Rubber Accumulation and Storage in Laticifer Cells of *Taraxacum kok-saghyz* Roots grown in Soil and Hydroponic Cultivation Systems

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Abstract

The commercialization of *Taraxacum kok-saghyz* (TK) as an alternative rubber crop requires fundamental knowledge of the storage mechanism of the rubber in the plants. Research in this area is important due to the lack of clarity in studies of rubber particle ontogeny, development and maturation. The mechanism of rubber accumulation and storage post rubber particle ontogeny was investigated in both soil- and hydroponically-grown roots. Even though rubber is formed as particles in aqueous cytosol (thus, in the form of latex) transmission electron micrographs indicate that, unusually, many of these aggregate and coagulate to form solid rubber in laticifer cells of living roots. Rubber particles from both cultivation systems coalesced sometimes while still surrounded by cytosol, and sometimes after the cytosol had been degraded due to the aging of the laticifer cells. However, laticifer cells in hydroponically-grown roots were often univacuolate, and a single very large rubber particle was contained within the vacuole. Soil-grown root laticifer cells were multivacuolate and many small rubber particles aggregated to form large rubber masses, not a single large particle. Also, these large masses were formed to a greater extent in cells with intact cytoplasm but also formed in cells after the cytosol had largely degraded.

Main conclusions.

As laticifer cells matured, rubber particles coalesced with each other and filled up the laticifer cells as solid rubber. This occurred in laticifers of both soil- and hydroponically-grown roots. A single very large rubber particle formed in the univacuoles of laticifer cells in hydroponically-grown roots, in contrast to large aggregated rubber masses in soil-grown roots.

Keywords: Cell development. Hydroponic system. Latex. Laticifer cell. Rubber particles. Solid rubber

Abbreviations

ASE	Accelerated solvent extraction	LQ	Latex quantification
TEM	Transmission electron microscopy	TK	*Taraxacum kok-saghyz*

Introduction

Taraxacum kok-saghyz (TK) is an herbaceous species native to central Asia that has been introduced as a new potential rubber crop for the temperate regions of the US and Europe (Cornish, 2017). The introduction of this crop, along with a semi-arid, rubber-producing shrub, *Parthenium argentatum* (guayule), will help secure the natural rubber (NR) supply (van Beilen and Poirier, 2007) and reduce the world rubber source dependency on the tropically grown rubber tree, *Hevea brasiliensis* (Cornish *et al*, 2013. Cornish, 2017). Domestication and commercialization of TK in the United States and Europe will benefit industrial sectors, and is expected to provide rubber for conventional industrial, non-medical products (Mooibroek and Cornish, 2000; Cornish *et al*, 2015). As TK is still in the early stage of domestication, fundamental knowledge on botany and rubber biosynthesis is useful to guide the crop commercialization process. This information can be used to improve agronomic practices and protect rubber properties (Munt *et al*. 2012).

Structurally, TK roots contain laticifer cells which produce rubber in the form of rubber particles in the cytosol. The TK laticifer characteristics are similar to those of *H. brasiliensis*, although most laticifer cells in *H. brasiliensis* are found in the tree bark which allows the latex tapping process used to harvest rubber from this species. Little is known about TK rubber particle ontogeny in its laticifers and the mechanisms involving rubber accumulation in the laticifers have not yet been explored. Recent studies of morphology and localization indicate that TK rubber particles are developed directly from the endoplasmic reticulum-Golgi vesicular complex (unpublished).

What happens during development and maturation is still unclear. However, unlike *H. brasiliensis* and *P. argentatum*, in which the rubber particles in healthy tissues are maintained in latex form throughout healthy plant growth and development, most of the particles in TK coagulate *in vivo*. The mechanism of latex coagulation in TK involves polyphenoloxidase (PPO) (Wahler *et al*, 2009) and plants genetically engineered to have low PPO activity freely bleed latex when the roots (and laticifers) are cut (Gronover *et al*, 2011). A loss of rapid coagulation is likely to lead to losses during root harvest as roots accrue damage rather than increase harvestable yield. In other rubber latex producing plants, a wounding-induced coagulation process has been noted and has provided bio-inspiration for the development of self-healing materials and polymers (Speck *et al*, 2013).

Fundamental studies of rubber particle formation, accumulation and storage, and their coagulation mechanisms, may help our understanding of the biological system, further the selection or creation of elite germplasm, and contribute to the development of novel rubber products (Bauer *et al*, 2014). Several agronomic practices have been used to cultivate TK including in outdoor and indoor raised beds and boxes, in fields, and in greenhouse hydroponics (Kopicky, 2014). Hydroponic systems were tested for production of *Taraxacum mongolicum* (Chinese dandelion), a species of medicinal value in Chinese medicine (Yarnell and Abascal, 2009), due to the limited land area and soil pollution, as well as to produce and market high quality plants (Chen *et al*, 2015). A comparison of field and hydroponically-grown TK may help diversify the agronomic and horticultural options for TK cultivation, and inform how rubber particles are accumulated and stored in laticifers. The main objective of this study was to histologically investigate the mechanisms of rubber particle accumulation and storage in laticifer cells using transmission electron microscopy (TEM) in TK grown in soil and hydroponics.

Materials and methods

Soil grown mixed genotypes.

The TK seeds, from open pollinated mixed seed lines collected in 2014 and 2015 from greenhouse grown plants, were germinated in trays of PRO-MIX® BX, in a greenhouse at The Ohio State University, Ohio Agriculture and Research Development Center (OARDC), Wooster OH. After 2 months, the seedlings were transferred into outdoor shallow raised beds containing soil composed of 20% parboiled rice hulls, 30% peat moss, 25% cow manure compost from OARDC campus facilities, and 25% Wooster silt loam field soil. The planting density was 400,000 plants/acre. Plants were sampled for TEM after 4, 8 and 12 months of growth in the beds. Samples of 4-month-old plants were from TK planted in April 2016. Samples of 8 and 12 month-old plants were from TK planted in October 2015.

Hydroponically- and soil-grown clones of one genotype.

