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Seed germination of *Cistanche armena* (Orobanchaceae), a rare endangered holoparasitic species endemic to Armenia

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Abstract

The obligate root parasite *Cistanche armena* is a recently rediscovered, extremely rare endangered species endemic to Armenia, specifically parasitizing camelthorn (*Alhagi maurorum*, Fabaceae) and saltwort (*Salsola dendroides*, Chenopodiaceae). Its populations are reputedly declining due to habitat destruction and biotic impacts. Since the only known means of its reproduction is via the seeds, understanding the mechanisms of breaking *C. armena* seed dormancy and germination along with the related aspects of the species' biology is highly important both from fundamental (functional ecology and evolution) and applied (conservation and management) perspectives. Here, we present the first *in vitro* seed germination protocol for *C. armena* involving fluridone, a systemic herbicide targeting the carotenoid biosynthetic pathway. In addition, the seed micromorphology of *C. armena* is described using both light microscopy and lignin autofluorescence visualized by confocal laser scanning microscopy. The actin cytoskeleton in radicle cells of germinated *C. armena* seedlings is described for the first time, being the proof of seed viability. Further elaboration and application of the proposed germination protocol with the cultivation of *C. armena* on susceptible hosts are altogether seen as a valuable tool for the conservation of this species.

Introduction

The obligately root-parasitic Orobanchaceae, renowned for their host specificity, reportedly gain much evolutionary success from the production of a plethora of tiny (ca. 1 mm long) 'dust seeds' (Eriksson and Kainulainen, 2011). Unlike the non-parasitic angiosperms, whose germination requires mostly an optimal combination of abiotic factors (such as temperature, light and moisture), the holoparasitic Orobanchaceae naturally germinate only in close proximity to the host root and (predominantly) in response to the root exudates containing special germination stimuli – the carotenoid-derived phytohormones strigolactones (Yoneyama et al., 2008; Bouwmeester et al., 2021). In turn, *in vitro* germination of these plants typically requires a conditioning period, usually in darkness and under the temperature mimicking natural growth conditions of a species followed by the treatment of the conditioned seeds with the specific host root exudates, root extracts and/or artificial chemicals to break their physiological dormancy (Pouvreau et al., 2013; Matusova et al., 2014; Bouwmeester et al., 2021). Other bioactive compounds such as gibberellic (GA) and abscisic (ABA) acids are also important for breaking Orobanchaceae seed dormancy (Bao et al., 2017). Such a complicacy of initial stages of the life cycle is presumed to secure against the premature 'suicidal' germination, when no susceptible hosts are available nearby the parasitic seeds in the soil. Combating this protection by adding strigolactone-containing growth regulators to the soil will pave the way for integrating the suicidal germination approach in sustainable root-parasitic weed management strategies (Jamil et al., 2022).

The Old World holoparasitic genus *Cistanche* (Orobanchaceae), commonly named 'ginseng of the desert', contains about 25 species, favouring arid, semi-arid and halophytic habitats across Eurasia and North Africa. These impressive yet barely understood root-holoparasites often specialize on the species of Chenopodiaceae, Zygophyllaceae and Tamaricaceae as hosts (Piwowarczyk et al., 2019). The herbaceous stems of some *Cistanche* species (e.g. *C. salsa*, *C. deserticola*, *C. sinensis*, *C. tubulosa*, etc.) have been used for centuries in the traditional Chinese medicine as a herbal tea and functional food supplement with a

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plethora of confirmed medicinal functions due to the increased amounts of phenylethanoid glycosides with antioxidant properties, the content of which is host- and organ-dependent (Li et al., 2016; Piwowarczyk et al., 2020). Consequently, most *Cistanche* species are endangered and declining because of their overharvesting, aggravated by the extensive utilization of some of their host plants, such as a saxaul (*Haloxylon ammodendron*) used as firewood. The high demand for this product has stimulated multiple attempts of *Cistanche* domestication and further cultivation in some regions of China (Xu et al., 2009). Therefore, habitat protection along with the studies of various aspects of *Cistanche* biology (e.g. germination, haustorium formation and host associations) would contribute to the multi-level strategy of its conservation.

