Journal of Applied Botany and Food Quality 79, 89 - 93 (2005)

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Bioactivity of bryophyte extracts against *Botrytis cinerea, Alternaria solani* and *Phytophthora infestans*

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(Received March 7, 2005)

Summary

Plant extracts can be used as crude extracts, alternative sources of known fungicides, new leads for fungicides and resistance inducers for an integrated pest managment strategy. They are the point of interest in discovery of organic pesticides. Though several phytochemical constituents and medicinal uses of bryophytes are known, their potentials in the course of crop protection remained yet little or unexplored. The scope of this work was to study extracts from 17 bryophytes as source of potential antifungal agents under *in vitro* and *in vivo* circumstances. Results showed that bryophyte extracts from *Bazzania trilobata, Diplophyllum albicans, Sphagnum quinquefarium, Dicranodontium denudatum* and *Hylocomium splendens* with high level of inhibition (>50%) of the mycelial growth of *Botrytis cinerea* and *Alternaria solani*. Extracts of *B. trilobata* and *D. albicans* significantly reduced disease severity of *Phytophthora infestans* on tomatoes. In conclusion, the study indicated that extracts from bryophytes can be used as natural sources for alternative pest managment tools.

Introduction

Before discovering synthetic pesticides, plant-derived insecticides and fungicides were used as plant protection compounds (Jacobson and Crosby, 1971) such as pyrethrins produced from flowers of *Chrysanthemum cinerariaefolium* (McCallan, 1969), azadirachtin from the families of Meliaceae and Rutaceae, ryanodins from *Ryania speciosa* and a carbamate group of plant-derived insecticides (physostigmine) from *Physostigma venenosum* (Dukec, 1990) which are among a few examples of pesticides of natural origins. Similarly, mainly from micro-organisms fungicides had been developed such as pyrrolnitrin, methylacrylate, strobilurin A, oudemansin, hedacidin and thiolutin (Buchenauer, 1996).

Natural products are being considered as alternatives to the arsenal of synthetic pesticides, because of their relatively short environmental half-time and less suspect for toxicology (Dayan et al., 1999). More over, antifungal activities of bioactive substances originated from organisms are in nature certainly guaranteed from the sources unlike that of compounds synthesised in a laboratory with unknown bioactivity. Thus, an isolation and chemical characterisation of active compounds from plants with strong biological activities have received major research endeavours (Dukec, 1990). Due to these facts, there exist ever growing interests to find those active substances from plants, which enable the reduction of the amount of pesticide active ingredients applied in an ecosystem. In general, plant extracts can be used as crude extracts, alternative sources to known fungicides, new chemical structures for fungicides and resistance inducers.
Bryophytes are non woody lower vascular plants and taxonomically placed between Algae and Pteridophyta. About 20 000 species of mosses are described world wide and divided into the classes Anthocerotae (hornworts), Hepaticae (liverworts) and Musci (mosses).
So far, several active phytochemical constituents and medicinal uses of products from bryophytes are commonly known. Especially, liverworts are a rich sources of diverse bioactive secondary metabolites characterised by a large variety of sesqui-, di-, and triterpenes together with different phenols (Asakawa and Heidelberger, 1982; Zinsmeister et al., 1994; Becker, 1995). Nevertheless, their potential for the management of plant pathogenic fungi seems to be largely undiscovered.
Hence, this paper provides some insights on preliminary bioactivity screening achievements on the use of bryophyte extracts and their future perspectives to control fungal pathogens in an integrated pest management strategy in crop production.

Materials and methods

Host plant cultivation

Tomatoes, *Lycopersicon lycopersicum* (L.) Farw. cv. 'Rheinlands Ruhm' and green pepper, *Capsicum annuum* L. cv. 'Yolow Wonder' were used in the investigations. Sowing of certified seeds raised test plants in commercial potting soil. All plants were cultivated at the following growth conditions in the glasshouse: 16 hours light period (approx. 7000 lx), 60 to 80 % RH, 20 ± 2°C, irrigation twice per day and a weekly supply of a 2 % nutrient solution.

