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Flavonoids induce germination of basidiospores of the ectomycorrhizal fungus *Suillus bovinus*

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Abstract Under laboratory conditions, spores of ectomycorrhizal fungi usually germinate very poorly or not at all. In a previous study, we showed that spores of the ectomycorrhizal fungus *Suillus bovinus* germinated through the combination of activated charcoal treatment of media and co-culture with seedlings of *Pinus densiflora*, which suggested that some substances contained in root exudates induced the germination. Among the compounds reported from root exudates, flavonoids have been elucidated to play various and substantial roles in plant–microbe interactions; we therefore investigated the effects of flavonoids on basidiospore germination of *S. bovinus* by the diffusion gradient assay on water agar plates pretreated with charcoal powder. Seven out of the 11 flavonoids tested, hesperidin, morin, rutin, quercitrin, naringenin, genistein, and chrysin, had greater effects than controls, whereas flavone, biochanin A, luteolin, and quercetin showed no positive effects. The effective concentration presumably corresponded to several micromolar levels, which was equivalent to those effective for pollen development, *nod* gene induction, and spore germination of *F. solani* f. sp. *pisi* and AM fungi. The results suggest that flavonoids play a role as signaling molecules in symbiotic relationships between woody plants and ectomycorrhizal fungi.

Keywords Basidiospore germination · Ectomycorrhizal fungi · *Suillus bovinus* · Flavonoids · Signaling molecules

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Introduction

Propagation of ectomycorrhizal fungi occurs primarily by spore dispersal and vegetative growth of mycelia. Since the last decade, with the development of polymerase chain reaction-based DNA analysis, molecular ecological studies have been intensively conducted and have revealed mainly from clone sizes that their relative importance depends on the species studied. It has been concluded that basidiospores are the main means for dispersal and initiation of infections in some ectomycorrhizal species of relatively small genet sizes (Gryta et al. 1997; Gherbi et al. 1999; Fiore-Donno and Martin 2001; Redecker et al. 2001; Huai et al. 2003; Liang et al. 2005; Bergemann and Miller 2002). However, under laboratory conditions, spores of ectomycorrhizal fungi usually germinate very poorly or not at all (Bowen and Theodorou 1973; Fries 1984), and thus, comprehensive explanations of life cycles of ectomycorrhizal fungi in forests cannot be based solely on clone sizes.

Although spores are assumed to require stimulatory factors for germination, these conditions are still unknown in most ectomycorrhizal species. Among ectomycorrhizal basidiomycetes, three main types of germination activators have been reported (Fries 1987): (1) various types of non-ectomycorrhizal microorganisms such as colonies of the yeast *Rhodotorula glutinis* (Frensen.) Harrison (Fries 1976, 1978), the filamentous fungus *Ceratocystis*

fagacearum Brents (Hunt; Oort 1974), and some bacterial isolates obtained from sporophores, mycorrhizae, or soil (Ali and Jackson 1988); (2) a mycelium of the same species as the spores (Fries 1978, 1983a; Iwase 1992); and (3) roots of higher plants (Melin 1962; Fries and Birraux 1980; Birraux and Fries 1981; Kope and Fortin 1990; Fries 1989). Generally, the effects are caused by exudates from various microorganisms or root exudates of plants, and these exudates presumably contain compounds possessing the capacity to trigger spore germination. However, only a few of the chemical substances active in induction of basidiospore germination in ectomycorrhizal fungi have been identified. They include *n*-butyric acid and related compounds for *Tricholoma matsutake* (Ito & Imai) Singer and several other species (Ohta 1986, 1988), gluconic acid for *T. robustum* (Alb. & Schwein.) Ricken (Iwase 1992), and abietic acid and some resin acids for *Suillus* species (Fries et al. 1987; Fries 1988).

In a previous study, we showed that spores of *Suillus bovinus* (Pers.) Roussel germinated only by the combination of activated charcoal treatment of media and co-culture with seedlings of *Pinus densiflora* Sieb., but that abietic acid, a genus-specific inducer of spore germination for *Suillus* species (Fries 1988), showed no positive effect; this result suggested that some substances other than abietic acid contained in root exudates induced germination of basidiospores of *S. bovinus* (Kikuchi et al. 2006).

