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ORIGINAL PAPER

Differential effects of ephemeral colonization by arbuscular mycorrhizal fungi in two *Cuscuta* species with different ecology

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Abstract Seedlings of parasitic *Cuscuta* species are autotrophic but can survive only a short period of time, during which they must locate and attach to a suitable host. They have an ephemeral root-like organ considered not a "true" root by most studies. In the present study, two species with contrasting ecology were examined: *Cuscuta gronovii*, a North American riparian species, and *Cuscuta campestris*, an invasive dodder that thrives in disturbed habitats. The morphology, structure, and absorptive capability of their root-like organ were compared, their potential for colonization by two species of arbuscular mycorrhizal fungi (AMF) was assessed, and the effect of the AMF on seedling growth and survival was determined. The root of both species absorbed water and interacted with AMF, but the two species exhibited dissimilar growth and survival patterns depending on the colonization level of their seedlings. The extensively colonized seedlings of *C. gronovii* grew more and survived longer than non-colonized seedlings. In contrast, the scarce colonization of *C. campestris* seedlings did not increase their growth or longevity. The differential growth responses of the AMF-colonized and non-colonized *Cuscuta* species suggest a mycorrhizal relationship and reflect their ecology. While *C. gronovii* roots have retained a higher ability to interact with AMF and are likely to take advantage of fungal communities in riparian habitats, the invasive *C. campestris* has largely lost this ability possibly as an adaptation to disturbed ecosystems. These results indicate that dodders have a true root, even if much reduced and ephemeral, that can interact with AMF.

Keywords Parasitic plants · Dodder · Mycorrhizal fungi · Root · Function · Seedling survival

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Introduction

Cuscuta (dodders, Convolvulaceae) includes ca. 200 species of annual, obligate stem parasites with a sub-cosmopolitan distribution (García et al. 2014; Costea et al. 2015); they are found in nearly all terrestrial habitats (Yuncker 1932; Costea et al. 2015). The study of this genus is important for at least three reasons. First, similarly to other parasitic plants, dodders are keystone species in their ecosystems because they impact multiple trophic levels and may even alter the abiotic environment by altering biogeochemical cycling as well as nutrient and water availability (reviewed by Press and Phoenix 2005). Second, *Cuscuta* is one of the most economically important groups of parasitic plants, as infestation by some of its species can result in significant yield losses in numerous agricultural and horticultural crops (Parker and Riches 1993; Dawson et al. 1994; Costea and Tardif 2006). Last but not least, an estimated 30–50 % of dodder species require conservation measures (Costea and Stefanović 2009).

Seedling establishment is a crucial stage in the life history of annual plants because it affects the persistence and dynamics of their populations (Grubb 1977; Harper 1977). As for other plants, seedlings of *Cuscuta* face abiotic and biotic challenges (e.g., Maun 1994; Maestre et al. 2003; Isselstein et al. 2002), but in addition, they have to locate compatible hosts, circumvent their defenses, and successfully establish a haustorial connection with them (Dawson et al. 1994; Costea and Tardif 2006). This host "hunt" takes places under an implacable deadline; if seedlings cannot find a suitable host within a certain period of time, they will die (Dawson et al. 1994;

Costea and Tardif 2006). Thus, the non-parasitic (seedling) stage in *Cuscuta* represents a significant ontogenetic population bottleneck.

The survival of *Cuscuta* seedlings and factors affecting it have received little or no attention because most studies conducted during the autotrophic stage have concentrated on the parasite–host interactions (reviewed by Dawson et al. 1994; Costea and Tardif 2006). Survival periods are usually reported anecdotally (i.e., they did not result from a study): 8 days for *Cuscuta campestris* (Parker and Riches 1993), 13–19 days for *Cuscuta chinensis* (Marambe et al. 2002), 3 weeks for *Cuscuta europaea* (Heide-Jorgensen 2008), 4 weeks for *Cuscuta pedicellata* (Lyshede 1985), or up to 7 weeks for *Cuscuta gronovii* (Heide-Jorgensen 2008). While these diverging numbers suggest that seedling survival may be a species trait in *Cuscuta*, it is unknown why and how the seedlings of a certain species survive longer than those of others.

It has been suggested from previous structural studies that dodders lack a "true" root and that this organ is a modification of the shoot base (e.g., Haccius and Troll 1961; Truscott 1966; Sherman et al. 2008). As a result, *Cuscuta* seedlings are often referred to as "rootless" (e.g., Lanini and Kogan 2005; Runyon et al. 2006; Albert et al. 2010), generating the false idea that a root-like organ is entirely absent. In *Cuscuta*, the radicular organ lacks a root cap and meristem; it is devoid of exodermis, endodermis, and pericycle, and its vascular system is very simple (Truscott 1966; Lyshede 1986; Lee et al. 2000; Sherman et al. 2008). Several days after germination (e.g., 5 days in *C. gronovii*; Truscott 1966), the root-like organ starts to degenerate acropetally through an irreversible senescence-like process (Sherman et al. 2008). In general, the function of this organ as a root has been regarded as questionable (e.g., Haccius and Troll 1961; Truscott 1966; Sherman et al. 2008). Interestingly, one study (Khalid and Iqbal 1996) reported that the "underground part" of *Cuscuta reflexa* seedlings was colonized by arbuscular mycorrhizal fungi (AMF). Although Khalid and Iqbal's study (1996) had a limited scope and somewhat unclear methodology (no information was provided about the colonized organ, its structure, fungal identity, penetration, or colonization), it highlighted that, despite its short life, the root-like organ of *Cuscuta* is capable to interact with microorganisms. This observation led us to hypothesize that if *Cuscuta* seedlings are able to form a relationship with AMF, they may have a true root after all, and that a transitory relationship with mycorrhizal fungi may play a role in the differential survival of dodder seedlings.

Thus, the general aim of the present work was to explore the potential effects of AMF colonization on the growth and survival of *Cuscuta* seedlings. To achieve this, two *Cuscuta* species with different ecology were selected: *C. gronovii*, a common riparian dodder in North America (Costea et al. 2006a; Costea and Tardif 2006), and *C. campestris*, a nearly cosmopolitan invasive weed of North American origin (Holm et al. 1997; Costea et al. 2006b). The morphology, structure, and absorptive capability of their root-like organ were compared, their potential for colonization by two species of arbuscular mycorrhizal fungi (AMF) was assessed, and the effect of the AMF on seedling growth and survival was determined.

Material and methods

Seed collection and plant growth conditions

Seeds of *C. gronovii* were collected in 2012 from a natural population growing on the banks of the Grand River (43° 30′ 12.02" N, 80° 29′ 37.97" W), Ontario, Canada; the host plant was *Solidago canadensis*. *C. campestris* seeds were sampled from a herbarium specimen [USA, CA, Sonoma Co., Rosa, 38° 13′ 26.60" N, 122° 49′ 42.10" W, abundant on *Xanthium strumarium*, Sep 2007, *Cadman* et al. *2832*]. To propagate the latter, seeds were germinated on wet filter paper in Petri plates, and seedlings were transferred to pots containing the hosts *Ocimum vulgare* and *X. strumarium*. The seeds of *C. campestris* thus produced were used in the following experiments. Herbarium vouchers for both species are kept in the Wilfrid Laurier University Herbarium. Harvested seeds were dry stored in the fridge at 4 °C in glass vials.

