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Full Length Research Paper

Utilisation of organic and inorganic nitrogen sources by *Scleroderma sinnamariense* Mont. isolated from *Gnetum africanum* Welw.

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The ability of *Scleroderma sinnamariense* Mont. to utilise some organic and inorganic nitrogen (N) sources for growth was examined in axenic liquid cultures. *Pisolithus tinctorius* Pers. was included for comparison. Both fungi species produced measurable biomass on all the N sources used in this experiment. The growth of *S. sinnamariense* on ammonium-N was better than that observed on glycine and bovine serum albumin (BSA), comparable to that on glutamic acid and nitrate, but inferior to that observed on arginine, alanine and peptone. *P. tinctorius* showed optimal growth on ammonium-N, which was comparable to alanine and glutamic acid but greater than that observed on glycine, arginine, BSA, nitrate and peptone. This *Scleroderma* isolate thus has the potential to utilise nitrogen from organic substrates and this may be important for the nitrogen nutrition of its ectomycorrhizal host plants in tropical forests.

Key words: *Scleroderma sinnamariense*, organic nitrogen utilisation, ectomycorrhiza.

INTRODUCTION

Nitrogen (N) is an important element necessary for the synthesis of amino acids, proteins, nucleic acids, enzymes and energy transfer materials such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP). Shortage of N will therefore lead to the cessation of growth and reproduction in fungi. Ectomycorrhizal (EM) fungi can enhance host plant growth by improving N nutrition, and this can be achieved via an increase in absorptive surface area provided by the fungal hyphae, greater efficiency in uptake or by increasing access to various N sources which are unavailable to non-mycorrhizal plants (Finlay et al., 1992; Hogberg, 1989). Such fungi have been shown to directly exploit N contained in substrates representative of those occurring in their natural environments (Read and Perez-Moreno, 2003).

Several studies have demonstrated the ability of EM fungi in pure culture to use different N sources such as amino acids, nitrate and some proteins. Abuzinadah and Read (1988) evaluated the ability of three EM fungi, *Suillus bovines* Rouss., *Amanita muscaria* Pers. and *Hebeloma crustuliniforme* Quel. to grow on protein, acidic, basic and neutral amino acids in comparison to ammonium and nitrate. Although all fungi produced measurable biomass on all N sources tested, glutamic acid and alanine promoted the greatest growth. The growth of the fungi on these amino acids was comparable to that on ammonium-N. This finding was also upheld by Tibbett et al. (1998). Nonetheless, strain D of the *Hebeloma* sp. used showed no growth on ammonium-N, in both high and low carbon media. Lysine compared poorly as an N source for all strains of the *Hebeloma* spp. Furthermore, Prima Putra et al. (1999) found that *Scleroderma verrucosum* Pers. produced higher biomass on nitrate than on ammonium. It was also noticed that when this fungus was grown in presence of equal amounts of nitrate and ammonium, uptake rate for ammonium was considerably higher than that of nitrate. They concluded that limitation in ammonium concentration was a prerequisite for nitrate uptake,

Table 1. The different N sources and the corresponding amounts added to 1 L of basal MMN medium giving a C:N ratio of 20:1.

Nitrogen (N) source	Weight of N source added (g)	Weight of glucose added (g)
$Ca(NO_3)_2.4H_2O$ (NO_3^{2-})	0.504	3.004
$(NH_4)SO_4$ (NH_4^+)	0.284	3.004
Glutamic acid (Glu)	0.632	2.216
Arginine (Arg)	0.224	2.764
Alanine (Ala)	0.380	2.528
Glycine (Gly)	0.324	2.688
Peptone (Pep)	0.380	2.100
Bovine serum albumin (BSA)	0.340	2.620
No Nitrogen (No N)	0.000	3.004

probably because nitrate reductase was inducible by nitrate and repressed by ammonium. Meanwhile, most of the *Pisolithus* isolates studied by Anderson et al. (1999) showed a preference for ammonium over nitrate, and some showed no significant preference for either inorganic source.

