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A global assessment using PCR techniques of mycorrhizal fungal populations colonising *Tithonia diversifolia*

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Abstract *Tithonia diversifolia* (Mexican sunflower) is a shrub commonly used as a green manure crop in Central and South America, Asia and Africa as it accumulates high levels of phosphorus and other nutrients, even in depleted soils. In root samples collected from the global distribution of *Tithonia*, we examined the degree of mycorrhizal colonisation and estimated the families of associated arbuscular mycorrhizal (AM) fungi. No colonisation by ectomycorrhizas was found. The degree of colonisation by AM fungi was on average 40%, but ranged between 0 and 80%. No mycorrhizal colonisation was found in the samples collected from the Philippines or in one each of the Rwandan and Venezuelan samples. Throughout its global distribution (Costa Rica, Nicaragua, Indonesia, Honduras, Mexico, Kenya and Rwanda), *Tithonia* forms mainly associations with *Glomaceae*. Only in one location in Nicaragua were associations with another family (*Acaulosporaceae*) found.

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Introduction

Tithonia diversifolia (Hems.) Gray, commonly known as the Mexican sunflower, is a shrub belonging to the family Asteraceae. As its common name implies, *Tithonia* is native to Mexico, but is now also common in Central and South America, Asia and Africa (Jama et al. 2000). Interest in *Tithonia* stems from its ability to accumulate high levels of essential nutrients, such as phosphorus (P), nitrogen (N), potassium (K), calcium (Ca) and magnesium (Mg) in its tissues, even in nutrient-depleted soils (Nziguheba et al. 1998; Gachengo et al. 1999; Jama et al. 2000; Cobo et al. 2002a). *Tithonia* biomass used as a green manure rapidly releases accumulated nutrients into the soil, making them available to crops (Jama et al. 2000 and references therein; Cobo et al. 2002b). The mechanisms facilitating the accumulation of these solutes to high levels in *Tithonia* tissues remain poorly understood. In other plant species, however, it has been demonstrated that mycorrhizal associations have the potential to greatly promote nutrient uptake, in particular P uptake, into plant tissues (Jakobsen et al. 1992).

The majority of terrestrial plants investigated to date form mycorrhizal associations of one type or another. Specificity between host and fungal symbiont has not been demonstrated (Smith and Read 1997) and it is widely believed that arbuscular mycorrhiza (AM) are prolific in nature and will target any suitable host root system (Bonfante and Perotto 1995). However, there is increasing evidence of competition between AM fungi colonizing roots (Pearson et al. 1993; Jacquot-Plumey et al. 2001).

Identification of AM fungi in soils has been carried out mainly by analysis of spores or, more recently, by PCR methods (Jacquot et al. 2000). However, spore number and diversity do not always correlate to root colonisation (Clapp et al. 1995; Smith and Read 1997). Identification

of AM fungi within a root system is, therefore, challenging (Dodd et al. 2000). Due to advances in molecular biology and an increased understanding of the phylogeny of AM fungi, molecular methods of fungal identification are being utilised (Redecker et al. 2000; Martin 2001; Schüßler et al. 2001a, 2001b), largely using PCR technology. Wyss and Bonfante (1993) generated genetic fingerprints using RAPD-PCR (randomly amplified polymorphic DNA) and found that banding patterns differed between species and even between different fungal isolates of the same species. Selected RAPD-PCR bands have been used to isolate specific PCR primers (Abbas et al. 1996). Ribosomal DNA (rDNA) sequences have been extensively utilised in the generation of AM-specific PCR primers. Sequence differences have been highlighted in the small subunit (SSU)/18S gene (Simon et al. 1993), the large subunit (LSU)/28S gene (Van Tuinen et al. 1998), internal transcribed spacer (ITS) regions and intergenic (IGS) regions (Egger 1995). Using these differences, 18S gene family-specific PCR primers have been developed (Simon et al. 1993; Redecker 2000). Recently, primers specific for several species of *Glomus* have also been obtained using PCR products from 25S rDNA (Turnau et al. 2001; Jacquot-Plumey et al. 2001).

In this study, the extent and nature of symbiotic fungal colonisation in *T. diversifolia,* within its global distribution, was investigated by visual and molecular means. Family-specific PCR primers described by Simon et al. (1993) were used to identify (to the family level) the AM fungi colonising *Tithonia* roots in different countries and regions.

