**Short Communication**

A New Fungal Endophyte, *Helminthosporium velutinum*, Promoting Growth of a Bioalcohol Plant, Sweet Sorghum

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A new fungal isolate that grows endophytically in sweet sorghum was identified as *Helminthosporium velutinum* Link ex Ficinus & Schubert. Light-microscopy of cross-sections of colonized sweet sorghum roots showed that the intercellular, pigmented hyphae of the fungus was mostly limited to the epidermal layer and formed outer mantle-like structures. This endophyte has the ability to significantly increase sweet sorghum biomass. This is the first report of *Helminthosporium* as an endophyte and could help realize sustainable the biomass production for biofuel purposes.

**Key words:** endophyte, *Helminthosporium*, sweet sorghum

There is growing interest in biofuel production as an alternative to non-renewable energy sources (2). All renewable fuels of biological origin including fuel wood, bioalcohol and biogas, and purpose-grown crops such as sugarcane, maize, sweet sorghum, and rape seeds, represent an important source of biomass to satisfy increasing demand, raising hopes but also concerns related to competition (water and land) for food production. Assigning abandoned or non-arable lands to the production of purpose-grown biomass could help eliminate risks. Such a strategy would require growth-promoting measures.

Dark septate endophytic fungi (DSE) live in symbiosis with certain plants, improving the ability of these plants to tolerate unfavorable conditions (8, 14). When isolated in pure cultures, colonies of DSE are generally non-descript, range in color from olivaceous to brown or almost black, and often lack conidia or other taxonomically distinctive characteristics (9). Taxa that have sporulated in culture include *Phialocephala fortinii* Wang & Wilcox, *Phialocephala sphaeroides* Wilson, *Cadophora finlandica* (Wang & Wilcox) Harr & McNew (as “finlandica”), *Leptodontidium orchidicola* Sigler & Currah and *Heteroconium chaetospira* (Grove) Ellis. *P. fortinii* is the most commonly reported representative of this group. All these fungi have the ability, through mechanisms such as the transfer of nitrogen and uptake of phosphorus, to provide nutrients to host plants (6, 15, 16). The group has a broad host range encompassing approximately 600 plant species, suggesting little or no host specificity (9). *H. chaetospira* is able to colonize the roots of plants from eight families (4, 12). Members of the *Gramineae* family are no exception, but one gramineous crop, barley, is not suitable for *H. chaetospira* and *P. fortinii* which have little the ability to grow in the roots of axenically grown plants (12).

The purpose of the present study was to identify fungal endophytes which promote the growth of their host plants, particularly sweet sorghum. To obtain such endophytes from the natural environment, it is necessary to choose an appropriate method of isolation with respect to the targeted parts of plants. Therefore, surface sterilization was used to isolate fungal endophytes from living roots, leaves-stems and winter buds, known to be inhabited endophytes (1, 7, 10). We performed a pathogenicity test as a first screening for endophytes and examined any endophytism accordingly. Finally, we provide the sweet sorghum growth-promoting capability of a selected isolate and compare the isolate with known fungal endophytes under laboratory conditions.

Samples to isolate endophytic fungi were collected from October 2006 to June 2007 from a diversity of plants, mainly woody plants within the campus and Field Science Centre of Ibaraki University in Ami, Ibaraki, Japan. Entire leaves, stems and roots were collected. In the winter, mostly winter buds were collected.

Samples were first cut into approximately 5.0×5.0 mm fragments excluding any visible damage and embalmed in gauze prior to their disinfection. The disinfection process consisted of immersion in a 70% (v/v) solution of ethanol for 30 s and in a solution of sodium hypochlorite (1% available chlorine) for 2 min and three rinses in sterile distilled water. The segments were then air-dried on sterile filter paper in 9-cm Petri dishes on a clean bench overnight. For each sample, fifteen segments from each plant species were chosen at random and plated in Petri dishes (three segments per dish) with cornmeal agar medium [cornmeal infusion (Difco, Becton Dickinson [BD], Sparks, MD, USA), 1 g L⁻¹; and yeast extract (BD), 7.5 g L⁻¹] for isolating fungi. For purposes of identification, single fungal colonies were grown on cornmeal malt yeast agar medium [cornmeal infusion, 8.5 g L⁻¹; Bacto agar, 7.5 g L⁻¹; malt extract (BD), 10 g L⁻¹; and yeast extract (BD), 2 g L⁻¹] in 6-cm Petri dishes.

Fungal isolates were identified based on the morphology of sporulating structures or, in the case of non-sporulating fungi, grouped according to colony color. A total of approximately 1,200 fungal isolates were obtained from 36 plant species. The majority of these fungal isolates (56.5%) were collected from leaves-stems, whereas 25.5% were collected from winter buds, and 18.0% from roots. The DSE were...
the most prevalent with the greatest number of isolates (274 isolates, approx. 23%). Other taxa were recovered in much smaller numbers from the plants. Unidentified species of Cladosporium, Fusarium, Mortierella, Paecilomyces, Penicillium, Pythium, and Trichoderma were mostly isolated within 5 days of placing plant parts in the medium. Helminthosporium velutinum, H. chaetospira, L. orchidica, Meliniozymes variabilis, P. fortinii, DSE and a sterile isolate with white mycelia (SWM) were mostly isolated after 1 to 3 weeks.

