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A Competitive ELISA for Quantification of Protein A in Culture Medium

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Keywords: protein A, competitive ELISA, IgG, *Staphylococcus aureus*

ABSTRAK

Satu asai imunterjerap enzim berangkai secara bertanding untuk pengukuran protin A yang dihasilkan oleh Staphylococcus aureus *A676 (rintang terhadap metisilin) adalah berasaskan kepada pertandingan untuk terikat kepada molikul IgG arnab antara protin A yang ditandakan dengan enzim fosfatase beralkali dan protin A yang tidak bertanda. Kepekatan IgG yang optima bagi tujuan adalah diantara 2 dan 4 µg/ml. Masa pengeraman yang optima untuk pembentukan warna dengan penggunaan substrat ρ-nitrofenol fostat adalah diantara 20 dan 30 minit. Kepekatan protin A yang terendah dapat diukur dengan menggunakan asai imunterjarap enzim berangkai secara bertanding yang telah dioptimakan adalah 20 ng/ml dan kepekatan maxima adalah 2 µg/ml. Jumlah protin A yang dihasilkan didalam medium infusi otak-hati telah bertambah secara eksponen ketika fasa log pertumbuhan sel dan mencecah kepekatan maksima pada of 22.5 µg/ml selepas pengkulturan selama 15 jam.*

ABSTRACT

A competitive enzyme-linked immunosorbent assay (ELISA) for quantification of protein A produced by Staphylococcus aureus *A676 (a methicillin-resistant strain) was based on competitive binding to rabbit IgG molecules between alkaline phosphatase-labelled protein A and unlabelled protein A. The optimum IgG concentration required for coating was 2-4 µg/ml. The optimum incubation time for colour development using (ρ-nitrophenol phosphate substrate was 20-30 min. The lowest protein A concentration that could be measured using the optimized competitive ELISA was 20 ng/ml, and the maximum 2 µg/ml. The amount of protein A produced in brain-heart infusion medium increased exponentially during log phase of cell growth, reaching a maximum concentration of 22.5 µg/ml after 15 h cultivation.*

INTRODUCTION

Protein A is a 42 kDA polypeptide protein produced by most *Staphylococcus aureus* strains (Lind *et al.* 1970; Hjelm *et al.* 1972). This protein is capable of binding to human immunoglobulin and of forming precipitin lines in gel diffusion serological test (Lofkvist and Sjoquist 1962). Protein A has four homologous binding sites for Fc receptors; each site consists of approximately 60 amino acid residues. These binding sites are present at the N-terminus, which does not bind to the peptidoglycan of the *S. aureus* cell wall (Sjodahl 1977). However, due to the structural configuration only two binding sites can bind simultaneously to the complementary receptor sites (Langone *et al.* 1978).

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Protein A reacts mainly with the Fc receptor of IgG (Forsgen and Sjoquist 1969). Among IgG subclasses, IgG_1, IgG_2 and IgG_4 have a high affinity to protein A, but IgG_3 does not (Kronvall and Frommel 1970; Arkerst *et al.* 1974). Other immunoglobulin classes to which protein A binds are IgA, IgM and polyclonal IgE (Heremans 1974; Harboe and Folling 1974; Brunda *et al.* 1977). Due to their high binding affinity towards the Fc region of human, rabbit and guinea pig immunoglobulins, protein A and staphylococci-bearing protein A have been used in a wide variety of immunoassays such as agglutination, radioimmunoassay (O'Keefe and Bennett 1980; Richman *et al.* 1982) and enzyme-linked-immunosorbent assay (ELISA) (Engvall 1976; Buchanan *et al.* 1981; Ahmad *et al.* 1988; Zainal-Abidin *et al.* 1992). Protein A has also been used on agarose as ligand in affinity chromatography for purification of immunoglobulins (Goding 1978; Jaton *et al.* 1979; Gentile *et al.* 1984).