Seeds were surface sterilized and scarified using the methodology of García et al. (2006) with the difference that the treatment with 3 % active chlorine solution (i.e., bleach) was prolonged to 3 min. Sterilized seeds were kept at 35 °C overnight in sterile water to initiate scarification. The next day, they were submerged in 18 M sulfuric acid for 2 h, washed several times with sterile cold water, soaked in 3 % active chlorine solution for 30 s, and rinsed with sterile water 6–8 times afterwards. For the morphology and physiology studies, seeds were germinated on sterile filter paper in round Petri plates (diameter, 15 cm) and harvested when necessary (see below). For the study of AMF colonization, seeds were planted into square pots (10 cm) filled with PRO-MIX®BX MYCORRHIZAE™ (Premier Tech Ltd.; Rivière-du-Loup, Québec, Canada), a commercial mycorrhizal substrate containing *Rhizophagus intraradices* (also known as *Glomus intraradices*; Krüger et al. 2012) propagules. The pots were placed in a Biotronette growth chamber 850H under the following conditions: 22/30 °C, 8/16 h, dark/light (light intensity 39.6 μmol m^{-2} s^{-1}) respectively. Alternatively, some seeds were grown in plates of chicory (*Cichorium intybus*) root organ culture (ROC) consisting of Ri T-DNA transformed chicory roots colonized with *Rhizophagus irregularis* (also known as *Glomus irregulare* DAOM 197198; Krüger et al. 2012). New ROC plates (15 cm Petri dishes) were prepared by transferring segments of mycorrhizal chicory roots onto freshly made M medium (Bécard and Fortin 1988) solidified with

5 g Phytagel® (Sigma-Aldrich, Oakville, ON, Canada). The plates were sealed with Parafilm® strips and kept at 22 °C in the dark until they were needed. *Cuscuta* seeds were placed on the ROC and pushed gently until they were levelled with the Phytagel® surface; they were positioned in an area where fungal hyphae were visible. To facilitate germination, one drop of sterile water was added on top of each seed. The plates were then sealed with Parafilm® and kept in the same conditions as above.

Morphology, structure, and ability of *Cuscuta* root-like organ to absorb solutes

For each *Cuscuta* species, 15 embryos were dissected from rehydrated seeds and examined using a Nikon SMZ1500 stereomicroscope to compare their morphology; special attention was given to their radicular ends. Since Sherman et al. (2008) had reported that 1-day-old seedlings provided structurally the most consistent samples, 15 seedlings of each species were harvested at this age to study the root. For light microscopy (LM), 15 roots fixed in 50 % ethanol were embedded in LR White using a method modified from O'Brien and McCully (1981), where the infiltration time was increased to 3 h. The roots were transversally sectioned at 2 μm thickness with a Sorvall MT-1 ultra-microtome. Sections were mounted on glass slides and stained with 0.05 % toluidine blue O (TBO) pH 4.4 for 1 min. To study their ultrastructure, the roots of 1-day-old seedlings were fixed in 3 % glutaraldehyde and 2 % paraformaldehyde in 0.025 M sodium phosphate buffer (pH 6.8). The samples were then dehydrated through an ethanol series and embedded in Spurr's resin (Ma and Peterson 2000). The samples were transversally cut with a diamond knife at 0.5–1 μm thickness for LM and 80–100 nm thickness for transmission electron microscopy (TEM). Sections were mounted on glass slides for LM and on Formvar and carbon-coated copper grids for TEM observations. They were stained with Epoxy Tissue Stain (Electron Microscopy Sciences, catalogue number 14,950; a mixture of Toluidine blue and basic fuchsin; pH 8.2) and 5 % uranyl acetate for 10 min and Reynolds lead citrate for 5 min (Reynolds 1963), respectively. Observations were made with a JEOL 2011 Transmission Electron Microscope at 200 kV, and images were taken with a Gatan Ultrascan digital camera supplemented with "Digital Micrograph" software (Gatan Inc. 2007, Pleasanton, CA).

To test the ability of the two *Cuscuta* species root to absorb, the roots of 15 1-day-old seedlings were placed for 12 h in 1.5 ml Eppendorf tubes filled with 5 % Brilliant Blue FCF (commercial blue food dye and colorant # 2; McCormick Canada), a non-toxic tracer of solutes (e.g., Flury and Flühler 1995; Mader et al. 2003). Samples were washed with water to remove the excess stain, mounted in water, and observed under LM.

AMF colonization

C. gronovii and *C. campestris* seeds were planted into three pots filled with PRO-MIX®BX MYCORRHIZAE™. To ensure that a sufficient number of seeds germinated simultaneously to provide seedlings of an identical age, five seedlings were grown in a pot, but only two were chosen for observations; these were harvested at 10 days after emergence. The experiment was performed three times so that the total number of studied seedlings was 18 per species. To assess AMF colonization, roots of inoculated plants were stained with an ink-vinegar method modified from Vierheilig et al. (1998). Roots were gently washed with water and cleared with 10 % KOH aqueous solution for 8 min at 90 °C. Once cleared, the samples were covered with 10 % aqueous vinegar for 2 min and then incubated for 8 min in 5 % Sheaffer ink (Black, Sheaffer; BIC USA Inc.) in acetic acid (*v*/*v*) at 90 °C, after which the staining solution was replaced with 5 % vinegar for 10 min. Samples were washed twice with water and mounted in 50 % glycerol. To confirm fungal penetration and colonization of *Cuscuta* roots, observations were also made on seeds of *C. gronovii* and *C. campestris* grown on ROC plates of *R. irregularis*. Seedlings were harvested 10 days after germination and stained for fungal colonization as above. All prepared samples were observed under LM. The spatial distribution of *R. intraradices* inside the root and the rate of AMF colonization were determined with a 10-mm square reticle. The length of the reticle was considered as a single unit consisting of 10 columns of 1 mm each. The root-like organ was divided into reticle units starting from its distal end. In addition, one reticle unit covered the adjacent shoot part. The root and neighboring shoot units were examined and scored for the presence (1) or absence (0) of fungal hyphae in each reticle column. The numbers were then added to estimate the fungal colonization for each unit and then for each morphological region.

Assessment of seedling growth and survival

To assess seedling growth, seeds of both *Cuscuta* species were planted in ten pots: five filled with PRO-MIX®BX MYCORRHIZAE™ substrate (*R. intraradices*) and five with autoclaved PRO-MIX®BX MYCORRHIZAE™ substrate. A bacterial sieving of the inoculum was not added to the autoclaved control substrate because PRO-MIX®BX MYCORRHIZAE™ has no added beneficial bacteria. A separate experiment was conducted to verify the sterilization success of the PRO-MIX®BX MYCORRHIZAE™ substrate, and it was confirmed that no root colonization occurred in seedlings grown in autoclaved PRO-MIX®BX MYCORRHIZAE™ substrate. Pots were randomly placed in the growth chamber. Three seedlings from each species were sampled every 4 days, up to 20 days after emergence for *C. gronovii* and up to 16 days after emergence for

C. campestris. The length and the dry weight of seedlings were measured. This experiment was repeated three times. At each harvesting date, a test with Neutral Red (NR, PFAL TZ and BAUER, Inc., 375 Fairfield Ave. Stamford, Conn, USA) was performed to assess seedling viability (Dubrovsky et al. 2006; Timmers et al. 1995). The entire seedlings were incubated in 0.4 μmol (pH 5.5) NR aqueous solution for 2 h and then mounted directly in this solution; their roots and shoots were observed using fluorescence microscopy (filter combination R NX 96321).

