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The penetration of sunflower root tissues by the parasitic plant *Orobanche cumana* is intracellular

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HAL Authorization

1 ***Title***

2 **The penetration of sunflower root tissues by the parasitic plant *Orobanche cumana* Wallr.**
3 **is intracellular.**

4

5 Auriac MC, Griffiths C, Robin-Soriano A, Legendre A, Boniface MC, Muños S, Fournier J,
6 and Chabaud M.

7

8

9 ***Key message***

10 Combination of *in vivo* confocal, large field and transmission electron microscopy approaches
11 revealed how intimate the relationship between the parasitic plant broomrape (*Orobanche*
12 *cumana* Wallr.) and its sunflower host (*Helianthus annuus* L.) is at very early stages of their
13 interaction.

14

15

16 ***Key words***

17 Live-cell imaging, parasitic plant, *Orobanche cumana*, *Helianthus annuus* L., plant-plant
18 interaction.

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22 **Introduction**

23 Sunflower broomrape (*Orobanche cumana*) is one of the main pests for sunflower crops. This
24 holo-parasitic plant, is specific to sunflower crops. Broomrape seeds perceive their host thanks
25 to germination stimulants present in sunflower root exudates (Bouwmeester et al., 2021). Once
26 germinated, the broomrape radicle grows toward the host root (Krupp et al. 2021) and develops
27 papillae, which adhere to the host root and secrete mucilaginous compounds (Joel and Losner-
28 Goshen, 1994). Subsequently, epidermal cells at the tip of the haustorium, a specific parasitic
29 organ, differentiate into intrusive cells that penetrate the host root (Masumoto et al., 2021). This
30 penetration combines physical pressure and degradation of sunflower root cell walls thanks to
31 pectolytic activity enzymes released by the parasitic plant (Shomer-Ilan, 1993; Losner-Goshen
32 et al., 1998). Intrusive cells make their way toward the host root vessels, crossing the successive
33 host root tissues. Transcriptomic analyses showed that, in the case of a susceptible interaction,
34 defense genes were activated only transiently and at a low level (Letousey et al., 2007; Dos
35 Santos et al., 2003a, b). In addition, the expression of the putative defense suppressor gene *Par1*
36 of various parasitic plants at the early stages of interaction (Yang et al., 2020; Qiu et al., 2022),
37 suggests manipulation of their host by parasitic plants. Once in contact with the host xylem
38 vessels, intrusive cells differentiate into vessel elements and vascular connections are
39 established (xylem as well as phloem), to insure the nutrient supply of the parasite (Krupp et
40 al., 2019). Although numerous studies have been performed on parasitic seed germination and
41 haustorium development (Yoshida et al. 2016), most of them were focused on the parasitic
42 plants, and the host cellular mechanisms involved during the intrusive cell development were
43 poorly described (Mutuku et al., 2021). How the host cells behave during the massive expansion
44 of the haustorium tissues across the outer root cell layers remained quite unknown. A few
45 studies published in the 70-90s explored the host cellular reorganization during the early stages
46 of the haustorium penetration of various *Orobanchaceae* parasitic plant species. It was shown
47 that haustorium development is accompanied by unusual host cell proliferation (Kuijt 1977;
48 Dörr and Kollmann, 1974). Whether the penetration is intra or intercellular in the root host was
49 rarely stated. Dörr and Kollman (1974) and Kuijt (1977) mentioned intercellular growth only
50 of the haustorial cells, with no observations of plasmodesmata interconnecting host and parasite
51 cells for the interactions *O. crenata*/ *Vicia faba* and *O. ramosa*/ *Cannabis sativa*. Intercellular
52 penetration between two cortical cells was shown during the interaction between *Striga*
53 *gesnerioides* (another *Orobanchaceae* species) and cowpea (*Vigna unguiculata*; Reiss and
54 Bailey, 1998). By contrast, the work by Dörr (1969) on the stem parasitic plant species *Cuscuta*

55 (*Convolvulaceae* family) on the host *Pelargonium zonale* revealed intracellular as well as
56 intercellular penetrations preceding the vascular connection between the host and the parasite
57 (Press et al., 1990). In addition, Musselmann and Dickinson (1975) showed an example of an
58 intrusive cell of the parasitic plant *Agalinis aphylla* (*Orobanchaceae* family) penetrating
59 intracellularly a cortical cell through a small opening in the cell wall. Thus, whether sunflower
60 root penetration by the broomrape haustorium is intra and/or inter cellular remained an open
61 question. This knowledge is required in the perspective of subsequently investigate and
62 understand the sunflower cellular mechanisms associated with resistances to *O. cumana*. In this
63 work, using an efficient selection of the early stages, and combining various microscopy
64 approaches including live-cell imaging of transgenic fluorescent host tissues, we re-investigated
65 the relationships between host and parasitic tissues at the cell level during the early stages of
66 haustorium penetration. The questions we addressed were: (i) do intrusive cells penetrate the
67 host root inter or intra-cellularly? (ii) do the sunflower root cells in the vicinity of the intrusive
68 cells die or stay alive? (iii) are sunflower cell divisions induced at the very early stages of the
69 penetration, and which are the root tissues involved?

70 ***Early stage kinetics of the sunflower/ broomrape interaction in rhizotrons***

71 To answer these questions, we needed to observe attachments at very early stages, *i.e.*
72 haustorium penetration sites sampled before the establishment of vessel connections. To this
73 end, we used a dedicated growth and inoculation device called rhizotron, a plexiglass home-
74 made box, which facilitates the observation of inoculated sunflower roots and selection of
75 attachment sites (Le Ru et al., 2021, **Notes S1**). For large field and transmission electron
76 microscopy (TEM) observation of stained longitudinal sections of attachments, we used root
77 fragments from young inoculated wild-type sunflower plantlets (*i.e.* non-transformed). In
78 addition, to get more information on the living status and the sub-cellular organization of the
79 penetrated cells, we observed attachments using *in vivo* confocal imaging of living inoculated
80 transgenic composite sunflower plants, *i.e.* obtained by *Agrobacterium rhizogenes*-mediated
81 transformation. This method generated plants with fluorescent roots, expressing the Green
82 Fluorescent Protein (GFP) targeted to the endoplasmic reticulum (ER) (**Fig. S1-S2; Table S1**).
83 Observation of attachments was performed from 4 to 8 days after inoculation (dai) (**Table S2**).
84 Broomrape rarely penetrated the host root before 6 dai, while most of the haustoria had reached
85 the inner root tissues (inner cortex to the vessels) at 8 dai. The kinetics were very similar
86 whatever the type of plants and microscopy approach. Interestingly, similarly to our

87 observations, Joel and Losner-Goshen (1994) observed the first stages of attachments at 5-7
88 dai.

89 ***Broomrape enters into living sunflower root tissues intracellularly***

90 Germinated broomrape seeds developed papillae at the tip of the radicle when contacting the
91 host root (**Fig. S2i**, Joel and Losner-Goshen, 1994). Mechanical pressure of the broomrape in
92 contact with sunflower root epidermal cells led to cell wall deformation (**Fig. 1a-c**).
93 Differentiated intrusive cells at the broomrape radicle tip were strongly stained by toluidine
94 blue O. They displayed a very dense cytoplasm, a reduced vacuole and a large nucleus
95 containing a darkly stained nucleolus, suggesting a high metabolic activity (**Fig. 1a, d, g, j**).
96 Imaging early stages of broomrape penetration revealed that intrusive cells penetrated the
97 epidermal layer as well as the successive outer cortical layers intracellularly (**Fig. 1d-i**). In our
98 culture system, sunflower roots had 4 to 5 cortical cell layers between the epidermis and the
99 endodermis (**Fig. S3**). Intracellular penetration of sunflower root cells was observed in all the
100 analyzed penetration sites (21 sites for large field microscopy and 21 sites for confocal
101 microscopy, **Table S2**). The use of the GFP-ER construct provided information about both the
102 cytoplasmic organization and the nucleus position, thanks to the ER outline labelling the nuclear
103 envelope (Genre et al. 2005). In many cases, the nucleus of the penetrated cell was strikingly
104 positioned close to the intrusive cells (**Fig. 1b, c, h, i**). The nucleus re-positioning close to the
105 intruder, is reminiscent of the cellular reorganization of plant cells during bacterial and fungal
106 symbiotic or pathogenic interactions (Fournier et al., 2008; Genre et al., 2005, 2008; Genre et
107 al., 2009). It suggests that the host nucleus perceives the intrusive cell, either through the
108 exerted mechanical pressure (Genre et al. 2009) and/or through unknown chemical signals.
109 However, in contrast to root penetration by symbiotic (Genre et al., 2005; 2008) or pathogenic
110 biotrophic fungi (Koh et al., 2005; Kankanala et al., 2007; Genre et al., 2009), no cytoplasmic
111 aggregation, nor specific ER re-organization were observed ahead of the penetration process.
112 Interestingly, ER was surrounding the broomrape intrusive cells (**Fig. 1e, f, h, i, k, l**), showing
113 active, though not massive, host intracellular re-organization along with the penetration
114 process. These results suggested active membrane synthesis around intrusive cells requiring
115 nucleus and ER activity in the host cell, and showed that the sunflower penetrated cells
116 remained alive. Deeper root tissues (endodermis and pericycle) were also penetrated
117 intracellularly by intrusive cells (**Fig. 1j**). In most cases, haustoria penetrated the host root with
118 minimal host cell damage. However, the live-cell imaging approach revealed a few cases of cell
119 death as shown by the absence of fluorescence (2 sites, **Fig. S4a, b**), or a severe ER disruption