Clonal planting materials were developed from root cuttings of a single mother plant produced as described (Cornish *et al*, 2016). After two months, ramets were translocated into nutrient film (NFT) growing channels (March 2015) inside a greenhouse (#115) at The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH. The temperature in the greenhouse was set to 21-23°C during the day and 17-20°C at night. The plants were grown under natural light during summer. Each growing channel was 122 cm long and was equipped with a top cover with six square holes. In one NFT table, six growing channels were used to grow 36 TK plants, 6 per channel. The plants were grown in nutrient solution circulated using water pumps. Two stock solutions were made. The first stock was 640.9 mM greenhouse grade Ca $(NO_3)_2$, 327.1 mM Multi-K greenhouse grade KNO_3 (both from Haifa Chemicals Ltd., Matam-Haifa, Israel), and 1.53g/L 11% DTPA iron chelate (CropKing, Lodi, OH). The second stock was 327.1 g/L Multi-K greenhouse grade KNO_3 (Haifa Chemicals Ltd., Matam-Haifa, Israel), 206.3 mM KH_2PO_4 (Sigma Aldrich, St. Louis, MO), 408.3 mM $MgSO_4$ and 20 g/L Micromix (both from CropKing, Lodi, OH). Each stock was diluted before use by co-mixing 11.25 ml/L of each with water. The nutrient solution was replenished with the same addition of both stock solutions after 2 weeks to prevent leaf yellowing. After four weeks, the nutrient solution was replaced with fresh solution to reduce algae growth and replenished after 2 weeks as before. This cycle was repeated throughout the experiment. The pH was maintained at 5.7±0.3 by adding 25% hydrochloric acid when necessary. After 4 months, 12 plants were randomly selected for microscopy studies, latex and analyte quantification (0 day), followed by additional samplings after 25 and 50 days. As ramet numbers from the same phenotype were limited, soil cultivation of ramets was established later in the year (December 2015). The ramets were grown inside a greenhouse (house 112) at The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH. Soil composition and planting density in the raised bed inside the greenhouse was similar to the outdoor beds. The temperature in the greenhouse was set to 21-23°C during the day and 17-20°C at night with 16 hours under high-intensity discharged (HID) light. Plants were harvested 6 months later for analyte and latex quantification.

Analyte quantification

Analytes were gravimetrically quantified in 0.25 g dried and ground root biomass extracted by accelerated solvent extraction (ASE) (Thermal Fisher Dionex ASE 350) using three solvents sequentially, distilled water (inulin and other minor water soluble components), acetone (resin) to eliminate resin contamination of the hexane extract, and hexane (rubber). The same pressure (10.34 MPa or 1,500 psi) was used for all extractions with water injected first into each cell (at 95°C), followed by acetone (at 23°C) and hexane (at 120°C). Extracts were collected, poured into tared aluminum pans, dried and weighed. Analyte concentrations and total analyte per root were calculated. Because inulin is the predominate component of the water extract (Ramirez-Cadavid *et al*, 2017) this extract is referred to simply as inulin throughout the remainder of this paper.

Latex quantification (LQ)

Latex is the sub-fraction of rubber that is still in the form of individual particles in aqueous cytosol, and in TK it can be measured using a published LQ method (Cornish *et al*., 1999) with minor modification. Briefly, three replications of 1 ml TK root homogenate were analyzed for each sample. Each 1 ml sample was centrifuged for 5 minutes at 17,000 xg. The latex rose to the top of the centrifuge tubes and was coagulated using glacial acetic acid, re-centrifuged, collected, dried and then weighed. In addition, three separate 1 ml homogenate samples were dried and weighed to measure total solids within the root homogenates. In order to calculate the total rubber (latex + solid rubber), rubber was quantified in the leftover (latex-free) homogenate. The homogenate was dried, ground and residual rubber quantified by ASE as described above.

Tissue preparation and staining for TEM

Roots were cut 2 cm below the crown. Samples (approximately 0.5 cm in length) were collected at the middle of the roots based on the total length of each root. Samples were fixed in 3% glutaraldehyde, 2% paraformaldehyde and 0.1 M potassium phosphate buffer (PB) overnight. Samples were then post-fixed in 1% osmium tetroxide and 1% uranyl acetate for 1 h and then rinsed 3x with double distilled water. The fixed root samples were dehydrated using a series of ethanol concentrations (25%, 50%, 70% and 90%), then infiltrated with series of propylene oxide and EM Bed-812 resin mixtures with the ratio of 2:1 for an hour, 1:1 and 1:2 each for 2 hours. Lastly, each sample was embedded in 100% EM Bed-812 resin and left to dry in an oven overnight (60°C). The resin blocks were sliced (70nm thick) using a LEICA EM-UC6 Ultra Microtome (LEICA, Vienna, Austria). Reynold's lead citrate and 3% aqueous uranyl acetate were used to stain the sectioned tissues. The samples were then examined using a TEM microscope (Hitachi H-7500, Tokyo, Japan). All chemicals for tissue preparation and staining were obtained from Electron Microscopy Sciences (EMS, Hatfield, PA, US).

Statistical analysis

Comparisons between means were evaluated by ANOVA and determined using Least Significant Difference (LSD) tests ($\alpha = 0.05$) using SAS software, Version 9.4(SAS Institute Inc., Cary, NC, USA).

Results

Laticifer cells and rubber particles in soil- and hydroponically-grown roots

Rubber particles in TK laticifer cells from both cultivation systems were produced in the cytoplasm and translocated into the vacuoles (Fig.1a and 4f), similar to previous observations in *Parthenium argentatum* (Backhaus and Walsh, 1983) and *Asclepias curassavica* (Giordani, 1996). Rubber particles began to increase in number while the laticifers were very young (Fig. 1c). Multivacuolate laticifers were observed mostly in soil-grown roots (Fig. 1a) whereas univacuolate lactifiers predominated in hydroponically-grown roots (Fig. 1b). Typical laticifer cells in soil-grown plants contained two types of rubber particles, identified by their locality, shape and how they were produced. Plastidic rubber was produced from small rubber particles that originated from the endoplasmic reticulum-Golgi vesicular complex and which later accumulated in laticifer plastids and merged, whereas cytoplasmic rubber was formed without these plastids (unpublished). Most cytoplasmic rubber was found in the cytosol adjacent to the tonoplast (Fig. 1a). Plastidic and cytoplasmic rubber were also produced in hydroponically-grown roots although they were sometime less osmiophilic than rubber particles in soil-grown roots. However, these hydroponically-grown roots also produced single very large rubber particles not found in soil grown roots (Fig.4). In both cultivation systems, younger laticifer cells were characterized by a large amount of cytosol (Fig. 1a) whereas mature laticifer cells had much greater relative vacuolar volume/cell (Fig. 1d) as observed in *Parthenium argentatum* (Backhaus and Walsh, 1983).

Rubber particle accumulation in soil grown roots

Coagulated rubber formed after the laticifer cells became mature. These mature cells were characterized by degradation of the cytosol, followed by thickening of the cell wall. At the same time, the rubber particles aggregated and merged forming solid rubber masses in the laticifer. The coagulation of rubber particles into solid rubber masses occurred in two ways: (i) the number of globular shaped rubber particles increased after cytoplasmic degradation (Fig. 2a) followed by coalescence of the particles (Fig. 2b, big arrow), which later merged to form large irregular masses (Fig. 2b, small arrow); (ii) proliferation of rubber particles in intact cytoplasm, followed by coalescence (Fig. 3a), without production of intermediate globular particles (Fig. 3b), then cytoplasmic degradation left coagulated rubber masses (solid rubber) in the laticifer cell (Fig. 3d). As the laticifer cells matured, perforation and anastomisation of the laticifer cells enabled the solid rubber masses to merge with those in neighboring laticifer cells (Fig. 2c).