In addition, glutamic acid was identified as a good source of N for most EM fungi, but four out of the 37 *Pisolithus* isolates tested by Anderson et al. (1999) were unable to utilise this amino acid. This study also demonstrated that though bovine serum albumen (BSA) did not support growth of most of the fungal isolates, prolonged maintenance on axenic culture could influence this response, indicating that N utilisation could change with prolonged storage in axenic culture. Studies by Ahmad et al. (1990), Finlay et al. (1992), Keller (1996), Rangel-Castro et al. (2002) and Sawyer et al. (2003) have all demonstrated a similar pattern of N utilisation irrespective of the time the test fungi have been maintained on axenic media. However, these studies all showed some differences in N source utilisation between and within the species of EM fungi studied and most were carried out on temperate species. Inorganic N supplied by mineralization is insufficient to meet the demand of plant and microbial communities (Kielland, 1994). Assessing growth of EM fungi on mineral N sources only, would therefore be underestimating the part that these important organisms play in nutrient cycling in ecosystems. Other authors such as Abuzinadah and Read (1988), Keller (1996), Tibbett et al. (1998), etc, have looked at the ability of some species of EM fungi to utilise single amino acids and proteins as sole N source.

There is presently no information on the growth and utilisation of N sources by *Scleroderma sinnamariense*. *S. sinnamariense* an EM fungal symbiont of *Gnetum* spp. in Cameroon, has also been reported with *Hopea* sp. and *Shorea* sp. in Malaysia, as well as with Dipterocarps in China (Sims et al., 1999; Lee et al., 1997). The aim of this experiment therefore was to determine whether *S. sinnamariense* would grow better on amino acid-N, BSA-N and peptone-N, compared to ammonium or nitrate-N. An isolate of *Pisolithus* was included in the study for comparative purposes.

MATERIALS AND METHODS

Fungal culture and assay medium

S. sinnamariense was isolated from *Gnetum africanum* EM root tips as described in Bechem (2004). An isolate of *P. tinctorius* was included in the study, and this was because it is a fungus with a widespread global distribution (Marx, 1977), easily grown *in vitro*, studied widely, has a broad host range and belongs to the same family of Sclerodermataceae as *S. sinnamariense* (Chambers and Cairney, 1999).

Modified Merlin-Norkran (MMN) culture medium (Marx, 1969) was used in this assay because the *Scleroderma* isolate had shown better growth on this medium when compared to Pachlewski agar, Hagem agar, malt extract agar or potato dextrose agar, in an earlier growth screening experiment. Most pure culture studies on mycorrhiza fungi reported in literature were carried out on MMN; hence it would be possible to compare our observations with those of other researchers in similar studies. We used MMN liquid medium excluding the N source as basal medium. Different N sources were added individually to give a C: N ratio of 20:1. The chosen N sources and corresponding amounts of added glucose are shown in Table 1.

The inorganic N sources were added prior to autoclaving to MMN basal medium and pH adjusted to 5.0 using 0.1 M NaOH or 0.1 M H_2SO_4. Autoclaving was done at 121°C for 15 min. Organic N sources were dissolved in a small portion of the basal medium, pH adjusted to 5.0 and filter sterilised with a 0.2 µM membrane filter. This was then added to the remainder of the already autoclaved basal medium. All organic N sources were supplied by Sigma®, whilst the inorganic N sources were supplied by BDH®. BSA had a molecular weight of 67000 with 16% (w/w) N content and peptone had an N content of 11% (w/w) as given by the manufacturers' product details.

Inoculation

An amount of 25 ml of each media type were poured into 9 cm diameter sterile plastic Petri dishes. Mycelial plugs of 5 mm diameter were cut from the margin of actively growing fungal colonies and used as inoculums. All treatments were replicated

Table 2. Dry weight yields (mg) after 30 days of growth of *Scleroderma* and *Pisolithus* on liquid MMN media containing different nitrogen sources.

N source	*S. sinnamariense*	*P. tinctorius*
Calcium nitrate	4.2 ± 0.1	3.0 ± 0.2
Ammonium sulphate	7.2 ± 1.1	16.8 ± 2.7
Glutamic acid	8.8 ± 1.2	16.1 ± 0.9
Arginine	12.9 ± 1.9	5.3 ± 0.2
Alanine	11.2 ± 0.6	14.0 ± 0.4
Glycine	2.8 ± 0.5	4.0 ± 0.3
Bovine Serum Albumin	3.5 ± 0.2	6.2 ± 1.2
Peptone	16.7 ± 1.4	4.3 ± 0.2
No Nitrogen	2.5 ± 0.1	3.0 ± 0.1

Values are means ± standard errors of mean. Number of replicates (n) = 3. Least significant difference at 95 % (one-way comparison) for *Scleroderma* was 3.324 and 3.825 for *Pisolithus*.

three times for each N source and for each isolate. Plates were incubated at 30°C in the dark. Harvesting was done at 0, 10, 20 and 30 days.