Fungal culturing and maintenance

Isolates of *Botrytis cinerea* Pers. ex. Fr., *Alternaria solani* (Ellis et Martin) Sorauer and *Phytophthora infestans* (Mont.) de Bary were used as assay indicators. Culturing and maintenance of *B. cinerea* and *A. solani* was performed on Potato-Dextrose-Agar (PDA) (39g medium in 1000 ml Aqua dest.) at 20 ± 5°C. *P. infestans* was cultured on vegetable juice-calcium carbonate agar (200ml V_8 juice; 3 g $CaCO_3$; 16 g agar; 800 ml Aqua dest.) and maintained at 18°C in the dark.

Source of bryophyte extracts

Bryophyte species of the Hepaticae such as *Scapania undulata* (L.) Dum., *Bazzania trilobata* (L.) S.F. Gray, *Pellia epiphylla* (L.) Corda and *Diplophyllum albicans* (L.) Dum. and of the Musci such as *Hylocomium splendens* (Hedw.) B.S.G., *Bryum pseudotriquetrum* (Hedw.) Schwaeger, *Philonotis fontana* (Hedw.) Brid., *Sanionia uncinata (*Hedw.) Loeske, *Mnium hornum* Hedw., *M. undulatum* Hedw., *M. affine* Funk, *Plagiothecium undulatum (*Hedw.) B.S.G., *Dicranodontium denudatum* (Brid.) Britt., *Sphagnum quinquefarium* (Braithw.) Warnst., *Rhytidiadelphus loreus* (Hedw.) Warnst., *Polytrichum commune* Hedw. and *Dicranum scoparium* Hedw. were collected in the Vosges Mountains (France). The taxonomic identity of individual species was checked by Frahm and Frey (1992).

Bryophyte extraction

10 g dry-weight of each sample was finally pulverised with a coffee mill and diluted in 250ml ethanol (70 %). Thereafter samples were extracted under reflux condition in a magnetic stir and warm-water-bath at 60°C for 2 hours. The supernatants and pellets were separated by a hydraulic vacuum filtration (KURT, 1997).

Bioassays for screening of active bryophyte extracts against mycelium growth *in vitro* and *in planta*

Extracts from 17 bryophytes, each at 0.1% m/v, were tested against the mycelial growth of *B. cinerea* and *A. solani*. Each extract was added into the growing media (PDA) in Petri-dishes on which agar discs with one-week-old mycelium of the fungi were placed and incubated lateron at 20 ± 5°C. Untreated or with 70% ethanol treated agar plates were used as standard control (BLAESER et al., 1998). All treatments were replicated five times in a CR design and the experiments with each fungus were conducted two times. The rate of radial mycelium growth (cm) of test fungi was measured 2 and 4 days after the inoculation (dpi).

Preliminary screening of bryophyte extracts was conducted on the *B. cinerea*/green pepper pathosystem. Three weeks old green pepper plants were protectively sprayed with ethanolic extracts from 17 moss species each at 1% mv^{-1} (in distilled water containing 0.0125% Tween 20 as surfactant) and with the fungicide dichlofluanide (50 ppm). Untreated plants were compared for efficacy determination. Extracts were applied 4 hours before inoculation with the pathogen. Each treatment was replicated four times in a completely randomised design. A conidia suspension counting 1x 10^6 conidia ml^{-1} of *B. cinerea* was used for a uniform inoculation of test plants. Inoculated plants were incubated in a moist chamber at 20 ± 5°C and 95% relative humidity for 48 hours. Advanced screening of extracts from bryophytes (*B. trilobata, D. albicans, S. quinquefarium* and *D. denudatum)*, each at 0.1, 0.5 and 1% m/v in 100ml Aqua dest. with 0.0125% Tween 20, were retested against the infection density of *P. infestans* (8' 10^4 zoospores/ml) on tomatoes. Test plants were sprayed with bryophyte extracts 24 hours, 2 and 4 days before the challenge inoculation. As a standard, plants sprayed with water were accommodated. Treatments were arranged in a Block Balanced Design and each treatment was replicated 4 times. The bioassay was repeated for confirmation of results from the initial experimental outcomes.

Disease evaluation, efficacy computation and statistical analysis

The mycelial growth inhibition activity of each test substance was measured *in vitro* based on radial growth (cm) of the test fungi and calculated as follows:

Inhibition (%) = 100 [(Ut-Tr)/Ut], whereby, Ut = mean value for untreated control and Tr = mean value for treated with moss extracts (ABBOTT, 1925).