Materials and methods

Biological materials

Root samples were collected from *T. diversifolia* populations of more than 50 individuals in Costa Rica, Nicaragua, Honduras, Mexico, Colombia, Venezuela, Ecuador, Indonesia, the Philippines, Kenya and Rwanda (Table 1). These areas cover the global distribution of *T. diversifolia.* Within each population, a soil block was excavated from around a large structural root about 50 cm from the stem of each of five plants where possible, although at some sites fewer than five plants were sampled. The blocks were stored in polythene bags for transport to the laboratory, where they were carefully washed to separate fine roots from the soil. Root samples were rinsed briefly then fixed in a 50:50 volume glycerol:ethanol mixture and transferred to Bangor, UK, for estimation of the degree of mycorrhizal infection and estimation of mycorrhizal families colonising the roots using PCR techniques.

Mycorrhizal staining and colonisation

Mycorrhizal colonisation was determined on five samples of roots from each soil block using the magnified intersection method described by McGonigle et al. (1990) after staining in trypan blue (Koske and Gemma 1989). Only soil blocks were considered to be independent replicates.

DNA extraction

Using five samples of roots from each soil block, DNA was extracted from colonised roots by grinding in liquid nitrogen followed by a CTAB buffer extraction (Doyle and Doyle 1990).

PCR amplification using fungal-specific primers

Fungal DNA was extracted from the more abundant plant DNA by PCR using fungal-specific primers (Simon et al. 1993) in 25 µl of reaction mixture consisting of Taq DNA polymerase (1.5 units), deoxynucleotides, reaction buffer, magnesium chloride and an inert red tracer dye (ReadymixREDTaq PCR Reaction Mix with $MgCl_2$; Sigma R2523), 22µl deionised water, 1 µl (2 µl in total) of each oligonucleotide primer (Operon Technologies, VhBio Ltd.) and 1 µl DNA template. The PCR mix was overlaid with mineral oil (Sigma M5904) and run on a Hybaid, Omnigene thermal cycler.

A two-step PCR reaction was used (Simon et al. 1993). Step-one primers VANS1 and VANS22 were utilised to amplify a 720-bp length of fungal-ribosomal DNA from the 18S gene region (Simon et al. 1993). The PCR product from the step-one reaction was diluted 100 times and used as DNA template in a step-two PCR reaction with the fungal primers, VAGLO, VAGIGA and VAACAU, considered as specific to the *Glomaceae*, *Gigasporaceae* and *Acaulosporaceae*, respectively (Simon et al. 1993). All family-specific fungal primers were used in conjunction with the primer VANS1 (Simon et al. 1992, 1993). Each primer pair was positively controlled prior to use, using DNA extracted from a standard root mixture collected from a field in Bangor, UK.

Table 1 Regions where *Tithonia diversifolia* roots were collected and the numbers of samples taken

Site	Code	Latitude, longitude	Number of soil blocks sampled
San Pablo, Heredia, Costa Rica	CR-HE	10° 05′N, 84° 05′W	5
Matagalpa, Nicaragua	NI-MA	12° 59′N, 85° 55′W	6
Batu, Malang, Indonesia	IND-MA	7° 59′S, 112° 43′E	2
Valle de Angeles, Honduras	HO-VA	14° 07′N, 87° 07′W	5
Union Roja, Tapachula, Mexico	ME-TA	15° 01′N, 92° 13′W	5
Simojovel to Huitiupán, Mexico	ME-SI	17° 09′N, 92° 42′W	5
Lantapan, Philippines	PH-LA	8° 02′N, 125° 02′E	3
Malava, Kenya	KE-MA	0° 27′N, 34° 51′E	5
Nyabeda, Kenya	KE-NY	0° 08′N, 34° 24′E	5
Buye, Rwanda	RW-BU	2° 36′S, 29° 44′E	5
Ngoma, Rwanda	RW-NG	2° 04′S, 29° 55′E	1
Gikongora, Rwanda	RW-GK	2° 29′S, 29° 34′E	1
Sevilla, Colombia	CO-SE	4° 10′N, 75° 50′W	1
Pescador, Colombia	CO-PE	2° 48′N, 76° 33′W	1
Oripoto, Venezuela	VEN-OR	10° 26′N, 66° 53′W	1
Tapipa, Venezuela	VEN-TA	10° 14′N, 66° 19′W	1
Nanigalito, Ecuador	ECU-NA	0° 10′S, 79° 38′W	1

PCR cycling conditions were: 35 cycles at 94°C for 60 s, 50°C for 45 s and 72°C for 60 s, followed by a single cycle at 72°C for 10 min (Simon et al. 1993). PCR products were run on a 1.3% agarose gel at −80 V for 2 h and visualised by staining with ethidium bromide.

