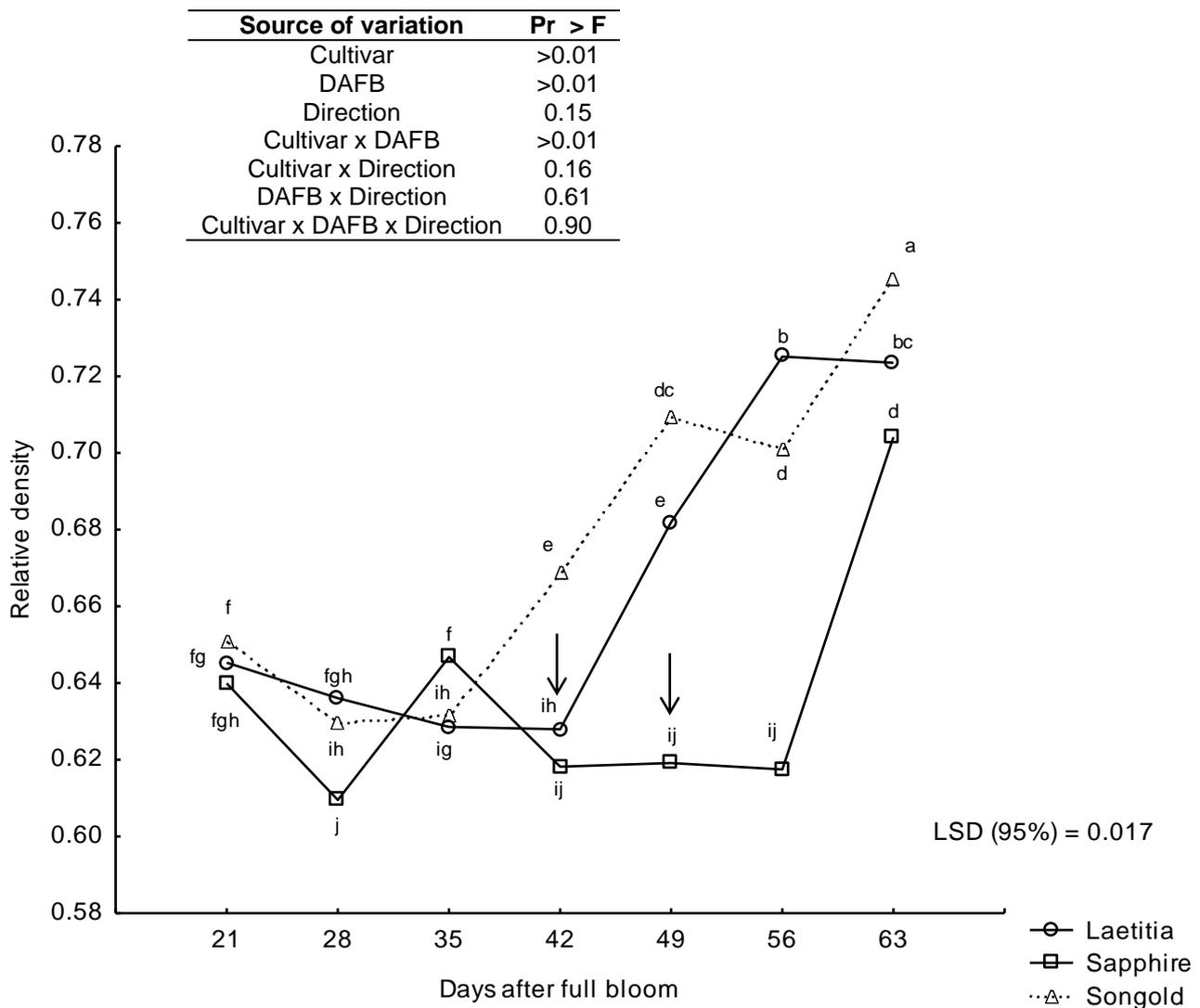
Summary of temperature and rainfall data, indicated as averages with standard deviation, for the 2013/2014 and 2014/2015 seasons, obtained from the Rustenberg weather station in the Stellenbosch area (33°53S 18°54E).

<b>Season</b>	<b>Cultivar</b>	<b>Average temperature (°C) (0-30 dafb)</b>	<b>Average temperature (°C) (0-63 dafb)</b>	<b>Total spring rainfall (mm)</b>	<b>Broken stones at harvest (%)</b>
2013/2014	'Laetitia'	15.45 ± 3.30	16.71 ± 3.38	308 ± 0.74	32.69
	'Songold'	15.28 ± 3.30	16.70 ± 3.38		7.23
2014/2015	'Laetitia'	13.97 ± 3.55	16.22 ± 3.75	147.6 ± 0.83	7.00
	'Sapphire'	13.63 ± 4.41	15.18 ± 4.74		14.00
	'Songold'	14.85 ± 3.82	16.46 ± 3.74		15.00

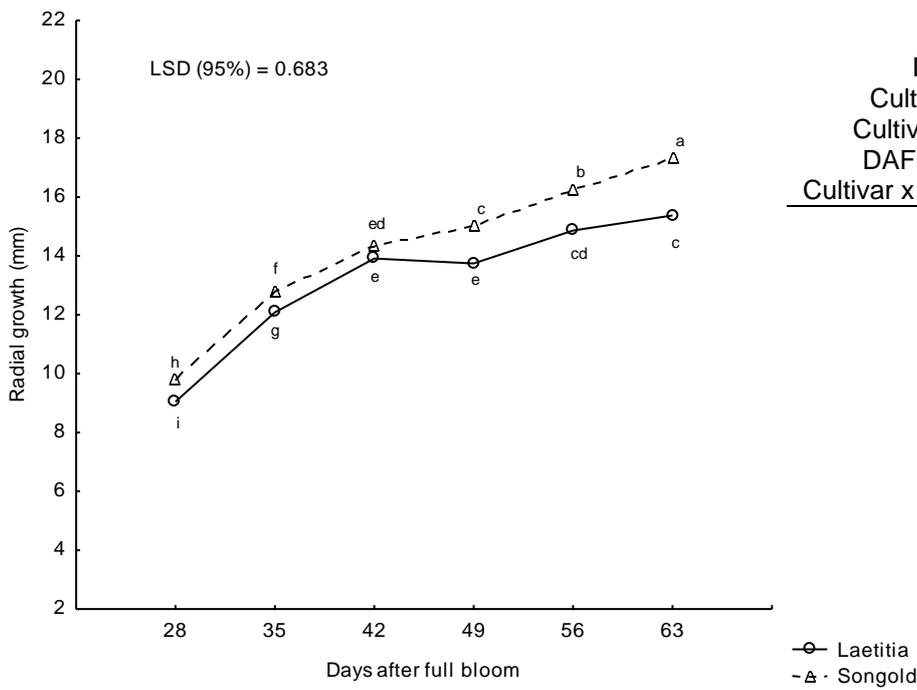
Source of variation	Pr > F
DAFB	>0.01
Cultivar	0.08
Direction	>0.01
DAFB x Cultivar	0.06
DAFB x Direction	>0.01
Cultivar x Direction	0.08
DAFB x Cultivar x Direction	0.19



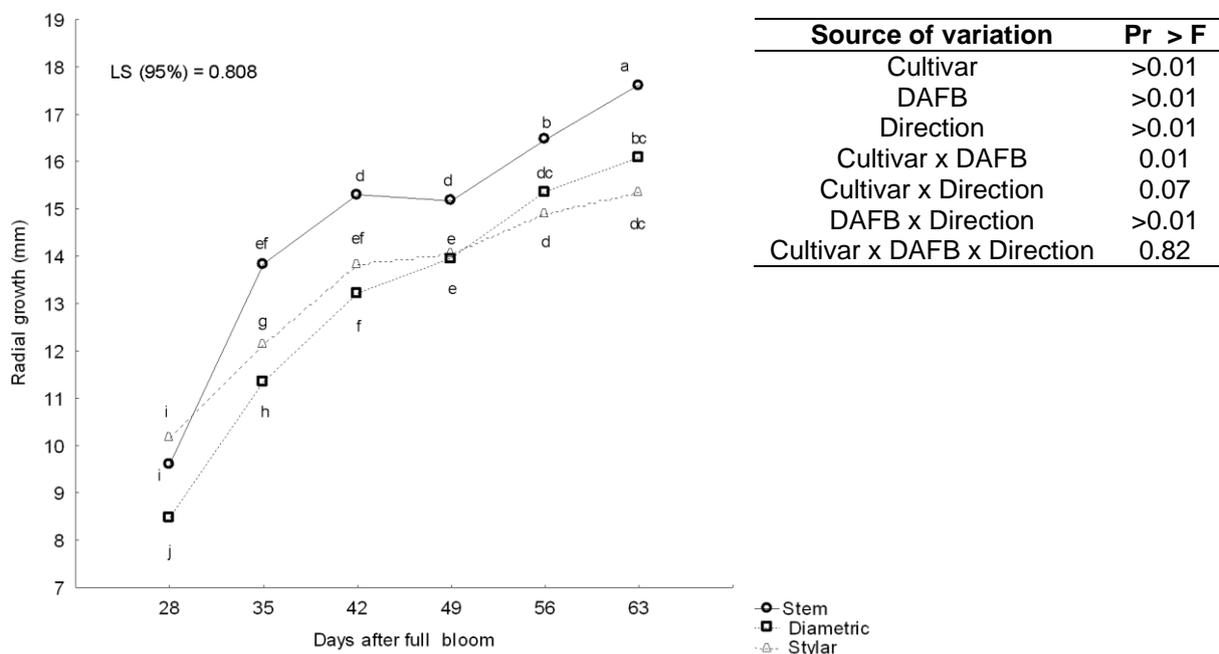
**Fig.1.** Progression of stone hardening, as measured by a change in relative stone density (expressed as a ratio between the average grey value of the stone and that of a reference material of constant density), in ‘Laetitia’ and ‘Songold’ plums during the 2013/2014 season. Significant differences are indicated in lower case letters. The table indicate the source of variation. Broken stones were first observed at 35 dafb for ‘Laetitia’ and 42 dafb for ‘Songold’.



