The Relationship Between Oil Gland and Fruit Development in Washington Navel Orange 
(Citrus sinensis L. Osbeck)

TOBY G. KNIGHT, ANDREAS KLIEBER and MARGARET SEDGLEY*

Department of Horticulture, Viticulture and Oenology, Waite Agricultural Research Institute, Adelaide University, Glen Osmond, South Australia 5064, Australia

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Changes in structure, size and number of oil glands located in the fruit rind were assessed in developing fruit of the Washington Navel orange (Citrus sinensis L. Osbeck) from pre-anthesis to fruit maturity. Initiation of oil glands was found to be restricted to early fruit development. Glands continued to develop throughout fruit growth, until all reached maturity by a fruit size of 30 to 50 mm diameter. Mature glands continued to enlarge with fruit growth. Mature fruit had between 8,000 and 12,000 oil glands. Anatomical studies of the fruit rind were carried out using light microscopy on samples prepared by different tissue processing methods. Glands were found to develop from a cluster of cells adjacent to the epidermis, into a structure consisting of a central cavity surrounded by several layers of epithelial cells. All glands were joined to the fruit epidermis, irrespective of their stage of development. Neither lignin nor suberin was present in the gland. Gland cavity formation appeared to involve schizogeny.

Key words: Washington Navel orange, Citrus sinensis L. Osbeck, fruit development, secretory cavity, oil gland, image analysis, light microscopy.

INTRODUCTION

Secretory cavities occur naturally in all species of the family Rutaceae (Fahn, 1979). In the genus Citrus they are commonly referred to as oil glands and occur in the stem, mesophyll of leaves, all parts of the flower except the stamens, and the fruit, where they are positioned in the exocarp or ‘flavedo’ layer of the rind amongst compact subepidermal parenchyma tissue (Mauseth, 1988).

Oil glands have been observed in the fruit as early as the ovary stage e.g. in Eureka lemon (Ford, 1942), Washington Navel orange (Holtzhausen, 1969) and Mediterranean mandarin (Bosabalidis and Tsekos, 1982a). Gland initiation in Citrus species has been reported by some to be confined to early stages of fruit development (Ford, 1942; Bain, 1958), whilst others have suggested continuous formation with fruit growth (Schneider, 1968). Glands are also reported to develop and enlarge up to fruit maturity (Bain, 1958; Holtzhausen, 1969).

Structural aspects of gland development have been reported in the floral ovaries of Citrus deliciosa Ten. (Mediterranean mandarin) (Bosabalidis and Tsekos, 1982a, b), but to date, detailed anatomical studies of the oil gland in Navel oranges are confined to the leaves (Thomson et al., 1976). A contentious issue of gland development is whether the central cavity forms by lysigeny or schizogeny. Cavity formation in Citrus and related species has been reviewed by Turner (1999). He points out the difficulties in distinguishing between a fixation artefact and a true lysigenous process, and suggests that schizogeny be favoured over lysigeny in cases where there is dispute.

There is controversy in the literature regarding the timing of gland initiation and development in fruit of Citrus species, and no study has quantified or investigated anatomical aspects of gland development in Washington Navel orange fruit throughout development. In this paper we examine oil gland structure and quantify gland changes in the developing fruit of Washington Navel orange, from pre-anthesis to maturity. The timing of gland initiation, development and enlargement in relation to fruit growth is examined, and mature gland anatomy is investigated at different fruit ages.

MATERIALS AND METHODS

Plant material

Fruits were collected from two 23-year-old Washington Navel trees on Poncirus trifoliata rootstock, located in the Alverstoke orchard, Adelaide University, Australia. The trees were positioned 3 m apart, in a north-south facing row. Fruits were harvested at monthly intervals from pre-anthesis to fruit maturity. At each harvest, six fruits were picked from each tree, comprising two replicates of three size classes. Each harvest was carried out at the same time of day to avoid possible discrepancies due to diurnal changes.

