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SHORT NOTE

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Arbuscular mycorrhizas in cycads of southern India

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Abstract Root and soil samples of three potted or ground-grown cycads (*Cycas circinalis*, *C. revoluta*, *Zamia* sp.) were collected between November 1999 and June 2000 and surveyed for arbuscular mycorrhizal (AM) colonization and spore populations. AM fungi were associated with all root systems and rhizosphere samples examined. Root colonization was of a typical Arum type and AM colonization levels differed significantly between species and between potted and ground-grown cycads. Mycorrhizal colonization levels were inversely related to root hair number and length. Spores of nine morphotypes belonging to three genera (*Acaulospora*, *Glomus*, *Scutellospora*) were extracted from soil. The percentage root length colonized by AM fungi was not related to soil factors, but total AM fungal spore numbers in the rhizosphere soil were inversely related to soil nitrogen and phosphorus levels. AM fungal spore numbers in the soil were linearly related to root length colonized. The co-occurrence of septate non-mycorrhizal fungi was recorded for the first time in cycads. These observations and the relationship between plant mycorrhizal status and soil nutrients are discussed.

Keywords Cycads · Arbuscular mycorrhizas · Arum type · Arbuscule · *Glomus*

Introduction

Arbuscular mycorrhizal (AM) fungi are believed to have facilitated colonization of terrestrial environments by vascular plants in Siluro-Devonian times and mycotrophy is considered to be an ancestral condition (Cairney 2000). The cycads, a group of primitive slow-growing, woody plants which appeared in the upper Triassic, are restricted entirely to tropical and subtropical regions (Jones 1993). Cycads are unique in possessing cyanobacteria as nitrogen (N)-fixing symbionts in their corolloid roots (Zimmerman and Rosen 1992). Although AM association in N-fixing legumes and actinorrhizal trees is well documented, little is known about the mycorrhizal association in plants with cyanobacteria as N-fixing symbionts.

Ecto- and endomycorrhizal associations have been reported widely in several gymnosperms (see Smith and Smith 1997), but little information is available on the mycorrhizal status of cycads (Brundrett and Abbott 1991; Lamnot 1982). The IUCN has classified more than half of the 182 species of the order Cycadales (Stevenson and Osborne 1993) as endangered, vulnerable or rare (Gilbert 1984). Four cycad species, *Cycas circinalis* L., *C. pectinata* Griff., *C. rumphii* Miq. and *C. beddomei* Dyer occur naturally in India (Pant 1973). Of these, only *C. circinalis* occurs in southern India. In addition, *C. revoluta* Thunb., a native of Japan, and a species of *Zamia*, native to tropical and temperate regions of Africa, Australia and America, are introduced and are widely cultivated in gardens (Henry et al. 1989). In view of the paucity of information on the mycorrhizal status of cycads, the present investigation was carried out on three cycads occurring in southern India to elucidate the role of root morphology and edaphic factors on their mycorrhizal status.

Materials and methods

Eighteen root and soil samples were collected from three potted or ground-grown cycad species (*Cycas circinalis*, *C. revoluta*, *Zamia* sp.) from November 1999 through June 2000; one sample contained *C. circinalis* occurring naturally in Nilgiris of Western Ghats, southern India. To assess mycorrhizal colonization and AM fungal spores, 3-cm-diameter × 10-cm-deep (100 g) soil samples were taken at three random points between the stem and pot periphery in potted cycads. Similarly, for ground-grown cycads, the samples were collected at three random points 15 cm away from the stem around each cycad.

Root material for each sample was removed manually from the soil and washed in water to remove debris. Since root morphology is known to influence the mycorrhizal status, the length and number of root hairs per mm of root were determined. Samples of 10

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Table 1 Soil characteristics and mycorrhizal status (mean ± S.E.) of cycads

