The effectiveness of stilbenes in resistant *Vitaceae*: ultrastructural and biochemical events during *Plasmopara viticola* infection process

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Abstract

Leaves of different *Vitis vinifera* L. cultivars, susceptible or resistant to downy mildew, Chasselas, Solaris, IRAC 2091 (cvs. Gamaret x Bronner) and *Muscadinia rotundifolia* were inoculated with *Plasmopara viticola*. Samples were then examined by scanning and transmission electron microscopy, by light microscopy and for their ability to synthesise stilbenes. These phytoalexins were strictly analysed at infection sites. In the susceptible Chasselas, *P. viticola* colonises, at 72 hours post infection (hpi), all of the spongy mesophyll with functional haustoria and produces mainly the non toxic piceide. No necrotic zone was observed on Chasselas leaves. The ultrastructural response to downy mildew infection is different in each of the other three resistant grape cultivars. In Solaris, where leaf necrosis are rapidly induced, the infection is restricted to the upper part of the loose spongy mesophyll, and associated with a rapid cell wall disruption and the dispersion of cytoplasmic content along with the production of viniferins. In IRAC 2091, leaf necrosis are quite similar to those observed on Solaris but the infected plant cell, as well as the haustoria, show high electron dense cellular particles without any recognisable organelles, probably related to the effect of the toxic compound pterostilbene, which is synthesised in this grape cultivar. In *M. rotundifolia* leaf necrosis are much more scarce and smaller than in other cultivars, but pathogen and plant cells are both strongly affected, with concomitant expulsion of cytoplasmic materials through the stomata after *P. viticola* penetration. In this cultivar, the concentration of all identified stilbenes exceeds $1*10^3$ µmol mg^{-1} FW. The critical role of stilbenes in the resistance of *Vitis* spp. is discussed.

Keywords: downy mildew, grapevine, leaf, stilbenes, stomata, resistance, ultrastructure.

1. Introduction

Plasmopara viticola (Berk & Curt.) Berl. & de Toni, the biotrophic agent of downy mildew of *Vitaceae* and one of the major worldwide pathogens of vineyards [1], belongs to the Oomycetes and *Peronosporaceae* family. The disease is controlled using specific fungicides applied eight to ten times, depending on meteorological conditions [2]. Fungicides increase the costs of production and carry the risk of facilitating the emergence of resistant strains of *P. viticola* [3,4]. Therefore, breeding programs were initiated in different countries to develop grapevine genotypes resistant to downy mildew [5,6].

Most of the cultivated grapevine varieties (*Vitis vinifera* L.) are susceptible to *P. viticola,* while resistance to the pathogen is found in some other *Vitis* species [7]. The mechanisms of resistance in *Vitis* are complex and involve constitutive substances that act as fungicide-like molecules [8]. Grapevines can produce a variety of factors in response to infection by *P. viticola*, including callose synthesised in stomata [9], induced peroxidases [10], and factors involved in lignification processes [11]. However, stilbenic phytoalexins production is one of the most important responses to fungal infections in grapevine [12-16]. Different stilbenes produced after either biotic or abiotic stresses have been characterised [14], such as ε- and δ-viniferin, which are resveratrol dimers synthesised via peroxidase activity [17]. Stilbene synthase genes from different grape (*Vitis vinifera*) cultivars have been cloned [18].

Previous works include qualitative and quantitative studies of stilbene production [6,11-13,17,19], and histological observations [7,9,20-22] of grapevine leaves after inoculation with *P. viticola*. Apart from these works, no data on the relationship between the effect of stilbenes both on plant cells and on the pathogen has been reported yet.

The aim of this work is to identify the link between the infection process at the cellular level and the synthesis and accumulation of stilbenic phytoalexins in both the pathogen and the host in three different *Vitis* genotypes, and in *Muscadinia rotundifolia*, which is considered to be totally resistant to *P. viticola* infections [23].

2. Results

2.1. Leaf anatomy

The cellular organisation of the leaves of the four studied cultivars was observed by light and transmission electron microscopy (Fig. 1). The limb of a non-inoculated leaf from any of the cultivars presents an adaxial epidermis, without stomata, formed by elongated cells and coated on the outer side with a cuticle. Beneath this epidermis lies the palisade mesophyll, made up of tubular, tightly packed cells, normally arranged in one layer. These cells contain many peripheral chloroplasts, particularly in *Muscadinia* (Fig. 1K and L), showing a high electron-dense content. Large starch granules are present in IRAC 2091 and *Muscadinia* (Figs. 1I and 1L). Immediately beneath the palisade mesophyll is the spongy mesophyll, noticeably thicker in Chasselas than in the other three cultivars studied (Fig. 1A). The spongy mesophyll consists of 4-5 cell layers of irregular cells that contain fewer chloroplasts than palisade mesophyll cells contain. It also includes large intercellular air spaces connected with the substomatal cavity in the abaxial epidermis. The abaxial epidermis is a one-cell-thick layer like the adaxial epidermis, but containing more sinuous cells.

Ultrastructural observations were done on the stomatal apertures (Fig. 2). In all cases, the stomata present a central pore surrounded by two guard cells. This pore has an outer cuticular rim that is a protrusion of the abaxial cuticle over the stomata and is continuous with the cuticle. An internal cuticular rim, at the neck region of the substomatal cavity, is only present in *M. rotundifolia* (Fig. 2D).

2.2. Ultrastructure of the infection process

The development of the pathogen was followed between 24 and 72 hours post-infection (hpi) by scanning and transmission electron microscopy (Fig. 3-6). At 24 hpi, it was possible to observe in all cultivars the encystment of the released zoospores on the stomata (Fig. 3A, 4A, 5A, 6A). In cross sections, germinating zoospores produce a penetrating germ tube reaching the substomatal cavity, accompanied by the differentiation of the infective vesicle in Chasselas only (Fig. 3B-D). In Solaris and IRAC 2091, the presence of atypical vesicles can be observed (Fig. 4C-D, 5E), while no vesicles are present in *M. rotundifolia* (Fig. 6B-C). In Chasselas, from 24 hpi, the hyphal development reaches the whole spongy mesophyll, with the formation of numerous haustoria (Fig. 3E-H), and the accumulation of electron-dense material around the neck of penetration in host cells (Fig. 3H). In the other three

cultivars, the infection process is quite different. In Solaris, development of downy mildew is restricted to the upper part of the spongy mesophyll, contains few haustoria. A rapid mycelia cell destruction (Fig. 4E) with the concomitant expulsion of cell content (Fig. 4G) can be observed, particularly around the haustorium (Fig. 4E-F). In IRAC 2091, parasite development is also restricted to the first cell layer of the spongy mesophyll (Fig. 5C-E). The initiation of haustoria formation and cell infection takes place 24 hpi (Fig. 5C-D). However, contrary to Solaris, no cellular deconstruction is observed, but the infected plant cell as well as the haustorium are considerably modified, showing high electron dense cytoplasm, without any recognisable organelles (Fig. 5F-H). In *M. rotundifolia*, just after penetration, the downy mildew and the plant cell strtuctures are destroyed and extrusion of cytoplasmic material through the stomata occurs (Fig. 6D-H).

2.3. Stilbenes quantification and sporangia density

Six days after infection, an average of 90 sporangia per mm^2 was spectrophotometrically measured on Chasselas (Fig. 7B), whereas no sporulation occurred on the other three cultivars. Solaris and IRAC 2091 present characteristic necrotic areas from within the infection droplets with a mean surface of 20 mm^2 (Figs. 7D and 7F). In *Muscadinia*, the necrotic areas are much more delimited, and consist in a lot of small necrotic spots (mean of 35 per 20 mm^2, with an average surface area of 0.0028 mm^2) under the infection droplets surface (Fig. 7H).

