Physiologia Plantarum 147: 396–406. 2013



# Physiological and morphological changes during early and later stages of fruit growth in *Capsicum annuum*

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Received 12 May 2011;
revised 23 May 2012

doi:10.1111/j.1399-3054.2012.01673.x

Fruit-set involves a series of physiological and morphological changes that are well described for tomato and Arabidopsis, but largely unknown for sweet pepper (*Capsicum annuum*). The aim of this paper is to investigate whether mechanisms of fruit-set observed in Arabidopsis and tomato are also applicable to *C. annuum*. To do this, we accurately timed the physiological and morphological changes in a post-pollinated and un-pollinated ovary. A vascular connection between ovule and replum was observed in fertilized ovaries that undergo fruit development, and this connection was absent in unfertilized ovaries that abort. This indicates that vascular connection between ovule and replum is an early indicator for successful fruit development after pollination and fertilization. Evaluation of histological changes in the carpel of a fertilized and unfertilized ovary indicated that increase in cell number and cell diameter both contribute to early fruit growth. Cell division contributes more during early fruit growth while cell expansion contributes more at later stages of fruit growth in *C. annuum*. The simultaneous occurrence of a peak in auxin concentration and a strong increase in cell diameter in the carpel of seeded fruits suggest that indole-3-acetic acid stimulates a major increase in cell diameter at later stages of fruit growth. The series of physiological and morphological events observed during fruit-set in *C. annuum* are similar to what has been reported for tomato and Arabidopsis. This indicates that tomato and Arabidopsis are suitable model plants to understand details of fruit-set mechanisms in *C. annuum*.

## Introduction

Successful completion of pollination and fertilization is essential to trigger fruit-set in most flowering plants (Gillaspy et al. 1993). Fertilization induces the development of an ovary into a fruit, while failure of fertilization triggers senescence of floral organs and finally leads to flower abscission (Nitsch 1970, Talon et al. 1992). Fruit-set involves a coordination between signaling compounds, i.e. auxin, gibberellins, ethylene and cytokinin (Vriezen et al. 2007). Several independent observations have indicated that auxin is important for fruit-set and development. For example, auxin supplied either exogenously or ectopic expression of

*Abbreviations* – DAA, days after anthesis; DAB, decolorized aniline blue; DIC, differential interference contrast; HPP, hours post pollination; IAA, indole-3-acetic acid; MS, mass spectrometry.

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genes encoding enzymes of auxin biosynthesis induces fruit-set in tomato and Arabidopsis (Vivian-Smith and Koltunow 1999, Spena and Rotino 2001). However, it is still an important question how auxin signaling enables the coordinated developmental change from pistil/gynoecium into fruit. Prior to anthesis, carpel development is arrested and pollination/fertilization induces processes such as vascular development and differentiation within the carpel to assist the development of the pistil/gynoecium into a fruit (Gillaspy et al. 1993, Griffiths et al. 2006). Modulations in the auxin signaling pathways have been reported to occur within 24 hours post-pollination (HPP) in tomato, and this results in developmental changes and changes in carbon partitioning (Vriezen et al. 2007). In Arabidopsis, the vascular strands between ovule and carpel connect within 54 HPP in a fertilized ovule, while they remain separated in an unfertilized ovule (Fuentes and Vivian-Smith 2009). This vascular connection has been reported to occur also in the absence of fertilization in the Arabidopsis parthenocarpic *fruit without fertilization (fwf/arf8)* mutant (Vivian-Smith 2001). These findings support the idea that auxin signals might transfer from the ovule to the carpel, triggering vascular connection and fruit-set.

Sweet or bell pepper (*Capsicum annuum*) is an important vegetable fruit crop. In *C. annuum*, various physiological aspects have been studied including fruit-set and flower abortion. Factors influencing these processes are the plant hormones auxin and ethylene (Wien et al. 1989, Huberman et al. 1997, Heuvelink and Körner 2001), assimilate availability (Marcelis et al. 2004), assimilate utilization and dominance of competing fruits (Aloni et al. 1996), reduced metabolic activity due to heat stress (Aloni et al. 1995) and cultivar susceptibility for flower abortion (Wubs et al. 2009).

In *C. annuum*, successful pollination and fertilization at optimal growth condition result in seeded fruits while failure of pollination and fertilization at suboptimal growth condition (high or low night temperature) result in seedless fruit (Tiwari et al. 2007, 2011). The physiological and morphological changes that occur in a developing ovary to regulate fruit-set are not well studied in *C. annuum*. The aim of this paper is to study the physiological and morphological changes during early and later stages of the fruit growth in *C. annuum* and to investigate whether the mechanisms of fruit-set observed in Arabidopsis and tomato are also applicable to *C. annuum*. To do this, we accurately timed the physiological and morphological changes such as in vivo pollen tube germination and pollen tube growth in the ovule vicinity, and vascular connection between ovule and carpel. To understand the role of auxin in fruit-set and fruit development, endogenous indole-3-acetic acid (IAA) levels were measured in seeded and seedless fruits obtained from pollinated and unpollinated flowers, respectively. To check the contribution of cell division and cell expansion to fruit growth, we measured fruit diameter, cell number/number of cell layers or cell diameter in a developing ovary at early (0–72 HPP) and at later [0–40 days after anthesis (DAA)] stages of fruit growth. The results indicate that tomato and Arabidopsis are suitable model plants to understand details of fruit-set mechanisms in *C. annuum*.