Growth determination

At each harvest, all fungi mycelium in each plate was collected and placed in a pre-weighed aluminium boat. The boats were placed in an oven at 80°C overnight, thereafter cooled in a desiccator and weighed. The final pH of the growth medium was determined at each harvest. The intensity of diffusible pigments (visual assessment) were noted following the rankings used in Keller (1996), and modified during this study, in which (0) = no pigmentation, (1) = trace pigmentation, (2) = weak pigmentation, (3) = moderate pigmentation, (4) = strong pigmentation and (5) = very intense pigmentation.

Statistical analysis

One-way analysis of variance was run on the data collected at each harvest time. The least significant difference at each harvest time was also determined. Two-way analysis of variance was run on data collected at 30 days so as to compare the growth of the two fungi species on the different N sources.

RESULTS

Dry weight yield

The mycelium dry weight produced by *S. sinnamariense* following growth on the inorganic and organic N sources is shown in Table 2 and Figure 1a. As shown in the table, *S. sinnamariense* produced measureable biomass on all the N sources used in the experiment. Growth of *S. sinnamariense* on ammonium-N (7.2 mg) on day 30 was comparable to that of glutamic acid (8.8 mg) and nitrate (4.2 mg), but inferior ($P<0.05$) to that on arginine (12.9 mg), alanine (11.2 mg) and peptone (16.7 mg) with the greatest difference being observed on peptone. *S. sinnamariense* also showed growth on ammonium, which was superior to that observed on glycine (2.8 mg), BSA (3.5 mg) and absence of an N source (2.5 mg) (Table 2). Overall, the growth of *Scleroderma* on ammonium was greater ($P<0.05$) than that observed on the control, whilst growth on nitrate was comparable to that of the experimental control (no N source). Growth of *S. sinnamariense* on all organic N sources assayed was greater ($P<0.05$), than that in absence of N source, with the exception of glycine and BSA, which were both comparable to growth in absence of N source. This *Scleroderma* isolate produced optimum biomass of 16.7 mg on peptone-N.

Similarly, the *Pisolithus* isolate showed growth on all the N sources used in the experiment (Table 2 and Figure 2a). *P. tinctorius* in this study grew best in medium containing ammonium-N (16.8 mg), and this was comparable to growth on alanine (14 mg) and glutamic acid-N (16.1 mg) but greater ($P<0.05$) than that observed on glycine (4.o mg), arginine (5.3 mg), BSA (6.2 mg), nitrate (3.0 mg), peptone (4.3 mg) and in the absence of an N source (3.0 mg). There was no difference in the mean dry weight of the two fungi averaged over all N sources (Table 4). However, N source, and the interaction between N source and species had highly significant effects on the dry weight yield (Table 4). Comparison of the utilisation of each N source, *P. tinctorius* grew better than *S. sinnamariense* on glutamic acid and ammonium, but *S. sinnamariense* grew better than *Pisolithus* on arginine and peptone. Generally, fungal growth in the absence of N was slow with values of 2.5 mg and 3.0 mg dry weight for *S. sinnamariense* and *P. tinctorius*, respectively. A summary of the analysis of variance is shown in Tables 3 and 4.

pH changes

The results of pH changes observed following growth on different N sources are shown in Figure 1b and 2b for *S.*

