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Comparative study of factors influencing seed germination and seedling longevity in Cuscuta (dodder, Convolvulaceae)

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Comparative study of factors influencing seed germination and seedling longevity in *Cuscuta* (dodder, *Convolvulaceae*)

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THESIS

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Abstract

Cuscuta (dodder), the only parasitic genus of *Convolvulaceae* family, is a significant plant from ecological, economical, and conservational point of views. The genus consists of *c.* 200 described species with wide ranges of ecological distributions. *Cuscuta* spp. are categorized as stem parasitic plants with reduced (or lost) photosynthesis-related genes. The filamentous coiled embryo, embedded in the endosperm, lacks cotyledons and is covered with a multiple-layered seed coat. The seedlings carry a vestigial root-like organ which is not considered a 'true' functional root. Members of the genus differ in their germination rate and seedling survival. It is known that seed germination, as a vital stage for seedling establishment, is influenced by the impermeable seed coat in most *Cuscuta* species. However, anatomical data are still lacking; for example, it is unknown how seed coats of *Cuscuta* species differ from one another, and if the differences (if any) affect the germination rates. In the seedling stage, *Cuscuta* lives for a short period of time if the plant cannot attach to a proper host(s). The seedling lifespan varies widely among *Cuscuta* species; however, the factors causing the variations have not received much attention. Thus, the aims of this study are: 1) to examine the structure of *Cuscuta* seed coats in relation to the germination success using three *Cuscuta* species, 2) to study the capability of *Cuscuta* vestigial root to absorb water and nutrients, and 3) to investigate factors affecting seedling growth and longevity, including mycorrhizal association, in two *Cuscuta* species. To achieve these goals, various microscopic techniques were employed to examine the structure of the seed coat. Furthermore, the interaction of two mycorrhizal fungal species with the roots and the effects of fungal colonisations on seedling growth and survival of two *Cuscuta* species were studied. Moreover, morphological, structural and absorptive capabilities of the root were investigated. Structural analysis of the seed coats suggested that palisade layers and the overall

seed coat thickness likely have significant effects on the germination rate. The absorptive capabilities and mycorrhizal associations of the *Cuscuta* seedling roots led me to consider the vestigial root-like organ of the *Cuscuta* seedlings as a 'true' root although it is an ephemeral organ. I noted that *Cuscuta* species interact differently with the fungal species causing dissimilarity in the growth and longevity of their seedlings. This suggests that the fungal specificity may play a role in the ecological distributions of *Cuscuta* species. The work presented in this thesis is significant because it brings to light a relationship between species survival and ecological distribution.

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Chapter 1

General Introduction

1.1 Parasitic plants

The total number of angiosperms is estimated to be *c*. 420,000 species (Heide-Jorgensen, 2008). Among them, *c*. 4500 species (*c*. 1%) belonging to 20 families are categorized as parasitic plants (Heide-Jorgensen, 2008), meaning that they rely on host plant(s) for their food acquisition (Kuijt, 1969; reviewed in Press & Phoenix, 2005). The invasion by parasitic plants occurs in the root or shoot of the host plant(s). It allows parasitic plants to acquire partial or total of their water, nutrients, and assimilated carbohydrates via the production of a specific organ called haustorium which forms a physiological bridge between the parasitic plant and the host plant(s) (Kuijt, 1969; reviewed in Press & Phoenix, 2005; Heide-Jorgensen, 2008). Parasitic plants play influential roles in their ecosystems as keystone species impacting the growth and development of their host plants (reviewed in Press & Phoenix, 2005). They also affect hosts' reproductive performance, and as a result, they alter the frequency of the plants within the plant communities, population dynamics, vegetation cycling and zonation (reviewed in Press & Phoenix, 2005).

1.2 *Cuscuta* **spp.**

Cuscuta, otherwise known as dodders, is the only parasitic genus within the *Convolvulaceae* family (Kuijt, 1969; Stefanović *et al*., 2002), consisting of *c*. 200 species and 70 varieties (Costea & Stefanović, 2010; Costea *et al*., 2015). Dodders are categorized as stem parasitic plants (Kuijt, 1969). Although photosynthesis-related genes have been reduced (or lost) as a result of the

evolution to parasitism, some species of *Cuscuta* are considered cryptically photosynthetic and may perform very reduced degree of photosynthesis (Van der Kooij *et al*., 2000; McNeal *et al*., 2007; Krause, 2008; Braukmann *et al.,* 2013). However, even the most active photosynthetic species belonging to subgenus *Monogynella* derive 99.5% of their carbon from their hosts and can be considered functionally holoparasitic (Jeschke *et al.*, 1994; Hibberd *et al.*, 1998), i.e., they entirely depend on their hosts.

1.3 Classification, ecology and distribution

Cuscuta is nearly cosmopolitan and it can occur from tropical to temperate regions (Dawson *et al.,* 1994). Based on morphological and anatomical differences, as well as phylogenetic evidence, the genus is subdivided into four subgenera, including *Grammica*, *Cuscuta, Monogynella* (Yuncker, 1932), and *Pachystigma* (García *et al.,* 2014; Costea *et al.,* 2015). The most abundant subgenus is *Grammica* which is found on all continents (except Antarctica), with the highest occurrence of its species in the Americas; the subgenus *Cuscuta* inhabits the Mediterranean regions from west of Asia to Africa and Europe; the subgenus *Monogynella* is mostly Eurasian; finally, the subgenus *Pachystigma* inhabits South Africa. The species that grow in Canada include: *Cuscuta gronovii*, *C. campestris* (often mistakenly referred to as *C. pentagona*), *C. umbrosa, C. epithymum, C. epilinum, C. pacifica* (previously known as *C. salina), C. cephalanthi, C. coryli, C. polygonorum, C. compacta, and C. indecora* (Scoggan, 1979; Costea *et al*., 2004). In particular, *C. gronovii* and *C. umbrosa* which are native species, as well as *C. campestris* which is an introduced species, may occur as weeds in Canada; *C. epithymum* and *C. epilinum* may also occur as weeds but only occasionally (Costea & Tardif, 2006).

Of interest to this study are 3 species that belong to the subgenus *Grammica*: *Cuscuta gronovii, C. campestris,* and *C. nevadensis*. *Cuscuta gronovii* grows in temperate and subtropical North America, and prefers riparian ecosystems (Costea & Tardif, 2006; Dawson *et al.,* 1994; Costea *et al.*, 2006a). It has three varieties: var. *gronovii* ‑ the focus of the present study ‑ var. *latiflora,* and var. *calyptarata* (Costea *et al.,* 2006a). *Cuscuta gronovii* var. *gronovii* is the most common species in North America (Yuncker, 1932; Costea *et al.*, 2006a). In Canada, in can be found in Ontario (the most common local *Cuscuta* species) (Crins & Ford, 1988), and in all provinces except Manitoba and Saskatchewan (Costea *et al.*, 2006a). *Cuscuta campestris* can grow in temperate and subtropical areas; however, it prefers agricultural ecosystems or habitats with a degree of anthropomorphic disturbance (Costea & Tardif, 2006). This species may be the most widespread and troublesome *Cuscuta* species in the world (Holm *et al.,* 1997; Costea *et al.,* 2006b). It is found in the U.S.A., as well as Europe, Asia, Africa, South America and Australia (Yuncker, 1932). In Canada, it occurs in Ontario, Alberta, British Columbia, Saskatchewan, Manitoba, Nova-Scotia and Quebec (Costea *et al.,* 2006b). *Cuscuta nevadensis* is a native species to southern U.S.A., with a viviparous nature, thus its germination occurs while the seeds are in the fruits (capsules), still attached to the parent plants (Costea *et al.,* 2005). It is found in California and Nevada (Costea *et al.,* 2005) and it inhabits desert regions.

1.4 Significance of the genus

Dodders are keystone species in natural ecosystems. They can reduce the host biomass, alter the host allocation patterns (De Bock & Fer, 1992), and change the structure of plant communities (reviewed in Pennings & Callaway, 2002). Some species of *Cuscuta* have been widely used for treating infertility problems in traditional Chinese medicine (Chao *et al.,* 2003). *Cuscuta chinensis* (Nisa *et al.,* 1985 as cited by Dawson *et al.,* 1994), *C. chilensis* (Backhouse *et*

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al., 1996), and *C. campestris* (Agha *et al.,* 1996) have an anti-inflammatory action, while *Cuscuta reflexa* has an antibacterial effect (Pal *et al.,* 2006). In contrast, some *Cuscuta* species are important weeds/agricultural pests. These dodder species invade agricultural or horticultural crops and they can have negative economic impacts (Dawson, 1989). For instance, *Cuscuta gronovii* may reduce the yield of cranberry crop by 80% (reviewed in Sandler, 2010). It can also infest a broad range of woody and herbaceous plants such as *Salix* and *Populus*, as well as carrot, mint, potato, and sweet potato (Parker & Riches, 1993; Costea *et al.,* 2006a). *Cuscuta campestris* is known as one of the most important weeds in 55 countries (Holm *et al.,* 1997). It can infest alfalfa (Yuncker, 1932; Parker & Riches, 1993), and when left uncontrolled, led to an 85% reduction of the yield (Dawson, 1989; reviewed in Dawson *et al.,* 1994; see Parker & Riches, 1993). *Cuscuta campestris* can also infest sugarbeet leading to *c*. 40% of root weight and *c*.15% of sugar content reductions (Parker & Riches, 1993; Tóth *et al.,* 2006), tobacco, red clover (80% field infestation), and many vegetables, including carrot, asparagus, and tomatoes (Parker & Riches, 1993; Kroschel, 2001; reviewed in Costea & Tardif, 2006). It may also infect some ornamental plants such as *Tanacetum* spp. (reviewed in Costea & Tardif, 2006). *Cuscuta nevadensis* is not a weedy species; its host range includes dicotyledonous shrubby plants such as *Graya spinosa*, *Lycium pallidum*, *Ambrosia dumosa*, *Psorothamnus fremontii*, and *Atriplex* sp. (Wallace *et al.,* 1976; Costea *et al.,* 2005).

Moreover, because the size and shape of *Cuscuta* seeds are similar to those of some crops (Kuijt, 1969), including small-seeded host legumes such as alfalfa and clovers, dodders can contaminate commercial seed lots (Kuijt, 1969; Musselman, 1982, 1986; Knepper *et al.,* 1990; Rowarth *et al.,* 1990), and this is considered the major cause that leads to the dispersal of *Cuscuta* species as agricultural pests (Knepper *et al.,* 1990). Despite the fact that only a relatively

reduced number of *Cuscuta* species are weedy or invasive (only *c*.15 *Cuscuta* species are weeds; Parker & Riches, 1993), and more species need to be conserved (Costea & Stefanović, 2009), all the *Cuscuta* spp. are classified as weeds throughout the world. Contaminated commercial seed lots with *Cuscuta* seeds are not permitted the entry into most countries including Canada (reviewed in Costea & Tardif, 2006). In Canada, *Cuscuta* sp. are categorized in block as noxious weeds in Ontario, Manitoba, British-Columbia and Quebec, and as restricted weeds in Alberta (Invader database system, 2013; reviewed in Costea & Tardif, 2006).

1.5 *Cuscuta* biology

1.5.1 Seed and germination

Cuscuta seeds consist of an embryo that is devoid of cotyledons but is embedded in endosperm, and of a seed coat (Kuijt, 1969; Lee *et al.,* 2000; Heide-Jorgensen, 2008). Morphologically, the embryo has a simple shoot apex at one end, and a radicular organ at the other end (Lyshede, 1992). Anatomically, the embryo comprises an epidermis with a thickened outer cell-wall covered by a cuticle, a cortex with thin-walled globular cells, and central cells that have a procambial nature (Lyshede, 1992). The endosperm exhibits an external aleurone layer that contains proteins and lipids, and the rest of the endosperm cells, depending on the species, can be totally or partly filled with starch (Lyshede, 1984). The seed coat (testa) results from the integument of the ovule. A mature testa is generally composed of a cutinized epidermal layer with papillae, two palisade layers, and 2-4 parenchymatous layers. The external palisade layer (hypodermis) is formed by cells with thin walls while the internal palisade layer (sub-hypodermis) is composed of layer of linear sclereids with thick walls (Gaertner, 1950; Kuijt, 1969; Lyshede, 1984; Lyshede, 1992; reviewed in Costea & Tardif, 2006; Jayasuriya *et al*.,

2008; Rodriguez-Pontes, 2009). Seemingly, these characteristics are shared among most *Cuscuta* species with some differences in the three outermost layers (Lyshede, 1984). The impervious seed coat causes physical dormancy in the mature dried seeds of *Cuscuta*; the palisade layers (Hutchison & Ashton, 1979) or the epidermal layer (Lyshede, 1984) layers have been recognized for this process.

The effect of various treatments on the germination rate of *Cuscuta* sp. has been studied in laboratory conditions (Gaertner, 1950; Tingey & Allred, 1961; Hutchison & Ashton 1979; Srivastava & Chauhan, 1977; Benvenuti *et al.,* 2005; Jayasuriya *et al.,* 2008; Sarić-Krsmanović *et al.,* 2013). However, to date, studies have rarely been undertaken to compare the structural variations of the intact seed coats in relation to the germination success of different *Cuscuta* species.

Cuscuta spp. germinate from May to June in southern Canada (reviewed in Costea & Tardif, 2006). A temperature of 30°C is optimal for *C. campestris* (Benvenuti *et al.,* 2005) and *C. gronovii* (Konieczka *et al.,* 2009) germination. During germination, water uptake causes the seed coat epidermis to become mucilaginous because of its pectin content (Lyshede, 1984; reviewed by Costea & Tardif, 2006) and the outer epidermal wall bulges because of mechanical pressure (Lyshede, 1984). The endosperm becomes soft and the small radicular root emerges from the cracked seed coat (*c*. 1-2 mm in length) (Sherman *et al.,* 2008). Harvested and stored seeds of *Cuscuta* do not readily germinate because dormancy occurs after the seed coat becomes dry, hard, and impervious a few days after harvesting (Gaertner, 1950; Lyshede, 1992). In order to get high germination rate, *Cuscuta* seeds need to be scarified (Gaertner, 1950; Benvenuti *et al.,* 2005). Seeds may survive for a long time depending on the species and storage conditions. For

example, *C. campestris* can survive for 10 to 20 years and *C. gronovii* can survive about 30 years in a dry storage (Gaertner, 1950; reviewed in Costea & Tardif, 2006).

1.5.2 Seedling stage

The filamentous *Cuscuta* seedlings are usually yellow/orange in color and slightly green at the shoot tip (Sherman *et al.,* 2008); they consist of a shoot at one end and a tuberous structure (Lyshede, 1985, 1986) identified as a root (Lyshede, 1989) at the other end. The shoot surface is covered with a thin cuticle and has scale-like, alternate leaves (Lyshede, 1985). Structurally, the shoot consists of an epidermal layer with the cells containing chloroplasts, vacuolated cortical cells with starch grains and chloroplasts, and a central vascular strand (Lyshede, 1985, 1986). The seedling length varies depending on the species; for instance, *C. gronovii* can grow up to 35 cm whereas *C. campestris* can grow about 5-10 cm (see Parker & Riches, 1993).

One of the unique characteristics of *Cuscuta* is that it does not possess a permanent root system. Dodder root primordia resemble those of other vascular plants; however, at germination this similarity ends (Sherman & Vaughn, 2003). The lack of a root cap and the absence of an apical meristem are the most prominent characteristics of the *Cuscuta* root (Truscott, 1966; Lee *et al.,* 2000; Sherman *et al.*, 2008); it means that the root is not capable of growth and development (Truscott, 1966; Parker & Riches, 1993; Sherman & Vaughn, 2003; Sherman *et al.,* 2008). However, root hairs develop in the root of most *Cuscuta* species (Truscott, 1966). During the seedling stage, *C. gronovii* has root hairs (Truscott, 1966), whereas *C. campestris* possesses a smooth epidermis (Lyshede, 1985; reviewed in Costea & Tardif, 2006), with only some root hairs around the root tip.