Rubber particles in hydroponically-grown roots

Small individual rubber particles in the cytosol of hydroponically-grown roots averaged 0.67 ±0.07 µm (n=10) in diameter whereas the rubber particles in soil-grown roots averaged 0.86 ± 0.06 µm (n=30). However, single large rubber particles usually occupied the univacuoles of the laticifer cells (Fig. 4b, 4f) and the size of these unique rubber particles averaged 7.18 ± 0.06 µm (n=10). Large rubber particles were formed by the coalescence of smaller globular shaped rubber particles in the cytoplasm (Fig. 4a). As for all other TK rubber particles produced in the cytoplasm, these large rubber particles were translocated into the vacuoles. This process began with the invagination

of the cytoplasm and the splitting of the tonoplast (Fig. 4c). This process allowed the rubber particle to be squeezed through the tonoplast split (Fig. 4d) until the entire particle was translocated (Fig. 4e, 4f). The schematic diagram of the whole process is presented in Figure 5. The formation of solid rubber in the hydroponically-grown roots was similar to that in soil-grown roots. Rubber particles increased in number and coalesced while the cytosol was still intact, but the shape of these particles differed between hydroponically- (Fig. 6a, 6b) and soil-grown roots (Fig. 3a). As the globular shaped rubber particles became denser, an even spread of irregular solid rubber masses developed in hydroponically-grown roots leaving fewer rubber-free areas in the laticifer cells (Fig. 6c) than in the soil-grown roots (Fig. 3d). Globular shaped particles continued to increase in number as the cytosol degraded (Fig. 6d, 6e). Following this degradation, the rubber particles coalesced and formed an irregular mass (Fig. 6f) not a single large particle. We also noticed that the thickening of the cell walls was less apparent in hydroponically-grown roots than in soil-grown ones.

Plant growth and rubber production

Fresh hydroponically-grown roots were of similar weight at 4 months old and 50 days later, but were significantly larger ($P<0.05$) than the roots of soil-grown plants (Fig. 7). Root growth may have been restricted by the NFT system because roots continued to grow in a hydroponic system with much larger volume available for root growth (Kopicky 2014). Inulin/root (total inulin) was lowest in soil-grown plants (Fig. 8) and significantly lower than in hydroponically-grown plants of similar age ($P<0.05$). Inulin/root declined from 4 months over the next 25 days with little change thereafter but remained higher than the soil-grown roots. This was matched by a significant decline in inulin concentration ($P<0.05$). Total latex and solid rubber (per root) among the hydroponic samples was not significant (Fig. 9). The mean amount of rubber appeared to be inversely correlated to the mean amount of inulin in roots of the hydroponically-grown plants ($r^2 = -0.869$, d.f.=1), and more rubber was produced in hydroponically-grown roots than in soil-grown roots ($P<0.05$). Rubber concentration was higher in soil-grown roots ($P<0.05$), and did not change over time during hydroponic cultivation (Fig. 10).

Discussion

Coagulation of rubber particles *in vivo* in *cis*-polyisoprene producing species is uncommon but has been previously reported in *Eucommia ulmoides*, a *trans*-polyisoprene producing tree (the Chinese rubber tree). This species produces *trans*-rubber particles which coagulate into solid *trans*-rubber in fibrous strands in the laticifers (Nakazawa[a] *et al*, 2009). The formation of solid *trans*-polyisoprene in *E. ulmoides* appears similar to the formation of solid *cis*-rubber in TK as the laticifer cells aged. *E. ulmoides* begins to form solid rubber after the granular-shaped *trans*-polyisoprene particles produced in the laticifer cytosol fill the inner space of the laticifer (Nakazawa[b] *et al*, 2009). Fibrous *trans*-polyisoprene strands and irregularly shaped fibrous masses are formed by the fusion of the granules. In contrast, in *H. brasiliensis*, although fusion of small (0.1 μm) rubber particles may contribute to the formation of large (1 μm) particles, these particles increase in number as laticifers mature but, in healthy trees, do not coalesce or fuse with each other

to create masses. Instead, as the particles pack densely in the laticifer cells they stack into polygonal shapes but maintain their individuality (de Faÿ *et al*, 1989). Similarly, densely packed particles in the vacuoles of mature *P. argentatum* cells do not coagulate, even though vacuolar pH is usually acidic (acid is used to coagulate latex into solid rubber). However, *H. brasiliensis* rubber particles have been observed to aggregate and coagulate into masses similar to those in TK and *E. ulmoides* in trees suffering from brown bast syndrome (Gomez *et al*, 1990) or more severe tapping panel dryness (TPD) (de Faÿ *et al*, 1989). It is known that the composition of the *H. brasiliensis* rubber particles changes as TPD progresses, including proteins involved in latex coagulation which likely affects particle stability (Krishnakumar *et al*, 2001).

In soil-grown TK (Figs 2 and 3) and *H. brasiliensis* (de Fay *et al*, 1989), the laticifer cell wall thickens as laticifers mature. However, before rubber particles are released between *H. brasiliensis* laticifer cells, the thickened cell walls between two adjacent laticifer cells thin by hydrolysis (de Fay *et al*, 1989) then break to release rubber from both sides. Most of these rubber particles retain their spherical shape and do not fuse with each other. However, in soil-grown TK, the thickened cell walls do not thin before perforation, and the rubber particles only cross between latificers after they have coalesced to form large masses in their original laticifer cell. During fusion, the rubber particle monolayer biological membranes (Cornish *et al.* 1999) surrounding the TK rubber particles dissipate, similar to what was reported in *E. ulmoides.*

However, the formation of single large rubber particles in the root laticifer univacuoles of hydroponically-grown rubber-producing plants has not been previously observed. These large particles persisted in the univacuole for at least 50 days after formation. Univacuolate cells contain one central vacuole surrounded by cytoplasm and in *P. argentatum* this is characteristic of a mature parenchyma cell (Goss, 1991). However, *P. argentum* packs many normal sized particles (with mean particle size of 1.4µm (Wood and Cornish, 2000)) into the vacuole without generating a single large particle (Backhaus and Walsh, 1983).