PCR product sequencing and phylogenetic analysis

From every successful amplification, the products obtained in step-two PCR were cloned and sequenced commercially by DNASHEF Technologies, Edinburgh (www.dnashef.com). The BLAST program (Altschul et al. 1997) was used to search for sequence similarities in the published database (www.ncbi.nlm.nih.gov).

Phylogenetic analysis

rDNA sequences were aligned using Clustal W (version 1.81) and alignments were altered manually where appropriate. A neighbourhood joining tree was drawn using PHYLIP. Bootstrap analysis was carried out using SEQBOOT to generate 100 random combinations.

Results

Root colonisation by visual analysis

T. diversifolia root samples were entirely AM; no evidence of ectomycorrhizal colonisation was found. Analysis of hyphal, arbuscular and vesicular colonisation was undertaken (Fig. 1). The degree of vesicular colonisation was less than 5% in all samples (data not shown). The most abundant fungal structures were hyphae, followed by arbuscules and vesicles, which were the least abundant fungal structures in each case. Colonisation ranged from 0 to 80% root length, with a mean value of 40%. No mycorrhizal colonisation was found in the samples collected from the Philippines, and in one each of the Rwandan and Venezuelan samples. The highest colonisation value of 80% was observed in samples taken from Indonesia and Honduras.

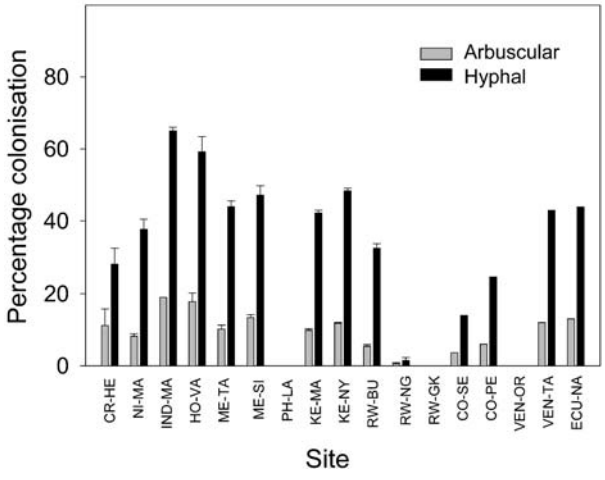

Fig. 1 Mean root length colonization by fungi in *Tithonia diversifolia* samples from Costa Rica (*CR-HE*), Nicaragua (*NI-MA*), Indonesia (*IND-MA*), Honduras (*HO-VA*), Mexico (*ME-TA* and *ME-SI*), the Philippines (*PH-LA*), Kenya (*KE-MA* and *KE-NY*), Rwanda (*RW-BU*, *RW-NG* and *RW-GK*), Colombia (*CO-SE* and *CO-PE*), Venezuela (*VEN-OR* and *VEN-TA*) and Ecuador (*ECU-NA*). Error bars represent standard error, n= 5, except for NI-MA, IND-MA, RW-GK which have n values of 6, 2 and 3, respectively. Samples CO-SE, CO-PE, VEN-OR, VEN-TA and ECU-NA all have an n value of 1