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Seen as a transversal section, the root has a simple structure. It consists of a cutinized (*c*. 0.1µm; Sherman *et al.,* 2008) epidermal layer with vacuolated cells (with a thin rim of cytoplasm), some of which develops into root hairs; a cortex with highly vacuolated, thin-walled cells connected to each other via plasmodesmata, and a central vascular tissue (Lyshede, 1985, 1989) that extends to the very tip of the root and ends bluntly (Sherman *et al.,* 2008). The root tip cells do not have meristematic characteristics (Sherman *et al.,* 2008). The root starts wilting a few days (5 days; Truscott, 1966) after germination (Sherman & Vaughn, 2003; Sherman *et al.,* 2008). The root epidermal and cortical cells begin to degenerate while the peripheral cells of the central vascular strand may remain alive for a longer time (Lyshede, 1989); the vascular tissue appears as a region of the root persisting at the time of degeneration (Sherman *et al.,* 2008). The root cells lose their integrity through a senescence-like process (Sherman *et al.,* 2008) rather than apoptosis (Sherman & Vaughn, 2003). Because of its ephemeral nature and the absence of any of the structures that characterize typical dicotyledonous roots, such as exodermis, endodermis, or pericycle, there has been a general reluctance to consider this organ a 'true' root (Haccius & Troll, 1961 cited by Sherman *et al.,* 2008; Truscott, 1966; Sherman *et al.,* 2008). Although absorption and metabolization of phosphates in *C. gronovii* (Fer, 1976) and the effects of nitrogen fertilizers on *C. reflexa* seedling growth (Srivastava & Chauhan, 1977) suggest that the root is capable of absorption, the root has been interpreted either as a highly modified stem (Sherman *et al.,* 2008) or as a radicular tuberous end, which would have resulted from the evolutionary reduction of the root (Lyshede, 1986). This organ supposedly anchors the seedling, stores nutrients (Sherman *et al.,* 2008), and is likely capable of supplying water for the young seedling (Koch, 1880 cited by Lyshede, 1986). However, to date, no direct study has been

carried out to illustrate clearly the absorbent ability of the root. Thus, the root is thought not to have a critical absorbent role (Sherman *et al.,* 2008).

Cuscuta seedlings emerge from the soil having a hook shape (Kuijt, 1969), at which point they start circumnutating (counterclockwise) and searching for a host (Kuijt, 1969). The unattached seedlings continue to grow but lie down on the soil after a few days; they forage in their vicinity to find a suitable host (Lyshede, 1985). There may be two outcomes to this stage. First, the seedling may find a suitable host and twine around its stem. Second, if the seedling cannot find a compatible host, it will die after several days through the progressive degeneration of basal structures (Sherman *et al.,* 2008). In the 1st case, the twined seedling produces haustorial protuberances 2-4 days after attachment (Dawson *et al.,* 1994). Cytoplasmic-enriched and modified epidermal cells elongate to form the haustorium and attach to the host surface by secreting cement-like substances (de-esterified pectin) (Vaughn, 2002). Eventually the haustorium penetrates (searching hyphae) into the host plant, a process which involves both mechanical and enzymatic processes (Vaughn, 2002). The haustoria become coated by the stretched cell walls of the host plant and this results in the formation of new, chimeric, cell walls obtained from the fusion of the walls of the two plant cells; those walls will contain secondary plasmodesmata that connect the cell cytoplasm of the two species (Vaughn, 2003). Thus a physiological bridge of vascular tissues gets established between the host and the parasite. Rapid cell divisions and elongation cause a strong increase in the shoot apical growth (Lyshede, 1985). The scale leaves and axillary buds are differentiated from the shoot apex and a mature plant is formed. Mature *Cuscuta* plants produce flowers and fruits that exhibit a large morphological diversity among species, this diversity is used for species identification (Kuijt, 1969).

As for the 2nd case, the duration that a seedling survives without a host varies widely among different *Cuscuta* species, from several days up to 7 weeks. For instance, Dawson *et al.* (1994) mentioned that seedlings of *Cuscuta* spp. have several days to find a host. *Cuscuta campestris* seedlings were reported to lose their ability to attach to the host and die in about 8 days, whereas *C. gronovii* can survive for several weeks (see Parker and Riches, 1993), even 7 weeks (Heide-Jorgensen, 2008). *Cuscuta pedicellata* seedlings can survive up to four weeks (Lyshede, 1985); a 13-19 days survival period was reported for *C. chinensis* (Marambe *et al.,* 2002), and 3 weeks for *C. europaea* (Heide-Jorgensen, 2008). The factors affecting these survival rates are unknown. Taking into account that the root organ is considered non-functional, and even though some nutrients are available from the seed endosperm, it is not clear how the seedling of *Cuscuta* can survive these periods of times without a supply of water and nutrients. The only logical explanation is that *Cuscuta* root organ has at least some limited capacity to perform the functions of a normal root (e.g., capable to absorb water, interact with microorganisms, etc.), which may vary in extent from one species to another.

1.6 Mycorrhizal fungi in parasitic plants

Plant survival and growth are significantly impacted by biotic factors, including mycorrhizal fungi. Mycorrhizal fungi belonging to the phylum *Glomeromycota* associate with the roots of *c.* 90% of land plants in a mutualistic relationship (Schübler *et al.,* 2001; reviewed in Parniske, 2008; reviewed in Gutjahr, 2014). Fungi improve nutrient and water uptake in the colonized plants; in return, they obtain carbohydrates from the plants (Harley & Smith, 1983; Bago *et al.,* 2003; reviewed in Parniske, 2008; reviewed in Gutjahr, 2014). Since parasitic plants obtain their nutrients and water (partially or entirely) from the host plant(s), they are expected to be non-mycorrhizal (Lesica & Antibus, 1986; Li & Guan, 2008). However, some of them, such as

Pedicularis species, a root hemiparasite in the *Orobanchaceae* (Heide-Jorgensen, 2008), have been shown to be mycorrhizal (Li & Guan, 2008). Among the 29 different species of *Pedicularis* analyzed, 23 were mycorrhizal and 3 likely mycorrhizal (only fungal hyphae were observed in the latter). Based on these results, it was suggested that the mycorrhizal *Pedicularis* may survive by using a mycorrhizal association as an alternative nutritional strategy in the absence of a proper host plant (Li & Guan, 2008). Although arbuscular mycorrhizas (AM) may help *Pedicularis* sp. grow better by facilitating P translocation from the soil (Li *et al.,* 2013), AMs may also reduce the ability of the parasitic plant to invade potential hosts by limiting the initiation and formation of haustoria. For instance, *Glomus mosseae* and *G. intraradices* were shown to decrease the number of haustoria in *P. tricolor* parasitizing *Hordeum vulgare* (Li *et al.,* 2012; Li *et al.,* 2013); *Glomus mosseae*, also, strongly inhibited the invasion of *P. kansuensis* parasitizing *Elymus nutans* (Sui *et al.,* 2014).

A tripartite association has also been reported in a holoparasitic plant. In *Cytinus hypocistis* (*Cytinaceae*), an endophytic root holoparasite, and its host species *Cistaceae*, a high level of AM colonization was observed with coiled hyphae and vesicles in both roots of the parasitic plant and its host (De Vega *et al.,* 2010).

1.6.1 Mycorrhizal interaction in *Cuscuta*

One single study (Khalid & Iqbal, 1996) reported that AM colonized seedlings of *C. reflexa*, a species of subgenus *Monogynella*, had a higher dry weight and survived 2 days longer than seedlings grown on a sterile substrate (Khalid & Iqbal, 1996). Sanders *et al.* (1993) also suggested that arbuscular mycorrhiza helps indirectly *Cuscuta* grow better; the authors found that

C. pentagona had a greater growth on a mycorrhizal host, *Abutilon theophrasti* colonized by *Glomus intraradices,* than on the non-mycorrhizal host (Sanders *et al.,* 1993).

1.7 Rationales of the study

➢ Germination rate play a significant role in survival rates of plants. Like in other plants, *Cuscuta* seed germination is influenced by various environmental and structural factors (e.g., Gaertner, 1950; Tingey & Allred, 1961; Hutchison & Ashton, 1979; Srivastava & Chauhan, 1977; Benvenuti *et al.,* 2005; Jayasuriya *et al.,* 2008; Sarić-Krsmanović *et al.,* 2013). However, the relation between the intact structure of the seed coat and the germination success among *Cuscuta* species has been rarely considered. To study such a relation may help us to better understand how some species evolved adaptations to survive in different ecosystems.

➢ The absorbent ability of the vestigial root of *Cuscuta* seedlings is unknown. Despite a couple of reports indicating the potential role of *Cuscuta* root in mineral uptake (Fer, 1976; Srivastava & Chauhan, 1977), an absorption role is considered questionable (Truscott, 1966; Lyshede, 1985; Haccius and Troll, 1961 cited in Sherman *et al.,* 2008; Sherman *et al.,* 2008). Thus, an investigation of the ability of the *Cuscuta* root to absorb water is required.

➢ Because of the parasitic nature of *Cuscuta* (Kuijt, 1969; Heide-Jørgensen, 2008) and its ephemeral root system (Lyshede, 1989; Sherman *et al.,* 2008), the effect of biotic factors including soil microorganisms such as mycorrhizal fungi on *Cuscuta* seedling growth performance and survival has not been fully investigated and understood. Mycorrhization has been studied in the root of hemiparasitic plants (Lesica & Antibus, 1986; Li & Guan, 2008; Li *et al.,* 2012; Sui *et al.,* 2014) and rarely reported in holoparasitic plants (Khalid

& Iqbal, 1996; De Vega *et al.,* 2010). In the case of *Cuscuta*, little is known about its mycorrhization. Although Khalid and Iqbal (1996) reported a positive effect of the AM colonisation on *C. reflexa* growth and survival, the authors did not provide clear mycological information, such as fungal identification, structures, and penetration, nor fungal colonization rate. Furthermore, their methodology was unclear. Therefore, the symbiotic interaction and its effect on the growth and survival of *Cuscuta* sp. during the non-parasitic phase of its life-cycle need to be further investigated. Potentially, these results may help explain the different ecology of various *Cuscuta* species.

1.8 Objectives

➢ To examine the morphological/structural variations of the seed coat in relation to the germination success of different *Cuscuta* species belonging to the subgenus *Grammica* including *C. gronovii*, *C. campestris*, and *C. nevadensis*.

➢ To determine if the rudimentary root of *C. gronovii* and *C. campestris* is capable of absorption.

➢ To investigate the mycorrhizal colonization and its effects on the growth and survival of two ecologically different *Cuscuta* species, *C. gronovii* and *C. campestris,* during the non-parasitic phase (autotrophic) of their life-cycle (the seedling phenophase).

Chapter 2

A comparative study of seed coat characteristics in three *Cuscuta* species in relation to their germination success

2.1 Introduction

Germination, i.e., the emergence of the radicle from the seed, is an essential stage in a plant lifespan (Bewley & Black, 1985; Meulebrouck *et al.,* 2008) because it initiates seedling establishment and development in a new environment (Raven & Johnson, 2002; Donohue *et al.,* 2010). It has significant effects on plant survival, persistence of plant populations and dynamics (Willis *et al.,* 2014), as well as plant evolutionary history (Donohue *et al.,* 2010). In many plant species, when their seeds are subjected to favourable germination conditions including sufficient water, suitable range of the temperature, and normal composition of atmosphere, they do not germinate simultaneously; this is termed delayed germination (Crocker, 1906; Mayer & Poljakoff-Mayber, 1963). Seed germination normally occurs at various rates from a few days to months depending on the species (Crocker, 1906; Barton, 1965). The differences in germination rates among different species are caused by the production of seeds that may be affected by different types of dormancies, such as physiological dormancy (e.g. *Helianthus annuus*, Bazin *et al.,* 2011; *Stipa tenacissima*, Gasque & Garćia-Fayos, 2003) or physical dormancy (Mayer & Poljakoff-Mayber, 1963; Barton, 1965; Baskin & Baskin, 1998, 2004). For instance, the *Leguminosae* (e.g., *Lotus unifoliolatus*, *Lupinus albicaulis*, and *Trifolium willdenovii*; Russell, 2011) and some species of other families such as the *Convolvulaceae* (*Cuscuta australis*, Jayasuriya *et al.,* 2008; *Ipomoea lacunosa*, Jayasuriya *et al.,* 2009), the *Geraniaceae* (*Geranium pratense*, Van Assche & Vandelook, 2006), the *Malvaceae* (*Malva sylvestris*, Van Assche &

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Vandelook, 2006), and the *Chenopodiaceae* (*Chenopodium album*, black seeds, Yao *et al.,* 2010) are known to produce seeds subjected to physical dormancy. These seeds are surrounded by impervious seed coats with various degrees of impermeability (Barton, 1965). The impervious seed coat is caused by the presence of palisade layers in the seed coat of many species (Baskin & Baskin, 2004).

Cuscuta, the only parasitic genus in *Convolvulaceae* family (Kuijt, 1969), is a widespread genus around the world (see the general introduction) (Dawson *et al*., 1994). The parasitism of *Cuscuta* on a wide range of woody and herbaceous plants (Parker & Riches, 1993; Dawson *et al.,* 1994; Holm *et al.,* 1997) has made the genus ecologically (De Bock & Fer, 1992; Pennings & Callaway, 2002; Press & Phoenix, 2005) and economically (Dawson *et al.,* 1994) significant. Seed formation in *Cuscuta* (Macpherson, 1921; Truscott, 1966; Johri & Tiagi, 1952; Tiagi, 1966; Govil & Lavania, 1980; Rodriguez-Pontes, 2009) is relatively similar to that of other *Convolvulaceae* species (Corner, 1976; *Ipomoea lacunosa*, Jayasuriya *et al*., 2007). However, seeds vary among different *Cuscuta* species in terms of their size and ability to germinate (Gaertner, 1950, 1956). A mature *Cuscuta* plant produces thousands of seeds (e.g., *c*. 16000 seeds in *C. campestris*, Costea & Tardif, 2006) which mostly lose their germination abilities a few days after harvesting because of a mechanical obstruction of their seed coats (Gaertner, 1950; Tingey & Allred, 1961; Hutchison & Ashton, 1979; Lyshede, 1984); this seed coat impermeability causes very low germination rates (Hutchison & Ashton, 1979) in most *Cuscuta* species. In species such as *C. europaea*, the germination is independent of the seed coat structure; to germinate, its seeds need to be stored at cold temperature (0 °C) for at least 3 months immediately after harvesting (Gaertner, 1956). Previous researchers have investigated considerably the variations of the seeds of *Cuscuta* species for both their morphological (e.g.,

well-described by Costea *et al.,* 2005, 2006a,b) and anatomical (Lyshede, 1984, 1992; Rodriguez-Pontes, 2009; Jayasuriya *et al.,* 2008) properties. Moreover, the various dormancy breaking methods affecting *Cuscuta* germination were also studied (Gaertner, 1950; Tingey & Allred 1961; Hutchison & Ashton, 1979, 1980; Jayasuriya *et al.,* 2008; Meulebrouck *et al.,* 2008; Ghantous & Sandler, 2012). While these studies suggested that the seed coat structure is basically similar in all *Cuscuta* species, certain differences became apparent, particularly in the three outermost layers of the seed coats (Gaertner, 1950, Lyshede, 1984, Jayasuriya *et al.,* 2008). These variations are likely the cause of the different germination rates among *Cuscuta* species. To my knowledge, the relationship between the intact seed coat and the germination rate that naturally occurs in nature has been rarely (only Lyshede, 1984, but the germination data were not shown) studied among *Cuscuta* species. Thus, the purpose of this study is to examine the morphological/structural variations of seed coats in relationship with the germination success of different *Cuscuta* species belonging to the subgenus *Grammica*.

2.2 Materials and methods

2.2.1 Seed collection

Seeds of *C. gronovii* were collected in 2012 from a population growing on the banks of the Grand River: Canada, Ontario, Waterloo, Claude Dubrick Trail, 43°30'12.02"N, 80°29'37.97"W. The dominant species of the plant community is *Solidago canadensis*, which is the preferred host; other local, less frequent host species include: *Glechoma hederacea*, *Calystegia sylvatica*, and *Symphytum officinale*. *Cuscuta campestris s*eeds were removed from a herbarium specimen (see below) and germinated; the seedlings were grown in Waterloo on *Ocimum vulgare* and *Xanthium strumarium* in 2013 to produce the seeds used in the experiments. This specimen came

with the following herbarium information: U.S.A., California, Sonoma Co., Rosa, between the channel and Sanford Road, ca. 0.5 mi N of Occidental Rd, 38°13'26.60"N, 122°49'42.10"W, abundant on *Xanthium strumarium*, in seasonally flooded freshwater wetland, clay soil; *Rumes crispus*, *Aster chilensis*, Sep 2007, *Cadman & al. 2832* (WLU). *Cuscuta nevadensis* seeds were collected by Dr. Sasa Stefanovic in 2013 (herbarium voucher: U.S.A., California, Inyo Co., Panamint springs, on Hwy # 190. Death Valley NP, 36°20'23"N, 117°28'04"W, part of Death Valley national park, the hottest and driest place in North America with precipitation average of 50-59 mm annually, between Old toll Rd. and Panamint Valley Rd. reaching to Majavo desert. *Larrea tridentate*, *Atriplex*, and Mesquite are abundant species on the area; July 2013, *SS.13.30* (U of T). Thus, all the seeds used in the experiment were one year old.

2.2.2 Seed coat micromorphology

Seeds (n = 16) of *C. gronovii, C. campestris,* and *C. nevadensis* were examined to determine their morphological variation. Dried and rehydrated (with deionised (DI) water) seeds were passed through an ethanol dehydration series (30% 50%, 70%, 85%, 95%, and 100%; each for 1h). Seeds were then subjected to a hexamethyldisilazane (HMDS) treatment as an alternative method to critical point drying (Wright *et al*., 2011), and mounted on specimen stubs, which were then coated with 30 nm of gold using an Emitech K550 sputter coater. Examination was conducted with a Hitachi SU-1500 Scanning Electron Microscope (SEM) at 3 KV.

