FUNCTIONAL AND IMMUNO-CHARACTERIZATION OF SEBACINALES: FUNGI WITH A BROAD MYCORRHIZAL POTENTIALS

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Abstract: The novel plant-growth promoting symbiotic fungi *Piriformospora indica* and *Sebacina vermifera* sensu belonging to Sebacinales (Basidiomycetes) were characterized for maize plant growth promotion and was compared with Zygomycetous arbuscular mycorrhizal fungus *G. mosseae*. Polyclonal antibodies were raised against total homogenate and cell wall/membrane fractions. Based on enzyme-linked immunosorbent assay (ELISA), immunoblotting and immunofluorescens studies of *P. indica* and *S. vermifera* sensu were found to be serologically closely related. ELISA analysis indicated that *G. mosseae, G. mosseae* 376 and *G. proliferum* were close to *P. indica*. Whereas, polyclonal antibodies raised from *S. vermifera* sensu showed affinity to *G. intraradices, G. geosporum, G. mosseae, G. proliferum* and *G. lamellosum*. This phenomenon was recorded only when the antibodies were raised from total homogenate and not so distinct from cell wall and membrane fractions. No affinity was recorded with members of Basidiomycetes and Ascomycetes. Antisera from *G. mosseae* and *G. proliferum* also significantly recognized the members of Sebacinales.

Immunoblots antiserum of *P. indica* and *S. vermifera* sensu (total homogenate) showed strong affinity with each other. Both fungi showed strong affinity with *G. mosseae, G. proliferum, C. albicans* and *S. cerevisiae*. Similarly, antiserum of *P. indica* and *S. vermifera* sensu (cell wall/membrane) showed strong affinity with each other. Again *P. indica* antiserum (cell wall/membrane) showed affinity with *G. mosseae, G. proliferum, G. lamellosum, S. vermifera* sensu, *C. albicans* and *S. cerevisiae*. Antiserum from *S. vermifera* sensu (cell wall/membrane) also recorded affinity with *G. mosseae, G. proliferum, G. lamellosum, S. vermifera* sensu and *Sebacina* spp. Further, antisera from *G. mosseae* and *G. proliferum* showed affinity with *P. indica, S. vermifera* sensu, *Sebacina* spp. and *C. albicans*. Both the antisera recognized each other strongly. Antiserum of *G. proliferum* showed greater affinity with *G. lamellosum*. The antisera of *P. indica* and *G. mosseae* showed differential binding pattern with inter- and intracellular hyphal structures and chlamydospores of each other as evident by immunofluorescens assay.

Key words: symbiotic fungus; arbuscular mycorrhizal fungus; polyclonal antibodies; ELISA; immunoblot; immunofluorescens.

A novel plant promotional root colonizing fungus, *Piriformospora indica* Verma *et al.* has been isolated from a desert soils in northwestern part of India (1). The fungus was able to grow axenically on a variety of simple and complex media. Electron microscopy and genomic studies employing the analysis of a part of 18s and 28s rRNA placed it in Hymenomycetes (Basidiomycota) (1, 2). *Sebacina vermifera* sensu (Warcup and Talbot) which was isolated from Bavaria, South Germany (3, 4) also occupied the same taxonomic position as that for *P. indica*. Based on 28s and internal transcribed spacer (ITS) data, *Neottia nidus-avis*, was found to be closely related *P. indica* and *S. vermifera* sensu (5).

P. indica colonizes the roots of a diverse range of host plants including legumes, cereals, medicinal plants and some bryophytes asymtomatically, and forms a mycorrhiza with terrestrial orchids (2, 6, 7, 8, 9, 10). These properties are normally reported for arbuscular mycorrhizal fungi. This communication reports a comparative plant promotional properties of several symbiotic fungi and their immunological characteristics.

MATERIAL AND METHODS

Photo-Mycobionts Interactions. The maize seeds were surface sterilized and germinating seedlings were transferred to earthern-ware pots. Growth was monitored for four months in an environmentally controlled green house as standard method described (2).