To study the effects of AMF colonization on the survival of seedlings, seeds of *C. gronovii* and *C. campestris* were potted in the same conditions as above. A total number of 60 seedlings (30 colonized and 30 non-colonized) per species were selected as statistically representative and the experiment was repeated twice. The lifespan of seedlings was determined as the number of days until seedlings died. "Living" seedlings were those with an intact, green shoot tip, while "dead" ones were those with brown, dried, and deformed shoot tips. Observations were conducted with a Nikon SMZ1500 stereomicroscope.

Statistical analyses

Analysis of variance (ANOVA) using the statistical software Minitab version 16.2.4.3 was performed on the fungal colonization data of the two *Cuscuta* species using either values obtained for the entire root or from specific regions (e.g., absorbent hairs area, swollen area, adjacent shoot area) and particular reticle units (e.g., of the absorbent hairs area). ANOVA was also used to analyze the effect of AMF presence/absence on seedling growth (length and dry weight) of the two species. Growth trends were fitted with a quadratic polynomial equation using the computed R^2. It was not possible to statistically compare the effect of AMF colonization between the two species because the seedlings of the two species have a different life span. Instead, the life span between AMF-colonized and non-colonized seedlings was compared within each species using the Student's *t* test.

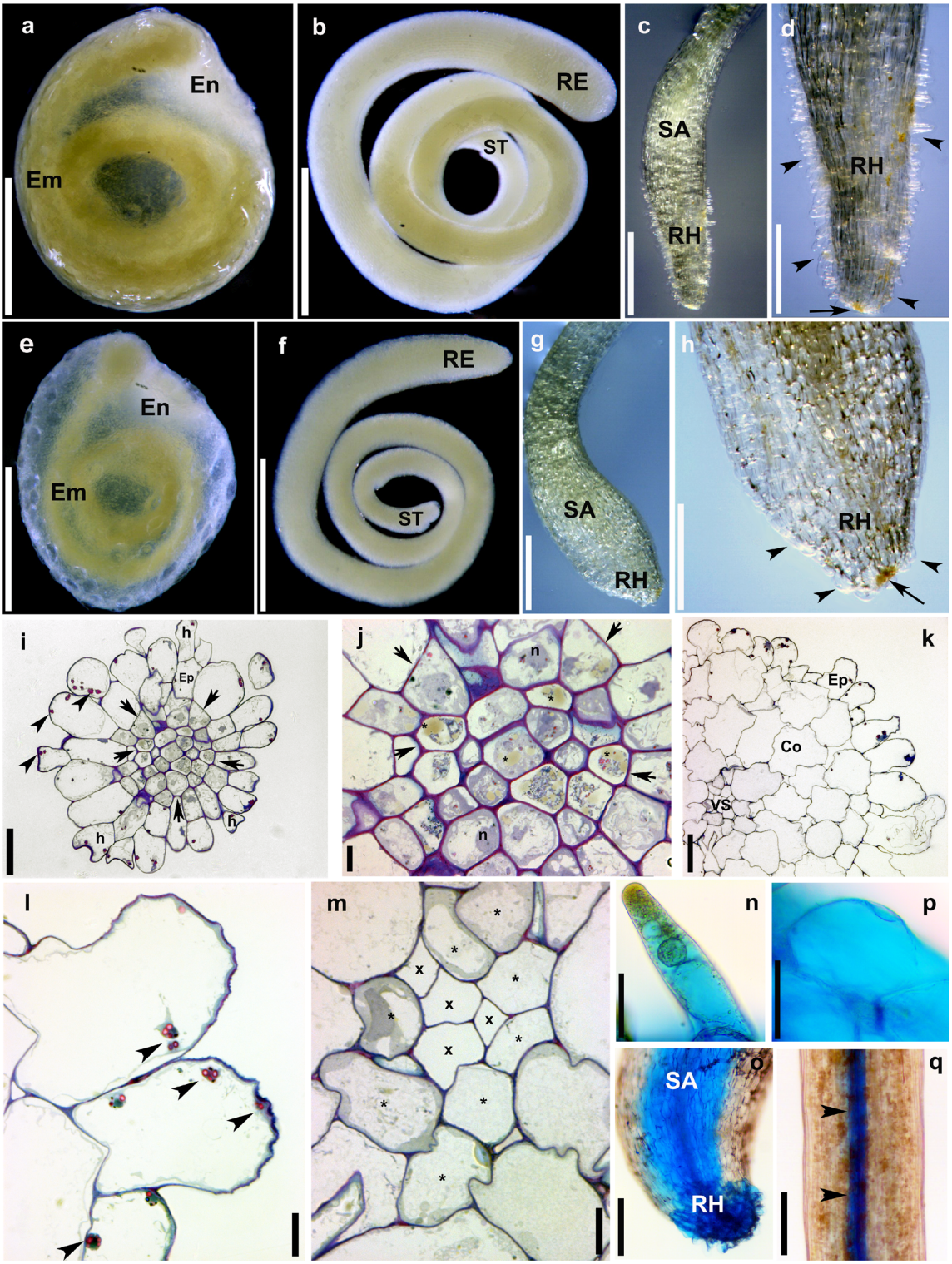
Results

Morphology, structure, and ability of *Cuscuta* roots to absorb

The embryos of both *Cuscuta* species are morphologically identical. They are filiform and coiled two to three times in the endosperm (Fig. 1a, e), which is entirely consumed during germination. Thus, the size of the seeds and the amount of endosperm present in them most likely do not influence the survival of the seedlings. The root-like organ of *C. campestris* emerges from the seed 2 to 3 days after sowing and 1 or 2 days later for *C. gronovii*. Simultaneously, the shoot grows within the empty seed coat, which is discarded soon afterwards. The entire seedling exhibits a minimal morphological differentiation of the traditional organs, i.e., root and shoot, and there is no sharp morphological boundary between the two; there is no hypocotyl or epicotyl because *Cuscuta* seedlings are devoid of cotyledons (Fig. 1b, f).