2.2.3 Seed coat structure

Rehydrated seeds (n = 3) of *C. gronovii, C. campestris,* and *C. nevadensis* were cut in half through the hilar region, perpendicular to the hilum scar. Half of the seeds were prepared as previously described for SEM microscopy (Wright *et al.,* 2011). The other half were fixed in a

mixture of Formaldehyde, Acetic acid, and Ethanol (FAA; 2:1:10), and then passed through an ethanol series (50%, 70%, 85%, 95%, and 100 %; each for 1h); once the series was completed the ethanol was exchanged for LR White resin (Hard; London Resin Company, U.K; Sigma-Canada). Samples were passed through various mixtures of ethanol and LR White resin (3:1, 2:1, and 1:1 each for 1-2 h), and then into pure resin which was changed twice a day for 3 days. Infiltrated specimens were placed in capped capsules containing fresh resin and kept at 60°C for 8 h until the resin became completely polymerized (modified from O'Brien & McCully, 1981). Samples were sectioned at two μm thickness using a Sorvall MT-1 ultra-microtome; sections were stained with 0.05% Toluidine Blue O (TBO) (pH4.4) for 1 min. Structural examination of the seed coat was conducted using a Nikon Eclipse 50i with a PaxCam Arc digital camera and Pax-it 7.5 software (MIS Inc. 2012, Villa Park, IL). In addition, the thickness of the seed coat in the hilar region and in the rest of the seed was estimated.

2.2.4 Germination test

To investigate the effect of the seed coat structure on germination rate (GR), the three *Cuscuta* species, *C. gronovii, C. campestris,* and *C. nevadensis,* were used. Sterilized seeds (n = 36) of each species were placed in sterile Petri dishes (six Petri dishes / six seeds per dish; one dish was considered as a replication) which contained moistened sheets of No.1 Whatman filter paper. The plates were sealed with Parafilm[®] strips to reduce water evaporation, and randomly placed in the growth cabinet set at 30°C. The germination test was conducted in complete darkness. Because of the photo-insensitivity of *Cuscuta* (negatively/or non photoblastic) (Hutchinson and Ashton, 1980, Ebrahimi *et al.*, 2012), the light did not affect germination during observation. The same six plates were observed every day over a period of 21 days. If required, five ml of sterile water were added to the Petri dishes at the observation times. The seeds were

considered germinated once one tip of the embryo had emerged approximately 1 mm from the seed coat.

2.2.5 Statistical analysis

Analysis of variance (ANOVA) and mean comparison were performed using the statistical software Minitab version 16.2.4.3 for germination rates at three different days after sowing date (2nd day, 7th day/1st week, and 21st day/3rd week) among the three species and at the level of each individual species. Germination percentage was also calculated for each species.

2.3 Results

2.3.1 Seed coat morphological variations

Seed shape varied from ovoid or oblong-ovoid to subglobose and the seed surface was almost similar in the three species (Appendix Fig. 1). In *C. gronovii*, the seeds are reddish brown to brown; obliquely ovoid to broadly ovate; and dorsoventrally compressed to indistinctly angled (Fig. 1a). The hilar region is a distinct oval with striation (Fig. 1a), and the hilum is linear vertical to slightly oblique (Fig. 1d). The seed surface is alveolated when dried (Fig. 1g) and with papillae when hydrated (Fig. 1h). The number of seeds per capsule varied between 1 to 4 seeds. In *C. campestris*, the seeds were yellowish-brown to yellow and slightly ovoid to obovoid (Fig. 1b). The hilar region was ovate with a distinct striation (Fig. 1b). The hilum was short, linear, and vertical (Fig. 1e). The seed surface was alveolated when dried (Fig. 1i) and with papillae when hydrated (Fig. 1j). The number of seeds was 2-4 per capsule. In *C. nevadensis*, the seeds are light brownish to brown and globose to slightly ovoid (Fig. 1c). The hilar region was indistinct, concaved, and round (Fig. 1c). The hilum was sunken and with a pinpoint shape (Fig. 1f). The seed surface was alveolated when dried (Fig. 1k) and with papillae when hydrated (Fig.

1l). The number of seeds was 1 per capsule; the seed coat being strongly attached to the wall of the fruit.

2.3.2 Anatomy of seed and seed coat

Mature seeds of *C. gronovii* and *C. campestris* were highly similar anatomically; they consisted of a multiple-layered seed coat, an endosperm with layers of parenchymatous cells, and a yellowish, filamentous, 2-3 coiled embryo times embedded in the endosperm (see Fig. 1, chapter 3). The thread-like embryo morphologically consisted of a shoot tip at one end and a slightly thickened radicular structure at the other end (see Fig. 1, chapter 3). In contrast, *C. nevadensis* had a mature seed which was composed of a seed coat (see below) and an embryo with the shoot tip at one end and an enlarged globose structure at the other end. The latter structure differed from that of the other two species. Furthermore, the mature seed of *C. nevadensis* lacked the endosperm, of which only a thin layer remained to cover the entire embryo.

The structure of the seed coat of *C. gronovii* and *C. campestris* were almost similar, but differed from that of *C. nevadensis* in terms of the composition of the seed coat layers. In *C. gronovii*, the seed coat consists of an epidermis, two palisade layers (Fig. 1m, Fig. 2a,b,c), and layers of compressed parenchymatous cells (Fig. 2a,c). The epidermis comprises thin-walled cells, covering the seed (Fig. 2a,b,c). Within the region of the hilum, these cells have developed thickened walls that fully impregnate the cell lumen. They are tightly arranged shaping a counter palisade layer (Fig. 2a). There are two palisade layers (Fig. 2a,b,c). The first one is thinner than the second one and composed of sclereid cells (osteosclereids) with cell walls entirely occupying the cell lumens at the hilar area.

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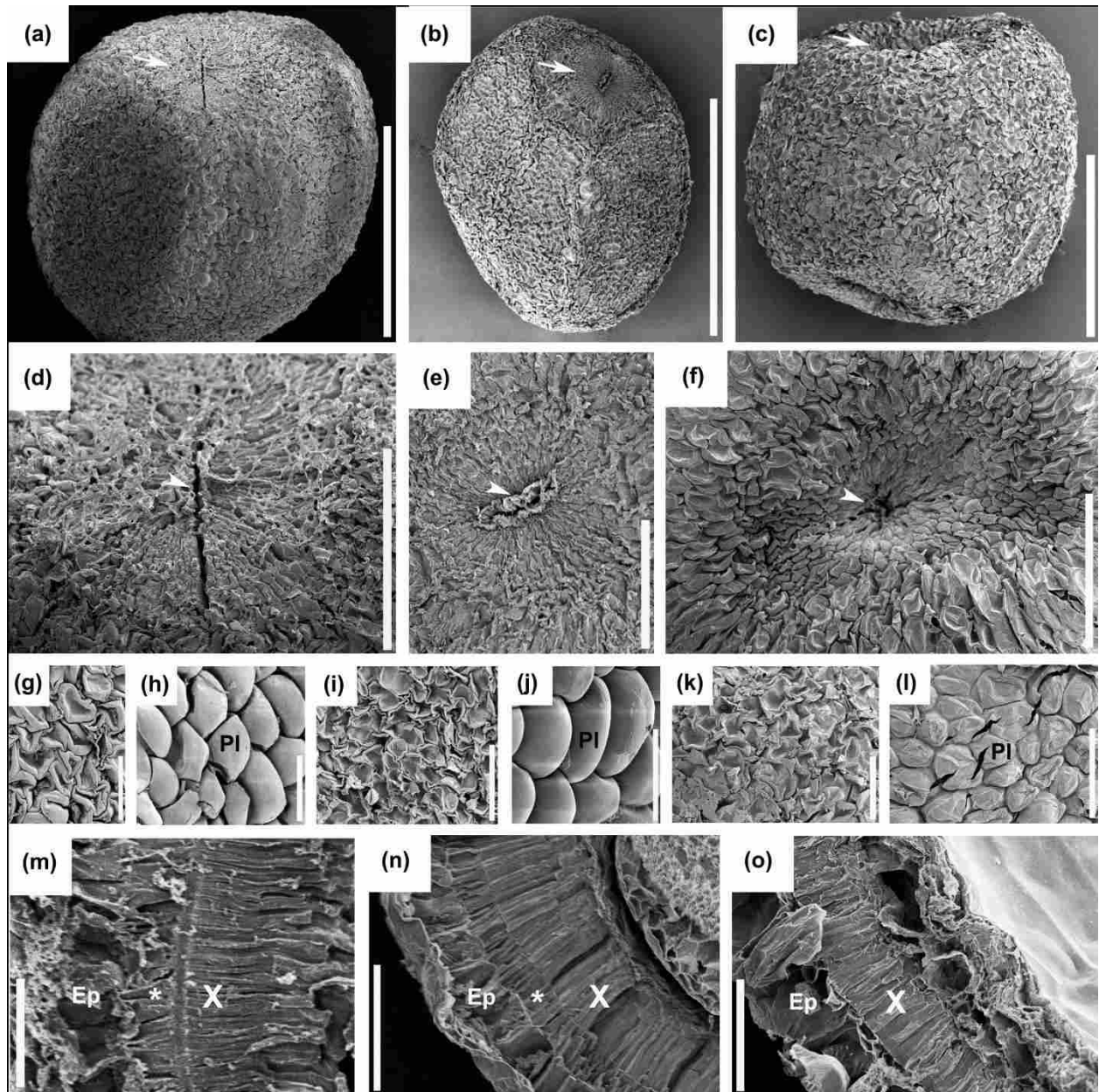
Fig. 1 Micrographs of morphological (a-l) and structural (m-o) variations of seeds of the three *Cuscuta* species. Seeds of (a) *C. gronovii*, (b) *C. campestris*, (c) *C. nevadensis*. Note: Hilar region is indicated by an arrow for each species. Hilum (arrowhead) of (d) *C. gronovii*, (e) *C. campestris*, (f) *C. nevadensis*. Seed epidermal cells (alveolate vs. papillae cells, respectively): (g,h) *C. gronovii*, (i,j) *C. campestris*, (k,l) *C. nevadensis*. Seed coat structures of: (m) *C. gronovii*, (n) *C. campestris*, (o) *C. nevadensis*. Pl: Papillae, Ep: Epidermis, asterisk: The first palisade layer, X: the second palisade layer. Scale bars: 1mm in a, b; 500 μm in c; 400 μm in d, 200 μm in f; 100 μm in e, g, i, k, l, m, n; 50 μm in h, j, o.

The second palisade layer consists of long sclereid cells with thickened walls filling the cell lumens. In addition, 2-3 layers of cuboid to polygonal sclerotic cells are present in the second palisade layer at the hilar region (Fig. 2a,b); these layers increase the thickness of the seed coat in this region compared to that of the areas from the rest of the seed (Fig. 2c). The entire seed coat thickness was *c*. 185 µm at the hilar region and *c*. 137 µm in the areas away from hilum.

In *C. campestris*, the seed coat exhibits layers similar to those seen in *C. gronovii*; however, it is thinner than that of *C. gronovii* (Fig. 1n, Fig. 2d-f). The epidermal layer (Fig. 1n, Fig. 2e,f) consists of thin-walled cells tightly arranged into one another. The wall of these cells is, however, thickened at the hilar region (Fig. 2d) to form a counter palisade layer which does not look as tight as in *C. gronovii*. The first and second palisade layers are located below the epidermis (Fig. 1n, Fig. 2d,e,f). The former is composed of short sclereid cells (osteosclereids) with thickened wall completely filling the cell lumens at the hilar area (Fig. 2d,e) and thin wall with visible lumens elsewhere in the seed coat (Fig. 2f). The inner anticlinal wall adjacent to the second palisade layer seems to be slightly thicker than the other walls (Fig. 2d,e). The latter palisade layer consists of linear sclerotic cells with impregnated cell lumens and thick walls (Fig. 2d,e,f,). At the hilar area, one to 2 layers of shorter sclereid (cuboid) cells are present below the second palisade layer increasing the thickness of the seed coat (Fig. 2 d). Compressed parenchymatous cells are also seen facing the second palisade layer (Fig. 2d). The entire seed coat thickness was *c*. 140 µm at the hilar region and *c*. 94 µm in the areas away from the hilum.

Fig. 2 Seed coat structures (a-f) and germination patterns (g,h) of *Cuscuta gronovii and C. campestris*. Note: The sections were embedded in LR White and stained with TBO. (a-c) Longitudinal section of *C. gronovii* seed and (d-f) *C. campestris* seed. (g) *C. gronovii* and (h) *C. campestris* germinated seeds. *C. gronovii* (a) and *C. campestris* (d) seed coats consist of an epidermis (Ep), the first (*) and second (X) palisade layers with some short sclereid cells (SC), and compressed parenchyma (CP) cells. Note: In both species epidermal cell walls at hilar region (HR) are thickened forming a counter palisade layer which seems more tightly arranged in *C. gronovii* than in *C. campestris* (double arrowheads). Higher magnification of the seed coat of (b) *C. gronovii* and (e) *C. campestris* in the area flanking the hilum. Seed coat structure of (c) *C. gronovii* and (f) *C. campestris* in the area away from the hilar region. Emerged radicular ends from the seeds of (g) *C. gronovii* and (h) *C. campestris*. Arrow: anticlinal thickened walls of the first palisade layer in *C. campestris* seed coat (d,e), En: endosperm, R : radicular end of the embryo, S: seed. Scale bars: 1 mm in g, h; 100 μm in a, b, c, d, e, f.