The recently rediscovered endemic species from Armenia (Western Asia), *Cistanche armena* (K. Koch) M.V. Agab., is an obligate root parasite of a camelthorn (*Alhagi maurorum* Medik., Fabaceae) and saltwort (*Salsola dendroides* Pall., Chenopodiaceae), flowering from May to early June and fruiting from June to July (Piwowarczyk et al., 2017, 2019). It is known only from the Ararat and Armavir provinces in Central Armenia, in the Arax River valley and foot of Mount Ararat, on the border between Armenia, Turkey and Nakhchivan (Piwowarczyk et al., 2019; Fig. 1a–f). The species is currently confirmed only at two locations in one of the hottest and extremely arid regions of Armenia (Piwowarczyk et al., 2019). Both the habitat and the range of *C. armena* have a total area of less than 10 km^2, with only several hundreds of individuals remaining because of the habitat degradation, intense amelioration works and arable land expansion (Piwowarczyk et al., 2017, 2019). Besides the abiotic stresses, *C. armena* suffers from extreme parasitization by the hoverfly larvae (*Eumerus mucidus* Bezzi, Syrphidae), while its key host, *A. maurorum*, is highly infested with the stem-parasitic Eastern dodder (*Cuscuta monogyna* Vahl., Convolvulaceae) (Piwowarczyk et al., 2018; Piwowarczyk and Mielczarek, 2018). Consequently, *C. armena* is considered critically endangered (Piwowarczyk et al., 2019). Largely because of the extremely narrow distribution range and rarity, the biology and ecology of *C. armena* are barely known. No studies have addressed seed germination in *C. armena*, with only few published works being known that have challenged this aspect of biology in congeneric species such as *C. deserticola* (Li et al., 1989; Niu et al., 2006; Chen et al., 2009, 2012; Zhang et al., 2008, 2009; Wang et al., 2017), *C. salsa* (Qiao et al., 2007) and *C. tubulosa* (Yang et al., 2007; Chen et al., 2011, 2016). Therefore, establishment of the working protocol of *C. armena* germination, unravelling its early developmental stages, biology and distribution mechanisms, as well as search for the new easy-culturable potential hosts (particularly in the Fabaceae and Chenopodiaceae) are seen as relevant and important steps in developing a complex strategy of the species' conservation with possible cultivation for medicinal purposes.

The aim of this work was to design a feasible protocol for breaking *C. armena* seed dormancy and confirm viability of germinated seedlings by actin cytoskeleton visualization. For germination experiments, fluridone (1-methyl-3-phenyl-5-[3-trifluoromethyl (phenyl)]-4(1*H*)-pyridinone), a systemic herbicide known to act as an ABA-biosynthesis inhibitor, was used. It has been reported to regulate seed conditioning and germination in parasitic and some non-parasitic plants (Ali-Rachedi et al., 2004; Chae et al., 2004; Song et al., 2005; Chen et al., 2016; Wang et al., 2017). Fluridone reduced the conditioning period prior to exposure of *S. asiatica* seeds to the natural strigolactone strigol (10^{-10} M) and, when applied alone, induced haustorium formation (Kusumoto et al., 2006). To the best of our knowledge, we have designed the first protocol for breaking seed dormancy and germination of *C. armena*.

Materials and methods

Plant material

The mature seeds of *C. armena* parasitizing *A. maurorum* and *S. dendroides* were harvested in south-eastern Armenia, Ararat province, Ararat valley near Khor Virap (39°53″01′N, 44° 34″49′E, 818 m a.s.l.) on halophytic vegetation in a semi-desert in May 2016 and July 2017. After the seed harvesting, the material of *C. armena* (whole individuals with several host-plant branches) was dried under the natural conditions and deposited in the Herbarium KTC (Jan Kochanowski University in Kielce, Poland). In addition, the seeds of two other widespread Orobanchaceae species were used in the experimental design (see below): seeds of the lesser broomrape (*Orobanche minor* aggr.), growing on an asteraceous host, were collected in 2011 in Porto Cevro (Sardinia) and deposited in the Herbarium KTC, and those of the purple witchweed (*Striga hermonthica* (Delile) Benth.) from an unknown monocot host were collected in 2007 in Sudan and kindly provided by Prof. Binne Zwanenburg (Radboud University Nijmegen, Netherlands).

Germination assay: seed surface sterilization, conditioning and stimulants

The seeds of *C. armena* were surface-sterilized for 6 min in 4% v/v sodium hypochlorite and 0.1% v/v Triton X-100 solution under vigorous stirring, then thoroughly washed five times in laminar flow cabinet with MilliQ water using vacuum pump (VACUUBRAND, Germany), a fritted borosilicate glass S3 (Sinter, Czechoslovakia) with a rubber stopper sterilized by UV- and 96% ethanol, and an autoclaved 500 ml Büchner flask. Two layers of filter paper (Whatman, 32 mm in diameter) were placed into each well of a sterile Greiner CELLSTAR 12-well cell culture plate (Greiner Bio-One, Austria) and moistened with 1 ml of ½ Murashige and Skoog medium (½ MS; Duchefa, Netherlands) with 1% w/v sucrose (Murashige and Skoog, 1962). Two sterile glass fibre filter paper disks (Whatman, GF/D, 10 mm in diameter) were put into each well and sterile seeds after their sedimentation were dispersed on each disk using a sterile wooden toothpick. The moisture inside the plates was kept by adding the sterile MilliQ water in all spaces between the wells. Plates were thoroughly sealed with parafilm and wrapped in aluminium foil to provide darkness. The seeds of *C. armena* were exposed to cold stratification at 4°C in darkness for 4 d and then placed into an incubator at 21°C. The same procedure was followed for *O. minor* and *S. hermonthica* seeds, with the difference that they were not cold-stratified but were conditioned (Pouvreau et al., 2013). Prior to treatments with germination stimulants, the seeds were conditioned (i.e. warm-stratified and imbibed in ½ MS medium) for 7–10 d at 21°C in darkness (except *S. hermonthica*, which requires conditioning at 30°C). Then, the discs were transferred to a sterile filter paper, aseptically dried for 15 min under flow hood, and placed back to plates. Stock solutions of fluridone (Fluka; Sigma-Aldrich, USA) were preliminary