The root-like organ consists of two morphological areas: the absorbent hairs area and the tuberous area referred to here as root hair (RH) region and swollen area (SA), respectively (Fig. 1c, g and Fig. 2). Furthermore, at the distal end of the RH region, a group of darker-colored, tightly adhering cells is visible even under the stereomicroscope (Fig. 1d, h). Cross-sections through this region revealed that most of the cells in this group are alive in 1-day-old seedlings (Fig. 1i and Fig. S1). These cells possess thick pectinaceous walls, a large nucleus, dense cytoplasm, plastids with starch grains, mitochondria, smooth endoplasmic reticulum, lipid droplets, and one or two vacuoles (Fig. 1j and Fig. S1). The RH region is, on average, three times longer in *C. gronovii* (1540 μm) than in *C. campestris* (470 μm) (Fig. 1c, g and Fig. 2a). Its structure is quite simple: an epidermis with the absorbent hairs, a parenchymatous cortex, and a central vascular strand (Fig. 1k). In *C. gronovii*, nearly all the epidermal cells develop into root hairs (Fig. 1d) whereas in *C. campestris* root hairs are scarce

Fig. 1 Embryos and roots of **a**–**d** *Cuscuta gronovii* and **e**–**h** *C. campestris*. **a**, **e** Embryos (*Em*) enveloped in the endosperm (*En*). **b**, **f** Embryos coiled from their radicular end (*RE*) to their shoot tip (*ST*). **c**, **g** Root composed of root hair region (*RH*) and of swollen area (*SA*). **d**, **h** The RH region of **d** *C. gronovii* possesses elongated root hairs (*arrowheads*); that of **h** *C. campestris* is much shorter and exhibits dome-like root hairs (*arrowheads*). The RH region terminates in both species with a small group of colored cells (*arrow*) which is interpreted as a remnant of root cap. **i**, **j** Transversal section of a 1-day-old root tip of *C. gronovii* embedded in Spurr's resin and stained with Epoxy Tissue Stain. **i** The cells at the center of the tip are organized in a circular manner (*delineated by the arrows*). These putative root cap cells are alive and contain a dense cytoplasm. Towards the outside are highly vacuolated epidermal cells (*Ep*), **h** some of which have already developed into root hairs; amyloplasts (*arrowheads*) may be seen at the periphery of these cells. **j** Higher magnification of the central cells; the *arrows* are located at the exact same place as in **i**. Cells are tightly joined by a thick, pectinaceous cell wall and contain nuclei (*n*) and large lipid droplets (*asterisk*). **k**–**m** Transversal section of a root of a 1-day-old seedling of *C. gronovii* embedded in Spurr's resin and stained with Epoxy Tissue Stain. **k** Low magnification of the root hair region consisting of a layer of epidermal cells (*Ep*), several layers of cortical cells (*Co*), and a central vascular strand (*VS*). **l** Highly vacuolated root hairs with amyloplasts (*arrowheads*). **m** Detail of the vascular strand in which tracheary-like cells (*X*) are surrounded by cells (*asterisks*) with a dense cytoplasm and a large vacuole. **n**, **q** Photographs illustrating root absorption. **n** An elongated root hair of *C. gronovii* and **p** a dome-shaped root hair of *C. campestris* absorbing the stain (*blue color*). **o** Whole mount of *C. campestris* root illustrating the absorption of the stain at the root hair (*RH*) region. It is difficult to distinguish the proper path of the stain in the swollen area (*SA*) region. **q** Whole mount of a *C. gronovii* shoot illustrating the stain transported through the vascular strand (*arrowheads*). Scale bars: 1 mm in **a**–**c** and **e**–**g**; 500 μm in **d**, **h**, **q**; 100 μm in **o**; 50 μm in **i**, **k**, **n**, **p**; 10 μm in **j**, **l**, **m**



(Fig. 1h). In addition, while the root hairs of *C. gronovii* are elongated, cylindrical, and reminiscent of those present on the roots of typical higher plants (Fig. 1d), those of *C. campestris* are dome-shaped or globular (Fig. 1h). Plastids with starch grains are present both in the root hair cells (Fig. 1l) and the cortex (data not shown). A typical endodermis is absent, but the cells bordering the vascular system have a denser cytoplasm (Fig. 1m). Phloem is absent from the vascular system, which consists of a strand of empty vessel elements with non-lignified, primary cell walls (Fig. 1m). As for the SA region, it

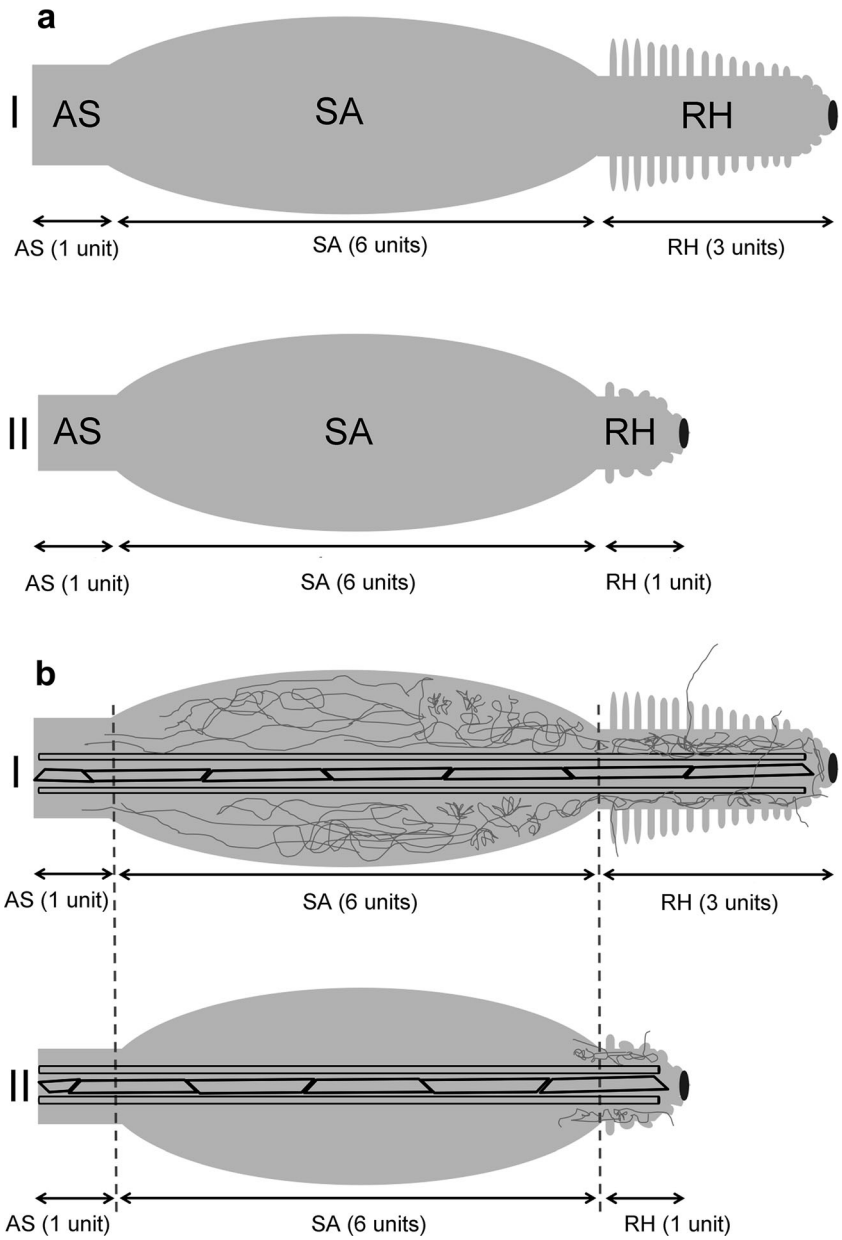


Fig. 2 Conceptual schematic diagram of roots, whether non-mycorrhizal (**a**) or mycorrhizal (**b**) of *Cuscuta gronovii* (I) and *C. campestris* (II) with the adjacent shoots (*AS*). Each root includes a root hair (*RH*) region and a swollen area (*SA*). The size of the root is based on reticle units (see "Material and Methods"), and their quantitative measurements are given below each root. For the simplicity of the diagram, the vascular strand has been omitted in **a**. Fungal penetration occurs in the RH regions of the two species (**b**). In *C. gronovii* (I), fungal hyphae colonize the cortex of both the RH and SA regions. The hyphae may also enter in the distal part of the adjacent shoot. In *C. campestris* (II), fungal hyphae colonize the cortex of the short RH region but just barely penetrate the AS region