## Materials and methods

### Plant material and growth conditions

Seeds of sweet pepper (*C. annuum* L.) cultivar 'Bruinsma Wonder' were obtained from Plant Research International (PRI: 2004001). Four weeks after sowing, 20 seedlings were transplanted into pots of 2 l size with potting soil and transferred into a climate room. Nutrient solution (Voogt and Bloemhard 1993) was supplied regularly. Flower emasculation (removal of outer floral organs: petals and anthers) was performed 2 days before the expected date of anthesis to avoid accidental self-pollination. Two experiments were conducted, where the first experiment includes in vivo pollen tube growth, vascular development between ovule and carpel and cell number and cell diameter in the carpel from 0 to 72 HPP. The second experiment includes the measurement of endogenous IAA levels, number of cell layers and cell diameter in the carpel from 0 to 40 DAA.

### In vivo pollen tube growth, vascular development, cell number and cell diameter

Plants were grown in a climate room with a constant temperature of 24°C day/night temperature. From the previous experiments we know that emasculation of flowers in this growth condition results in flower abortion (Tiwari et al. 2011). Plants were exposed to 16 h day-length (from 08:00 to 24:00 hours) by using high pressure mercury lamps (Philips HPI 400W, Eindhoven, the Netherlands) providing a minimum photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$ at plant level. Pots were arranged on a table of 1.20-m height and were allowed to grow without any pruning. At anthesis, all emasculated flowers were hand pollinated by using fresh pollens from other flowers of the same plant and date and time of the pollination was registered.

To determine the in vivo pollen tube growth, flowers were collected at 0, 2, 4, 6, 8, 10, 12, 14, 15, 20 and 28 HPP. For each stage, 5–15 flowers were harvested

and fixed immediately in an acetic acid alcohol (1:3) solution for overnight. Flowers were rinsed three times with water, replaced with 10% sodium sulphite solution (w/v) and autoclaved at 121°C for 5 min. After cooling down, flowers were again rinsed three times with water and finally replaced with decolorized aniline blue (DAB)/glycerol (DAB: mix 0.2 g of aniline blue and 4.6 g $K_3PO_4$, stored in dark overnight). A stock solution of 0.1% DAB is prepared by decolorizing water-soluble aniline blue (Fisher Scientific) in aqueous phosphate (0.1 *M* $K_3PO_4$). Flowers were dissected to reveal the ovary, and observations of pollen tube growth on stigmas and elongation into the style were made using an epifluorescence microscope (Leica, Dialux 20EB with BP340-380 excitation filter, RKP400 beam splitting mirror and LP430 barrier filter). Pollen tubes contain callose (ß1, 3 glucan) in their wall as well as in their plugs that segment growing tubes. Callose plugs were highly fluorescent under the microscope; therefore, presence of callose plugs was used to locate the pollen tubes in the stylar tissue and in the ovule vicinity.

To evaluate vascular development in the fertilized and unfertilized ovules, ovaries (n = 3–5) obtained after pollination or emasculation were collected at 48 HPP. Same numbers (n = 3–5) of ovaries from both treatments were allowed to grow on the plants to see whether they will set into fruit or abort. Collected ovaries were cleared by using Hoyers solution (Zhang and Somerville 1997: 100 g chloral hydrate, 5 ml glycerol and 30 ml $H_2O$). Cleared ovaries were dissected under a simple microscope and observed under a Zeiss Axioplan 2 fluorescence/differential interference contrast (DIC) microscope (Sliedrecht, the Netherlands). Photographs were taken with a Zeiss AxioCam MRc 5 digital color camera (Sliedrecht, the Netherlands). The vascular connectivity was also observed in a hot pepper cultivar (*C. annuum*: 'Fireflame'; F1 hybrid, De Ruiter Seeds, Bergschenhoek, The Netherlands) grown under the same growth conditions as Bruinsma Wonder. cv. Fireflame has a simple anatomy (ovules are concentrated around a vein type of placenta), which facilitates easy, clear and convincing observation of vascular connection compared with Bruinsma Wonder (ovules are concentrated around a centrally located axile type of placenta).