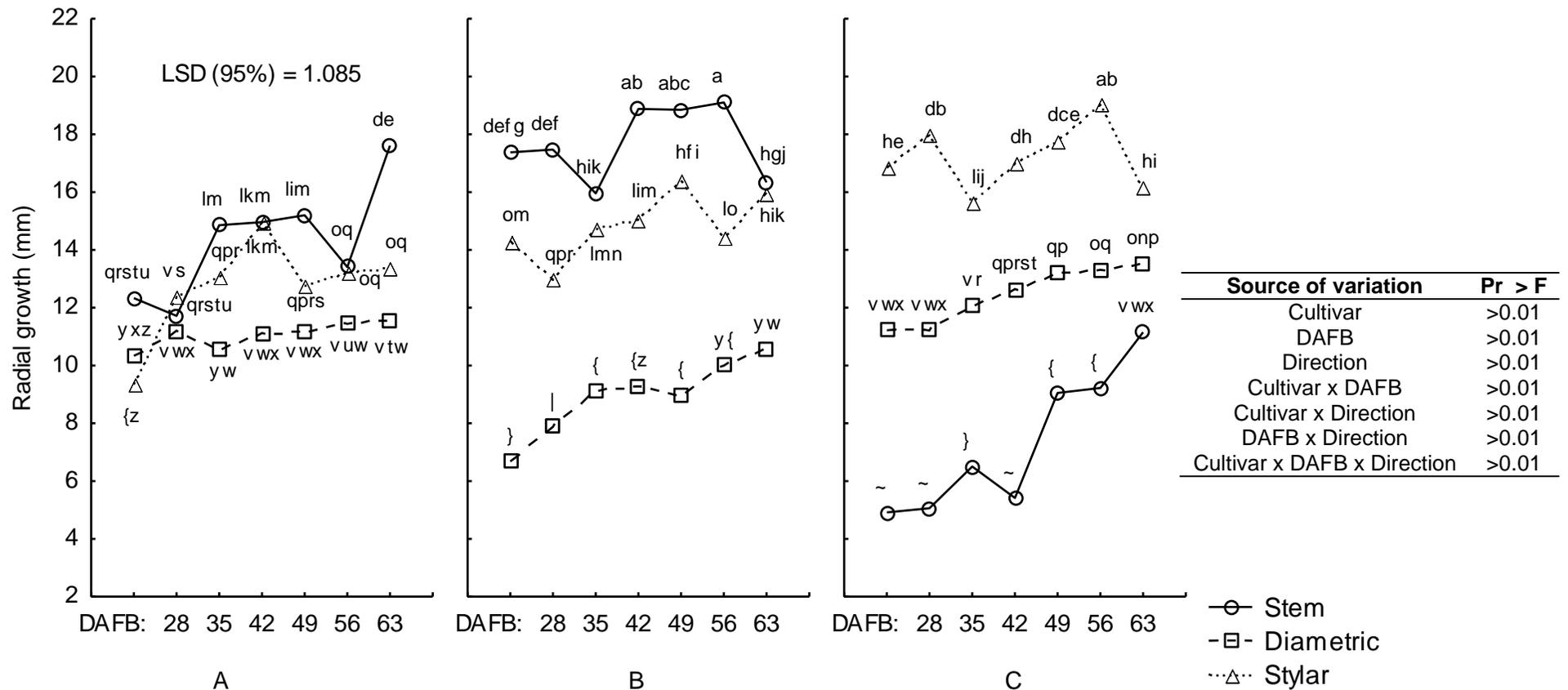
**Fig. 2.** Progression of stone hardening, as measured by a change in stone density (expressed as a ratio between the average grey value of the stone and that of a reference material of constant density), in ‘Sapphire’, ‘Laetitia’ and ‘Songold’ plums during the 2014/2015 season. Significant differences are indicated in lower case letters. The table indicates the source of variation. Arrows indicate the dates when the first broken stones were observed for the respective cultivars (for ‘Songold’ broken stones were only observed at harvest).



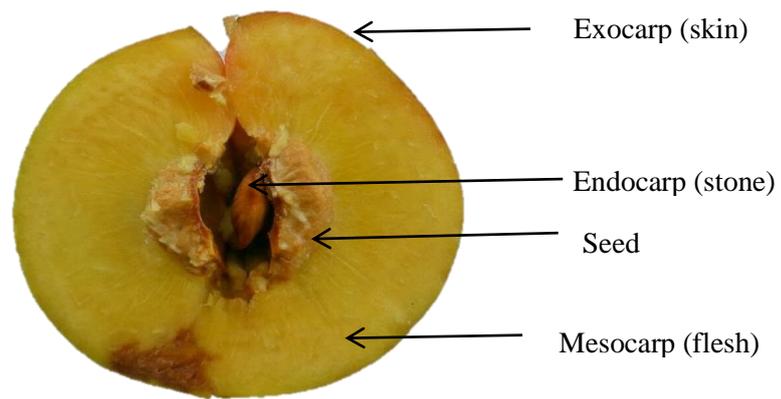
**Fig. 3.** Progression of radial growth in relation to days after full bloom of ‘Laetitia’ and ‘Songold’ plums in the 2013/2014 season. Data is pooled for direction of growth. Significant differences are indicated in lower case letters. The table indicates the source of variation.



**Fig. 4.** Progression of radial growth of pooled ‘Laetitia’ and ‘Songold’ plums in relation to days after full bloom during the 2013/2014 season. Significant differences are indicated in lower case letters. The table indicates the source of variation.



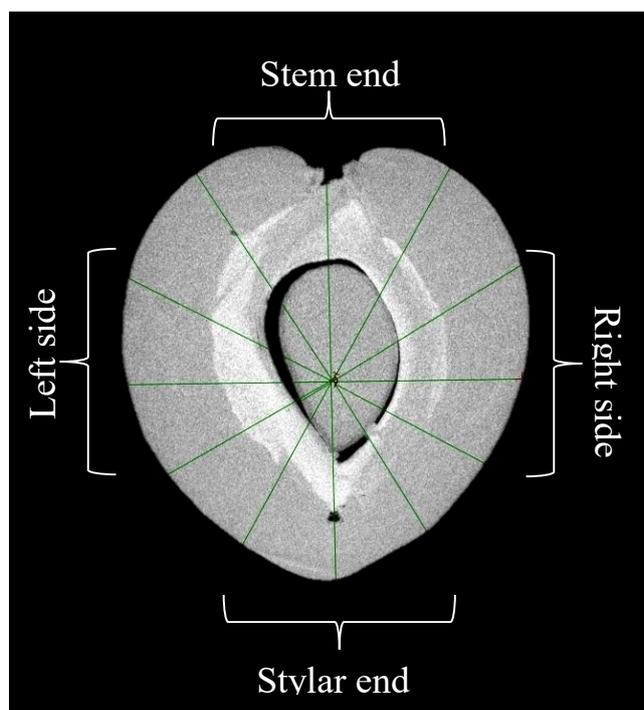
**Fig. 5.** Progression of radial growth of ‘Laetitia’ (A), ‘Sapphire’ (B) and ‘Songold’ (C) plums in relation to days after full bloom and direction of growth (diametric, towards the stem end and styler end of the fruit) during the 2014/2015 season. Significant differences are indicated in lower case letters. The table indicates the source of variation. In order to present the third order interaction more clearly, the effect of the cultivar was divided between Panels A, B and C. Therefore, the three graphs should be seen as a unit and not as three separate sets of data.



**Fig. 6.** Split pit in peach fruit indicating how the stone has split along the suture.



**Fig. 7.** A broken stone in the Japanese plum cultivar 'Laetitia' – unlike what is observed with peaches, the stone did not split along the suture, but is broken near the stilar end of the fruit (from personal observation)