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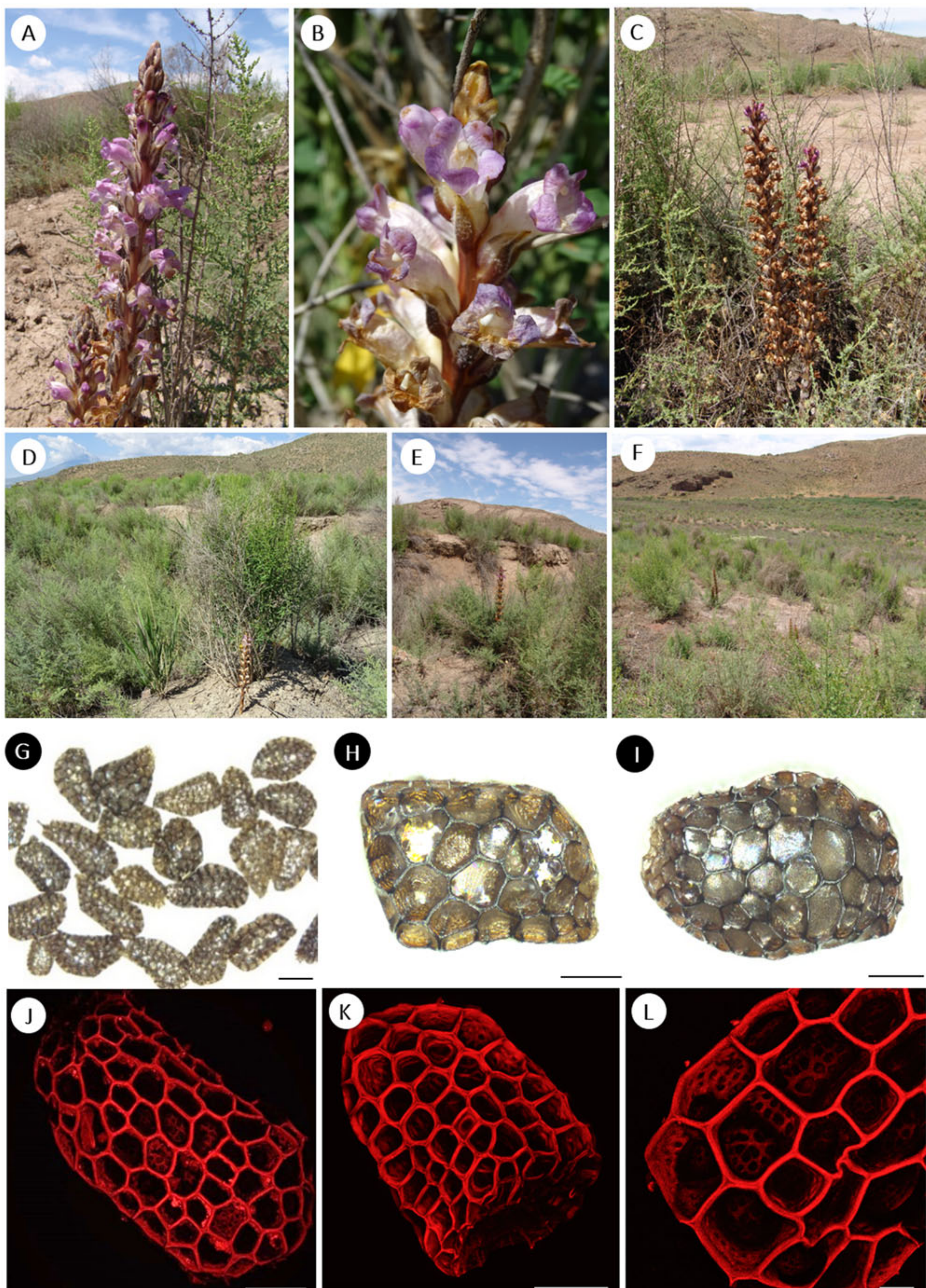

Fig. 1. General appearance, habitat and seed micromorphology of *C. armena*: flowering (a,b) and fruiting (c) individuals on their host plants in semi-desert habitats in Armenia (d–f); seed polymorphism observed with ZOOM stereomicroscopy (g–i); lignin autofluorescence revealed by CLSM (j–l). Scale bars: (g–i) 200 μm; (j,k) 100 μm; (l) 50 μm.