To evaluate cell division and cell expansion in the carpel, pollinated ovaries (n = 3–5) were harvested at 12, 20, 30, 40 and 72 HPP. Length and diameter of each ovary was measured using digital Vernier calipers. Horizontal and vertical cross sections of the carpel were mounted, stained with 'safranin O' (stock solution: 2.5 g safranin O Certistain® in 100 ml of 96% ethanol, working solution: 1:100 dilution in water) and observed under a Zeiss Axioplan 2 fluorescence/DIC microscope. Photographs were taken with a Zeiss AxioCam MRc 5 digital color camera. Cell diameter, measured by using UTHSCSA image tool version 3.00 (http://ddsdx.uthscsa.edu/dig/itdesc.html), was used as a measure of cell expansion, cell number and cell division. For each treatment, 20–30 cells were observed from 4 to 5 sections per fruit. To measure the diameter of individual cells we used the same four to six layers in the mesocarp starting from layer 5 from the exterior (Fig. 3A, box). From these data we estimated the total number of cells (Smith 1950). Carpel cell number = (fruit volume-placental cavity volume)/average cell volume, where volume (sphere) = $4\pi/3(r)^3$. Average cell diameter (calculated from the mesocarp) was used to estimate the average cell volume.

### Endogenous IAA levels and number of cell layers and cell diameter in the carpel

Plants of *C. annuum* cultivar 'Bruinsma Wonder' were grown in a climate room, as described above, at 20.4/20.2°C day/night temperature. Parthenocarpy is a genetic trait in *C. annuum* and emasculation of flowers at the favorable growth condition resulted in parthenocarpic fruit-set (approximately 30%) in cv. Bruinsma Wonder (Tiwari et al. 2011). Fruits obtained after emasculation can be true seedless fruits only when they have reached at least 50% of the weight of seeded fruits, while the remaining fruits were considered as knots or pseudo-fruits (Tiwari et al. 2007). This definition was taken into account only for the mature fruits, but not for the immature fruits. Plants were exposed for long day (from 06:00 to 22:00 hours) by using fluorescence tubes (Philips TL 50W, color 84, Eindhoven, the Netherlands) with a light intensity of $200 \pm 2\ \mu mol\ m^{-2}\ s^{-1}$.The terminal flowers were removed from all plants at anthesis to support vegetative growth. The twin-branch system was applied, resulting in two stems per plant, with all side shoots restricted to one leaf and flower. Emasculated flowers were hand pollinated at anthesis (day 0) and ovaries (n = 2–3) were collected at 0, 2, 4, 6, 8, 10, 15, 20, 30 and 40 DAA. The same numbers of ovaries were collected at the same time (DAA) for pollinated and un-pollinated flowers. After measuring fresh weight of each ovary, these were used for the measurement of endogenous IAA and for the evaluation of the number of cell layers and cell diameter in the carpel.

For the measurement of endogenous IAA, carpel and placenta were collected (10–20 mg fresh weight) from ovaries in 1.5 ml micro centrifuge tubes

(http://www.sarstedt.com/php/main.php) with conical bottom and stored at −80°C. Endogenous level of IAA was quantified using an isotope dilution mass spectrometry (MS) technique (Edlund et al 1995). To each sample 1–6 ng [$^{13}C_6$] IAA (Cambridge Isotope Laboratories, Woburn, MA) was added as an internal standard. Analysis was performed by gas chromatography-selected reaction monitoring-MS, using a JMS-SX/SX102A instrument (JEOL, Tokyo, Japan).

For the evaluation of the number of cell layers and cell diameter, rectangular wedge was cut from the equatorial section from each fruit in duplicates. Samples were collected for each stage except for 0 and 2 DAA, where whole fruits were fixed by using paraffin method technique (Bunger-Kibler and Bangerth 1983). Thin sections (4–6 μm) were obtained from pericarp samples using a rotary microtome and stained with aniline blue. Samples were analyzed under a light microscope (Leitz Aristoplan). Photomicrographs were then obtained using a camera (Nikon digital camera DXM1200) mounted on the microscope and analyzed by using image tool (version 3.00). Cell diameter as a measure of cell expansion was determined by averaging diameter of 10–20 cells from 3 to 5 sections per fruit from exocarp, mesocarp (described above, Fig. 3A, box) and endocarp layers. The number of cell layers was counted in the mesocarp as a measure of cell division.

### Statistical analysis

Cell number and cell diameter at each developmental stage was tested by using one way ANOVA while IAA level was tested by using two-way ANOVA (time as a second factor). Mean separation was done by Student's *t*-test (LSD) based on the ANOVA mentioned above. Data processing and statistical tests were carried out with SPSS (Statistical Package for the Social Sciences) 15.0.

## Results

### Fertilization induces vascular connection between ovule and replum to initiate fruit-set

To establish the timing of fertilization, pollen tube growth and their movement in the vicinity of the ovule was observed after pollination. Pollen tubes started their germination on the surface of the stigma already at 2 HPP. However, none of the pollen tubes reached the base of the style upto 8 HPP. Only a few pollen tubes reached the base of the style at 12 HPP, but many did so at 15 HPP, at which time the first pollen tubes were observed in the ovule vicinity. At 20 and 28 HPP many pollen tubes were observed close to the ovules, suggesting that the majority of ovules were fertilized by that time (Fig. 1).

