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# Germination of the fully myco-heterotrophic orchid *Cyrtosia septentrionalis* is characterized by low fungal specificity and does not require direct seed-mycobiont contact

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ABSTRACT

We examined the level of specificity exhibited by seeds of *Cyrtosia septentrionalis* for germination-inducing fungi. Three wood-decomposing *Armillaria* species (*A. gallica*, *A. mellea* subsp. *nipponica* and *A. tabescens*) known to colonize adult plants and an unidentified species of Polyporales isolated from naturally growing protocorms were tested. *Xylobolus annosus*, a free-living decomposer of Russulales, was included as a control. Seed germination occurred in a sawdust-based medium in sealed and unsealed containers in the presence of all fungi, indicating low mycorrhizal specificity in germination. Moreover, germination occurred even in modified containers in which the fungus was physically isolated from the orchid seeds, indicating that direct seed-fungus contact is not required. Higher germination percentages were observed in sealed containers in which a modified atmosphere, consisting of a lower $O_2$ and a higher $CO_2$ concentration, had been established in the air above the inoculated medium as a result of the saprophytic activity of the fungus. In nature, atmospheric conditions more effective for seed germination might be established by the action of mycobionts in decomposing wood. Seeds germinated in the presence of the unknown Polyporales species underwent further growth and development, as compared with the other fungi tested, when covered with soil.

## 1. Introduction

Orchids establish mycorrhizal symbioses by enlisting an appropriate, free-living fungus to colonize their germinated seeds (Waterman and Bidartondo 2008). The orchid's subsequent subterranean growth is sustained by the mycobiont, which supplies all of the plant's mineral and organic nutrient requirements until its first green leaf emerges. After emergence,

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partial myco-heterotrophic orchids, or mixotrophs, continue to obtain carbon from their fungal partner(s) to supplement what they themselves fix through photosynthesis (Merckx et al. 2009). Fully myco-heterotrophic orchids, however, lack the ability to photosynthesize and so remain entirely dependent upon their mycobionts throughout both subterranean and emergent life history phases. Because of this heightened dependency, it has been argued that full myco-heterotrophs exhibit greater specificity in their interactions with their mycobionts as compared with partial myco-heterotrophs (Waterman and Bidartondo 2008). Several investigations support this hypothesis by identifying narrow lineages of ectomycorrhizal or free-living fungi associated with emergent individuals of a range of different fully myco-heterotrophic orchid species (e.g. Ogura-Tsujita and Yukawa 2008; Barrett et al. 2010; Kennedy et al. 2011; Okayama et al. 2012).

The level of specificity exhibited by fully myco-heterotrophic orchids during seed germination, however, remains unclear. Stable, *in vitro* co-culture of seeds with a putative mycobiont isolated from field samples is required in order to confirm whether the association is necessary and sufficient for germination in nature. Although several investigations that have successfully demonstrated such an association appear to indicate high mycorrhizal specificity (e.g. Xu and Guo 2000; Yagame et al. 2007), other studies have revealed a generalist requirement on the part of the orchid for several different germination-inducing fungi (e.g. Umata et al. 2000; Bidartondo and Read 2008).

Several explanations for this apparently ambivalent picture have been put forward, including inaccurate or uncertain identification of mycobionts (Leake 1994), variable co-culture conditions (e.g. differing substrate composition or temperature) giving rise to differing mycobiont performance (Rasmussen 1995), varying specificity on the part of the mycobiont at different stages in the development of the orchid (e.g. Zelmer et al. 1996; McCormick et al. 2004), and technical difficulties associated with confirming the role of ectomycorrhizal fungi in orchid germination due to their resistance to pure culture (e.g. McKendrick et al. 2000). To explore this topic further, we hypothesized that the mechanism for symbiotic germination, encompassing the release of stimulatory factors and the establishment of enabling microconditions by the mycobiont, must be consistent where different mycobionts with differing ecologies are able to induce the same germination response in the same orchid species. We sought to test this hypothesis using the fully myco-heterotrophic terrestrial orchid *Cyrtosia septentrionalis* (Rchb.f.) Garay for which symbiotic seed germination has not yet been reported, despite over 70 years of study as a model of mycorrhizal symbiosis in adult plants (Hamada 1939).