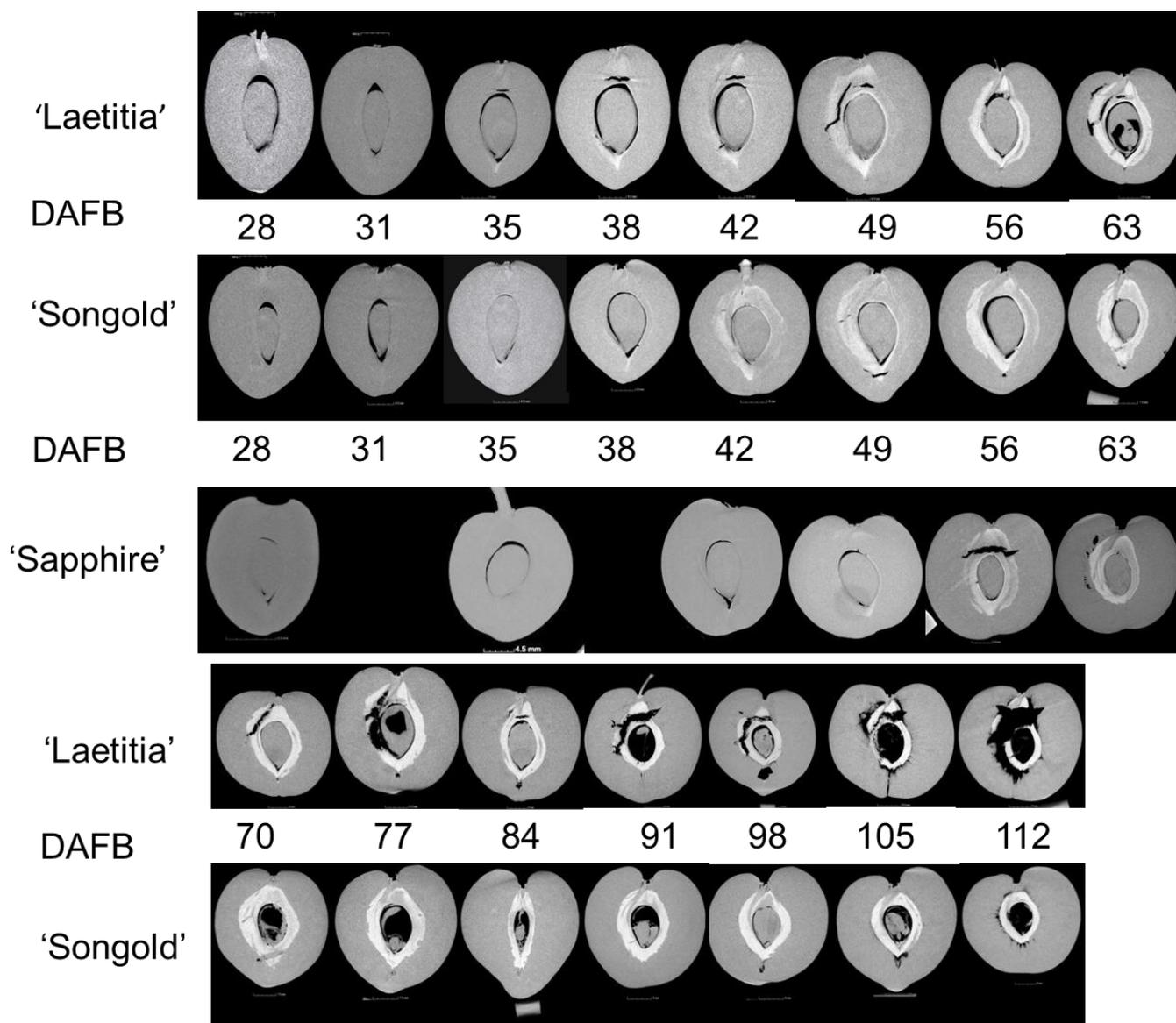
A



B

**Fig. 8A.** 2D slice made along the suture of Japanese plum fruit used for three dimensional computed tomography scans. Lines drawn on the fruit indicate the different directions at which radial growth were measured (at  $30^\circ$  angles to each other) and the brackets indicate which measurements were grouped together to represent the growth at the stem end, stylar end, right side and left side of the fruit.

**Fig. 8B.** Three dimensional computed tomography scan of a 'Songold' plum with a view of the suture. At the bottom of the image is a piece of plastic with a known density which was included in each scan to use as a reference when determining the density of the stone.



**Fig.9.** CT-scans showing the progression of endocarp lignification between 'Laetitia' and 'Songold' over the period of stone hardening in the 2013/2014 season and 'Sapphire' in the 2014/2015 season. For 'Sapphire', scans were only performed during the period of stone hardening and not for the entire season. Lighter colours depict denser areas, whereas darker colours indicate areas of lower density.



**Fig. 10.** 'Laetitia' plums exposed to a Phloroglucinol-HCl solution, which stains tissue pink in the presence of lignin, to indicate the progression of lignification and hence stone hardening in relation to days after full bloom.

## PAPER 3

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### **Effect of foliar and root applications of calcium and silicon on broken stones and fruit quality in Japanese plum cv. 'Laetitia'**

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#### **ABSTRACT**

Broken stones are observed in plums and are defined as a condition that occurs when a piece(s) of the stone breaks off during fruit development. Fruit affected by broken or split stones may not be exported as Class I, even though these fruit adhere to all other Class I quality standards, and thus the presence of broken stones can lead to significant financial losses for South African fruit growers. The exact causes of stone breakage/splitting are not fully understood; both genetic and environmental factors are thought to be involved. Knowing the roles of both calcium and silicon in strengthening the cell wall, this study set out to determine whether foliar and root applications of such products could reduce the incidence and severity of broken stones in the Japanese plum cultivar Laetitia which is very prone to this condition. It was found that neither foliar application of  $\text{Ca}(\text{NO}_3)_2$  and  $\text{K}_2\text{O}_3\text{Si}$ , post-harvest  $\text{Ca}(\text{NO}_3)_2$  applications, nor root  $\text{K}_2\text{O}_3\text{Si}$  application had an effect on the incidence of broken stones. Future studies should determine whether application of these mineral treatments should be started earlier, applied at shorter intervals or at higher concentrations before the onset of stone hardening to determine whether it will lead to a reduction in the incidence of broken stones. Currently neither calcium nor silicate treatments applied as in this paper, can be recommended for reducing broken stones in plums.

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#### ***Keywords:***

Broken stones; Calcium, Endocarp, Plums, Silicate

## 1. Introduction

Broken stones are observed in plums, prunes and peaches and is defined as a condition that occurs when a piece(s) of the stone has broken off during fruit development (HORTGRO, 2012). On the other hand, when the stone of a fruit is broken along its longitudinal axis, it is defined as split pit. According to standards set by the Organisation for Economic Co-operation and Development (OECD), fruit affected by broken or split stones may not be exported as Class I, even though these fruit adhere to all other Class 1 quality standards (OECD, 2002). As a reduced price is paid for Class 2 fruit, the presence of broken stones can lead to significant financial losses for fruit growers. The exact causes of stone breakage/splitting are not fully understood; both genetic and environmental factors are thought to be involved (Claypool et al., 1972; Engin et al., 2010). As fruit continue to grow after breakage or splitting has occurred, cavities often form within the flesh (Chatzitheodorou et al., 2004; Engin et al., 2010). Furthermore, the extent of splitting or breakage has been found to vary greatly among cultivars and between seasons (Sotiropoulos et al., 2010; Woodbridge, 1978).

Traditionally, early maturing peach cultivars are more prone to broken stones than later maturing cultivars ( Barcelon et al., 1999; Chatzitheodorou et al., 2004 Claypool et al., 1972; Tani et al., 2007). This is because the stone hardening (Stage II) and final swell (Stage III) phases of fruit growth occur relatively close together due to the shortened Stage II of early cultivars. During the hardening and lignification processes of Stage II, the stone starts to lose flexibility and becomes very rigid while the flesh is still tightly attached to it (Tani et al., 2007). In early ripening cultivars, Stage III of growth commences before the attachment between the stone and the flesh has weakened (Tani et al., 2007). Growth during Stage III then creates internal forces that pull on the stone. If these forces are strong enough, they can pull the stone apart along its weakest parts, because during this stage, the stone is not strong enough to withstand such external forces (Ragland, 1934; Tani et al., 2007).

Ragland (1934) hypothesized that split stones in peaches may be caused by inadequate calcium translocation within the fruit, because broken stones resulted from cell wall fractures. Calcium plays an essential role in strengthening and stabilizing the cell walls by binding to pectin to form a tight calcium pectate complex (Marchner, 2002; Taiz and Zeiger, 2010). Calcium is generally immobile in the phloem and is thus transported from the roots via the xylem (Sotiropoulos et al., 2010; White and Broadley, 2003). Translocation of calcium to fruit and leaves is, therefore, dependent on the transpiration rate of these tissues (White and Broadley, 2003). The transpiration rates of fruit as well as young leaves are usually quite low, and as a consequence, only small amounts of calcium are imported into fleshy fruit (Saure, 2005; White and Broadley, 2003). Calcium uptake is quite slow during the first 3 weeks after bud-break and, therefore, early growth is dependent on the calcium reserves stored within the permanent structures of the tree (Stassen and Stadler, 1988). However, from 3 weeks after full bloom calcium accumulates rapidly until harvest. The calcium content in the permanent structures of the tree remains

relatively constant, which indicates that new growth is entirely dependent on calcium uptake during the growing season (Stassen et al., 1983).

Foliar applications of calcium, applied directly to the surface of young fruit, can penetrate the fruit through trichomes and stomata and is then transported via diffusion through the apoplast (i.e. the cell walls and intercellular spaces) (Saure, 2005). In addition to calcium, silicon (in the form of amorphous silica) also enhances the strength and rigidity of the cell walls by interaction with pectins and polyphenols (Marchner, 2002). Furthermore, silicon also increases cell wall elasticity during extension growth (Marchner, 2002) and the coating formed by silicon foliar applications on the surface of leaves have been found to act as a physical barrier to pathogen penetration (Menzies et al., 1992). Similar to calcium, silicon is transported in the xylem and is dependent on transpiration for movement through the plant.

While studies have been conducted on the effect of calcium and silicon on the incidence of broken/split stones in peaches (Evert et al., 1988; Sotiropoulos et al., 2010), to our knowledge, no such studies have been conducted on plums. Knowing the roles of both calcium and silicon in strengthening the cell wall, in this study we set out to determine whether foliar and root applications of these products could reduce the incidence and severity of broken stones in the Japanese plum cultivar 'Laetitia' which is very prone to this condition. In addition, we also aimed to determine whether these treatments would affect post-harvest and post-storage quality.

## **2. Materials and Methods**

### **2.1 Site description**

In the 2013/2014 season the trial was conducted on 'Laetitia' plums (*Prunus salicina* Lindl.) at the Welgevallen experimental farm, Stellenbosch University, Stellenbosch, Western Cape, South Africa (33°56'49.8"S 18°52'16.6"E). Trees (on 'Marianna' rootstocks) were planted in 1998, with a north-south row direction, a planting distance of 4.5 x 1.5 m and trained according to a palmette system. This trial will be referred to as Trial 1 in this paper.

In the 2014/2015 season, Trial 1 was repeated in the same orchard. Since we had already laid out the trial with an additional postharvest calcium application which was applied after the harvest in the 2013/2014 season, and since we subsequently decided to include potassium-silicate root applications, an additional trial (Trial 2) was conducted in an adjacent orchard. Trees (on 'Mariana' rootstocks) used for Trial 2 were planted in 1992 at a spacing of 4.5 x 1.25m. The row orientation was north-south and the trees were also trained according to a palmette system and were managed similar to Trial 1.

