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Phytochemistry 62 (2003) 1145–1151
PHYTOCHEMISTRY
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Effects of seven pure flavonoids from mosses on germination and growth of *Tortula muralis* HEDW. (Bryophyta) and *Raphanus sativus* L. (Magnoliophyta)

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Received 1 July 2002; received in revised form 30 October 2002

Abstract

Dried mosses (five moss species) were progressively extracted and subjected to a four-step Craig distribution. Seven pure flavonoids were isolated and identified. The flavonoids were the flavones apigenin, apigenin-7-*O*-triglycoside, lucenin-2, luteolin-7-*O*-neohesperidoside, saponarine and vitexin; and the biflavonoid bartramiaflavone and they were submitted to biological tests. The tests were performed in vitro on spore germination and protonemal growth of the moss *Tortula muralis* and on seed germination and root growth of *Raphanus sativus*. Flavonoids caused a decrease in the percentage of spore germination, protonemal development and root growth. In addition they caused morphological alterations, such as forked tips, swollen apices, rounded cells and early formation of brood cells in the protonemata. Data were discussed in relation to the presence of allelochemicals in mosses.

Keywords: Mosses; Flavonoids; Biflavonoids; Moss allelochemicals; Moss development; Root growth

1. Introduction

Flavonoids are widely found in plants and, as shown by recent studies, also in mosses (Asakawa, 1981, 1982). Among the monoflavonoids, apigenin, luteolin, kaempferol and orobol derivatives are those usually found in mosses (Zinsmeister and Mues, 1980; Zinsmeister et al., 1991; Markham, 1988; Mues and Zinsmeister, 1988; López-Sáez et al., 1996b). Biflavonoids from apigenin, luteolin and eryodictiol are also an important source of secondary metabolites from mosses (Geiger et al., 1988; Geiger and Quinn, 1988; Geiger, 1990; Markham, 1990; López-Sáez, 1994; López-Sáez et al., 1996a,b). Flavonoids are frequently involved in allelopathic effects (Harborne, 1988). In particular, the flavonoid quercetin isolated from infestant plant *Pluchaea lanceolata* inhibited the growth of *Vigna unguicuolata* (Inderjit and Dakshini, 1996) flavonoid coumarin extracted from *Ruta graveolens* inhibited seed germination of *Raphanus sativus* L. (Aliotta et al., 1993; Aliotta and Cafiero, 1999).

This paper deals with the potential allelochemical activity of flavonoids (apigenin, apigenin-7-*O*-triglycoside, bartramiaflavone, lucenin-2, luteolin-7-*O*-neohesperidoside, saponarine and vitexin) isolated from five moss species, on spore germination and sporeling growth of the moss *Tortula muralis* and on a standard system for detection of allelochemicals: root development in *R. sativus* L.

2. Results and discussion

2.1. Test on T. muralis

2.1.1. Morphogenetic alterations

In control cultures germination starts with the emergence of a single protonemal tube (chloronema) with lenticular chloroplasts and transverse septa (Fig. 1a). The basal cells emerging directly from the spore may appear more swollen than the others, but the diameter of newly formed cells immediately decreases, forming a