The equatorial and polar diameters of each fruit were measured; an average of these two measurements is used when referring to fruit diameter. Fruit surface area was extrapolated according to Turrell (1946). In this method the fruit is considered a spheroid, and the difference between polar and equatorial diameters is incorporated into an equation to calculate surface area.

For microscopy, a minimum of three rind tissue samples was collected from the equatorial region of each fruit.

* For correspondence. Fax +61 (0)8 8303 7116, e-mail margaret.sedgley@adelaide.edu.au

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Gland counts

Gland density was measured on fruit ranging in diameter from 9 to 88 mm. Within a sector of 180 mm² along the equator of the fruit (or an adjusted area for fruit smaller than this dimension), glands visible on the surface using a dissecting microscope were counted. Glands with deeper-seated cavities, difficult to detect from surface examination, were counted by dissection of the tissue along the flavedo/albedo boundary. The sum of these two counts gave the total number of glands within the sector. For all fruit, gland density was expressed as number of glands per 180 mm².

The total number of glands per fruit was estimated using values of gland density and fruit surface area: total gland number = gland density x (fruit surface area/180 mm²).

Light microscopy

Two tissue processing methods were employed: aldehyde fixation; and aldehyde fixation plus osmium tetroxide postfixation. The first method is similar to that of Feder and O'Brien (1968). Rind samples approx. 5 x 5 mm were fixed overnight at 4°C in 3 % glutaraldehyde in 0.025M phosphate buffer (O'Brien and McCully, 1981). Samples were dehydrated in an alcohol series and embedded in glycol methacrylate (GMA) (Sigma Chemical Co., St Louis, USA). Sections (4 μm thick) were cut with a Reichert-Jung 2050 Supercut Microtome using a glass knife, fixed to microscope slides and stained with Periodic Acid/ Schiff's (PAS) and Toluidine Blue O (TBO) (O'Brien and McCully, 1981). Material processed using this method was used predominantly in this study. Histochemical staining of this material for lignin using Phloroglucinol (Gurr, 1965) and suberin (O'Brien and McCully, 1981) was used to outline the anatomical gland development in fruit of Washington Navel orange, as follows: stage 1, a cluster of up to ten cells adjacent to the epidermis. These gland initials were slightly radially elongated and lacked starch; they could thus be distinguished from surrounding parenchyma cells (Fig. 1A). Stage 2, a larger cluster of ten–30 cells (Fig. 1B). Stage 3, a larger cluster beginning to differentiate into boundary and inner cells (Fig. 1C). Stage 4, a differentiated gland with flattened boundary cells enclosing polyhedral inner cells; nuclei of the inner cells appear enlarged (Fig. 1D). Stage 5, a differentiated gland with flattened boundary cells enclosing polyhedral inner cells whose walls appear to form the cavity (Fig. 1E). Stage 6, a mature gland with an expanded central cavity (Fig. 1F).

Statistical analysis

Gland volume data were analysed using the statistical package Genstat for Windows version 4.1 (Lawes Agricultural Trust, IACR Rothamsted). A completely randomized design was applied to the data. Statistical differences due to fruit age were tested at the 95 % confidence level.

RESULTS

Anatomy of gland development

From light microscopy observations of aldehyde-fixed tissue samples, a series of six stages was chosen to outline anatomical gland development in fruit of Washington Navel orange, as follows: stage 1, a cluster of up to ten cells adjacent to the epidermis. These gland initials were slightly radially elongated and lacked starch; they could thus be distinguished from surrounding parenchyma cells (Fig. 1A). Stage 2, a larger cluster of ten–30 cells (Fig. 1B). Stage 3, a larger cluster beginning to differentiate into boundary and inner cells (Fig. 1C). Stage 4, a differentiated gland with flattened boundary cells enclosing polyhedral inner cells; nuclei of the inner cells appear enlarged (Fig. 1D). Stage 5, a differentiated gland with flattened boundary cells enclosing polyhedral inner cells whose walls appear to form the cavity (Fig. 1E). Stage 6, a mature gland with an expanded central cavity (Fig. 1F). Differences in the appearance of surrounding parenchyma cells can be attributed to differences in fruit age. This outline was used to quantify gland development in relation to fruit growth. The anatomy of cavity-containing glands (stages 5 and 6) was examined more closely in aldehyde plus osmium tetroxide postfixified tissue (Fig. 6A and B).