Stilbene production was quantified in infected grape leaves between 24 and 72 hpi. In Chasselas, a rapid increase in the amount of piceide in the leaf occurs during the infection process, reaching a value of 245 μmol mg^{-1} FW (Fig. 7A). In Solaris, resveratrol and especially δ- and ϵ-viniferins are induced during pathogenesis showing at 72 hpi values around 57, 73 and 322 μmol mg^{-1} FW, respectively (Fig. 7C). In IRAC 2091, at 48 hpi, pterostilbene is the major induced stilbene, reaching 118 μmol mg^{-1} FW at 72 hpi (Fig. 7E). In *Muscadinia*, a large amount of stilbenes are already induced 24 hpi (Fig. 7G). Comparison with the other grape cultivars indicate that at 72 hpi, *Muscadinia* produces 42 times more piceide than Chasselas ($1.04 * 10^4$ μmol mg^{-1} FW), 32 and 22 times more ϵ- and δ-viniferins, respectively than Solaris, ($1.04 * 10^4$ and $1.6 * 10^3$ μmol mg^{-1} FW, respectively), and 80 times more pterostilbene than IRAC 2091 ($9.4 * 10^3$ μmol mg^{-1} FW). Non-infected controls do not show any induction of stilbenes.

3. Discussion

In this study, ultrastructural investigations have shown that *P. viticola* cannot complete its life cycle on the resistant cultivars Solaris and IRAC 2091, even if some infective haustoria are initially formed. In Chasselas, 72 hours post infection, downy mildew has colonised all of the spongy mesophyll, has reached even the palisade mesophyll, and contains numerous fully developed haustoria. Number of infective encysted zoospores are different within the same cultivar as well as within cultivars. Consequently, the number of zoospores presented in this work is not significant of the resistance level. Chasselas shows the characteristic pattern of susceptible cultivars to downy mildew, with the production and accumulation of the non-toxic piceide compound during the infection process [17] while the cellular integrity is maintained. In Solaris, the content of the fungal cell structures (germ tube, atypical vesicles) is rapidly modified. In this cultivar, at 72 hpi, the ultrastructure of haustoria cell cytoplasm is disorganised, as well as in the infected plant cell. Moreover, there is an outflow of cytoplasmic matter of the infected plant cell reaching the stomatal apertures. Previous work [9] has shown that Solaris, in response to downy mildew infection, can secrete callose in the form of deposits in and around the infection site and stomatal openings. The present results suggest that those deposits are cellular debris with a high polysaccharide content. However, the ultrastructural destructuration of downy mildew cells could be due to the toxic activity of δ-viniferin, as it is present at a concentration five times its ED_{50} [15]. Before and/or in parallel to the induction of phytoalexin accumulation, production of large amounts of ROS and other reactive compounds [10] could lead to rapid cell destruction and expulsion of their content. In IRAC 2091, there is also a rapid disorganisation of cell membranes and organelles of both fungal and infected plant cells, but no cell debris were observed. This disorganisation could be due to the toxic effect of pterostilbene on cells of both the host plant and the pathogen [24]. These authors have shown that low concentrations of pterostilbene are able to coagulate cytoplasmic material and disorganise organelles and cellular membranes in less than 30 min. In the case of IRAC 2091, the concentration of pterostilbene at 72 hpi is effectively ten times more than its ED_{50} [15], which explains the observed cellular disorganisation and the total inhibition of downy mildew development.

In *Muscadinia rotundifolia*, the infection process is stopped before the development of either vesicles or infective structures in the spongy mesophyll, confirming previous results [11]. According to Boubals [25], highly resistant plants can rapidly accumulate flavonoids (polyphenolic compounds, such as catechins) in the stomatal tissues and in the cell walls around the stomata. The present work show a rapid accumulation of stilbenes, in considerable amount. All stilbenes are not equally toxic to *P. viticola*, as shown previously [17]. δ-viniferin and pterostilbene are considered to be the most toxic against downy mildew, but usually pterostilbene is absent or at too low concentration to have a significant effect. In *Muscadinia* at 24 hours after infection, δ-viniferin and pterostilbene are present at levels 24 and 42 times higher than their respective ED_{50}'s [15]. Therefore the most important step in the total inhibition of disease in *Muscadinia* may be the rapid induction, upon infection, of metabolic responses, before any haustorium can appear. Other work dealing with nonhost responses to *P. viticola* has shown defence responses in resistant grapevines that are triggered only upon the appearance of functional haustoria [22]. However, the synthesis of stilbenes, which is a typical hypersensitive response (host resistance) following the establishment of functional infective structures [7], is one of the effective defence mechanisms of *Muscadinia* against downy mildew. The presence of this mechanism in *Muscadinia* could signify that haustorium formation is not the only key stage for the establishment of biotrophy, and consequently for specificity and host recognition.

Resistance mechanisms involved in the inhibition of *P. viticola* disease development are not completely understood. Furthermore, many studies have focused on induced resistance traits, either on molecular [18,26-28] or on biochemical events [9,11,17]. It is interesting to consider constitutive elements to be part of the innate resistance of grapevine to downy mildew, as these elements are still considered to be in other studies [20]. Ultrastructural observations of non-infected leaves show that only *Muscadinia* has an inner cuticular rim that is supposed to play a role during the infection process. This inner cuticular rim could be involved in impairing or delaying the development of the germ tube, as previously shown by Jürges et al. [29] on *Vitis* species from North America, Asia and Europe. Semi-thin sections show that the spongy mesophyll of Solaris and IRAC 2091 is looser, with bigger intercellular spaces, than that of Chasselas. An implication of this structural difference is that the hypha of *P. viticola* would have to cover a longer distance to target the cells and develop infectious structures in Solaris and IRAC 2091 than in Chasselas. In *Muscadinia*, cells of the palisade and

spongy mesophyll contain important electron-dense deposits, which are probably phenolic accumulations [30]. These could represent highly toxic compounds, like oligomeric and polymeric tannins, which are readily excreted and play an important role in the inhibition of some enzymatic activities, as shown in *Botrytis cinerea* [31,32].

The present work shows that resistant grapevine cultivars react rapidly to *P. viticola* infections by producing high concentrations of stilbenes at the site of infection. For Solaris and IRAC 2091, this synthesis occurs on the entire surface of inoculation. Each of these two cultivars preferentially synthesises one of the two most toxic stilbenes against *P. viticola*, viniferins in Solaris and pterostilbene in IRAC 2091. *Muscadinia*, which is totally resistant to *P. viticola* [23], accumulates large concentrations of all stilbenes, localised at the exact sites of infection. These results confirm the critical role and effectiveness of stilbenic phytoalexins in grapevine resistant to downy mildew.

4. Material and methods

4.1. Plant material and culture

Grafted plants of two *V. vinifera* L. cultivars, the susceptible Chasselas (old cultivar) and the resistant Solaris [Merzling x (Saperavi severneyi x Muscat Ottonel)], as well as IRAC 2091 (Gamaret × Bronner) obtained at Agroscope ACW, and *Muscadinia rotundifolia* cv. Carlos (kindly provided by Professor Alain Bouquet, France) were cultivated in the greenhouse as described in Pezet et al. [17]. At the 10-developed-leaf-stage, the 4th-5th leaves from the top were detached and placed in humid chambers. *Plasmopara viticola* was grown on leaves of rooted cuttings of cv. Chasselas in pots growing in climate chambers, according to Gindro et al. [9]. Sporangia were collected by vacuum aspiration from sporulating lesions and suspended in a plastic tube (Falcon; 50 ml), each tube containing 30 ml of distilled water (concentration 4×10^5 sporangia ml^{-1}). They were then slowly stirred at room temperature. When the release of zoospores had begun, 100 droplets (20 µl, each covering 20 mm^2 of leaf surface) of the suspension were deposited on the abaxial leaf surface of three leaves per cultivar, in humid chambers. Control consisted of leaves of each cultivar treated with distilled water only.