In *C. nevadensis*, in which no endosperm remains in a mature seed, the epidermis of the seed coat is composed of thin-walled cells (Fig. 1o, Fig 3b-e). These epidermal cells are smaller than those of *C. gronovii* and *C. campestris*. The first palisade layer is reduced and only present at the hilar region (Fig. 1o, Fig. 3a). The cells composing it are thick-walled (Fig. 3c-e). The second palisade layer consists of linear, thick-walled, sclereid cells with an almost uniform thickness; it extends throughout the seed coat (Fig. 1o, Fig. 3a,c,d,e). Unlike in the other two species, short (cuboid) sclereid cells were not observed in the second palisade layer at the hilar region (Fig. 3a,b). As in the other two species, compressed parenchymatous cells were observed between the inner palisade layer and the embryo (Fig. 2a,b,e) The entire seed coat thickness was *c*. 77 µm at the hilar region and *c*. 40 µm at the areas away from the hilum.
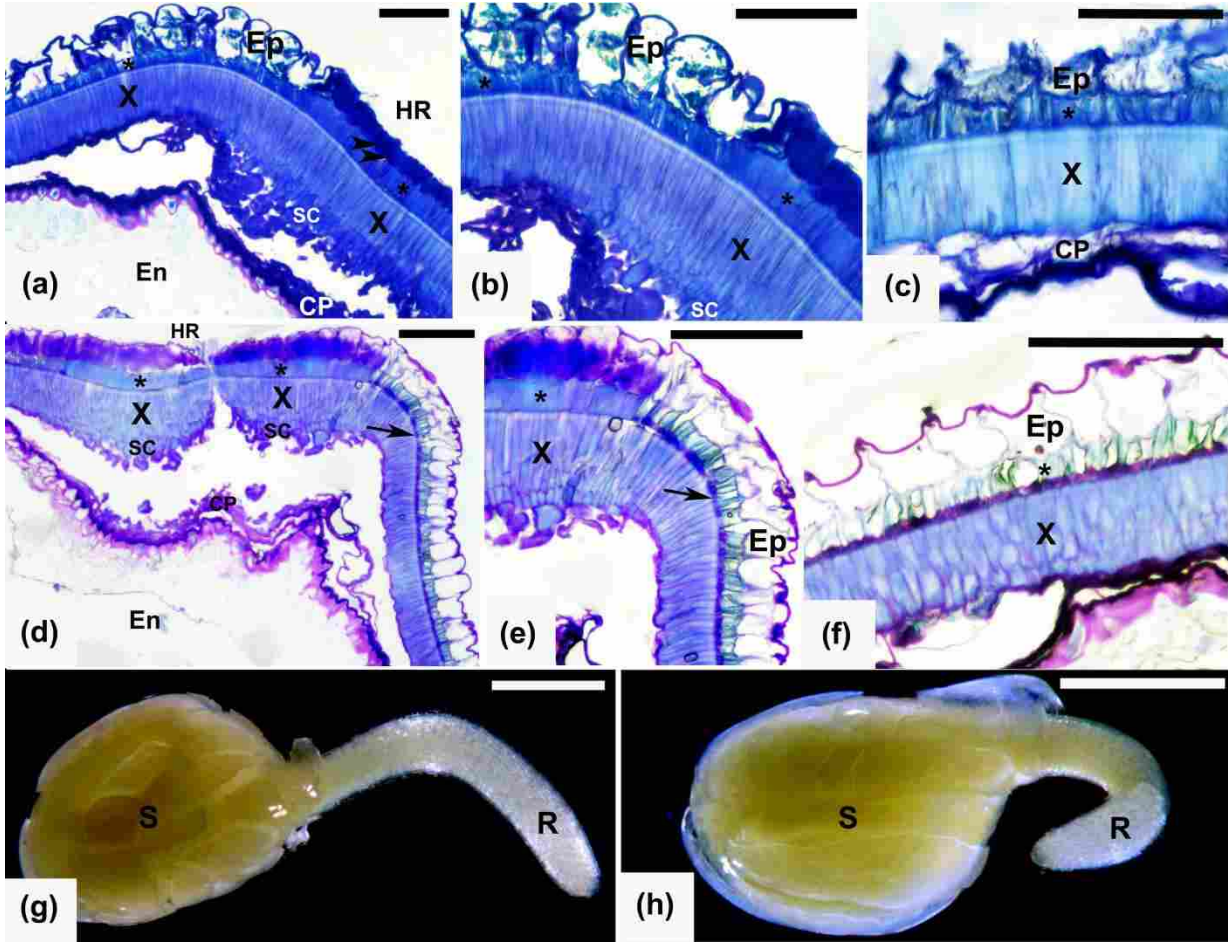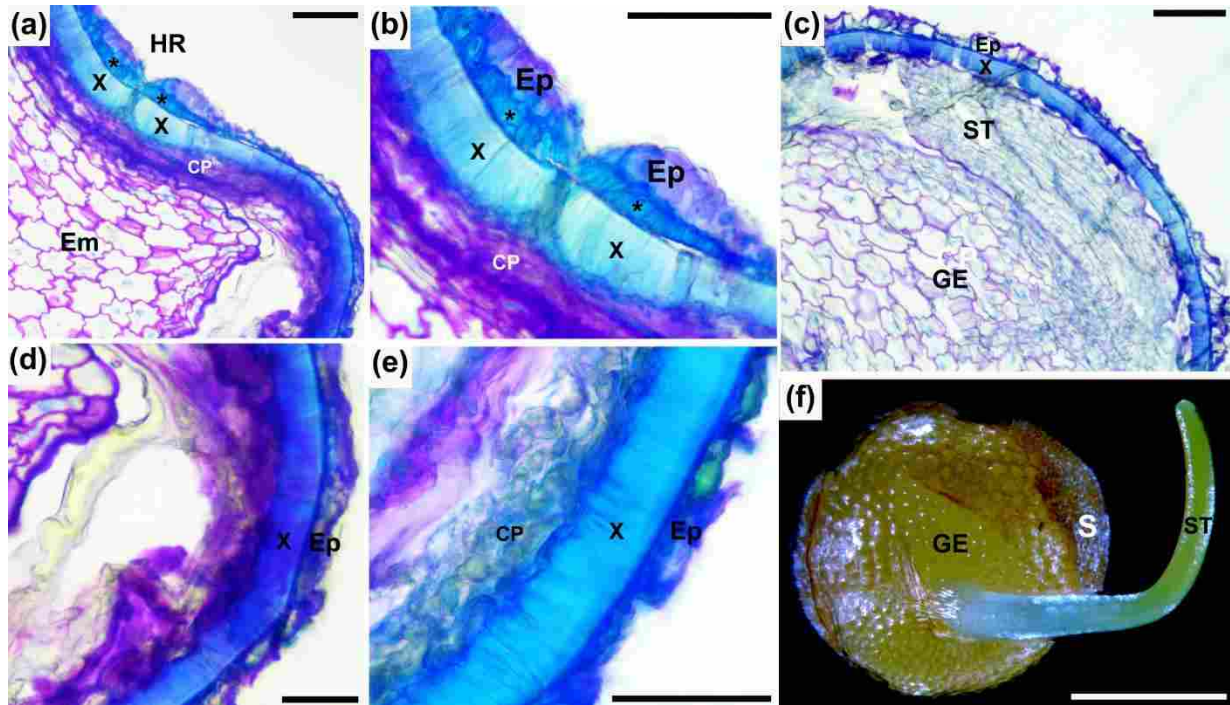
Fig. 3 Seed coat structure (a-e) and germination pattern (f) of *Cuscuta nevadensis*. Note: The sections were embedded in LR White and stained with TBO. (a) The seed coat consists of the epidermis (Ep), the first palisade layer (*) which is only present at the hilar region (HR), the second palisade layer (X), and compressed parenchyma cells (CP). (b) Higher magnification of the seed coat at the hilar region. (c) Seed coat structure away from the hilar region. (d) Higher magnification of the area flanking the hilar region. (e) Higher magnification of the area away from the hilar region. (f) Emerged shoot tip of *C. nevadensis* from the seed. Em: embryo, GE: globose end, S: seed, ST: shoot tip. Scale bars: 1mm in f; 100 µm in a, b, c, d, e.

2.3.3 Seed germination

C. gronovii and *C. campestris* have a typical germination pattern; their seed germination occurs by emergence of the radicular end of the embryo from the seed coat (Fig. 2g,h). *C. nevadensis* seeds exhibited a different germination pattern. In stark contrast, the shoot tip of the embryo emerges first from the seed coat (Fig 3f). Once imbibed, the seeds of the three species become relatively gelatinized especially in *C. nevadensis*.

Interspecifically, the total germination rate (GR) significantly differed among the selected species at each harvesting date (Fig. 4a-c). The highest GR was seen in *C. nevadensis* followed by *C. campestris* and *C. gronovii* at all observation dates (Fig. 4a-c). Many *C. nevadensis* seeds germinated 2 days after sowing, with an average of three germinated seeds per dish (50% total germination) which was significantly ($P \leq 0.001$) higher than the other two species with an average of 1 for *C. campestris* and 0 for *C. gronovii* per dish at the same time (Fig. 4a). Despite an increase in *C. campestris* and *C. gronovii* GRs by the first week, their GRs were still significantly lower than that of *C. nevadensis* (Fig. 4b). The same was true for the third week ($P \leq 0.001$), with the highest GR in *C. nevadensis* with an average of 5.6 germinated seeds per dish (94.45% total germination), followed by *C. campestris* with an average of 2.1 and *C. gronovii* with an average of 1 germinated seeds per dish (Fig. 4c).

Intraspecifically, the most rapid germination occurred in *C. nevadensis* while *C. gronovii* had the most delayed germination (Fig. 4d-f). Germination of *C. nevadensis* is rapid (Fig. 4d) and occurs within 7 days after sowing. In *C. campestris*, the GR increases insignificantly over time ($P = 0.225$) and reaches 36.11% at day 21st (Fig. 4e). In *C. gronovii*, no germination
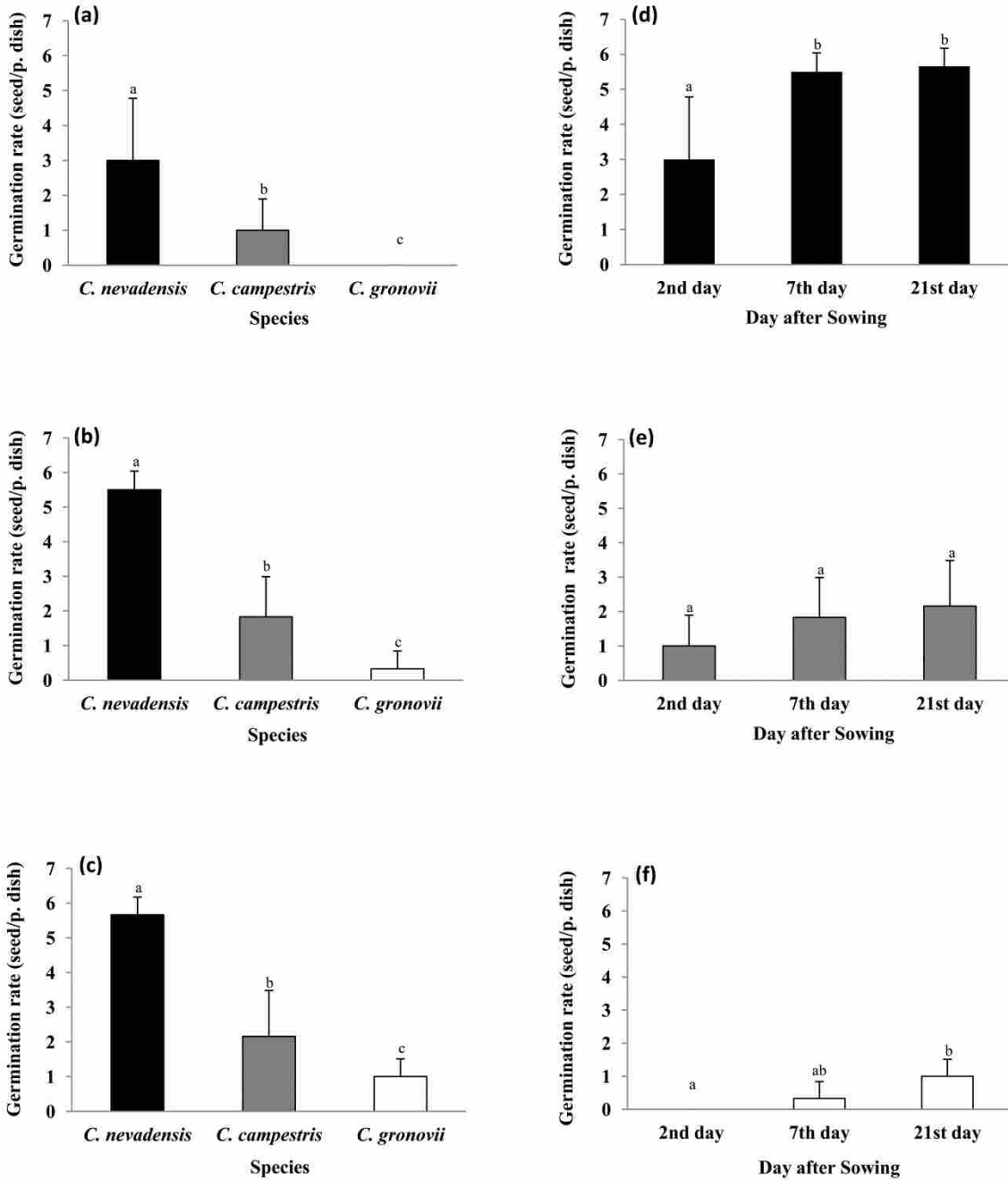
Fig. 4 Differential germination rates of the three species at different harvesting date (a-c). (a) Day 2nd, (b) Day 7th, (c) Day 21th. Germination rates of each species at different harvesting dates (d-f). (d) *C. nevadensis*, (e) *C. campestris*, (f) *C. gronovii*. (n = 36).

occurred at 2nd day (Fig. 4f). The species exhibited very low GR within the first week (5.5% total germination); the GR increased to 11.1% by day 21st.

2.4 Discussion

Among the three species studied, the seed coats and germination rates of *C. gronovii* and *C. campestris* exhibited similarities. *Cuscuta nevadensis,* however*,* had a high degree of dissimilarity compared to the other two species in all parameters observed and measured: its seed coat structure, germination pattern, rate and timing of germination. The results are in agreement with the previous morphological (e.g., Lyshede, 1984; Costea *et al.,* 2005, 2006a,b) and anatomical findings of *Cuscuta* seed coat (e.g., Hutchison & Ashton, 1979; Lyshede, 1984,1992; Jayarusiya *et al.,* 2008; Rodriguez-Pontes, 2009). However, the findings related to *C. nevadensis* seed coat are regarded here as novel. The three species share, however, one common characteristic in their seed coat, the hydrophobic property. The dried invaginated and water-induced bulged walls of the epidermal cells of *Cuscuta* seeds seen in the three species are similar to those of most *Cuscuta* species (Knepper *et al.,* 1990; Costea & Tardif, 2006) with the exception of species in the subgenus *Monogynella* (Knepper *et al.,* 1990). The hydrated bulging cells have some degree of morphological similarities to those of other *Convolvulacea* seed coats (Jayasuriya *et al.,* 2007); they may function to increase the surface of the seed since at the hydration time they form a papillous layer. Moreover, because of the presence of pectin in the epidermal cell walls, water easily adheres to the seed surface allowing the formation of a mucilaginous layer around the seeds (Lyshede, 1984, Costea & Tardif, 2006). The coating aids the seed germination in low water conditions (Young & Evans, 1973) by drawing water into the seeds (Lyshede, 1984) and protects the seeds from water loss (Harper & Benton, 1966).

The impermeability of the seed coat caused by the palisade layers has been well investigated in different plant species (e.g., *Anacardiaceae*, Li *et al.,* 1999); these two layers have been suggested to restrict mechanically seed germination in *Cuscuta* (Hutchison & Ashton, 1979). The seed coat thickness is thought also to play a significant role in germination probably by restricting water uptake and gas diffusion into the seed resulting in preventing the radicle protrusion (Mohamed-Yasseen *et al.,* 1994). On the basis of these results, I suggest that seed germination in *Cuscuta* species is likely influenced negatively by the existence of thick and dense seed coat layers. Thus, on one hand the high germination rate seen in *C. nevadensis* would be explained by its seed lacking the first palisade layer and possessing a thinner coat than the other two species. The thin seed coat may also provide more space for the globose end of the embryo, characteristic of that species, which is considered as a storage organ for water and nutrient (Costea *et al.,* 2005). On the other hand, *C. gronovii* seed with its two thick palisade layers with highly thickened cell walls and a counter palisade layer with tightly arranged cells at the hilar region has the lowest GR, and the most delayed germination. In *C. campestris*, which has an intermediate GR all seed coat layers are present but the coat is thinner, probably causing less strength in term of mechanical restriction. In contrast to the other species of *Convolvulaceae* where the bulges adjacent to the micropyle are the point of water entry to the seed (Jayasuriya *et al.,* 2007), the hilum is the main water gap (entry point) in *Cuscuta* seeds (Jayasuriya *et al.,* 2008). The mechanical pressure caused by the turgid cells of the endosperm and the embryo causes an opening in the hilum; a slit forms in the hilar pad (Jayasoriya *et al.,* 2009) allowing the water to penetrate better into the seed. Thus, the stronger (thicker) seed coat of certain species such as *C. gronovii* persists against the force and retains its impermeability. Overall these findings are in agreement with the results of Hutchison and Ashton (1979) who suggested the

role played by the palisade layers in *Cuscuta* seed coat impermeability. These seed characteristics may strongly influence germination rate and consequently survival of plants (Navarro & Guitián, 2003).

From an ecological perspective, species living in highly specific habitats often produce seeds with highly specialized adaptation (Navarro & Guitián, 2003). For instance, species living in desert habitats produce seeds that are able to germinate rapidly during the short rainy season (Gutterman, 1972) which is a vital adaptation strategy for species survival (Went, 1953 cited by Gutterman *et al.,* 1995). In agreement with this, a high rate and rapid germination of *C. nevadensis* seeds are likely a strategy to survive in arid regions with very low frequency of precipitation; it would allow the plant to establish its population in a short period of time once it has received water. Since *C. nevadensis* has a viviparous nature (Costea *et al.,* 2005) and a surprising germination pattern (shoot emerging first), its seed germination does not occur in the soil. This is likely another strategy of *C. nevadensis* to survive in its extreme habitat. This species lives in an environment dominated by shrubby plants, and its seedling, if germinated in the soil, would have difficulty to find or attach to a proper host plant. Likely its soil seed bank persistency (Bewley & Black, 1985) is not high and the species evolved to produce seeds with capabilities of rapid germination for fast completion of its life cycle. The other two species, considered as weedy species (especially *C. campestris*), invade a wide range of woody and herbaceous plants in different habitats. They likely have a persistent soil seed bank, which they may need to protect their seeds against harmful microorganisms and undesired environmental conditions (Mohamed-Yasseen *et al.,* 1994). Thus, their thick seed coat would be efficient in this case. Also, a low rate and gradual germination would likely increase their chances to find a proper host plant(s). In fact, these features ensure the survival of these species in their plant

communities. Here, I propose that one of the significant survival strategies of these species for their population persistency is their seed coat characteristics (3 outer layers). Because a variation in germination rate is usually considered as a reflecting adaptation strategy to a certain ecological condition (Venable & Lawlor, 1980; Grime *et al.,* 1981), I can also suggest that the great difference seen in germination rates is caused presumably by specific sets of traits for species to become adapted to their ecosystems. To my knowledge, *C. nevadensis* (most likely the entire section *Denticulatae* in general) stand alone among the species belonging to the *Grammica* subgenus because its seeds possess a single palisade layer and because its shoot tip emerges first. The seed coat structure along with the unique embryo of *C. nevadensis* are specific traits likely important from an evolutionary point of view that need to be further investigated.

2.5 Conclusion

The results of this study suggest that the different germination pattern occurring in *C. nevadensis* seeds may be an adaptation strategy of this species for a rapid access to a host plant. The absence of the first palisade layer in this species is expected to decrease the strength of the seed coat and consequently increase the seed permeability and germination. In contrast, the multiple palisade layers, especially at the hilar region, seen in the other two species likely cause a delayed germination which may help them survive better and increase their seed bank persistency in the soil. In conclusion, seed coat features are specific traits which influence the germination of a species, allowing it to better adapt to its habitat.