In Arabidopsis, before fertilization the vasculature in the funiculus is not connected to the replum and this connection becomes established after fertilization. This

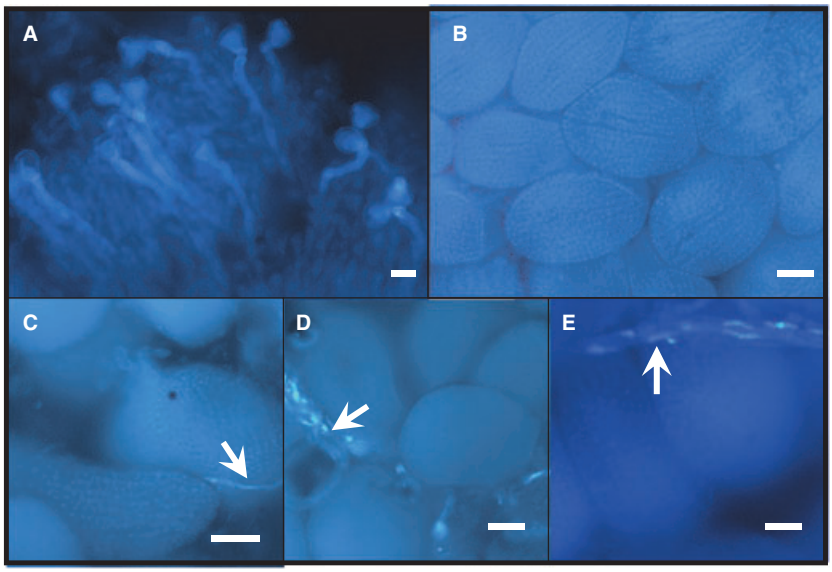


**Fig. 1.** *In-vivo* pollen tube growth in a pollinated ovary of *Capsicum annuum* cultivar 'Bruinsma Wonder'. (A) Pollen germination on the stigma of emasculated flowers at 2 HPP, (B) no pollen tubes in the ovule vicinity at 12 HPP, (C) a few pollen tubes in the ovule vicinity at 15 HPP (arrow head) and many pollen tubes in the ovule vicinity at 20 HPP (D) and 28 HPP (E). The bright spots are callose plugs, indicating the position of pollen tubes (arrow heads). Scale bar: 100 μm (A), 50 μm (B–E).

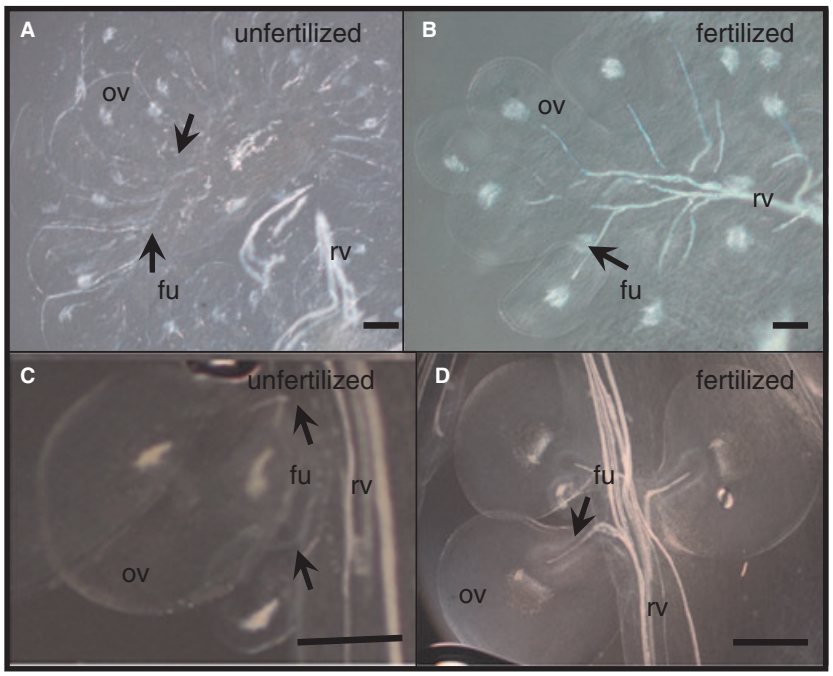


**Fig. 2.** Vascular development in unfertilized and fertilized ovules of *Capsicum annuum* at 48 HPP. (A, B) Vascular connectivity between funiculus and replum was absent in unfertilized ovules (arrow head, A) but present in fertilized ovules (B) of cultivar 'Bruinsma Wonder'. (C, D) Vascular connectivity was absent in unfertilized ovules (arrow head, C) but present in fertilized ovules (D) of cultivar Fireflame. Ov, ovule; rv, replum vasculature; fu, funiculus. Scale bar: 100 μm (A, B), 50 μm (C, D).

suggests that fertilization induced (auxin) signals that are crucial for fruit initiation might be transmitted from the ovule to the carpel via vascular development (Fuentes and Vivian-Smith 2009). To test the same in *C. annuum*, cleared fertilized and unfertilized ovules were evaluated under a fluorescence microscope. Vasculature prior to fertilization was not observed in *C. annuum*. Fertilized flowers set into fruits while unfertilized flowers aborted. At 48 HPP, no vascular connection between funiculus and replum was observed in an unfertilized ovule (Fig. 2A, C) and also none of the unfertilized ovaries set into fruit. In contrast, vascular differentiation in fertilized flowers resulted in a vascular element that extends from the base of the ovule funiculus toward the replum vasculature in Bruinsma Wonder (Fig. 2B) and Fireflame (Fig. 2D). These observations suggest that the vascular connection is an early indicator for successful fruit-set after pollination and fertilization in *C. annuum*.