120 (1 site, **Fig. S4c, d**). Similarly, change of the vacuole structure was observed using large field
121 microscopy for a few sites (7 sites among 21 penetration sites): appearing as a blue smear (**Fig.**
122 **1d; Fig. S4e**) or light blue material filling the cell (**Fig. S4e**). One or a few penetrated cells only
123 were affected, adjacent to the intrusive cells in outer root tissues. These results suggested that
124 in some cases, penetration of the intrusive cells got out of control and synchronization of the
125 penetration process and the sunflower cellular re-organization failed, leading to sunflower cell
126 death. This phenomenon remained cell-autonomous, without other defense reactions in the
127 surrounding or the deepest root tissues. Furthermore, penetration could result in the separation
128 of the host nucleus from the distal part of the penetrated cell, probably leading to cell death as
129 well. Strikingly, broomrape intrusion was thicker in outer root tissues (**Fig. 1**) than in inner root
130 tissues, in which only single elongated and separated intrusive cells were detected (**Fig. 1e, f,**
131 **h, i, k, l**). Similarly, Dörr et al. (1969) reported intracellular “searching hyphae” for the *Cuscuta*
132 stem parasite.

133 *Divisions are induced at very early stages*

134 Sunflower roots were known to swell locally at the site of broomrape attachment by means of
135 cell division (Kuijt, 1977) and as early as 7 dai (Dörr and Kollamn, 1994). In the present study,
136 sunflower root cell divisions were observed as early as 6 dai, close to attachments (respectively
137 9 and 7 sites for sections and live-cell imaging). Divisions were mostly anticlinal in the cortex,
138 and periclinal in the pericycle (**Fig. S5; Fig. 1d, e, h, i**). The number of dividing root cell layers
139 and the length of the dividing zone were highly variable (for example from 1 cortical cell to
140 more than 30 cells in a row). These divisions may account for root hypertrophy that was
141 previously observed at the site of 14-dai attachments in rhizotrons (Chabaud et al., 2022). These
142 divisions could be induced indirectly (host hormonal regulation) or directly by the parasitic
143 plant (hormonal release: such as auxin [Ishida et al., 2016] or cytokinin [Spallek et al., 2017]).
144 Whether germinated broomrape seed exudates would be sufficient for the induction of host cell
145 divisions remains an open question.

146 *Intrusive cells penetrate a new apoplastic compartment*

147 The interface between intrusive cells and the sunflower penetrated root cells at early stages of
148 the interaction was further characterized by TEM (**Fig. 2**). A 7 dai attachment with the
149 haustorium reaching the 3rd cortical cell layer is illustrated **Fig. 2a, b**. Starch grains, a sign of
150 the transition from the autonomous (germination stage) to the parasitical stage (Joel and Losner-
151 Goshen, 1993), were present in the central part of the attachment (**Fig. 2a, c**). In the outer root
152 cell layers, the interface appears as a thick layer surrounding broomrape (**Fig. 2b**), as already

153 described for *Striga* (Reiss and Bailey, 1998; Neuman et al., 1999). The intrusive cells were
154 easily distinguished from sunflower root cortical cells thanks to their dense cytoplasm,
155 containing Golgi stacks, large mitochondria as well as a reduced vacuole (**Fig. 2b, e, f**) as
156 already reported by Kuijt and Toth (1976) and Kuijt (1977). By contrast, the host cortical cells
157 whether penetrated or not, contained a large vacuole with a thin layer of surrounding cytoplasm
158 (**Fig. 2b, d**). Mitochondria in the penetrated host cells were present all along the host
159 plasmalemma, suggesting intense activity at the periphery of the host cell such as membrane
160 biosynthesis (**Fig. 2d**). This dense cytoplasm confirmed that the penetrated host cells were alive
161 at this stage. The parasitic cell wall was present all around the intrusive cells. By contrast, the
162 presence of a host matrix along the anticlinal interface of the host penetrated cell was not always
163 detectable and its appearance varied along the length of the haustorium. On the outermost
164 anticlinal side of the host penetrated cell, the host interface with the haustorium appeared as a
165 dark thick layer, in continuity with the existing periclinal host cell wall (**Fig. 2b, d**). It could
166 partly result from the invagination of the existing periclinal host cell wall pushed in by the
167 penetrating intrusive cells. The discontinuity of the staining suggests disorganization of this
168 host cell wall/ matrix. On the innermost side of the cell, the host matrix was either too thin to
169 be visible (**Fig. 2e, f**) or appeared as a low-density material (stars in **Fig 2. g, h**) separating the
170 host cell plasmalemma from the parasitic cell wall, and differing from the existing darker
171 periclinal host cell wall. The use of various fluorescent dyes to distinguish host cell wall from
172 newly-made matrix would be interesting, as done for the symbiotic nitrogen-fixing bacterial
173 infection thread (Rae et al., 2021). At the frontline of the haustorium penetration the existing
174 periclinal host cell wall seemed also disorganized (**Fig. 2i**), suggesting progressive local
175 enzymatic degradation of the host cell wall. This apparent dissolution of the nearby host cell
176 walls (Kuijt, 1977) or a partial digestion of the cell wall at the interface (Kurotani et al., 2020,
177 in the case of the interaction *Ptheirospermum japonicum/ Arabidopsis thaliana*) had been
178 reported previously. While cell-wall degrading enzymes which might contribute to this process
179 have been identified from the parasite (Shomer-Ilan et al., 1993; Loshen-Gosner et al., 1998),
180 there is no evidence at the moment of the direct involvement of host enzyme activities involved
181 in host cell wall degradation in this context (Mitsumatsu et al., 2015; Yang et al., 2020).
182 Nevertheless, the host cell plasmalemma seemed to remain undisturbed and continuous (**Fig.**
183 **2g; Fig. S6b**). Both host and parasitic plasma membranes were highly convoluted at the front
184 line of the haustorium (**Fig. S6b**), suggesting membrane synthesis for the haustorium
185 accommodation (host) and haustorium expansion (parasite). No plasmodesmata were observed
186 on the interface at these early stages, indicating that molecular exchanges between the parasite

187 and the host happened at later stages, or through vessel connections, as interspecific
188 plasmodesmata have been shown in the phloem (Krupp et al., 2019). In some cases, the
189 penetration led to disaggregation of the vacuole of the host cell (**Fig. S6c-d**), with disruption
190 of the host cell plasmalemma, leading to cell necrosis, as for *Striga* (Neuman et al., 1999).
191 However as mentioned above this was not very common and remained cell autonomous. In
192 addition, no evidence of cell death was observed at the later stages (Chabaud et al., 2022).
193 Altogether, these results showed that the parasitic intrusive cells penetrate the host root cells
194 intracellularly, as a result of degradation of the host cell wall and formation of a new host trans-
195 cellular apoplastic compartment for haustorium accommodation.

196 ***Concluding remarks***

197 Most striking among our findings has been the observation of intracellular haustorium
198 penetration of host root tissues, in contrast to most studies on *Orobanchaceae*. These studies
199 relied mainly on the observation of transverse sections, by contrast to the longitudinal sections
200 used in this work, which made it easier to distinguish between intra and intercellular processes.
201 Our work showed the intimate broomrape penetration into its host, through the formation of a
202 new apoplastic compartment. It suggested that although host cell wall integrity has been
203 damaged by parasitic cell wall degrading enzymes (Shomer-Ilan et al., 1993; Losner-Goshen et
204 al., 1998), only minor defense reactions were induced as previously reported for biotrophic
205 pathogenic fungi (Mendgen and Hahn, 2002; Bellincampi et al., 2014). In that respect, genes
206 encoding inhibitors of cell wall degrading enzymes could be good candidates for increasing
207 resistance to broomrape. In addition, as *HaOr7* (Duriez et al. 2019) and *HaOr_{Deb2}* (Fernandez-
208 Aparicio et al., 2022) encode Leucine-Rich-Repeat Receptors Like Proteins, providing
209 resistance to various *O. cumana* races, it would be of outstanding interest to characterize the
210 cellular processes involved in these incompatible interactions. Comparing the cellular processes
211 for various *O. cumana* races could highlight common or different mechanisms. Finally, using
212 these approaches on other major parasitic plant species such as *Striga* will be of great interest
213 for future resistance development in a larger host range.

214

215

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227

228 *Competing interests*

229 None.

230

231 *Authors and contribution*

232 MCA performed cytological experiments (large field microscopy and TEM). CG, ARS and AL
233 established sunflower transformation experiments. MCB produced sunflower and broomrape
234 resources. SM: coordinated the ICSG, assisted with the construction of the project and the
235 writing of the manuscript. JF gave technical and scientific advice, assisted with the construction
236 and the writing of the manuscript. MC designed the experiments, carried out confocal
237 microscopy and cytology experiments, wrote the manuscript.

