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Axenic culture of a vesicular-arbuscular mycorrhizal fungus

K. K. Janardhanan*, M. L. Gupta and Akhtar Husain†
Plant Pathology Division, Central Institute of Medicinal and Aromatic Plants, Post Bag No. 1, Lucknow 226 016, India
†Present address: Vice-Chancellor, Hamdard University, Hamdard Nagar, New Delhi 110 062, India

A vesicular-arbuscular mycorrhizal fungus, *Glomus aggregatum* Schenck & Smith, associated with palmarosa (*Cymbopogon martinii* var. *motia*), was cultured and maintained on a synthetic medium. Mycorrhizal association of the isolate was successfully established in callus culture, axenic plants and potted plants. The studies demonstrate for the first time that *G. aggregatum*, which is considered as an obligate symbiont, can be grown in axenic culture. The finding is a major breakthrough in mycorrhizal research and has potential application in agriculture.

MYCORRHIZAE are symbiotic associations between fungi and plants. The majority of vascular plants have mycorrhizal associations[1]. Soil fungi that penetrate roots and form morphologically distinct structures called vesicles and arbuscules within the cortex are called vesicular-arbuscular mycorrhizal (VAM) fungi. VA mycorrhizae increase the absorption of diffusion-limited nutrients, such as phosphorus, copper and zinc, and thus enhance the growth of crop plants[2]. They have also been found to play an important role in plant–water relations and thus enhance drought resistance[3]. In addition, VAM may increase resistance to certain root-infecting pathogens[4]. The beneficial effects of mycorrhizae in plant growth and their application in agriculture and forestry have been recognized in recent years. However, VAM fungi are considered as obligate symbionts and have not, so far, been grown on artificial media[5]. They are usually maintained and multiplied in pot culture[6]. Several alternatives to pot culture have been proposed—in axenic plants in agar medium[7–9], sand culture[10], solution culture[11–14] and root organ culture[15, 16]. The inability to culture VAM fungi has been the major limiting factor in their application in agriculture[17]. In this communication, we describe experiments leading to the isolation and culturing of a VAM fungus, *Glomus aggregatum*, on a synthetic medium.

*For correspondence

Methods

Palmarosa (*Cymbopogon martinii* var. *motia*) is an important essential oil-bearing plant cultivated in India. Microscopic examination of the roots of plants growing in the experimental farm revealed extensive colonization by VAM fungi. The morphology and other diagnostic features of the spores extracted from the soil indicated the presence of two species of *Glomus*, namely *Glomus aggregatum* Schenck & Smith and an undetermined species of *Glomus*[18]. Soil samples were collected and spores were extracted by wet sieving[19]. Spores of *G. aggregatum* were picked up separately under a microscope. A monoculture of *G. aggregatum* was established by inoculating palmarosa plants in steam-sterilized pots, with spores. Infected roots collected from these plants were used for the isolation of the fungus on White's medium[20] supplemented with 1 g l^{-1} yeast extract. Isolation was carried out by two steps using 1–1.5 cm root segments from six-month-old plants. Root segments surface-sterilized with sodium hypochlorite (1–2% available chlorine) were transferred aseptically to petri plates (10 cm) containing 20 ml medium. The petri plates were incubated in the dark at 25 ± 1°C. After 10 days the root segments were again transferred to fresh medium. This process was repeated twice in order to keep the root pieces moist. Each root piece was lifted from the petri plates, transferred to sterile distilled water, cut into two pieces, and aseptically placed approximately 1 cm away from the roots of axenic plants raised from surface-sterilized palmarosa seeds on White's medium in 150-ml Erlenmeyer flasks. The isolate that emerged from the root pieces was transferred, free from the parent root segments, to White's medium (supplemented with 1 g l^{-1} yeast extract) in 10-cm petri plates after 15 days of growth.

In order to verify whether the organism isolated from the roots was a VAM fungus, a number of axenic palmarosa plants raised on White's medium, callus cultures established on Murashige and Skoog (MS) medium[21] supplemented with 1 g l^{-1} yeast extract, 0.25 mg l^{-1} kinetin, 2.0 mg l^{-1}

2,4-dichlorophenoxyacetic acid (2, 4-D) and 8 g l^{-1} agar in 150-ml Erlenmeyer flasks, and axenic plants differentiated from callus cultures on MS medium devoid of 2,4-D, but containing 0.25 mg/l kinetin, 1 g l^{-1} yeast extract and 8 g l^{-1} agar, were inoculated with a 20-day-old subculture of the isolate. Thirty days after inoculation, infected callus and roots were examined for VAM association after staining with trypan blue[22]. Symbiotic nature of the isolate on callus cultures and axenic plants was tested thrice.