differs from the yellow-green shoot only in its swollen appearance and its yellow-cream color. Its structure is similar to that of the RH region, but root hairs are absent, cortical cells are very large, and the vascular tissue consists of both xylem and phloem (data not shown).

Despite its minimalistic, reduced structure, the root-like organ of both species is capable of absorption. The dye solution was taken up by the root hairs (Fig. 1n–p) passed throughout the cortex (Fig. 1o), entered into the xylem elements, and was channelled upward to the shoot (Fig. 1o, q).

AMF colonization

The penetration and the incipient stages of AMF colonization were observed 4 days after seedling emergence in *C. gronovii* and 6 days after emergence in *C. campestris*. In both species, penetration of the fungus occurred through the root hairs (Fig. 3b) or typical epidermal cells (Fig. 3c, n). However, the two species exhibited differential qualitative and quantitative patterns of AMF colonization, which became most obvious 10 days after germination (Fig. 2b). Moreover, while *C. gronovii* roots were colonized by both *R. intraradices* and *R. irregularis* (PRO-MIX®BX MYCORRHIZAE™ substrate and ROC plates, respectively), *C. campestris* roots were colonized only by *R. intraradices* in the commercial substrate and not by *R. irregularis* when grown on ROC plates.

In *C. gronovii* growing in the commercial substrate containing *R. intraradices*, after penetration, the hyphae spread profusely within the intercellular spaces of the RH region (Fig. 2b I, reticle units 1–3). Subsequently, hyphae extended

rapidly into the cortex of the SA region (Fig. 3a), and some even reached into the adjacent shoot portion (Fig. 2bI, units 4–10). Hyphae were able to enter the cortex, where they branched, became thinner, and formed an intracellular structure somewhat reminiscent of coiled hyphae or an incomplete arbuscule (Fig. 3d, e). However, the hyphae never filled the entire cell lumen (Fig. 3d). In the ROC system, with mycorrhizal chicory roots (Fig. 3f), the *R. irregularis* hyphae were attracted and grew towards the root of *C. gronovii* (Fig. 3g); fungal penetration occurred as for *R. intraradices*. The hyphae extended intracellularly into the cortex of the entire root (Fig. 3h, i), and as characteristic of AMF, they branched at sharp angles (Fig. 3j) within the intercellular space, following the contour of the cortical cells. When intercellular hyphae penetrated into cortical cells, they formed an intracellular coil-like structure (Fig. 3k, l). Vesicles, spores, or typical arbuscules were not observed in seedlings grown either with *R. intraradices* within PRO-MIX®BX MYCORRHIZAE™ or with *R. irregularis* in the ROC system.

As for *C. campestris*, initial colonization by *R. intraradices* was similar to that of *C. gronovii*. When seedlings were colonized, hyphae kept mainly to the region of absorbent hairs (Fig. 2b II, reticle unit 1), with only a few hyphae entering the adjacent portion of the SA region (Fig. 2b II, reticle unit 2). No hyphae were ever observed in the remaining part of the swollen area or in the shoot (Fig. 2b II, reticle units 3–9). The hyphae of *R. intraradices* penetrated the root in the RH region (Fig. 3m), and the intracellular hyphae formed structures which resembled "attempted" arbuscules (Fig. 3o).

AMF colonization levels were significantly higher in *C. gronovii* than in *C. campestris* ($P \leq 0.001$) at the level of the entire root (Fig. 4a). The same significant difference in colonization was seen when the two morphological regions, RH and SA, were compared independently between the two species (Fig. 4b, c, respectively). Only the colonization level of the adjacent shoot unit did not differ significantly between the two species ($P=0.12$), despite the fact that only *C. gronovii* contained hyphae in this area (data not shown). The higher level of AMF colonization of the RH region in *C. gronovii* is attributable to the increased length of this region in this species (Fig. 2b I); while one reticle unit was enough to cover the entire absorbent hair area of *C. campestris*, three units were necessary in *C. gronovii* (Fig. 2b). When colonization levels in the one reticle unit of *C. campestris* were compared to only one of the three units of *C. gronovii*, differences between the two species were not significant (data not shown). In contrast, because the swollen area has a similar length in both species (covered by six reticle units), the significantly higher colonization level obtained for *C. gronovii* was the result of a higher density of hyphae. In *C. gronovii*, the highest level of AMF colonization was observed in the swollen area, followed by the absorbent hair area and the adjacent shoot region. In *C. campestris*, the fungi were restricted to the region of absorbent hairs (Fig. 2bII).