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243

244 *Data availability statement*

245 The original contributions presented in the study are included in the article/Supplementary
246 Material. Further inquiries can be directed to the corresponding author.

247

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- 338

339 *Legends*

340 **Fig.1. Broomrape haustorium penetrates the sunflower root tissues intracellularly.**

341 Broomrape attachments (6-7 dai) were observed at the cellular level, using 2 approaches: (i)
342 large field microscopy of thin sections of resin-embedded and toluidine blue O-stained
343 attachments (right column, **a, d, g, j**); (ii) confocal microscopy of broomrape-inoculated
344 transgenic fluorescent sunflower roots expressing the GFP targeted to the endoplasmic
345 reticulum (ER) (middle and left columns, **b, c, e, f, h, i, k, l**). Confocal microscopy images show
346 fluorescence alone (combined GFP fluorescence and auto-fluorescence channels, middle
347 column, **b-e-h-k**) or fluorescence with the corresponding bright field image (left column, **c, f,**
348 **i, l**).

349 **a-c.** Deformation of the epidermal cell wall (arrow) due to the mechanical pressure exerted by
350 the haustorium. Re-positioning of the nuclei of the deformed epidermal cells in the vicinity of
351 the haustorium tip (**b.** white arrowheads). **d-i** and **k-l.** Intrusive cells penetrated the root
352 epidermal and cortical layers intracellularly. The penetrated cortical cells had wavy (**d.** black
353 double arrowheads) or deformed (**g.** black arrow) cell wall in contact with the haustorium. ER
354 surrounded the intrusive haustorium tip (**h, k.** white double arrowheads). Nuclei were
355 positioned close to the intrusive cells (**h.** white arrowheads). **j.** Intrusive cells reached the host
356 xylem vessels (xylem: black cross), and crossed intracellularly the deepest cortical cell layer as
357 well as the endodermis and the pericycle. Divisions in the cortex (mostly anticlinal, squares)
358 and the pericycle (mostly periclinal, double squares) in the vicinity of the haustorium (**d, e, j**).
359 **c, f, i, l.** White dots outline the broomrape haustorium visible on the bright field image. **b-c, e-f**
360 **, k-l** are z axis projections of serial optical sections.

361 Scale bar = 50 μm (**a, b, c, d, e, f, g, j**); 20 μm (**h, i, k, l**).

362

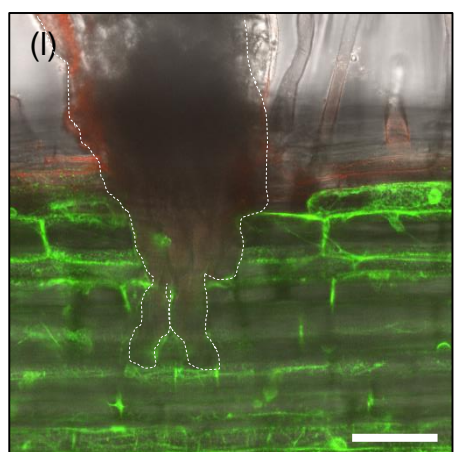
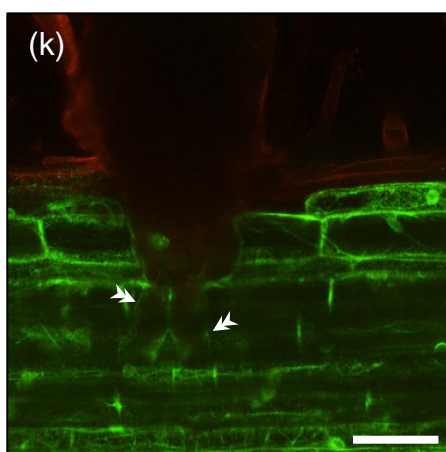
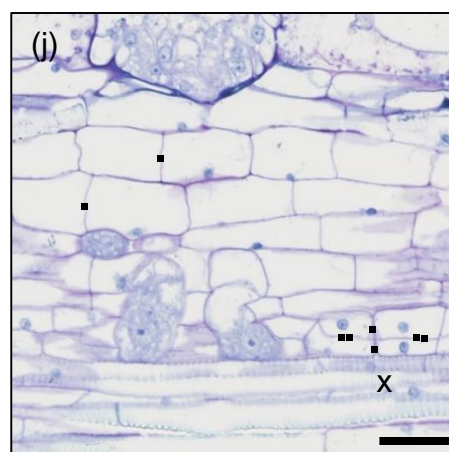
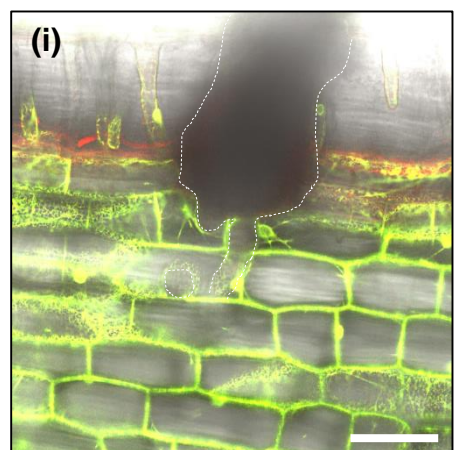
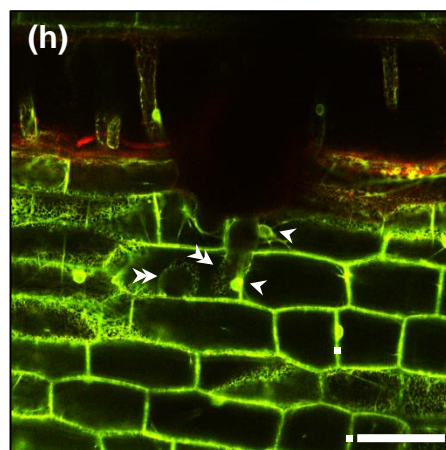
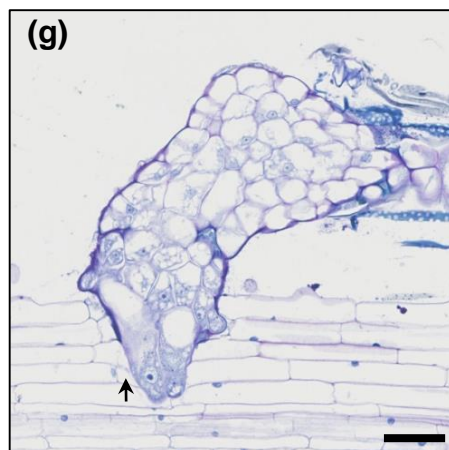
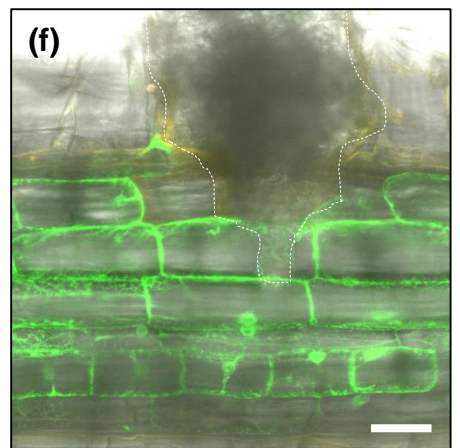
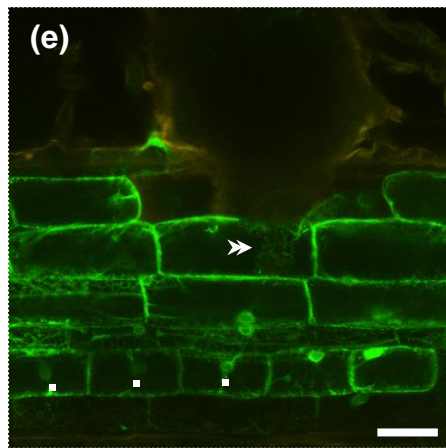
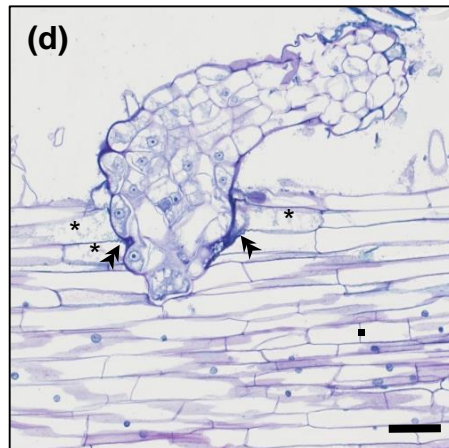
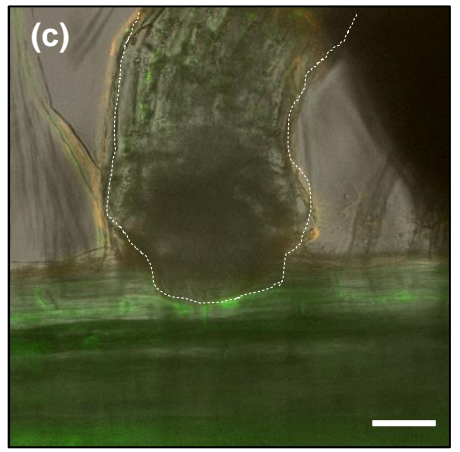
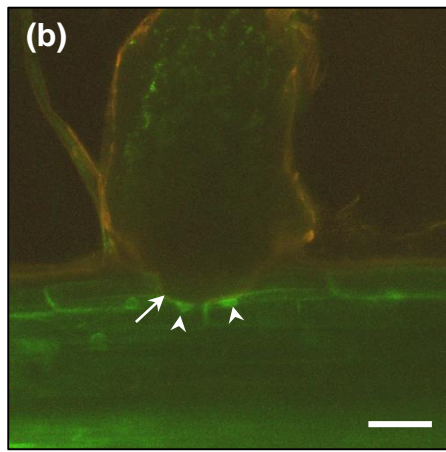
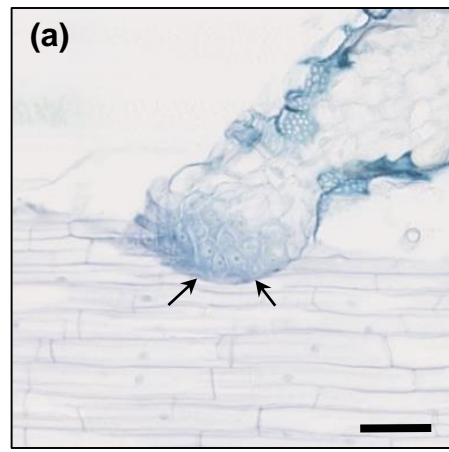
363 **Fig.2. Creation of a new apoplastic compartment for haustorium accommodation.**