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PII: S0031-9422(02)00659-3

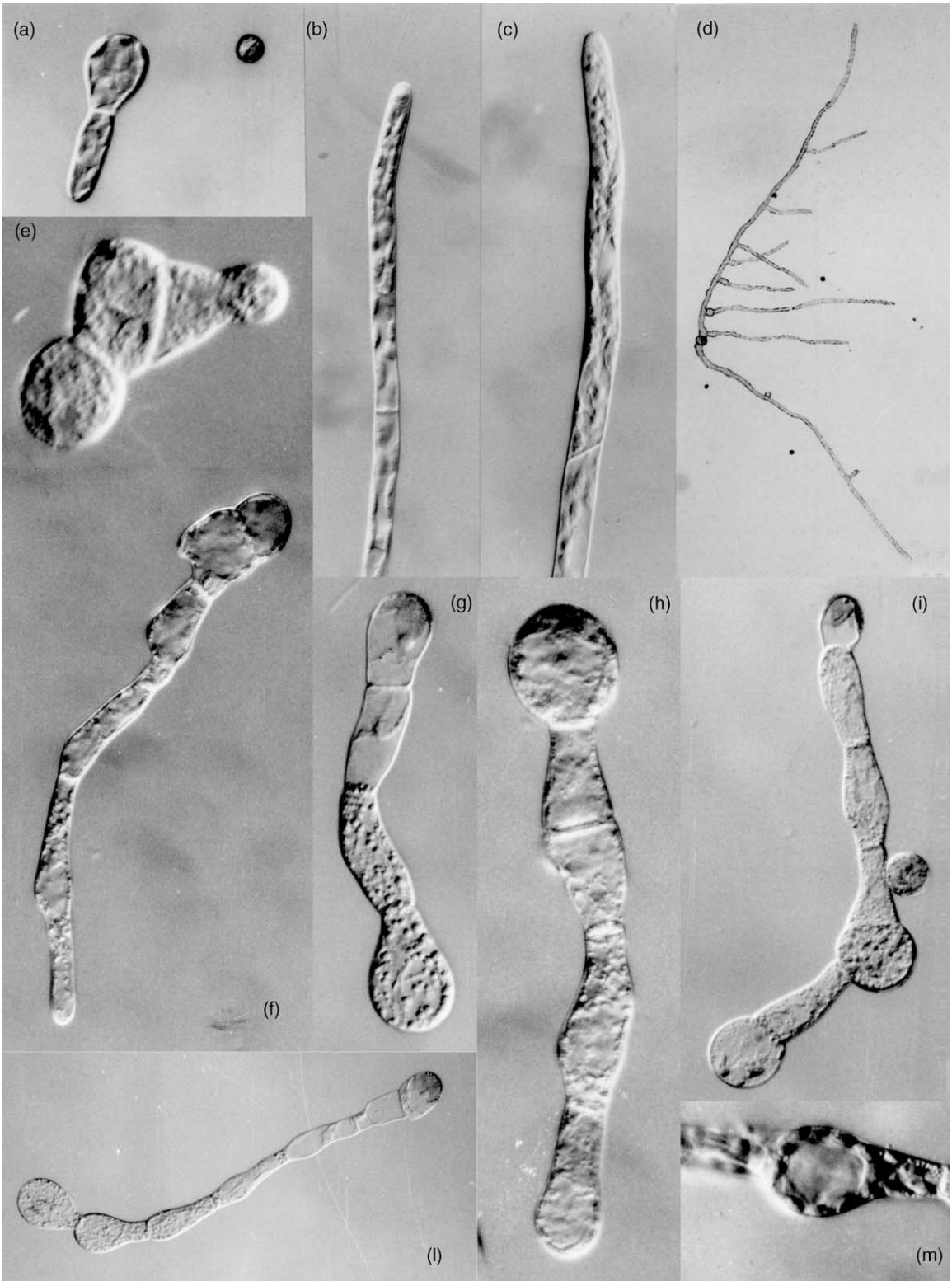

Fig. 1. Protonemal development from *Tortula muralis* spores grown in control and in flavonoids containing culture media: (a–d) Control samples; (e–m) samples of protonemata treated with flavonoid saponarin at the 0.01 μg/ml; (d) light microscopy with conventional illumination, (a–c,e,f) phase contrast micrographs. (a) Normal germination pattern after 3 days in culture. The spore, with a marked cell polarity, forms a germinating, cylindrical tube (× 300); (b) apical cell from control chloronemata after 7 days of culture (× 300); (c) apical cell from control caulonemata after 14 days of culture (× 300); (d) normal pattern of germination of control protonemata after 14 culturing days (× 120); (e) abnormal germinating spores in saponarin treated samples after 14 culturing days (× 500); (f) abnormal development of saponarin-treated samples after 21 culturing days (× 300); (g–l) typical appearance of flavonoid-treated samples after 14 culturing days, showing delayed development, swollen apex and a non-uniform cell diameter along the filament (g) × 350; (h) ×450; (i) ×320; (l) ×250; (m) brood cells in saponarin-treated samples after 14 culturing days (×300).

tapering tip. Germination is mostly unipolar or sometimes bipolar. After 21 days, other germinating tubes emerge from the original spore, giving a bi-multipolar protonema with chloronemata and caulonemata (Fig. 1b–d). After 30 days brood cells are still absent.