The *in vivo* antifungal activity of plant extracts was based on visually rating a percentage (%) of infected leaf area in relation to total healthy tissues of sampled leaves. Samples of 4 leaves per replication and 16 per treatment were used in assays on green pepper. Totally, four leaves of tomatoes (each bearing 12 leaflets per replication and 48 leaflets per treatment) were sampled for data collection. The mean disease severity per replication was calculated to conduct statistical analysis and efficacy determination in all experimental cases.

The percentages of disease severity data were subjected to logarithmic transformation to affirm the basic assumptions during the statistical variance analysis. Thereafter, the analysis of variance and multiple separation of means (t-test and Tukey-test, a <0.05, p <0.05) were determined by using Sigma Stat (SPSS, Version 2, 1997, USA).

Results

Inhibition of radial mycelium growth of *A. solani* and *B. cinerea*

The screening of ethanol extracts from different bryophytes showed significant differences in the mycelium growth of the tested fungi. Significant differences between the moss extracts and untreated checks were also detected. More than 50% of the tested substances showed low efficacy in the range of 0-25% inhibition of mycelial growth for *B. cinerea* and *A. solani* (Fig.1, 2). *M. affine, S. uncinata* and *S. undulata* were among the extracts which showed low level of mycelium growth inhibition against *B. cinerea.* Similarly, a weak inhibitory activity was found due to extracts of *S. undulata, M. undulata, P. epiphylla* against *A. solani.* Extracts from *R. loreus, P. epiphylla, P. commune* and *M. undulatum* exhibited moderate ranges of 25-50% antifungal activities. The higher level of mycelial growth inhibitory activity of bryophyte extracts was recorded by using extracts from *B. trilobata, D. albicans, D. denudatum* and *S. quinquefarium.*

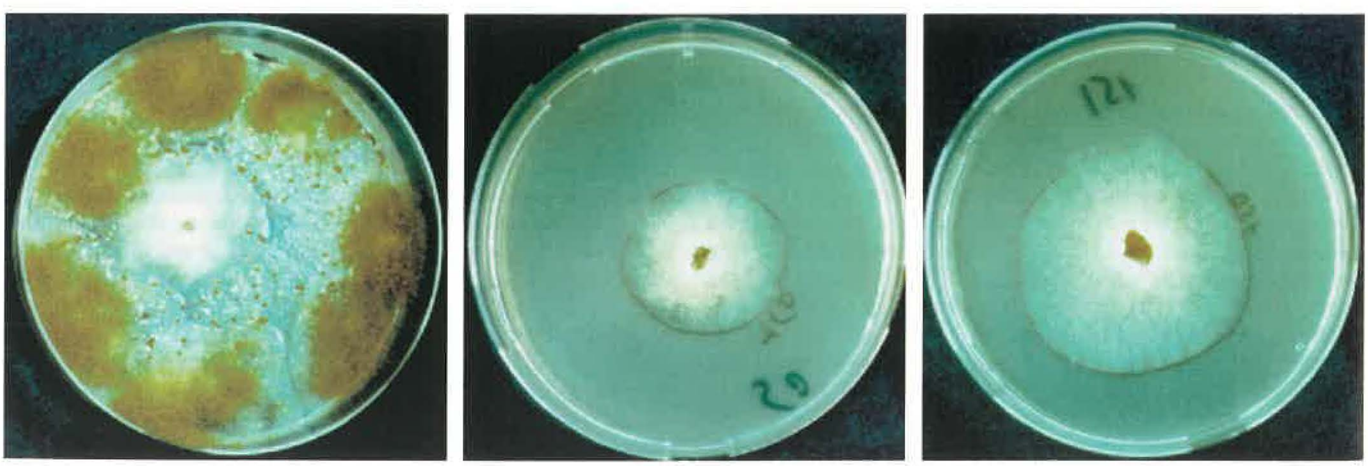

Fig.1: Effects of extracts from the leafy liverworts *Diplophyllum albicans* and *Bazzania trilobata* on growth characteristics of *Botrytis cinerea.*