Chapter 3

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

3.1 Abstract

Seedlings of the parasitic plant *Cuscuta* (dodder) are autotrophic but can survive only a short period of time during which they must locate and attach to a host. Seedlings have an ephemeral root-like organ considered not a 'true' functional root by most studies. We studied two species with contrasting ecology, *C. gronovii* and *C. campestris*, and compared the morphology, structure and absorptive capability of their root-like organ, assessed their potential for mycorrhizal colonization using two fungal substrates, and determined the effect of the symbiotic relationship on the growth and survival of their seedlings. The root organ of both species was capable to absorb and form mycorrhizae but the two species exhibited dissimilar growth and survival patterns depending on the mycorrhizal status of their seedlings. The extensively colonized seedlings of *C. gronovii* grew more and survived longer than non-mycorrhizal seedlings. In contrast, the scarce colonisation of *C. campestris* seedlings did not increase their growth or longevity. Our results strongly indicate that dodders have a 'true' root, even if much reduced, ephemeral, and partially functional. The different mycorrhizal associations reflect the ecology of the two species and suggest that fungal specificity plays an important role in the evolution and biogeography of dodders.

3.2 Introduction

Cuscuta (dodders, Convolvulaceae) includes *c*. 200 species of annual, obligate stem parasites with a sub-cosmopolitan distribution (García *et al.,* 2014; Costea *et al.,* 2015); it is found in nearly all the terrestrial habitats (Yuncker, 1932; García *et al.,* 2014). 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 (reviewed by Press & 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 & Riches, 1993; Dawson *et al.,* 1994; Costea & Tardif, 2006). Last but not least, an estimated 30-50% of the dodder species require conservation measures (Costea & 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 the seedlings of other plants, *Cuscuta* seedlings face abiotic and biotic challenges (e.g., Maun, 1994; Isselstein *et al.,* 2002; Maestre *et al.,* 2003) 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 & 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 & Tardif, 2006). Thus, the non-parasitic (seedling) stage in *Cuscuta* represents a significant ontogenetic population bottleneck.

The survival of *Cuscuta* seedlings and the factors affecting it have received little or no attention because most of the studies conducted during the establishment stage have concentrated on the parasite-host interactions (reviewed by Dawson *et al.,* 1994; Costea & Tardif, 2006).

Survival periods reported are often anecdotal and vary widely among species: eight days for *C. campestris* (Parker & Riches, 1993), 13–19 days for *C. chinensis* (Marambe *et al.,* 2002), three weeks for *C. europaea* (Heide-Jorgensen, 2008), four weeks for *C. pedicellata* (Lyshede, 1985) or up to seven weeks for *C. gronovii* (Heide-Jorgensen, 2008). These diverging preliminary numbers suggest that seedling survival may be a species trait in *Cuscuta* but if that is true, it is unknown why and how the seedlings of a certain species survive longer than those of others.

Authors of structural studies suggested that dodders lack a 'true' root and that this organ is a modification of the shoot base (e.g., Johri & Tiagi, 1952; Haccius & Troll, 1961; Truscott, 1966; Dawson *et al.,* 1994; Sherman *et al.,* 2008). As a result, *Cuscuta* seedlings are often referred to as "rootless" (e.g., Lanini & Kogan, 2005; Runyon *et al.,* 2006; Albert *et al.*, 2010), generating the false idea that the root-like organ is entirely absent. In fact, the radicular organ lacks a root cap and meristems; it is devoid of exodermis, endodermis, 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., five days in *C. gronovii*; Truscott, 1966), the root-like organ of *Cuscuta* 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 & Troll, 1961; Truscott, 1966; Sherman *et al.,* 2008). Interestingly, one study (Khalid & Iqbal, 1996) reported that the 'underground part' of *C. reflexa* seedlings was colonized by arbuscular mycorrhizal (AM) fungi. Although Khalid and Iqbal's study (1996) had a limited scope and somewhat unclear methodology (e.g., the authors did not provide information about the colonized organ, the fungus identity, its structures, penetration, and colonization), it highlighted that, despite of its short life, the root-like organ of *Cuscuta* is capable to interact with microorganisms. This latter result led us to hypothesize that the

transitory relationship with fungi may play a role in the differential survival of dodder seedlings. Also if they are capable of forming mycorrhizae, *Cuscuta* seedlings may have a 'true' root after all.

Thus, our general aim was to explore the potential effects of mycorrhizal colonization on the growth and survival of *Cuscuta* seedlings. To achieve this, we selected two *Cuscuta* species with different ecology, *C. gronovii*, a common riparian dodder in North America (Costea *et al.,* 2006a; Costea & Tardif, 2006), and *C. campestris*, a nearly cosmopolitan invasive weed (Holm *et al.,* 1997; Costea *et al.,* 2006b). We compared the morphology, the structure and the absorptive capability of their root-like organ, assessed their potential for mycorrhizal colonization, and determined the effect this symbiotic relationship had on the growth and survival of their seedlings.

3.3 Material and methods

3.3.1 Seed collection and plant growth conditions

Seeds of *Cuscuta gronovii* were collected in 2012 from a natural population growing in Ontario, Canada, on the banks of the Grand River (43°30'12.02"N, 80°29'37.97"W); the host was *Solidago canadensis*. *Cuscuta campestris* seeds were removed from a herbarium specimen [U.S.A., California, Sonoma Co., Rosa, 38°13'26.60"N, 122°49'42.10"W, abundant on *Xanthium strumarium*, Sep 2007, *Cadman & al. 2832*]; to propagate this species, its seeds were germinated on wet filter paper on Petri plates. The seedlings were then transferred to pots containing the hosts *Ocimum vulgare* and *Xanthium strumarium* for *C. campestris* to complete its life-cycle. The seeds thus produced were used in the following experiments. Herbarium vouchers for both species are kept in the Wilfrid Laurier University Herbarium (Waterloo, Ontario, Canada). Harvested seeds were stored in the fridge at 4°C in dried glass vials.

Seeds were sterilized and scarified using the methodology of García *et al*. (2006) with some modifications. Seeds were soaked in sterile water for 1h, submerged in 3% active chlorine solution (i.e., bleach) for 3 min, and rinsed with sterile water 6–8 times. 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 sec, 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 on fungal colonization, the seeds were planted into square pots (10 cm) filled with Promix® (Premier Tech Ltd., Rivière-du-Loup, Québec, Canada), a commercial mycorrhizal substrate containing *Rhizophagus intraradices* (also known as *Glomus intraradices*; Krüger *et al.,* 2012) fungal propagules. The pots were placed in a Biotronette growth chamber 850H under the following conditions: 22:30°C, 8:16 h, dark: light respectively. Alternatively, some seeds were grown onto plates of chicory (*Cichorium intybus* L.) Root Organ Culture (ROC) consisting of Ri T-DNA transformed chicory roots inoculated with the arbuscular mycorrhizal species *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 a freshly-made M medium (Bécard & 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 onto the plates and pushed gently into the Phytagel® until they were levelled with its 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.

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

For each species, 15 embryos were dissected from rehydrated seeds and examined using a Nikon SMZ1500 stereomicroscope to compare their morphological differences; special attention was given to their radicular ends. Sherman *et al.* (2008) had reported that one-day old seedlings provided the most consistent-structurally samples; therefore, fifteen seedlings of each species were harvested at this age to study the root. For light microscopy (LM), roots fixed in 50% ethanol were embedded in LR White using a method modified from O'Brien and McCully (1981) whereby the infiltration time was increased to 3 h. The roots were transversally sectioned at 2 μm thickness with a Sorvall MT-1 ultra-microtome, and sections were stained with 0.05% Toluidine Blue O (TBO) pH 4.4 for 1 min. To study the root ultrastructure, the roots of 1 day-old seedlings were fixed in 3% glutaraldehyde and 2% paraformaldehyde in 0.025M sodium phosphate buffer (pH 6.8). The samples were then dehydrated with an ethanol series, and embedded in Spurr's resin (Ma & Peterson, 2000). The samples were transversally cut with a diamond knife at 0.5-1 μm thick for LM and 80–100 nm thick for transmission electron microscopy (TEM). Sections were mounted on glass slides for LM observations and onto Formvar and carbon-coated copper grids for TEM observations. They were stained with Epoxy Tissue Stain (Electron Microscopy Sciences, catalogue number 14950; 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 *Cuscuta* root to absorb, 1-day old seedlings had their root organ 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 & Flühler, 1995; Albrecht *et al.,* 2002; Mader *et al.,* 2003). Samples were washed with water to remove the excess stain, mounted in water and observed under LM.

3.3.3 Mycorrhizal colonization

Cuscuta gronovii and *C. campestris* seeds were planted into three pots filled with Promix®. To ensure that a sufficient number of seeds germinated simultaneously and provided 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. To assess fungal infection, roots of inoculated plants were stained with ink (Black, Sheaffer; BIC USA Inc.) - vinegar using a method modified from Vierheilig *et al.* (1998). The 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% vinegar aqueous solution for 2 min and then incubated for 8 min into a mixture of 5% Sheaffer ink- Acetic acid (v:v) at 90°C. 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 infection of *Cuscuta* roots, observations were also made on seeds of *C. gronovii* and *C. campestris* grown on ROC plates. Seedlings were harvested 10 days after germination and stained for fungal infection as above. All prepared samples were observed under LM. The spatial distribution of *R. intraradices* inside the root and rate of mycorrhizal 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 one 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 colonisation for each unit and then for each morphological region.

3.3.4 Assessment of seedling growth and lifespan

Seeds of both species were planted in ten pots: five filled with Promix® substrate (with *R. intraradices*) and five with autoclaved Promix® substrate. A separate experiment was conducted to verify the sterilisation success of the Promix® substrate, and it was confirmed that no fungal infections occurred in the roots of seedlings grown in autoclaved Promix® substrate. Pots were randomly placed in the growth chamber. Three seedlings from each species were sampled every four 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.

To study the effects of fungal 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 mycorrhizal and 30 non-mycorrhizal) per species were selected as statistically representative. The lifespan of seedlings were recorded up to 16 and 24 days after emergence for *C. campestris* and *C. gronovii*, respectively.

At each harvesting date, a test with Neutral Red (NR, PFALTZ & BAUER, Inc., 375 Fairfield Ave. Stamford, Conn, USA) was performed to assess seedling viability (Timmers *et al.,* 1995; Dubrovsky *et al.,* 2006). Entire seedlings were incubated into 0.4 μM (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). 'Living' seedlings were

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considered 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.

3.3.5 Statistical analyses

Analysis of variance (ANOVA) using the statistical software Minitab version 16.2.4.3 was performed on the fungal colonization rates of the two species using either the data 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 mycorrhiza presence/absence on the seedling growth (length and dry weight) of the two species. Growth trends were fitted with a quadratic polynomial equation with the computed R^2. Because the seedlings of the two species have a different lifespan, we could not compare statistically the effect of mycorrhiza-formation between the two species. Instead, within each species, we compared the lifespan between mycorrhizal and non-mycorrhizal seedlings using the Student's t-test.

3.4 Results

3.4.1 Morphology, structure and ability of *Cuscuta* root to absorb

Embryos of both species are identical morphologically. They are filiform and coiled two-three times in the endosperm (Fig. 1 a,e) which is entirely consumed during germination. The root-like organ emerges from the seed two to three days after sowing in *C. campestris* and one or two days later in *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., the root and the shoot. There is no sharp morphological boundary between the shoot and the root-like organ; there is no hypocotyl or epicotyl because *Cuscuta* seedlings are devoid of cotyledons (Fig. 1 b,f).

The root-like organ consists of two morphological areas: the absorbent hairs area and the tuberous area which we called root hair (RH) region and swollen area (SA), respectively (Fig. 1c,g; Fig. 2a). 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 one-day old seedlings (Fig. 1i). These cells possess thick pectinaceous walls, a large nucleus, dense cytoplasm, a few amyloplasts, droplets of lipids, and one or two vacuoles (Fig. 1j; Appendix Fig. 2a,b). The RH region is on average three times longer in *C. gronovii* (1540 µm) than in *C. campestris* (470 µm) (Fig. 1c,g ; 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. 1h). In addition, while the root hairs of *C. gronovii* are elongated, cylindrical and reminiscent of those present on the roots of typical plants (Fig. 1d), those of *C. campestris* are
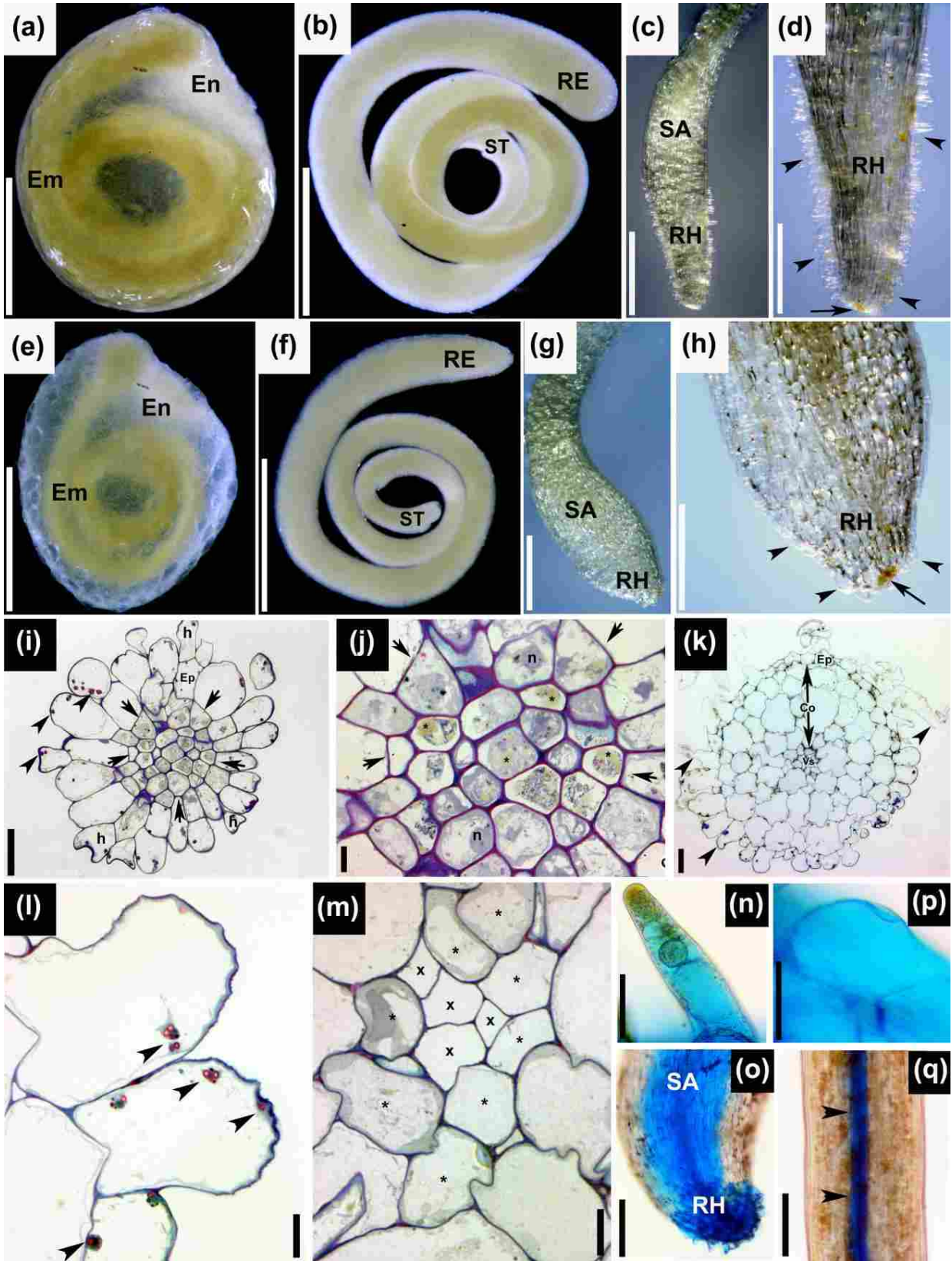
Fig. 1 Embryos and roots of *Cuscuta gronovii* (a-d) and *Cuscuta campestris* (e-h). (a,e) Embryos (Em) enveloped in the endosperm (En); (b,f) Embryos coiled from their radicular end (RE) to their shoot tip (ST); (c,g) Root composed of a root hair region (RH) and of a swollen area (SA); (d,h) While the RH region of *C. gronovii* possesses elongated root hairs (arrowheads), that of *C. campestris* is much shorter and exhibits dome-like root hairs (arrowheads). The RH region terminates in both species by a small group of colored root tip cells (arrow). (i,j) Transversal section of a root tip of a 1-day-old *C. gronovii* embedded in Spurr's resin and stained with Epoxy Tissue Stain. (i) The cells at the centre of the very tip are organized in a circular manner as delineated by the arrows. They are alive and contain a dense cytoplasm with many cell organelles. Towards the outside and more distal to the tip are highly vacuolated epidermal cells (Ep), some of which have already developed into root hairs (h); 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 Figure 1i. The cells are tightly joined with a thick, pectinaceous wall. One can recognize nuclei (n) and large lipophilic droplets (*). (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), some of which have developed into root hairs (arrowheads), several layers of cortical cells (Co) highly vacuolated and with wavy walls, and a central narrow vascular strand (Vs). (l) Higher magnification of the highly vacuolated root hairs with amyloplasts (arrowheads). (m) Higher magnification of the vascular strand where tracheary-like cells (X) are seen surrounded by cells (*) with a dense cytoplasm and a large vacuole. (n, q) Photographs illustrating root absorption. An elongated root hair of *Cuscuta gronovii* (n) and a dome-shaped root hair of *C. campestris* (p) absorb the stain (blue color). (o) Whole mount of *C.*