Root exudates contain a wide variety of compounds, including sugars, amino acids, organic acids, fatty acids, sterols, flavonoids, growth factors, and enzymes (Uren 2001). Among them, amino acids show some positive effects on germination of basidiospores of *Laccaria laccata* (Scop.) Fr. and *Suillus* species (Fries 1976, 1983a), although more effective treatments were reported later for both species: Co-culture with conspecific mycelium or seedlings of host plants stimulated germination (Fries 1978, 1983a, 1983b). Certain flavonoids induce transcription of nodulation genes in *Rhizobium* (Peters et al. 1986; Redmond et al. 1986) and promote spore germination and hyphal growth of arbuscular-mycorrhizal (AM) fungi (Gianinazzi-Pearson et al. 1989; Vierheilig et al. 1998). Flavonoids have been elucidated to enhance growth rates of *R. meliloti* (Hartwig et al. 1991). In two ectomycorrhizal fungi, *Pisolithus tinctorius* (Pers.) Cocker & Couch and *S. bovinus*, the flavonol rutin is effective at concentrations as low as 1 pM for their growth (Lagrange et al. 2001).

By analogy of the roles of flavonoids in the two major plant–microbe symbioses, i.e., legume–rhizobia and herbs–AM fungi, it is possible that flavonoids play roles in another major symbiosis between woody plants and ectomycorrhizal fungi. In this study, we investigated the effect of flavonoids on spore germination of the ectomycorrhizal fungus *S. bovinus*.

Materials and methods

Spore collection and seedlings of P. densiflora

Fruit bodies of *S. bovinus* were collected in a *P. densiflora* stand located in Kake, Hiroshima Prefecture, Japan in October 2005. Spore prints were obtained by fastening pieces of a cap under the lid of a sterilized plastic petri dish with vaseline so that the spores were cast into the bottom of the dish. After 2 to 3 h, the lid was replaced by another sterile lid, and several spore prints from the same fruit body were collected in separate petri dishes. The spore prints were stored in darkness at 4°C until use.

Seeds of *P. densiflora* were surface sterilized by 30% H_2O_2 for 5 min, washed with sterilized distilled water, and placed on one fifth strength Hamada medium [0.4% glucose, 0.04% yeast extract, 1.0% agar (*w/v*)].

Chemicals

Eleven flavonoids, specifically three flavones (flavone, luteolin, chrysin), four flavonols (quercetin, quercitrin, rutin, morin), two flavanones (naringenin, hesperidin), and two isoflavones (genistein, biochanin A), and abietic acid were tested to determine their effect on the germination of *S. bovinus* basidiospores. The flavonoids were selected among those investigated previously for their effects on spore germination of AM fungi (Gianinazzi-Pearson et al. 1989; Vierheilig et al. 1998) or hyphal growth of ectomycorrhizal fungi (Lagrange et al. 2001). All investigated flavonoids and abietic acid were available commercially: flavone, morin, narigenin, hesperidin were obtained from Sigma Aldrich (USA), luteolin, quercetin, quercitrin, rutin, biochanin A, and abietic acid from Wako Pure Chemical (Japan), and chrysin and genistein from Funakoshi (Japan).

Abietic acid and all flavonoids except for hesperidin were dissolved in absolute ethanol (Wako Pure Chemical) to provide 2 mM stock solutions. Hesperidin was dissolved in 0.1 N KOH to provide 2 mM stock solutions.

Pretreatment of water agar plates with charcoal powder

As reported by Bjurman (1984), an organic acid inhibitory to spore germination of ectomycorrhizal fungi is formed during autoclaving, and pretreatment of solidified agar with charcoal powder is effective for removal of the inhibitory factor.

Autoclaved (121°C, 10 min), molten 1% (*w/v*) water agar was poured into plastic petri dishes (φ90 mm), 15 ml per dish. When the agar had solidified, its surface was covered with a thin cellophane disk autoclaved (121°C, 15 min) in distilled water, and a thin layer of sterilized

(autoclaved at 121°C for 30 min) activated charcoal powder was sprinkled over the cellophane. After 2 weeks, the cellophane and the overlying charcoal powder were removed.