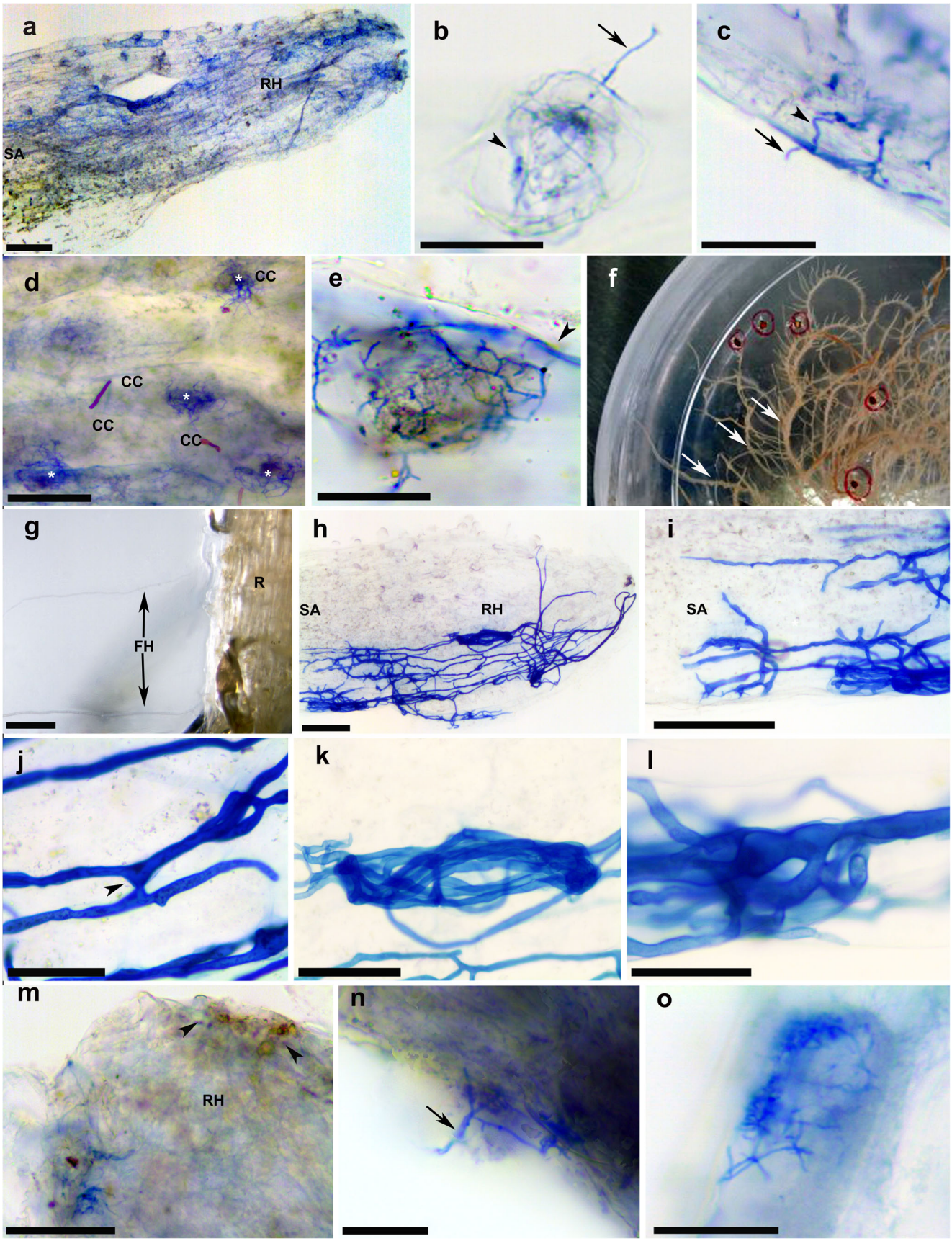
Effect of AMF colonization on seedling growth and survival

Not only the two *Cuscuta* species differed in their AMF colonization levels (Fig. 4a–c), but they also exhibited dissimilar growth (Fig. 4d–g) and survival patterns (Fig. 4h–i), depending on whether seedlings were colonized or not by the AMF. Colonized seedlings of *C. gronovii* were significantly longer than non-colonized ones ($P<0.01$ at day 20; Fig. 4d). Both AMF-colonized and non-colonized seedlings attained their maximum length 12 days after emergence (30.58 and 24.28 cm, respectively) at which time the colonized seedlings were ca. 20 % longer than the non-colonized ones. No further increase in length occurred in older seedlings (Fig. 4d). The dry weight (DW) of both AMF-colonized and non-colonized *C. gronovii* seedlings increased by day 4 (Fig. 4e). Whereas the DW of the latter declined abruptly, those of the former continued to increase slowly until day 8 with a gradual reduction afterwards (Fig. 4e). These dissimilar growth patterns resulted in significant DW differences between colonized and non-colonized seedlings at 8 and 12 days after emergence ($P<0.01$ and $P \leq 0.001$, respectively). As seedlings aged, these DW differences became insignificant (e.g., at day 20; Fig. 4e) because of the progressive degeneration of the root and basal parts of the shoot.

The seedling growth indicators (length and DW) for *C. campestris* were not significantly different between AMF-colonized and non-colonized seedlings at any harvest time (Fig. 4f, g). For example, the length of colonized seedlings at day 16 (12.21 cm) did not differ significantly ($P=0.311$) from that of non-colonized seedlings (11.74 cm) (Fig. 4f). The DW increased by day 4 after emergence and then decreased similarly in both colonized and non-colonized seedlings (Fig. 4g).

Seedling longevity of the two *Cuscuta* species was affected differently by the presence/absence of AMF. In *C. gronovii*, colonized seedlings lived, on average, 2 days and a half longer than non-colonized seedlings (20.53 versus 18.13 days, respectively), a difference that was statistically significant ($P<0.01$; Fig. 4h). In contrast, the lifespan of *C. campestris* seedlings was not affected by AMF colonization, and the differences between colonized and non-colonized seedlings were insignificant. The average lifespan of *C. campestris* seedlings reached a maximum of 15.5 days, regardless of the mycorrhizal condition. Although not compared statistically, the longevity of *C. gronovii* seedlings was clearly higher than that of *C. campestris* in both AM and sterile substrates.

The duration of the root-like organ in the two *Cuscuta* species followed the same trend as the lifespan of their seedlings. In *C. gronovii*, the first signs of cellular degeneration were observed 8 days after emergence in AMF-colonized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◀ **Fig. 3** Colonization of a *Cuscuta gronovii* root by **a**–**e** *Rhizophagus intraradices* and **f**–**l** *R. irregularis*, and colonization of the *C. campestris* root by **m**–**o** *R. intraradices*. The fungal hyphae stained with the ink-vinegar method appear blue. **a** Low magnification of a root illustrating fungal colonization of the root hair (*RH*) region and the distal part of the swollen area (*SA*) region. The hyphae extend extensively into the intercellular space of the cortex. **b**, **c** An extraradicular hypha (*arrow*) entering the root via either **b** a root hair or **c** an epidermal cell; in neither case, a hyphopodium is present. Once inside the root, the hyphae (*arrowhead*) branched. **d** Once in the SA, the fungal hyphae enter the cortical cells (*CC*) and form in each cell a structure reminiscent of a hyphal coil or an incomplete arbuscule (*asterisk*). **e** These AMF structures do not take the appearance of a true arbuscule. **f** *Cuscuta gronovii* seeds (*surrounded by a red circle*) on ROC plates in the vicinity of transformed chicory roots (*arrows*) which served as a host to the fungus *R. irregularis*. **g** Fungal hyphae (*FH*) growing towards the root (*R*) of a germinated seedling. **h**, **i** The fungus has entered the cortex of **h** the RH region and is spreading via intercellular spaces towards **i** the SA region; hyphal coils are present in some cortical cells. **j** As the fungal hypha spreads through the intercellular space, it delineates clearly the shape of the cells it surrounds and, characteristic of mycorrhizal fungi, branching occurs at sharp angles (*arrowhead*). **k**, **l** Higher magnification of two hyphal coils seen in **h**. (**k**) The hyphal coil fills most of the cellular space. **l** The hypha not only coils but also branches. **m** *R. intraradices* colonizing the RH region of *C. campestris* seedling. Note the root cap-like structure (*arrowheads*) at the root tip. **n** An extraradical hypha (*arrow*) branches on the surface of the root and penetrates into an epidermal cell. **o** A cortical cell containing an arbuscule-like structure; the diffuse appearance of the hypha suggests profuse branching within the cell. Scale bars: 300 μm in **a**, **g**, **h**; 200 μm in **j**; 100 μm in **m**; 50 μm in **b**, **d**, **i**, **k**, **l**,; 25 μm in **c**, **n**, **o**; 20 μm in **e**

seedlings and 4 days in non-colonized seedlings (data not shown). Ultimately, the root organ of colonized seedlings lasted longer (12 days) than that of non-colonized ones (7–8 days). In *C. campestris*, the complete degeneration of the root-like organ was recorded at 12 days after emergence, irrespective of the AMF colonization status of its seedlings.