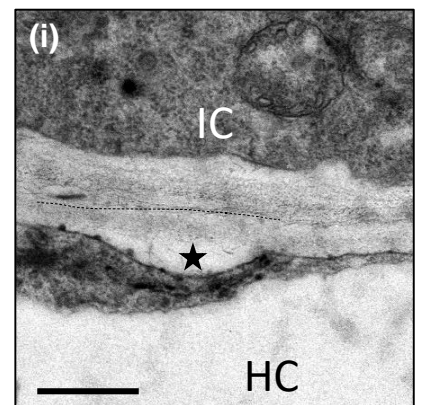
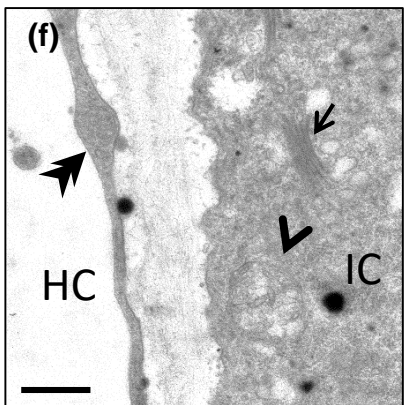
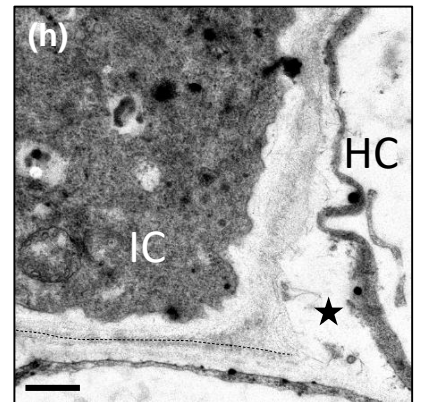
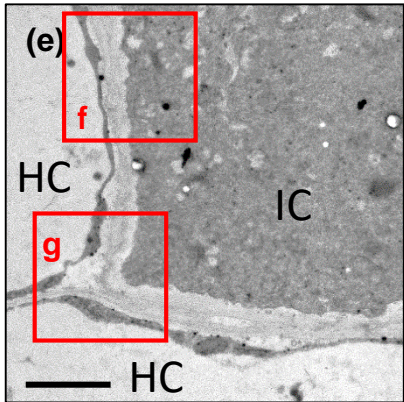
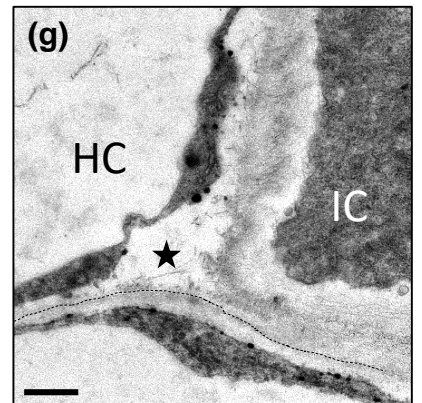
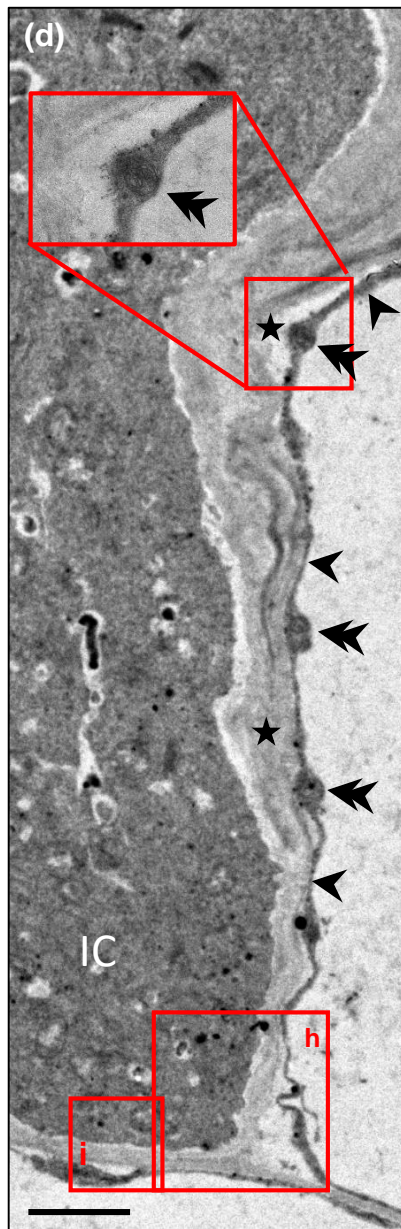
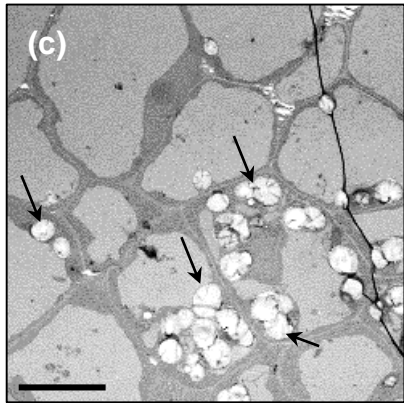
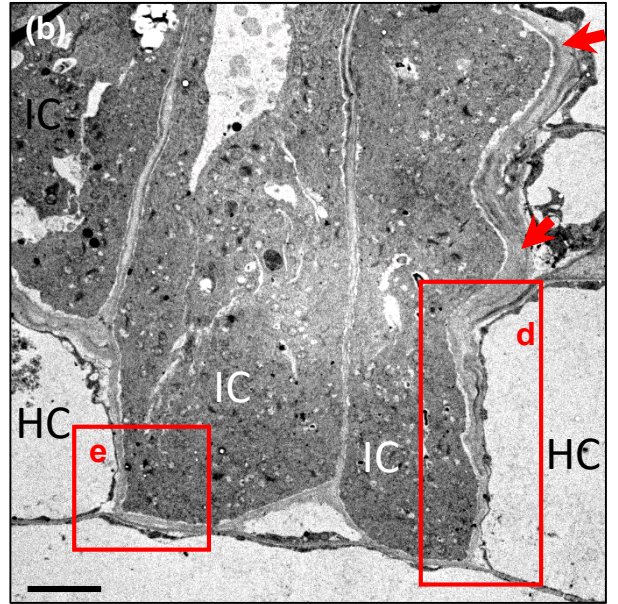
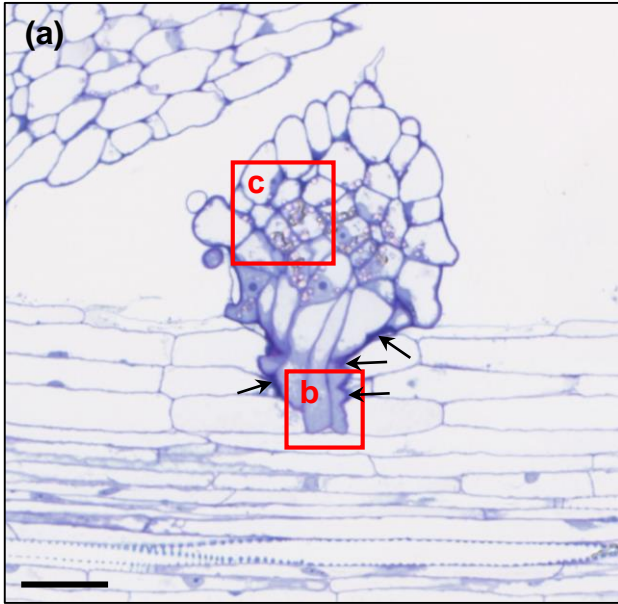
364 A 7 dai attachment, with intrusive cells reaching the 3rd cortical cell layer was imaged. **a.**
365 General view of the attachment using large field microscopy. **b-i.** Transmission electron
366 microscopy. IC intrusive cell, HC host cell.