Langone (1982) reported that more than 95% of *S. aureus* strains produce protein A in varying amounts. This protein was reported as a cell wall constituent, which is covalently linked to the peptidoglycan (Sjoquist *et al.* 1972). Some *S. aureus* strains are capable of secreting protein A into culture medium (Forsgen and Sjoquist 1969; Masuda *et al.* 1975). For example, the strain Cowan I secretes 30% of protein A, and methicillin-resistant strains secrete almost all protein A synthesized by the cell into the culture medium (Forsgen and Sjoquist 1969; Masuda *et al.* 1975). The-cell wall bound protein A can be isolated by using enzymes such as lysozyme, DNAase and lysostaphin (Yoshida *et al.* 1963; Sjoquist *et al.* 1972). However, the yields produced by lysozyme and DNAase are variable and heterogeneous compared to lysostaphin (Bjork *et al.* 1972).

This paper describes a competitive ELISA technique for quantification of extracellular protein A in culture medium produced by a methicillin-resistant strain of *Staphylococcus aureus*, strain A676. Competitive ELISA is a heterogeneous enzyme immunoassay where the antigen-antibody complexes physically separate an antibody from free antigen using a solid phase system (Engvall 1976). This assay system is very specific and sensitive, and can be used for measuring either antigen or antibody. A competitive ELISA system, which is based on a competitive binding between enzyme-labelled protein A and unlabelled protein A with human IgG, was first described by Goding (1978). Human or rabbit IgG was selected because of its high binding affinity to the Fc region by protein A through extensive hydrophorbic interaction to the binding sites CH_2 and CH_3 at the constant regions of heavy-chain (Endresen and Grov 1978; Zikan 1980; Gentile *et al.* 1984). This ELISA alkaline phosphatase system was chosen to be conjugated with protein A because it is stable and the activity has a linear relationship with substrate concentrate compared with horseradish peroxidase enzyme (Voller *et al.* 1976).

MATERIALS AND METHODS

Cultivation of Bacteria and Source of Protein A

Staphylococcus aureus strain A676, a methicillin-resistant strain, was provided by Prof. C. Brown of Heriot-Watt University, Edinburgh, UK. The bacterium, isolated from a single colony, was sub-cultured on nutrient agar containing 0.2% methicillin (Sigma, St. Louis, MO) at 37°C for 24 h, and then transferred to 10 ml of nutrient broth in a 100-ml flask for 10 h at 37°C with continuous shaking at 150 rpm (Centromat, B. Braun, Germany). The culture was centrifuged at 3000 rpm for 10 min and the pellet was resuspended in phosphate-buffered saline (pH 7.4) and

optical density (OD) adjusted to 0.6 at 600 nm. The brain-heart infusion medium (pH 7.4) was inoculated with the bacterial suspension at a final concentration of 1% (v/v) and incubated at 37°C in a shaking water bath at 150 rpm. A 5-ml sample was removed at 2-h intervals and centrifuged at 10,000 xg for 5 min.

Preparations of IgG and Purification of IgG

IgG was purified according to the method of Clark and Adam (1977). Blood from white New Zealand rabbits was allowed to clot at room temperature for 60 min and kept overnight at 4°C. Serum was separated from blood cells by centrifugation at 3000 rpm for 10 min. An equal volume of 40% saturated ammonium sulphate was added and the mixture was again centrifuged at 2000 rpm for 10 min. The precipitate was dissolved in 5 mM phosphate buffer (pH 7.4) and dialysed with three changes of the same buffer. Immunoglobulin G in serum was chromatographed on DEAE-cellulose which was pre-swollen in 5 mM phosphate buffer (pH 7.4) and packed into a column (1.6 × 30 cm) and equilibrated with 1 l of the same buffer. One m of partially purified IgG was applied and eluted with a gradient of increasing ionic strength of phosphate buffer (5-50 mM). Fractions from the first peak were collected and pooled. The concentration was determined at 280 nm and stored at -20°C.

Competitive ELISA (CELISA)

Optimization of the ELISA method was developed with respect to IgG concentration and incubation time for colour development. To determine the optimum concentration of IgG, solutions of various concentrations (1, 2, 4, 6 and 8 µg/ml) in carbonate buffer (pH 9.6) were dispensed at 150 µl per well of a 96-well microtitre plate (Nunc). The plate was incubated overnight at 4°C and then washed three times with PBS containing 0.05% Tween 20 (Merck) PBS-T). The wells were blocked with 150 µl of 3% bovine serum albumin (BSA) in PBS and incubated at 37°C for 1 h after a subsequent washing with PBS-T.