Effects of flavonoids on spore germination

Effects of flavonoids on spore germination were evaluated by the diffusion gradient assay (Bjurman and Fries 1984) with slight modification. A hole, approximately 2 mm in diameter, was punched in the center of each plate with a sterilized Pasteur pipette; the pipette tip was inserted into the agar, the contents were removed by suction (Nagahashi and Douds 1999), and then the flavonoid or control solution was injected into the hole.

In the experiments, controls were injected with equal volumes of absolute ethanol (for chemicals other than hesperidin) or 0.1 N KOH solution (for hesperidin) into the hole made on non-pretreated and pretreated plates [water agar plates (WA) and water agar plates pretreated with charcoal powder (WAC), respectively]. As positive controls, seedlings of *P. densiflora* were placed on pretreated plates, one seedling per plate (referred to as Pd).

After several hours, the solution dried up, and a suspension of spores containing ca 4×10^6 spores per milliliter in sterilized distilled water was spotted at 0.1 ml per plate and spread over the plate with a glass rod. The petri dishes were sealed with parafilm and incubated at 23°C in the dark.

Experiment I. Effects of different flavonoids on spore germination

The effect of each flavonoid on germination was evaluated by injecting 20 μl of 2 mM solution of each flavonoid. From preliminary experimentation, it was determined that 20 μl was an adequate volume for showing clear effects among injection volumes of 5, 10, 20, and 40 μl.

Experiment II. Effects of different volumes of injected 2 mM morin solution on spore germination

To find an optimal flavonoid concentration for germination, effects of different volumes of flavonoids on germination were evaluated by injecting 2, 5, 10, 20, 40, and 100 μl of 2 mM solution of morin. Morin was selected because it produced the largest levels of spore germination in the preliminary experiment.

Spore collections from five fruiting bodies were used due to limited spore quantities from each collection; one collection was used in both experiments, and two additional collections were used in each experiment. Five plates were prepared for each treatment.

Germination rates

Germination rates were scored once a week, via photomicrographs, in four directions perpendicular to each other across a hole made by a Pasteur pipette. In the Pd treatment, photomicrographs were taken and germination was measured along two perpendicular lines at the middle and lower one fourth part of the root of each seedling. As each direction was considered independent, even on the same plate, the number of replicates was 20 (five plates × four directions) per treatment. Unfortunately, as a result of contamination or death of pine seedlings, the number of plates of Pd examined in experiments I and II (two and three, respectively) and the number of repeats (8 and 12, respectively) were low.

Experimental data were analyzed by analysis of variance, and a multiple comparison was conducted with Holm's method using the software R ver.2.3.1 (R Development Core Team 2005).

Results

Observations were made up to 2 weeks because on the plates of treatments that showed positive effects, the germ tubes grew so long that it became difficult to distinguish among germinating spores after 2 weeks (Fig. 1). In both experiments, no spores were observed to germinate on WA plates, whereas spontaneous germination was observed on WAC plates (Figs. 2 and 3).

Here, spore collections from five fruiting bodies were used: One collection was used in both experiments and two other collections in each experiment. However, in each experiment, the spores of only one of the additional spore collections germinated. Therefore, the results for experiments I and II are based on two different spore collections.

Experiment I. Effects of different flavonoids on spore germination

Germination rates observed in experiment I are shown in Fig. 2. The two control treatments, i.e., the ethanol treatment and the 1 N KOH treatment, had similar effects (data not shown). Thus, we included only the values of the ethanol treatment in Fig. 2.

Except for flavone, biochanin A, luteolin, and quercetin germination rates were significantly higher than the control ethanol treatment. Abietic acid showed no positive, rather an inhibitory effect. Among the flavonoids tested, hesperidin and morin showed the considerable effects on germination, which was in accordance with the result of preliminary experiments in which two spore collections were used (data not shown).