TK roots co-produce rubber and inulin, a polyfructose with a degree of polymerization around 20 (Ramirez-Cavidad *et al.*, 2017). Inulin is a major storage carbohydrate in TK roots (van Beilen and Poirier, 2007). In a study of *T. brevicorniculatum*, a close relative of *T. kok-saghyz* but which makes little rubber, transgenic plants with complete inhibition of rubber biosynthesis increased inulin content by up to 20% (Post *et al*, 2012), possibly due to diversion of excess assimilated carbon. It has previously been reported that carbon from catabolized inulin, during post-harvest cold storage of hydrated roots, can eventually lead to the production of additional rubber (Cornish *et al*., 2013). In the current hydroponic study, as inulin declined with age (Fig. 8), rubber did increase (Fig. 9) although the differences were not statistically significant. This is consistent with the hypothesis that the catabolizable sink (inulin) and the terminal carbon sink (rubber) are inter-related with respect to assimilate partitioning. The higher concentration of rubber in soil-grown roots is partly due to their slightly lower water content (84 % versus 87%) while the higher rubber per root in the hydroponically-grown roots is primarily due to the larger size of these roots. TK can be grown in soil or in hydroponics (Kopicky, 2014). The advent of vertical farming raises the possibility of TK rubber and inulin production in this manner. Such a crop would be weed- and soil-free and may allow multiple harvests of the same plants. In an analogous production system,

P. argentatum plants are often harvested multiple times by clipping or pollarding the crop, then re-growing more shoot biomass on the same stand (Foster and Coffelt, 2005) to reduce time to harvest and cost of crop establishment.

***Author contribution statement*:** MAAG planned the study, conducted experiments, analyzed and interpreted the images, interpreted the data and drafted the manuscript. KC contributed to conception of the work and data interpretation, and extensively edited and finally approved the manuscript for submission.

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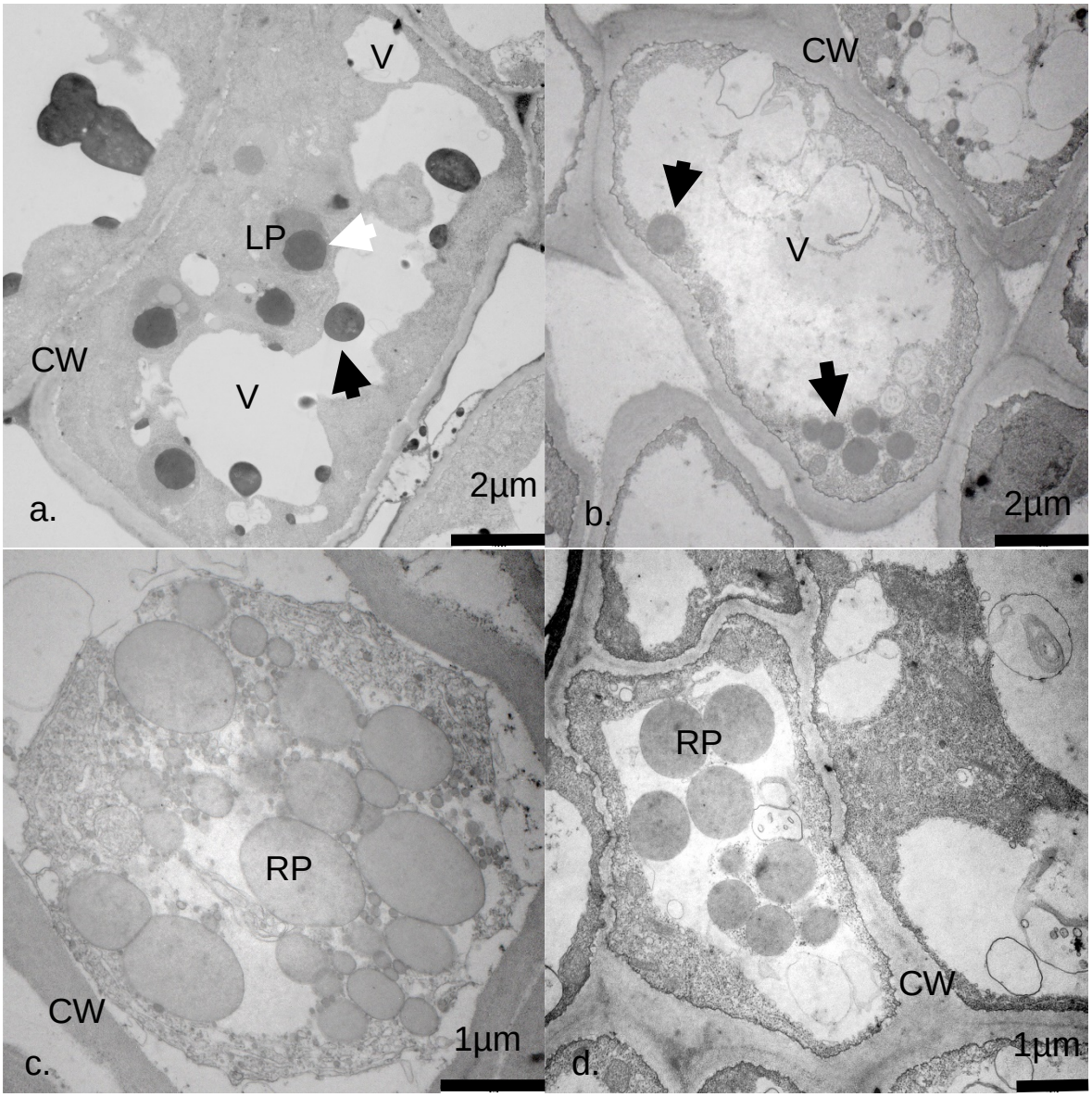

Fig.1 a Typical multivacuolate laticifer cell in a 12 month-old root from a soil-grown plant. **b,c,d** laticifer cell from a 6 month old hydroponically-grown plant with **b.** Univacuolate; white arrow indicates plastidic rubber; black arrows indicate cytoplasmic rubber. **c.** Large numbers of rubber particles present in a laticifer cell with intact cytosol **d.** large vacuole with rubber particles *Key: Cell wall (CW); laticifer plastid (LP); rubber particle (RP); vacuole (V)*