All flavonoids tested induce a general slowing of growth and a considerable decrease in spore germination percentage (Fig. 2). Both effects are dose-dependent. In particular, concentrations 1 and 0.1 μg/ml completely inhibited spore germination. At 0.01 μg/ml all flavonoids showed strong inhibition of spore germination and protonemal growth, with evident morphogenetic alterations (Fig. 1e). In the presence of saponarin, the most active flavonoid, germination was 2–4–8–10% after 6–10–14–18 days respectively, and the protonema was on average 200 μm long also after 18 culturing days. In the presence of vitexin, the least effective, germination was 10–30–36–38% after 6–10–14–18 culturing days and the protonema was 400 μm long after 18 days. Apigenin, apigenin-7-*O*-triglycoside, bartramiaflavone, lucenin-2, and luteolin-7-*O*-neohesperidoside showed intermediate activity (Figs. 2 and 3). All flavonoids employed induced dose-dependent alterations.

Swollen tips can be found frequently (Fig. 1g–l) and protonemal intercalary cells are also swollen and short (Fig. 1f), profoundly altering the general appearance of

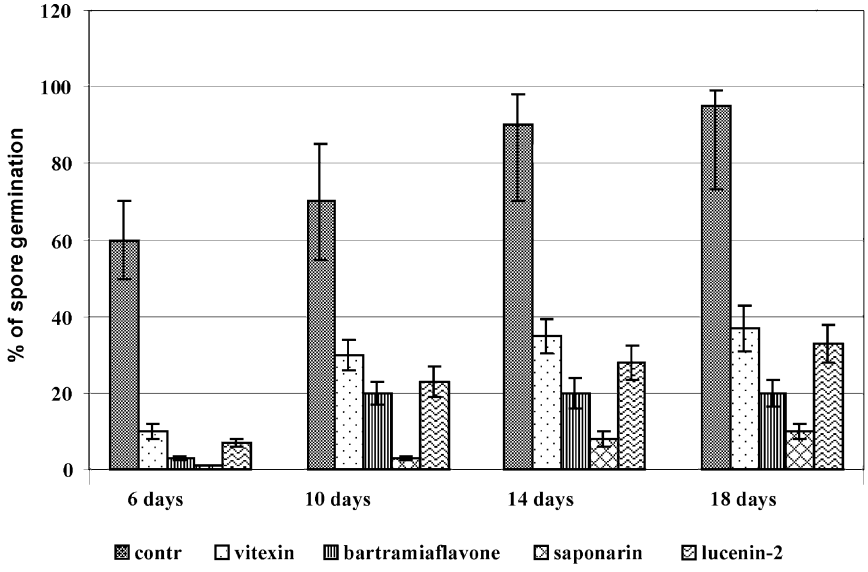

Fig. 2. Effect of flavonoids on spore germination of *Tortula muralis* cultured on light at the concentration of 0.01 μg/ml. Germination percentages were evaluated by examining about 300 spores per replicate, and protonemal length was measured on 30 protonemal filaments per replicate at 6, 10, 14 and 18 days from inoculation.

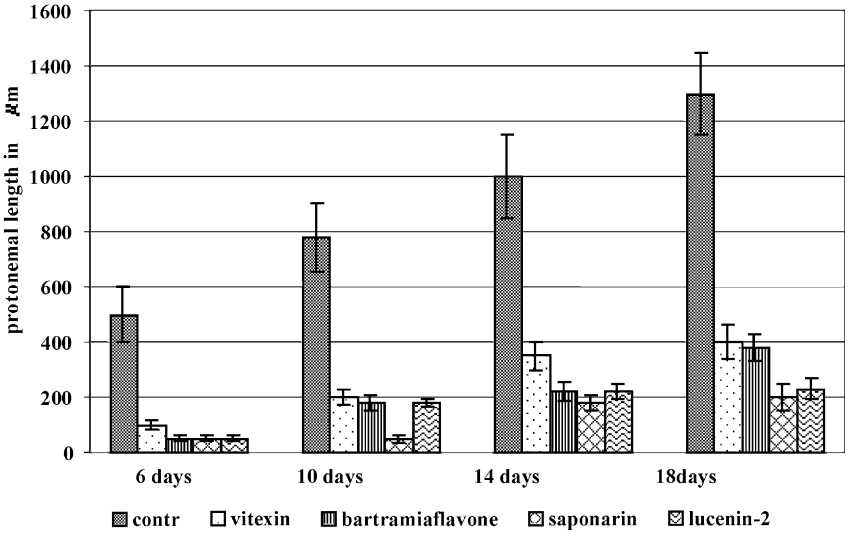

Fig. 3. Effect of flavonoids on protonemal growth of *Tortula muralis* cultured on light at the concentration of 0.01 μg/ml. Germination percentages were evaluated by examining about 300 spores per replicate, and protonemal length was measured on 30 protonemal filaments per replicate at 6, 10, 14 and 18 days from inoculation.

the protonema especially during the first days of culture (Fig. 1e). The altered appearance is frequently retained even after 3 weeks (Fig. 1l). All morphological alterations are dose-dependent and do not occur in specimens transplanted and cultured in flavonoid-free fresh medium.