For the assay, 200 µl of unlabelled protein A (Sigma) at various concentrations (0.005 - 4 µg/ml) in PBS or BHIB complex medium (Oxoid) with the addition of 200 µl protein A alkaline phosphate (Sigma) at a concentration of 1 mg/ml was used. The plate was incubated at 37°C for 2 h and washed three times with PBS-T. Fresh para-nitrophenyl phosphate (Sigma) substrate prepared at a concentration of 1 mg/ml was added to each well and kept in the dark for 15, 20, 25 or 30 min. The reaction was stopped by the addition of 50 µl of 3 M NaOH. The OD value was read using an ELISA reader (Bio-Tek Instruments, USA) at λ = 405 nm. The standard curve was established by plotting the OD values at Y axis and protein A at X axis. The concentration of samples was then estimated by using the Kinetic-Calc program on a computer linked to the ELISA reader (Bio-Tek Instruments, USA). For logit-log plot, the logit values for Y axis were calculated by using the formula: Logit $[(^{OD}\text{maximum})/^{OD}\text{maximum}-^{OD}\text{standard or sample})]$, versus log standard protein A in X axis.

RESULTS AND DISCUSSION

The optimum concentration of rabbit IgG at which unlabelled protein A effectively competed with the enzyme-labelled protein A was 2-4 µg/ml (*Fig. 1*). The binding of protein A-alkaline phosphate to the IgG molecules adsorbed to the wells was reduced when the concentration of unlabelled protein A in the standard or samples was increased. The absorbance values of alkaline-phosphate-protein A bound to the IgG were measured 20 min after additions

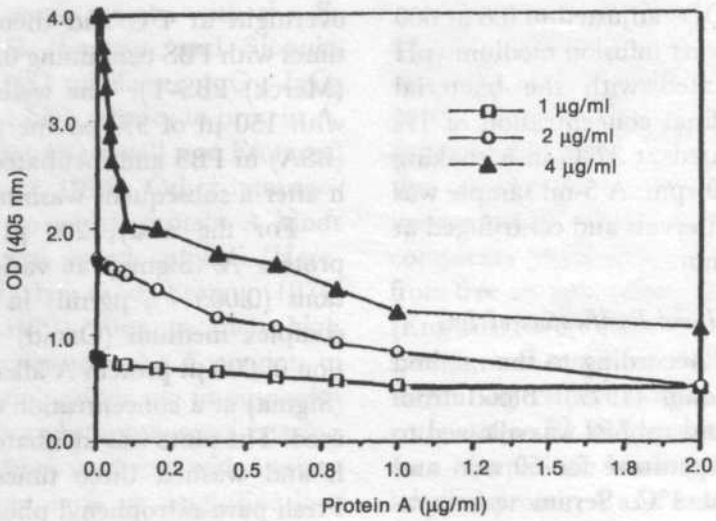

Fig. 1. Standard curves of protein A (Wells were coated with IgG at 1, 2 or 4 µg/ml and the competitive ELISA performed as described in the text)