367 **a, b.** Intrusive cells intracellular penetration in the host outer cortex cell. A thick stained layer
368 surrounded the penetrating intrusive cells in outer host tissues (epidermis and outer cortex;
369 black arrows in **a**, red arrows in **b**). **c.** Magnification of the central part of the broomrape, with
370 starch grain-containing cells. **d.** In the outermost side of the anticlinal interface between the
371 intrusive cell and the penetrated host outer cortical cell, disorganization of the host cell

372 wall/matrix (star). Continuous thin layer of cytoplasm in the host cortical cell (black
373 arrowheads), containing numerous mitochondria (double arrowheads). **e, f.** Presence of the
374 parasitic cell wall but no visible host cell matrix in the outermost anticlinal side of the penetrated
375 cell. **e.** Magnification of **b.** **f.** Magnification of **e.** **f.** The intrusive cell cytoplasm contains Golgi
376 (thin arrow) and mitochondria (arrowhead). Mitochondria are also present in the thin host
377 cytoplasm layer (double arrowhead). **g-h.** At the tip of the haustorium, presence of a host matrix
378 (star) with fibrillae fragments. Convolution of the plasmalemma of the host cortical cells
379 adjacent to the intrusive cells. **i.** At the front line of the intrusive cell tips (periclinal interface),
380 local disorganization of the host cell wall (star), the plasmalemma of the host cell remaining
381 intact and continuous. The dotted line shows the separation between the parasitic and host cell
382 walls (**g-i**).

383 Scale bar = 50 μm (**a**); 5 μm (**b**); 10 μm (**c**); 2 μm (**d-e**); 0.5 μm (**f, g, h, i**).





1 **Notes S1. Details of the materials and methods.**

2

3 ***Plant genotypes, bacterial strains, and constructs***

4 We used the susceptible cultivated *H. annuus* L., XRQ (Chabaud et al., 2022; Badouin et al.,
5 2017) as the sunflower host for experiments in rhizotrons and for root transformation
6 experiments. The *O. cumana* Wallr. population used in this study was the French race E-BOU
7 of *O. cumana*, with a virulence level classified between E and F, and harvested in 2017 in
8 Bourret (Tarn et Garonne, France; reference: LIPM-20734).

9 The *Agrobacterium rhizogenes* strain K599 (Savka et al. 1990) was used for sunflower root
10 transformation. The binary vector used was derived from pBIN19 (Bevan, 1984) and carried
11 the construct p35S-GFP-ER coding for the Cauliflower Mosaic Virus 35S promoter (p35S)
12 driving the constitutive expression of the Green Fluorescent Protein targeted to the endoplasmic
13 reticulum due to the presence of ER-targeting sequences (N-terminal signal peptide) and ER-
14 retention (C-terminal tetra-peptide HDEL; Haseloff et al. 1997). Bacteria were grown on LB
15 medium supplemented with Streptomycin 100 mg/l (bacterial selection) and kanamycin 50 mg/l
16 (plasmid selection)

17

18 ***Cultivation of sunflower plants in rhizotrons***

19 Two types of sunflower plants were used: wild-type (non-transformed, non-fluorescent)
20 sunflower plantlets for large field and TEM microscopy, and transgenic composite plants
21 (expressing the fluorescent marker in their roots only) for confocal microscopy.

22 **Wild-type (*i.e.* non-transformed) sunflower plants**

23 Sunflower seeds of wild-type plants were surface sterilized for 10 min in a 4.8 % sodium
24 hypochlorite solution, rinsed 3 times and sown in a 1/1 v/v mixture of sand/ vermiculite in
25 alveoli. Seedlings were grown at 22 °C, 60% humidity, 16 h light 118 $\mu\text{E}/\text{m}^2/\text{s}$ for 7 days.
26 Rhizotrons, 12x12 cm home-made plexiglass boxes were assembled as described in Le Ru et
27 al. (2021), with water-soaked sterile rock-wool, a sterile glass fiber paper and a 7-day old
28 sunflower plantlet. Rhizotrons were then watered with a ½ Long Ashton solution (Hewitt, 1966)
29 containing 370 μM phosphate.

30 **Transgenic composite sunflower plants**

31 To transform sunflower roots and obtain composite plants (with a non-transformed aerial part
32 and transgenic roots), we used a modified version of the protocol from Parks and Yordanov
33 (2020, **Fig. S1-S2**). Sunflower seeds were decontaminated and grown in alveolus-trays as above
34 for 10 days. Transformation of plantlets was performed in soaked pre-cut rock-wool cubes

35 (reference ALR02G from GRODAN) with bacterial solution [final OD= 0.25 in ¼ (MS +
36 Gamborg vitamins B5)], in Magenta boxes. After 3 days, Magenta boxes were slightly opened.
37 Six days after transformation, plantlets were transferred to hydroponics, using sterile 1000µl
38 cone boxes, as described in Morel et al. (2018) for another 6 days of culture in ¼ MS liquid
39 medium (without vitamins; Sigma reference MS 5524). Culture in hydroponics, a new step
40 compared to Parks and Yordanov's protocol (2020) was very beneficial to newly developed
41 root growth. Finally, 14 to 18 days following transformation (6-7 days in rock-wool cubes and
42 7-12 days in hydroponics; **Fig. S1**), transformation efficiency was recorded by measuring the
43 percentage of plants with fluorescent roots and the number of fluorescent roots/ transformed
44 plant (**Table S1**). Fluorescence expression was observed by epifluorescence microscopy using
45 a stereomicroscope (Axiozoom V16; Zeiss), equipped with a GFP Long Pass filter (excitation
46 485/12 nm and emission from 515 LP). Composite plants were transferred to rhizotrons as for
47 non-transformed plantlets (see above).

48 Four experiments were performed. In the first experiment, 17-day-old plantlets were used for
49 transformation, and the transfer to rhizotrons was done 18 days later, resulting in large 35-day-
50 old plants that were difficult to handle under the confocal microscope. Hence the length of
51 culture was progressively reduced in the following experiments to 10-day-old plantlets for
52 transformation, culminating with transfer to rhizotrons as soon as 14 days later in experiment
53 No. 4 (**Fig. S1**). The reduction of the age of the plants used for transformation facilitated the
54 manipulation of the composite plants when they were removed from the rock-wool cubes and
55 limited wounding of the transgenic roots. It also facilitated their transfer into rhizotrons and use
56 in confocal microscopy.

57

58 ***Inoculation of sunflower plants in rhizotrons***

59 Broomrape seeds were surface sterilized for 5 min in a 3.2 % sodium hypochlorite solution,
60 rinsed 3 times using a 40 µm cell strainer, and water-conditioned for 7 days in a 50 ml sterile
61 tube at 23 °C in the dark, at a final concentration of 10 mg/ 3 ml of water. Each rhizotron was
62 inoculated with 10 mg (3 ml) of conditioned broomrape seeds.

63 Seven day-old wild-type plants were inoculated the day of transfer in rhizotron. For transgenic
64 composite plants the day of inoculation varied among the transformation experiments from 1
65 to 8 days after the transfer in rhizotrons (*i.e.* 27 to 36 days after sunflower sowing; **Figure S1**).

66

67 ***Microscopic observation of young *O. cumana* attachments***

68 Young wild-type sunflower plantlets were inoculated and used for large field and transmission
69 electron microscopy (TEM) observation of stained longitudinal sections of attachments.
70 Selected composite sunflower plants with fluorescent roots were inoculated and used for *in vivo*
71 confocal microscopy images of attachments.

72 **Large field microscopy observations**

73 This approach was inspired from the work of Xiao et al. (2014) on early symbiotic nodule
74 development. Four to 8 days after inoculation (dai), root samples were prepared for cytological
75 studies as described in Chabaud et al. (2022). Fixed samples were embedded in Technovit 7100
76 resin (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer's
77 recommendations. Thin (4-5 μm) sections, longitudinal to both haustorium and host root, were
78 made using a microtome (2040 Reichert Jung), stained in 0.2% toluidine blue for 3 min,
79 mounted in DePeX mounting medium (BDH Laboratories, Poole, England) and scanned using
80 a Nanozoomer (NDP, Hamamatsu).