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prepared in 0.96% v/v ethanol to 0.3 mM concentration and stored at −20°C for 14 d maximum (Barua et al., 2012).

Seed germination was stimulated by imbibition with 100 µl of 0.03 and 0.3 mM fluridone per disk with the final acetone concentration of 0.1% v/v in 100 µl of ½ MS medium. Although sterile MilliQ water can serve an efficient substitute of the ½ MS medium for seeds' imbibition, we kept the conditions as close as possible to the physiological ones to enable visualization of actin filaments, which are very sensitive to the cultivation medium content and usually degrade when seeds are germinated in water.

A synthetic strigolactone GR24 (Chiralix, Netherlands) was dissolved *ex tempore* in anhydrous acetone to prepare a 10 mM stock solution and used at 1 µM final concentration. Gibberellin (GA_3) stock was prepared in DMSO and diluted 1:1000 in ½ MS to a final solvent percentage of 0.1 (v/v) (Halouzka et al., 2020).

For double treatments of seeds, the disks imbibed with fluridone (as described above) were kept at 21°C (30°C for *S. hermonthica*) in darkness overnight. Next day, the fluridone was carefully washed away with ½ MS medium, the disks were dried for 15 min, and 100 µl of 1 µM GA_3 (in 0.1% acetone) per disc was added.

All treated seeds were placed back into the incubator and cultivated under 21 or 30°C and darkness for up to 1.5 months, checking their germination status. To examine the germination efficiency and radicle morphology, the seeds were captured with Axio Zoom.V16 Stereo Zoom microscope (Carl Zeiss, Germany) in bright-field illumination (objective lenses PlanApo Z 1.5x, FWD = 30 mm) and processed in ImageJ software using Fiji macros (Schneider et al., 2012). Error bars represent standard deviation in three biological repetitions ($n \geq 150$).

F-actin visualization

Actin filaments in the radicle cells were revealed by Alexa Fluor 568-conjugated phalloidin (Thermo Fisher Scientific) following Panteris' et al. (2006) protocol with minor modifications. Germinated *C. armena* seeds (4th–7th day post-germination (dpg)) were incubated in 300 µM *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), prepared from a 300 mM DMSO stock, in PEM with 0.1% (v/v) Triton X-100 in darkness for 30 min for actin filament stabilization. Subsequently, they were fixed in 4% (w/v) PFA + 5% (v/v) DMSO and 1:400 phalloidin, rinsed thoroughly and extracted with 5% (v/v) DMSO + 1% (v/v) Triton X-100 and 1:400 phalloidin during 1 h. The staining itself was performed with 1:40 phalloidin in the PBS buffer at 37°C for 2 h. DNA was counterstained with 250 µg ml^{-1} 4,6-diamidino-2-phenylindole (DAPI, Sigma) in PBS for 10 min and after final washing in PBS the specimens were mounted in an antifade solution (0.5% (w/v) *p*-phenylenediamine in 70% (v/v) glycerol in PBS or 1 M Tris-HCl, pH 8.0) or in the commercial antifade mounting medium VECTASHIELD™ (Vector Laboratories).

Microscopy

General seed morphology was imaged by Axio Zoom.V16 Stereo Zoom system (Carl Zeiss, Germany) in bright-field illumination (objective lenses PlanApo Z 1.5x, FWD = 30 mm). For testa micromorphology imaging based on lignin autofluorescence in non-fixed samples, we employed a LSM710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) argone laser line with excitation of 568 nm and emission spectra 603 nm for red fluorescence using a 20× Plan-Apochromat (0.8 numerical aperture) objective. Laser excitation intensity did not exceed 2% of the laser intensity range. Images were presented as maximum intensity projections of Z-stacks processed in Zen Blue 2012 software package (Carl Zeiss, Jena, Germany). For the imaging of phalloidin-labelled actin, the fluorescent signal from Alexa Fluor 568 (Thermo Fisher Scientific) was visualized with 20×/0.8NA Plan-Apochromat, 40×/1.40NA and 63×/1.40NA Plan-Apochromat objectives with oil immersion. The DAPI fluorescence signal in nuclei was imaged using excitation laser line 405 nm and emission spectra 410–495 nm.