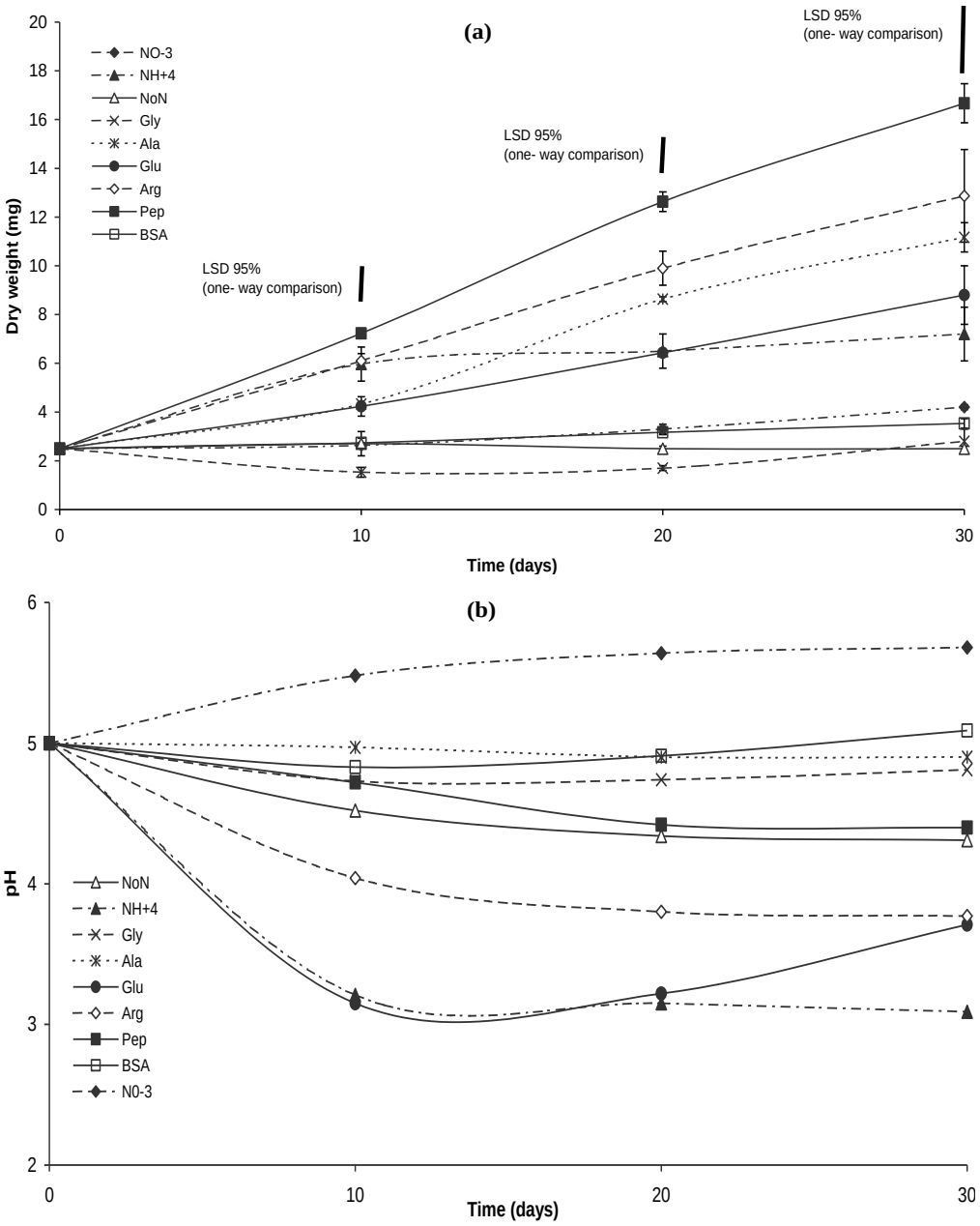

Figure 1. Dry weight yield (a) and residual pH of growth medium (b) following growth of *Scleroderma* on organic and inorganic N sources. Each point is a mean of three replicates. Vertical bars on (a) represent standard error of mean.

sinnamariense and *P. tinctorius* respectively. Both species showed a slow increase in pH on nitrate-N as opposed to the sharp fall in pH on ammonium-N. A decrease in pH with growth in medium containing alanine-N was also observed. Initially, on glycine, BSA and glutamic acid-N, there was a fall in pH during the first 10 days which was followed by a gradual increase whilst *Scleroderma* showed a decrease in pH following growth on arginine and peptone-N, *Pisolithus* showed on these N-sources an initial decrease in pH during the first 10 days followed by an increase up to day 30.