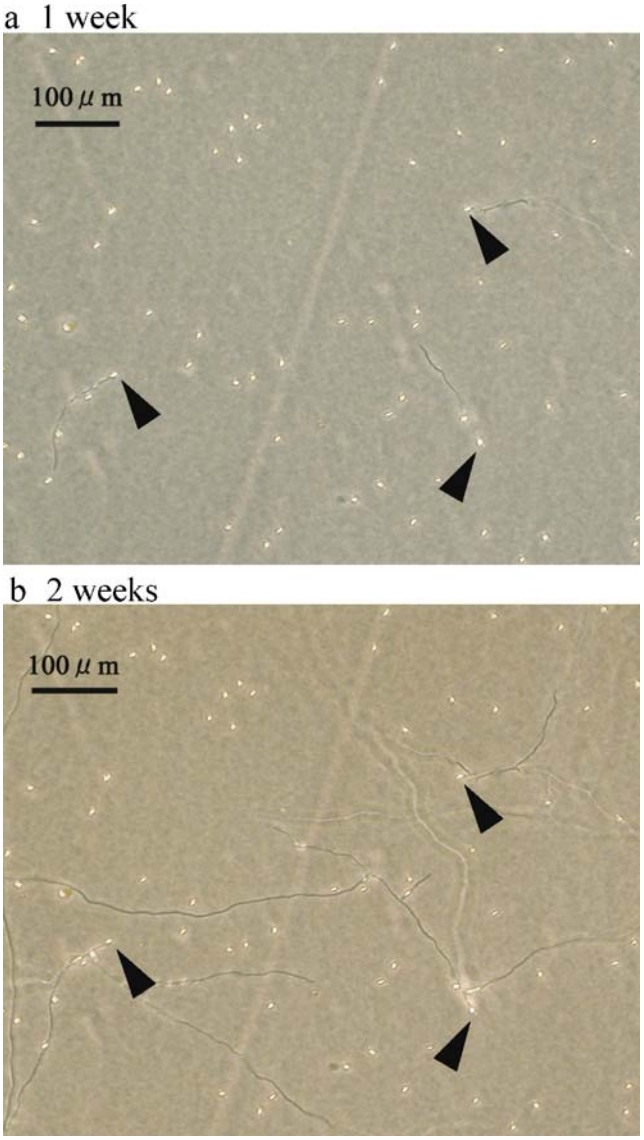

Fig. 1 Photomicrograms of a plate injected with hesperidin. The two photomicrograms are of the same location 1 week (**a**) and 2 weeks (**b**) after spore inoculation. Germinating spores are indicated by *arrows*. Germ tubes grew so long that germinating spores became difficult to distinguish

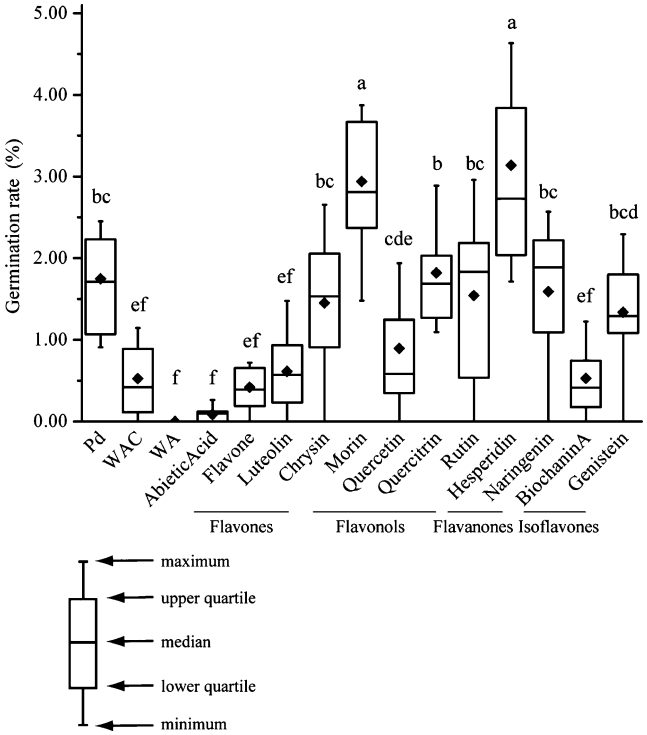

Fig. 2 Effects of flavonoids on spore germination of *S. bovinus*, 2 weeks after inoculation. *Boxes with different letters* indicate significantly different germination rates ($p<0.05$). *Closed diamonds* represent the average germination rates. *Pd* Co-culture with a seedling of *P. densiflora*, *WA* water agar plate, *WAC* water agar plate pretreated with charcoal powder

Experiment II. Effects of different volumes of injected 2 mM morin solution on spore germination

Germination rates observed in experiment II are shown in Fig. 3. In the control ethanol treatment, volumes of ethanol applied did not significantly affect germination rates (data not shown). Therefore, we included only the results of the 20-μl injection in Fig. 3 as the control.