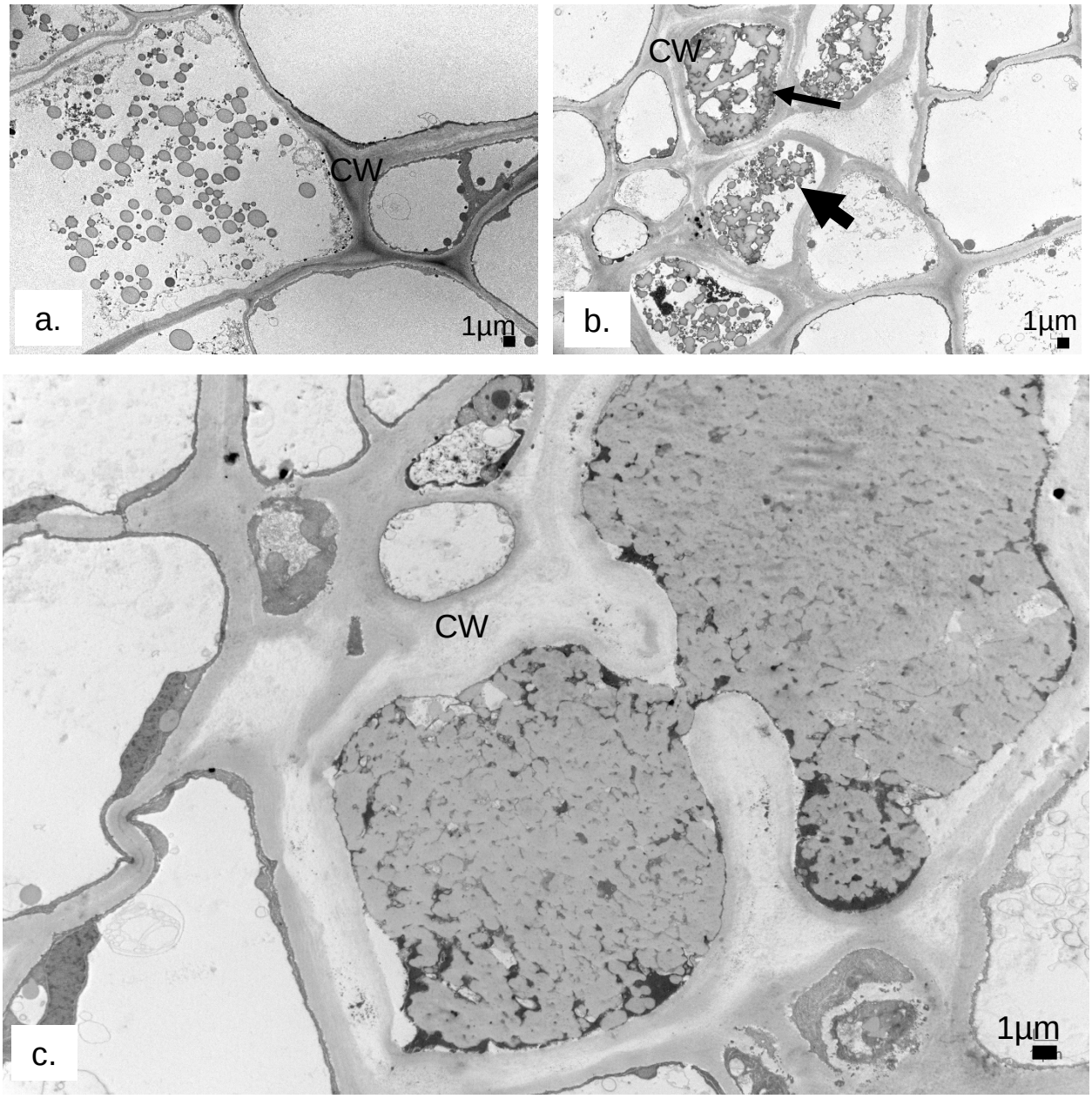

Fig. 2 Solid rubber formation from the globular shaped rubber particles after the degradation of the cytosol in 4 and 8 months old soil-grown roots: **a** Early stage of solid rubber formation after the cytosol degraded; **b** Degradation left only coalescing globular rubber particles with each other (big arrow) and later formed large irregular masses of solid rubber in the cell (small arrow); **c** Perforation and the anastomisation of laticifer cells allowed the solid rubber to cross into neighboring laticifer cells. *Key: Cell wall (CW)*

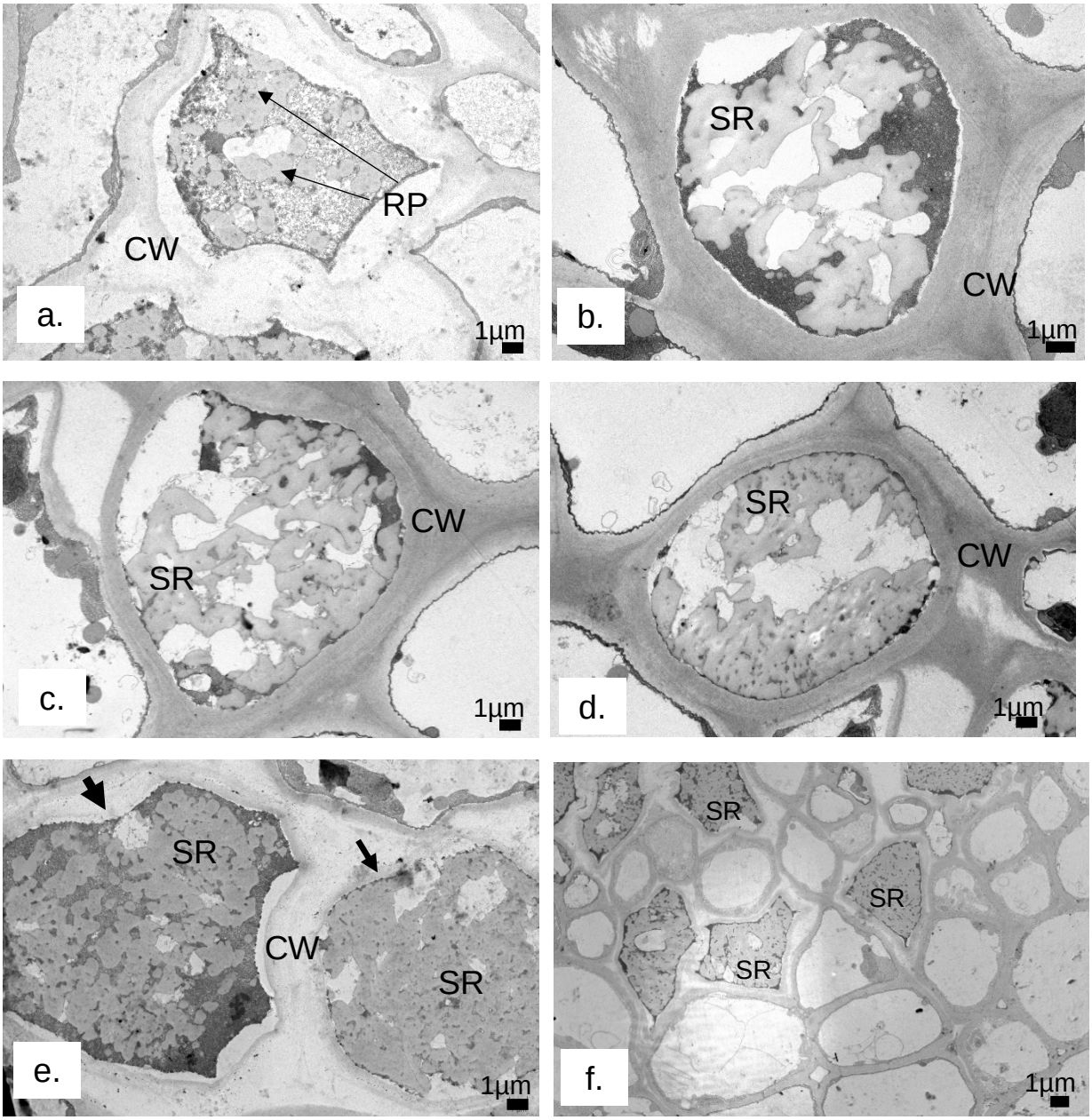

Fig. 3 Solid rubber formation in the presence of cytosol, in 4 and 8 months old soil-grown roots: **a** Early stage of solid rubber formation with irregular rubber formed in the cytosol; **b, c** Cytosol degradation; **d** Large irregular masses of solid rubber in the laticifer cell; **e** side by side comparison of solid rubber with (big arrow) and without cytosol (small arrow); **f** A lower magnification view of solid rubber formed in multiple laticifer cells. *Key: Cell wall (CW); rubber particle (RP); solid rubber (SR)*