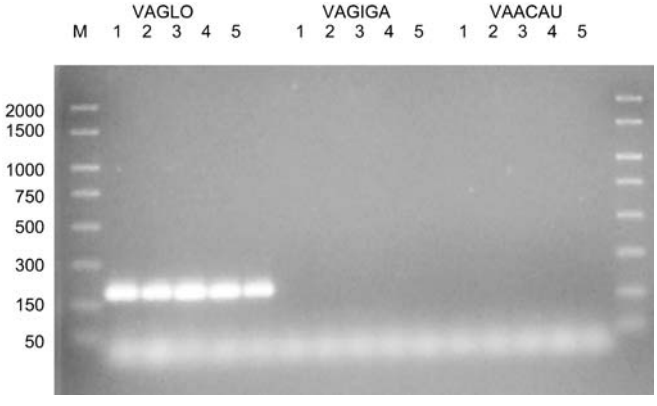

Fig. 2 The identification of mycorrhizal fungi to family level using PCR with family-specific fungal primers in *T. diversifolia* roots from Kenya, provenance KE-MA. *M* is the PCR ladder (bp), *numbers 1 to 5* refer to the sample number. The primers VAGLO, VAGIGA and VAACAU were used (in conjunction with primer VANS1). Primers VAGLO, VAGIGA and VAACAU target the fungal families *Glomaceae*, *Gigasporaceae* and *Acaulosporaceae*, respectively

Identification of AM fungi

Fungal identification using the family-specific primers described by Simon et al. (1993) generated a single PCR product 150–200 bp in length, which corresponded to the presence of a particular family of AM fungi (see example shown in Fig. 2). A summary of the PCR data obtained is given in Table 2. All five Kenyan root samples from Malava tested positive for the family *Glomaceae*.

Positive PCR results for the family *Glomaceae* were obtained in samples from Costa Rica (2 from 5 blocks), Nicaragua (6 from 6), Indonesia (2 from 2), Honduras (1 from 5), Mexico (Tapachula 2 from 5, Huitiupan 1 from 5), and Rwanda (Buye 2 from 5, Ngoma 2 from 5). No PCR product bands were generated for the family *Gigasporaceae,* and *Acaulosporaceae* was detected only in one Nicaraguan sample.

Sequence analysis of PCR products

Using the BLAST database, several high alignment scores were obtained for the PCR products generated (Table 3). Most of the sequences showed similar alignment scores with more than one probable homologue. The samples could be separated into four groups. For the first 10 samples listed, in group A (CR-HE4 to RW-NG3) the highest alignment scores were found with published sequences of *Glomus intraradices* and *Glomus vesi-*

Table 2 A summary of the fungal PCR analysis on *T. diversifolia* roots (*Yes* a positive PCR result, *N/D* none detected)

Country	Provenance	*Glomaceae*	*Gigasporaceae*	*Acaulosporaceae*
Costa Rica	CR-HE	Yes	N/D	N/D
Nicaragua	NI-MA	Yes	N/D	Yes
Indonesia	IND-MA	Yes	N/D	N/D
Honduras	HO-VA	Yes	N/D	N/D
Mexico	ME-TA	Yes	N/D	N/D
Mexico	ME-SI	Yes	N/D	N/D
Philippines	PH-LA	N/D	N/D	N/D
Kenya	KE-MA	Yes	N/D	N/D
Kenya	KE-NY	N/D	N/D	N/D
Rwanda	RW-BU	Yes	N/D	N/D
Rwanda	RW-NG	Yes	N/D	N/D
Rwanda	RW-GK	N/D	N/D	N/D
Colombia	CO-SE	N/D	N/D	N/D
Colombia	CO-PE	N/D	N/D	N/D
Venezuela	VEN-OR	N/D	N/D	N/D
Venezuela	VEN-TA	N/D	N/D	N/D
Ecuador	ECU-NA	N/D	N/D	N/D

Table 3 BLAST analysis best homologues for samples from different sites. The letter code denoting the country and site is as given in Table 1, followed by the numerical sample number from each site. Nucleotide alignment scores are indicated in parentheses as percentages

Best homologue	Group A	Best homologue	Group B	Best homologue	Group C	Best homologue	Group D
Glomus	CR-HE4 (86)	*Glomus sinosum*	RW-BU5 (94)	*Glomus caledonium*	NI-MA4 (212)	*Acaulospora laevis*	NI-MA3 (198)
intraradices	HO-VA1 (121)						
vesiculiferum	IND-MA1 (165)			*geosporum*	NI-MA6 (216)		
	IND-MA2 (100)			*fragilistratum*	RW-NG2 (147)		
	KE-MA5 (94)			*mossae*			
	ME-TA1 (139)			*verruculosum*			
	ME-SI4 (141)						
	NI-MA1 (198)			+ *Acaulospora cerradensis*	KE-MA2 (139)		
	RW-BU3 (135)						
	RW-NG3 (103)			*Scutellospora heterogama*	KE-MA3 (98)		
				Scutellospora sp.			
+ *Glomus*	CR-HE5 (98)						
fasciculatum	ME-TA4 (109)						
+ *Glomus*	NI-MA2 (149)						
proliferum							