Results

Breaking seed dormancy in the Orobanchaceae seeds

Combined application of fluridone and GA_3 was most efficient in stimulating the germination of *C. armena* seeds (Fig. 2). Contrarily, the seeds of *O. minor* and *S. hermonthica* germinated best when treated with GR24 but showed very modest response to the application of fluridone alone and in combination with GA_3.

It is noteworthy that fluridone and GA_3 triggered *C. armena* seed germination only when added sequentially, not simultaneously. Without the conditioning and stimulation, less than 1% of *C. armena* seeds germinated spontaneously, which was lower than in *O. minor* (1–2%) and *S. hermonthica* (4–5%). Some of the spontaneously germinated seeds formed terminal haustoria without the addition of haustorium-inducing factors. However, neither stimulant was sufficient for the germination of *C. armena* seeds at 21°C without their cold pre-stratification for at least 4 d. Even the cold-stratified seeds of *C. armena* germinated with significantly longer delay (starting from the 22nd day and till the 40th day after fluridone and GA_3 treatment) as compared to *O. minor* and *S. hermonthica*, whose germination occurred within the 7th–14th and 4th–7th post-treatment day intervals, respectively.

Seed micromorphology and actin cytoskeleton architecture in radicle cells of C. armena

The seeds of *C. armena* are highly polymorphous, usually oblongoid to ovoid, rarely subrectangular (Fig. 1g–i), with clearly pitted, reticulate, highly fluorescent testa (Fig. 1j–l). The average length of testa perforations is 19.8 µm, and the width is 14.5 µm. The actin filaments were stained in different types of radicle epidermal cells, being a confirmation for the successful germination of *C. armena* seeds and the potential of further growth towards the host with intrusion into its tissues (Fig. 3).

Alexa Fluor 568-conjugated phalloidin staining revealed a dense network of long actin cables traversed by the individual filaments both in the central part of the germinated radicle (Fig. 3a–c) and the pole with apical meristem (Fig. 3e). F-actin filaments in papillate epidermal cells, which will form haustorial hairs, are coiling below their apices (Fig. 3d) as compared to non-papillate cells (Fig. 3a–c). Numerous nuclei stained by DAPI were abundant in *C. armena* radicle tips (Fig. 3e).

Proposed seed germination protocol for C. armena

Based on our experiments, the following protocol for *C. armena* seed germination has been designed, still requiring optimization for seed dormancy breaking and *in vitro* germination:

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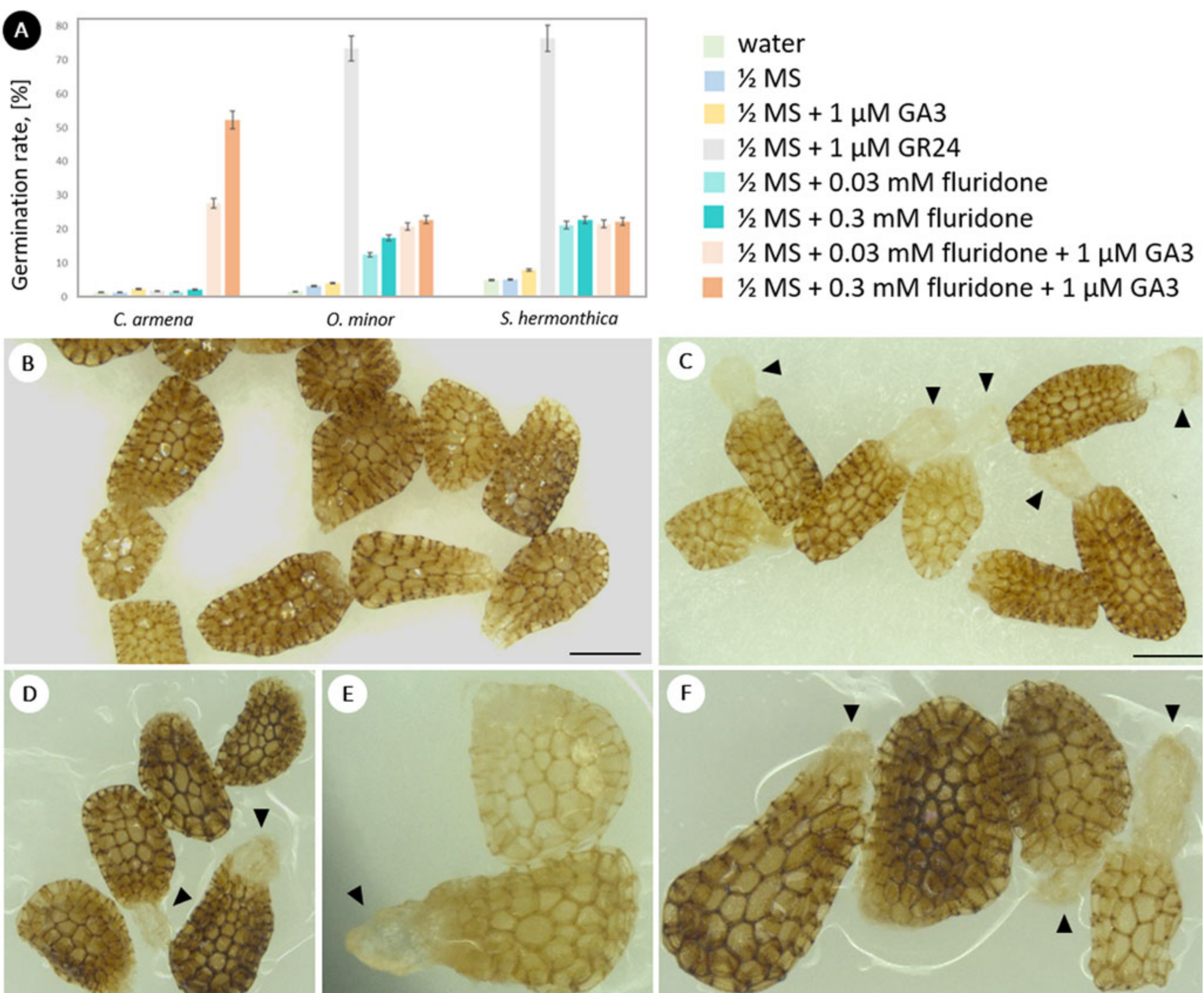