At each stage of gland development, glands were found to be joined to the fruit epidermis by a stalk-like structure.
The stalk was often conical in shape (Fig. 2A). The structure was solid rather than funnel-like, as revealed by tangential sectioning of the rind tissue (Fig. 2B). The stalk appeared to be an extension of the inner cells, uninterrupted by boundary cells, which met the epidermis at its apex. Stalk cells were round to oblong in shape, and were smaller and different in form to other cells of the gland complex and surrounding parenchyma cells of the rind.

**Timing of gland development**

Glands were present in the pre-anthesis floral ovary. Glands at various stages of development were observed in
The youngest sample collected, a flower bud measuring 8 mm in length from the base of the calyx to the petal tip, with an ovary approx. 2-6 mm in diameter. The most advanced stage of gland development observed in this sample was stage 4.

Gland initiation was found to be restricted to early stages of fruit development. Gland counts under the dissecting microscope showed an exponential decline in gland density up until fruit reached a size equating to a surface area of approx. 20 cm² (25 mm diameter). From this time onwards,
a more gradual decline was evident (Fig. 3A). To investigate possible effects of fruit growth on gland density, the total number of glands per fruit was assessed. Initially, total gland number per fruit appeared to increase, but then remained fairly constant with increasing fruit size, at around 8000 to 12,000 glands per fruit (Fig. 3B). The total number of glands in a mature fruit measuring 88 mm in diameter (247 cm² surface area) was similar to that in an immature fruit measuring 20 mm in diameter (13 cm² surface area). This confirms that gland initiation was confined to early stages of fruit development, until fruit reached approx. 20 mm in diameter. Results of the survey of gland age supported this finding, with gland initials (stage 1) comprising 27% of the gland population in the floral ovary at full bloom (stage A), declining to 11% in a 10 mm fruit (stage B) and disappearing thereafter (Fig. 4).

In the floral ovary, a high proportion of glands contained newly formed cavities (20%) and some showed a more advanced stage of cavity opening (3%). The proportion of mature glands increased from 23% in the floral ovary (stage A), to 64% in a 10 mm fruit (stage B), to 87% in a 32 mm fruit (stage C) and to 100% in a 52 mm fruit (stage D) (Fig. 4). Hence, all glands reached maturity in immature green fruit with a diameter of between 32 and 52 mm.

The mature gland

The mature gland continued to enlarge with fruit growth (Fig. 5). Mean gland volume varied significantly between the five stages from full bloom to mature fruit (P < 0.001). A large difference (P < 0.05) in mature gland volume was found between fruit of 52 mm (stage D) and 88 mm (stage E) diameter, suggesting a period of rapid gland enlargement. Gland size also varied within each fruit, as suggested by the high standard errors associated with gland volume (Fig. 5). Mature glands were not uniform in shape, as observed in tissue sections viewed under the light microscope (Fig. 2C and D).

The stalk of mature glands appeared to become less prominent as the fruit aged (Fig. 2E and F). In mature fruit (stage E), the stalk is reduced to only a few cell layers in depth below the epidermis (Fig. 2E). The orientation of the stalk in relation to the fruit surface also changed with fruit age. In young, rapidly growing fruit (stage B), gland stalks were positioned at an angle to the epidermal plane (Fig. 2F), whereas in older fruit the stalk was located in the medial section of the gland (Fig. 2E).