Sample no.	Species	Soil pH	Soil nutrients (mg kg^{-1})			AM colonization (%)	Spore number (per 25 g)			
			N	P	K		*Acaulospora*	*Glomus*	*Scutellospora*	Total
1.	*Cycas circinalis*[a]	7.3±0.01	1.31±0.03	0.71±0.01	7.31±0.08	53.2±1.31		20.01±5.03	3.00±1.15	23±1.53
2.	*C. circinalis*[a]	7.5±0.12	1.42±0.01	0.62±0.01	6.58±0.17	58.3±1.52		18.00±3.92		18±2.08
3.	*C. circinalis*[a]	7.5±0.08	1.31±0.02	0.95±0.01	7.39±0.21	55.1±1.04		24.67±3.35	3.33±0.67	28±2.08
4.	*C. circinalis*[a]	7.8±0.02	1.21±0.01	0.86±0.02	8.06±0.35	52.5±0.40		20.00±2.86		20±1.53
5.	*C. circinalis*[b]	8.1±0.10	2.61±0.02	1.21±0.03	12.31±0.73	62.3±1.29	13.33±2.03	7.00±1.85	3.00±1.00	24±1.15
6.	*C. circinalis*[a]	7.6±0.21	1.30±0.03	0.90±0.01	7.54±0.61	48.6±0.77		11.99±2.39		12±1.53
7.	*C. circinalis*[b]	7.5±0.18	3.15±0.09	1.50±0.03	8.95±0.83	45.2±0.66	10.00±1.73	5.00±0.58		15±1.15
8.	*C. circinalis*[b]	7.3±0.20	2.97±0.13	1.03±0.02	9.31±1.03	43.4±1.72	12.67±2.60	2.33±1.20	2.00±0.58	17±1.53
9.	*C. revoluta*[b]	8.0±0.15	3.15±0.21	1.13±0.01	11.06±1.13	48.1±1.45	6.00±1.15	4.00±0.58		10±1.00
10.	*C. revoluta*[b]	8.2±0.21	3.18±0.15	1.63±0.03	10.51±1.26	42.6±1.02	7.00±0.58	4.00±1.63		11±1.15
11.	*C. revoluta*[b]	7.9±0.30	2.91±0.18	1.90±0.01	9.79±0.82	53.4±1.45	6.67±0.67	2.33±1.28		9±1.53
12.	*C. revoluta*[a]	7.3±0.20	1.51±0.13	0.92±0.01	8.26±1.02	49.3±1.54		18.00±4.32		18±2.08
13.	*C. revoluta*[a]	7.2±0.31	1.03±0.00	0.81±0.02	7.32±0.83	36.6±1.47		7.67±3.48	7.33±1.45	15±1.53
14.	*C. revoluta*[a]	7.9±0.25	1.26±0.20	0.93±0.01	7.51±0.01	32.1±1.18		16.00±1.70		16±1.00
15.	*C. revoluta*[a]	8.1±0.41	1.31±0.06	0.99±0.02	6.38±0.81	35.3±1.05		3.67±3.13	9.33±2.19	13±1.53
16.	*Zamia* sp.[b]	7.5±0.30	3.05±0.28	2.10±0.05	10.15±0.91	33.1±100	4.00±1.00	0.67±0.67	2.33±0.67	7±1.58
17.	*Zamia* sp.[b]	7.8±0.40	3.31±0.17	1.83±0.02	9.32±.83	28.3±1.20	6.00±0.58	0.33±0.33	2.67±1.27	9±1.08
18.	*Zamia* sp.[b]	7.7±0.31	3.46±0.01	2.25±0.05	8.59±1.06	33.3±1.56	3.33±0.67	0.33±0.33	1.33±0.89	5±1.57

[a] Ground-grown; [b] Potted

tertiary roots taken 5 cm from the apex of each species were suspended in water on a microscope slide and root hair number and length were measured under a binocular microscope (Itoh and Barber 1983). Remaining roots were thoroughly washed, cut into 1- to 2-cm lengths, cleared in 2.5% KOH, acidified in 1 M HCl and stained in lactoglycerol-trypan blue (0.05%) (Muthukumar and Udaiyan 2000). Stained roots were mounted on microscope slides and root colonization levels were estimated by the magnified intersection method (McGonigle et al. 1990) using a compound microscope. Approximately 200 intercepts were examined per sample and presence or absence of fungal colonization was recorded at each intercept.