4.2. Scanning electron microscopy

At 24, 48 and 72 h post-infection (hpi), three pieces of leaf from within the droplet surface were cut from each inoculated and each control leaf. Leaf samples were fixed with osmium tetroxide vapours (aqueous solution of 2% OsO_4 [w/v] and 3% CrO_3 [w/v]) for 4 h at room temperature in a humid chamber. They were then dehydrated in a graded series of acetone solutions of 10-30-50-70-90-100-100% (v/v) (20 min on ice each step), dried in CO_2 according to the critical point drying method (Critical Point Dryer, Baltech) and coated with platinum. The samples were observed with a scanning electron microscope at 5 KV (Jeol JSM-6300 F).

4.3. Transmission electron microscopy

At 24 and 72 hpi three pieces of leaf from within the droplet surface were cut from each inoculated and each control leaf. The leaf samples were prepared according to Roland and Vian [33], prefixed with a solution of 3% glutaraldehyde-2% paraformaldehyde in 0.07 M pH 7 phosphate buffer, embedded in 2% agarose and postfixed with a solution of 1% OsO_4. They were then dehydrated in a graded series

of ethanol solutions of 30-50-70-95-100% (v/v) and embedded in LR White resin (14381-UC London Resin Company). After polymerisation (24 h at 60 °C), semi-thin (0.8 μm) and thin (0.08 μm) sections were cut and stained with a solution of 1% methylene blue, sodium tetraborate and azure II for the semi-thin sections, and 2% uranyl acetate followed by lead citrate according to Reynolds [34] for the thin sections. Semi-thin sections were observed using a light microscope (Leica DMLB) equipped with a Leica DFC 490 FX camera. Thin sections were observed with a transmission electron microscope (Philips CM10) with a Mega View II camera.

4.4. Sporangia density

Five leaf disks (diameter 1 cm) were excised from each cultivar, placed in humid chambers at room temperature and inoculated by spraying 1 ml of sporangia suspension (see above) on each five leaf disks. These leaf disks were used to determine sporangial density. To create control samples, two additional leaf disks were sprayed with sterile distilled water. Six days after inoculation, the sporulation density was measured by turbidimetry, using a spectrophotometer at 400 nm, according to Gindro and Pezet [35]. For this test, each leaf disk was put in a 1.5 ml plastic tube containing 1 ml of distilled water and shaken for 1 min. The resulting sporangial suspension was collected and used for measurements. The controls were treated in the same way and used for calibration. The results are expressed in units of sporangia per mm^2. The experiment was done in triplicate.

4.5. Stilbene analysis

At 24, 48 and 72 h post-infection (hpi), three pieces of leaf from within the droplet surface or to the developing necrotic zone were cut, using a micro scalpel, from each inoculated and each control leaf under a stereo microscope (Leica MS5). Three replicates were made for each sample time and each cultivar. Each leaf sample was weighed and placed in a microfuge tube (1.5 ml) with 50 μl of MeOH. The tightly closed tubes were placed in a thermo regulated shaker at 60°C for 10 min and then placed in an ice bath for 5 min. The methanol extracts (30 μl) were analysed for stilbenes as described by Pezet et al. [14]. Results are expressed as mean of 3 replicates in μmol mg^{-1} fresh weight (FW).

Acknowledgements

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Figure captions

Fig. 1: Cellular organisation of the uninfected leaves of the four studied cultivars, observed by light and transmission electron microscopy. A-C: *V. vinifera* cv. Chasselas; D-F: *V. vinifera* cv. Solaris; G-I: IRAC 2091; J-L: *Muscadinia rotundifolia*. Abe: abaxial epidermis, Ade: adaxial epidermis, chl: chloroplast, is: intracellular spaces, pm: palisade mesophyll, sm: spongy mesophyll, s: stomata, st: starch granule. Arrows:electron dense material.

Fig. 2: Stomatal ultrastructure on control leaves of the four grape cultivars. A: *V. vinifera* cv. Chasselas; B: *V. vinifera* cv. Solaris; C: IRAC 2091; D: *Muscadinia rotundifolia*. gc: guard cells, ir: inner cuticular rim, or: outer cuticular rim, s: stomata.

Fig. 3: Infection process of *Plasmopara viticola* on *Vitis vinifera* cv. Chasselas (susceptible) between 24 and 72 hpi by scanning and transmission electron microscopy. A: zoospores encystment; B-D: germ tube penetration and substomatal vesicle formation; E-F: hyphal development of downy mildew in the spongy mesophyll; G-H: haustoria formation in adjacent cells. gt: germ tube, h: haustorium, m: downy mildew, s: stomata, v: substomatal vesicle, z: encysted zoospore.

Fig. 4: Infection process of *Plasmopara viticola* on *Vitis vinifera* cv. Solaris (resistant) between 24 and 72 hpi by scanning and transmission electron microscopy. A: zoospores encystment; B-D: germ tube penetration and atypical substomatal vesicle formation; E-G: cellular destruction and expulsion of cell content (arrows) particularly around the haustorium; gt: germ tube, h: haustorium, m: downy mildew, s: stomata, v: substomatal vesicle, z: encysted zoospore.

Fig. 5: Infection process of *Plasmopara viticola* on IRAC 2091 (resistant) between 24 and 72 hpi by scanning and transmission electron microscopy. A: zoospores encystment; B: germ tube penetration and atypical substomatal vesicle formation; C-D: initiation of haustoria formation and cell infection; E-H: electron dense cytoplasm linked to cell disorganisation. gt: germ tube, h: haustorium, m: downy mildew, s: stomata, v: substomatal vesicle, z: encysted zoospore.

Fig. 6: Infection process of *Plasmopara viticola* on *Muscadinia rotundifolia* (very rersistant) between 24 and 72 hpi by scanning and transmission electron microscopy. A: zoospores encystment; B-C: germ tube penetration; D-H: deconstruction of the plant cellular content and extrusion of cell elements (arrows). gt: germ tube, m: downy mildew, s: stomata, z: encysted zoospore.
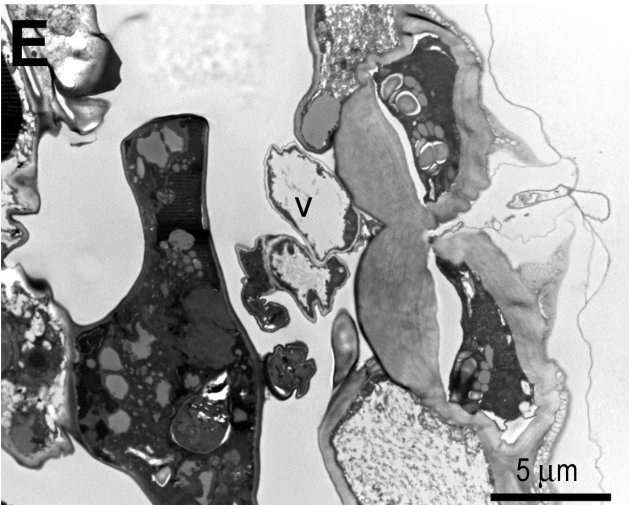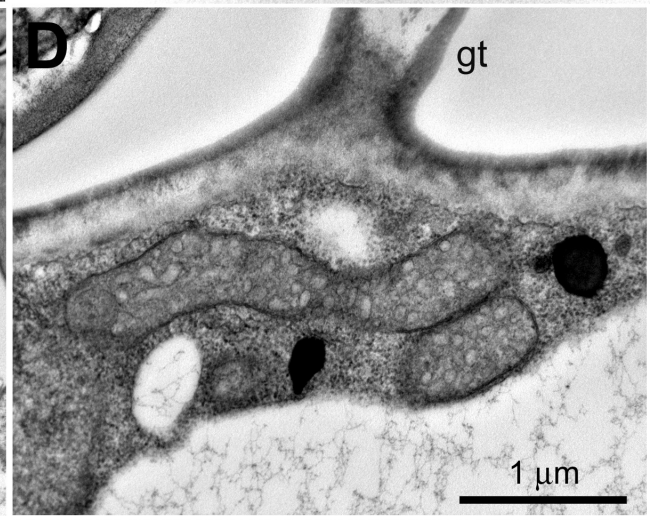
Fig. 7: Infection pattern (6 days post inoculation) and stilbene production 24, 48 and 72 hpi of four grape varieties infected with *Plasmopara viticola*. A-C-E-G: stilbene quantification in Chasselas, Solaris, IRAC 2091 and *Muscadinia rotundifolia*, respectively. B-D-F-H: infection pattern of Chasselas, Solaris, IRAC 2091 and *M. rotundifolia*, respectively. Scale bar=1 mm. The X-axis represents the time course of infection, the Y-axis represents the quantity of stilbenes expressed as mean of three replicates in µmol mg^{-1} FW and include standard error.