Further confirmation of the VAM nature of the isolate was accomplished by two glasshouse experiments. Palmarosa seedlings were raised from surface-sterilized seeds in pots filled with sandy loam soil and sterilized at 121°C for 2 h. A culture suspension of the VAM isolate was prepared in sterilized distilled water using a third subculture of the isolate on synthetic medium. One-month-old seedlings were uprooted, dipped in the culture suspension, and one seedling was transferred to each sterilized pot (15 cm) filled with sandy loam soil (pH 8.6, available phosphorus 10 ppm, organic carbon 0.12%). Uninoculated seedlings transplanted into sterilized pots served as controls. Five replications were maintained in the experiment. All pots were kept in the glasshouse and watered regularly using sterilized tap-water. Three months after inoculation, growth and biomass production were evaluated. VAM infection was also determined by microscopic examination of inoculated and uninoculated plant roots and soils. Phosphorus content of the plants was estimated[23] in order to determine if P uptake was enhanced by the cultured VAM fungus.

Establishment of the culture

Palmarosa root segments infected by *G. aggregatum* when placed on White's medium did not show any fungal growth after 30 days. However, when they were transferred to the proximity of roots of axenic plants, initiation of growth of fungal mycelium was observed from a few root segments after 20–25 days. The fungus, when transferred to White's medium (supplemented with 1 g l^{-1} yeast extract) in 10-cm petri plates, was found to be slow-growing and took nearly one month to grow into a 10-cm-diameter colony (Fig. 1, *a*). The subculture of the isolate at this stage was free from the parent root segments. The isolate was further maintained in culture by transfers to slants of White's medium supplemented with yeast extract (1 g l^{-1}) after every 30 days. Until the time of this report fifteen transfers of the fungus have already been made and, apparently, no change in its growth rate was observed. The fungus produced snow-white colonies. The mycelium was submerged in the early stages but the growth was aerial and cottony later on the surface of the medium. Microscopic examination showed aseptate, branched, hyaline mycelium. A few globose or spherical double-walled chlamydospore-like structures were observed in older cultures (Figure 1, *b*, *c*).

Inoculation experiments

When inoculated onto callus cultures the isolates produced large aggregations of fungal hyphae and spores in the callus tissue (Figure 1, *d*, *e*). The spores were mostly double-walled and showed definite similarity with those of *Glomus* spp. Similarly, examination of roots of inoculated axenic plants differentiated from callus culture (Figure 1, *f*) also showed colonization by the fungus and the presence of typical VAM spores in the cortical cells (Figure 1, *g*). The spores were pale yellow to light brown in colour, globose to subglobose, and mostly double-walled. The results of the experiments show that the fungus isolated and cultured from VAM-infected roots of palmarosa is a VA mycorrhizal fungus belonging to a species of *Glomus*. Thus, culture of a VAM fungus was successfully established on a synthetic medium.

Microscopic examination of the roots of potted plants inoculated with the cultured VAM fungus showed the presence of a large number of spores (Figure 2, *a*). Typical vesicles and numerous hyphal coils and aggregations of mycelia were also observed within the cortical cells (Figure 2, *b*, *c*). The fungus also produced a large number of extramatrical spores in inoculated soil (Figure 2, *d*). The spore morphology indicated that root colonization was produced by a pure culture of a *Glomus* sp. The spores produced within the roots of infected plants and inoculated soils were smooth, globose to subglobose, pyriform to irregular spores (17.5)–96.25–(175) µm, irregular spores (25)–110–(145) µm, light yellow to yellow-brown to orange-brown in colour, with one or two separable coloured, laminated walls, each (1)–2.5–(4) µm thick. Subtending hyphae were straight, curved occasionally, regular, up to 12.5 µm thick at spore base, otherwise (2.5)–6.25–(10) µm with (1)–1.5–(4) µm thick walls. The pore was usually open or closed by a thin, curved septum in the subtending hypha. Morphological characters showed significant similarity to *G. aggregatum*[24, 25]. The fungus was identified as *G. aggregatum* Schneck & Smith and the identification was confirmed by Dr R. E. Koske, The University of Rhode Island, Kingston, USA (personal communication). However, microscopic examination showed that the roots of uninoculated plants were devoid of mycorrhizal colonization. These findings provide further evidence that the fungus isolated from palmarosa roots and cultured on synthetic medium is a true VAM fungus.

Finally, the enhanced growth and phosphorus uptake of inoculated plants (Table 1) also establish the symbiotic nature of the fungus. Inoculated plants showed significantly higher growth and greater biomass production than uninoculated plants (Figure 2, *e*). Analysis of the plants showed marked increase in phosphorus content of inoculated plants compared to that of controls, indicating enhanced P uptake in the former. VAM association is known to increase plant growth and biomass production primarily by increasing P uptake. This characteristic behaviour of VAM association was also exhibited by the cultured VAM fungus in the present study.