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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 this swollen area (SA) region. (q) Whole mount of a *C. gronovii* shoot illustrating the stain being transported through the vascular strand (arrowheads). Scale bar: 1 mm in a-c and e-g; 500 μm in d, h, o; 100 μm in q; 50 μm in i, k, n, p; 10 μm in j, l, m.
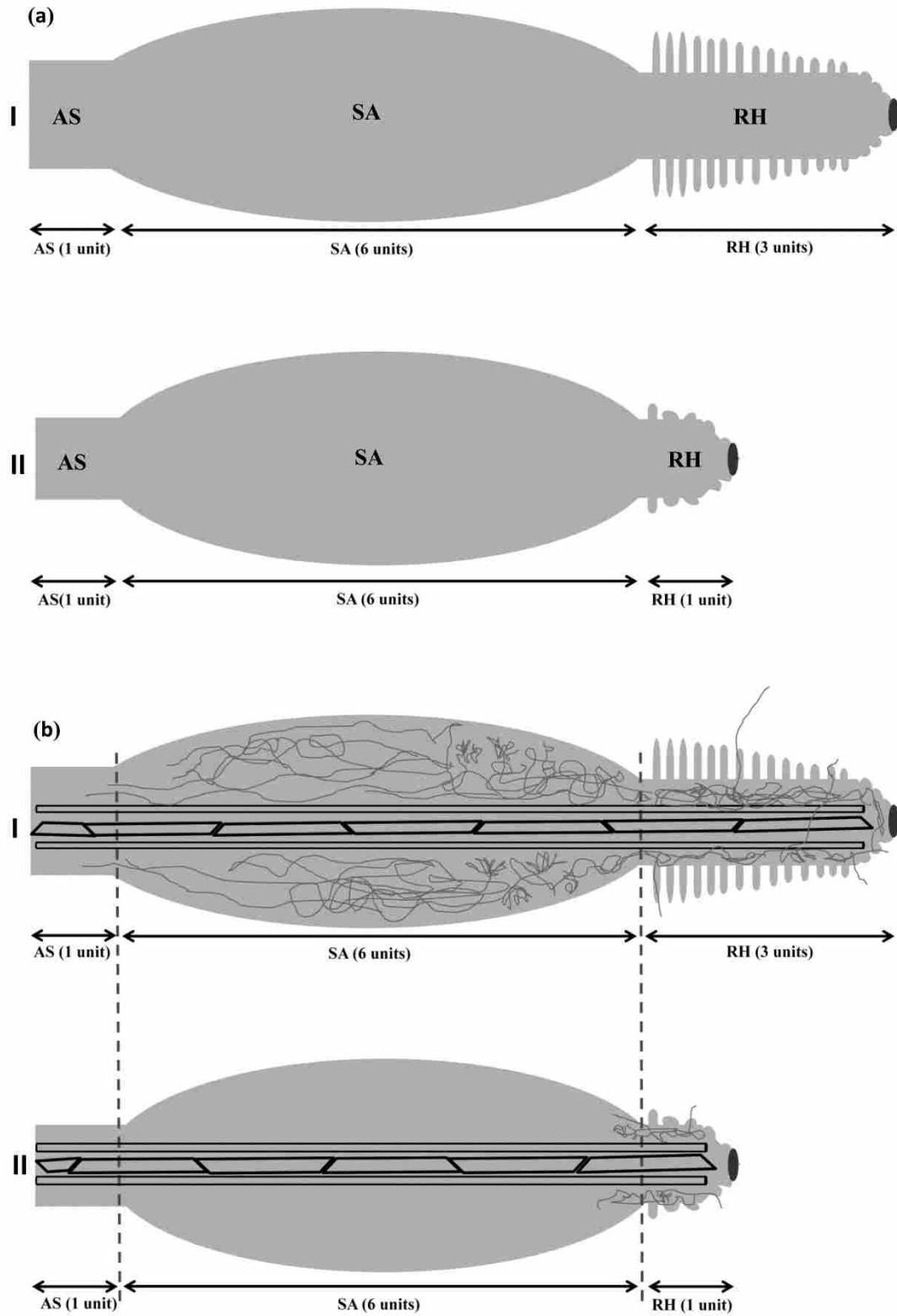
Fig. 2. Conceptual schematic diagram of roots, whether non-mycorrhizal (a) or mycorrhizal (b), of *C. gronovii* (I) and *C. campestris* (II) with the adjacent shoots (AS). Each root comprises a root hair (RH) region and a swollen area (SA). The size of the root is based on reticle units (see Materials and Methods section), and their quantitative measurements are given just below each root. For the simplicity of the diagram, the vascular strand has been omitted from the diagram. (b) Fungal penetration occurs in RH regions of the two species. In *C. gronovii* (I), fungal hyphae colonize the cortex of both the RH and the SA regions; in the distal part of the latter region, they may form peloton-like structures. 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.

dome-shaped or globular (Fig. 1h). Plastids with starch grains are present both in the root hair cells (Fig. 1l; Appendix Fig. 2c) and the cortex (Appendix Fig. 2d). A typical endodermis is absent but the cells bordering the xylem strand have a denser cytoplasm (Fig. 1m). The vascular system consists of a strand of empty vessel elements with un-lignified, primary cell walls (Fig. 1m). Phloem is absent. The SA 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 (Appendix Fig. 3).

Despite its 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 channeled upward to the shoot (Fig. 1q).

3.4.2 Mycorrhizal colonization

Mycorrhizal fungal penetration and the incipient stages of colonization were observed four days after seedling emergence in *C. gronovii* and six days after emergence in *C. campestris*. In both species, the penetration of the fungus occurred through the root hairs or typical epidermal cells (Fig. 3b,c,p). However, the two species exhibited differential qualitative and quantitative patterns of mycorrhiza-formation, which became most obvious 10 days after germination (Fig. 2b, Fig. 3a,o). Moreover, while *C. gronovii* roots were colonized by both fungi *Rhizophagus intraradices* and *R. irregularis* (i.e., in both Promix® substrate and ROC plates, respectively), *C. campestris* roots were colonized only by *R. intraradices* when seedlings were grown in Promix®.

In *C. gronovii*, after penetration, the hyphae spread profusely within the intercellular spaces of the RH region (Fig. 2b; reticle units 1–3). Subsequently, hyphae extended rapidly into the cortex of the SA region, and some even reached into the adjacent shoot portion (Fig. 2b, units 4–
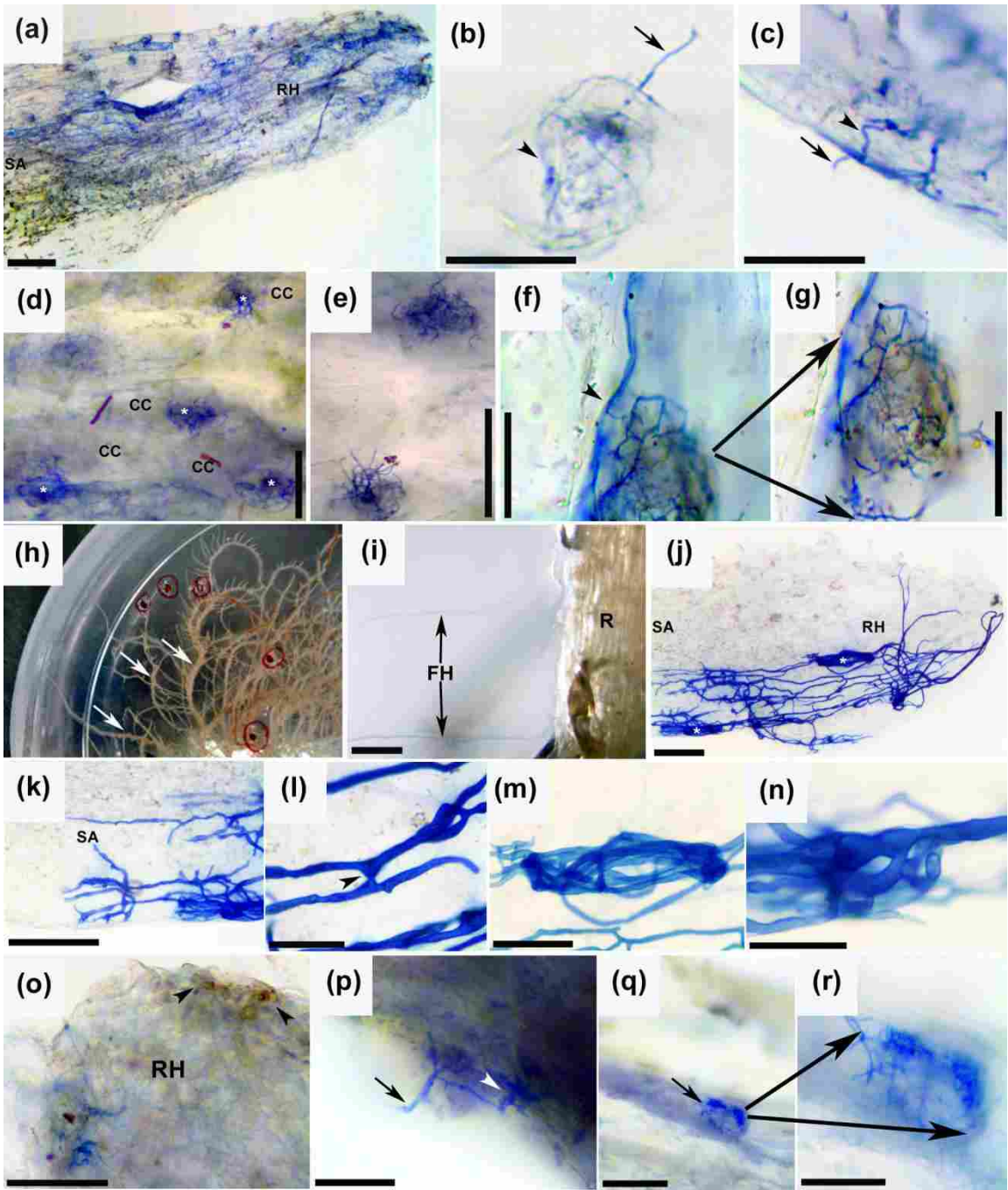
Fig. 3 Colonization of the root of *C. gronovii* seedling by *Rhizophagus intraradices* (a-g) and by *R. irregulare* (h-n), and colonization of the root of *C. campestris* by *R. intraradices* (o-r). The fungal hyphae are stained with the Ink-Vinegar method and appear blue. (a) Low magnification of a root illustrating the colonization by the fungus of the root hair (RH) region and the distal part of the swollen area (SA) region. The hyphae propagate extensively in the intercellular space of the cortex. (b,c) The extraradicular hypha (arrow) can enter the root via either a root hair (b) or an epidermal cell (c); in either case, no hyphopodium is present. Once inside the root, the intraradical hyphae (arrowhead) branch profusely. (d) Once in the SA, the fungal hyphae enter the cortical cells (CC) and form in each cell a structure reminiscent of a peloton (*). (e) Two peloton-like structures in which the hyphae branch very finely. (f,g) Higher magnifications illustrating the entry (arrowhead) of an intercellular hypha into a cortical cell. Note that the peloton-like structures do not take the appearance of a true arbuscule: No trunk is formed at the entry point (arrowhead) and the hyphae do not become thinner as they grow away from the entry point. On the contrary, they appear very fine but of equal diameter throughout the cortical cell. (h) *Cuscuta gronovii* seeds (surrounded by a red circle) seen onto the ROC plates in the vicinity of transformed chicory roots (arrows) which served as host to the fungus *R. irregulare*. (i) Fungal hyphae (FH) growing towards the root (R) of a germinated seedling. (j,k) The fungus has entered the cortex of the RH region (j) and is spreading via intercellular spaces towards the SA region (k). Note the presence of hyphal coils (*) in some of the cortical cells. (l) As the fungal hypha spreads through the intercellular space, it delineates clearly the shape of the cells it surrounds. Characteristic of mycorrhizal fungi, the branching occurs at sharp angles (arrowhead). (m,n) Higher magnification of the two hyphal coils seen in (j). (m) The coil, reminiscent of that formed in a *Paris*-type mycorrhizal association, fills most of the cellular

space. (n) The hyphae not only coils but also branches. Note the difference in width of the hyphae formed by the two fungi by comparing (n) and (e). (o) *R. intraradices* is capable of colonizing the RH region of *C. campestris* seedling. Note the root cap-like structure (arrowheads) at the root tip. (p) An extraradical hypha (arrow) branches at the surface of the root and penetrates into an epidermal cell (arrowhead). (q) A cortical cell with a peloton-like structure (arrow). (r) Higher magnification of the peloton-like structure seen in (q), illustrating the diffuse appearance of the hypha, which branches profusely within the cell. Scale bars: 300 μm in a, i, j; 200 μm in l; 100 μm in o; 50 μm in b, d, e, k, m, n, q ; 25 μm in c, p, r; 20 μm in f, g.

10). Hyphae were capable of entering the cortical cells, where they branched, became thinner, and formed an intracellular structure reminiscent of a peloton (Fig. 3d,e,f,g). However, the hyphae never filled the entire cell lumen (Fig. 3d,e). We were able to obtain a clearer view of the fungal colonization in *C. gronovii* when the seedlings were germinated on the ROC system (Fig. 3h). The fungal hyphae of *R. irregularis* were attracted and grew toward the root of *C. gronovii* (Fig. 3i); fungal penetration occurred as previously described. The hyphae extended intercellularly into the cortex of the entire root (Fig. 3j,k); as is characteristic of mycorrhizal fungi, they branched at sharp angles (Fig. 3l) within the intercellular space, following the contour of the cortical cells. When intercellular hyphae penetrated into the cortical cells, they formed a coil-like intracellular structure, with the hyphae coiling many times inside the cell (Fig. 3 m,n). That structure was reminiscent of that seen in a *Paris*-type mycorrhiza, although with no sign of fine branching. Neither vesicles nor typical arbuscules were ever observed in seedlings grown with *R. intraradices* within Promix® or with *R. irregulare* in the ROC system.

As for *C. campestris,* its initial colonization by *R. intraradices* was similar to that of *C. gronovii*, but its colonization by *R. irregulare* was never observed. When it was colonized, the hyphae kept mainly to the region of absorbent hairs (Fig. 2b, reticle unit 1), with only a few hyphae entering the adjacent portion of the SA region (Fig. 2b, unit 2). No hyphae were ever observed in the remaining part of the swollen area or in the shoot (Fig. 2b, units 3–8). The intracellular hyphae formed also peloton-like structures (Fig. 3q,r).

Fungal colonization rates were different between the two species. At the level of the entire root (Fig. 4a), the colonisation rate was significantly higher in *C. gronovii* than in *C. campestris* ($P \leq 0.001$). The same significant difference in colonisation rate was seen when the two morphological regions, RH and SA regions, were compared independently between the two

species (Figs. 4b,c respectively). Only the colonisation rate of the adjacent shoot unit did not differ significantly between the two species ($P = 0.12$) despite the fact that only *C. gronovii* exhibited hyphae in this area (data not shown). The higher colonisation rate of the RH region in *C. gronovii* is attributable to the increased length of this region in this species (Fig. 2b); while one reticle unit was enough to cover the entire absorbent hairs area of *C. campestris*, three units were necessary in *C. gronovii* (Fig. 2b). A comparison of the colonisation rates observed in the only reticle unit of *C. campestris* with either one of the three units of *C. gronovii* produced differences that were not significant between the two species (data not shown). Because the swollen area has a similar length in both species (covered by six reticle units), it was possible to assign the significantly higher colonisation rate obtained for *C. gronovii* to a higher density of hyphae. In *C. gronovii*, the highest colonization rate was observed in the swollen area followed by the absorbent hairs area and the adjacent shoot region. In *C. campestris*, the fungi were restricted in the region of absorbent hairs (Fig. 2b).