Fungal antigens. *P. indica* Verma *et al.*, of *S. vermifera* sensu (Warcup and Talbot) and *Sebacina* spp. were grown on aspergillus broth and agar medium (7, 12) at 30°C for 7 d (Fig. 1). Axenic cultures of *S. vermifera* sensu and *Sebacina* spp. were obtained from Dr. F. Oberwinkler, Germany. Soil cultures of *G. mosseae* (Nicol and Gerd.) Gerd. and Trappe and *G. intraradices* (Shenck and Smith) were obtained from Dr. F. Buscot, Germany. Isolated

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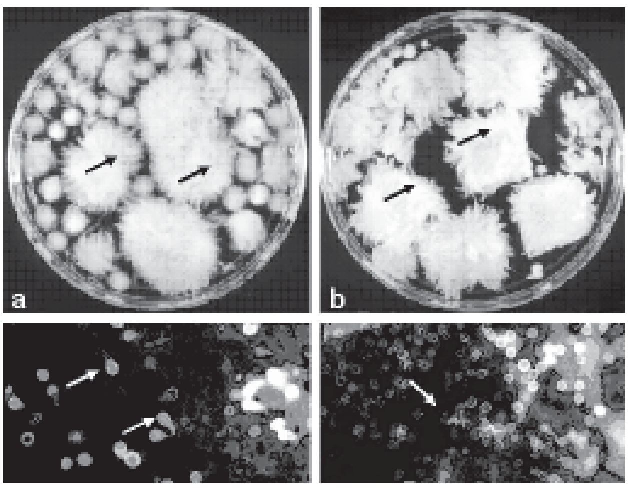
spores of *G. caledonium* (Nicol. and Gerd.) Trappe and Gerd., *G. geosporum* (Nicol. and Gerd.) Walker and *G. coronatum* (Giovannetti) were obtained from Dr. H. Dehne, Germany. Soil cultures of *G. entunicatum* (Becker and Gerd.), *G. fasciculatum* (Walker and Koske), *Gigaspora margarita* (Becker and Hall), *Scutellospora calospora* (Nicol. and Gerd.) Walker and Sanders, *S. gilmorei* (Trappe and Herd.) Walker and Sanders were obtained from Dr. A. Varma, India. Glomerales (AM-fungi) cultures were maintained on maize plants (*Zea mays* var. White). Carrot root organ cultures of *G. proliferum* (Dalpé and Declerck) and *G. lamellosum* (Dalpé, Koske and Tews) were obtained from Dr. S. Declerck, Belgium. *Lactarius torminosus* (Schaeff ex Fr.) Gray, *Pisolithus tinctorius* (Mich. ex Pers.) Coker and Couch, *Rhizopogon roseolus* (Corda) *Suillus variegatus* (Fr.) O Kunze were obtained from Dr. P. Mleczko, Poland (maintained on MMN medium, 25°C) (13). *Cunninghamella echinulata* (Thaxter) Thaxter ex Blakeslee, *Schizophyllum commune* (Fr. ex Fr.), *Rhizoctonia bataticola* (Taubenhaus) EJ Butler, *Sclerotinium solani* (Lib.) de Bary were supplied by Dr. G. Kost, Germany and Dr. A. K. Sarabhoy, India (maintained on Potato Dextrose Agar). Axenic cultures of *C. albicans* and *S. cerevisiae* were obtained from Dr. R. Prasad, India.

Antigen preparation. The spores of AM-fungi (Glomerales) were extracted from the soil by wet sieving and decanting technique (14) and were surface sterilized (15). The mycelia of all the other fungi were harvested from their respective media by centrifugation at 8000 rpm for 15 min, washed several times in 80 mM phosphate buffer (pH 7.5). The hyphae and spores were taken in a glass homogenizer and the extracts were prepared in 80 mM phosphate buffer (pH 7.5). The extent of fragmentation was judged by light microscopy. The cytoplasmic and cell wall/ membrane fractions from the hyphae of *P. indica, S. vermifera* sensu and *Sebacina* spp. were isolated following the method of Straker and Mitchell (16). Protein content of all the samples was measured by Coomassie dye method using bovine serum albumin as standard (17).

Morphological appearance of *P. indica and S. vemifera* sensu. Fungi were grown on aspergillus medium for 7 d in dark at 30±2°C. Black arrows show the origin of respective inocula. A magnified view of the fast growing corals-like out growth retrieved from Erlenmeyer flask. Incubated on GFL Shaker (Type 3019, Germany) at 144 rpm, maintained in dark at 30±2°C (arrows show the corals like out growth) (Fig. 1a,b). Fig. 1 c and d show the autofluorescent chlamydospores (Leitz Orthomat microscope, Germany).