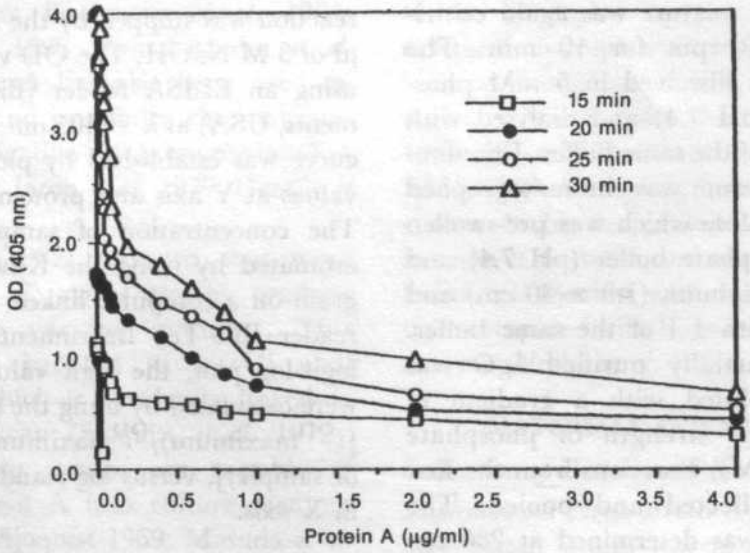

Fig. 2. Determination of optimum incubation time with IgG coated at 2 µg/ml (The IgG at 2 µg/ml was coated on the microtitre plate and the competitive ELISA performed; absorbance was measured every 5 min)

of the substrate. The absorbance at zero concentration of protein A was increased with increasing concentrations of IgG coated. The significant reduction of absorbance values was observed with the increase in unlabelled protein A for the wells coated with 2 and 4 µg/ml IgG. At higher IgG concentrations (4 µg/ml or above), no competition occurred because the binding sites for protein A were not limited. Conversely, at IgG concentrations lower than 1 µg/ml the number of protein A receptors was too small to allow any competition to occur between alkaline phosphatase-protein A and the unlabelled protein A molecules (Goding 1978).

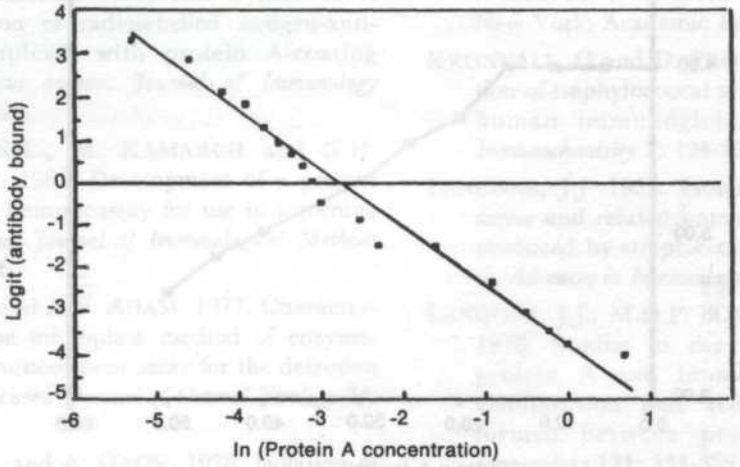

Fig. 3. Logit-log plot of standard curve using IgG coating at 2 μg/ml with a 25-min substrate incubation time

Fig. 2 shows the standard curve, which was plotted using absorbance values taken at time intervals of 15, 20 and 30 min after the addition of substrate. At 20 and 30 min the absorbance values were inversely proportional to a wide range of protein A concentrations. *Fig. 3* shows a linear standard curve using IgG coating at 2 μg/ml with incubation time of 25 min transformed into logit-log plot. This plot allows approximation of sample concentration in the region which is approaching saturation (Peterman and Butler 1989). The minimum detectable limit of protein A in this competitive ELISA system was determined by constructing the standard curve with a concentration range of 0-5 ng/ml using 4 μg/ml IgG coating. *Fig. 4* shows that the minimum detectable limit was 20 ng/ml.

The production of protein A from methicillin-resistant *S. aureus* A676 cultured in brain-heart infusion medium in the shake flask was measured using the competitive ELISA as described above. The supernatant of samples was obtained every 2 h for the first 10 h and subsequently every 10 h until 48 h of total incubation time. The sample was then diluted 10 × in PBS before measuring the protein A concentration. Using this competitive ELISA measurement, absorbance of the protein A standard and samples was performed in the same 96-well plate. *Fig. 5* shows concentration of protein A in the medium measured for a period of 48 h using competitive ELISA technique. The concentration of protein A produced in this experiment was correlated with increase in cell number. The production increased exponentially during log phase and reached maximum concentration of 22.5 μg/ml after 15 h cultivation. Thus, this techniques is successful in measuring protein A concentration in *S. aureus* cultures.