The labels visible in the figure panels:

A: Abe, s, sm, is, pm, Ade, 10 μm

B: chl, 10 μm

C: 0.5 μm

D: s, Abe, sm, is, pm, Ade, 10 μm

E: chl, 10 μm

F: 0.5 μm

G: s, Abe, is, sm, pm, Ade, 10 μm

H: chl, 10 μm

I: chl, st, 0.5 μm

J: Abe, s, is, sm, pm, Ade, 10 μm
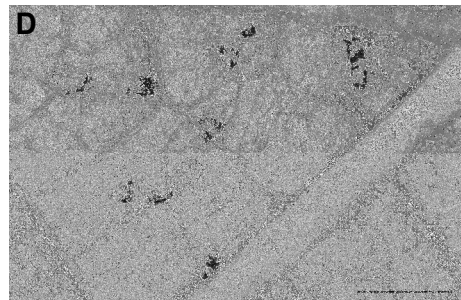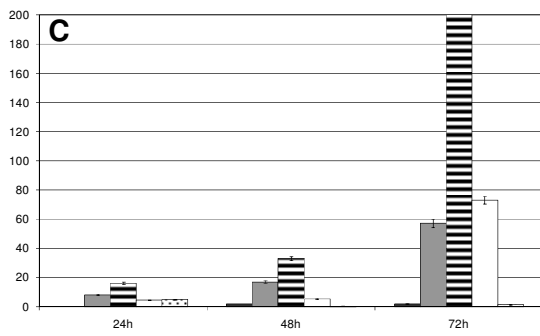
K: chl, 10 μm
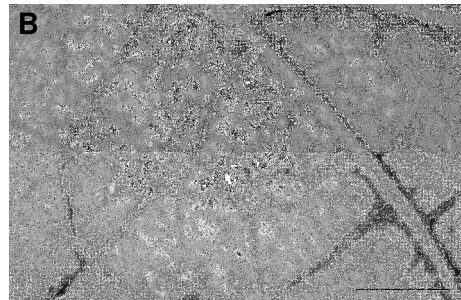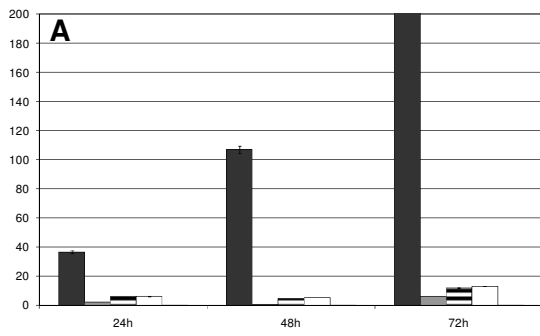
L: chl, st, 0.5 μm