At early stages of cavity formation, flattened boundary cells surrounded inner polyhedral cells whose walls appeared to form the cavity (Fig. 6A). Inner gland cells had large nuclei and many small vacuoles, and cells surrounding the cavity exhibited a densely stained cytoplasm. In mature glands, with an expanded cavity, inner cells appeared to be more flattened, but slightly swollen, with edges bulging into the cavity space (Fig. 6B). Cell walls were also observed to increase in thickness towards the gland perimeter. The thickened walls of boundary cells have been demonstrated in an aldehyde-fixed tissue section (Fig. 6C). Histochemical staining of aldehyde-fixed tissue sections and of fresh tissue was undertaken to show differences in cell wall composition, but failed to show the presence of lignin or suberin in the thickened cell walls. TEM examination of the cells surrounding the gland cavity showed the presence of bulbous extracellular pockets (Fig. 6D).

DISCUSSION

In this study, the effect of the different tissue preparation methods used has been taken into consideration. Aldehyde-fixed tissue facilitated the collection of large tissue samples and serial sectioning, which was required to establish the anatomical gland developmental series, and to carry out the gland age survey and gland volume measurements. Aldehyde fixation has been reported to cause artefactual swelling and rupture of gland cells in the foliar glands of *Citrus limon* (L.) Burm. F. (Turner *et al.*, 1998). For this reason, an improved fixation protocol was used to prepare tissue for the examination of mature gland anatomy and cavity formation. In addition, glands depicted were not damaged during sample collection and have been captured in medial section.

Prior to this study, gland development had not been examined in fruit of the Washington Navel orange. A general description of gland cell differentiation was made by Schneider (1968), and gland cavity opening was examined in foliar glands of Washington Navel orange by Thomson *et al.* (1976). To date, the most complete outline of gland development has been achieved in ovaries of *Citrus deliciosa* Ten. (Mediterranean mandarin) by Bosabalidis and Tsekos...
Our light microscope findings in Washington Navel orange fruit are similar to those made in *C. deliciosa* ovaries (Bosabalidis and Tsekos, 1982a). In both studies, gland initiation and cavity formation appeared to occur as two separate events.

To date, the presence of a gland 'stalk' has been described only by Bosabalidis and Tsekos (1982a), in the ovaries of *Citrus deliciosa*. In their study, the glandular structure was reported to consist of the 'main gland' and the 'stalk' at the completion of gland cell division. The stalk was described as 'conical' and was observed to connect the main gland with the epidermis, with the top of the stalk consisting of epidermal cells. These cells have been identified by surface examination of Washington Navel orange fruit and described as oil-gland cover cells (Scott and Baker, 1947). Oil-gland cover cells were reported to have an area proportional to the underlying gland and appear somewhat flattened and polygonal in surface view. In our study, the gland stalk was observed in all stages of differentiation of Washington Navel orange glands, as well as at different fruit ages. The stalk structure was less apparent in older fruit, which may be explained by a lateral flattening above oil glands or by the continual growth of cells in the epidermal, hypodermal and outer flavedo layer, causing a bulging effect around the gland stalk. The inconspicuous nature of the stalk in mature fruit may explain why the majority of previous studies have failed to mention the presence of a stalk structure in oil glands of mature citrus fruit. In addition, if tissue sections are being examined then the stalk can go unnoticed if a longitudinal tissue section does not pass directly through it, as pointed out by Bosabalidis and Tsekos (1982a).

Gland initiation, development and enlargement were all quantified with respect to fruit growth. Gland counts were localized to the equatorial region of the fruit, to correspond to microscopy samples. Gland initiation was found to be restricted to early fruit development, ceasing at a fruit size of approx. 20 mm diameter. This agrees with findings in Eureka lemon (Ford, 1942) and Valencia orange, where gland initiation was observed only in the 'cell division' stage of fruit development, which continued to a fruit size of approx. 16 mm (Bain, 1958). However, our findings refute...
the statement of Schneider (1968) who suggested that oil glands continued to form as the citrus fruit matured.