Fig. 2. Germination of the Orobanchaceae seeds: (a) germination rate (%) of *C. armena*, *O. minor* and *S. hermonthica* on the 22nd day after treatment with germination stimulants; (b) control, conditioned seeds incubated without any germination stimulants, 22nd day in ½ MS medium; (c–f) germinated seeds with emerged radicles (indicated by arrows) imaged by a ZOOM microscope at 22nd day after fluridone and GA_3 treatment. Scale bars: (b,c) 2000 µm; (d–f) 500 µm.

1. *Seed sterilization* (2% v/v NaClO + 0.1% v/v Triton X-100) for 5 min under stirring with further five times washing in the sterile MilliQ water using vacuum pump, sterile glass Buchner flask, and UV-sterilized frit.
2. *Positioning* of sterile seeds on two sterile glass fibre filter paper disks (Whatman, GF/D, 10 mm in diameter) on two layers of filter paper (Whatman, 32 mm in diameter) placed into each well of a 12-well plate and moistened with 1 ml of MilliQ water or the sterile ½ MS medium.
3. *Cold stratification* at 4°C in darkness (covered with aluminium foil) for 4 d.
4. *Conditioning* at 21°C in darkness for 7 d.
5. *Fluridone treatment* (0.3 mM fluridone in 96% EtOH, 100 µl per disk) applied on the preliminary dried disks at 21°C in darkness for 24 h.
6. *Fluridone washing* five times in sterile MilliQ water with further disk drying for 15 min.
7. *Gibberellic acid (GA_3) treatment* (100 µl of 1 µM GA_3 in 0.1% acetone per disk) applied on the preliminary dried disks at 21°C in darkness for 20 and more days.
8. *Moisture regime support* by adding 100 µl of water.
9. *Germination* starts after the 22nd day with the steady increase of the percentage of the germinated seeds after GA_3 treatment.

Discussion

Although the highest germination rates of *Cistanche* seeds have been obtained in long-term trials in nature (Wang et al., 2017), efforts to germinate them aseptically *in vitro* have also shown success (Baskin and Baskin, 2022), implying that *Cistanche* species may not necessarily rely on the external biotic factors (e.g. symbiotic microorganisms or host-emitted stimuli) as germination triggers. On the other hand, 'dust seeds' have commonly been associated with such reliance, attributing it to the insufficiency of the seed nutrient reserves and the need for a supplementary host-derived carbon to ensure germination and successful seedling establishment (Eriksson and Kainulainen, 2011). As a convergent trait, 'dust seeds' also characterize mycoheterotrophic plants, having similar life-history traits as the root parasites but relying upon fungal symbionts as hosts (Merckx et al., 2013). In