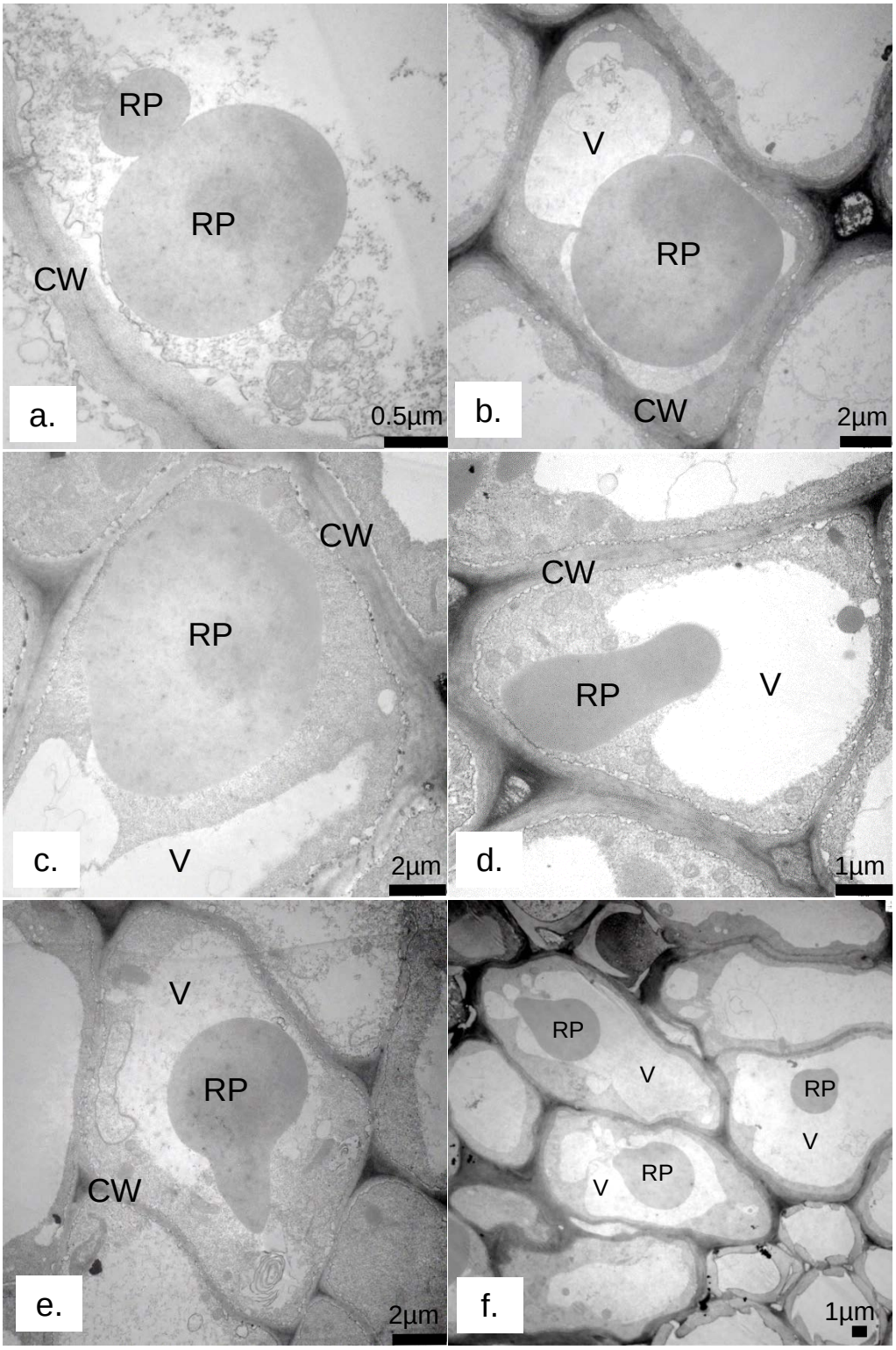

Fig. 4 Formation of a single large rubber particle: **a** Small rubber particles coalesce with one another; **b** A fully formed large rubber particle; **c** Invagination of the cytoplasm and the splitting of the tonoplast; **d** The first part of the rubber particle is pinched into the vacuole; **e** The rubber particle is ejected into the vacuole; **f** A lower magnification view of single large rubber particles in multiple cells in hydroponically-grown roots. *Key: Cell wall (CW); rubber particle (RP); vacuole (V)*