When 20, 40, or 100 μl of 2 mM morin solution were applied, overall germination rates were significantly higher than those in the control ethanol treatment, but they were not significantly different among the three volumes injected. This suggested the existence of a plateau in the relationships between concentration of flavonoids and germination rates and justified the volumes of morin solution applied in experiment I. When 2, 5, or 10 μl of the flavonoid solution were injected, although more basidiospores were observed to germinate at higher rates than in the control ethanol treatments (data not shown), germination rates were not significantly different among them.

Discussion

In this study, 7 out of 11 flavonoids investigated were effective in stimulating basidiospore germination of the ectomycorrhizal fungus *S. bovinus*. Including the results of the preliminary study, in which spore collections from two fruiting bodies were used, positive effects of flavonoids were shown in four spore collections. To our knowledge, this is the first report of a positive effect of flavonoids on the germination of basidiospores and spores of an ectomycorrhizal fungus.

Flavonoids as signaling molecules

Flavonoids are secondary plant products that include pigments (anthocyanins) and colorless or yellow com-

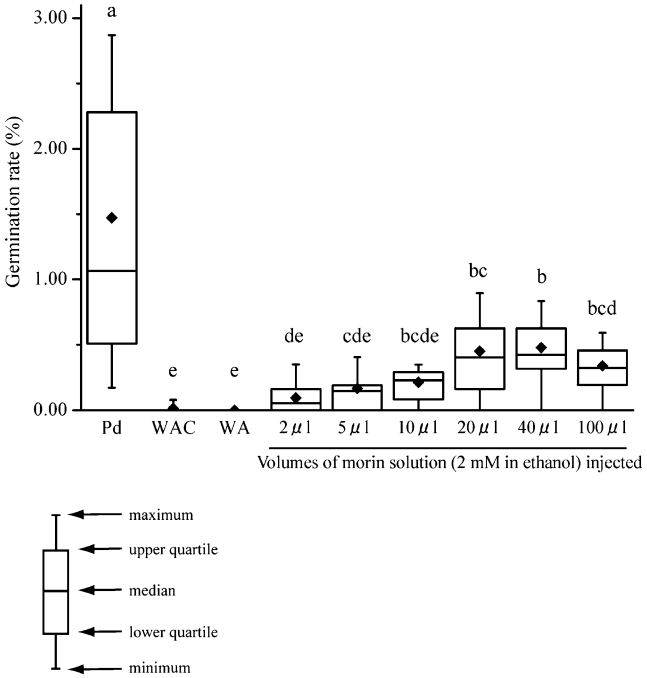

Fig. 3 Effects of injection volumes of morin solution (2 mM in ethanol) on spore germination of *S. bovinus* 2 weeks after inoculation. *Boxes with different letters* indicate significantly different germination rates ($p<0.05$). *Closed diamonds* represent average germination rates. *Pd* Co-culture with a seedling of *P. densiflora*, *WA* water agar plate, *WAC* water agar plate pretreated with charcoal powder

pounds (flavanones, flavones, and flavonols) involved in attracting animal vectors for pollination and seed dispersal (e.g., flower color), UV light protection, stress protection, and signaling in plant reproduction and plant–microbe associations (Gould and Lister 2005).

In plants, flavonols have a strong stimulatory effect on in vitro pollen development, pollen germination, and pollen tube growth in tobacco, maize, and petunia and are effective at concentrations in the micromolar range (Ylstra et al. 1992; Mo et al. 1992).