Pigmentation

Both isolates exuded pigments to varying degrees (Table 5) in the different N sources. The intensity of the exuded pigments seems to correlate to the mean dry weight observed after 30 days (Figure 3a, b). This correlation between pigmentation index and mean dry weight was

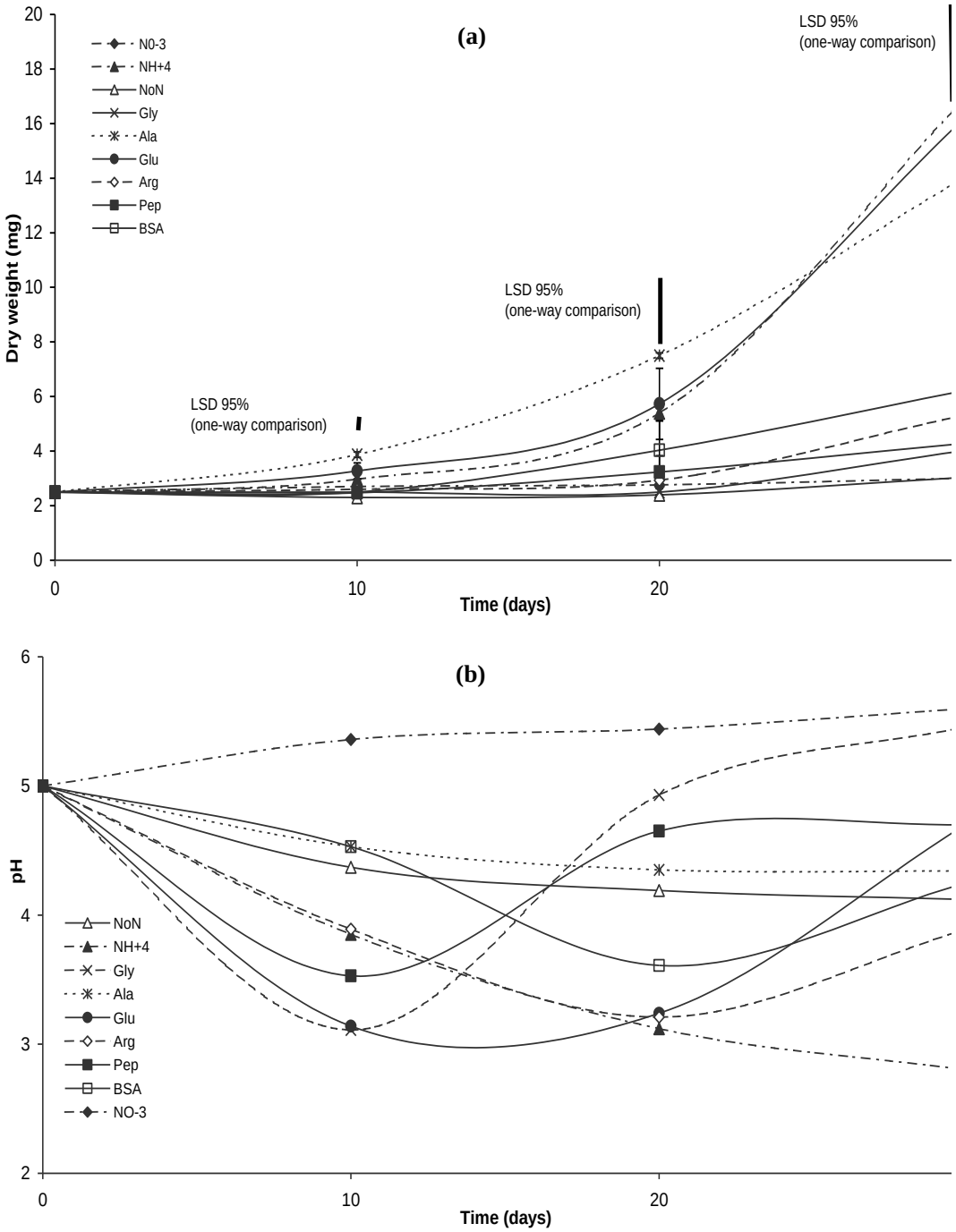

Figure 2. Dry weight yield (a) and residual pH of growth medium (b) following growth of *Pisolithus* on organic and inorganic N sources. Each point is a mean of three replicates. Vertical bars on (a) represent standard error of mean.

higher (R^2 = 0.629) for *Scleroderma* (Figure 3a) than for (R^2 = 0.347) *Pisolithus* (Figure 3b). Pigment exudation by both fungi was at its highest (5-very intense) with growth on alanine-N and was least (1-trace) in the absence of N. This was similarly observed (least) in the presence of nitrate-N. *Pisolithus* also showed very intense pigment exudation following growth in glutamic acid-N.