It is worth noting the early occurrence (after 15 days) of brood cells (Fig. 1m), along the protonemal filaments or at the tip, that in the control develop after longer culturing (2 months).

2.2. Test on R. sativus

Percentage of seed germination, root elongation and hair growth were inhibited by the flavonoids tested; inhibition was dose-dependent and saponarin was the most active flavonoid (Figs. 4, 5 and 6A–D). The most evident morphological alteration is the shape of the root apex that was larger (Fig. 6B). At the lowest concentration used, that allows root development, the width of the hair zone was reduced as well as hair growth; they correspond to previous developmental steps (i.e. 2 days' root is like that of 12 h).

All the results given, both on moss and on *R. sativus*, were obtained on light while on dark they were not shown as the differences with the control are not significant.

In a previous work on antibacterial activity of the same flavonoids, saponarin isolated from *Plagiomnium cuspidatum* shows the highest antibacterial activity with MIC between 4 and 2048 μg/ml (Basile et al., 1999). In

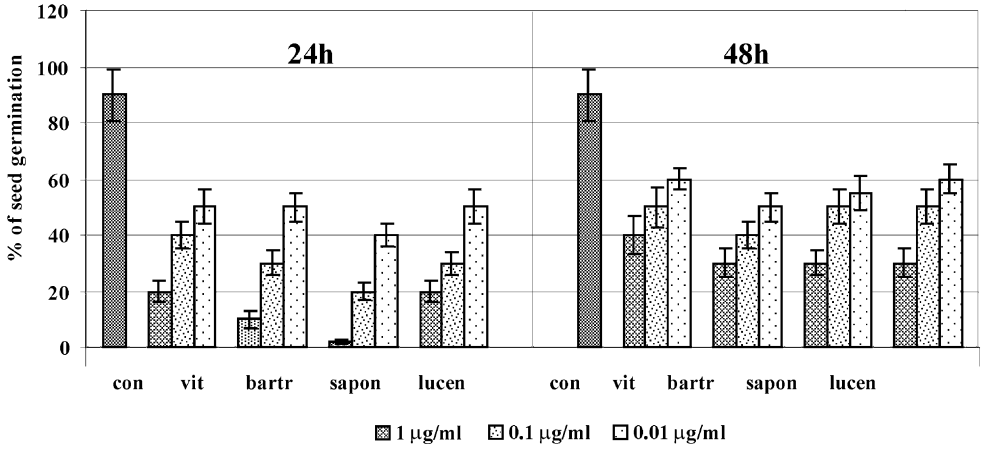

Fig. 4. Effect of flavonoids on seed germination of *Raphanus sativus* cultured on light. Germination percentages were evaluated by examining 30 seeds per replicate, and root and epicotile length was measured on 15 roots per replicate, at 24 and 48 h from inoculation. All experiments were carried out in triplicate and repeated three times. Data reported are mean values on all experiments. Observations were made by using a Leitz Aristoplan microscope equipped with differential interference contrast optics (Nomarski).