P. indica *S. vermifera* sensu

Fig. 1: Cultivation and morphological appearance

Antibody preparation. The polyclonal antibodies were raised against soluble spore extract of *G. mosseae* (soil culture) and *G. proliferum* (root organ culture). Similarly; polyclonal antibodies were raised against fractions of *P. indica, S. vermifera* sensu and *Sebacina* spp. cell wall/ membrane and total homogenate. The standard method was followed as described by Hahn *et al.* (18). Pre-immune serum and post immune serum were processed as described in a standard method (18).

.**Enzyme linked immunosorbent assay (ELISA).** The protein extracts were tested for cross reactivities by indirect ELISA (19). A secondary antibody (anti rabbit antibody peroxidase conjugate, Sigma St Louis, MO, USA) was used at a dilution of 1:1000. Control wells were run for every treatment using pre-immune serum in place of immune serum and the sample readings (a minimum of three replicates) were adjusted accordingly. The plate was read at 405 nm using ELISA Reader (Cambridge Technology, Inc.).

Statistical treatment of the data was done by a two-way analysis of variance (ANOVA). Two values were considered to be significantly different from each other when $P < 0.05$ (20). But in some cases, P value was very significant, and was considered higher than equal to 0.001. Such antigens were considered to be cross-reactive and were used in immuno-blotting experiments.

Western blotting. For SDS-PAGE, the protein extract of *P. indica* was prepared and run according to Laemmli (21) using 4 % stacking and 10 % separating gel in a vertical tank. Electrophoretic transfer of the resolved proteins from the gel to nitrocellulose (NC) membrane (Schnieder and Schuell, Germany, pore size 0.22 µm) was performed (22, 23) at room temperature for 2 h at 200 mA. After the transfer, the blots were incubated separately with polyclonal antibodies raised against spores and hyphal fractions of *P. indica, S. vermifera* sensu, *G. mosseae* and *G. proliferum* spores. An anti-rabbit peroxidase conjugated secondary antibody (1:1000, Sigma), was used for the detection of the reactive protein bands.

Immunofluoresens. The immunolabeling of the spores and hyphae of *G. mosseae* and *P. indica*, respectively was carried out in a small eppendorf tubes. After a blocking step using nonspecific IgG from goat (10 µg/ ml) for 30 min at 30°C, the polyclonal antisera raised

Surface sterile plastic pots of ½ Kg capacity, contained autoclaved sand and soil mixture (1:3) at pH 7.0. Individual fungal inocula for *P. indica* and *S. vermifera* sensu used applied at the rate of 1 % (w/v), which contained hyphae and chlamydospores. Control pots were treated with equal amounts of autoclaved fungi. *Z. mays* var white seeds were germinated on water agar medium. Initially five germinating seedlings were placed in each pot; after a week, they were thinned to two. Growth for four months was allowed in an environmentally controlled green house maintained at 30±2°C, 16 h light/ 8 h dark, relative humidity 65 %, and light intensity 1000 Lux. The plants were nourished by Hoagland solution (11) on every alternate week for 10 weeks. To keep the soil moisture level about 70 %, the plants were irrigated with sterile tap water on every alternate day. (a) Control. (b) *G. mosseae*. (c) *S. vermifera* sensu. (d) *P. indica*.

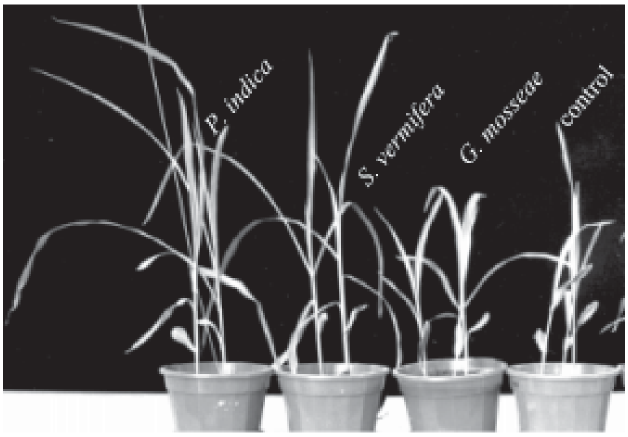


Fig. 2: Effect of symbiotic fungi on maize (*Zeya mays* white)

against total hyphal homogenate of *P. indica* and spores of *G. mosseae* were added at a dilution of 1:50 and the incubation took place overnight at 4°C. After a second blocking step as above, an anti rabbit antibody conjugated to FITC (Fluoresein isothiocynate, Sigma St. Louis, MO, USA) was added at a dilution of 1:50 and incubated for 30 min at 37°C. Between every step, the samples were washed three times with PBS (80 mM, pH 7.5), all the dilutions were done with PBS. The fluorescens microscopy was performed using Leitz Orthomat microscope, Germany (mercury short arc lamp HBO 200 W; filter excitation region: blue; exciter filter 470-490; beam split RKP 510; suppression filter LP 515).