## 2.3 Product specifications

YaraLiva™ Calcinit™ (Yara BU Africa, Fourways, South Africa) which contains 19.0% (w/w) calcium, 26.3% (w/w) calcium oxide and 15.5% (w/w) nitrogen, was used for the foliar calcium nitrate ( $\text{Ca}(\text{NO}_3)_2$ ) treatments. Approximately 2 L of solution was applied to each tree by spraying the tree for 30 s from both sides of the tree row, at a concentration of 675 mL/100 L of water. Applications were carried out early in the morning, with a motorised knapsack sprayer until run-off, only when wind speeds were less than  $4 \text{ m s}^{-1}$ . For the potassium silicate treatment, AgriSil™ K50 (PQ Corporation, Wolseley) containing 33 g/kg of potassium (as K) and 96 g/kg of silica (as Si) was used – applied at a  $5 \text{ kg ha}^{-1}$  rate. Trees were sprayed for 30 s from both sides of the tree row, thus applying about 2 L of solution per tree at a concentration of 170 mL/100 L of water. No surfactants were added to any of the treatments. For the post-harvest calcium root application, 15 g of YaraLiva™ Nitrabor™ granules (Yara BU Africa, Fourways, South Africa) were spread around the trunk of each tree (containing 15.4% nitrogen and 26% calcium). For the root potassium silicate ( $\text{K}_2\text{O}_3\text{Si}$ ) treatment, the same product and at the same concentration that was used for the foliar application, was applied to the roots. Two litres of solution was applied directly to the roots around the trunk of each tree.

## 2.2 Experimental layout

### 2.2.1 2013/2014 season (Trial 1):

A foliar  $\text{Ca}(\text{NO}_3)_2$  application and a foliar  $\text{K}_2\text{O}_3\text{Si}$  application were applied as treatments in addition to the control, with no supplemental sprays beyond those used in standard cultivation practices. A randomised complete block design with eight two-tree plots per treatment was used. Buffer trees were left between treatments and between rows to prevent spray drift of the foliar applications. Four consecutive calcium sprays were applied weekly from 21 days after full bloom (dafb) until the onset of stone hardening. Three potassium-silicate sprays were applied at 14 day intervals from 21 dafb until the start of stone hardening.

### 2.2.2 2014/2015 season (Trial 1 and Trial 2):

In the 2014/2015 season Trial 1 was repeated in the same orchard, but on different trees with the addition of a post-harvest calcium treatment. In the 2014/2015 season Trial 1 therefore had 4 treatments, namely a control, a postharvest calcium application (which was applied after harvest in the 2013/2014 season), a foliar ( $\text{Ca}(\text{NO}_3)_2$ ) application and a foliar potassium-silicate application. The post-harvest calcium applications consisted of two foliar applications, applied 14 and 21 days after harvest, and a calcium root application applied 21 days after harvest in the 2013/2014 season. Three consecutive foliar calcium sprays were applied weekly from 21 dafb until the onset of stone hardening, while two potassium-silicate

foliar sprays were applied at 14 day intervals from 21 dafb until the start of stone hardening. Since the onset of stone hardening was earlier in the 2014/2015 season, fewer foliar applications could be applied than in the 2013/2014 season. A randomised complete block design with eight one-tree plots per treatment was used. Buffer trees were left between treatments and between rows to prevent carry-over.

In Trial 2, four treatments were applied namely a control, with no supplemental sprays beyond those used in standard cultivation practices, a foliar calcium nitrate ( $\text{Ca}(\text{NO}_3)_2$ ) application, a foliar potassium-silicate ( $\text{K}_2\text{O}_3\text{Si}$ ) application, and a root  $\text{K}_2\text{O}_3\text{Si}$  application. Three foliar calcium sprays were applied weekly from 21 dafb until the onset of stone hardening, while two potassium-silicate sprays were applied at 14 day intervals from 21 dafb until the start of stone hardening. Root applications were applied every 4 weeks, from full bloom until the end of stone hardening, amounting to two applications. A randomised complete block design with eight two-tree plots per treatment was used.

Relative humidity and temperature was logged in both seasons at hourly intervals in the orchard from full bloom until harvest with a 'Tiny Tag' (TGP-4500) logger (Gemini Data Loggers Ltd, Chichester, West Sussex, UK). The logger was placed approximately 1.5 m above the ground in a white logger shield.

## 2.4 Measurements

On the commercial harvest date (28 Jan. 2014) in the 2013/2014 season, all the fruit from one tree per block per treatment were harvested to determine yield. After harvest, all fruit were cut open along the suture to determine the severity (size of the piece of stone that had broken off) and incidence of broken stones, as well as the size of the cavity formed as a result of stone breakage. Severity of stone breakage was graded on a scale between 1 and 4, with 1 being no broken pieces, 3 being large broken pieces and 4 used to classify instances where the entire stone had broken in half i.e. split stones (Fig. 1). Cavities were graded on a scale between 1 and 6 with 1 being no cavity, 5 a very large cavity and 6 was used for cases where the stone had broken completely in half (Fig. 2). In the 2014/2015 season, only 100 fruit per treatment, per block, were harvested to determine the incidence and severity of broken stones and cavity size.

Ninety fruit were selected randomly at harvest from each block per treatment in both seasons and sub sampled for the following analyses: Thirty fruit were used to determine fruit maturity and quality at harvest, while 60 fruit were packed into two count-30 pulp trays, placed into a carton (MO5I) and covered with a perforated, high density polyethylene (HDPE) shrivel sheet while still in the orchard. This was done to prevent moisture loss from the fruit as 'Laetitia' plums are prone to shrivel during storage. Directly after harvest, the fruit were transported to the laboratory at the Department of Horticultural Science, Stellenbosch University, using covered, uncooled transport. The cartons of packed fruit were immediately put into cold-storage and stored under an intermittent warming regime

(10 days at -0.5 °C, 7 days at 7.5 °C, 25 days at -0.5 °C plus a simulated shelf-life period of 7 days at 10 °C) used commercially. Fruit (30) were evaluated for quality parameters on the harvest date, after cold-storage and after the simulated shelf-life period. The plastic shrivel sheet was removed before the fruit were placed under shelf –life conditions to prevent condensation and fruit decay.

The following measurements were performed on the harvest date. Peel foreground colour (percentage red blush) was determined on all 30 fruit per treatment per block by comparison to an industry standard colour chart (PL 25, Unifruco, Belville), where 1= no red colour and 15 = full red colour. Hue angle ( $h^\circ$ ) was determined with a Minolta CR-400 Chroma meter (Konica Minolta, Japan) on both cheeks of 10 fruit per treatment per block.  $H^\circ$  was calculated from the coordinates obtained from the chroma meter (arctangent of  $b^*/a^*$ ), where  $0^\circ$  = red,  $90^\circ$  = yellow,  $180^\circ$  = green and  $270^\circ$  = blue (McGuire, 1992). Flesh firmness (N) was determined on both cheeks of 10 fruit per block per treatment using an electronic fruit texture analyser (GÜSS GS-20, Strand, South Africa) fitted with an 11 mm tip. Total soluble solids (TSS) were measured by pooling and juicing wedges of 10 fruit from each block and treatment separately and measuring the % Brix using a digital refractometer (Palette PR-32 AGAGO, Bellevue, USA). Titratable acidity (TA), expressed as the percentage malic acid present, was measured using 10 g of the pooled juice sample which was then titrated with 0.1 M NaOH to an end-point of pH 8.2 using a 719 S Titrino automated titrator, fitted with a Metrohm AG 760 sample changer (Herisau, Switzerland).

After cold-storage and after the simulated shelf-life period, respectively, 30 fruit per carton (15 from the top layer and 15 fruit from the bottom layer) were evaluated. All fruit were inspected individually for the presence of shrivel, which was determined subjectively and only recorded if shrivelled skin reached over the shoulder of the fruit. Flesh firmness and  $h^\circ$  were determined on 10 fruit per treatment per block. Internal defects (%) were determined for all fruit by cutting the fruit around the equatorial axis, and separating the two halves of the fruit. A gelatinous breakdown of the inner mesocarp tissue surrounding the stone, while the outer mesocarp tissue had a healthy appearance, was classified as gel breakdown. A brown discolouration of the mesocarp tissue, associated with a loss in juiciness, was classified as internal browning. Gel breakdown and internal browning values were combined to represent percentage fruit with chilling injury. Over-ripeness was recorded when the fruit were abnormally soft and excessive amounts of juice were present.

## 2.5 Statistical analysis

Two-way analysis of variance (ANOVA) were performed (95% confidence limit) to determine the effects of treatments on the different parameters for each trial. Then, for the first three treatments of each trial (control, pre-harvest foliar  $\text{Ca}(\text{NO}_3)_2$  and foliar  $\text{K}_2\text{O}_3\text{Si}$ , trial number was included as a factor. By doing this, the variation contributed by the orchards and seasons were removed from the analysis and only the effect of the control and foliar calcium and silicate treatments were established on the

various parameters measured. Differences were expressed as least square means (95% confidence limit). The analyses were performed using Statsoft STATISTICA version 12.

### 3. Results

#### 3.1 Incidence and severity of broken stones

The incidence of broken stones from the control trees was much higher in the 2013/2014 season (29.10%) compared to the 2014/2015 season (4.01% average) (Table 2 and 3). There was no significant difference on either the severity of broken stones or the size of the cavity formed within the flesh between the pre-harvest foliar Ca and Si treatments and the control for Trial 1 conducted in the 2013/2014 season (Table 1). The treatments did, however, differ slightly regarding the incidence of broken stones. While the  $K_2O_3Si$  treatment did not differ from the control, it had a significantly lower incidence of broken stones compared to the  $Ca(NO_3)_2$  treatment, which had the highest incidence of broken stones, but also did not differ significantly from the control. During season 2 (2014/2015), there were no significant differences between the treatments for either the incidence and severity of broken stone formation or the size of the flesh cavities (Table 2). Similarly, in Trial 2 (2014/2015), no significant differences between the treatments were observed with regard to broken stone incidence or severity or the size of the cavities in the flesh (Table 3).