DISCUSSION

S. sinnamariense and *P. tinctorius* produced measurable biomass on all the nitrogen sources used in this experiment, thus indicating that they have the ability to use a wide range of nitrogen sources. Biomass produced on ammonium-N was better than that produced on

Table 3. Summary of one-way analysis of variance of the effects of nitrogen source on growth of *Scleroderma* and *Pisolithus* on MMN.

Species	Source of variation	Time (days)	Df	F value	P value
Scleroderma	Nitrogen source	10	8	21.101	0.000***
		20	8	63.908	0.000***
		30	8	20.141	0.000***
Pisolithus	Nitrogen source	10	8	6.145	0.001***
		20	8	4.765	0.003**
		30	8	20.277	0.000***

Table 4. Summary of two-way analysis of variance of the effect of fungal species and source of N on dry weight yield following 30 days of growth of *Scleroderma* and *Pisolithus* on MMN nutrient solution containing a range of N sources.

Source of variation	Df	F value	P value
Fungal species	1	0.34	0.566^{ns}
N source	8	24.37	0.000***
Fungal species*N source	8	16.06	0.000***

Table 5. Index of pigmentation following growth of *Scleroderma* and *Pisolithus* on liquid media containing different N sources.

Isolate	Nitrogen source								
	No N	NH_4^+	NO_3^-	Gly	Ala	Glu	Arg	Peptone	BSA
Pisolithus	1*	2	1	3	5	5	3	3	2
Scleroderma	1	2	1	1	5	3	2	4	1

No N, No Nitrogen; NH_4^+, ammonium; NO_3^-, nitrate; Glu, glutamic acid; Arg, arginine; Ala, alanine; Gly, glycine; BSA, bovine serum albumin. Index of pigmentation: 0, no pigmentation; 1, trace; 2, weak; 3, moderate; 4, strong; 5, very intense.

nitrate-N and based on dry weight yields; both *S. sinnamariense* and *P. tinctorius* may be described as 'non-nitrate' fungi. This observation is consistent with previously published findings on other ectomycorrhizal fungi species from other habitat types. Out of the 30 fungal isolates of diversed species examined by Keller (1996), 21 (70%) were unable to use nitrate. Lundeberg (1970) observed that 15 (42%) of the isolates used in his work were extremely poor users of nitrate, whilst Finlay et al. (1992) reported that 6 (35%) of EM fungi species studied were poor users of nitrate. Other studies like, Abuzinadah and Read (1988), Anderson et al. (1999), Rangel-Castro et al. (2002) and Sawyer et al. (2003) reported variations in the utilisation of nitrate. Our results showed a slow increase in pH following growth on nitrate-N by both isolates, which suggested a limited assimilation of nitrate too.

S. sinnamariense grew best on peptone-N, producing a biomass which was more than twice the yield observed on ammonium-N, it could be described as a 'peptone-fungus'. Nonetheless, the pattern of utilisation of these two N sources was quite different. Growth in medium containing peptone-N led to a continuous increase in dry weight with time, whilst growth in ammonium-N led to an initial increase in dry weight during the first 10 days, followed by a decrease in weight during the next 20 days. On the other hand, *P. tinctorius* grew poorly on peptone-N giving a yield which was significantly lower than growth on ammonium-N, but the pattern of utilisation was similar. Such diversity in biomass production observed on peptone-N was also reported by Keller (1996), in which all isolates grew quite well on peptone-N except two *Lactarius rufus* isolates: La 5-3 and La 5-4.

Both *S. sinnamariense* and *P. tinctorius* produced significantly poor yields on BSA in comparison to ammonium. The isolates of *Scleroderma* and *Pisolithus* used in this study could be considered as 'non-protein fungi'. In the studies carried out by Anderson et al. (1999), optimum growth on BSA-N was observed on *Pisolithus* species W37 and NSW1, whilst in the same study, *Pisolithus* species BP03, KC02, R02, R04, W14, W16, and LJ30 showed no growth on this N source.