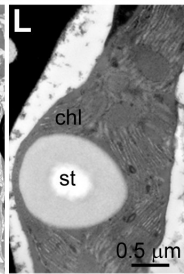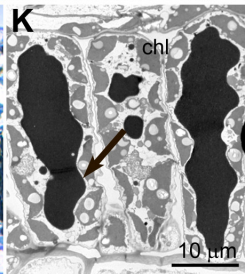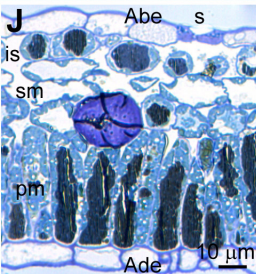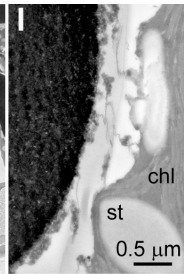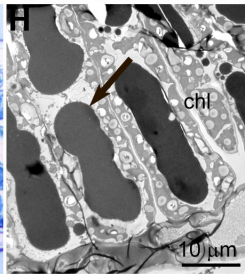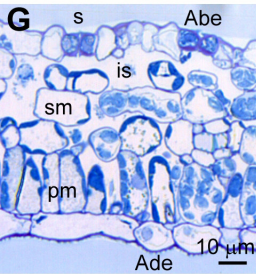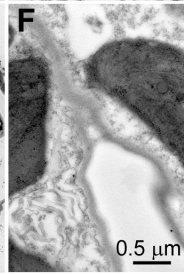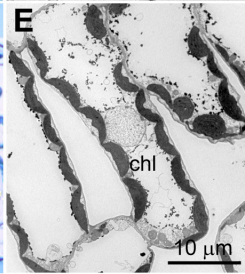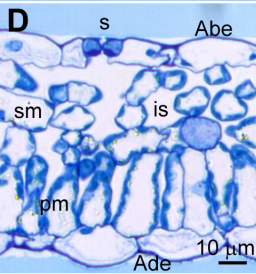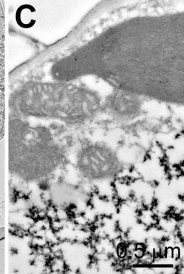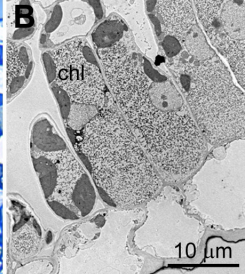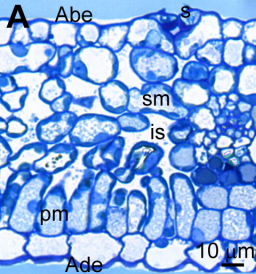