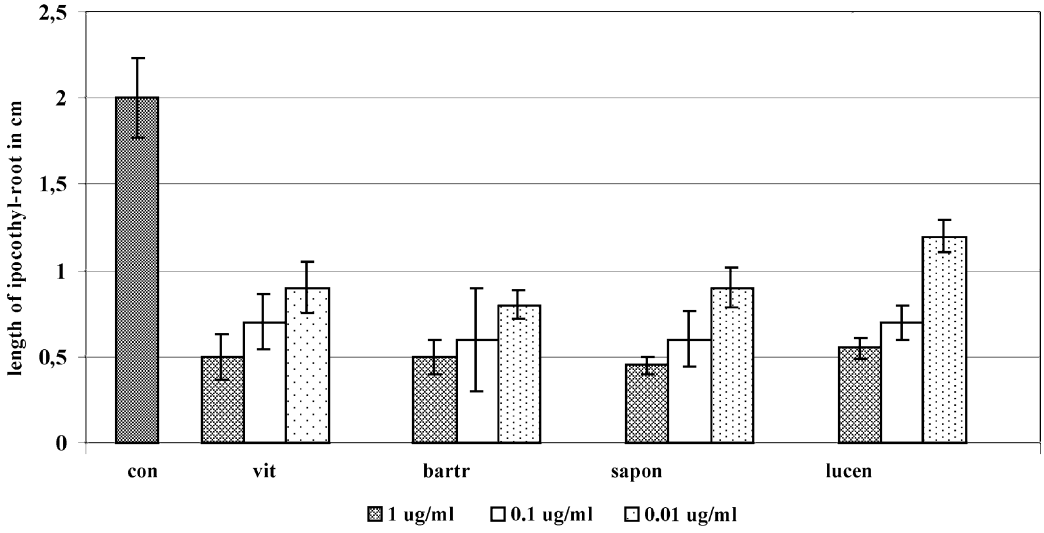

Fig. 5. Effect of flavonoids on plantlet growth of *Raphanus sativus* cultured on light. Germination percentages were evaluated by examining 30 seeds per replicate, and root and epicotile length was measured on 15 roots per replicate, at 24 and 48 h from inoculation. All experiments were carried out in triplicate and repeated three times. Data reported are mean values on all experiments. Observations were made by using a Leitz Aristoplan microscope equipped with differential interference contrast optics (Nomarski).

agreement with this finding our results show that saponarin is the most active among flavonoids tested in inhibiting spore germination and protonemal growth in *T. muralis* as well as root development in *R. sativus*. Moreover, it was reported that the same substance can show both phytotoxic and antibacterial activity (Vaughn, 1995).

This finding was also shown by flavonoids from *Castanea sativa* that inhibit both bacterial and root growth (Basile et al., 2000). Comparing the activity of flavonoids from *Castanea sativa*, obtained on the same model (*R. sativus* seeds) and in the same culture conditions, with the present data on flavonoid activity it is evident that flavonoids from mosses are more active.

As for the morphological alterations observed in protonemal development, they could be related to the toxic effects of flavonoids on membrane permeability