81

82 **Transmission electron microscopy (TEM) observations**

83 Samples were prepared as in Cerutti et al. (2017) with some modifications. Five to 7 dai,
84 broomrape attachments on sunflower root fragments were fixed under vacuum for 30 min with
85 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH = 7.2) containing 0.1 % Triton
86 X100, and then at the atmospheric pressure in the same solution without Triton X100, washed
87 in the same cacodylate buffer and post-fixed for one hour at room temperature with 1 % osmium
88 tetroxide (OsO_4) in the same buffer. They were then dehydrated in ethanol series, and embedded
89 in Epon. Thin (1 μm) or ultra-thin (80-90 nm) sections were prepared on UltraCut E
90 ultramicrotome (Reichert-Jung) equipped with a diamond knife (Reichert-Leica, Germany).
91 The histological organization of tissues was observed on thin sections stained in a 1 % borax
92 solution containing 0.2 % methylene blue and 0.1 % toluidine blue, rinsed in water and then in
93 an aqueous solution of 0.07 % basic fuchsin. Ultrastructural observation was done on ultrathin
94 sections stained with uranylless (Delta microscopy, Maressac France), and lead citrate (delta
95 microscopies, Maressac France) using the electron microscope Hitachi-7700 (Japan) operating
96 at 80 kV.

97

98 **Confocal microscopy**

99 This approach was inspired from the cytology work of Genre et al. (2005) for arbuscular
100 mycorrhizal symbiosis studies on *Medicago* and carrot. Six days after broomrape inoculation

101 (dai) in rhizotrons, inoculated sunflower composite plants were transferred to a 12x12 cm
102 square Petri dish containing 80 ml of solid medium ½ Long Ashton 370 µM phosphate, 3 g/l
103 Phytigel, with the aerial part outside of the dish (**Fig. S2g**). The root system was covered with
104 a gas-permeable plastic film (Lumox Film, Starsted) as described in Fournier et al. (2015).
105 Attachments were imaged with a Leica TCS SP8 AOBS confocal laser scanning microscope
106 equipped with a long-distance 25X HC FLUOTAR (numerical aperture, 0.95) water immersion
107 objective. The 488 nm argon laser line was used to excite GFP and auto-fluorescence. Specific
108 emission windows used for GFP and auto-fluorescence signals were 500 to 550 nm, and 580 to
109 650 nm, respectively, and emitted fluorescence was false-coloured in green (GFP), and red
110 (auto-fluorescence). The images shown are single confocal sections or maximal projections of
111 selected planes of a z-stack. Images were acquired and projected using Leica confocal software
112 and processed using Leica confocal software.

113

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Fig. S1. Sunflower transformation experiments and broomrape inoculation of composite plants for confocal microscopy observations.

The initial experiment (No.1) led to broomrape inoculation of 36-day-old sunflower plants, which were very developed and not easy to handle under confocal microscopy. Hence, the length of culture was progressively reduced in the following experiments to finally reach broomrape inoculation of smaller, 26-day-old plantlets in experiment No.4. This also allowed to generate two series of inoculated plants according to their development in experiments No. 2 and 4, using the most developed composite plants for the 1st inoculation and the less developed composite plants for a 2nd, delayed inoculation 6 days later. Confocal microscopy was performed 5 to 8 dai.

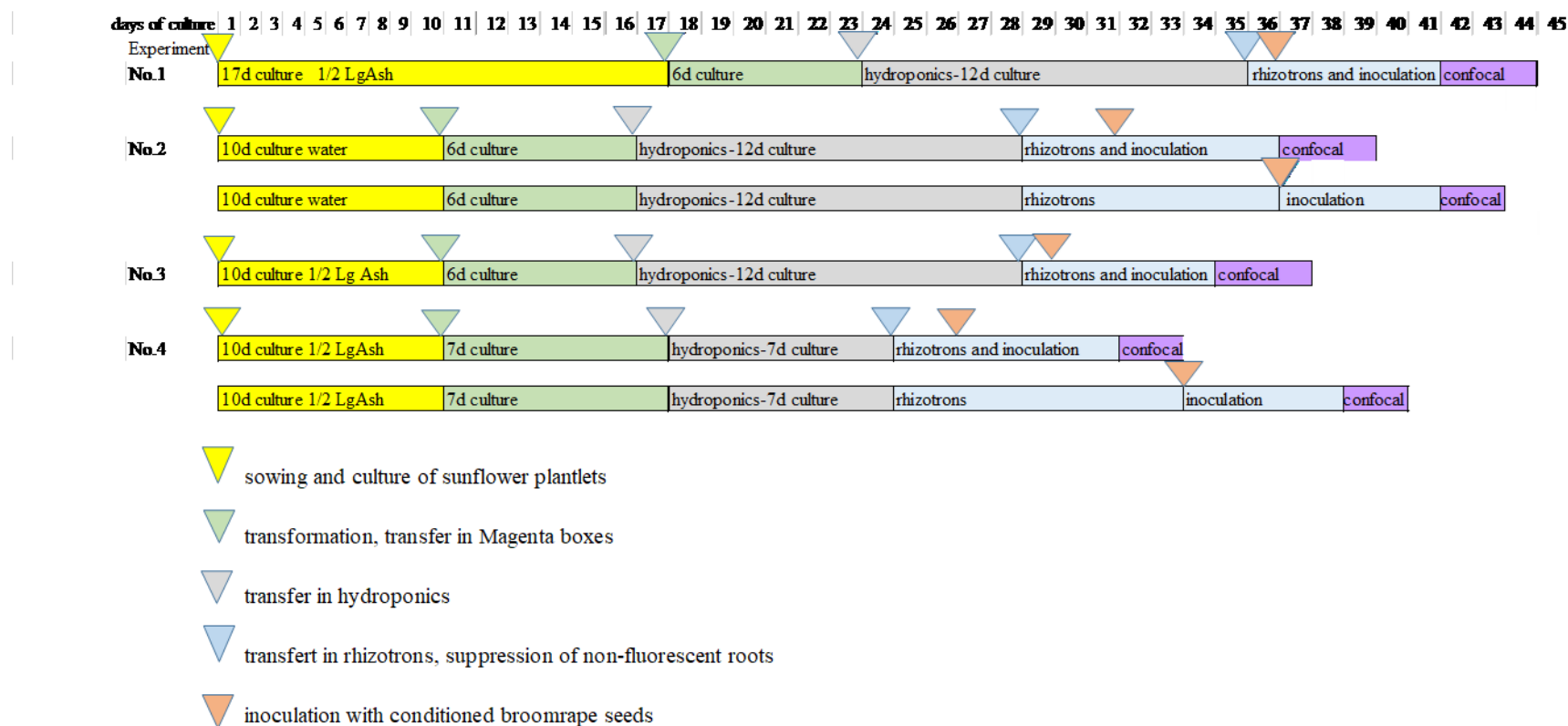


Fig. S2. Transformation of sunflower plants via *A. rhizogenes* and transfer of composite plants in rhizotrons for broomrape inoculation and observation using confocal microscopy.

Cuttings without cotyledons were excised from 10-day-old plantlets (a), and transformed in pre-soaked rock-wool cubes with *A. rhizogenes* solution (b). Six days later, plantlets were transferred in hydroponics (c) for another 8 days of culture. Fourteen days after transformation, green fluorescent transgenic roots were counted under a binocular microscope (d, Table S1). Non-fluorescent roots were removed and composite plants were transferred to rhizotrons (e) and inoculated with pre-conditioned broomrape seeds 1 to 2 days later (f). Five to 8 days after inoculation, inoculated composite plants were transferred to a Petri dish (g- 5 dai) and attachments were selected and imaged by confocal microscopy (h-i). i. Papillae development (arrows). Scale bar = 4 cm (a); 2 cm (b); 5 cm (c); 0.5 cm (d); 3 cm (e-g); 100 μ m (h); 10 μ m (i).