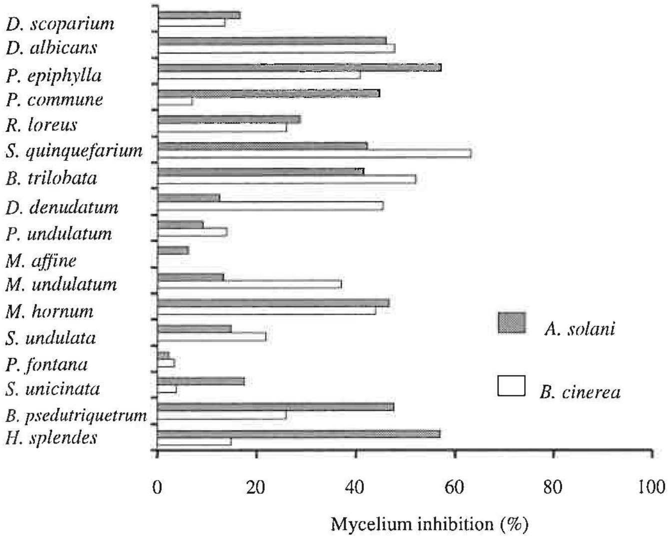

Fig. 2: The influence of moss extracts (0.1% m/v) on the mycelium growth of *Botrytis cinerea* and *Alternaria solani* on PDA applied four days after inoculation.

Plant protection activity against *Botrytis cinerea*

The screening of ethanolic extracts from 17 bryophytes as potential sources of active substances for protective application against infection density of *B. cinerea* on green pepper demonstrated statistically significant variations between the various treatments (Tab. 1). The potential in the efficiency of the preparations in reducing grey mould disease reached up to 23%. Extracts from *D. denudatum, D. albicans, R. loreus, S. quinquefarium and B. trilobata* only caused a significant reduction of grey mould (15 to 23%) as compared to untreated plants with a 100% damage of foliages. Significantly higher degrees of efficiency (92%) were recorded when applying the standard fungicide dichlofluanid.

Antifungal activity of bryophyte extracts against *Phytophthora infestans* on tomatoes

The screening response of the four bryophyte extracts against *P. infestans* on tomatoes displayed various types of variability in the efficacy. Extracts from leafy liverworts exhibited a significantly better effectiveness in reduction of foliage damages of tomatoes (Fig. 3, 4) compared to other tested bryophyte extracts (Fig. 5, 6). On average, the prophylactic application of *Bazzania* extracts reduced the disease severity of late blight of tomatoes by more than 70%, when the products were applied at least 2 days before inoculation (Fig. 3). A similar higher trend in the efficiency to control late blight was recorded in the application of the other liverwort *D. albicans* (Fig. 4). Products from mosses (*S. quinquefarium* and *D. denudatum*) resulted in non persistent responses showing only an efficacy range of 25 to 70 %, whereas the disease severity level on untreated control amounted to 83.3% (Fig. 5, 6). The increasing concentration of test substances from 0.1 to 1.0% m/v showed indifferent significant efficacy responses for all test compounds against the late blight pathogen.

Discussion

The results of the *in vitro* screening generally indicated that ethanolic extracts from bryophytes contain biologically active components. Extracts from *B. trilobata, D. albicans, S. quinquefarium* and *D. denudatum* inhibited the mycelium growth of *B. cinerea* and *A. solani*

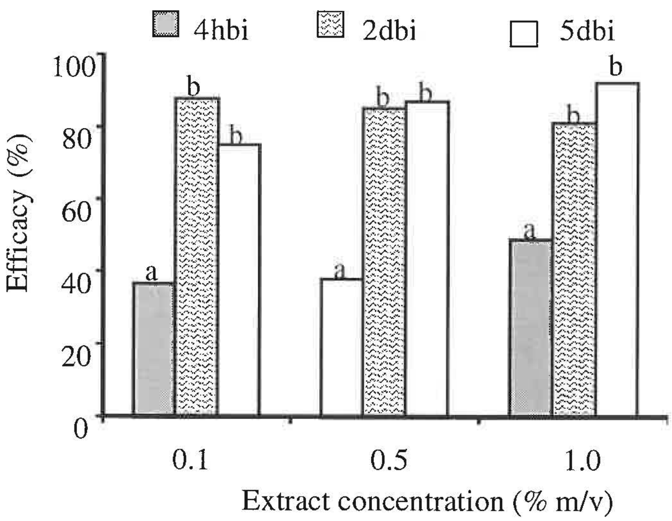

Fig. 3: The influence of extract concentrations on the efficacy of liverwort extracts from *B. trilobata* against *Phytophthora infestans* on tomatoes (a = 0.05, P =<0.001, Tukey Test).