Discussion

Based on the present results, the root organ of *Cuscuta* can fulfil, even if for a short period of time and in an impaired fashion, the roles of a typical root, an idea rarely put forward by other studies (Lyshede 1989). The root hairs of *Cuscuta* are certainly not typical, especially in *C. campestris*, but they do absorb solutes, as suggested by Koch (1880) more than a century ago. In addition, these root hairs can serve as an entry point for AMF, as they do in other plants (Guinel and Hirsch 2000; Novero et al. 2009). This implies that this organ can most likely employ the root signaling pathway necessary for establishing an appropriate cross talk with these fungi (e.g., Oldroyd 2013; Gutjahr 2014). Sherman et al. (2008) compared the developing dodder seedling to a treadmill: As the root-like organ gradually degrades, the reserves stored in its tissues are translocated to the expanding shoot. However, the current study showed that for a short period of time, the non-parasitic seedlings of *C. campestris* and *C. gronovii* not only elongate their shoot but also grow as biomass, implying carbon accumulation and suggesting a degree, even short-lived, of functionality for both the root and the shoot. The morphological differences observed between the roots of the two species, i.e., in their root hairs and size of the swollen areas, are likely to have a functional significance. In this context, *C. gronovii* roots with a larger RH region area covered by numerous normal-looking root hairs exhibited increased AMF colonization as compared to *C. campestris* roots, with few and bulbous root hairs.

The current results confirmed most of the structural findings of previous studies on the root-like organ of *Cuscuta* (Haccius and Troll 1961; Truscott 1966; Lyshede 1986, 1989; Sherman et al. 2008). However, the present study found that the group of cells located at the extreme tip of this organ was alive in 1-day-old seedlings, which is not in agreement with the conclusions of Truscott (1966) who considered these cells as dead. Sherman et al. (2008) proposed that these cells may represent the end of the vascular system of the root-like organ. We suggest an alternative interpretation to that of Sherman et al. (2008): Based on their position, thicker cell walls and lipid content, this tissue may be a vestigial root cap.

From an evolutionary point of view, if this organ were to be a modification of the shoot as hypothesized previously (e.g., Haccius and Troll 1961; Truscott 1966; Sherman et al. 2008), then two successive steps must have taken place during the evolution of dodders to parasitism: (1) the complete loss of the ancestral root of a putative *Convolvulaceae* ancestor and (2) the evolution of a novel structure analogous to a root at the base of the shoot. A question then arises: Why would a short-lived and partially impaired analogous root structure evolve if the true root was not needed in the first place? Parsimoniously, it is better to consider that this organ is the result of a series of reductions and alterations of the (true) root of a morning glory ancestor in a fashion similar to what occurred in the photosynthetic apparatus and plastome of the *Cuscuta* shoot (reviewed by Braukmann et al. 2013). As a structural study cannot provide an answer *per se*, transcriptome analysis and gene expression studies are required to confirm the root–organ identity at a tissue level (e.g., Kaufmann et al. 2010; Wolf 2013). Until then, considering this organ as a true, even if reduced, root as proposed initially by Lyshede (1986) is probably appropriate and avoids the pitfalls of the term "rootless seedlings."

Khalid and Iqbal (1996) reported that spores, vesicles, and arbuscules formed in the "underground parts" of *C. reflexa* seedlings within 7 days. No spores or vesicles were observed in *C. gronovii* and *C. campestris* with either *R. intraradices* or *R. irregularis*. The two *Cuscuta* species examined formed dissimilar AM structures with the different *Rhizophagus* species, but these structures could not be confidently placed into any of the known mycorrhizal types (e.g., Peterson et al. 2003).