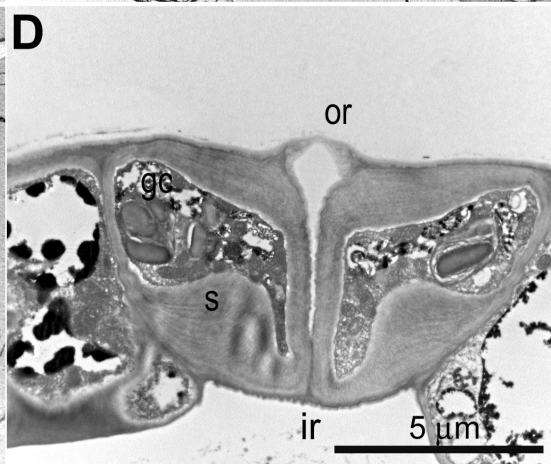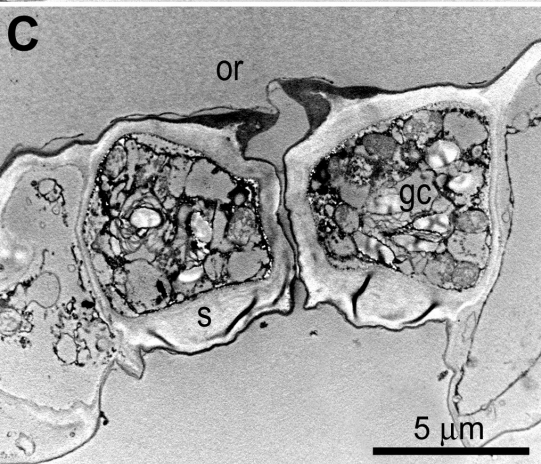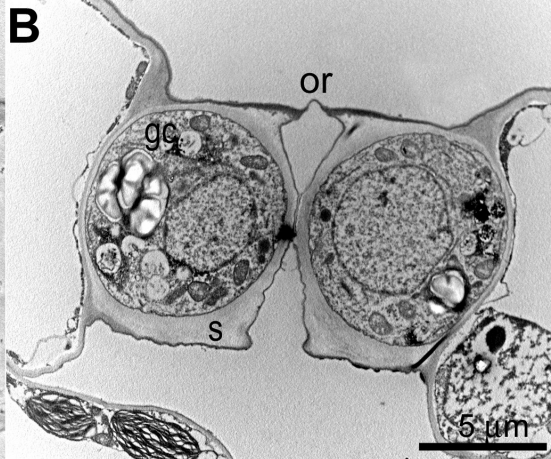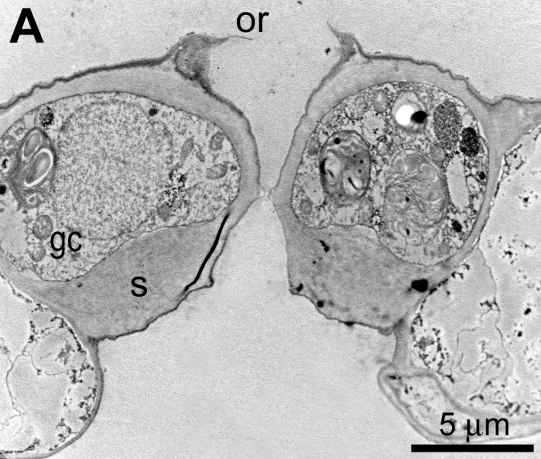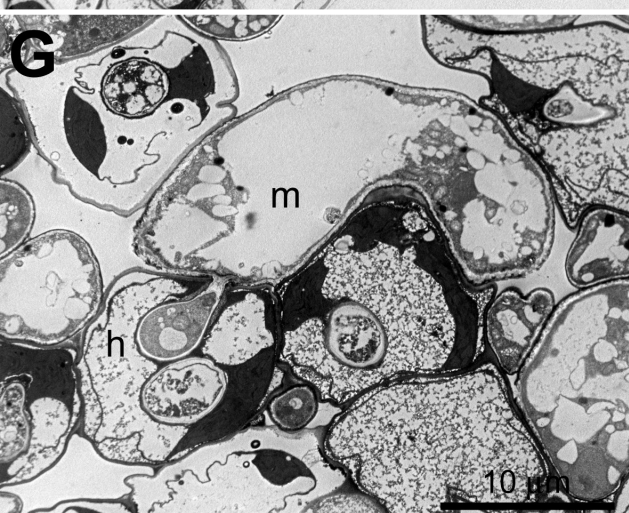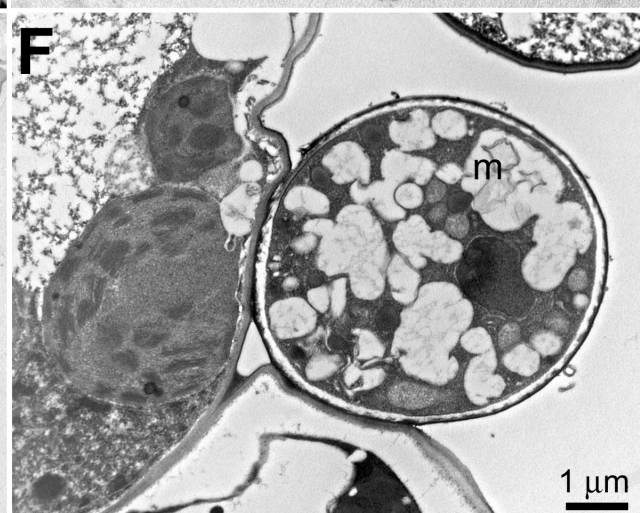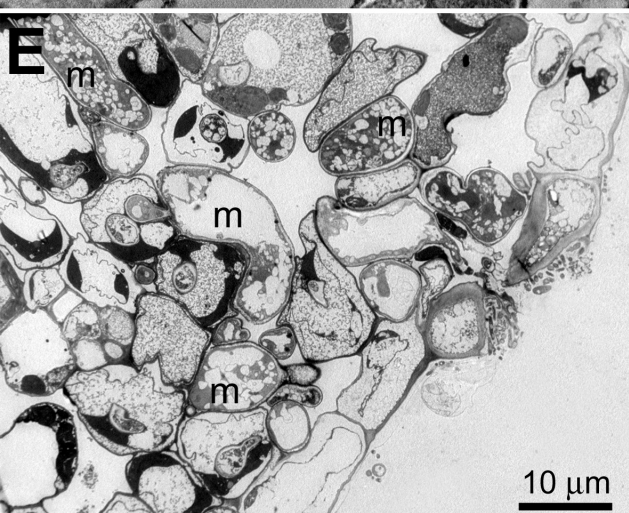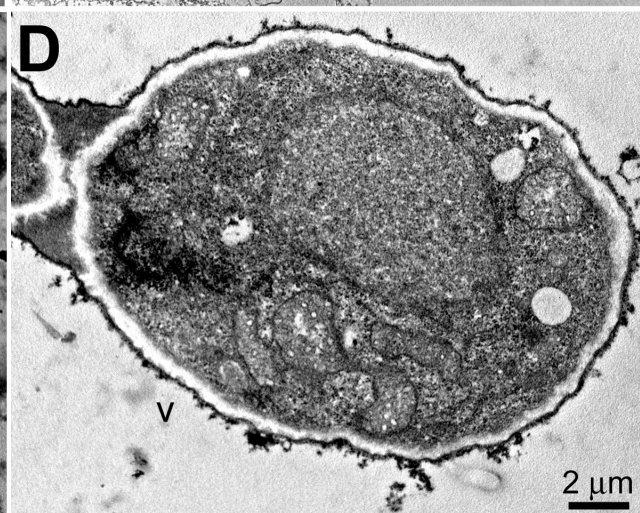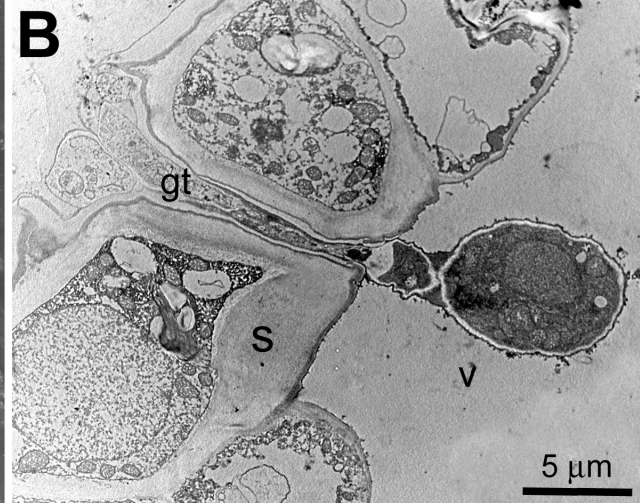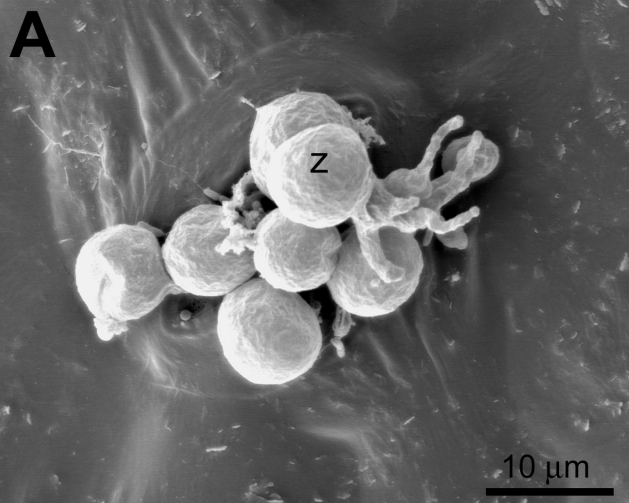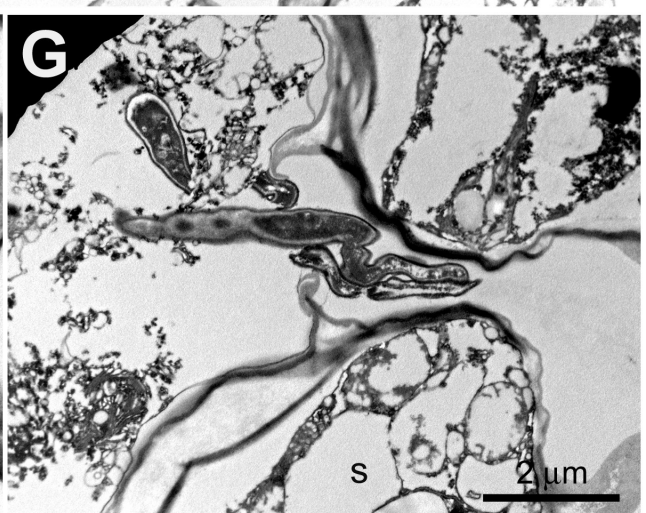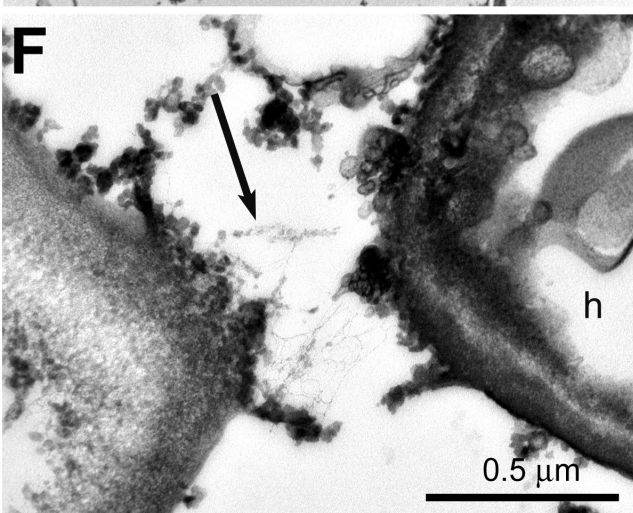