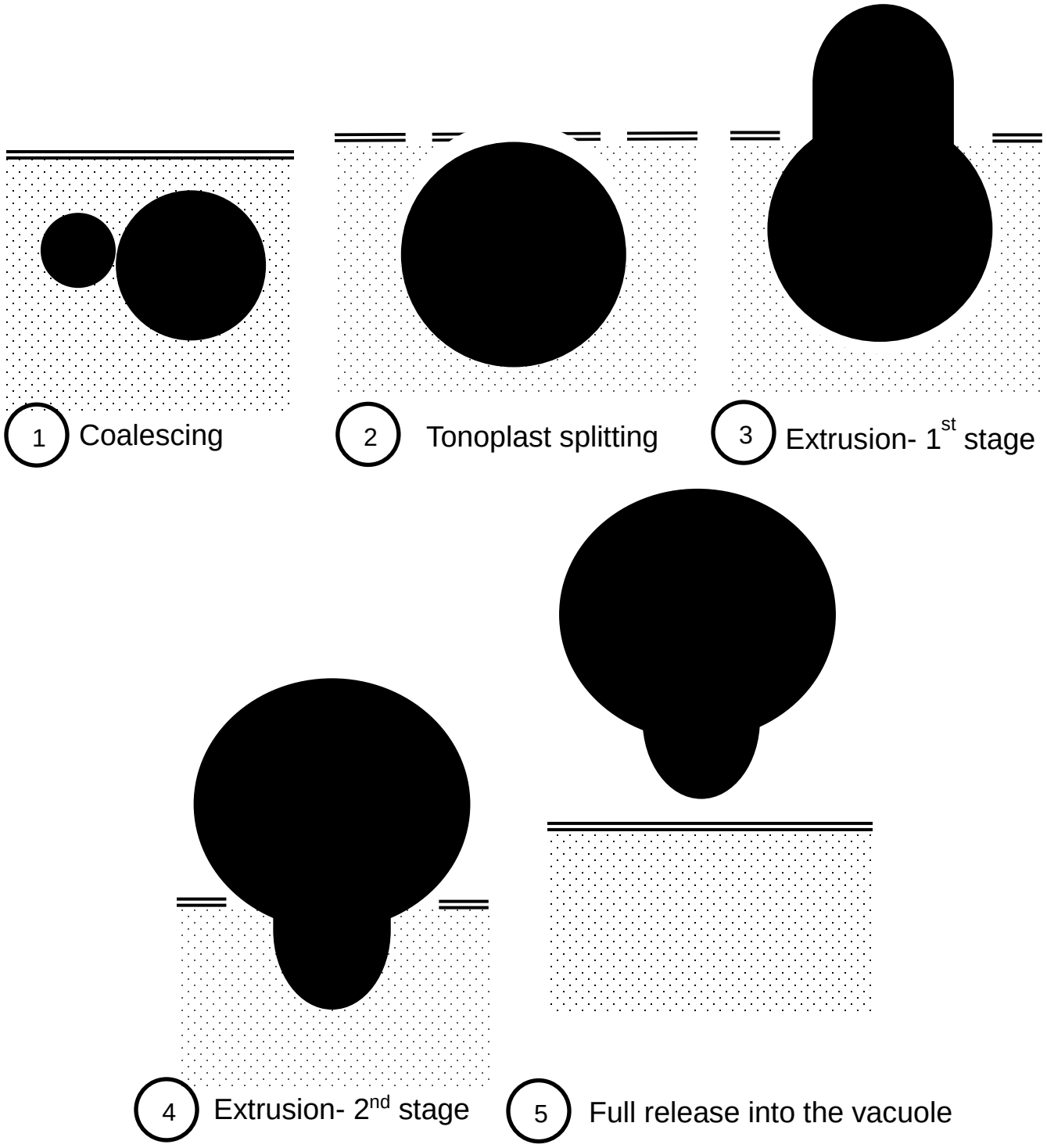

Fig. 5 Diagram of large rubber particle formation in a laticifer cell of a hydroponically-grown root.

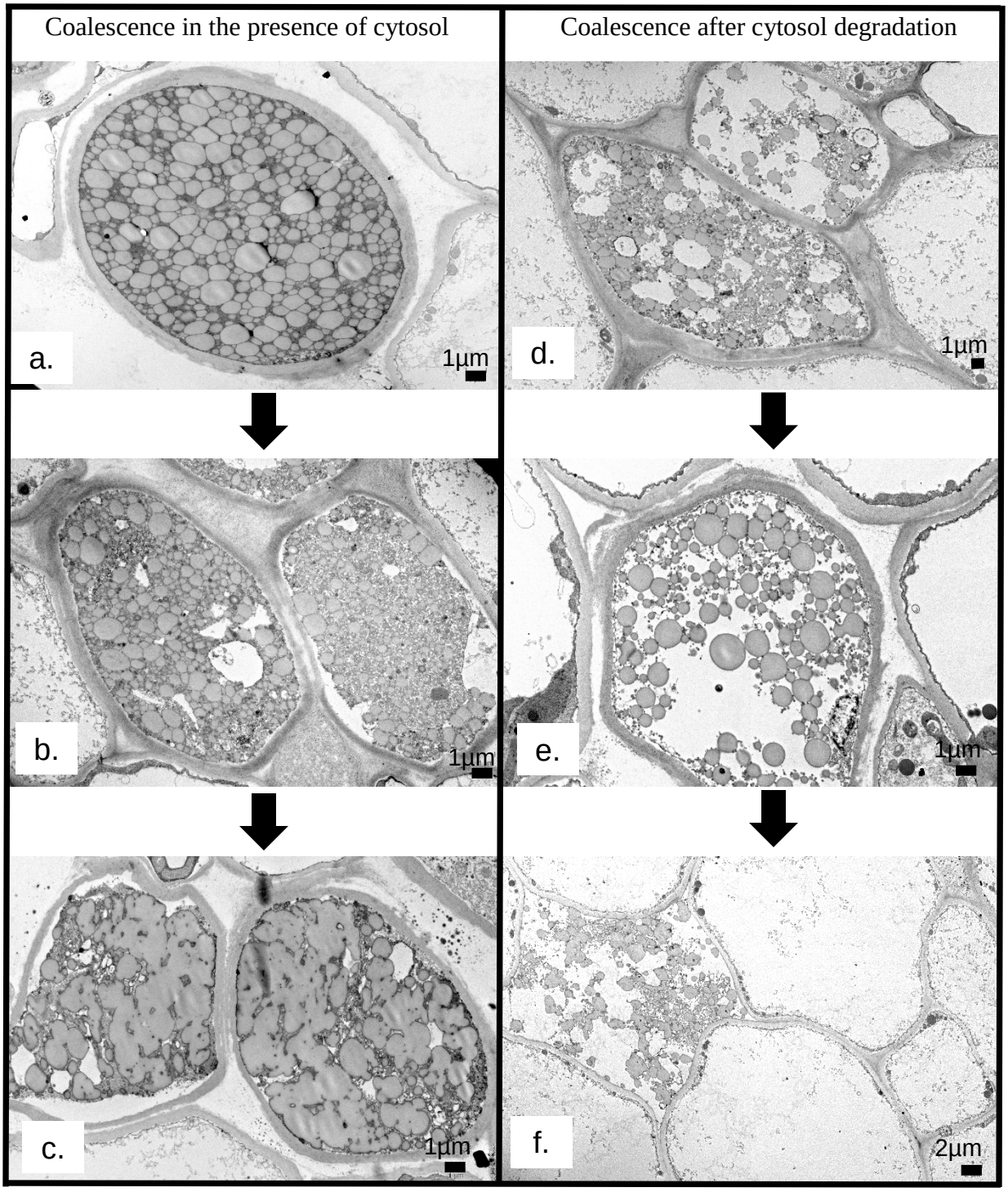

Fig 6. Solid rubber formation in the laticifer cells of hydroponically-grown roots **a,b,c** the formation of solid rubber with the cytosol present; **d,e,f** the formation of solid rubber after the cytosol degraded.