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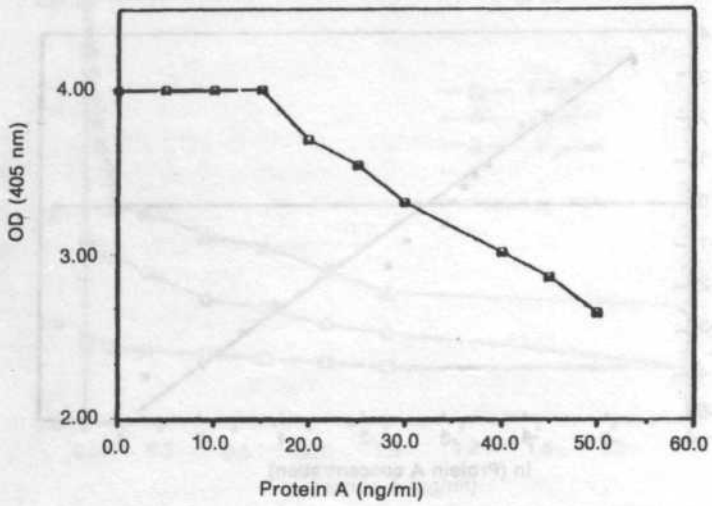

Fig. 4. Standard curve of protein A with a concentration range of 0-50 ng/ml using IgG coating at 4 µg/ml. (The OD was measured 25 min after substrate was added)

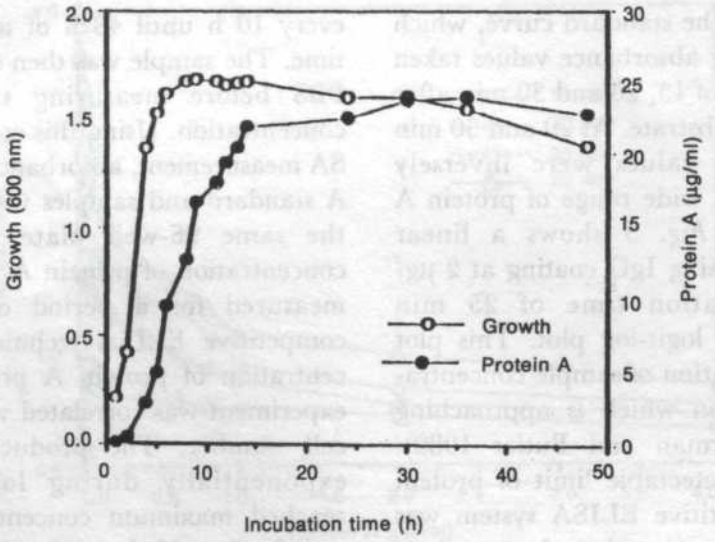

Fig. 5. Growth of S. aureus *A676 in BHIB medium and concentration of protein A produced when cultured in shake flask at 37°C for 48 hours*

Universiti Pertanian Malaysia. The authors would also like to thank Prof. C. Brown for supplying the culture.

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Effect of Irradiance on Growth, Physiological Processes and Yield of Melon *(Cucumis melo)* Plants Grown in Hydroponics

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Keywords: irradiance, melon cultivars, growth, stomatal conductance, photosynthetic rate, yield

ABSTRAK

Pengaruh radiasi yang berbeza ke atas tanaman tembikai wangi (Cucumis melo) *cv. Birdie, Charity Ball dan Jade Dew yang ditanam di dalam hidroponik telah dikaji. Tanaman diberi rawatan min radiasi yang berbeza iaitu 11.4, 8.2, 6.1 dan 3.0 MJ* m^{-2} *hari*$^{-1}$ *yang diperolehi dengan menggunakan teduhan. Hasil berat kering berhubung rapat dengan paras radiasi. Konduksi stomata dan kadar fotosintesis adalah tertinggi bila tanaman berada pada paras radiasi yang tertinggi. Tanaman yang ditanam di bawah radiasi 11.4 MJ* $m^{-2}h^{-1}$ *menghasilkan berat basah buah dan kandungan pepejal terlarut yang tinggi. Semua kultivar gagal untuk menghasilkan buah pada radiasi 3.0 MJ* $m^{-2}h^{-1}$.