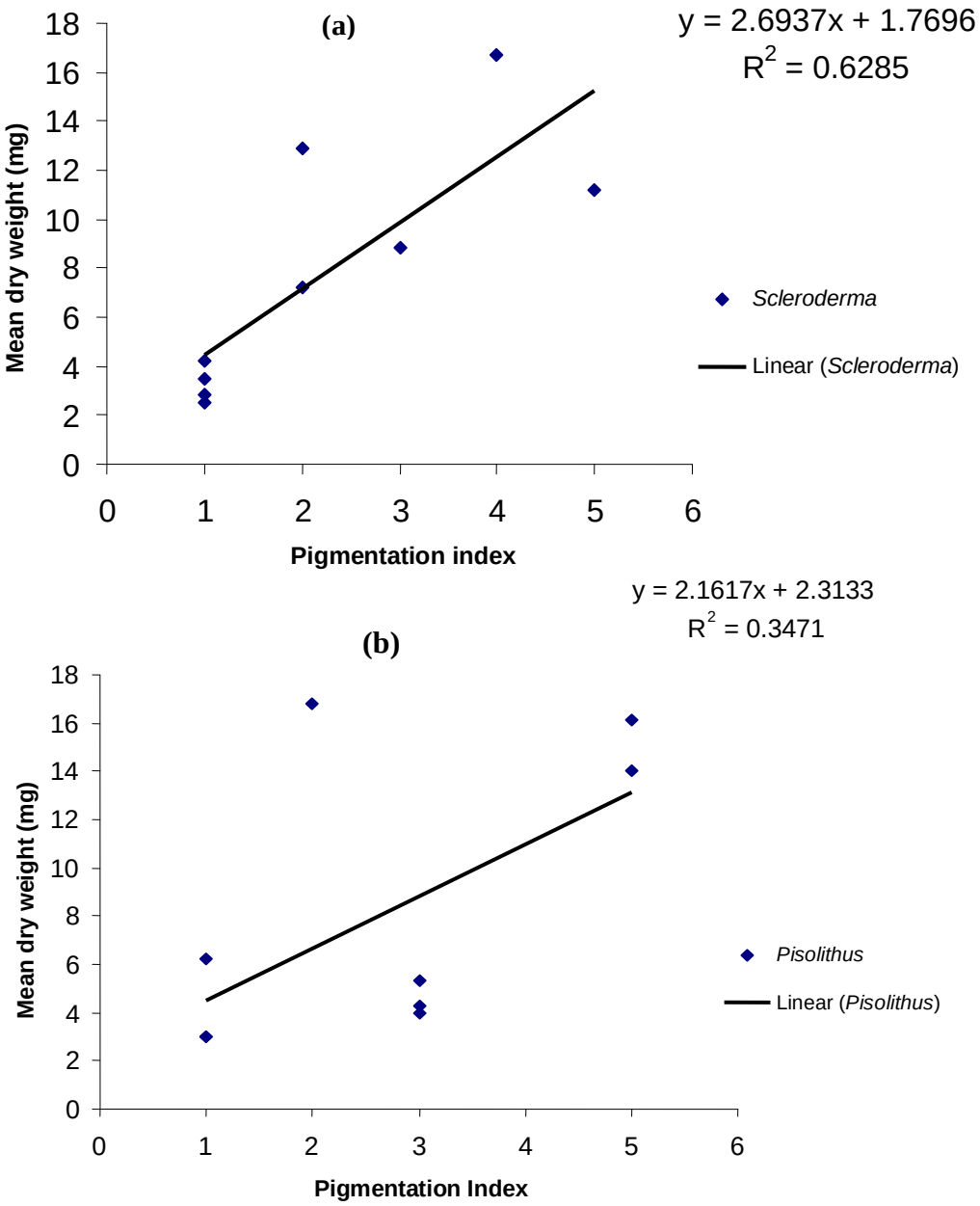

Figure 3. Scatter plot of correlation of dry weight and pigment exudation index of *Scleroderma* (a) and *Pisolithus* (b).

Abuzinadah and Read (1986) suggest that *Pisolithus* has an 'intermediate' ability to utilise proteins, but their observation was based on BSA utilisation by a single isolate. In the present study, *S. sinnamariense* could use peptone (partially hydrolysed protein) as N source over ammonium but not BSA. More experiments were necessary to explain the fact that the isolates of *Scleroderma* and *Pisolithus* used in this research could not utilise BSA, although this reaction does not necessarily imply that utilisation of other proteins is impossible. Such generalised classification of fungi, as 'protein' and 'non-protein' fungi can be misleading unless based on information derived by screening a large number of isolates and proteins.