Western blots developed against *P. indica* total homogenate antiserum. Diaminobenzaldehyde (DAB) was used as substrate for developing the blots. Wide range protein marker obtained from Sigma, St. Louis, USA was used for the molecular weight estimation of the reactive protein bands. Lane 1- *P. indica* (homologous antigen), Lane 2- *G. mosseae*, Lane 3- *G. proliferum*, Lane 4- *S. vermifera* sensu, Lane 5- *Sebacina* spp., Lane 6- *C. albicans*, Lane 7- *S. cerevisiae*, Lane 8- *S. solani* and Lane 9- *R. roseolus*.

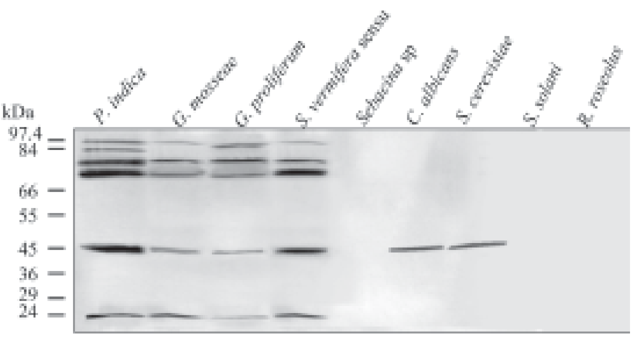


Fig. 3a: Western blot developed with *P. indica* total homogenate antiserum

RESULTS

Cultivation. Morphological appearances and characteristic features and of *P. indica* and *S. vermifera* sensu are given in Fig. 1 respectively. In the broth cultures they showed coral-like-out growth (Fig. 1a,b). Spores of the former was typically pear shaped, larger in size (16-25 µm) whereas in later it was round/oval and smaller in size (12-15 µm) (Fig. 1c,d).

Growth experiment. Fig. 2 shows a comparative aerial growth of maize plants treated with mycobionts. *P. indica* treated plants attained the maximum height (44.3±2.44 cm) followed by *S. vermifera* sensu (40.0±3.00 cm), *G. mosseae* (40.0±3.00 cm) and the lowest was recorded for controls (30.0±3.32 cm). The value for the fresh aerial biomass was 1.9±0.02, 1.7±0.34, 1.4±0.69 and 1.3±0.45 g, respectively for *P. indica, S. vermifera* sensu, *G. mosseae* and controls. Similarly, the value for the dry aerial biomass was 0.23±0.03, 0.13±0.03, 0.12±0.08 and 0.11±0.04 g, respectively for *P. indica, S. vermifera* sensu, *G. mosseae* and controls.

Immuno-characterization. Although, antisera diluted to 1:125600 recognized the respective antigens, however, the optimum reactivity at 405 nm was observed in antibody dilution of 1:800. Cross reactivity of the antisera with different classes of fungi: Zygomycetes (endomycorrhizae), Basidiomycetes (ectomycorrhizae,

Western blots developed against *P. indica* cell wall/membrane antiserum. Lane 1- *P. indica* (homologous antigen), Lane 2- *G. mosseae*, Lane 3- *G. proliferum,* Lane 4- *S. vermifera* sensu, Lane 5- *Sebacina* spp., Lane 6- *G. lamellosum,* Lane 7- *C. albicans,* Lane 8- *S. cerevisiae*, Lane 9- *S. solani* and Lane 10- *P. tinctorius.*