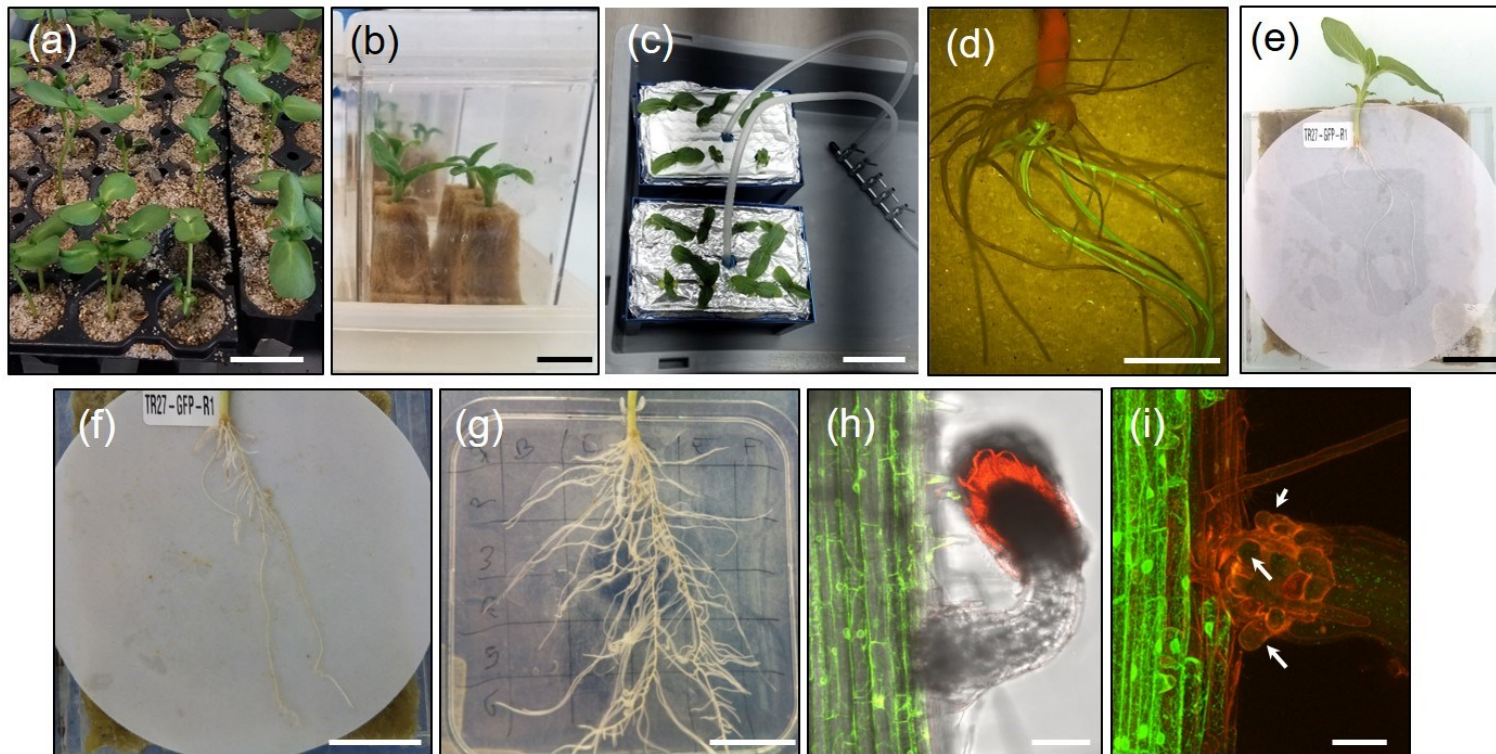


Fig. S3. Longitudinal section of a sunflower root.

A thin section of a sunflower root fragment, without broomrape attachment, resin-embedded and stained with toluidine blue O, was observed using large field microscopy. In our growth conditions, roots displayed 4 to 5 cortical layers.

Scale bar = 100 μm .

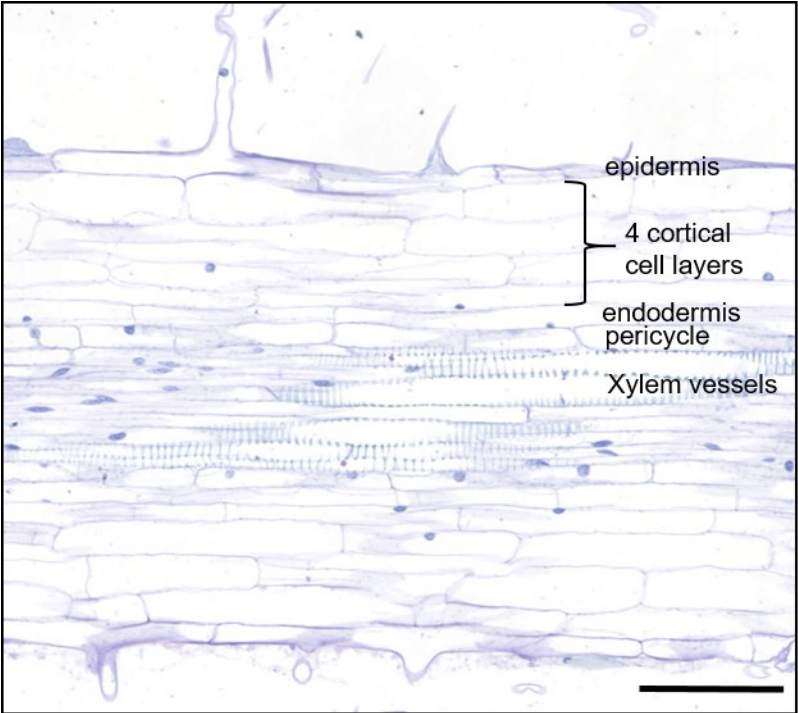


Fig. S4. Cell death or ER de-structuring associated with haustorium penetration.

Confocal microscopy imaging revealed that occasionally, sunflower cells in the vicinity of haustoria cells were either devoid of fluorescence (**a, b**, star) or showed a destructured ER (**c, d**, asterisk), suggesting cell death. **e**. Using bright field microscopy, similar events were observed as destructuring of the vacuole (blue smear, asterisk) or light blue material filling the cell (star). These observations suggested rare occurrence of dying or dead cells in contact or in the vicinity of the haustorium.

Scale bar = 10 μm (**a, b**); 50 μm (**c-e**).

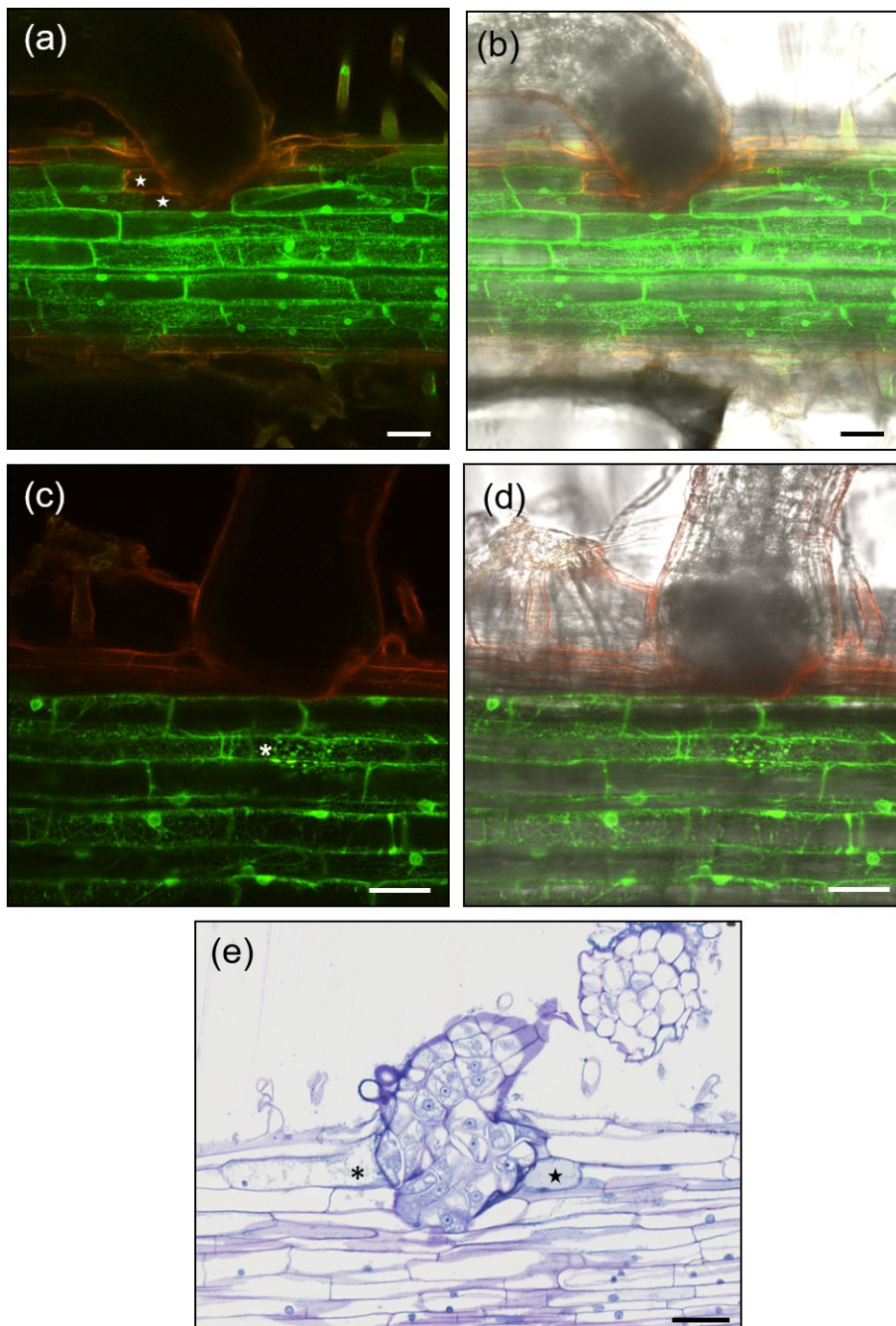


Fig. S5. Multiple divisions in the host root at the site of broomrape intrusion.

Lower magnification image of the section in **Fig. 1j**, showing a wider zone of the root. Cell division was observed around the intrusive cells (arrowheads). Anticlinal divisions affected the host cortex on both sides of the root (black square brackets). Periclinal divisions were visible in the pericycle (red square brackets).