3.4.3 Effect of mycorrhization on seedling growth and survival

Not only the two species differed in their colonization rate (Fig. 4a-c), but they also exhibited dissimilar growth (Fig. 4d-g) and survival patterns (Fig. 4h-i) depending on the mycorrhizal status of their seedlings. Mycorrhizal seedlings of *C. gronovii* were significantly longer than non-mycorrhizal ones (Fig. 4d). Both colonized and non-colonized seedlings attained their maximum length 12 days after emergence, 30.58 cm and 24.28 cm, respectively, at which time the mycorrhizal seedlings were *c*. 20% longer than the non-mycorrhizal ones. No further increase in length occurred in older seedlings (Fig. 4d). The dry weight (DW) of both colonized and non-colonized *C. gronovii* seedlings increased by day four (Fig. 4e). Whereas the DW of the latter declined abruptly, those of the former continued to increase slowly until day eight with a gradual

(a) (b) (c) Colonization rate (column/seedling) vs Species (*C. gronovii*, *C. campestris*)

(d) Length (cm) vs Day after emergence
$y = -1.7324x^2 + 17.889x - 14.761$
$R^2 = 0.9932$
$y = -1.5546x^2 + 15.461x - 12.593$
$R^2 = 0.9891$

(e) Dry Weight (mg) vs Day after emergence
$y = -0.0408x^2 + 0.2549x + 0.2964$
$R^2 = 0.9019$
$y = -0.02x^2 + 0.1057x + 0.4367$
$R^2 = 0.7509$

(f) Length (cm) vs Day after emergence
$y = -1.4563x^2 + 11.168x - 7.8355$
$R^2 = 0.9437$
$y = -1.3967x^2 + 10.675x - 7.3502$
$R^2 = 0.9359$

(g) Dry Weight (mg) vs Day after emergence
$y = -0.0121x^2 + 0.0501x + 0.1641$
$R^2 = 0.7209$
$y = -0.0067x^2 + 0.0179x + 0.1939$
$R^2 = 0.5423$

(h) (i) Seedling lifespan (day) vs Substrate (Myc+, Myc-)

Fig. 4 Colonization rate of *Rhizophagus intraradices* (based on distribution of fungal hyphae) in (a) the entire root, (b) the root hair area, and (c) the swollen area. (d-g) Length (d,f) and dry weight (e,g) of seedlings of *C. gronovii* (d,e) and *C. campestris* (f,g), grown over time in the absence (Myc-) or presence (Myc+) of *R. intraradices*. Note the growth trends were fitted with quadratic polynomial equations with computed R^2. (h) *C. gronovii* and (i) *C. campestris* seedling longevity. Note: analysis of variance (ANOVA) was performed on the fungal colonization and seedling growth performances of the seedlings of the two species. The seedling lifespan was compared between Myc+ and Myc- seedlings within each species using the Student's *t*-test.

reduction afterwards (Fig. 4e). These dissimilar growth patterns resulted in significant DW differences between colonized and non-colonized seedlings at eight and 12 days after emergence ($P \leq 0.01$ 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.

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

The seedlings' longevity of the two species was affected differently by the presence/absence of mycorrhizal fungi. In *C. gronovii*, mycorrhizal seedlings lived on average three days longer than non-mycorrhizal seedlings (20 days versus 17 days, respectively), a difference that was statistically significant ($P \leq 0.01$; Fig. 4h). Moreover, the minimum lifespan of mycorrhizal seedlings was 17 days versus 12 days for non-mycorrhizal seedlings. In contrast, the lifespan (Fig. 4i) of *C. campestris* seedlings were not affected by mycorrhizal colonization and the differences between mycorrhizal and non-mycorrhizal seedlings were insignificant. For example, the average lifespan of *C. campestris* seedlings was 15 days maximum and the minimum 12 days regardless of the mycorrhizal conditions. Thus, although not compared statistically, the longevity of *C. gronovii* seedlings was higher than that of *C. campestris* in both mycorrhizal and sterile substrates.

The duration of the root-like organ, based on the Neutral Red test, in the two species followed the same trend as the lifespan of their seedlings. In *C. gronovii*, the first signs of cellular degeneration were observed eight days after emergence in mycorrhizal seedlings and four days in non-colonized seedlings (data not shown). Ultimately, the root organ of mycorrhizal seedlings lasted longer (12 days) than that of non-mycorrhizal 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 mycorrhizal status of its seedlings.

3.5 Discussion

3.5.1 Mycorrhiza in *Cuscuta*: implications for the status and evolution of the root organ

The structure and role of the *Cuscuta* root-like organ have puzzled botanists since the earliest studies of this genus (Koch, 1880; Mirande, 1900). Subsequent embryology research found that the early separation of the proembryo into a shoot axis and a root axis seen in other dicots is not apparent in *Cuscuta* because a cotyledonary node does not form (Johri & Tiagi, 1952; Truscott, 1966). Thus, the root-shoot organ uncertainty is set developmentally as early as the proembryo stage. Since the *Cuscuta* root-like organ lacks most of the structural and developmental features present in the roots of other flowering plants, most studies available suggest that this transitory organ is not a root but a modification of the shoot base (e.g., Haccius & Troll, 1961; Truscott, 1966; Sherman *et al*., 2008). To accommodate this view, *Cuscuta* has been considered to lack a true root (e.g., Dawson *et al.,* 1994), and the root-like organ has been referred to as the 'tuberous radicular end' of the shoot (Lyshede, 1986; Sherman *et al.,* 2008). This is a potential source of confusion because it may be interpreted as a complete absence of the organ, as for example when *Cuscuta* seedlings are referred to as 'rootless' (e.g., Lanini & Kogan, 2005; Runyon *et al.,* 2006; Albert *et al.,* 2010).

Our results confirmed all the structural findings of previous studies (Haccius & Troll, 1961; Truscott, 1966; Lyshede, 1986, 1989; Sherman *et al.,* 2008). However, if the absence of many normal root features is a valid way to establish that this is not a 'true' root, then there are even fewer shoot characteristics to support a cauline origin hypothesis for this root-like organ. Furthermore, we found that the group of cells located at the extreme tip of this organ were alive in 1-day old seedlings (Figs. 1i,j); this result does not agree with that of Truscott (1966) who labelled these cells dead (their Figs. 11 and 18). Sherman *et al*. (2008) proposed that these cells may represent the end of the vascular system of the root-like organ (Sherman *et al.*'s Fig. 2b), although in their Fig. 3a, one can see that these cells are metabolically active and alive. We suggest an alternative interpretation to that of Sherman's (2008); that based on their position, thicker cell walls, and lipid content, this tissue may be a vestigial root cap.

The results of our study showed that the root organ of *Cuscuta* can fulfil even if for a short period of time the roles of a typical root, an idea put forward by very few other studies (Lyshede, 1989). The root hairs of *Cuscuta* are certainly not typical, especially in *C. campestris*, but they absorb solutes, as suggested by Koch (1880) more than a century ago. In addition, these root hairs can serve as an entry point for mycorrhizal fungi, as they do in other plants (Guinel & Hirsch, 2000; Novero *et al.,* 2009). The ability to form a mycorrhizal relationship implies that this organ must employ the root signaling pathway necessary for establishing a proper cross-talk with the fungi (e.g., Paszkowski, 2006; Oldroyd, 2013; Gutjahr, 2014). Also, because the AM fungi are obligate biotrophs, *Cuscuta* must be capable to supply them with carbon (Harley & Smith, 1983; Smith & Read, 1997; Peterson *et al.,* 2004). This is remarkable considering the 'cryptic' photosynthetic apparatus of the parasite (e.g., Hibberd *et al.,* 1998; Krause, 2008) and its own growth requirements during the seedling stage. 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, we show here that for a short period of time the 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 shoot. The morphological differences observed between the roots of the two species, i.e., in their root hairs and size of the root hair areas, have likely a functional significance. It cannot be a mere coincidence that *C. gronovii* roots, with their larger RH region area covered by numerous 'normal'- looking root hairs, exhibited an increased mycorrhizal colonization and an overall higher longevity of both the root organ and the entire seedling than *C. campestris* roots, with their few and bulbous root hairs.

From an evolutionary point of view, if this organ were to be a modification of the shoot, then two successive evolutionary 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 *per se* cannot provide an answer, 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 "rootless seedlings".

3.5.2 Mycorrhiza in *Cuscuta*: evolutionary and ecological implications

According to our knowledge, *Cuscuta* is the only holoparasitic plant capable of forming a mycorrhizal association. A more complex, tripartite association was reported among *Cytinus hypocistis* (*Cytinaceae*), an unknown AM fungus, and several host species from *Cistaceae*, but the interpretation of this relationship was controversial (De Vega *et al.,* 2010; Brundrett, 2011; De Vega *et al.,* 2011). Hemiparasitic plants such as *Pedicularis* can be mycorrhizal; such mycorrhizal status was reported to increase P availability (Li *et al.,* 2013) and may serve as an alternative nutritional strategy when appropriate host plants are not available (Li & Guan, 2008).

Khalid and Iqbal (1996) reported that spores, arbuscules, and vesicles formed in the underground part of *C. reflexa* seedlings within seven days. In our study, the lack of spores may be explained by the root degenerating before the fungal micro-symbiont completes its life cycle. As for the arbuscules or vesicles, we did not observe any in the mycorrhizal associations we studied, i.e., with the mycorrhizal fungi *Rhizophagus intraradices* and *R. irregulare*. This may not be surprising as it is the plant which controls the form and shape of the mycorrhizal structures it forms (Gutjahr, 2014). For example, *R. intraradices* forms *Paris*-type mycorrhizae in the plant *Panax quinquefolius* (Armstrong & Peterson, 2002) but *Aru*m-type ones in basil (*Ocimum basilicum*). Yet, in *Cuscuta*, the hyphae developed peloton-like structures (Fig. 3 d,q), reminiscent of mycorrhizal structures seen in some orchids (Rasmussen, 2002; Peterson *et al.,* 2004). The signaling in *Cuscuta* must differ from that of more typical plants.

The oddness of a mycorrhizal relationship in *Cuscuta* comes from its ephemeral duration. The rapid fungal colonization of the root cortex observed especially in *C. gronovii*, combined with the early biomass accumulation, suggest that the relationship is mutually beneficial at least in the beginning. However, later, the symbiosis may become parasitic when *Cuscuta* recycles

components from its short-lived roots and translocates them to the shoot. One may wonder if in fact *Cuscuta* cheats on the fungus as it may well recycle some of the fungal compounds too (Cox & Tinker, 1976; Smith & Read, 1997; Lee *et al.,* 2002; Koide & Mosse, 2004; Parniske, 2008).

As demonstrated here, the roots of *C. gronovii* and *C. campestris* exhibited different spatial and quantitative colonization patterns by the AM fungi, and the differential colonization was associated with dissimilar growth and longevities of their seedlings. The extensively colonized seedlings of *C. gronovii* were capable to grow more and survive longer than non-mycorrhizal seedlings. In contrast, the scarce colonisation of *C. campestris* seedlings did not increase their growth or longevity. All the dodder species, including those characterised as 'generalists', like *C. campestris* and *C. gronovii*, have a certain host range and preference, and their seedlings can establish a haustorial contact only with certain primary host plants (Gaertner, 1950; Parker & Riches, 1993; Costea & Tardif, 2006). Seedlings are capable of searching for suitable hosts in the plant community (e.g., reviewed by Costea & Tardif, 2006; Runyon *et al.,* 2006), but their success is predicated on the presence of appropriate hosts within their reach. Several days of prolonged survival may allow both a longer searching time and the potential emergence of suitable host plants in their proximity. But why would *C. gronovii* alone have retained the root capability to take advantage of the survival 'boost' provided by the mycorrhizal relationship while *C. campestris* would not? Both species belong to the subgenus *Grammica*, the last infrageneric group of *Cuscuta* to diversify (Stefanović *et al*., 2007; García *et al.,* 2014), and they are the most successful and widely distributed dodder species in North America (Dawson *et al.,* 1994; Costea & Tardif, 2006). The clade of *C. campestris* is known to be more recently derived (Stefanović *et al.,* 2007) and to have a slightly more degraded plastome than the clade of *C. gronovii* (Braukmann *et al*., 2013). Thus at first look, it may seem that the photosynthetic

apparatus and the root of *C. campestris* are too reduced phylogenetically to undertake an efficient mycorrhizal symbiosis. To this explanation, however, we prefer to propose that the answer to the above question lies in the different ecological strategy of the two species.

Cuscuta gronovii occurs in natural riparian habitats or mesic temperate forests; only in crops cultivated in these types of habitats, such as cranberry, it can become an aggressive weed (e.g., Costea & Tardif, 2006; Sandler, 2010). In contrast, *C. campestris* thrives in habitats with a degree of anthropomorphic disturbance, both ruderal and agricultural (Dawson *et al.,* 1994; Costea & Tardif, 2006). As a result, *C. gronovii* is currently restricted to 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 *et al.,* 2006b; Costea & Tardif, 2006). As with other mycorrhizal plants (e.g. Allen, 1991; Taylor *et al.,* 2004; De Long *et al.,* 2013), it is likely that the fungal relationship plays 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 become more ecologically versatile and therefore more extensively distributed geographically (e.g., Pringle *et al.,* 2009; Hempel *et al.,* 2013). This is probably the case of *C. campestris* which responded poorly to *Rhizophagus intraradices* and not at all to *R. irregulare*, in contrast to *C. gronovii*. In the former species, evolution has led to the loss of the survival boost provided by the mycorrhizal relationship, accompanied by further reductions of the root organ and photosynthetic apparatus, whereas in the latter species evolution banked on the fungal association to thrive.

The rarity versus abundance of various *Cuscuta* species has been explained as a consequence of their host range (Costea & Stefanović, 2009). Host-specialized species are restricted to a certain habitat and a geographical area, and are more likely to become rare or endangered

(Costea & Stefanović, 2009). The case of *C. gronovii* and *C. campestris*, both 'generalist' species capable to parasitize numerous hosts, strongly suggests that mycorrhizal specificity is also affecting the life history and biogeography of dodders. It can be predicted that a degree of mycorrhizal specificity is present in the numerous species of *Cuscuta* confined to a great variety of habitats, from temperate to tropical, desert to riparian, littoral to high mountains, grasslands, forests, saline, ruderal, and agricultural (Yuncker, 1932). For example, the roots of *C. gronovii* seedlings harvested from a natural population growing along the Grand River in Waterloo, Ontario, were colonized abundantly by several native unidentified species of fungi (Behdarvandi, pers. comm.). Thus, mycorrhizal specificity potentially adds another dimension of complexity to the evolution of *Cuscuta*. In the future, comparative studies using a broad sampling of both phylogenetically diverse *Cuscuta* species and their fungal partners from natural habitats will be necessary to unravel the evolutionary role of mycorrhiza in this genus.

Chapter 4

General Discussion

4.1 Contributions

In this study, I investigated the seed germination and seedling survival of three *Cuscuta* species belonging to the subgenus *Grammica* and having a different ecology. In doing so, I focussed on the non-parasitic phase of the life-cycle of these parasitic dodders. The major contribution of the present study was to understand some of the factors influencing germination, survival of the seedling and consequent population establishment. In previous studies, researchers discussed the *Cuscuta* seed coat structure and dormancy breaking methods (e.g., Gaertner, 1950; Tingey & Allred 1961; Hutchison & Ashton, 1979) but rarely considered the relation of the intact seed coat of *Cuscuta* seeds with their germination rates. Scientists also suggested controversially various seedling longevities as well as root absorption ability of *Cuscuta* (Koch, 1880, Fer, 1976; Srivastava and Chauthan, 1977; Lyshede, 1986, 1989; Sherman *et al.,* 2008) without providing reliable data for potential effective factors. Thus, in this study, I attempted to look into certain factors which could potentially influence *Cuscuta* life during its non parasitic phase. Among the new results of this study, clear indication of mycorrhizal colonization (see chapter 3) with identified fungal/plant species is one of the most significant findings. The subsequent effect of the fungal colonization on seedling growth and longevity of *Cuscuta* is also another major finding in agreement with the findings of Khalid and Iqbal (1996) in *C. reflexa,* which is a primitive species from the subgenus *Monogynella.* In fact, this is the first report of mycorrhizal colonization in the subgenus *Grammica* and the second report of mycorrhizal colonization in the entire genus. Furthermore, this study is the first precise report of

the seedling longevities in the two *Cuscuta* species, *C. gronovii* and *C. campestris*. The germination pattern and seed coat structure of *C. nevadensis* had never been described before and are not encountered to my knowledge in other *Cuscuta* species outside Sect. *Denticulatae*. The variations of germination pattern, the *Cuscuta* seed coat structure, as well as the qualitative and quantitative study of AM colonization in *Cuscuta* root will be beneficial and practical for further investigation of *Cuscuta* biology in the future. The findings also provide a new method of AM colonization quantification that may be useful for others dealing with atypical plants.

4.2 Integrative nature of the study

The interrelationships of organisms in environments have different levels of complexities, making it difficult to answer even a simple scientific biological question (Barbault *et al*., 2004). Finding rational responses to such an inquiry cannot be usually achieved by applying one discipline (Wake, 2008). Thus, biologists use multidisciplinary methods partaking what is known as 'integrative biology' (Wake, 2003). The broad meaning of the term can be interpreted differently depending on the people using it and the level of their studies (Wake, 2001). On the basis of the general definition of the term, I believe that the present study is integrative at its own level, although because of the time constraints of the Master's degree broad ranges of disciplines may not have been applied.