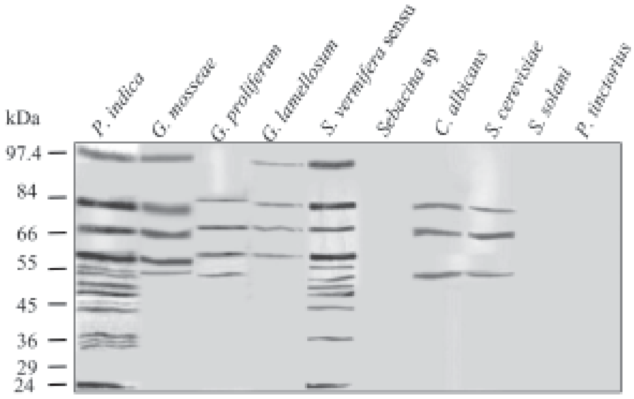


Fig. 3b: Western blot developed with *P. indica* cell wall/ membrane antiserum

non-mycorrhizae) and Ascomycetes (yeast) was carried out. The antisera recognized maximally the respective homologous antigens (Table 1). *P. indica* (total homogenate) showed highest cross reactivity with *S. vermifera* sensu and *G. mosseae.* Low cross reactivity was observed with *R. roseolus* and *P. tinctorius* (ectomycorrhiza) and the yeast. Very little activity was observed with *S. solani* (Basidiomycetes). Cross-reaction of antiserum of *P. indica* cell wall/membrane also showed a similar trend (Table 1).

Cross-reaction of antiserum *S. vermifera* sensu (total homogenate) showed highest cross reactivity with *P. indica.* Significant cross reactivity was observed with the members of Glomerales. *P. tinctorius*, *R. roseolus, S. solani* and yeast showed a very little cross reactivity (Table 2). Antiserum *S. vermifera* sensu (cell wall/membrane) showed similar activity except that there was very less cross reactivity with the members of Glomerales and Ectomycorrhizae (Table 2).

Antisera of *G. mosseae* and *G. proliferum* reacted with *P. indica*, *S. vermifera* sensu and members of

Western blots developed against *S. vermifera* sensu total homogenate antiserum. Lane 1- *P. indica*, Lane 2- *G. mosseae*, Lane 3- *G. proliferum*, Lane 4- *S. vermifera* sensu (homologous antigen), Lane 5- *Sebacina* spp., Lane 6- *G. lamellosum*, Lane 7- *C. albicans*, Lane 8- *S. cerevisiae*, Lane 9- *S. solani* and Lane 10- *R. roseolus.*

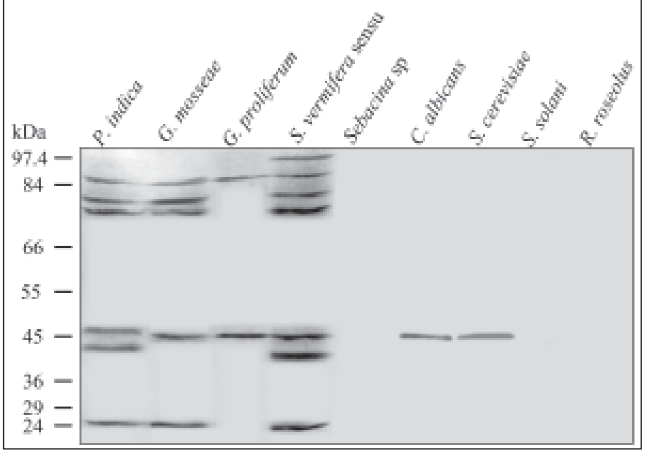


Fig. 4a: Western blot developed with *S. vermifera* sensu total homogenate antiserum

Table 1: Cross reactivity of polyclonal antisera raised against *Piriformospora indica* with different division of fungi

Antigens	**Δ $OD_{405\ nm}$ anti-*P. indica***	
	total homogenate	**cell wall/membrane**
Sebacinales		
P. indica	**0.60 ± 0.018**	**0.77 ± 0.070**
S. vermifera sensu	0.46 ± 0.038	0.29 ± 0.053
Sebacina sp	0.13 ± 0.003	0.27 ± 0.001
Glomerales		
Glomus proliferum	0.27 ± 0.006	0.02 ± 0.006
G. caledonium	0.20 ± 0.018	0.02 ± 0.006
G. mosseae	0.31 ± 0.023	0.05 ± 0.002
G. geosporum	0.23 ± 0.018	0.02 ± 0.001
G. mosseae 376	0.33 ± 0.022	0.05 ± 0.003
G. coronatum	0.16 ± 0.012	0.01 ± 0.001
G. lamellosum	0.02 ± 0.001	0.11 ± 0.009
G. intraradices	0.03 ± 0.013	0.01 ± 0.001
Basidiomycetes (Ectomycorrhiza)		
Lactarius torminosus	0.07 ± 0.003	0.04 ± 0.006
Pisolithus tinctorius	0.17 ± 0.013	0.10 ± 0.003
Rhizopogon roseolus	0.25 ± 0.036	0.07 ± 0.002
Suillus variegatus	0.03 ± 0.002	0.06 ± 0.003
Basidiomycetes (Non-mycorrhiza)		
Cunninghamella echinulata	0.04 ± 0.002	0.08 ± 0.001
Rhizoctonia bataticola	0.05 ± 0.001	0.08 ± 0.002
Schizophyllum commune	0.01 ± 0.001	0.04 ± 0.014
Sclerotina solani	0.10 ± 0.004	0.14 ± 0.001
Ascomycetes (Yeasts)		
Candida albicans	0.13 ± 0.015	0.03 ± 0.003
Saccharomyces cerevisiae	0.18 ± 0.021	0.05 ± 0.002