In plant–microbe interactions, detection of specific plant molecules by microbes is critical for recognition of hosts and subsequent colonization in a complex environment like soil. An early and essential event in most plant–microbe interactions involves chemotaxis toward plant root exudates to find infection zones. In members of the Rhizobiaceae, expression of nodulation (*nod*) genes is induced by flavonoids or isoflavonoids specific to the particular hosts at 1 to 0.1 μM and induction of the *nod* genes leads to production of a lipopolysaccharide (Nod factor), which triggers the formation of nodulation structure by plants (Spaink et al. 1998). Rhizobia are attracted to a wide range of chemicals such as sugars and amino acids in the milli- to micromolar range (Burg et al. 1982; Armitage et al. 1988), whereas several studies have shown that flavonoids also serve as chemoattractants in the micro- to nanomolar range for these bacterial symbionts (Aguilar et al. 1988; Armitage et al. 1988; Caetano-Anollés et al. 1988; Dharmatilake and Bauer 1992), with the exception of *Bradyrhizobium japonicum* (Parke et al. 1985; Kape et al. 1991; Barbour et al. 1991). It should be noted that concentrations of the attractants required for elicitation of chemotaxis in rhizobia are about 100- to 1,000-fold lower than those for inducing *nod* genes (Bauer and Caetano-Anollés 1990).

As for fungi, zoospores of most *Phytophthora* species are attracted to a variety of sugars and amino acids in the micromolar range and ethanol in the 0.2 to 20 mM range (Carlile 1983). In some plant-pathogenic oomycetes, zoospores exhibit positive chemotaxis in response to specific plant signals, mainly by isoflavonoids in the nanomolar range (Yokosawa et al. 1986; Sekizaki and Yokosawa 1988; Sekizaki et al. 1993; Horio et al. 1992; Morris and Ward 1992).

Flavonoids have been reported to play roles in the germination of propagules of some fungal species. Germination of macroconidia of the soilborne fungus *Fusarium solani* f. sp. *pisi* Snyder & Hansen (teleomorph *Nectria haematococca* Berk. & Broome) is strongly stimulated by some flavones and flavanones at micromolar levels, but not by flavanols or isoflavones (Ruan et al. 1995). Chlamydospore germination of *F. solani* is also stimulated by the same flavonoids effective in macroconidia germination, although the effect is smaller and variable (Ruan et al. 1995). Germination of spores of AM fungi has been reported to be stimulated or inhibited by flavonoids (Vierheilig et al. 1998), as two flavanones and one flavone were shown to stimulate germination of *Gigaspora margarita* Becker & Hall spores at 0.15 to 1.5 μM (Gianinazzi-Pearson et al. 1989).

Although previous studies have elucidated the roles of flavonoids as signaling molecules in plant–microbe interactions such as chemotaxis, growth promotion, and germination of propagules, to our knowledge, this is the first report of a positive effect of flavonoids on the germination of basidiospores and spores of an ectomycorrhizal fungus. In the case of spores of *F. solani* f. sp. *pisi* and AM fungi, spontaneous germination occurs commonly at high frequency. Germination rates of controls usually reach 30–40% or more (Ruan et al. 1995; Gianinazzi-Pearson et al. 1989; Scervino et al. 2005). These rates are much higher than those observed in ectomycorrhizal fungi, which are usually less than 0.1% on laboratory synthetic media (Bowen and Theodorou 1973) and, in our study, approximately 0.5 and 0.02% on WAC plates in experiments I and II, respectively (Figs. 2 and 3).

The effective concentrations reported previously for basidiospore germination of ectomycorrhizal fungi are as follows: 544.8 μM butyric acid for *T. matsutake* (0.005% *v/v*; Ohta 1986, 1988), 509.8 μM gluconic acid for *T. robusum* (0.01% *w/v*; Iwase 1992), and 0.33 μM, 330 μM, and 3.3 mM abietic acid for *Suillus granulatus* (L.) Roussel, *S. luteus* (L.)