Practically, this work is a collaboration of two different laboratories. Various types of microscopic techniques were applied in a 'Plant Systematics' laboratory to study broad ranges of topics from comparative morphology to ultra-structure in order to understand better the distinction existing between *Cuscuta* species. The study of mycorrhizal colonization using different types of AM fungi was done in a 'Plant Biology' laboratory to give knowledge of the

plant/soil microorganism interactions that may be involved in explaining the different ecologies of various *Cuscuta* species. Novelty is one of the main concepts of integrative biology (Wake, 2003), and new methods have been used in this study. For instance, ROC plates are commonly used to study the mycorrhizal association by placing the root of different species into a plate containing transformed roots. In this study, the *Cuscuta* seeds were directly placed into the plates and the seedlings were grown in the presence of the fungi and its host plant. Another novelty in this study was the method used to quantify fungal colonization; this method of scoring may help biologists later to deal with other plants. Moreover, because of its ephemeral nature, the *Cuscuta* seedling is highly vulnerable to damage during the experimental and analytical processes, thus, the embedding and staining methods had to be modified and adapted to this plant.

Moreover, this study investigated the interaction between two biological kingdoms (Plant/Fungi) and although this relationship is well-known in a typical plant (with AM fungi), it has been rarely reported and not fully understood in *Cuscuta*. This fungal/plant association in *Cuscuta* may help explain the various distribution of the *Cuscuta* species and eventually may lead to increase the knowledge of its control management. Finally, these new scientific data along with a selection of older sources (literature cited) are an effort to integrate structural and environmental factors (biotic) to better explain the seedling establishment and survival variations of *Cuscuta* species. These findings integratively cover the structural, developmental, and ecological aspects of plant biology. Consequently, I think this thesis entirely meets the fundamental concepts of integrative biology and its methodology and results can be extended and fitted to other biological studies.

4.3 Future directions and recommendations

Since the nature of scientific research is to answer some scientific questions thoroughly, this study attempted to produce a comprehensive investigation on the structure and development of *Cuscuta* in the non-parasitic phase of its life cycle. It focused on certain factors influencing seed germination and seedling growth/survival of only a few species. However, how these factors vary and act in other *Cuscuta* species is still unknown.

In this study, the morphology and structure of seed coat in three *Cuscuta* species were surveyed. One of the most important factors affecting seed germination is the chemical composition of the seed coat, which can be compared among different *Cuscuta* species with more advanced molecular and microscopic methods. A possible difference can be investigated among seeds (seed coats) of species with dehiscent versus indehiscence capsules. It may be hypothesized that species with indehiscent capsule have a thinner seed coat than those with dehiscent capsule. The variations (if any) may have effects on the seed germination and dispersal and consequently the establishment of the seedlings.

The other focus of the study involving the effect of mycorrhizal colonization on growth and survival of the seedling can also be extended. There are still many species, even among typical plants, that are not known as mycorrhizal plants only because of lack of research or insufficient samples and methods. Thus, one possible study can be an investigation of AM fungal interaction in species of the other *Cuscuta* subgenera while the AM colonization has been already reported in the subgenus *Monogynella* (*C. reflexa*; Khalid & Iqbal, 1996) and the subgenus *Grammica* (the present study). The comparison could be also extended between *Cuscuta* species producing dehiscent versus indehiscent capsules to figure out if this species-specific trait is involved in the

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fungi/plant interactions. In addition, plant hormones including strigolactones can also be studied to understand the potential role of the phytohormones for fungal specificity in *Cuscuta*. Furthermore, while this study provides evidence of mycorrhizal colonization and its effects on seedling growth and survival, the efficiency of mycorrhizal infection in this ephemeral relationship is still not fully understood but can be surveyed histochemically by measuring alkaline phosphatase activity. Finally, since this research was done under laboratory conditions in the absence of a host plant, further investigation can be considered in a natural habitat in the presence of native fungal species and host plants.

Although this study clearly states that *Cuscuta* seedling has a 'true' root which is capable of absorption and interaction with soil microorganisms, the precise region where the root ends and shoot begins is still unknown. This can also be studied using advanced molecular techniques to understand the related gene expression in the root compared to the shoot of *Cuscuta* seedlings.

In general, as the growth and survival of *Cuscuta* seedling had not been thoroughly investigated, this study tried to produce precise data on the seedling lifespan. All data presented here may eventually help scientists to have a broader understanding of *Cuscuta* biology and ecology.

4.4 Summary

➢ Different germination patterns occur in *Cuscuta* species with 'viviparous' nature.

➢ Seed coat structures vary among *Cuscuta* species and directly influence the seed germination rates. The external palisade layer is probably responsible for causing the impermeability of the seed coats (physical dormancy).

➢ The seed coat structure is likely a species-specific trait for a better adaptation of *Cuscuta* species to their habitats.

➢ The vestigial root of *Cuscuta* seedling is a 'true' root, capable of absorption and interaction with soil microorganisms, although it is a short-lived organ.

➢ AM fungal colonization occurs in the rudimentary root of *Cuscuta* species. However, different species of *Cuscuta* exhibit different fungal specificity and interact differently with the same AM fungus.

➢ Different fungal interactions of *Cuscuta species* result in dissimilar growth and survival of their seedlings. Abundant AM colonization in *C. gronovii* root increases the growth and survival of the seedling compared to a non-mycorrhizal seedling.

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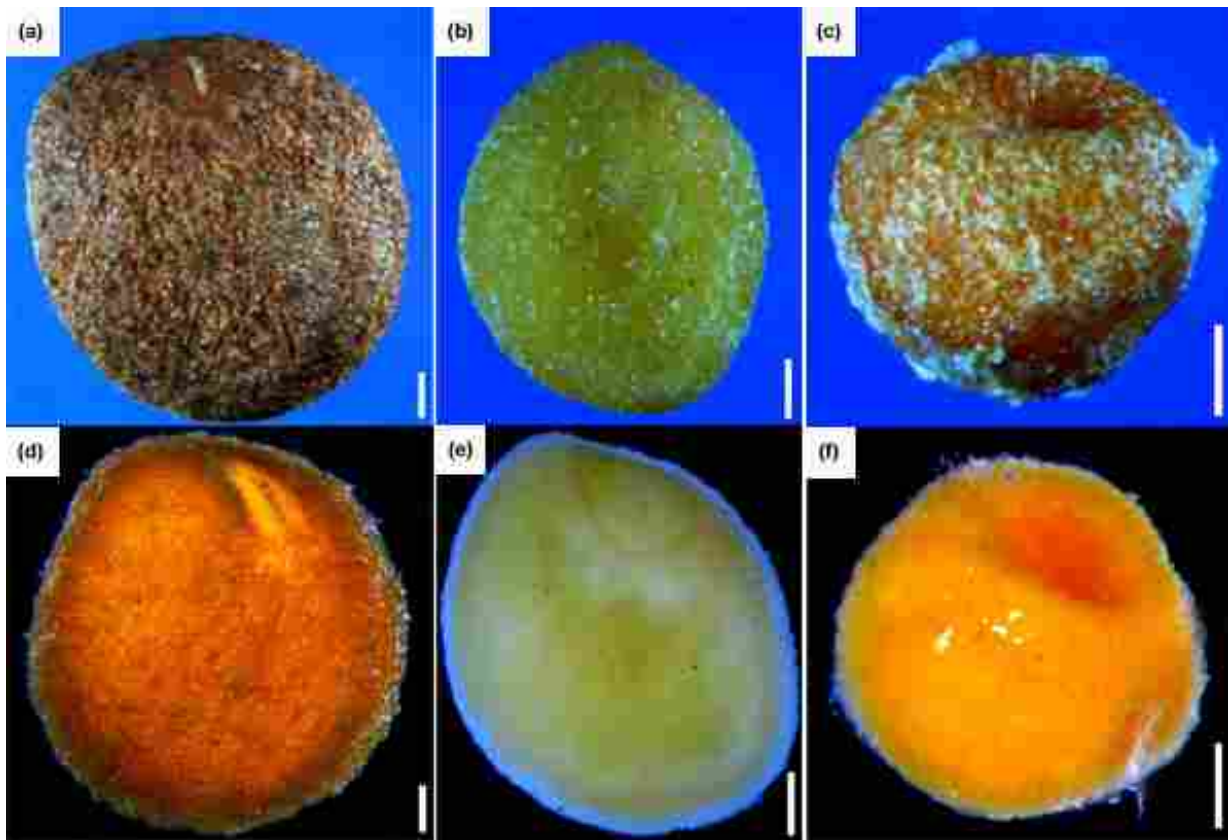
Fig. 1 Dry (a-c) and hydrated (d-f) seeds of three *Cuscuta* species. Dry seeds: a) *C. gronovii,* b) *C. campestris,* c) *C. nevadensis.* Hydrated seeds: d) *C. gronovii,* e) *C. campestris,* f) *C. nevadensis*. Note: The epidermal outer cell walls of the three species seeds are alveolated when dry and papillae when hydrated. Scale bars: 200 μm in a,b,d,e,f; 100 μm in c.
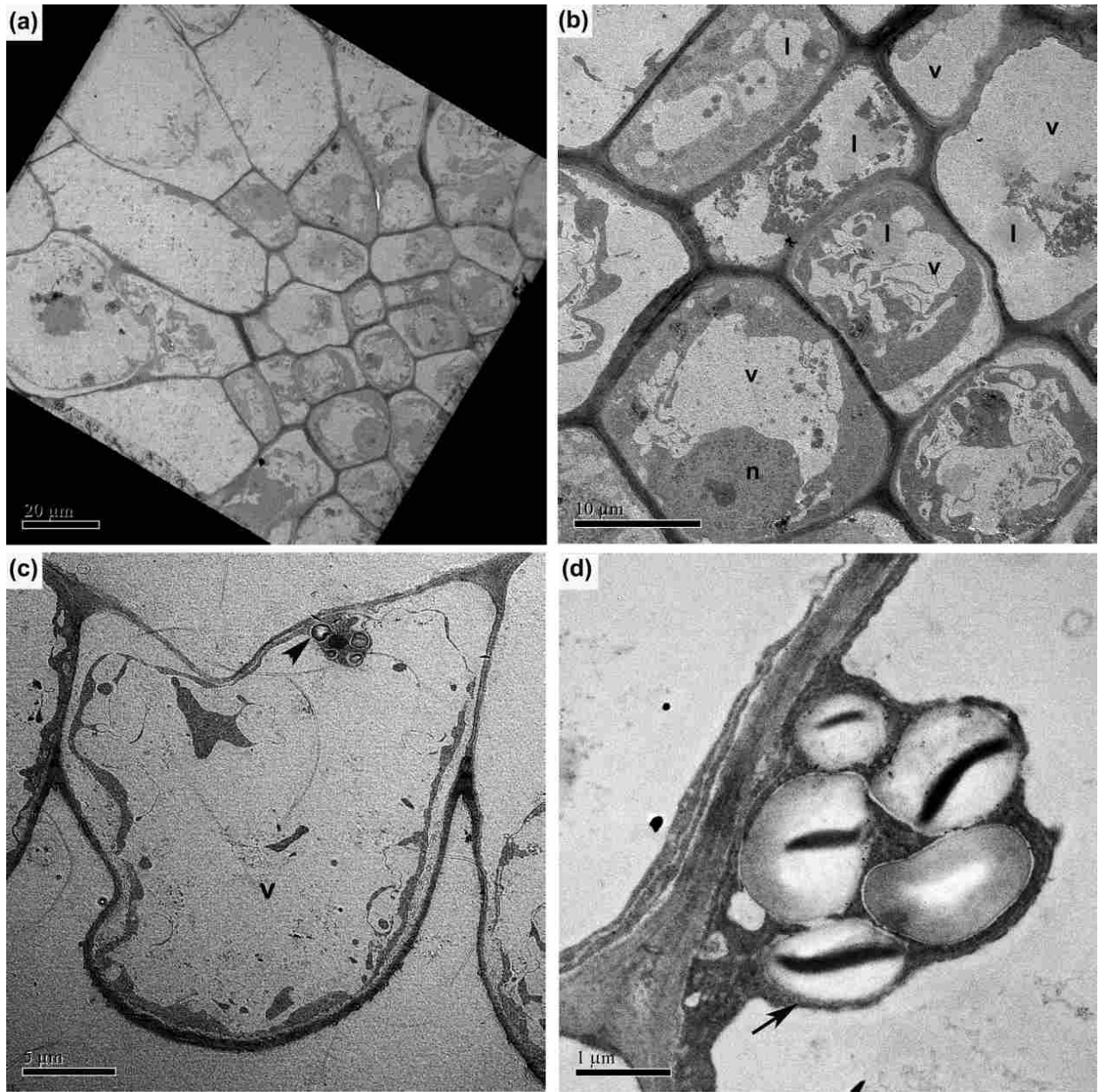
Fig. 2 Ultrastructural micrographs of *Cuscuta gronovii* root (a-d). Note: the root was embedded in Spurr's resin and stained with uranyl acetate and Reynolds lead citrate. a) *C. gronovii* root tip cells with cell contents, b) Higher magnification of the root tip cells, c) A single highly vacuolated root hair with starch grain (arrowhead), and d) A cortical cell of the root with starch grains (arrow). l: lipid droplet; n: nucleus; v: vacuole.
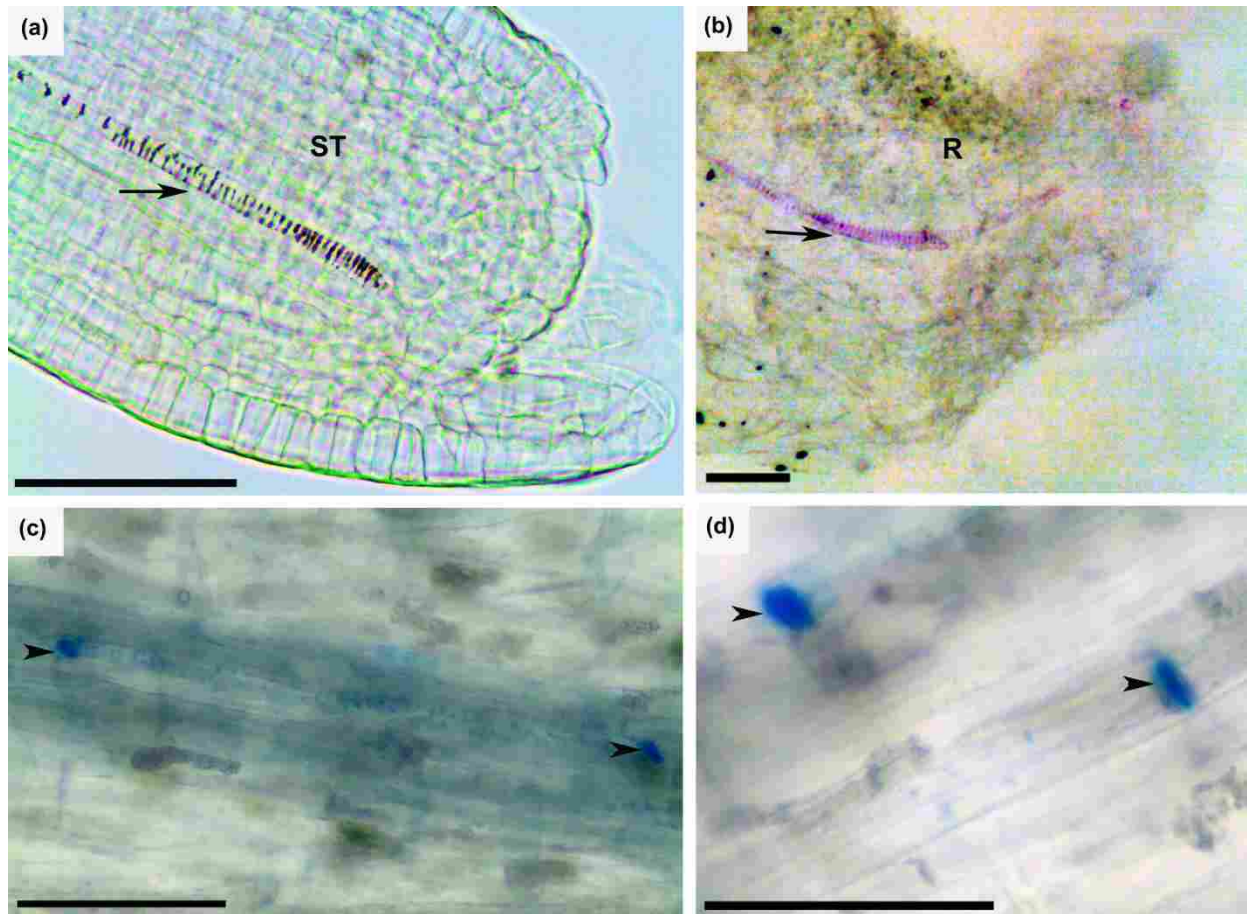
Fig. 3 Vascular system of *Cuscuta* seedling (a-d). Note: lignin in xylem elements and callose in phloem elements were stained with basic fuchsin and lacmoid, respectively. Spiral lignin thickenings (arrow) in a) *C. gronovii* shoot, and b) *C. campestris* root. Callose accumulation in the sieve plates (arrowheads) of c) *C. gronovii* root and d) *C. campestris* shoot. Scale bars: 200 μm in b; 100 μm in a,c,d.