When data from Trial 1 were pooled for seasons and orchards to remove the effect of season and orchard from the data set, there were still no significant differences between the control and treatments were observed for the incidence of broken stones, broken stone severity or the size of the cavity in the flesh (Table 4).

#### 3.2 Maturity indexing on harvest date

Maturity indexing performed on the harvest date in the 2013/2014 season indicated no significant differences between the treatments and the control for fruit firmness, TA, TSS or  $h^\circ$  (Table 5). Peel foreground colour was significantly influenced by the  $K_2O_3Si$  treatment, being significantly redder than both the control and the calcium treatment.

In the 2014/2015 season, the control and foliar  $Ca(NO_3)_2$  treatments in Trial 1 had significantly more red colouration compared to the foliar  $K_2O_3Si$  and postharvest  $Ca(NO_3)_2$  treatments (Table 6). Treatments did not differ significantly from the control for TSS, TA,  $h^\circ$  or fruit firmness. Trial 2 of the 2014/2015 season, indicated that the root silicate treatment differed significantly from the other treatments, having a lower %TSS (Table 7). Titratable acidity also differed significantly between the treatments, with both the foliar and root silicate applications being significantly lower than the control

and foliar calcium treatment. No significant differences were observed between the treatments for foreground colour,  $h^{\circ}$  or firmness (Table 7).

For the pooled data over the two seasons there were no significant differences between treatments regarding fruit firmness, TSS or  $h^{\circ}$  (Table 8). Peel foreground colour was significantly influenced by the treatments with the foliar  $K_2O_3Si$  treatment being significantly redder than the foliar calcium treatment and the control. Titratable acids also differed significantly between treatments, with the foliar calcium and silicate applications having significantly lower levels than the control.

### 3.3 Post-storage and post-shelf-life quality

After cold-storage and after the 7 day shelf life period, no significant treatment effect was observed for  $h^{\circ}$  for Trial 1 in the 2014/2014 season (Table 9). However, for all treatments, the  $h^{\circ}$  decreased over storage time from harvest (Table 5), which means that more red pigmentation developed during cold storage and/or were unmasked due to chlorophyll breakdown. Firmness and shrivel were also not significantly different between the treatments (Table 9). Though not significant, the foliar silicate treatment had a slightly lower incidence of shrivel after shelf life compared to the control and calcium nitrate treatment. No internal fruit defects were recorded after cold-storage or shelf-life (data not shown)

After cold-storage and after shelf-life, no significant treatment effect was observed for  $h^{\circ}$ , firmness, %shrivel, or the %internal defects for either Trial 1 or Trial 2 in the 2014/2015 season (Tables 10 and 11). However, for all treatments, both the  $h^{\circ}$  and flesh firmness decreased from harvest during the cold-storage period as fruit matured (Table 6). No post-storage decay or over-ripe fruit were observed (data not shown). The same results were observed when the data from Trial 1 were pooled over seasons and orchards (Table 12). No significant differences between the treatments were observed for any of the parameters.

## 4. Discussion

In the 2013/2014 season the incidence of broken stones was significantly higher than in the 2014/2015 season (30.41% versus 5.19%). However, there was no significant treatment effects on the percentage broken stones for either season (Table 1, 2, 3 and 4). These results are in contrast to results obtained by Sotiropoulos et al. (2010) who found that potassium silicate significantly decreased the amount of split-pit in peaches (also after applying three consecutive foliar sprays). They hypothesised that the increase in cell wall elasticity brought about by silicon might explain the lower incidence of split-pit. It is possible that, when the cell walls of the endocarp are more elastic, the stones are better able to resist pulling forces from the flesh during stages of rapid mesocarp growth, and are thus less likely to break. However, this could not be confirmed in our study. In peaches, the stone usually splits open along the suture/ seam

of the endocarp (Department of Agriculture Forestry and Fisheries, South Africa, 1990). However, with stone breakage in Japanese plums, the endocarp can disintegrate (break apart) anywhere, and often in multiple areas (Personal observation). As the way in which the stones break/split differ between plums and peaches, this might explain why we did not observe a similar reduction in broken stones after silicon treatments. Similar to our findings Evert et al. (1988) found that foliar  $\text{Ca}(\text{NO}_3)_2$  sprays, also applied before and during stone hardening, had no effect on the occurrence of split pit in peaches.

The combined results for both seasons (Trial 1) indicated that the both the foliar  $\text{Ca}(\text{NO}_3)_2$  and foliar  $\text{K}_2\text{O}_3\text{Si}$  treatments resulted in a significantly lower TA compared to the control (Table 8). These differences are not commercially relevant, because the difference between the treatments was so small that it would not have affected fruit taste. Similarly, in the 2014/2015 season, both the foliar  $\text{K}_2\text{O}_3\text{Si}$  and the root-applied  $\text{K}_2\text{O}_3\text{Si}$  treatments (Trial 2) had significantly lower TA compared to the control and the foliar  $\text{Ca}(\text{NO}_3)_2$  treatments (Table 7). However, the differences were not big enough to affect fruit taste. Furthermore, the root application of  $\text{K}_2\text{O}_3\text{Si}$  led to significantly lower TSS compared to other treatments (Table 7), but this difference was also not big enough to be commercially significant and will not be discussed further.

Foliar  $\text{K}_2\text{O}_3\text{Si}$  as well as post-harvest  $\text{Ca}(\text{NO}_3)_2$  had a significant effect on peel foreground colour, as in both cases, the treatments led to significantly less red foreground colour compared to the other treatments (Tables 5, 6 and 8). However the LSD values were only 0.63, 0.46 and 0.32, respectively – a difference in colour that would not be distinguishable by the human eye. This was confirmed by the non-significant differences in the  $h^\circ$  measurements between treatments (Tables 5, 6 and 8).

The changes in  $h^\circ$  values between harvest and cold-storage were expected and correlated with the findings from other plum studies (Abdi et al., 1997; Jooste, 2012; Singh et al., 2009).

The decrease in flesh firmness observed after harvest was expected as loss of firmness is associated with the ripening process of fruit (Khan and Singh, 2007). In contrast to our results, which show that firmness did not differ significantly between treatments, multiple authors have shown that fruit firmness is increased by application of calcium treatments in different crops (Ochei and Basiouny, 1993; Raese and Drake, 2000; Tzoutzoukou and Bouranis, 1997; Wojcik, 2001). Since differences between treatments were non-significant, no mineral analyses were performed to determine calcium or silicon concentrations in the fruit flesh. Hence, it is possible that in this study, no effect of calcium on broken stones or fruit quality was found due to a lack of calcium absorption by the endocarp, which was not quantified, or a significantly high level of these mineral elements rendering additional applications ineffective. In addition, studies on both avocado fruit and zucchini corroborate with our results that treatment of fruit with silicon does not have a significant effect on fruit firmness (Kaluwa et al., 2009; Savvas et al., 2009).

The small percentage of shrivelled fruit observed after cold-storage may be due to sub-optimal relative humidity in the cold-room (Jooste, 2012). Firstly, moisture loss from the fruit depends on the deficit between the saturated vapour pressure within the fruit and the actual vapour pressure of the atmosphere surrounding the fruit (Paull, 1999). This means that if the relative humidity within the cold-room was not ideal for plums, it would result in an increase in shrivel incidence over time as the fruit continue to lose moisture to the surrounding atmosphere. Secondly, fruit treated with calcium might be more prone to developing symptoms of shrivel as storage duration increases. Plich and Wojcik (2001) found that plums treated with calcium chloride sprays lost moisture faster than control fruit. This may explain why we observed a slightly higher (although not significant) incidence of shrivel with the foliar  $\text{Ca}(\text{NO}_3)_2$  treatment (Tables 9, 10, 12). In contrast,  $\text{K}_2\text{O}_3\text{Si}$  was reported to reduce transpiration from the cuticle (LiQun et al., 2006; Ma and Yamaji, 2006; Marchner, 2002). This may explain why slightly less shrivel was observed in plums treated with  $\text{K}_2\text{O}_3\text{Si}$  compared to the  $\text{Ca}(\text{NO}_3)_2$  treatment, as the fruit may have lost less moisture during storage. However, in this study, we focussed on the effects of treatments on broken stones and shrivelling will thus not be discussed further.

## 5. Conclusions

We set out to determine whether foliar and/or root applications of either calcium nitrate or potassium silicate would decrease the occurrence or severity of broken stones in Japanese plums. As it is known that both silicon and calcium increase the strength of the cell walls, it was hypothesized that either foliar applications or root applications of  $\text{Ca}(\text{NO}_3)_2$  and  $\text{K}_2\text{O}_3\text{Si}$  would strengthen the plum endocarp and thereby reduce the incidence of broken stones. However, no such effect was observed. Since no treatment effects were found, we did not quantify the uptake of either mineral in the fruit or stones. Studies in peaches have shown that calcium foliar applications have no effect on the incidence of split stones and our results are similar. In contrast, studies have found that potassium silicate lowers the incidence of split-pit in peaches, but our results with Japanese plums do not support this. This may either be due to the differences between the fruit kinds, between the two defects or the protocol followed with treatment application.

In future studies, foliar applications of Ca and/or Si could be applied earlier (before 21 dafb), be applied at shorter intervals (bi-weekly) or possibly be applied at higher concentrations before the onset of stone hardening to investigate whether it will lead to a reduction in the incidence of broken stones. Similarly, an investigation into the increase of the Ca reserve status in the tree by postharvest soil application of Ca and/or Si may be of interest. Currently neither calcium nor silicate treatments as applied according to this study can be recommended for reducing the incidence of broken stones in plums.