Glycine was a poor source of N for *S. sinnamariense* and *P. tinctorius*. The biomass produced by *Scleroderma* on glycine was about 1/3 of that produced on ammonium (7.2 mg). Of the three fungi species tested by Abuzinadah and Read (1988), at a final N concentration of 60 mg/L, *S. bovinus* produced a biomass on glycine which was about 1/6 of that produced on ammonium (30.7 mg). *A. muscaria* produced a biomass (26 mg) on

glycine which was comparable to that produced on ammonium, whilst *H. crustuliniforme* produced a biomass (25.3 mg) which was about 1/3 of that on ammonium. This study supported Chalot and Brun (1998) results that glycine utilisation is limited for EM fungi. However, with other diverse observations, this idea may not hold true for all EM fungi species as demonstrated in the study of Abuzinadah and Read (1988).

Furthermore, the growth of *S. sinnamariense* in glutamic acid was comparable to that in ammonium, whilst growth in arginine and alanine was better than in ammonium, an observation quite similar to that produced by the *S. bovinus* isolate assayed by Abuzinadah and Read (1988). This study suggests that free amino acid pools in soil as well as those released directly by the action of fungal protease might help to supplement the supply of mineral N. It is important ecologically that all fungi retain their ability to utilise organic N sources in a mycorrhiza association. The study by Bajwa and Read (1985) showed that colonisation significantly increased yield and N content in plants grown on peptides as sole N sources. The results in this study agrees with their observation, with the assumption that organic N sources present in soil solution or adsorbed on to soil colloids will be assimilated by the fungi; the nitrogen obtained being subsequently transferred to the host plant.

Detailed information on the availability and residence time of organic N sources in tropical rain forest soils of Cameroon is lacking, but soluble amino acids and proteins probably represent a significant pool. *S. sinnamariense* would therefore take up these N and make them available to its plant partner when in symbiosis. The data from this study indicate that the *S. sinnamariense* isolate studied has the potential to access a range of organic sources that might be present in the soil N pool, particularly amino acids and small peptides. It also demonstrate that there is interspecific variation in the ability of tropical EM fungi to use amino acids and proteins as sole N source. *S. sinnamariense* did not show the well-developed proteolytic capacity shown by EM fungi of the North temperate and boreal regions where litter breakdown was much slower than that in tropical forest. Due to the limited information about the dynamics and pool sizes of different soil organic N sources, it is therefore necessary to interpret results from axenic culture with caution. It is also possible that not all proteolytic capability observed in axenic culture would be retained by the fungus in symbiosis.

Ammonium has always been thought of as the best N source for the growth of micro organisms. During this study, the results show that organic N sources like peptone, arginine and alanine were better N sources for *S. sinnamariense*. Similarly, the growth of *Scleroderma* and *Pisolithus* has confirmed the findings of other researchers that some fungi can use amino acids as sole nitrogen source. It also showed that some species do prefer amino acids and proteins as N sources over ammonium, which is the conventional nitrogen in most media. The increase in pH on nitrate treatment and decrease on ammonium treatment was a common phenomenon which had been observed by several workers (Abuzinadah and Read, 1988; Read and Bajwa, 1985). The irregular pattern in pH changes observed with growth of *S. sinnamariense* on amino acid treatments was also similar to that of *S. bovinus* in the study of Abuzinadah and Read (1988).

In this study, *S. sinnamariense* and *P. tinctorius* exuded pigments when grown in all nitrogen treatments. This observation is contrary to that of Keller (1996) in which *A. muscaria* and *L. rufus* exuded no pigments on all N treatments used except trace pigmentation in peptone. Nonetheless, in Keller (1996), *Suillus plorans* isolates Su 2-4, 2-19, and 2-24 exuded intense pigmentation following growth on BSA, an observation which was different from that in the current study in which *S. sinnamariense* and *P. tinctorius* exuded only weak and trace pigmentations respectively. The intensity of pigmentation produced by *Scleroderma* and *Pisolithus* when grown on peptone was comparable to that exuded by all the isolates of *S. plorans, Suillus placidus* and *Paxillus involutus* in Keller's study.

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