*Cyrtosia septentrionalis* (=*Galeola septentrionalis*) is native to eastern China and Japan (Chen and Cribb 2009). Previous work on this orchid in Japan has revealed high specificity in its mycorrhizal associations as an adult plant, with mycobionts being restricted to certain wood decomposing species of the parasitic genus *Armillaria* (Agaricales), in particular *A. cepistipes* Velen., *A. gallica* Marxm. & Romagn., *A. mellea* (Vahl) P. Kumm. subsp. *nipponica* Cha & Igarashi, *A. jezoensis* Cha & Igarashi, and *A. tabescens* (Scop.) Emel. (Hamada 1939; Cha and Igarashi 1995, 1996; Terashita 1996). Aseptically grown rhizomes of *C. septentrionalis* have been shown to be capable of establishing a symbiotic association when subsequently co-cultured with certain *Armillaria* species (Terashita 1985; Terashita and Chuman 1989). Nakamura (1962) reported that seeds of *C. septentrionalis* can be germinated aseptically when cultured with the addition of potassium chloride (KCl) and other nutrients known to stimulate growth in autotrophic plants. Nakamura et al. (1975) further reported that certain atmospheric conditions in the culture container, namely, a reduced concentration of oxygen ($O_2$) or an elevated concentration of carbon dioxide ($CO_2$), were also effective for germination, but these results have not been verified in co-culture.

Another fully myco-heterotrophic orchid, *Gastrodia elata* Blume, is also known to associate with *A. mellea* (Kusano 1911). Interestingly, its germination was not induced by *A. mellea*, but by *Mycena osmundicola* Lange (Agaricales), while protocorms that had been induced by *M. osmundicola* needed colonization by *A. mellea* for normal onward growth. *Mycena osmundicola* was isolated from naturally growing protocorms while *A. mellea* was obtained from the adult orchid (Xu and Guo 2000). A similar succession of associations may occur in *C. septentrionalis*: mycobionts required for germination may be isolated from germinated seeds or juvenile plants, but not from adults. In an earlier study, Umata et al. (2006) carried out field sowing experiments to bait mycobionts of juvenile plants but found that germinated seeds contained no fungal pelotons. To date, no mycobionts have been isolated from germinated seeds of *C. septentrionalis*.

In the present investigation, we conducted field sowing and co-culture of *C. septentrionalis* seeds with a range of fungi, including *Armillaria* species, to clarify the enabling conditions for germination and the degree of specificity between the orchid and its inducing mycobionts, and to ascertain whether a succession of associations is necessary throughout the orchid's life cycle. A broadly defined *A. mellea* sensu lato (Kusano 1911; Hamada 1939; Xu and Guo 2000) is now widely recognized as comprising a complex of closely related species (Baumgartner et al. 2011). At least 11 and 14 *Armillaria* species have been reported so far from Japan and China, respectively (Nagasawa 1991; Cha et al. 1994; Ota et al. 1998, 2010; Kudo and Nagasawa 2003; Qin et al. 2007). To circumvent problems of fungal identification, we identified *Armillaria* mycobionts using molecular methods.

## 2. Materials and methods

### 2.1. *Field collection of* Cyrtosia septentrionalis *seeds*

Seed capsules were collected from wild populations of *C. septentrionalis* at two locations in Japan: Kirishima City in Kagoshima Prefecture on 28th October 2008 ('Kirishima seed') and Kyushu University's Shiiba Research Forest in Miyazaki Prefecture on 25th October 2009 ('Kyudai seed').

### 2.2. *Field baiting of* Cyrtosia septentrionalis *mycobionts*

Kirishima seed was used to bait naturally occurring mycobionts of *C. septentrionalis* in the field. Eight oblong nylon-mesh