## Cell number and cell diameter both contribute to early fruit growth

To know whether early fruit growth is because of the cell division or cell expansion or a combination of both, we carefully recorded the fruit diameter, cell number and cell diameter in a post-pollinated ovary. The three distinct layers found in carpels of other plant species, i.e. exocarp, mesocarp and endocarp could be clearly distinguished in *C. annuum* cv. Bruinsma Wonder. The exocarp being the outermost single cell layer, the mesocarp being multilayered and comprising of vascular bundles, and the endocarp consisting of a single layer of giant cells and a layer of small cells (Fig. 3A). In the exocarp and endocarp mainly anticlinal cell divisions (where new cell walls are inserted perpendicular to the surface of the fruit) were observed, whereas the orientation of cell division in the mesocarp was random (Fig. 3A, arrow). Cells in the exocarp and innermost endocarp remained small, which might be because of the extensive cell division in those layers, while the mesocarp and giant-endocarp cells underwent significant cell expansion. A clear gradient in cell size was observed from exterior to the interior in mesocarp layers (Fig. 3A, broken arrow). The cells were smaller and flat toward the exterior (close to exocarp), more rounded and relatively isodiametric in the middle and around the vascular bundle, and approaching the interior they became larger and elongated (Fig. 3A).

Multilayered mesocarp was used to evaluate the contribution of cell division and cell expansion in

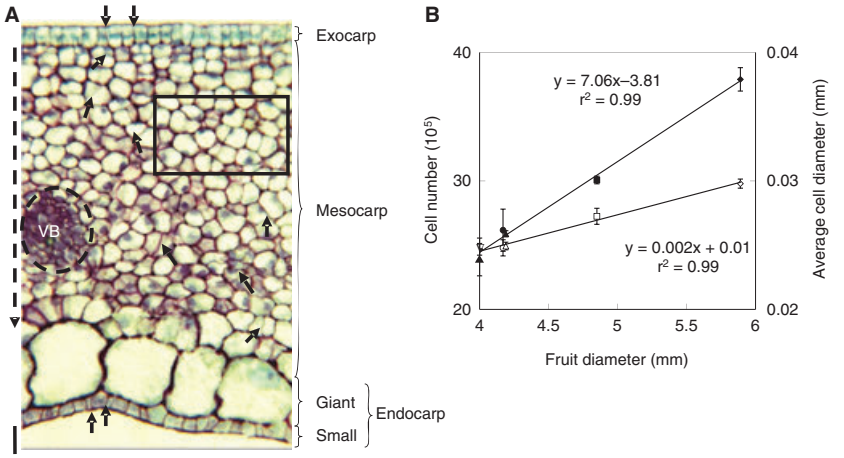


**Fig. 3.** Histological observation in a carpel of *Capsicum annuum* cultivar 'Bruinsma Wonder' at 12, 20, 30, 40 and 72 HPP. (A) Representative image of a horizontal cross section of the carpel (at anthesis) with indication of an area (box) in which diameter of cells were measured, three distinct layers, i.e. exocarp: single layered, mesocarp: multilayered and endocarp: double layered (giant and small). Vascular bundle (VB), anticlinal cell division in exocarp and endocarp (vertical arrows), random orientation of cell division in mesocarp (random arrow head). Smaller to bigger cell size gradient from exterior (above) to interior (below) direction in mesocarp (broken arrow). Scale bar: 50 μm. (B) Correlation between fruit diameter and cell number (closed symbols), and between fruit diameter and average cell diameter (open symbols) at 12 (▼, □), 20 (●, ○), 30 (▲, Δ), 40 (■, □) and 72(◆, ◇) HPP (n = 3–5 ovaries).