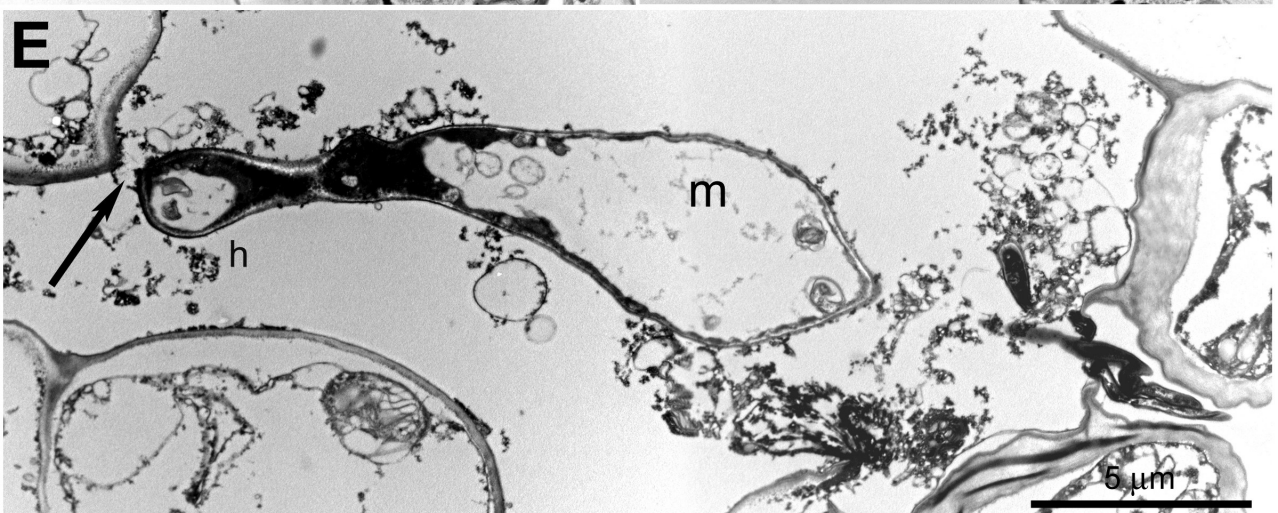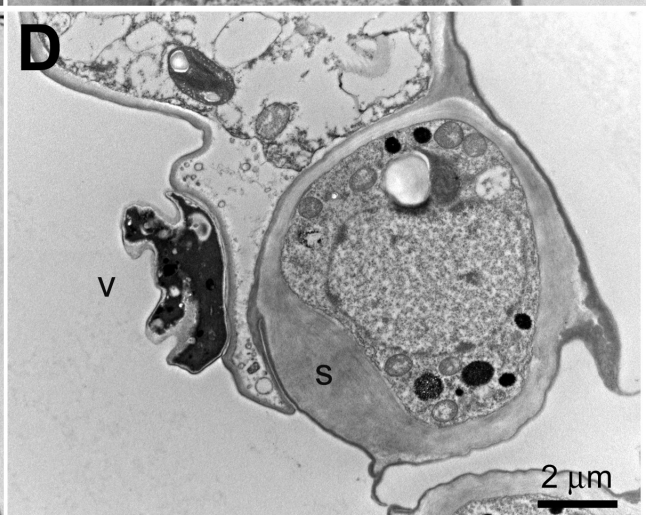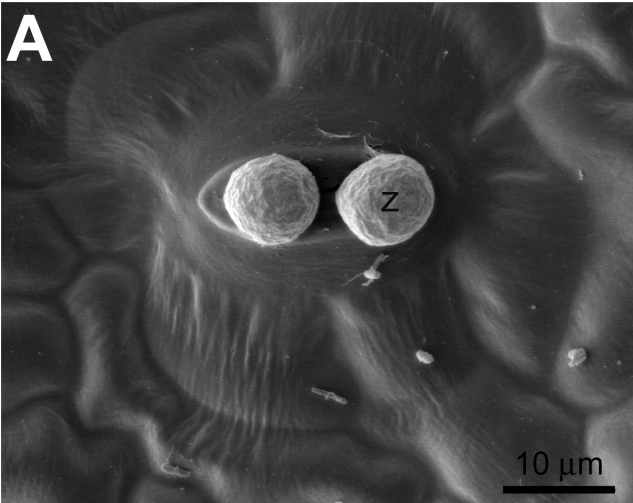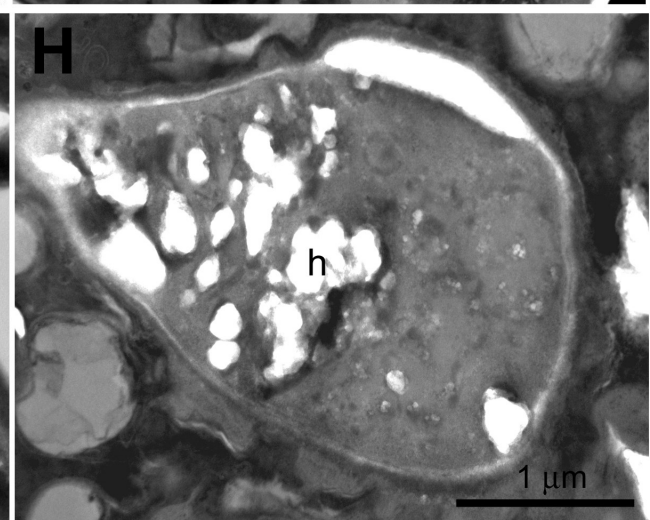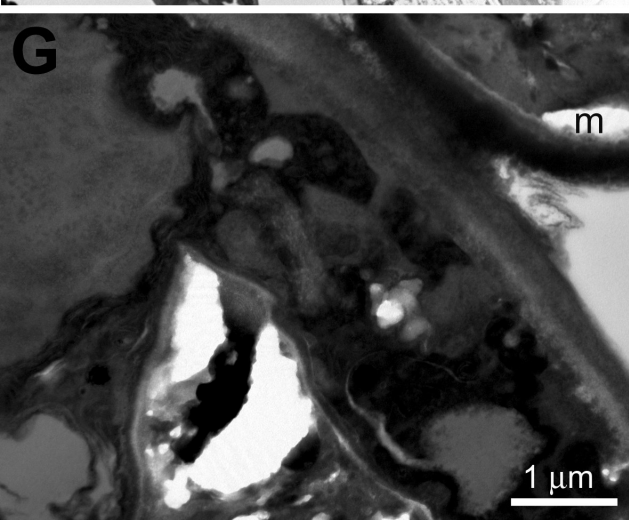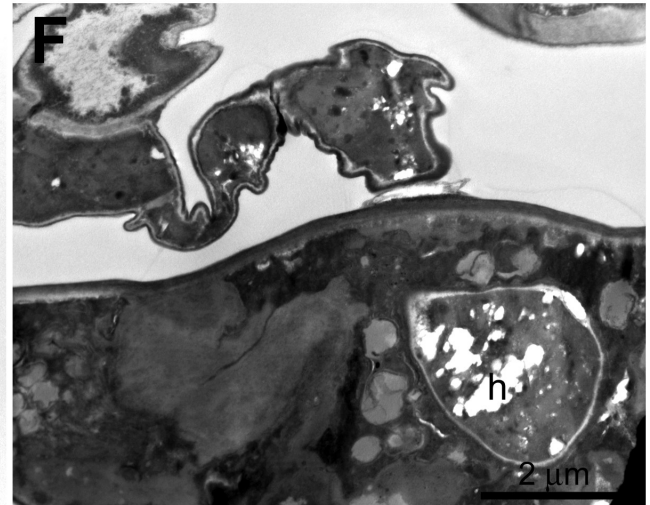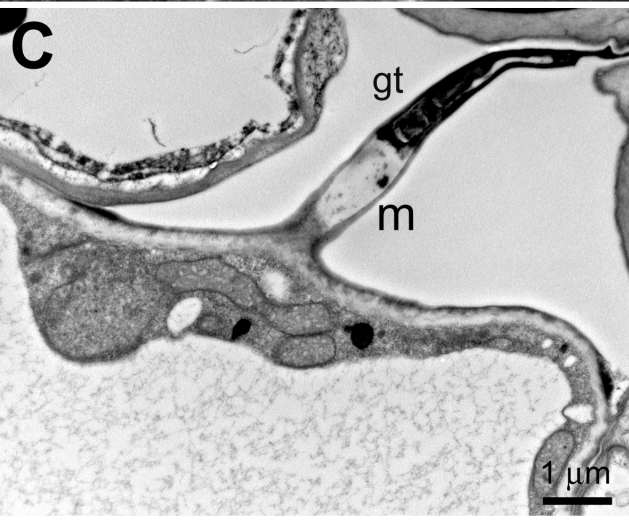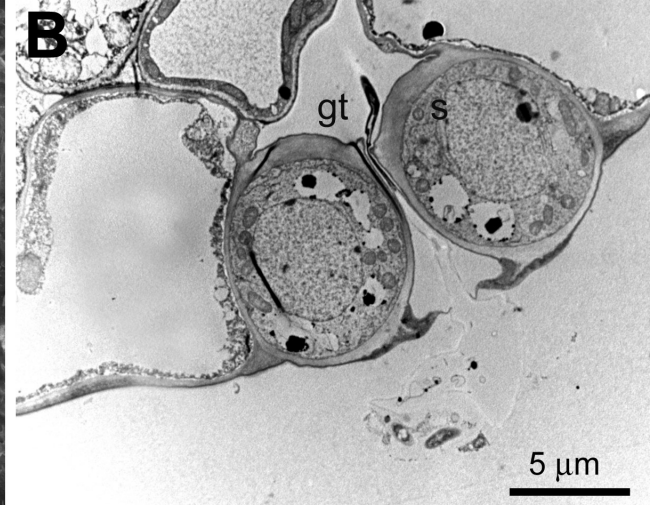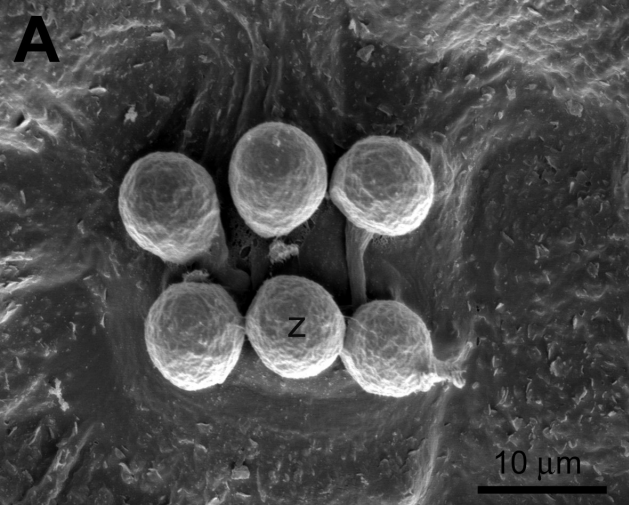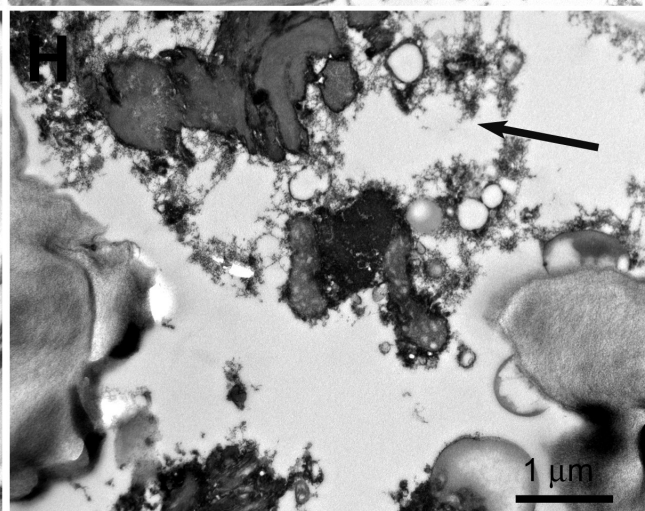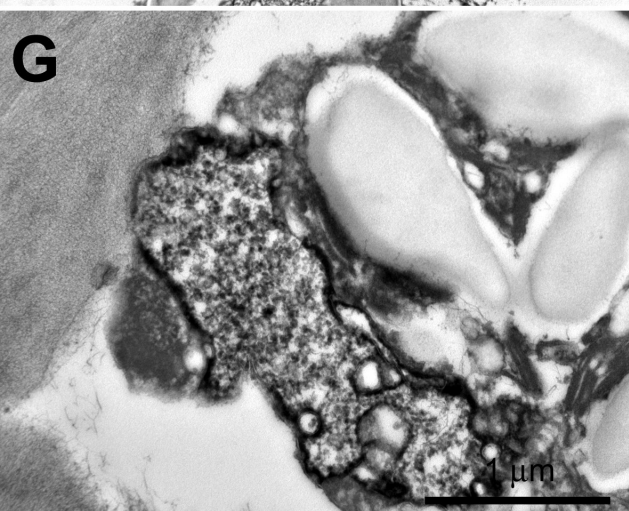