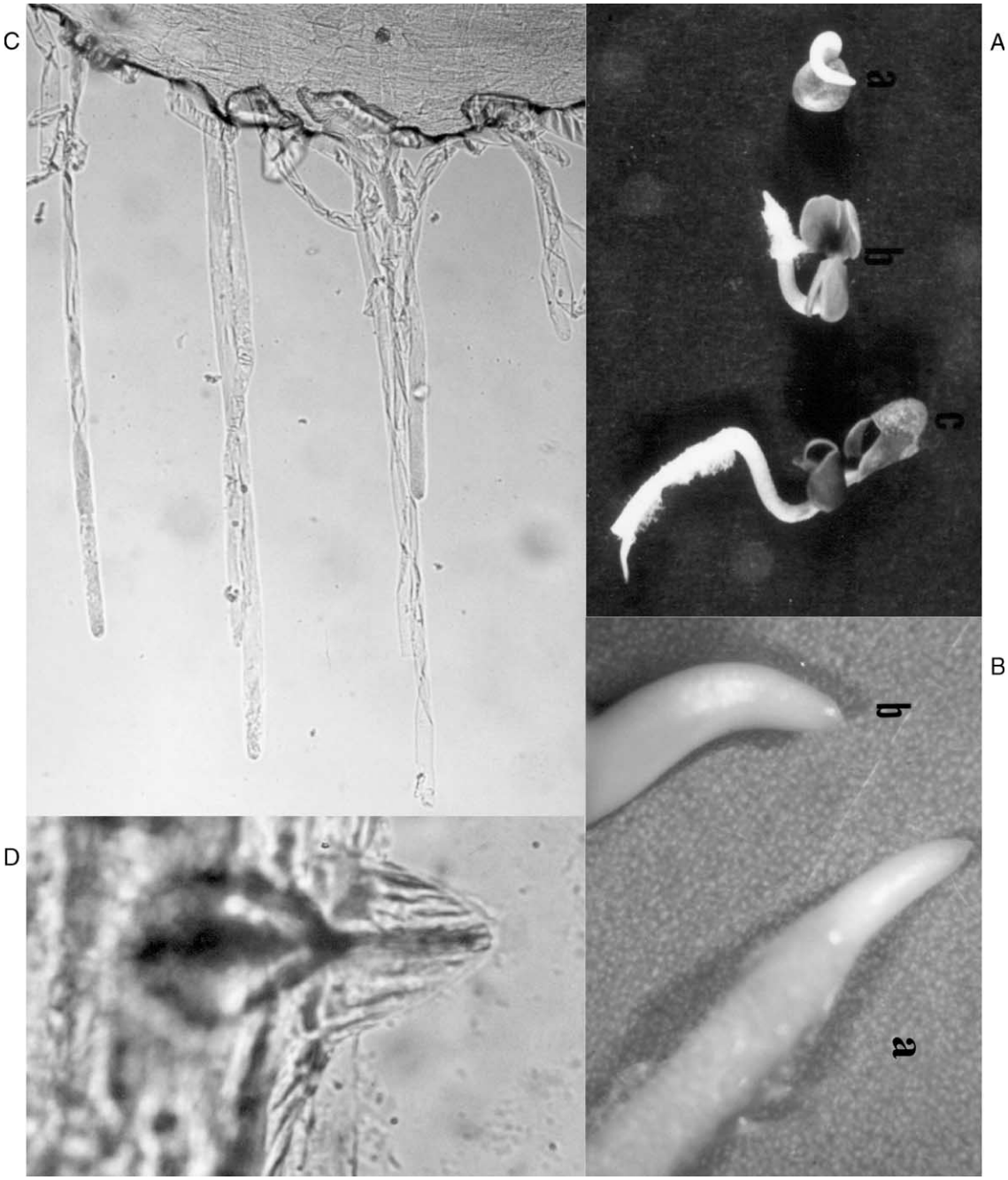

Fig. 6. Germination of *Raphanus sativus* seeds grown in control and in flavonoids containing culture media: (A and B) stereomicrographs; (C and D) light microscopy with conventional illumination. (A) Stereomicrograph of a germinating seed of *R. sativus* after 48 h treated with 1 μg/ml saponarin (a) 0.01 μg/ml saponarin (b) and in control conditions (c) (× 2.5); (B) stereomicrograph of radish seeds showing the root with normal apex in the control (a) and larger apex in saponarin (0.01 μg/ml) treated sample; (C and D) light micrograph of control hair zone with normal hair growth after 48 h (× 75) (C) and saponarin treated sample (0.01 μg/ml) showing reduced hair growth (× 300) (D).

(and therefore on cellular turgidity, that is essential in growth by extension).

An alteration of protonemal development pattern was shown in mosses (*F. hygrometrica, Bryum capillare* and *Pleurocherete squarrosa*) treated with lichen *Cladonia foliacea* extracts (Giordano et al., 1997, 1999).

As for early brood cell formation, it could be considered an adaptive response of the moss to the presence of toxic substances rather than an alteration. In fact, brood cells are spherical or ovoid cells which are produced in nature or in control cultures in the case of senescence or in stress conditions, such as desiccation or lack of nutrients in general and calcium ions in particular. Brood cells are structures of resistance due to the presence of a thick wall and a large number of lipid drops, and have been interpreted as diaspores with the task of propagating in adverse nutritional conditions (Duckett and Ligrone, 1992; Duckett et al., 1993; Goode et al., 1993, 1994). We previously reported the increased production of brood cells in response to lead stress in *F. hygrometrica* (Basile et al., 1995). In addition, the formation of brood cells in this moss was reported in the presence of Cu and Zn (Coombes and Lepp, 1974). Our data allow us to hypothesise that brood cell formation is a generalised resistance mechanism which occurs in response to stresses including toxic substances by transforming the filamentous protonema, with thin and highly permeable walls, into diaspores of resistance and propagation with thick walls and an abundant nutritional reserve.

The mechanism which triggers a ''brood cell morphogenetic program'' may be indirect: flavonoids lead to conditions of ''early aging'', which could be the signal for brood cell production. Alternatively, the flavonoids might affect membrane permeability to calcium by lowering its concentration, with consequent ''triggering'' of the brood cell program. When transplanted into control medium, the brood cells germinate and produce new and normal filaments.