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**Table 1**

The effect of pre-harvest calcium and pre-harvest silicate treatments on the incidence and severity of broken stones as well as the size of the cavity formed in the flesh due to stone breakage for Trial 1 of the 2013/2014 season.

Treatment	Broken stone severity <sup>z</sup>	Cavity severity <sup>y</sup>	Incidence of broken stones (%)
Control	0.6 ns	1.6 ns	29.1ab <sup>x</sup>
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	0.7	1.7	34.54
Foliar K <sub>2</sub> O <sub>3</sub> Si	0.5	1.5	23.0b
Pr>F <sup>x</sup>	0.07	0.29	<b>0.05</b>
LSD (95%)	0.20	0.19	8.92

<sup>z</sup> Broken stone severity was graded on a scale between 1 and 4, with 1 being no broken pieces, 3 being large broken pieces and 4 used to classify instances where the entire stone had broken in half (Fig. 1).

<sup>y</sup> Cavity severity was graded on a scale between 1 and 6 with 1 being no cavity, 5 a very large cavity and 6 was used for cases where the stone had broken completely in half (Fig. 2).

<sup>x</sup> Significant differences between values are indicated as lower case letters.

**Table 2**

The effect of pre- and postharvest calcium and pre-harvest silicate treatments on the incidence and severity of broken stones as well as the size of the cavity formed in the flesh due to stone breakage for Trial 1 of the 2014/2015 season.

Treatment	Broken stone severity <sup>z</sup>	Cavity severity <sup>y</sup>	Incidence of broken stones (%)
Control	0.1 ns	1.1 ns	3.6 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	0.1	1.1	3.4
Foliar K <sub>2</sub> O <sub>3</sub> Si	0.0	1.1	2.5
Post-harvest Ca(NO <sub>3</sub> ) <sub>2</sub>	0.1	1.1	3.1
Pr>F <sup>x</sup>	0.54	0.59	0.81
LSD (95%)	0.05	0.08	2.47

<sup>z</sup> and <sup>y</sup> For definitions of headings see Table 1

**Table 3**

The effect of pre-harvest calcium and silicate treatment on the incidence and severity of broken stones as well as the size of the cavity formed in the flesh due to stone breakage for Trial 2 of the 2014/2015 season.

Treatment	Broken stone severity <sup>z</sup>	Cavity severity <sup>y</sup>	Incidence of broken stones (%)
Control	0.1 ns	1.2 ns	4.5 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	0.1	1.3	5.6
Foliar K <sub>2</sub> O <sub>3</sub> Si	0.2	1.4	7.4
Root K <sub>2</sub> O <sub>3</sub> Si	0.1	1.2	11.5
Pr>F <sup>x</sup>	0.10	0.44	0.07
LSD (95%)	0.10	0.22	5.43

<sup>z</sup> and <sup>y</sup> For definitions of headings see Table 1.

**Table 4**

The effect of pre-harvest foliar calcium and silicate applications on the incidence and severity of broken stones as well as the size of the cavity formed in the flesh due to stone breakage over both seasons. Data of two seasons from the same orchard as well as of an additional orchard in the second season were pooled for the analysis to remove the variation created by season and orchard.

Treatment	Broken stone severity <sup>z</sup>	Cavity severity <sup>y</sup>	Incidence of broken stones (%)
Control	0.6 ns	1.1 ns	13.3 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	0.6	1.0	14.9
Foliar K <sub>2</sub> O <sub>3</sub> Si	0.6	1.0	12.6
Pr>F	0.91	0.59	0.61
LSD (95%)	0.09	0.11	4.52

<sup>z</sup> and <sup>y</sup> For definitions of headings see Table 1.

**Table 5**

Maturity indexing and quality parameters of ‘Laetitia’ plums measured on the harvest date for fruit which received pre- harvest calcium and silicate treatments (Trial 1) in the 2013/2014 season.

Treatment	TSS (%)	TA (%)	Foreground Colour <sup>2</sup>	Hue angle (h°)	Firmness (N)
Control	10.9 ns	1.4 ns	10.1b	46.3 ns	82.5 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	10.8	1.4	10.2b	54.2	82.1
Foliar K <sub>2</sub> O <sub>3</sub> Si	11.0	1.4	10.9a	49.7	81.3
Pr>F <sup>1</sup>	0.63	0.13	<b>0.05</b>	0.08	0.98
LSD (95%)	0.44	0.05	0.63	6.94	10.97

<sup>1</sup> Significant differences between values are indicated as lower case letters.

<sup>2</sup> Unifruco fruit colour plate PL25 (for Laetitia) for assessment of peel background colour according to a scale from 1 (no red colour) to 15 (full red colour).

**Table 6**

Maturity indexing and quality parameters of 'Laetitia' plums measured on the harvest date for fruit which received pre- and postharvest calcium treatments as well as pre-harvest silicate treatments (Trial 1) in the 2014/2015 season.

Treatment	TSS (%)	TA (%)	Foreground Colour <sup>2</sup>	Hue angle (h°)	Firmness (N)
Control	11.3 ns	2.0 ns	12.8a	32.3 ns	63.6 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	11.3	1.9	12.9a	30.0	61.4
Foliar K <sub>2</sub> O <sub>3</sub> Si	10.9	1.9	13.5b	28.1	52.9
Post-harvest Ca(NO <sub>3</sub> ) <sub>2</sub>	10.9	1.9	13.7b	26.6	57.1
Pr>F <sup>1</sup>	0.48	0.30	>0.01	0.13	0.58
LSD (95%)	0.67	0.12	0.46	4.91	17.05

<sup>1 and 2</sup> For definitions see Table 5.

**Table 7**

Maturity indexing and quality parameters of 'Laetitia' plums measured on the harvest date for fruit which received pre-harvest calcium and silicate treatments (Trial 2) in the 2014/2015 season.

Treatment	TSS (%)	TA (%)	Foreground Colour <sup>2</sup>	Hue angle (h°)	Firmness (N)
Control	11.7a	1.9b	12.9 ns	28.3 ns	58.6 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	11.8a	1.9b	12.9	29.8	62.3
Foliar K <sub>2</sub> O <sub>3</sub> Si	12.2a	1.7a	13.4	26.2	53.8
Root K <sub>2</sub> O <sub>3</sub> Si	10.3b	1.7a	12.1	28.8	60.3
Pr>F <sup>1</sup>	>0.01	>0.01	0.08	0.17	0.55
LSD (95%)	0.88	0.10	0.99	3.26	12.45

<sup>1 and 2</sup> For definitions see Table 5.

**Table 8**

Maturity indexing and quality parameters of 'Laetitia' plums measured on the harvest date over both seasons. Fruit received pre-harvest foliar calcium and silicate applications, respectively. Data of two seasons from the same orchard as well as of an additional orchard in the second season were pooled for the analysis to remove the variation created by season and orchard.

Treatment	TSS (%)	TA (%)	Foreground Colour <sup>2</sup>	Hue angle (h°)	Firmness (N)
Control	11.3 ns	1.8b	11.9a	35.6 ns	68.6 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	11.3	1.7a	12.0a	38.1	68.3
Foliar K <sub>2</sub> O <sub>3</sub> Si	11.4	1.7a	12.7b	34.8	62.4
Pr>F <sup>1</sup>	0.90	<b>0.01</b>	<b>&gt;0.01</b>	0.16	0.2
LSD (5%)	0.50	0.06	0.32	3.59	7.74

<sup>1</sup> and <sup>2</sup> For definitions see Table 5.

**Table 9**

Quality parameters measured after cold storage (10 days at -0.5 °C plus 7 days at 7.5 °C plus 25 days at -0.5 °C) and after shelf life (cold-storage plus 7 days at 10 °C) (Trial 1) in the 2013/2014 season.

Treatment	Hue angle (h°)	Firmness (N)	% Shrivel
<b>Post cold-storage</b>			
Control	21.4 ns	38.3 ns	0.0 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	22.1	34.9	1.5
Foliar K <sub>2</sub> O <sub>3</sub> Si	22.6	34.9	0.0
Pr>F	0.51	0.68	0.06
LSD (95%)	2.10	9.50	1.40
<b>Post shelf-life</b>			
Control	20.6 ns	26.9 ns	4.1 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	20.0	24.0	5.6
Foliar K <sub>2</sub> O <sub>3</sub> Si	19.1	23.0	1.6
Pr>F	0.31	0.50	0.12
LSD (95%)	1.95	7.02	3.57

**Table 10**

Quality parameters measured after cold storage (10 days at -0.5 °C plus 7 days at 7.5 °C plus 25 days at -0.5 °C) and after shelf life (cold-storage plus 7 days at 10 °C) (Trial 1) in the 2014/2015 season. Fruit received pre- and postharvest calcium applications as well as pre-harvest silicate treatments.

Treatment	Hue angle (h°)	Firmness (N)	% Shrivel	% Internal Defects
<b>Post cold-storage</b>				
Control	19.6 ns	33.9 ns	0.2 ns	0.9 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	18.5	34.0	0.4	0.4
Foliar K <sub>2</sub> O <sub>3</sub> Si	18.3	29.2	0.0	0.8
Post-harvest Ca(NO <sub>3</sub> ) <sub>2</sub>	18.9	32.3	0.3	0.9
Pr>F	0.92	0.64	0.48	0.71
LSD(5%)	3.88	8.72	0.51	0.91
<b>Post shelf-life</b>				
Control	20.9 ns	26.5 ns	0.0 ns	0.6 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	22.2	25.7	0.7	0.4
Foliar K <sub>2</sub> O <sub>3</sub> Si	20.9	23.7	0.6	0.8
Post-harvest Ca(NO <sub>3</sub> ) <sub>2</sub>	21.8	26.2	0.0	1.0
Pr>F	0.39	0.64	0.08	0.43
LSD (5%)	1.90	4.80	0.68	0.78

**Table 11**

Quality parameters measured after cold storage (10 days at -0.5 °C plus 7 days at 7.5 °C plus 25 days at -0.5 °C) and after shelf life (cold-storage plus 7 days at 10 °C) (Trial 2) in the 2014/2015 season. Fruit received pre-harvest calcium and silicate treatments.