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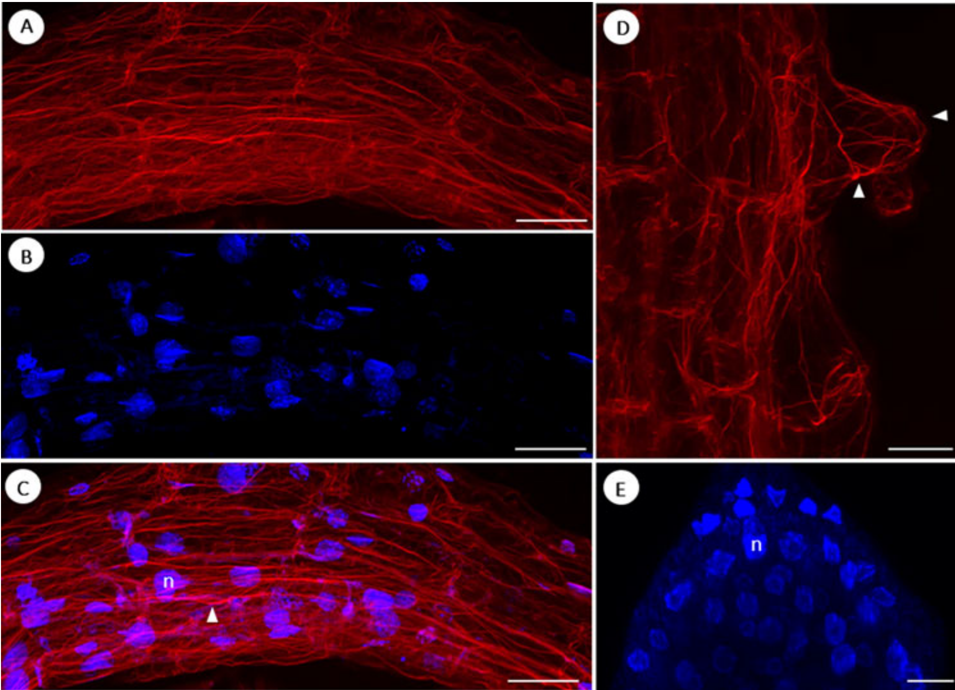

Fig. 3. Long F-actin cables, individual microfilaments and nuclei in epidermal cells of the elongating *C. armena* radicles, 1st day after germination. Labelling: red – Alexa Fluor 568-conjugated phalloidin (actin filaments); blue – DAPI (nuclei). Indications: n – nucleus; arrowheads point at the haustorial hair in the apical part of the radicle. Bars: (a–c) 50 μm; (d,e) 10 μm.

addition, soil microorganisms may play a role in establishing the orobanchaceous parasite–host interactions, and these relationships are known to be reciprocal (Fitzpatrick and Schneider, 2020). However, most of the studies have emphasized only the suppressive effect of soil microbes on parasitic seeds, either through direct antagonism or indirectly, for example, by affecting hormonal profiles and reducing strigolactone production in the autotrophic hosts (Hristeva et al., 2013; Müller-Stöver et al., 2016). The knowledge concerning communities and role of bacterial seed endophytes of holoparasitic plant species is still limited (Iasur Kruh et al., 2017; Huet et al., 2020; Durlik et al., 2021; Petrosyan et al., 2022). The recent study on endophytic bacterial communities in seeds of *C. armena* showed 256 bacterial genera. The plant growth-promoting (PGP) traits of these bacteria, such as production of indole, 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase and organic acids have the potential to improve plant tolerance against abiotic stresses. However, their benefits for the seed germination and seedling development are still unclear (Petrosyan et al., 2022).

Worth noting is that not all holoparasitic Orobanchaceae species require external chemical cues (e.g. strigolactones, host root exudates, GA_3, etc.) for the stimulation of germination. For instance, the seeds of the Indian broomrape (*Aeginetia indica*) can germinate just under the right combination of dormancy-breaking signals, such as light and temperature, after conditioning (Kato and Hisano, 1983; Kato et al., 1984; Chen and Hsiao, 2011), probably by producing endogenous gibberellins (Suwa et al., 1995).

Reported effects of the germination stimulants, tested in this study, on the seeds of *Cistanche* species have proved highly inconsistent under different experimental conditions being set. Qiao et al. (2007) reported that 0.1 mg l^{-1} fluridone treatment for 24–29 h in the temperature range 20–30°C was efficient for germinating the seeds of *C. tubulosa*, *C. deserticola* and *C. salsa*, whereas Wang et al. (2017) failed to germinate fresh *C. deserticola* seeds either in water or in a 10^{-5} M fluridone solution at any incubation temperature within 60 d. Furthermore, *C. deserticola* seeds stratified at 5°C for 6 months displayed no germination in the GA_3 and GR24 solutions and only 11% germination in the mixed fluridone/GA_3 solution (Wang et al., 2017). This contrasted with Chen's et al. (2009) results, showing >70% germination of GA_3-treated seeds that had been cold-stratified for 4–5 months. Conversely, Zhang et al. (2009) achieved up to 4.7% of germination of *C. deserticola* seeds with the GA_3 treatment only. In addition, Niu et al. (2006) stated that soaking seeds in water at 24–25°C for 30 d, rather than cold stratification at 4°C for 60 d, increased their sensitivity to exogenous GA_3. It has been suggested that the responsiveness of *C. deserticola* seeds to exogenous GA_3 may be determined by the physiological status of the seeds (Wang et al., 2017).