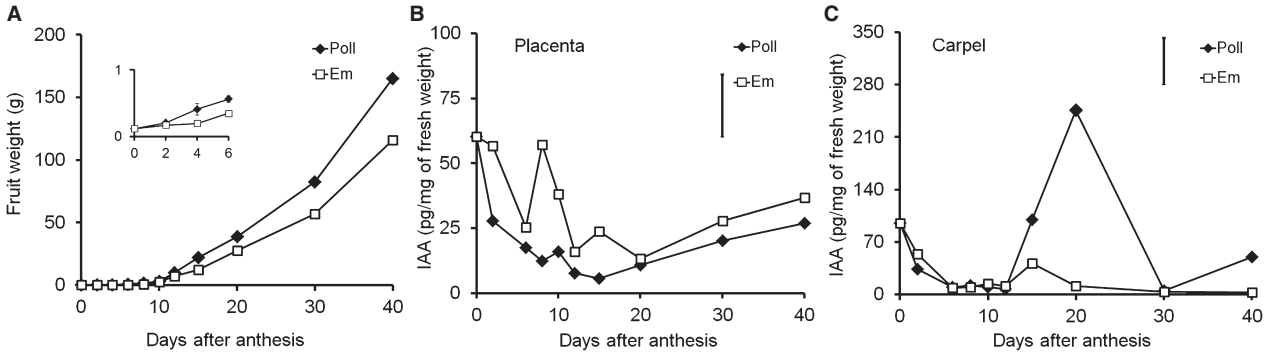


**Fig. 4.** (A) Fruit weight (g) (inset: fruit weight from 0 to 6 DAA) and endogenous IAA level for *Capsicum annuum* cultivar 'Bruinsma Wonder' in (B) placenta and (C) carpel at 0, 2, 6, 8, 10, 15, 20, 30 and 40 DAA from seeded (closed symbols) and seedless (open symbols) fruits obtained from pollinated (Poll) and emasculated (Em) flowers, respectively (n = 2–3). Vertical bars represents interaction LSD (0.05) values.

fruit growth. From 12 to 30 HPP no difference in cell diameter ($P = 0.941$), cell number ($P = 0.524$) and fruit diameter ($P = 0.125$) was observed. A significant increase in the cell diameter ($P < 0.001$), cell number ($P = 0.009$) and fruit diameter ($P = 0.009$) was observed between 30 and 40 HPP (Fig. 3B). Between 12 and 72 HPP there was a significant positive correlation between fruit diameter and cell number ($r^2 = 0.99$), and between fruit diameter and cell diameter ($r^2 = 0.99$; Fig. 3B), indicating that both cell division and cell expansion contribute to early fruit growth. The steepness of the curves indicates that the cell division contributed more to increased fruit diameter than cell expansion.

## Auxin is important in fruit-set and fruit growth

To evaluate the role of IAA in fruit-set and fruit growth, endogenous IAA levels were measured in the placenta and carpels of seeded and seedless fruits obtained from pollinated and unpollinated flowers, respectively. The development of both seeded and seedless fruits comprised an initial phase of slow growth between 0 and 10 DAA followed by a rapid growth (10–40 DAA). The ovary weight in seeded and seedless fruits was the same at 0 DAA, but increased in seeded fruits at 4 DAA ($P < 0.001$); whereas seedless fruit weight only significantly increased at 6 DAA ($P < 0.001$; Fig. 4A). At 40 DAA, seedless fruits were more conical in shape while seeded fruits were blocky in shape and fruit weight

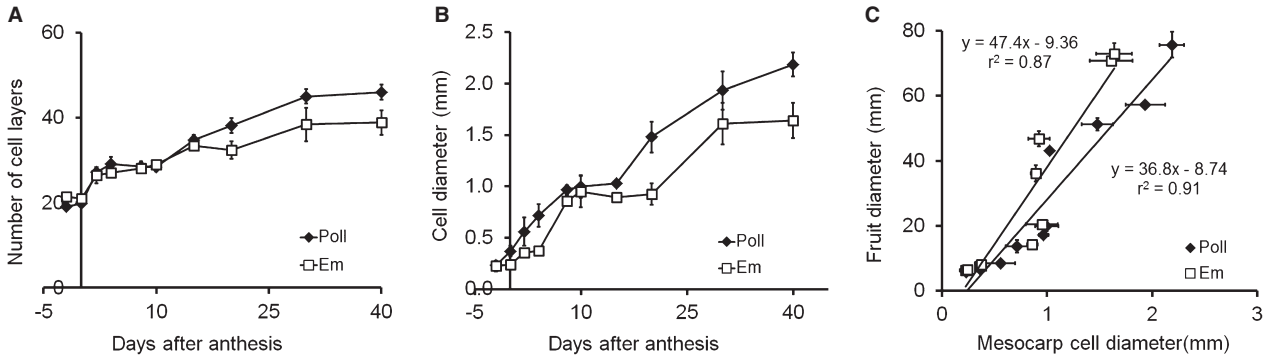


**Fig. 5.** Number of cell layers, cell diameter and fruit diameter in *Capsicum annuum* cultivar 'Bruinsma Wonder' (n = 3–5). (A) Average number of cell layers in the mesocarp, (B) average cell diameter in the mesocarp, (C) relation between fruit mesocarp cell diameter (mm) and fruit diameter (mm) in seeded (closed symbols) and seedless fruits (open symbols) obtained from pollinated (Poll) and emasculated (Em) flowers at 2, 0, 2, 6, 8, 10, 15, 20, 30 and 40 DAA, respectively. Vertical and horizontal bars indicate standard error of mean.

of seedless fruits was significantly smaller than seeded fruit ($P = 0.012$). In the placenta and in the carpels, the IAA levels were higher at or around anthesis (0 DAA) compared with 10–12 DAA in both seeded and seedless fruits. A significant peak of IAA was observed in the carpel of seeded fruits at 20 DAA ($P < 0.001$), whereas this peak was absent in seedless fruits (Fig. 4B, C).