culiferum. The DNA sequences generated from the samples from Costa Rica (CR-HE5) and Mexico (ME-TA4) had the highest alignment scores with *Glomus intraradices* and *Glomus vesiculiferum* and additionally *Glomus fasciculatum*. For the sample from Nicaragua (NI-MA2), the highest alignment score was found with 4 species: *Glomus intraradices*, *Glomus vesiculiferum*, *Glomus fasciculatum* and *Glomus proliferum.* In group C, three of the samples, two from Nicaragua (NI-MA4 and NI-MA6) and one from Rwanda (RW-NG2), showed the highest alignment with five species of *Glomus* different to group A. The two samples from Kenya (KE-MA2 and KE-MA3) showed the highest alignment with the five *Glomus* species plus two *Acaulospora* species and one *Scutellospora* species. The only sample testing positive with the VAACAU primer, which came from Nicaragua (NI-MA3), showed the highest alignment with one species, *Acaulospora laevis*.

The sequenced PCR products generated using the fungal-specific primers clustered within four groups (Fig. 3). One group (group 4) contained the sequence generated by the VAACAU primer (NI-MA3). Group 3 contained the sequences from samples from Kenya (KE-MA2 and KE-MA3) and Nicaragua (KE-MA2 and KE-MA3). Group 2 contained the sequences from samples Mexico (ME-TA1), Nicaragua (NI-MA1) and Rwanda (RW-NG2). The largest group, group 1, contained the samples from Costa Rica, Honduras, Indonesia, Kenya, Mexico, Nicaragua and Rwanda. The groups were supported by bootstrap values greater than 50. High bootstrap values were also evident within the groups.

A comparison of the species alignments in Table 3 and the tree in Fig. 2 shows a good correspondence between the alignment groups (Table 3) and the cluster groups (Fig. 2). In cluster group 3, all four sequences were generated from samples found in alignment group C.

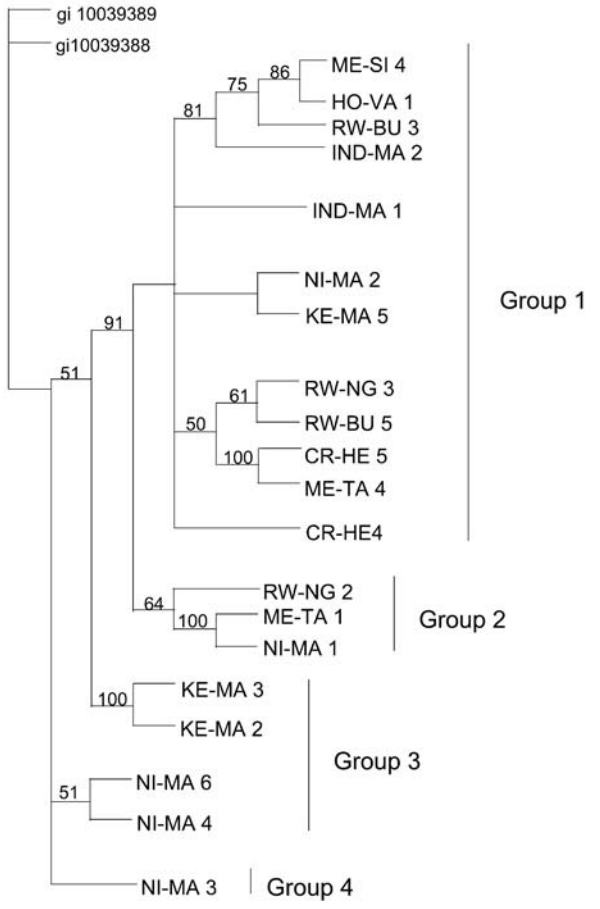

Fig. 3 Neighbourhood joining tree of PCR products from roots of *T. diversifolia*. Bootstrap values (%) of the neighbourhood joining analysis are shown above the branches (100 bootstraps). Only topologies with bootstrap support above 50% are shown. gi10039899 and gi10039388 are partial 18S RNA gene sequences from *Acaulospora* sp. (Saito et al. 2001)

Eleven out of the 12 sequences found in cluster group 1 (Fig. 2) were found in alignment group A (Table 3).