However, this fluridone/GA_3 triggered germination of *Cistanche* seeds might be mediated by endogenous strigolactones synthetized by the seeds. Fluridone, as a potent inhibitor of *de novo* carotenoid biosynthesis, inhibits phytoene desaturase, which catalyzes the desaturation step of phytoene to phytofluene in the carotenoid-biosynthesis pathway (Chae et al., 2004; Matusova et al., 2004). Nevertheless, already formed carotenoids are not targeted by fluridone, which might be used as a substrate for endogenous strigolactone production by biosynthetic machinery present in Orobanchaceae species (Das et al., 2015). Furthermore, carotenoid biosynthesis might also recover soon after the double treatment, since fluridone was washed out (see Materials and Methods) and might have been metabolized or degraded during further incubation. In addition, it has been shown for other Orobanchaceae species, whose germination is dependent on host-produced or artificial strigolactones, that the expression of strigolactone-biosynthesis genes significantly

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decreases during the conditioning period, making the seeds become highly sensitive to these regulatory molecules (Matusova et al., 2004; Das et al., 2015; Brun et al., 2018). It was found that fluridone also shifts ABA/GAs ratio in seeds due to the steady inhibition of ABA-biosynthesis with the increased concentration of GAs (Chen et al., 2016). In turn, GA signalling negatively regulates the endogenous levels of strigolactones (Ito et al., 2017).

Our results for *C. armena* partly conform with those of Wang et al. (2017) for *C. deserticola*, showing increased seed germination after the mixed fluridone/GA_3 treatment as compared to the treatments with each of the stimulants alone (Fig. 2). Taken together, results of the above studies imply that (1) the Orobanchaceae root parasites seem to display a species-specific response to different germination stimulants, modulated by the physiological condition of seeds and/or general ecophysiology of species; and (2) a number of abiotic factors, such as temperature, darkness and the season, play key role in the orobanchaceous seed germination. For instance, the seeds of alpine root-hemiparasitic *Euphrasia minima* and *E. salisburgensis* reportedly germinate both at constant (5°C) and varying temperatures (3–10°C), and never before spring after seed ripening (Liebst and Schneller, 2008). Cold stratification appears to be an important environmental factor also for *C. armena* (Fig. 2). Since this species naturally grows under the harsh semi-desert climate with contrasting day and night temperatures, its seeds are adapted to survive extreme temperatures but germinate only under optimal ones (21°C). Similarly, Qiao et al. (2007) reported the optimal germination temperature of 20–30°C and the absence of germination at sub- or supraoptimal temperatures (5 and 35°C) in *C. tubulosa*, *C. deserticola* and *C. salsa*. The promoting effect of darkness on seed germination may be attributed to the underground conditions under which the seeds are naturally stored in the soil. According to Pouvreau et al. (2013) and Matusova et al. (2014), seed conditioning under light exposure may cause photoinhibition of germination even after adding fluridone and GA_3. This is in line with our study, showing the successful germination of *C. armena* without illumination. In turn, the importance of season is likely related to a specialized kind of morphophysiological dormancy and/or critical size of embryo crucial for its further development. For *C. deserticola* seeds, the working protocol with over 50% germination rate included the incubation of the fresh seeds in 10^{-5} M fluridone solution in darkness in spring after they had overwintered on the soil surface in the natural habitat (Wang et al., 2017).

Cytoskeletal proteins, especially actin, are the driving force for cell processes such as tip growth, intracellular trafficking, exo- and endocytosis, cytoplasm streaming, organelle movement and cytokinesis (Šamaj et al., 2006; Szymanski and Staiger, 2018). Dynamic reorganization of actin cytoskeleton (F-actin cables, patches, individual filaments, rings and acquosomes) is a key prerequisite for growth and morphogenesis in higher plants (Smertenko et al., 2010), and seems to be important also for the germination and interaction of parasitic plants with their susceptible hosts (Kaštier et al., 2018). Therefore, we addressed for the first time the organization of the actin cytoskeleton in the radicle cells of *C. armena*.

In conclusion, this long-term *in vitro* approach of *C. armena* germination may contribute to its successful propagation and become a part of the conservation strategy of this rare species. The germination of *C. armena* seeds, using susceptible host's root exudates, up to the formation of the functional haustoria requires further investigations both *in vitro* and *in vivo*. The hormonal mechanism of the stimulatory effect of fluridone on the Orobanchaceae seed germination was not addressed here, since ABA concentration in the seeds was not measured, and hence, it is yet to be discovered.

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Data availability. All data generated or analysed during this study are included in this published article.

Conflict of interest. The authors declare that they have no competing interests.

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