Although there have been unconfirmed claims[26, 27], at

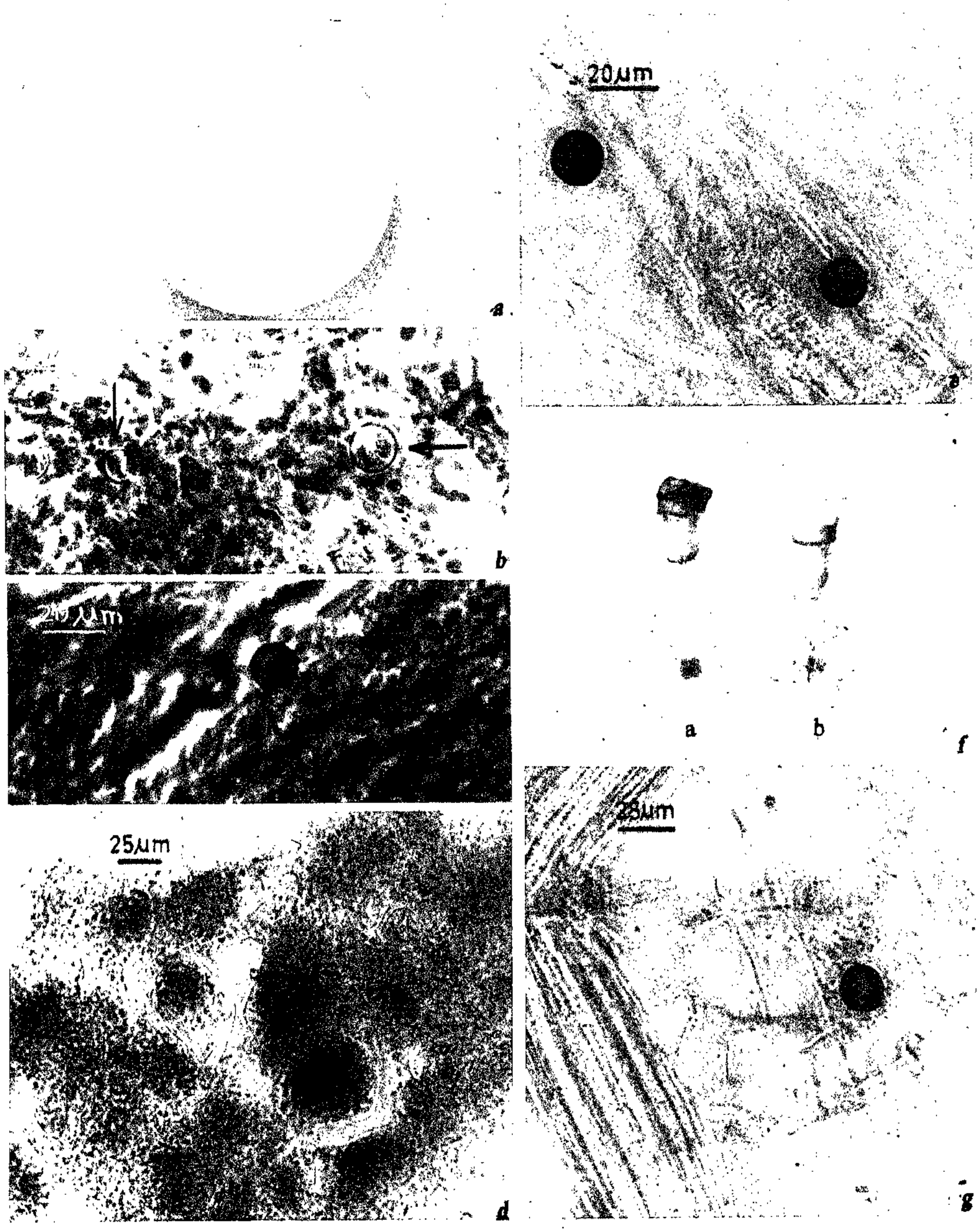

Figure 1. *a*, Culture of VAM fungus (*G. aggregatum*) growing on White's medium supplemented with 1 g l^{-1} yeast extract (30 days old). *b* and *c*, Mycelia and chlamydospore-like structures produced by the isolate in culture. Note the two-walled spore in *c*. *d*, Inoculated palmarosa callus with the isolate. Note aggregation of mycelium in callus cells. *e*, VAM spores produced by the isolate in the inoculated callus tissue of palmarosa. *f*, Axenic plants of palmarosa differentiated from callus: (i) inoculated with the VAM isolate, (ii) control. *g*, A VAM spore produced by the isolate in root of inoculated axenic plants.