Roussel, and *S. variegatus* (Sw.) Kuntze, respectively (Fries 1988). In contrast to a report by Fries (1988) that abietic acid at 330 μM resulted in poor germination of spores of *S. bovinus*, our study showed that the addition of 20 μl of 2 mM flavonoid solution (i.e., 40 nmol in 15 ml water agar), which presumably corresponds to several micromolar levels, was sufficient for inducing germination of *S. bovinus* spores. This concentration was equivalent to those effective for pollen development, *nod* gene induction, and spore germination of *F. solani* f. sp. *pisi* and AM fungi. Therefore, the result suggests a possible role of flavonoids as signaling molecules in basidiospore germination of *S. bovinus*.

In experiment II, germination rates were lower than those in experiment I, and co-culture with *P. densiflora* seedlings was the most effective treatment, in contrast to experiment I (Figs. 2 and 3), although morin showed a significant positive effect on germination in both experiments. Spore collections from different basidiocarps within the same species often differ markedly in the duration of germinability and germination frequency (Fries 1976, 1983a, 1984). Moreover, in some cases, the rate of germination may vary not only among different basidiocarps from the same species, but also among different parts of the hymenium in the same basidiocarp (Fries 1984). Miller et al. (1993) assessed germinability and viability of spores of ectomycorrhizal species by spore inoculation to pine seedlings and vital staining of spores, respectively. Based on their results, Miller et al. (1993) suggested that different basidiomes and different populations of the same ectomycorrhizal species might express various abilities or strategies of dormancy, activation, and germination of spores. Therefore, the contradictory results from experiments I and II might be explained by intraspecific physiological variation and imply that the effective flavonoid(s) contained in *P. densiflora* root exudates is not morin. Further investigation of flavonoid profiles in root exudates will clarify the cause of this contradiction.

Flavonoids as a determinant of host specificity

Contrasts in responsiveness to various flavonoids at inter- or intraspecific levels have been reported for rhizobia (Rolfe 1988), chemoattraction of zoospores (Yokosawa et al. 1986; Morris and Ward 1992), and germination of macroconidia of *F. solani* (Ruan et al. 1995) and spores of AM fungi (Scervino et al. 2005; Vierheilig et al. 1998), suggesting that flavonoids play a role in determining host ranges.

In basidiospore germination of ectomycorrhizal fungi, host-specific effects of root exudates have been reported. Basidiospores of *Thelephora terrestris*, *Rhizopogon luteolus*, *S. luteus*, and *Russula adusta* showed considerably faster germination at a higher percentage when induced by roots of host woody plants, but not by those of non-host woody plants or herbs, with some exceptions for other ectomycorrhizal species (Melin 1962; Birraux and Fries 1981; Fries and Swedjemark 1986; Theodorou and Bowen 1987; Fries 1989).

Among the phenomena of flavonoid-mediated plant–microbe associations, relationships between flavonoids and nodulation by rhizobia have been most intensively studied, and flavonoids have been shown to play a role in the determination of host range through interaction with the nodD protein (Cooper 2004; Brencic and Winans 2005). Inducers and anti-inducers co-exist in root exudates of a single legume species, and inhibitory effects can be overcome by increasing the concentration of inducers (Peters and Long 1988). The hypothesis that levels of *nod* gene induction depend on the net outcome of positive and negative flavonoid effects has been proposed (Peters and Long 1988; Rolfe 1988). This hypothesis was supported by the findings of Peck et al (2006). A similar mechanism might operate in host-specific effects of root exudates on spore germination of ectomycorrhizal fungi, as effective and non-effective flavonoids on germination of *S. bovinus* spores were present (Fig. 2).

Conclusion

We showed that certain flavonoids induce basidiospore germination of the ectomycorrhizal fungus *S. bovinus*. This suggests the stimulant for germination of spores of ectomycorrhizal fungi to be one of the roles of flavonoids as signaling molecules in plant–microbe interactions. However, as no reports have been published on flavonoid profiles in root exudates of woody plants except for a legume woody plant, *Robinia pseudoacacia* (Scheidemann and Wetzel 1997), whether morin or hesperidin, which were the most effective for basidiospore germination of *S. bovinus*, act as stimulants in the field remains unclear. For further study, identification and quantification of flavonoids in root exudates of woody plants and evaluation of the effects of each flavonoid on spore germination of *S. bovinus* and other ectomycorrhizal species will contribute to further interpretation of symbiosis and co-evolution between woody plants and ectomycorrhizal fungi.

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