Readings were determined by ELISA. Optical density (OD) values are given as mean of three replicates after correction of control (Δ OD) ± standard deviation at 1:800 dilution. Statistical analysis was done by ANOVA (Bailey 1995).Observation were based on independent triplicate analysis ± standard deviation. Dilutions of 1:1600 and 1:3200 also presented the same pattern, values were lower, respectively. Data significant at $P < 0.05$. The cross reactivity with the homologous antigen is presented in bold.

kDa, with identical cross reactive bands with *G. mosseae, G. proliferum, G. lamellosum.* Yeast also showed cross-reactive bands of 55, 66 and 84 kDa.

S. vermifera sensu antiserum showed common cross-reactive bands with *P. indica* and *G. mosseae,* bands of 24, 45, 72, 77, and 84 kDa (Fig. 4A). Like-wise *G. proliferum, C. albicans* and *S. cerevisiae* also exhibited common cross-reactive band of molecular weight of 45 kDa. The immunoblot showed that *P. indica* and *S. vermifera* sensu had highest affinity to each other and followed by *G. mosseae, G. proliferum* and the yeast, respectively.

Antiserum raised from *S. vermifera* sensu (cell wall/ membrane) showed common cross reactive bands of 20.1, 29, 78, 80, 82 and 84 kDa with *P. indica* (Fig. 4B). Similarly, *G. mosseae, G. proliferum, G. lamellosum* and *Sebacina* spp. exhibited common cross-reactive band of molecular weights of 45 kDa. The immunoblot showed highest cross-reactive bands with *P. indica.* Thus,

Western blots developed against *G. mosseae* spore homogenate antiserum. Lane 1- *P. indica*, Lane 2- *G. mosseae*, Lane 3- *G. proliferum*, Lane 4- *S. vermifera* sensu, Lane 5- *Sebacina* spp., Lane 6- *C. albicans*, Lane 7- *S. solani* and Lane 8- *R. roseolus*.

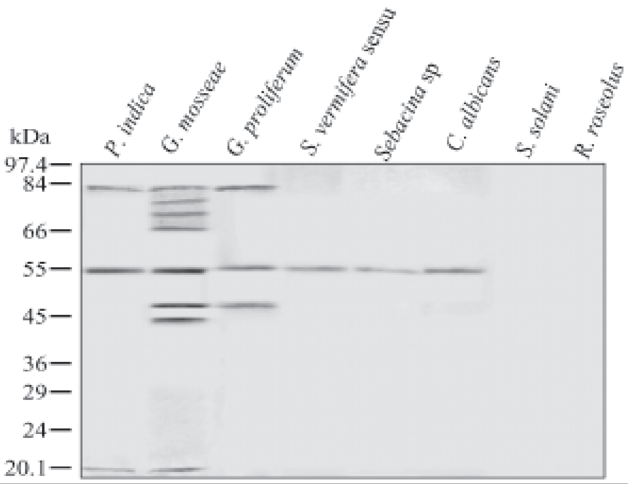


Fig. 5: Western blot developed with *G. mosseae* antiserum

Table 3: Cross reactivity of polyclonal antisera raised against Glomerales (pot/root organ cultures) with different division of fungi

Readings were determined by ELISA. Optical density (OD) values are given as mean of three replicates after correction of control (Δ OD) ± standard deviation at 1:800 dilution. Statistical analysis was done by ANOVA (Bailey 1995). Observation were based on independent triplicate analysis ± standard deviation. Dilutions of 1:1600 and 1:3200 also presented the same pattern. Data significant at $P < 0.05$. The cross reactivity with the homologous antigen is presented in bold.