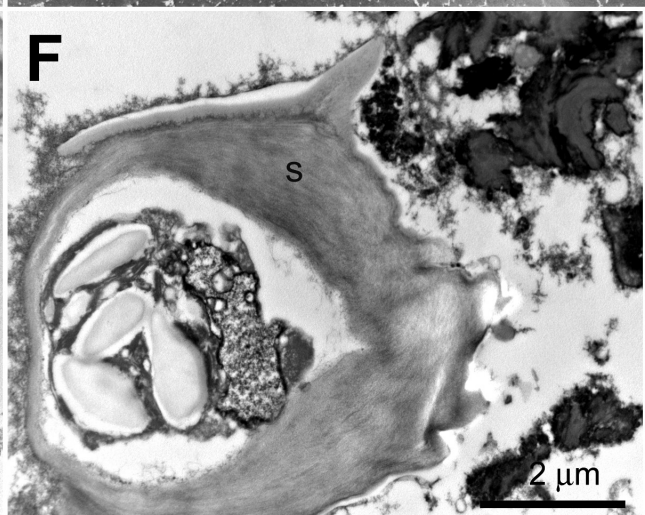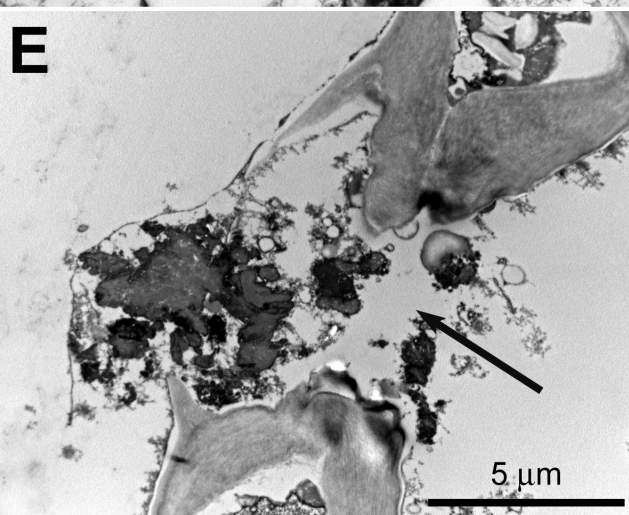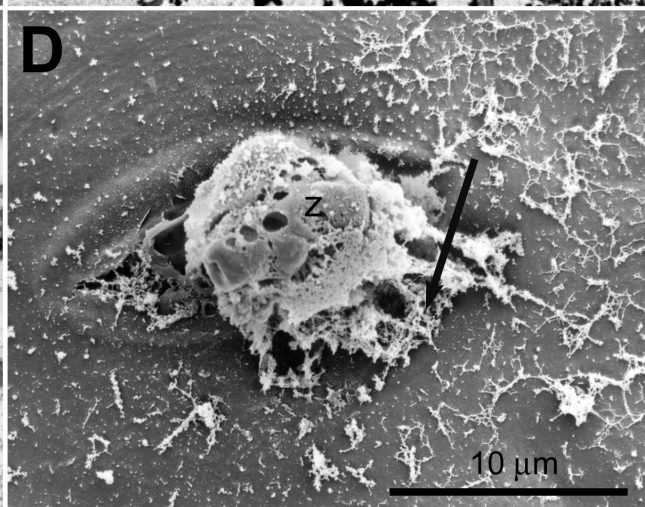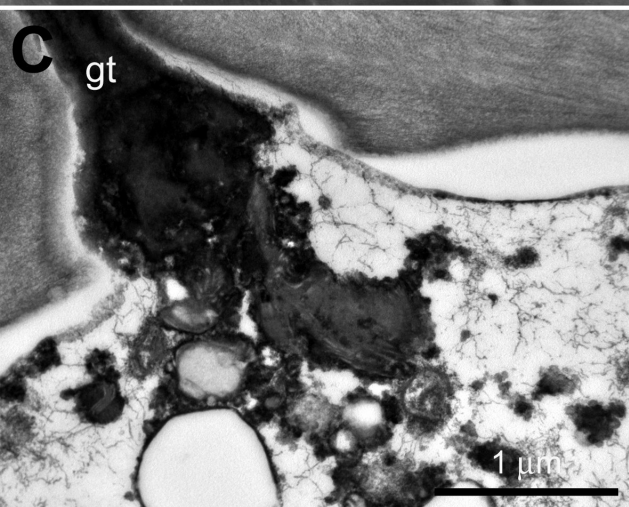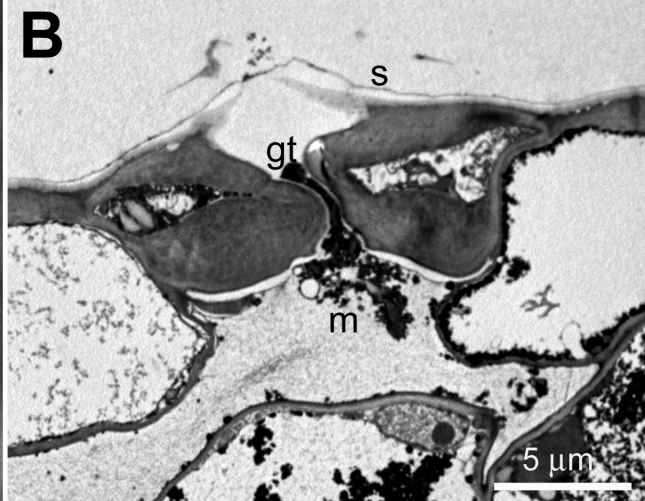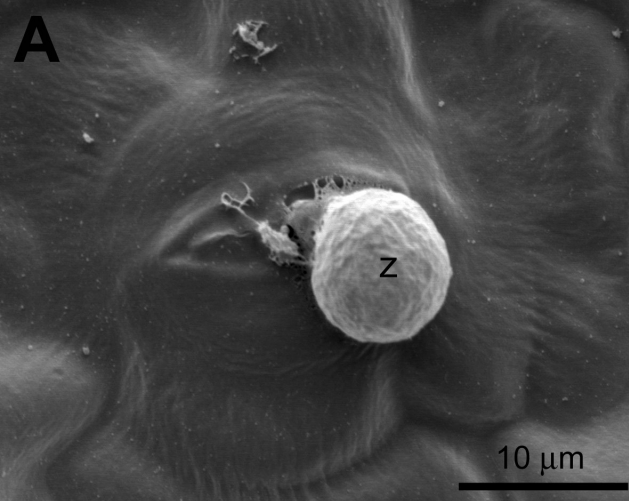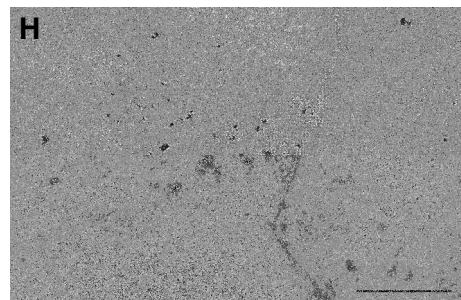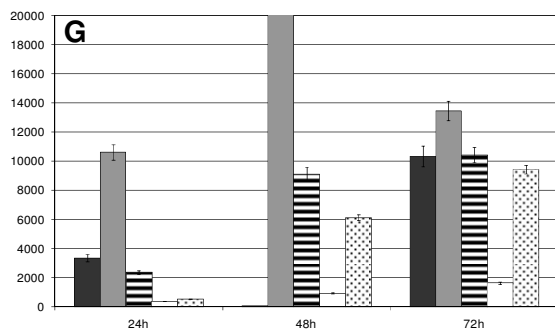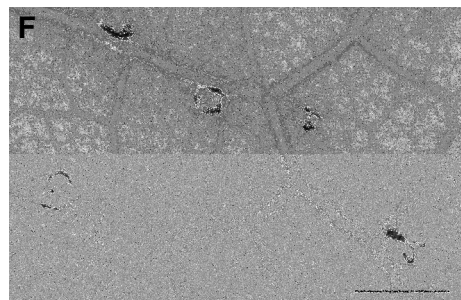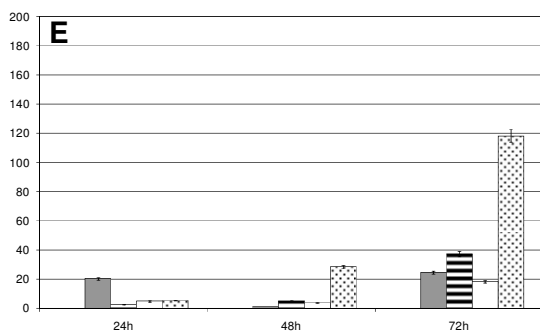
Legend:
■ Piceid ▨ Resveratrol ▤ epsilon-viniferin □ delta-viniferin ▨ Pterostilbene