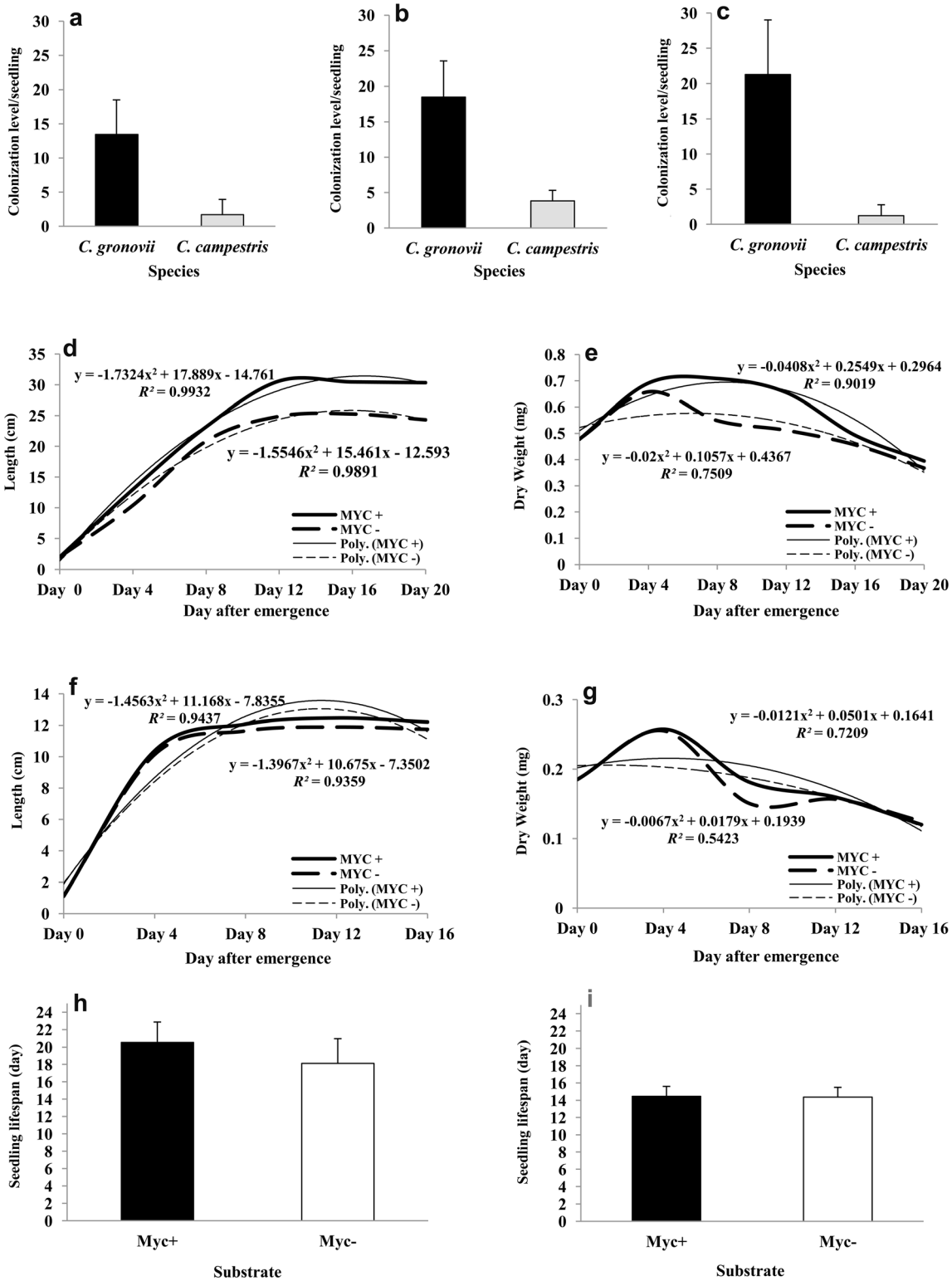

Fig. 4 Colonization levels of *Rhizophagus intraradices* (based on the distribution of fungal hyphae) in the entire root (**a**), the root hair area (**b**), and the swollen area (**c**). **d**–**g** Length (**d**, **f**) and dry weight (**e**, **g**) of seedlings of *Cuscuta gronovii* (**d**, **e**) and *C. campestris* (**f**, **g**) grown over time in the absence (Myc−) or presence (Myc+) of *R. intraradices*. Growth trends were fitted with quadratic polynomial equations with computed R^2. **h**–**i** Seedling lifespan of *C. gronovii* (**h**) and *C. campestris* (**i**) in Myc+ versus Myc− substrates

Although no structurally typical arbuscules were observed, both dodders formed AM structures with *R. intraradices* that may be interpreted either as an arbuscule "attempt" or as a hyphal coil structure reminiscent of AM in some orchids.

This differs from the case of AMF colonization of certain non-mycorrhizal plants (e.g., Veiga et al. 2013) because *C. gronovii* exhibited a positive growth response when colonized by *R. intraradices*. The absence of typical arbuscules and vesicles in *C. gronovii* and *C. campestris* may not be surprising as it is known that the plant partially controls the form and shape of the mycorrhizal structures it forms (Gutjahr 2014); thus, whereas *C. reflexa* would form typical mycorrhizal structures, *C. gronovii* and *C. campestris* may not. Phylogenetically, *C. reflexa* belongs to the most ancestral group of dodders (subgenus *Monogynella*; García et al. 2014; Costea et al. 2015), while *C. gronovii* and *C. campestris* are part of the most recently derived infrageneric group (subgenus *Grammica*; García et al. 2014; Costea et al. 2015). The presence of the full set of mycorrhizal structures in *C. reflexa* and their partial absence from *C. gronovii* and *C. campestris* may parallel multiple reductions (e.g., of the photosynthetic apparatus and root) that have accompanied the evolution from the more hemiparasitic species of subg. *Monogynella* to the holoparasitic species of subg. *Grammica* (Braukmann et al. 2013).

The oddness of the relationship that AMF establishes with *Cuscuta* comes from its ephemeral duration and the reduced root that hosts it. The rapid fungal colonization of the root cortex observed especially in *C. gronovii*, combined with the early dodder biomass accumulation, indicates that the relationship may be mutually beneficial in the beginning. This would be remarkable considering the limited photosynthetic capability of *Cuscuta* (e.g., Hibberd et al. 1998; Krause 2008) and the parasitic plant's own growth requirements during the seedling stage. Alternatively, the AMF colonization may rely on an external carbon source (e.g., from spores or propagules in the PRO-MIX®BX MYCORRHIZAE™ inoculum or the chicory roots in the ROC system). Regardless of the fungal source of carbon during the early stages, the host–fungus relationship likely loses its symbiotic nature when *Cuscuta* recycles components from its short-lived roots and translocates them to the shoot. One may wonder if *C. gronovii* also cheats on the fungus by recycling some of the fungal compounds (Lee et al. 2002; Koide and Mosse 2004; Parniske 2008).

As demonstrated in this study, the roots of *C. gronovii* and *C. campestris* exhibited different spatial and quantitative colonization patterns by the AMF, and the differential colonization was associated with dissimilar growth and survival rates of seedlings. The extensively colonized seedlings of *C. gronovii* were able to grow more and survive longer than non-colonized seedlings. In contrast, the scarce colonization of *C. campestris* seedlings did not increase their growth or longevity. Dodder seedlings are able to search for suitable hosts in the plant community (e.g., reviewed by Costea and Tardif 2006; Runyon et al. 2006), but their success depends on the presence of appropriate hosts within their reach. Two or three days of prolonged survival would allow a longer searching time and perhaps the eventual emergence of seedlings of suitable hosts in their proximity.

The differential AMF colonization of the two *Cuscuta* species may be related to their ecology. *C. gronovii* occurs in natural riparian habitats or mesic temperate forests, and it can become a weed only in crops cultivated in these types of habitats, such as cranberry (e.g., Costea and Tardif 2006; Sandler 2010). In contrast, *C. campestris* thrives in habitats with both ruderal and agricultural anthropomorphic disturbance (Dawson et al. 1994; Costea and Tardif 2006). As a result, *C. gronovii* is currently restricted in North America (Costea et al. 2006a), while *C. campestris* has spread to become the most widely distributed and aggressive invasive dodder pest worldwide (Dawson et al. 1994; Holm et al. 1997; Costea and Tardif 2006). As for typical mycorrhizal plants (e.g., Allen 1991; Taylor et al. 2004; De Long et al. 2013), the fungal relationship may play a significant role in the habitat specialization of different *Cuscuta* species. Numerous invasive plants are non-mycorrhizal or act as facultative macro-symbionts, which allows them to be more ecologically versatile and therefore more extensively distributed geographically (e.g., Pringle et al. 2009; Hempel et al. 2013). This could be the case of *C. campestris* which responded poorly to *R. intraradices* and not at all to *R. irregularis*, in contrast to *C. gronovii*. In the former species, evolution may have led to the loss of the survival boost provided by AMF accompanied by further reductions of the root organ and photosynthetic apparatus, whereas in *C. gronovii* evolution has ensured preservation of the beneficial fungal association.

Hemiparasitic plants such as *Pedicularis* are known to be mycorrhizal, and the mycorrhizal status has been reported to increase P availability (Li et al. 2013) serving as an alternative nutritional strategy when appropriate host plants are not available (Li and Guan 2008). However, when entering the realm of holoparasitic plants, the existence of a mycorrhizal relationship becomes debateable. In another study, de Vega et al. (2010) reported a complex, tripartite association between *Cytinus hypocistis* (*Cytinaceae*), an unknown AMF, and several host species from the *Cistaceae*, but the interpretation of this relationship remains controversial (Brundrett 2011; de Vega et al. 2011). Although the current study does not provide evidence for a balanced mycorrhizal relationship, it shows that *Cuscuta* seedlings benefited from AMF colonization of their roots. It has also demonstrated that *Cuscuta* species with different ecology respond differently to AMF. In general, the reductions and modifications that accompany the evolution to holoparasitism are so drastic (e.g., Heide-Jorgensen 2008) that even if present, a symbiotic relationship with AMF cannot be expected to possess the characteristics of typical mycorrhiza. More research is necessary to determine the limits of the relationship between AMF and *Cuscuta* roots and the putative transfer of nutrients between the partners, as well as to explore the evolutionary implications on the plant side.

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