Treatment	Hue angle (h°)	Firmness (N)	% Shrivel	% Internal Defects
<b>Post cold-storage</b>				
Control	17.4 ns	26.2 ns	0.6 ns	2.3 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	16.9	28.0	0.0	0.9
Foliar K <sub>2</sub> O <sub>3</sub> Si	14.2	25.8	0.5	2.9
Root K <sub>2</sub> O <sub>3</sub> Si	16.9	27.2	0.0	2.8
Pr>F	0.13	0.8	0.46	0.44
LSD (5%)	2.86	5.00	0.75	2.32
<b>Post shelf-life</b>				
Control	19.6 ns	22.1 ns	0.6 ns	2.5 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	19.0	20.4	0.4	1.5
Foliar K <sub>2</sub> O <sub>3</sub> Si	18.1	19.6	0.9	2.5
Root K <sub>2</sub> O <sub>3</sub> Si	18.9	20.5	0.6	2.4
Pr>F	0.34	0.16	0.60	0.60
LSD (5%)	1.60	2.16	0.75	1.77

**Table 12**

Quality parameters measured after cold storage (10 days at -0.5 °C plus 7 days at 7.5 °C plus 25 days at -0.5 °C) and after shelf life (cold-storage plus 7 days at 10 °C) on fruit which received preharvest foliar calcium and silicate applications, respectively. Data of two seasons from the same orchard as well as of an additional orchard in the second season were pooled for the analysis to remove the variation created by season and orchard.

Treatment	Hue angle (h°)	Firmness (N)	% Shrivel	% Internal Defects
<b>Post cold-storage</b>				
Control	19.6 ns	33.1ns	0.9 ns	5.8 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	19.2	32.3	1.1	2.1
Foliar K <sub>2</sub> O <sub>3</sub> Si	18.4	30.1	0.6	6.3
Pr>F	0.39	0.42	0.57	0.23
LSD (5%)	1.69	4.80	1.21	4.42
<b>Post shelf-life</b>				
Control	20.5 ns	25.3 ns	4.1 ns	5.8 ns
Foliar Ca(NO <sub>3</sub> ) <sub>2</sub>	20.4	23.4	5.6	5.0
Foliar K <sub>2</sub> O <sub>3</sub> Si	19.3	21.6	1.9	6.9
Pr>F	0.10	0.08	0.118	0.66
LSD (5%)	1.27	3.04	3.569	3.81



Fig. 1. Chart used to grade the severity of broken stones. A ranking of 0 was given when no breakage was present, 3 when a large piece of broken stone was present and 4 when the entire stone was split in half along the suture (i.e. split stone).

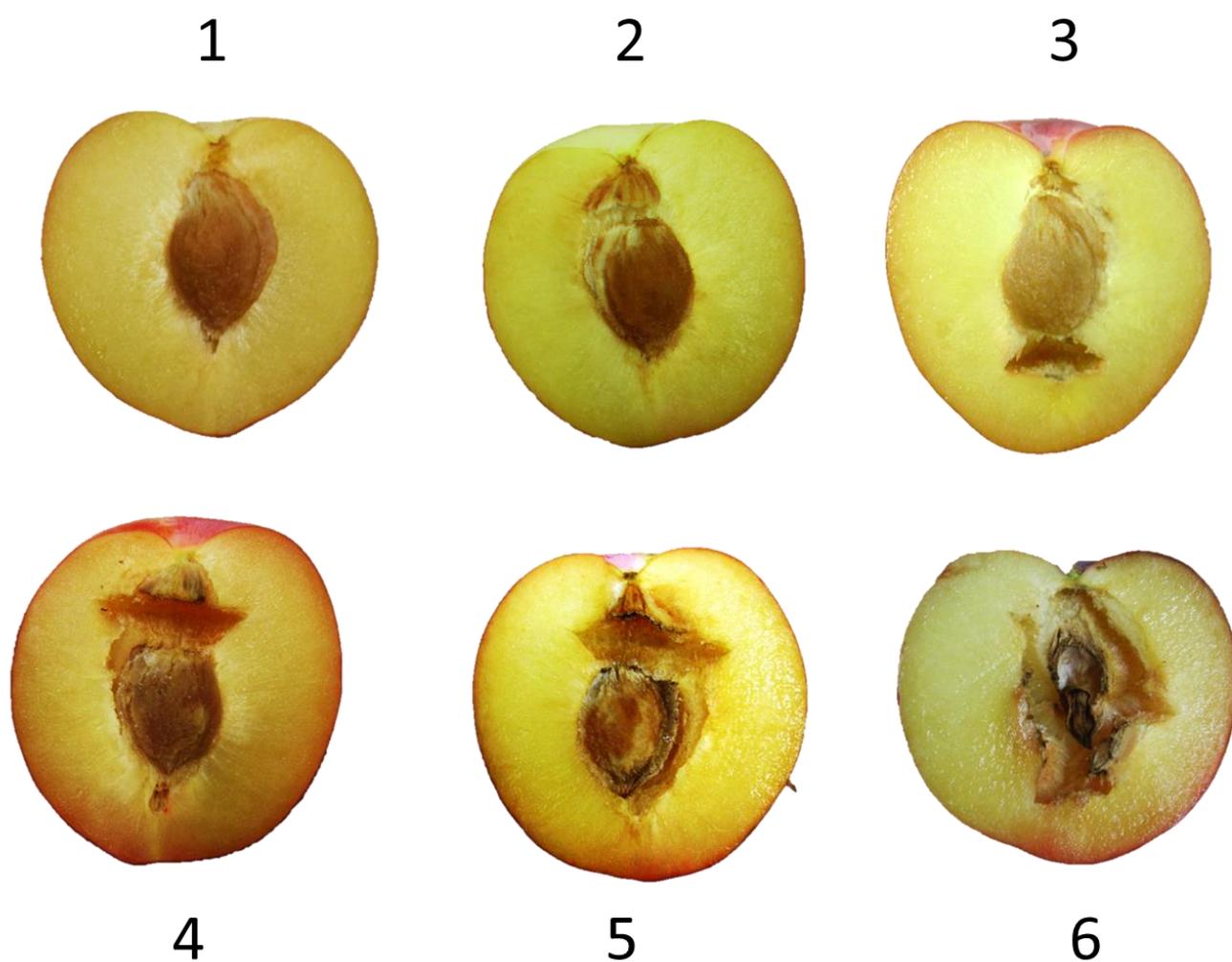


Fig. 2. Chart indicating the size of the cavity formed within the flesh, with 1 indicating no cavity, 5 indicates a very large cavity and 6 used for instances where the entire stone had broken in half (i.e. split stone).

## GENERAL DISCUSSION AND CONCLUSION

In order to export South African plums to overseas markets, strict quality standards must be maintained. According to the standards of the Organisation for Economic Co-operation and Development (OECD), plums destined for export are classified into classes according to internal and external quality (Organisation for economic co-operation and development, 2002.). Among these quality classifications are specifications about the presence of cavities and pieces of broken stone/pit within the flesh of the fruit. Under Class I classification a small cavity at the stem and/or styler end of the stone is allowed, but only if the flesh is not discoloured. In addition, it is also permissible for the stone to be broken while still clinging to the flesh. However, if the size of the cavity is large or stone breakage more severe, fruit have to be classified as Class II, even though they adhere to all other Class I specifications. Thus, if more than 10% of the fruit in a carton are affected by broken stones, the fruit are marketed as Class II. The 'Laetitia' plum cultivar is very prone to stone breakage, and as this cultivar is one of the main cultivars produced in South Africa, it is clear that stone breakage is a serious problem. A substantial volume of 'Laetitia' fruit destined for export is affected by broken stones and thus has to be marketed as Class II. Lower prices are attained for Class II fruit, and therefore the presence of broken stones has a detrimental effect on the income generated from these fruit. Therefore, the main aim of this study was to gain a better understanding of broken stones in Japanese plums.

The aim of Paper 1 was to investigate whether climatic differences between growing areas would affect the incidence of broken stones and whether certain parameters, measured throughout the growing season, were related to the incidence of stone breakage at harvest. The same pattern in the incidence of broken stones was observed in both seasons and in both growing areas – stone breakage was observed as soon as stone hardening was initiated. In peaches, stone splitting generally occurs during or just before stone hardening (Ragland, 1934; Woodbridge, 1978). Ragland (1934) and Tani et al. (2007) found that the hardening peach stones are not able to resist the forces of the rapidly expanding flesh that pulls on it and are subsequently pulled apart at its weakest parts. Our results indicate that the same holds true for Japanese plums, as the onset of stone breakage in our study coincided with the start of stone hardening. At the start of stone hardening the parts of the stone that are still 'soft' are not strong enough to resist pulling forces of the growing mesocarp on it and the stone is subsequently pulled apart. We found that the stones generally broke near the stem end of the fruit, which is the last part of the stone to harden (lignification proceeds from the styler end towards the stem end of the stone). However, no significant differences were observed in the incidence of broken stones between areas or seasons.

Regression analysis indicated that lengthwise growth of the fruit, fresh weight of the endo- and mesocarp, minimum orchard temperature and orchard night temperature, and RH early in the growing season (especially between 42 and 52 dafb) was related to the incidence of broken stones at harvest.