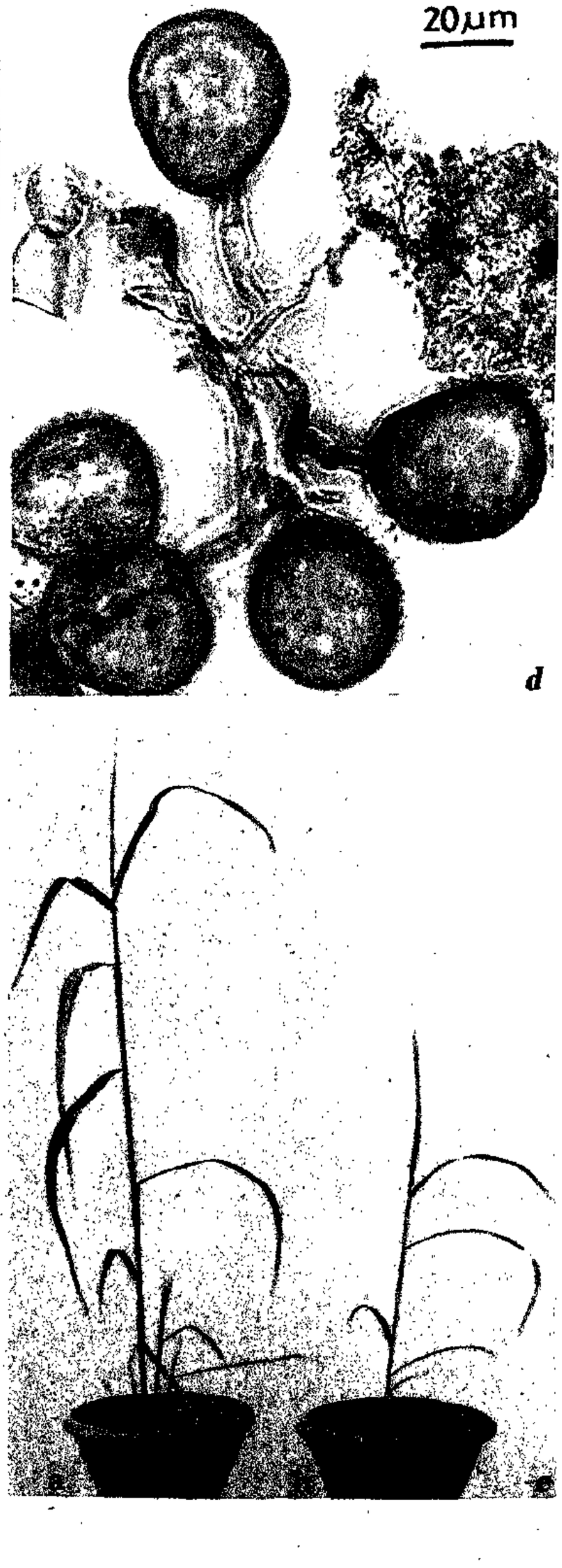

Figure 2. ***a***, Spores produced by VAM isolate on colonized roots of inoculated plants. ***b***, Production of typical vesicles in colonized roots of inoculated plants by VAM isolate. ***c***, Production of mycelial coils in the root cortical cells of inoculated plants by VAM isolate. ***d***, Extramatrical spores produced by the VAM isolate in soil. Note that the morphology of the spores is identical to that of spores of *G. aggregatum*, and the similarity of the young spore to structure of spores produced in culture. ***e***, Palmarosa seedlings: (i) inoculated with VAM isolate, (ii) uninoculated control. Seedlings are three months old. Note the better growth of inoculated seedling.

Table 1. Effect of *G. aggregatum* grown in culture on growth and phosphorus uptake of palmarosa plants.

Treatment	Height (cm)	Per cent root length colonized	Biomass production (g) Fresh	Dry	Phosphorus content (%)
Inoculated with VAM isolate	85**	90	21 **	8 **	0.143*
Control	35	0	6	2	0.122
S.E.*	7.791	—	1 .449	0 .524	0.00458

Growth was evaluated three months after inoculation. The data presented are the average of 5 replications. Phosphorus content was estimated (in triplicate) following the method of Jackson[23]. Per cent root length colonized was determined by the method of Bailey and Safir[29].
*Significance of difference: $*P < 0.05$, $** P < 0.001$.

present there are no other reports that any of the VAM fungi has been successfully cultured and maintained in axenic culture[28]. Hence we believe that this is the first report of culture of a VAM fungus on a synthetic medium. The present studies thus demonstrate for the first time that VAM fungi, which are considered to be obligate symbionts, can be grown in culture on a synthetic medium. The finding is a major breakthrough with far-reaching scientific and economic importance because of its significance for agriculture.

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