Western blots developed against against *G. proliferum* spore homogenate antiserum. Lane 1- *P. indica*, Lane 2- *G. mosseae*, Lane 3- *G. proliferum*, Lane 4- *G. lamellosum*, Lane 5- *S. vermifera* sensu, Lane 6- *Sebacina* spp., Lane 7- *C. albicans* and Lane 8- *S. solani.*

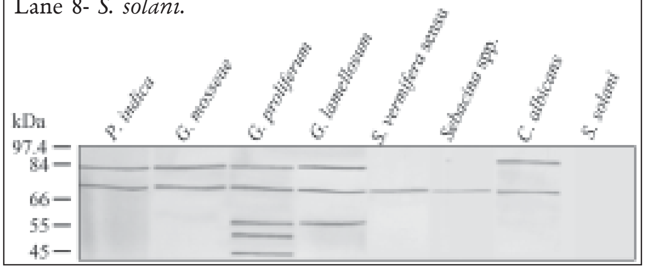


Fig. 6: Western blot developed with *G. proliferum* antiserum

Shows the mmunofluorescens behavior of treated and untreated (control) spores. Fluorescein-labeled hyphae and spores of *P. indica* and *G. mosseae* as viewed under UV reflected light microscope (Leitz Orthomat microscope, Germany). Left panel was control (a,c,e and g) and right treated with antibodies (b,d, f and h). (b) fluorescein labeled *P. indica* hyphae with antiserum raised against total hyphal homogenate. (d) Fluorescein labeled surface of *G. mosseae* spore with antiserum against soluble spore extract; note the spotty nature of the fluorescein label. (f) *P. indica* hyphae labeled with antiserum against soluble spore extract of *G. mosseae*. (h) *G. mosseae* spores labeled with antiserum raised against total hyphal homogenate of *P. indica*.

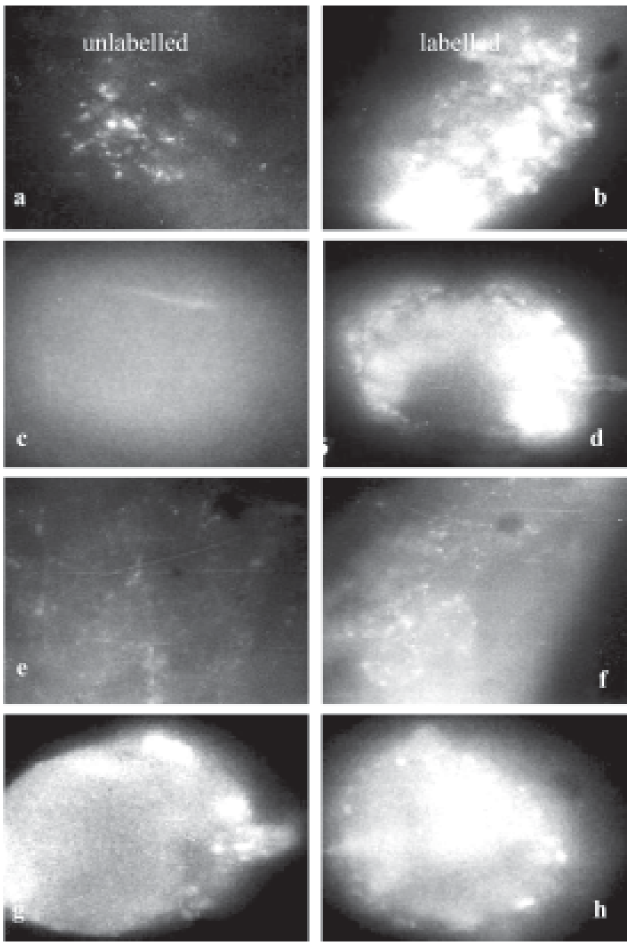


Fig. 7: Immuno-fluorescence: Fluorescein-labelled hyphae and spores of *P. indica* and *G. mosseae*

indicating much closer affinity to *S. vermifera* sensu followed by *G. mosseae, Sebacina* spp., *G. lamellosum* and *G. proliferum*, respectively.

Western blot analysis of G. *mosseae* (antiserum raised from spores screened from soil substratum) showed a common cross reactive band of 55 kDa with *P. indica, G. proliferum, S. vermifera* sensu, *Sebacina* spp. and the yeast. *P. indica* (55, 84 kDa) and *G. proliferum* (48, 55, 84 kDa) indicating closer affinity to *G. mosseae* (Fig. 5).