Spores were extracted from 50 g rhizosphere air-dried soil using a modified wet-sieving technique (Muthukumar and Udaiyan 2000). Spores were recovered by filtering the sieved fraction onto a filter paper. The filter paper was then spread over a glass plate and intact spores were counted according to morphologically distinct types and recorded as totals per sample under a dissecting microscope. Where intact spore types were found, slides were prepared and diagnostic features were recorded. Colour and dimensions of intact spores were assessed under the dissecting microscope using incident illumination. Spores were then mounted on microscope slides in polyvinyl-lactic acid-glycerol with or without Melzer's reagent and carefully crushed. The specimens were identified to genus using published descriptions (Schenck and Perez 1990). Although some could be identified to species, identification of field-collected spores is generally unreliable due to the lack of fine taxonomic characters or the presence of only a few spores. Therefore, the occurrence and statistical analysis of AM fungal spores was restricted to the genus level and morphologically distinct types within each genus were designated as morphotypes. For calculations, sporocarps or loose multispore groups were considered as one unit.

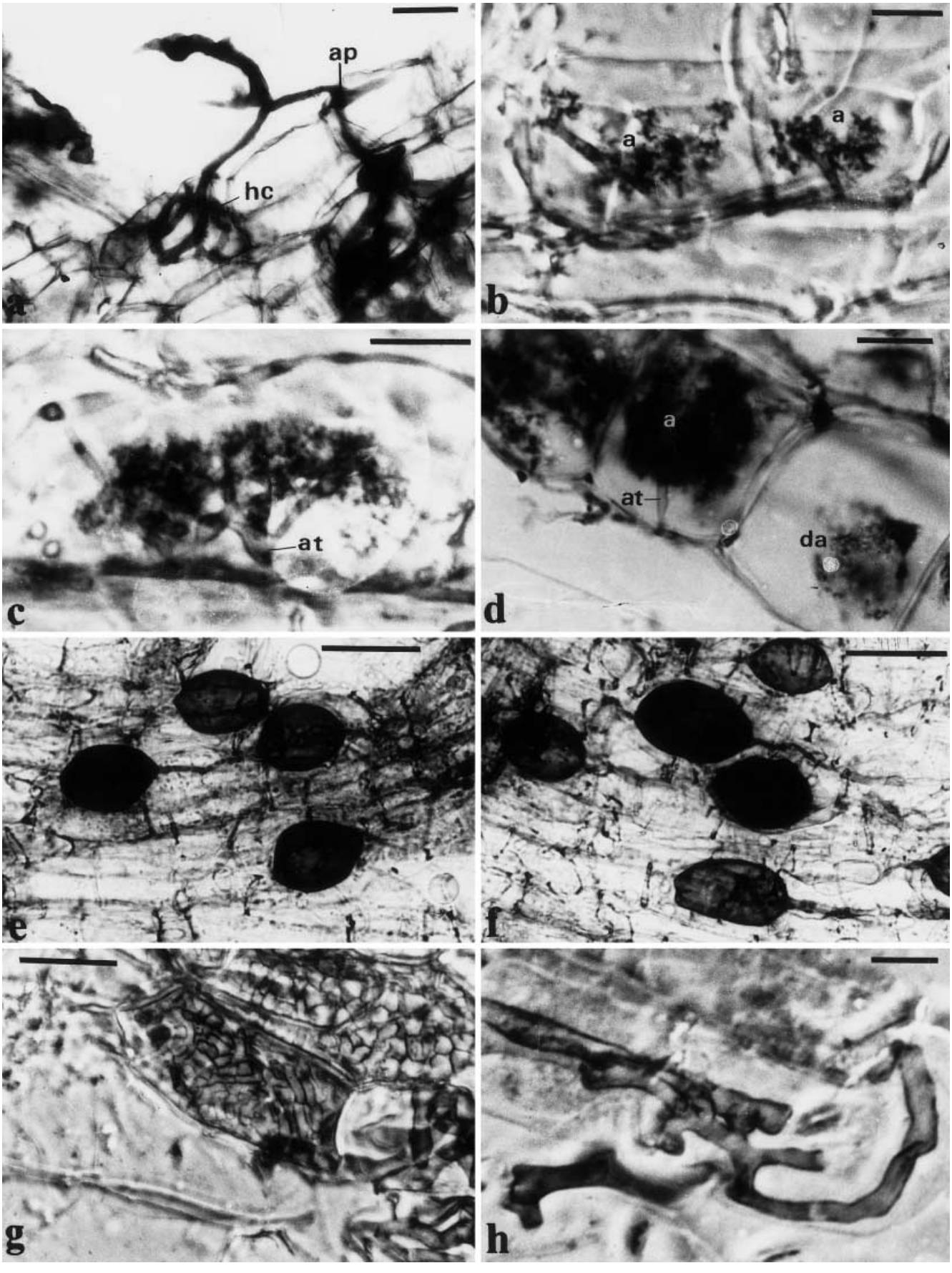
Soil pH (1:1, soil: water) was determined soon after the soil samples were brought to the laboratory. Dry soil samples were sieved to remove gravels >2 mm and weighed. Total N, P and available K were determined according to Jackson (1971).