To investigate the differences between seeded and seedless fruits at the cellular level, cell division (number of cell layers) and cell expansion (cell diameter) were quantified for both fruit types. The number of cell layers in the carpel was the same for seeded and seedless fruits at 0–12 DAA, while this was significantly higher in seeded fruits from 20 DAA onward, indicating more cell divisions in seeded fruits compared with seedless fruits (Fig. 5A). At 40 DAA, the mesocarp cell diameter was significantly higher in seeded fruits compared to seedless fruits ($P = 0.016$), while no difference was observed in exocarp ($P = 0.840$), giant endocarp ($P = 0.723$) and small endocarp ($P = 0.742$). A positive relation between mesocarp cell diameter and fruit diameter was observed in seeded ($r^2 = 0.91$) and seedless fruits ($r^2 = 0.87$; Fig. 5C) indicating the contribution of cell expansion to later fruit growth. The increase in mesocarp cell diameter at 20 DAA coincided with the peak in IAA levels in the carpel of seeded fruits (Figs 5B and 4C), suggesting that IAA stimulates cell expansion to trigger a major increase in fruit growth.

## Discussion

### Fertilization induces vascular connection between ovule and carpel to initiate fruit-set

After pollination, fruit-set requires pollen germination and pollen tube growth in the stylar tissue towards the ovule and into the embryo sac to enable the fusion between male and female gametes (Weterings and Russell 2004). Our study indicates that in *C. annuum* the majority of ovules are fertilized at 20–28 HPP. This is similar to the timing of fertilization observed in tomato (Iwahori 1966), but significantly longer compared with Arabidopsis, where fertilization occurs around 5 HPP (Faure et al. 2002).

Gene expression and mutant studies have indicated the important role of auxin in fruit-set in Arabidopsis and tomato (Wang et al. 2005, Goetz et al. 2007, Vriezen et al. 2007, de Jong et al. 2009). Moreover, the fact that ovule-specific expression of a bacterial auxin biosynthesis gene under the *DEFH9* promoter can induce fruit-set without fertilization in tomato and several other plant species (Rotino et al. 1997, Ficcadenti et al. 1999, Mezzetti et al. 2004, Yin et al. 2006) suggests that production of free auxin in the ovule is a rate-limiting step in fruit initiation. Enhanced expression of the auxin responsive *DR5*::*GFP* reporter has been observed in Arabidopsis ovules 5–24 HPP (Dorcey et al. 2009, Fuentes and Vivian-Smith 2009), suggesting that fertilization rapidly induces either de novo auxin biosynthesis or hydrolysis of auxin conjugates. In *C. annuum* we do not observe a clear increase in free IAA levels in the placental tissue (Fig. 4B), but rather a decrease. As our earliest time point was 2 days after pollination, we may have missed this initial burst of auxin production. Alternatively, such increase may not be detectable when the entire placenta is used as source tissue instead of ovules.

Immediately after fertilization, a vascular connection has been observed between ovule and carpel in Arabidopsis (Fuentes and Vivian-Smith 2009). The importance of auxin transport for vascular development (Scarpella et al. 2006) suggests that the vascular

connection between ovule and carpel is mediated by transport of fertilized ovule-generated auxin to the carpel. This process also occurs in *C. annuum* where fruit-set and vascular connection between ovule and replum was observed in fertilized ovaries, whereas the vasculature in the funiculus remained disconnected from the replum in unfertilized ovaries. The correlation between vascular development and fruit-set was observed in both a sweet and a hot pepper cultivar, i.e. Bruinsma Wonder and Fireflame, which strongly suggests that in *C. annuum* also vascular connection between ovule and replum is mediated by transport of newly produced free IAA from the ovule to the carpel, and is a determinant factor for sustained fruit growth after fertilization. This fertilization-induced vascular biogenesis does not appear to be restricted to the ovule–carpel junction, but also appears to occur elsewhere in the flower, e.g. vascular biogenesis in the pedicel has been related with fruit development in citrus (Bustan et al. 1995), apple (Drazeta et al. 2004) and Prunus (Else et al. 2004).

### Both cell division and cell expansion contribute to early fruit growth in *C. annuum*

During fruit development in *C. annuum*, the exocarp and endocarp layers comprise of small but mitotically active cells, whereas a substantial increment in the cell size was observed in the mesocarp (Fig. 3A). Anticlinal divisions contribute to increase in surface area and periclinal divisions (new cell walls are inserted parallel to the surface of the fruit) contribute to increase in tissue thickness in *Vitis vinifera* (Considine and Knox 1981). In *C. annuum*, mesocarp comprises both anticlinal and periclinal types of cell divisions, which contribute maximum to the fruit growth. The same pattern of cell division and expansion was reported in tomato and *Lagenaria leucantha* (Varga and Bruinsma 1976, Joubès and Chevalier 2000, Yu et al. 2001, Cheniclet et al. 2005). A gradient in cell size was observed in *C. annuum* mesocarp layers of the expanding ovary (Fig 3A, broken arrow). Interestingly, in tomato, genes controlling the cell expansion process (tonoplast intrinsic protein: TIP and pro-rich protein : PRP) or its regulation (hormone synthesis, signaling and response) are expressed along a gradient from the inner part to the outer part of the fruit, resulting in a gradient in cell size (Lemaire-Chamley et al. 2005).