Scale bar = 100 μm .

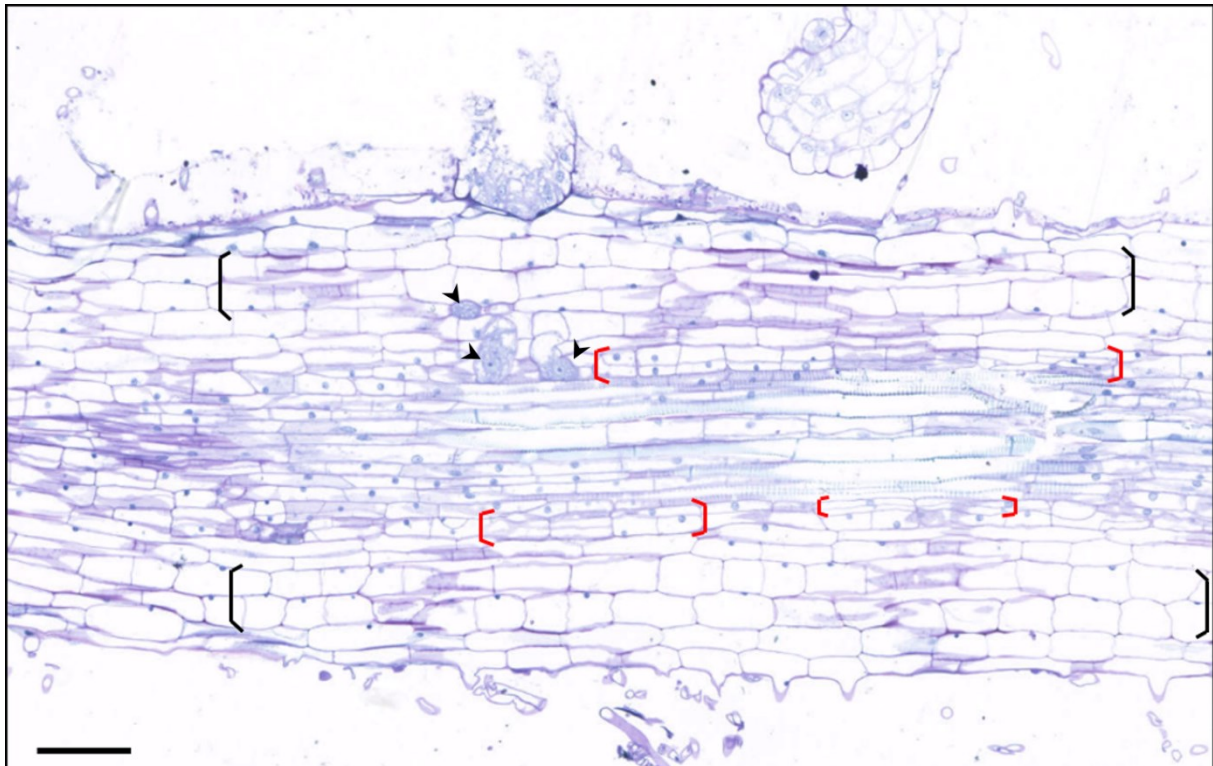


Fig. S6. Transmission electron microscopy of a 7 dai attachment.

The attachment shown in Fig. 2 was sectioned *c.* 9 μm further. **a.** General view of the attachment using large field microscopy. **b-d.** TEM.

a. Intrusive cells showed a dense cytoplasmic content and a big nucleus (arrow), containing a large nucleolus (dark blue). **b.** Assembly of 3 TEM pictures at the interface of the penetrating intrusive cells. The periclinal cell wall of the host cortical cell contacted by the tip of the haustorium was deformed (stars), potentially digested by parasitic pectolytic enzymes. By contrast the parasitic cell wall surrounding the intrusive cells (dotted line) appeared intact and continuous. The plasmalemma of the host cell showed convolution, suggesting active membrane synthesis, preparing the apoplastic compartment for subsequent accommodation of the haustorium. Electron dense granules were present along the host plasmalemma. **c, d.** One of the penetrated cortical cells, adjacent to the attachment, showed abnormal accumulation of granular material inside the vacuole. Electron microscopy of this cell indicated disruption of the tonoplast and plasmalemma as well as cytoplasm degradation, likely causes of cell death. Scale bar = 50 μm (**a**); 5 μm (**b**); 1 μm (**c-d**).

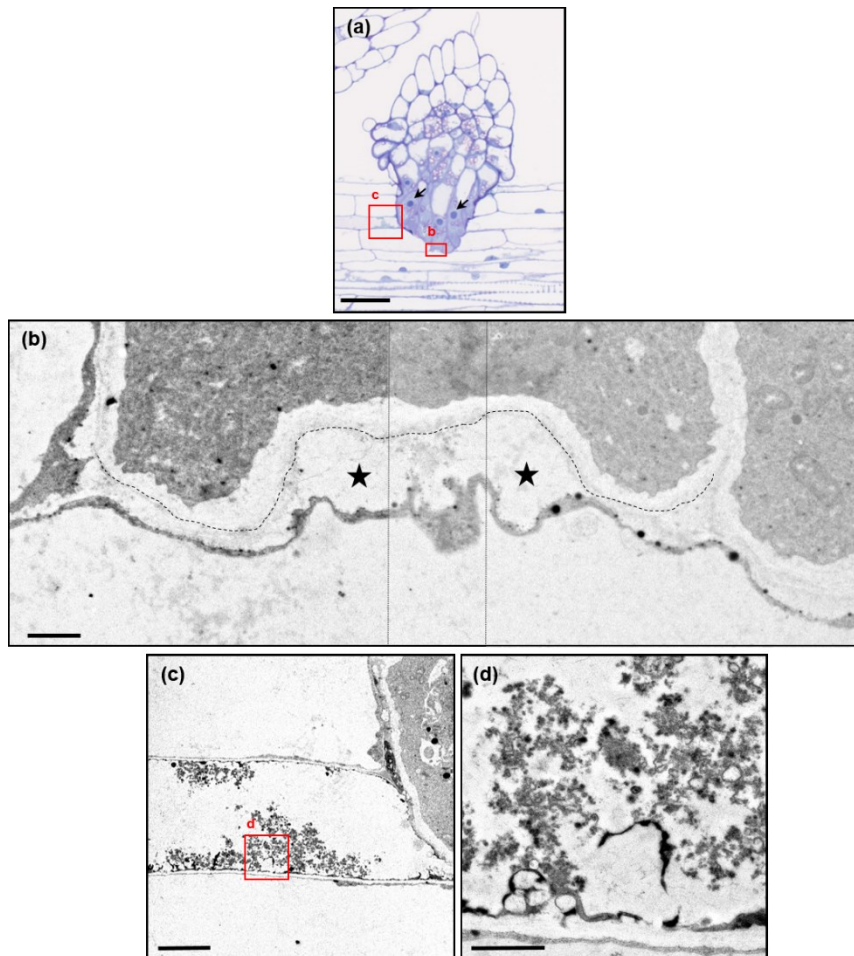


Table S1. Efficiency of sunflower transformation via *A. rhizogenes*.

Four experiments of sunflower transformation were performed, using the p35S-GFP-ER construct (**Fig. S1**). Transgenic fluorescent roots were observed using a stereomicroscope equipped with a Long Pass GFP filter, which allowed to discriminate transgenic green roots from non-transformed orange auto-fluorescent roots. Plants were counted as transformed when at least one green fluorescent root had developed.

NA: data not available. In experiment No.1, composite plants were too developed and consequently transgenic roots were intermingled and difficult to distinguish from one another.

experiment	number of plants	% of transformed plants	number of fluorescent root/ transformed plant	% of fluorescent root/ transformed plant
No.1	20	95	NA	NA
No.2	7	86	4,6	63
No.3	9	100	5,4	51
No.4	10	100	4,9	49

Table S2. Overview of observed sites.

Twenty five attachments (4 to 8 dai) from inoculated wild-type (non transformed) sunflower plants were scored using large field microscopy: 21 sites of penetration and 4 sites without penetration yet. Thirty eight attachments (5 to 7 dai) were scored under confocal microscopy on inoculated transgenic composite plants: 21 sites of penetration and 17 sites without penetration yet. Sites are organized according to time of observation (dai) and stage of colonization (*i.e.* deepest sunflower root cell layer reached by the intrusive cells).

Time	Stage of colonization	microscopy	
		large field	confocal
4 dai	no contact	1	
	epidermis	1	
5 dai	contact but no penetration		3
6 dai	contact but no penetration	3	10
	epidermis	1	1
	1st outer cortex	1	3
	2nd or 3rd outer cortex	1	8
	inner cortex	1	2
	vessels	1	
7 dai	contact but no penetration		4
	epidermis		1
	1st outer cortex	2	2
	2nd/ 3rd outer cortex	4	3
	inner cortex	3	1*
	vessels	1	
8 dai	1st layer outer cortex	2	
	2nd/ 3rd outer cortex		
	inner cortex	1	
	endodermis	1	
	vessels	1	
total number of sites		25	38

*: inner cortex or deeper