Tab. 1: Potentials for protective application of different bryophyte extracts against infection density of *Botrytis cinerea* on green pepper.

Source of bryophyte extracts	Disease severity (%)*	Source of bryophyte extracts	Disease severity (%)*
D. denudatum	77.3 *b*	*M. undulatum*	91.7 *bcd*
D. albicans	79.0 *b*	*P. epiphylla*	92.1 *bcd*
R. loreus	84.4 *bc*	*P. fontana*	93.5 *bcd*
S. quinqefarium	84.6 *bc*	*H. splendens*	94.2 *cd*
B. trilobata	85.0 *bc*	*B. pseudotriquetrum*	94.8 *cd*
P. commune	86.5 *bcd*	*M. affine*	95.8 *cd*
M. hornum	89.0 *bcd*	*S. uncinata*	96.0 *cd*
H. splendens	90.0 *bcd*	*D. scoparium*	98.6 *cd*
S. undulata	90.0 *bcd*	Dichlofluanid 50 ppm	8.5 *a*
P. undulatum	90.2 *bcd*	Untreated control	100 *d*

*mean disease severity indicated by same letters are significantly indifferent from each other according to t- test a= 0.05, p = <0.001.

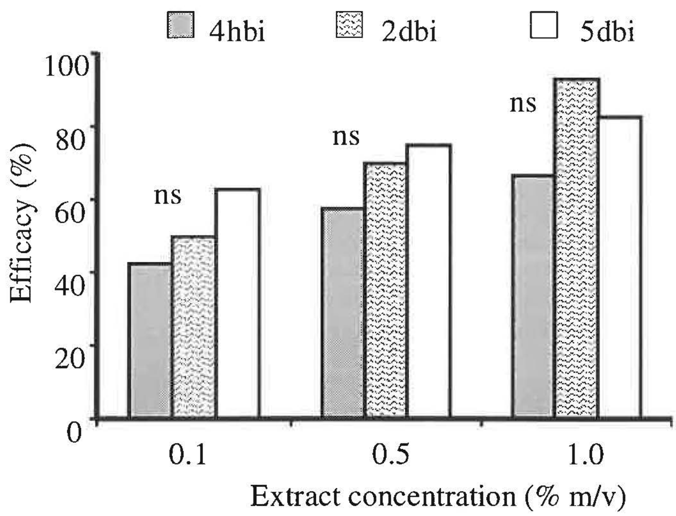

Fig. 4: The influence of extract concentrations on the efficacy of liverwort extracts from *D. albicans* against *Phytophthora infestans* on tomatoes (a = 0.05, P =<0.001, Tukey Test).

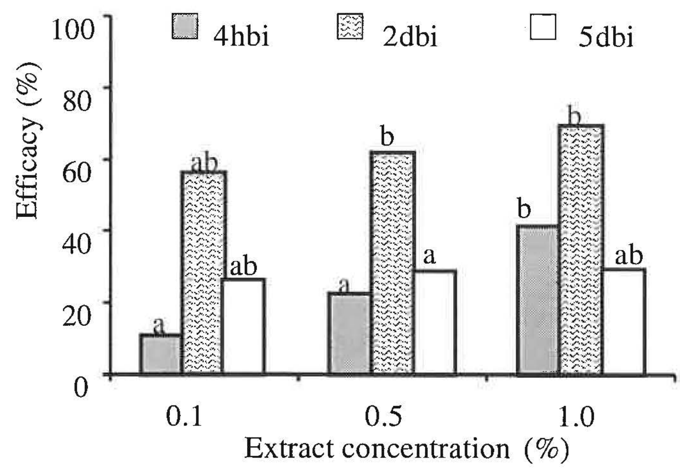

Fig. 6: The influence of extract concentrations on the efficacy of a leafy moss extracts from *D. denudatum* against *Phytophthora infestans* on tomatoes (a = 0.05, P =<0.001, Tukey Test).