The selection of endophytes requires the elimination of pathogens and other saprophytic fungi for future practical usage. Nineteen isolates were randomly selected as representative from each isolated fungal species, DSE, and SWM group (at least one isolate was selected for each species or group). Chinese cabbage c.v. Musou (Brassica campestris L.) (Takii Seed, Kyoto, Japan) known for its sensitive responses to fungal infections (13), was selected for the testing. The procedure was described by Narisawa et al. (13). Briefly, the fungus was grown in 6-cm Petri dishes filled with oatmeal agar medium [Oatmeal, 10 g L−1, and Bacto agar, 18 g L−1] enriched with nutrients [MgSO4·7H2O, 1 g L−1; KH2PO4, 1.5 g L−1; and NaNO3, 1 g L−1] until the plates were covered by fungal colonies. Disinfected seeds of Chinese cabbage were then sown (three per plate) on the growing colonies, and the whole set placed into sterile culture bottles (CB-1, As One, Osaka, Japan) and incubated for two to three weeks at 23°C with 18 h (light)-6 h (dark) (light: 180 µmol m−2 s−1) conditions. Symptoms were evaluated according to an index of 0 to 3 (0: no visible symptoms; 1: light yellowing; 2: yellowing and late growth; 3: wilting or death).

The results of the pathogenicity tests showed a broad range of sensitivity of the Chinese cabbage to the 19 isolates tested when both dry biomass and growth index were compared. Most ineffective isolates, once re-inoculated in axenically-grown Chinese cabbage seedlings, caused extreme yellowing of leaves and suppression of plant growth. Most of these plants died. Only one of the isolates (isolate 41-1) did not cause typical external symptoms on leaves, including etiolation. The dry biomass of the plants inoculated with 41-1 (36.9±3.5 mg) showed no significant difference with the control value (37.5±12.5 mg) (P<0.05).

Isolate 41-1 which was obtained from winter buds of Prunus lannesiana showed dark-septed mycelia, growing slowly and producing septate conidia ending with a hyaline structure (Fig. 1). The number of septates of the conidia varied from 3 to 4 with an average of 3 septates per conidium. There was also variation in the size of these conidia from 30.4 to 64.2 µm, with an average of 40.9 µm, in length, and from 4.90 to 7.69 µm, with an average of 6.12 µm, in width. These morphological characteristics strongly suggested that the isolate belongs to H. velutinum (DDBJ/GenBank Accession No. AB551948 based on the sequence of the internal transcribed spacer region of ribosomal RNA). This species, most abundant in temperate regions, is reported "on dead stems of herbaceous plants and twigs and branches of many different kinds of trees" (3) and other species of the genus are known to cause diseases such as leaf spot on oats (11) and leaf blight on rice, but not known as an endophyte. We have demonstrated for the first time the ability of H. velutinum to grow as an endophyte in Chinese cabbage without causing any symptoms of disease.

The ability of the isolate to support plant growth was investigated using sweet sorghum (Sorghum bicolor [L.] Moench) c.v. ‘FS902’ (Snow Brand Seed, Chiba, Japan). Fourteen other fungal endophytes (available from K. Narisawa) including C. finlandica (AY606313, PK34 and 608), H. chaetospira (J1HE1, BH2M and BC2HB2), P. fortinii (J2P4M4 and J2P2M2) and other unidentified DSE (YG45, T39, 309-4, 309-2, 309-8 and 312-6) collected in Canada and Japan were also tested (12, 13). Endophytes were grown on 6-cm Petri dishes filled with oatmeal agar medium. After two weeks, 1-d-old seedlings were transferred (2 seedlings per petri dish) on the growing colonies, kept in culture bottles, and incubated in the growth chamber for 28 d. Plants were harvested, oven-dried at 40°C for 48 h, and weighed for comparison with control plants (no fungal inoculation).

Of the 15 isolates tested, only H. velutinum significantly increased the biomass of sweet sorghum (P<0.05). The percentage of dry biomass increased to 7%, significantly higher than the values obtained with other fungal endophytes, including frequently reported representatives of DSE, such as C. finlandica, H. chaetospira and P. fortinii.

The roots inoculated with the fungal isolate were stained with 0.05% (w/v) cotton blue in 50% (v/v) acetic acid to examine the growth of the endophyte in host root tissues. Hyphae of the H. velutinum isolate extensively colonized the roots of sweet sorghum seedlings without causing any visible symptoms. Light-microscopy of cross-sections of colonized roots showed the intercellular, pigmented hyphae of the isolate mostly be limited to the epidermal layer (Fig. 2A). Hyphae grew along the surface of the root and formed outer mantle-like structures on/in the epidermal layer (Fig. 2A). Hyphae were rare in the outer cortical cells and middle or inner cortical cells (Fig. 2A). Conversely, no intercellular hyphae of H. velutinum extending into the inner cortical cells were present even in the vascular cylinder (Fig. 2A). No visible signs of the host reactions were seen in the root cells colonized by the fungus. In the control, most of the epidermal and cortical cells remained intact during the observation periods (Fig. 2B).

Studies have showed that mutualistic interactions with fungal endophytes can enhance the growth of plants. The root endophyte H. chaetospira, which significantly increases the biomass of Chinese cabbage due to nitrogen transfer (15), is one example. However, there is no report of a Helminthosporium species promoting an increase in biomass. This increase can be considered small when compared to the 50% increase in height achieved in cotton inoculated with the root endophytic fungus Cladorrhinum foecundissimum (6) or the 50% fresh biomass increase of the shrub, Artemisia annua L. inoculated with another root endophytic fungus, Piriformospora indica (5, 16). However, the finding reveals a sustainable way of increasing sweet sorghum biomass
without the use of chemical fertilizers. We suggest that *H. velutinum* can potentially increase sweet sorghum biomass for bioethanol production not only in the field but also in cultivated areas. Taking advantage of DSE for improving the production of selected crops and sweet sorghum would help achieve sustainable biofuel production.

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References

Endophytic Helminthosporium for Sweet Sorghum