Root colonization data were arcsine square root transformed and spore counts were logarithmically ($\log_{10}$) transformed prior to statistical analysis. Variances were compared by non-parametric ANOVA. Correlation analysis was used to assess the relationships between root colonization, total spore counts, spore densities from individual AM fungal genera and edaphic factors.

Results

The incidence and the proportion of root length colonized by AM fungi, AM fungal spore numbers and soil characters are presented in Table 1. Mean root hair density and root hair length were 17.9±1.60 mm^{-1} and 0.13± 0.009 mm, respectively, in *C. circinalis*, 26.2±1.14 mm^{-1} and 0.163±0.005 mm in *C. revoluta* and 37.8±1.14 mm^{-1} and 0.19±0.004 mm in *Zamia* sp. There were significant differences between species in root hair density ($F_{2,27}$= 132.5; P<0.001) and root hair length ($F_{2,87}$=231.2; P<0.001). Appressoria normally formed on the surface of the epidermal cells and infection hypha then penetrated into the cell. Following cell penetration, the hyphae coiled before entering an adjacent cell (Fig. 1a). Intercellular and intracellular hyphae, arbuscules and vesicles formed in the cortex in a manner typical of an Arum type (Fig. 1b–f). Intraradial hyphae were coarse (2 to >5 µm), with a smooth-walled or irregular outline with peg-like projections and 'H' junctions. Arbuscules and intercellular hyphae were the most frequently observed AM fungal structures in the colonized roots. Older roots of *C. circinalis* and *C. revoluta* were colonized by septate non-mycorrhizal fungi which formed microsclerotia-like structures within cortical cells (Fig. 1g, h).

Fig. 1a–h Colonization by mycorrhizal and non-mycorrhizal fungi of cycad roots. **a** Appressorium (*ap*) and hyphal coil formation (*hc*) in a *Cycas circinalis* root; *bar* 20 µm. **b, c** Phase-contrast images of arbuscules (*a*) in *C. circinalis* arbuscule trunk (*at*); *bars* 30 µm (b), 10 µm (**c**). **d** Mature (*a*) and degenerated (*da*) arbuscules in cortical cells of *C. revoluta*; *bar* 20 µm. **e** Vesicles in the root cortex of *Zamia* sp; *bar* 50 µm. **f** Vesicles in the root cortex of *C. revoluta*; *bar* 50 µm. **g** Septate non-mycorrhizal fungi forming microsclerotia like structures in a root of *C. circinalis*; *bar* 50 µm. **h** Hyphae of non-mycorrhizal fungi in the cortex of *C. revoluta*; *bar* 10 µm ▶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AM fungal colonization levels were moderate, ranging from 28.3% to 58.3% root length, except in one sample with 62.3% (Table 1). Mean colonization level varied significantly between species ($F_{2,51}$=34.39; P<0.001) and was highest in *C. circinalis* (52.3%). Similarly, AM fungal colonization was higher in ground-grown cycads than potted cycads ($F_{1,43}$=52.16; P<0.001). Mean colonization levels were inversely related to root hair density (r=–0.998, P<0.05, n=3) and root hair length (r=–1.000, P<0.001, n=3). AM fungal spores extracted from the rhizosphere soils were found at a frequency of 7–28 spores per 25 g of air dry soil. Overall spore number did not differ significantly between species or between ground-grown and potted cycads.