Discussion

Only AM associations were found in *T. diversifolia*. The extent of AM fungal colonisation in *Tithonia* was similar for most samples collected. The degree of hyphal colonisation was 30–50% for 9 out of 17 sample sites. This is a level similar to numerous species (McGonigle et al. 1990). In this work, family-specific primers were used to attempt to estimate the range of fungi forming mycorrhizal associations in *T. diversifolia*. If the positive amplifications using three family-specific primers are used as an indication of the presence of different genera of AM fungi, then *T. diversifolia* is globally almost only colonised by *Glomus* species. Only one sample generated a PCR product with one of the other two family-specific primers used. This result suggests an unusually high specificity for one family of AM fungi. However, it cannot be ruled out that only the most frequent fungal species representatives were most frequently detected. Generally, it is assumed that AM fungi have a broad host range, with only a few exceptions showing a degree of specificity (Giovannetti and Hepper 1985). However, these assumptions are usually based on compatibility studies under controlled conditions. Only the development of molecular identification methods has allowed studies under field conditions (Helgason et al. 1998; Turnau et al. 2001). In an investigation of the molecular diversity of AM fungi colonising *Hyacinthoides non-scripta,* Helgason et al. (1998) showed that roots were colonised by fungi generating 9 discrete clusters on a neighbour-joining tree, with representatives from the *Acaulosporaceae*, *Gigasporaceae* and *Glomaceae*. A similar range of genotypes of AM fungi was determined in roots of *Hyacinthoides non-scripta* (Merryweather and Fitter 1998).

In the past, identified AM fungal species have largely been classified according to the morphological properties of their spores (Morton and Benny 1990; Walker and Trappe 1993). Advances in molecular techniques, in particular PCR and sequencing technologies, have highlighted a complexity and diversity between fungi. This has led to modification and in some cases reorganisation of the AM classification system (Schüßler et al. 2001b). As understanding of fungal relationships at the molecular level increases, the development of family/genus/species-specific PCR primers as a tool for fungal identification in situ can be continuously modified and improved (Redecker 2000; Schüßler et al. 2001a, 2001b). The use of family-specific primers gives limited information. Furthermore, the VANS1, VAGLO, VAGIGA and VAACAU primers used in the work presented here appear to be less specific than previously thought. Schüßler et al. (2001b) reported that VANS1 is not specific for the Glomales, and that VAGLO, VAGIGA and VAACAU primers do not exhibit strict specificity for the families *Acaulosporaceae*, *Gigasporaceae* and *Glomaceae*. Even more advanced primers such as AM1 (Helgason et al. 1998) may not be entirely fungal specific (Schlüßler et al. 2001a). Schlüßler et al. (2001a) suggest that VANS1 is homologous only to a subgroup of the of AM fungi. Thus, the primers used here may have underestimated AM fungal diversity in *T. diversifolia.*

Four groups of sequences were found in a neighbour-joining tree of AM fungi in roots of *T. diversifolia*. With the exception of groups 3 and 4, these groups corresponded to four groups of species with highest homologies using BLAST analysis. The species listed by the BLAST must be viewed with great caution. In the neighbour-joining tree, the branches within a group are often supported by high bootstrap values. This may be due to different species within a group or, more likely, to variation in the targeted region of the SSU rRNA gene (Simon 1996; Helgason et al. 1998). If it is assumed that this corresponds to variation in the target gene, and similarly assuming that the groups shown in the BLAST analysis are in reality only one or two species, then *T.*

diversifolia would appear to be colonised by only a few species of AM fungi throughout its range. However, a higher AM fungal diversity was shown in one sample from Nicaragua. In field-grown *Medicago truncatula*, the majority of roots were infected by only one or two *Glomus* species (Jacquot-Plumey et al. 2001). A number of arable crops have also been shown recently to be colonised mainly by *Glomus* species (Daniell et al. 2001). The dominance by *Glomus* was suggested to be a consequence of soil disturbance. The soils of all of the sites where *Tithonia* was collected will have experienced some degree of disturbance as they are used for cultivation, but the soils were not subject to annual ploughing. Thus, although it is unknown how quickly fungal biodiversity recovers from soil disturbance, it is unlikely that soil disturbance is the sole reason for the potentially low number of species colonising *Tithonia* roots. Rather our data suggest that *T. diversifolia* shows a higher frequency of association with *Glomus* than with other families of AM fungi.

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