Peaches that show a high increase in growth during the second growth phase are more vulnerable to splitting, because during this time the stone is not yet able to resist the external forces of the rapidly expanding mesocarp and can be pulled apart (Claypool et al., 1972; Nakano, 2006; Tani et al., 2007). It is, therefore, possible that rapid lengthwise extension during the early parts of the stone hardening period, as observed in this study, pulls the stone apart at the interface between the parts of the stone that have started to lignify and the parts that are still 'soft'. Furthermore, our results indicate that the fresh weight of both the endocarp and mesocarp were at a maximum at 52 dafb, which was when these variables showed a strong relationship with the incidence of broken stone at harvest. One would expect that the rate of fresh weight accumulation of the endocarp would slow down during the lignification process (Boerjan et al., 2003; Evert et al., 1988), however, this was not the case in the 2014/2015 season and it slowed down only slightly in the 2013/2014 season. Thus, our results suggest that stones with higher moisture content tend to be more susceptible to breaks, especially if this is coupled with increased growth of the mesocarp, which was observed in terms of lengthwise fruit growth.

The onset of stone hardening started approx. 4 days later in the 2014/2015 season (which experienced cooler minimum orchard temperatures and night temperatures) compared to the 2013/2014 season. This finding agrees with that of Lopez and DeJong (2007) who found a negative correlation between temperatures during the first 30 dafb and the time between full bloom and stone hardening of peaches, i.e. lower temperatures during this period lengthened the time needed by the stone to harden completely. If the same is true for Japanese plums, lower temperatures during early spring may contribute to a higher incidence of stone breakage, because the lower temperatures will cause less competition between the growing plant organs and will aid in the development of larger fruit, which are more vulnerable to stone breakage (Grossman and DeJong, 1995; 1994). However, in this study a higher, though not statistically significant, percentage of broken stones were observed in the 2013/2014 season which experienced warmer temperatures during stone hardening. Therefore, the current plum data do not support the peach data that less broken stones could be expected after a warmer spring. However, these results agree with our results from Paper 2, which also showed a higher incidence of broken stones in the 2013/2014 season.

After gaining some understanding into the environmental factors and fruit growth parameters that influence the incidence of broken stones in 'Laetitia' plums, we investigated whether susceptible and non-susceptible cultivars differ in fruit growth rates during Stage II in Paper 2. Since the occurrence of broken stones in Japanese plums has a pronounced economic impact on the South African plum export industry, it is of the utmost importance to obtain a better understanding of the reasons why the defect only develops in certain cultivars in order to develop protocols to reduce the incidence of the defect. Thus, we compared the growth characteristics of 'Laetitia' and 'Sapphire' plum cultivars (which are prone to stone breakage) to 'Songold' (which is not susceptible to stone breakage) non-destructively by using computed tomography (CT) scans.

For all three cultivars used in this study, the tip of the stone at the stylar end started to lignify first. After the entire area of the stone had become slightly more dense (seen as a light area on the CT images), density further increased as the light parts of the images became even lighter. Ryugo (1961) observed that lignification of the individual cells begins in the middle lamella and then continues to the cell walls in peach fruit. The inner lining of the stone hardens first, after which lignification continues radially from the inside of the stone towards the margins. Our observations confirmed this.

Clear differences in the incidence of broken stones were observed between cultivars and seasons. This was expected as it is known that split/broken stones in peaches varies greatly among cultivars and seasons (Davis, 1933; Sotiropoulos et al., 2010; Woodbridge, 1978). For ‘Laetitia’ there was a clear difference in the incidence of broken stones between seasons, as fewer broken stones were observed in the 2014/2015 season (7% versus 32.69% in the 2013/2014 season). It was interesting to note that stone breaks were mostly observed at the stem end of ‘Laetitia’ fruit, while they were more often noticed at the stylar end of the ‘Songold’ stones. It seems that the stones tend to break at the interface between high and low density areas, which explains why ‘Laetitia’ stones tend to break mostly at the stem end, while ‘Songold’ stones broke at the stylar ends. For ‘Laetitia’ these breaks were observed as soon as the stones started to lignify (35 dafb in 2013/2014 and 42 dafb in 2014/2015). This agrees with the findings of Paper 1.

It seems that a semi-rigid stone combined with rapid radial fruit growth in the direction where the stone is least dense, is the cause of stone breakage in both ‘Laetitia’ and ‘Songold’. However, for ‘Sapphire’ the first signs of stone breakage were observed before stone hardening commenced, which indicates that the non-lignified stone was probably not able to resist the strong pulling forces created by the expanding mesocarp and was subsequently pulled apart. This corroborates with observation in early maturing peach cultivars where split pit is often observed, because fruit enlargement does not slow down during Stage II of fruit development to allow the stone to harden properly and consequently it cannot resist the pulling forces of the expanding fruit flesh and is pulled apart (Claypool et al., 1972; Ognjanov et al., 1995; Ragland, 1934; Scorzal et al., 1991; Tani et al., 2007; Tukey, 1936).

Since clear differences were seen in the incidence of broken stones, as well as endocarp density between seasons, it seems that environmental conditions also contribute to the incidence of stone breakage. A study comparing a ‘Stoneless’ prune cultivar (a cultivar that forms an incomplete endocarp) to a ‘normal’ cultivar found that the lignification pathways were similar in both cultivars, but that the ‘Stoneless’ cultivar displayed decreased and abnormal endocarp formation (significantly lower endocarp dry weight compared to the ‘normal’ cultivar) (Callahan et al 2009). Furthermore, endocarp development differed between seasons, suggesting that it might also be environmentally regulated. In years when higher spring temperatures were observed, fruit tended to contain more complete stones (Dardick and Callahan, 2014). It is possible that cultivars such as ‘Laetitia’ and ‘Sapphire’, which are susceptible to stone breakage, might also show abnormal endocarp formation during cooler springs.

Hence, higher spring temperatures may also lead to the formation of more complete endocarps (more endocarp cells) in plum cultivars susceptible to broken stones. Higher temperatures during early fruit growth stages enhance the rate of apple fruit growth and increase the cell numbers of different regions of the fruit, because temperature affects the rate of cell division (Bergh, 1980; Greybe et al., 1988; Stanley et al., 2000). A higher number of endocarp cells would certainly increase the density (and hence, rigidity) of the stone, and if this is coupled with rapid radial growth in a specific direction before the stone has hardened completely, stone breakage could occur at the interface between high and low density areas in the stone. Since the average temperature during the stone development period (0 to 63 dafb) in the 2013/2014 season was higher than that of the 2014/2015 season, it is suggested that 'Laetitia' was able to form a more complete stone, and hence, more stone cells which lignified to form denser, more rigid stones. Such a scenario would create a more pronounced interface between already hardened and less hardened stone parts during Stage II of fruit growth, which, in combination with rapid increases in radial fruit growth during Stage II, might serve as an explanation as to why 'Laetitia' had a higher percentage of broken stones in the 2013/2014 season compared to the 2014/2015 season. It is furthermore suggested that 'Songold' (less susceptible to broken stones) forms a complete endocarp irrespective of spring temperatures, but that the enzymes responsible for lignification are influenced by spring temperatures. Hence, the cultivar has a more flexible stone, resisting stone breakage, in a warmer spring when lignification is less pronounced and forms a much more rigid stone in a cooler spring when lignification is upregulated as suggested by the results of loquat, mangosteen and poplar cutting research (Dangcham et al., 2008; Hausman et al., 2000; Jincheng et al., 2006). A more rigid stone would form a more pronounced interface between already hardened and less hardened stone parts during Stage II of fruit growth, and in combination with rapid fruit growth during stone hardening, result in stone breakage.

Finally, in Paper 3, the effect of calcium and silicon foliar and root applications on the incidence of broken stone at harvest, was investigated in 'Laetitia'. As it is known that both silicon and calcium increase the strength of the cell walls, it was hypothesized that either foliar applications or root applications of  $\text{Ca}(\text{NO}_3)_2$  and  $\text{K}_2\text{O}_3\text{Si}$  would strengthen the plum endocarp and thereby reduce the incidence of broken stones. In the 2013/2014 season the incidence of broken stones was significantly higher than in the 2014/2015 season (30.41% versus 5.19%). However, there was no significant treatment effects on the percentage broken stones for either season. These results are in contrast to results obtained by Sotiropoulos et al. (2010) who found that potassium silicate significantly decreased the amount of slit-pit in peaches (also after applying three consecutive foliar sprays). They hypothesised that the increase in cell wall elasticity brought about by silicon might explain the lower incidence of split pit. It is possible that, when the cell walls of the endocarp are more elastic, the stones are better able to resist pulling forces from the flesh during stages of rapid mesocarp growth, and are thus less likely to break. However, this could not be confirmed in our study. In peaches the stone usually splits open along the suture/ seam of the endocarp (Department of Agriculture Forestry and Fisheries, South Africa, 1990). However, with stone breakage in Japanese plums, the endocarp can disintegrate (break

apart) anywhere, and often in multiple areas (Personal observation). As the way in which the stones break/split differ between plums and peaches, this might explain why we did not observe a similar reduction in broken stones after silicon treatments. Similar to our findings Evert et al. (1988) found that foliar  $\text{Ca}(\text{NO}_3)_2$  sprays, also applied before and during stone hardening, had no effect on the occurrence of split pit in peaches.

In conclusion, this study contributed towards our understanding of the occurrence of broken stones in Japanese plums. It was shown that stone breakage occurs at the onset of stone hardening and that it is affected by environmental factors as well as differences in fruit growth characteristics. This study also found distinct differences in endocarp density between seasons and between cultivars that differ in their susceptibility to broken stones. Hence, it is clear that both genetics and environmental factors play a role in the development of broken stones in Japanese plums. The phenomenon is more complex than previously thought and, unfortunately there is no easy solution in terms of calcium or silicate treatments yet. Validation and broadening of these findings to other cultivars and over more seasons might enable breeders to rather develop new cultivars that are genetically less susceptible to broken stones. Gaining understanding into the effect of early season environmental conditions and seasonal differences to be expected in the incidence of broken stones will further enable growers to plan their marketing strategies in accordance, and this will hopefully lead to a reduction in the financial losses due to the export of fruit affected by broken stones.

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