In the present survey, although AM fungal root colonization was not related to soil factors, total spore numbers were inversely related to soil N (r=–0.588, P<0.05, n=18) and P (r=–0.714, P<0.001, n=18). AM fungal spores, grouped into a total of nine morphotypes belonging to *Acaulospora*, *Glomus* and *Scutellospora*, were extracted from the indigenous soil. Spore density of *Acaulospora* morphotypes was significantly (P<0.001) and positively correlated to soil N (r=0.765, n=18) and K (r=0.664, n=18). In contrast, spore density of *Glomus* morphotypes was significantly and negatively correlated to soil N (r=–0.791, P<0.001; n=18), P (r=–0.734, P<0.001; n=18) and K (r=–0.491, P<0.05; n=18).

Discussion

The cycads observed in the present study were moderately mycotrophic, which is consistent with the reports of widespread occurrence of mycorrhizal associations in gymnosperms (Smith and Smith 1997). The inverse relationship between root morphology and AM colonization levels in *C. circinalis*, *C. revoluta*, and *Zamia* sp. is consistent with the view that the extent of root length colonized by AM fungi is proportional to the length and density of root hairs (Muthukumar et al. 1999). Since mycorrhizas function as extensions of the root system, it is possible that species with fewer and short root hairs are more dependent on AM than species with more abundant and long root hairs (Peat and Fitter 1993). In addition, symbiotic N-fixation is characterized by a high demand for P (Bethlenfalvay 1992), which might also contribute to the mycorrhizal dependence of this group.

The AM in cycads corresponds to the Arum type, characterized by intercellular hyphal growth with intracellular arbuscule formation, in contrast to the Paris type with extensive intracellular hyphal coils described in several gymnosperms (Smith and Smith 1997). Imhof and Weber (1997) suggested that Paris type mycorrhizas are more advantageous to plants than Arum type under extreme conditions. As the coiled hyphae could be a sign of major control by the plant over fungal development; they considered it to be more advanced than the Arum type (Weber et al. 1995). Smith and Smith (1997) questioned the advanced character of the Paris type because of its frequent occurrence in pteridophytes and gymnosperms. However, the presence of an Arum type in cycads, an intermediate type in Ginkgoaceae and a purely Paris type in Podocarpaceae and Taxaceae (Smith and Smith 1997) appears to suggest phylogenetic progress in mycotrophic patterns within gymnosperms.

Spore abundance of *Acaulospora* and *Glomus* morphotypes associated with the three cycads was found to be significantly influenced by soil factors, which is in accordance with studies reporting the influence of soil nutrients on AM fungal species (Brundrett et al. 1999; Cuenca and Meneses 1996). In an experiment using soil nutrient levels ranging from severely deficient to levels sufficient for maximal plant growth, Brundrett et al. (1999) found that species of *Glomus* were highly competitive at low soil fertility levels, while species of *Acaulospora* were able to adjust faster to changing soil conditions. Contrary to the reports in ecosystem studies, we demonstrated a strong relationship (r=0.655, P<0.01, n=18) between mycorrhizal root length and spore numbers in the soil. Although, it is believed generally that mycorrhiza formation and spore production are influenced by an array of host, fungal and environmental factors (Brundrett 1991), the present investigation suggests that factors influencing mycorrhiza formation can also influence sporulation.

Although non-mycorrhizal fungal endophytes have been identified in other gymnosperm families like Cupressaceae, Pinaceae and Podocarpaceae (Jumpponen and Trappe 1998), this is the first report of their occurrence in cycads. The common presence of non-mycorrhizal fungi along with AM fungi and the apparent healthiness of roots indicate that these are not pathogenic. It has been suggested that root colonizing non-mycorrhizal fungi function as mutualistics taking part in nutrient acquisition and providing a back-up system during periods when mycorrhizal fungi are inhibited by environmental conditions (see Jumpponen 2001). The actual physiological and ecological significance of their association in cycads needs to be ascertained, as does the extent to which cycads benefit from mycorrhizal associations.

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