Between 30 and 40 HPP the fruit diameter started to increase, and at the same time an increase in the mean cell diameter and increase in mesocarp cell number was observed (40 HPP), suggesting that early fruit growth is because of the coordination of cell division and cell expansion. Our results are in agreement with those in Arabidopsis and tomato where a combination of cell division and cell expansion promotes carpel growth immediately after pollination and fertilization (Nitsch 1970, Cheniclet et al. 2005).

Cell division is dominant in early fruit growth in *C. annuum* (Fig. 3B) similar to what has been reported in mandarin, tomato and *L. leucantha*, where early fruit growth is primarily the result of cell division (Guardiola and Lazaro 1987, Yu et al. 2001, Bertin 2004). Cell expansion is the main determinant of later fruit growth in *C. annuum* (Fig. 5B, C) similar to what has been reported in tomato, cucumber and pear where after few weeks of early fruit growth, the cell division ceases and subsequent growth of the fruits is mainly supported by cell expansion and the formation of intercellular space (Gillaspy et al. 1993, Marcelis and Baan Hofman-Eijer 1993, Matsumoto et al. 2008).

### Auxin-enhanced cell expansion is important for maximal fruit growth

Fruit growth in *C. annuum* follows a sigmoid growth pattern: an initial phase with slow growth, an intermediate phase with rapid growth and a last phase or ripening phase with no further growth (Nielsen et al. 1991). Also in our measurements we observed a sigmoid growth pattern, despite the fact that the last stages of fruit development were not included (Fig. 4A). At 40 DAA, seedless fruits were smaller than seeded fruits, which seem to be because of the limitation of both cell division and cell expansion as number of cell layers and cell diameter in mesocarp layers were significantly reduced in seedless fruits compared with seeded fruits (Fig. 5A, B). This result is in the agreement with tomato where fruit size is determined by both cell number and cell size (Ho 1996).

It has been suggested that auxin promotes cell expansion in fruits (Rayle and Cleland 1992, Gillaspy et al. 1993). Auxin presumably causes an increase in the extensibility of cell walls and induces uptake and retention of water and solutes (Masuda 1969, Cleland 1995). In seeded fruits of *C. annuum*, a steep increase in cell diameter was observed at 20 DAA correlating with a clear peak in IAA levels in the carpel (Figs 4C and 5B). The increase in cell size was significantly reduced in seedless fruits, and also no clear peak in IAA levels was observed here, explaining why seedless fruits are smaller than seeded fruits. These results suggest that the observed IAA peak in the carpel of seeded fruits at 20 DAA induces a major increase in cell diameter, which is essential to obtain maximum fruit size. Similar to our observations in pepper, a significant peak in auxin levels

has been reported in seeded tomato fruits around 30 DPP, coinciding with the phase of cell expansion during fruit growth (Sjut and Bangerth 1982/1983, Gillaspy et al. 1993); whereas this peak in auxin levels was not observed in seedless tomato fruits. Although we did not measure gibberellin levels, it is likely that auxin-induced gibberellin biosynthesis contributes to this increase in cell expansion (Ngo et al. 2002, Ozga et al. 2003, Serrani et al. 2008).

## Conclusion

Here we show that early fruit development induced by fertilization in *C. annuum* coincides with the development of a continuous vascular connection between ovule and replum, while without fertilization this vascular connection is absent and flowers abort. These results indicate that, as in Arabidopsis, vascular development is an early determinant of fruit-set after fertilization in *C. annuum*, suggesting that transport of newly produced auxin from the ovule is an early event in fruit development. Increase in cell number and cell diameter both contribute to early fruit growth in *C. annuum* after pollination and fertilization. Increase in cell number is more prominent at early stage of fruit growth while increase in cell diameter is more dominant at later stages of fruit growth. As in tomato, the increase in cell size appears to coincide with a peak in IAA levels. The observed similarities in fruit-set and initiation processes between *C. annuum* and tomato and Arabidopsis, suggests that the available knowledge from these model plants might help to understand fruit-set and development process in *C. annuum* and to improve fruit quality and introduce traits such as parthenocarpy in this crop species.

*Acknowledgements* – We thank Hans Dassen, Maarten Peters and Myckel E. J. Habets for their help with the laboratory measurements and greenhouse experiments. This research was funded by the Dutch Technology Foundation (STW), which is the applied science division of the Netherlands Organization of Scientific Research (project number LPB 6822).

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Edited by Y. Helariutta