G. proliferum (carrot root organ culture) antiserum showed common cross reactive band of molecular weight of 72 kDa with *P. indica, G. mosseae, G. lamellosum, S. vermifera* sensu, *Sebacina* spp. and *C. albicans.* The immunoblot showed highest affinity towards the *G. lamellosum* (55, 72, 84 kDa) (root organ culture). A similar kind of cross reactivity bands of 72, 84 kDa was recorded with *P. indica, G. mosseae* and *C. albicans* (Fig. 6).

Immunofluorescens. This study further confirmed the observations made by ELISA and western blot. Fig. 7 shows a pictorial view of the interactions with members of AM fungi. In general, the unlabelled samples showed weak fluorescens (left panel). The polyclonal antibodies raised against the total fraction of *P. indica* strongly labeled the hyphal surface of homologous antigen of *P. indica* (Fig. 7b). At places where the hyphae were crushed, some cytoplasmic material of unknown nature was also labeled. In a separate experiment, cross-reactivity of the antiserum raised against *G. mosseae* spores was also checked with *P. indica* antigens. The antiserum could recognize the homologous antigen as well as the hyphae of *P. indica* (Fig. 7d,f). At places the label was observed to be spotty on the outer spore wall (Fig. 7d). When the cross-reactivity was checked with *G. mosseae* antigens, the antibody also labeled the spore surface of *G. mosseae* substantially (Fig. 7h). Non-significant labeling of cytoplasm material was observed at places where the spore was burst opened.

DISCUSSION

Weiss *et al.* (24) made comprehensive Baysian molecular phylogenetic analyses based on nuclear rDNA coding for the D1/D2 region of the large ribosomal subunit. Their results confirmed that the Sebacinaceae is a monophyletic group that occupies a basal position within the Hymenomycetidae. The family is divided into two subgoups. *Sebacina vermifera* sensu Warcup and Talbot consists of a broad complex of species, including the anamorphic *P. indica* Verma *et al.*, and possibly also mycobionts of jungermannioid and ericoid mycorrhizae. Taxonomically, they elevate Sebacinaceae to a new order, Sebacinales, and give an emendation of the Auriculariales. *P. indica* and *S. vermifera* sensu are genomically highly evolved members of Basidiomycetes (24).

Functionally, these fungi exhibited many common features typically recorded for most primitive

Zygomycetes-arbuscular mycorrhizal fungi (2, 25, 26). Among them the most important are: mutualistic interaction with diverse plants, plant growth promotion, restricted to the roots (not passing through endodermis, pericycle and vascular bundles), excessively proliferating the root system, never traversing through the plant aerial part, agent of biological hardening of tissue culture-raised plants (early callus differentiation, prolongs the life span of callus tissues), phosphorous solubilizer and transporter, and biological control of plant pathogens. In our plant growth experiments, maize plants were promoted by *P. indica* and *S. versifera* sensu, better than AM fungi. this phenomenon was also recorded for a large number of higher plants (7, 9, 27).

In an independent study (5) symbiotic germination and development of the fully myco-heterotrophic orchid *Neottia nidus-avis* were studied in contact with adult plant *Fagus sylvatica*. The distribution and identity of the fungal partner (s) of *N. nidus-avis* were investigated by internal transcribed spacer (ITS)-restriction fragment length polymorphism (RFLP) and sequence analysis of part of the 28s gene in fungal DNA extracted from adult plants from the UK and Germany, and from seedlings germinated *in situ.* Germination commenced in the spring, but only in the presence of a specific fungus, and occurred most frequently in plots containing adults of *N. nidus-avis*. Seedlings grew beat in packets in which a large number of seeds germinated. Adults and seedlings of UK origin contained the same fungal partner whose 28s sequence most closely matched *Sebacina dimitica*. Plants of German origin contained a closely related, but distinct, fungus. The results provided the first definitive chronology of the development of *N. nidus-avis* and established its critical dependence upon, and specificity for, the locally distributed *Sebacina*-like fungus that is required for germination and growth. The highly conserved 5.8s gene regions of the *Neottia* fungi were statistically similar to GenBank entries for many fungi. *S. vermifera* and *P. indica* were most similar to the two *Neottia* fungi in the 5.8s gene region (5). Earlier we have reported the strong positive interaction of *P. indica* with terrestrial orchids viz., *Dactylorhiza fuchsii, D. maculata, D. majalis, D. purpurella* (26).

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