As for the light-dependent effect, Moreland and Novitsky (1988) showed flavonoids interacting with electron transport and phosphorylation light induced in the chloroplast.

Flavonoids from mosses seem to be broad spectrum substances, potentially able both to protect them from bacteria and help them in competition with other plants. Further studies are moving from standard organisms like *R. sativus* and *T. muralis* to organisms sharing the same habitats with those where flavonoids occur.

3. Experimental

Air-dried plant material from moss species (*Bartramia pomiformis*, 250 g; *Dicranum scoparium*, 285 g; *Plagiomnium affine* and *P. cuspidatum*, 150 g each; *Hedwigia ciliata*, 210 g, of which voucher specimens are deposited in the MACB Herbarium, Madrid, Spain) were extracted with methanol and acetone (8:2) at room temperature (Geiger, 1990). The combined extracts were subjected to a four-step Craig distribution (Geiger et al., 1988; Geiger, 1990; López-Sáez, 1994). The combined lower phases were reduced in vacuo to a thin syrup. The flavonoids apigenin (71 mg) and vitexin (86 mg) from *P. affine*, saponarine (95 mg) from *P. cuspidatum*, bartramiaflavone (92 mg) from *B. pomiformis*, lucenin-2 (53 mg) from *H. ciliata*, apigenin-7-*O*-triglycoside (43 mg) and luteolin-7-*O*-neohesperidoside (61 mg) from *D. scoparium* were isolated by column chromatography on polyamide-6 and purified on sephadex LH-20 (Geiger, 1990). They were identified by co-chromatography (TLC, HPLC), MS and NMR (Geiger et al., 1988; Geiger, 1990; López-Sáez, 1994). Only for *Bartramia* species and *D. scoparium* are NMR and MS data available from our previous research that has been published elsewhere (Geiger et al., 1993; López-Sáez et al., 1995a,b).

Flavonoids were dissolved in two drops of DMSO (dimethyl sulfoxide); Mohr medium was added to a concentration of 1 μg/ml. The solutions were sterilized by millipore filtration (0.45 μm). Serial 10-fold dilutions were prepared adding Mohr medium up to 0.01 μg/ml. Evaluation of effects was performed as reported below.

3.1. Biological assay

Mature capsules of *T. muralis* Hedw. and seeds of *R. sativus* L. were surface sterilized by 70% ethanol (2 min) and 2% NaClO with the addition of a few drops of Triton X-100 (5 min) and subsequently washed (10 min) with sterile distilled water. As for *T. muralis* the contents of 10 capsules were suspended in 10 ml of sterile distilled water. Samples (200 μl) of the moss sporal suspension were inoculated in modified Mohr medium (11), pH 7.5 [KNO_3 100 mg, $CaCl_2.4H_2O$ 10 mg, $MgSO_4$ 10 mg, KH_2PO_4 136 mg, $FeSO_4$ 0.4 mg and 1 ml of BBM (Bold Basal Medium) solution (Nichols, 1973) to 1000 ml distilled water] used as control and in the same medium with the addition of isolated flavonoids.

As for *R. sativus,* 30 seeds per Petri dish were inoculated in the same medium.

Both cultures were kept in a climatic room with a temperature of 16±1 °C, 70% constant relative humidity, and 16 h light (2000–5000 lx)/8 h dark photoperiod or 24 h dark and observed after 6, 10, 14 and 18 days.

3.2. Effect evaluation

With regard to moss bioassays, germination percentages were evaluated by examining about 300 spores per replicate, and protonemal length was measured on 30 protonemal filaments per replicate at 6, 10, 14 and 18 days from inoculation. Effects of the flavonoids on the

morphogenesis of the protonemal apparatus were evaluated by considering the following parameters: the kind of filaments (caulonema, chloronema, rhizonema), cell number in the main filaments, order of branching, cell number in I, II and III order branches, cell number without ramification from the apex. With regard to *Raphanus sativus*, germination percentages were evaluated by examining 30 seeds per replicate, and root and epicotile length was measured on 15 roots per replicate, at 24 and 48 h from inoculation.

All experiments were carried out in triplicate and repeated three times. Data reported are mean values on all experiments. Observations were made by using a Leitz Aristoplan microscope equipped with differential interference contrast optics (Nomarski).

3.3. Statistical analysis

Statistical analysis was performed using ANOVA one way test.

Acknowledgements

The authors wish to thank Dr. Mark Walters for reviewing the English.

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