Glands continued to develop until all had reached maturity in an immature green fruit of diameter 32 to 52 mm. This finding contradicts that of Holtzhausen (1969), who inferred that glands in Washington Navel orange fruit continued to develop until fruits were ripe. Mature glands enlarged with fruit growth, as has been observed previously in Washington Navel orange (Holtzhausen, 1969) and Valencia orange (Bain, 1958).
of rapid gland enlargement observed between fruit of 52 and 88 mm diameter roughly coincides with the period of cell enlargement in the rind of Washington Navel orange fruit described by Bouma (1959). Mature glands of different sizes were also found within each fruit examined, which may be explained by the non-uniform timing of both gland initiation and maturation in relation to fruit development.

Gland shape was found to be non-uniform. The oil glands of Citrus have been described as varying from 'oblate to spherical or ovoidal to pyriform' (Bartholomew and Reed, 1943). It has also been suggested that glands are located at different depths in the rind (Bartholomew and Reed, 1943; Ting and Attaway, 1971). In our study, although all glands were found to link to the epidermis by the gland stalk, gland cavities did occur at different depths due to size differences alone, often extending into the albedo layer.

Mature gland anatomy consisted of flattened epithelial cells increasing in thickness towards the perimeter of the gland. Observations suggest that inner gland cells are modified into boundary cells, as has been reported previously in glands of Citrus (Thomson et al., 1976; Bosabalidis and Tsekos, 1982b). In this study, the thickened walls of the boundary cells were found not to contain lignin or suberin. The same observation was made in foliar secretory cavities of Citrus limon (L.) Burn. F. (Turner et al., 1998). However, suberin has been detected in fruit secretory structures, such as the oil-containing cells of avocado (Persea americana) (Scott et al., 1963).

There is considerable contention in the literature regarding the mode of cavity formation (lysigeny or schizogeny) in the oil glands of Citrus species. Lysigeny refers to a process of cell degeneration, and schizogeny to a process of cell wall separation, to form a tissue cavity. This topic has been reviewed by Turner (1999). In an examination of the foliar secretory cavities of Citrus limon (L.) Burn. F. (Turner et al., 1998), the lysigenous appearance of glands was concluded to be a fixation artefact and a protocol for improved tissue preservation was recommended. Their method involved initial tissue fixation with osmium tetroxide vapour followed by fixation in an aldehyde plus osmium tetroxide solution, fixation in aldehyde alone, and finally, an osmium tetroxide solution postfixation. Using osmium vapour as a primary fixative was reported to reduce vacuolar swelling and improve lipid preservation. In our study, however, this protocol resulted in extremely poor tissue preservation of Washington Navel orange fruit samples. Instead, aldehyde fixation plus osmium tetroxide postfixation was employed for our examination of gland cavity formation. At the light microscope level, both methods were reported by Turner et al. (1998) to give similar structural preservation of intact glands. Using this method, epithelial cells surrounding the gland cavity showed no signs of structural degeneration. At an early stage of cavity formation, cells surrounding the cavity appeared structurally intact; however a number of them exhibited a densely stained cytoplasm. TEM examination of these cells showed the presence of bulbous pockets along the cell walls. Similar observations have been made in foliar glands of Citrus sinensis prepared by similar methods (Thomson et al., 1976). In their study, the formation of bulbous extracellular pockets was used as evidence of schizogenous cavity formation.

In conclusion, gland initiation was restricted to early stages of fruit development, and all glands matured in an immature fruit but continued to enlarge throughout fruit growth. All glands were joined to the fruit epidermis by a stalk, irrespective of their stage of development or location of the cavity within the rind. Light and transmission electron microscopy suggest that glands of Washington Navel orange fruit are anatomically similar to those of other Citrus species, and that early stages of cavity formation involve schizogeny. In addition, findings have been used as the basis of a study into the rind disorder oleocellosis, which is caused by rupture of the oil glands and whose incidence is suggested to vary with fruit maturation.

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Literature Cited