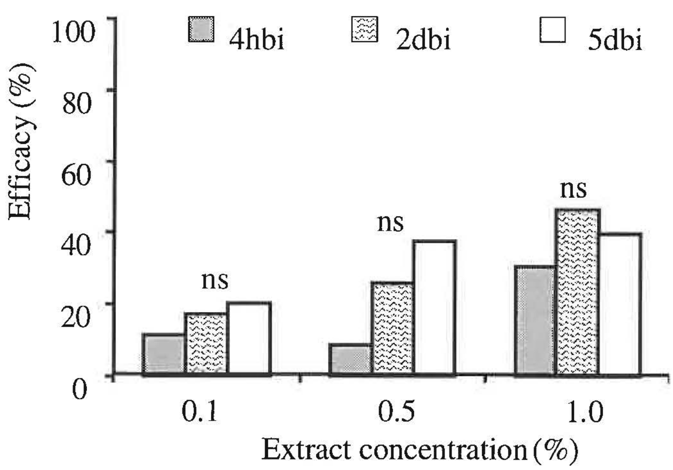

Fig. 5: The influence of extract concentrations on the efficacy of a leafy moss extracts from *S. quinquefarium* against *Phytophthora infestans* on tomatoes (a = 0.05, P =<0.001).

better than other extracts from bryophytes. Such variations might be resolved by adopting an appropriate extraction procedure for each species. *In vitro* antimicrobial activities of bryophyte extracts and the detection of differences between species in their active control behaviour have been similarly reported previously (FLOWERS, 1957; WOLTERS, 1964; MCCLEARY and WALKINGTON, 1966; DING; 1980; ASAKAWA and HEIDELBERGER, 1982).

Our observation in *in vivo* screening of extracts from different bryophytes applied 4 hours before inoculation demonstrated inefficient plant protection activities against the grey mould of green pepper. The result appeared in contrast to the high *in vitro* antifungal activity detected against *B. cinerea*. However, the low *in vivo* efficacy level of the extracts in our investigation might be evolved in the very high inoculum density of the pathogen with a 25 ml conidia suspension (1´10^6 conidia /ml) per leaf. Additionally, the tested compound might have had not a direct, but a more indirect antifungal effect, which usually requires an adequate time gap between the application of products and inoculation of the pathogen on the host. The results produced an existing lack of research information in supporting *in vivo* disease protective activities of bryophyte extracts in crop protection. In contrast, different authors reported of direct protective activities of extracts from higher plants (FRANK and MICHAEL, 1977; BLAESER et al., 1998).

A further study on antifungal activities of extracts from *B. trilobata, D. albicans, S. quinquefarium* and *D. denudatum* against late blight of tomatoes varied significantly, the extracts from the former two liverworts were more efficient to control the pathogens (MEKURIA et al.,1998a, b, 2003). An agreement could be achieved with a comparable degree of effectiveness from extracts reported from other higher plants (FRANK and MICHAEL, 1977; JEYARAJAN et al., 1986; ROVESTI et al., 1992; BLAESER et al., 1998). For instance, MOSCH and KLINGAUF (1989) and MOSCH and ZELLER (1989) reported of only 24 plant extracts from a total of 131 extract sources with bactericidal effects against the fire blight *Erwinia amylovora* of fruit trees. KLINGAUF and HERGER (1985) reported of some 73 plants as effective antifungal sources from 130 plants used for isolation of bioactive substances against powdery mildew of barley.

In our findings, the differences between species of Hepaticae and Musci in potentials and degrees of inhibition of biological activities in tested fungi may imply the existence of diverse sources of crop protection substances, though extracts from *B. trilobata* and *D. albicans* shared mostly similar patterns of antifungal effects. In general, extracts from liverworts, especially from *B. trilobata* and *D. albicans*, could effectively reduce the disease severity of *P. infestans* on tomatoes at all ranges of tested dosages when applied prophylactic. High levels of efficacy were achieved after application of the extracts 2 days before inoculation. That means both compounds might naturally have a uniform spectrum of antifungal activities, either due to unequivocally sensitivity of fungal pathogens to the product like the conventional copper fungicides, and/or incompatibility of host plants for disease development. Whether or not these findings are in agreement to the theory of systemic induced aquired resistance (SAR) activity (STEINER and SCHÖNBECK, 1997) it still requires future biological investigations. In conclusion, the current study suggests that active components of extracts from *B. trilobata* and *D. albicans* might open an ample opportunity on the further development of biotic products for ecological friendly crop protection agents.

Acknowledgements

Thanks are forwarded to Dr. P. Blaeser for his close collaboration and readiness to assist during laboratory and greenhouse investigations.

The KAAD (*Katholischer Akademischer Ausländer-Dienst*; Germany) provided a scholarship grant to the first author.

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