Coalescence in the presence of cytosol

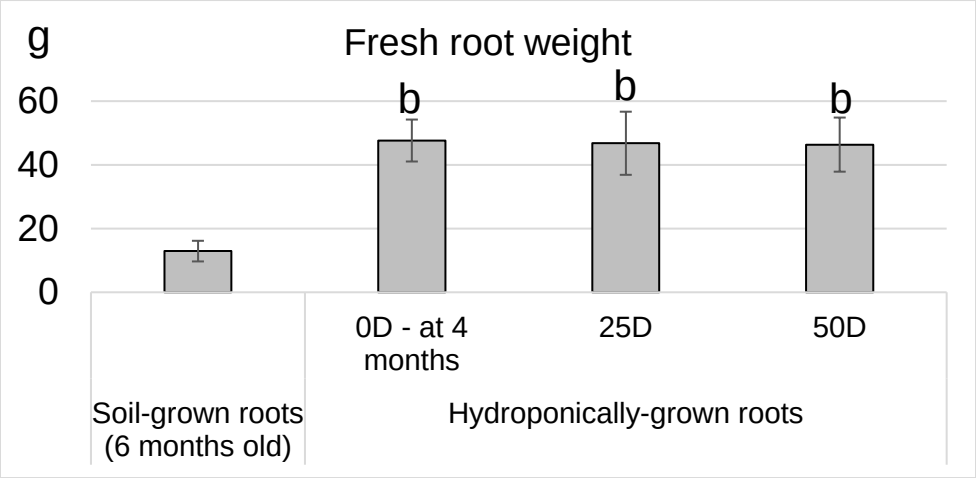

Figure 7. Mean fresh weight of roots of clonal plants produced from a single genotype (means of 12 ± se except for ambient after 25D with 6 ± se). Means with the same letter(s) are not significantly different at $p<0.05$ (LSD test).

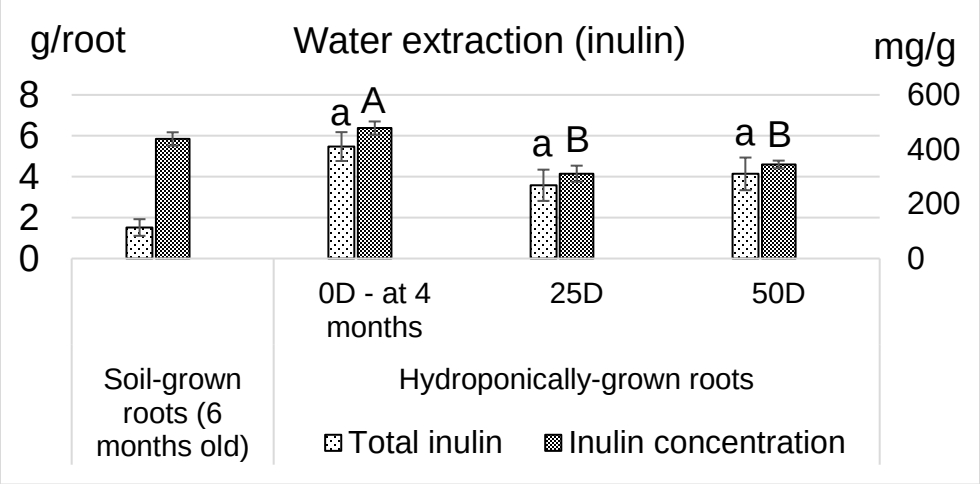

Figure 8. Total inulin (left axis) and inulin concentration (right axis) in roots of clonal plants produced from a single genotype (means of 12 ± se except for ambient/25D with 6 ± se). Means with the same letter(s) are not significantly different at $p<0.05$ (LSD test).

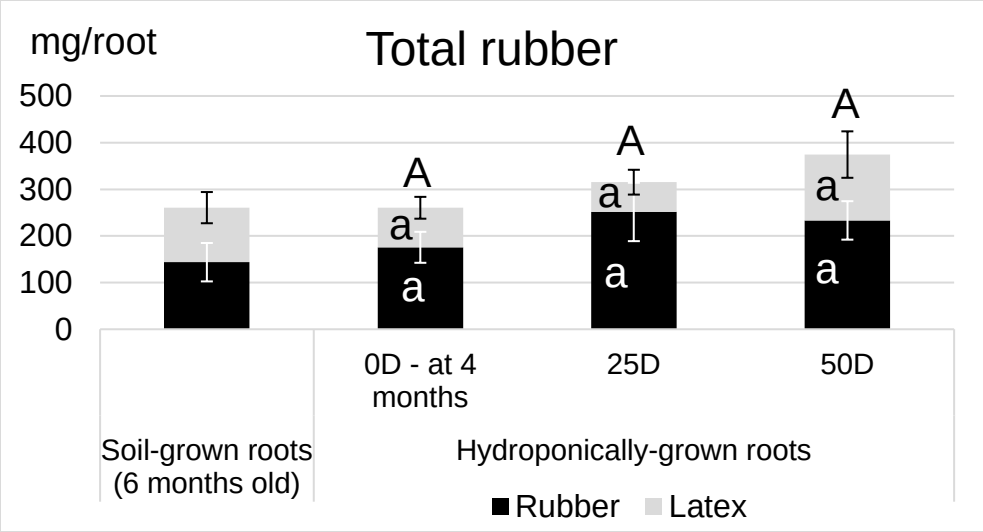

Figure 9. Total rubber (latex + solid rubber) in roots of clonal plants produced from a single genotype (means of 12 ± se except for ambient after 25D with 6 ± se). Means with the same letter(s) are not significantly different at $p<0.05$ (LSD test).

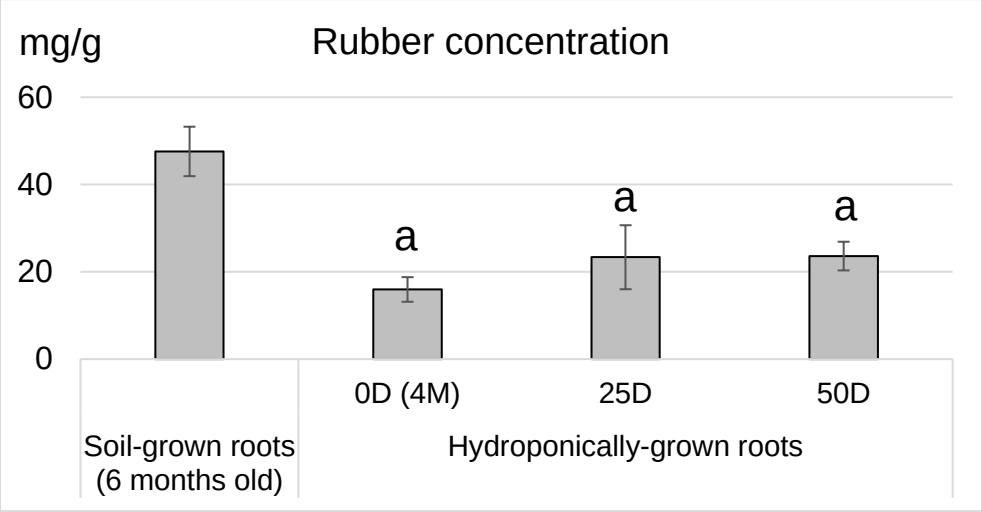

Figure 10. Rubber concentration in TK roots (means of 12 ± se except for ambient 25D with 6 ± se). Means with the same letter(s) are not significantly different at $p<0.05$ (LSD test).