ABSTRACT

*The effect of different irradiance levels on melon (*Cucumis melo*) cv. Birdie, Charity Ball and Jade Dew grown in hydroponics was investigated. Plants were exposed to mean daily irradiance levels of 11.4, 8.2, 6.1 and 3.0 MJ* m^{-2} *day*$^{-1}$ *achieved by using different levels of shade. The dry matter yield appeared to be directly proportional to the irradiance level received by plants. Stomatal conductance and photosynthetic rate were highest when the plants were grown under the highest irradiance level. Plants grown under 11.4 MJ* $m^{-2}d^{-1}$ *had the highest fruit fresh weight and total soluble solids. All cultivars failed to fruit when grown under irradiance of 3.0 MJ* $m^{-2}d^{-1}$.

INTRODUCTION

In Malaysia, the area of cultivation of horticultural crops under protected environment expanded rapidly in the late 1980s. This development has been encouraged by many factors such as the unpredictable weather conditions, the demand for quality produce and the introduction of soilless culture. As for open field cultivation, crop productivity under protected environment agriculture is dependent upon optimum environmental factors.

It is a common assumption that light is generally not limiting for the cultivation of crops in the tropics. This assumption is not always true. Malaysia, for example, often experiences periods of haze, which reduce radiation interception by almost 30-40% and this is even more pronounced under rain shelters (Mohd Razi 1991, 1994). Apart from these changes, different designs of rain shelter result in 18-50% reduction in radiation interception (Yeoh 1991). Robinson (1990) also reported that different types of plastic used as roofing material cause variation in light interception.

Nearly all previously reported experiments showing benefits of increased irradiance have involved plants growing in glasshouses in temperate regions, where low levels of radiation are more critical during winter (Hurd and Thornley 1974; Gislerod *et al.* 1989; Cockshull *et al.* 1992). In glasshouses in the tropics, Mohd Razi

and Ali (1994) found NFT-grown tomatoes failed to fruit when plants received less than 8.5 MJ $m^{-2}d^{-1}$ despite a 5°C reduction in temperature in the plant canopy under glasshouse conditions in Malaysia.

Melon (*Cucumis melo* L.) of the reticulatus type is a high value crop which can be grown successfully by hydroponics under rain shelters. Apart from a report by Bouwkamp *et al.* (1978), little information is available on the irradiance requirement for the production of melon in the tropics, especially when water and nutrient supply are not limiting factors in crop production, as is the case in hydroponics.

The present study was conducted to examine the effects of different levels of irradiance on growth, stomatal conductance, photosynthesis rate and yield of three melon cultivars, and, based on growth and yield data, to determine the optimal irradiance level for production of melon under protected environment in the tropics.

MATERIALS AND METHODS

The effects of irradiance on three melon (*Cucumis melo*) cultivars grown in a Kyowa deep culture system (Lim and Wan 1984) were investigated at the Hydroponic Unit, Universiti Pertanian Malaysia. Uniform, three-week-old melon plants (cv. Birdie, Charity Ball and Jade Dew) were grown under different shade regimes which gave varying levels of irradiance. Various levels of shade were achieved by placing an increasing number of layers of plastic film of ethylene vinyl acetate (EVA) copolymers over the plant canopy. EVA copolymers are transparent to visible light and allow all wavelengths essential for photosynthesis to pass through (Robinson 1990). Mean irradiance received by plants under various shade levels was 11.4, 8.2, 6.1 and 3.0 MJ $m^{-2}d^{-1}$ as recorded by solarimeters (Delta-T Device, Cambridge, UK). Air temperature and relative humidity in the plant canopy were between 25-37°C and 60-72%, respectively. The plants were supplied with a nutrient solution containing the ion concentrations given by Cooper (1979) with electrical conductivity maintained between 2.4-2.6 mS cm^{-1}. Plants were arranged in a completely randomized design in a split-plot arrangement where irradiance and cultivar were assigned as main plot and subplot, respectively. Each plot contained 12 plants, which were replicated 4 times.

At harvest, leaf length and breadth were measured with a ruler and the leaf area determined using an automatic leaf area meter (Delta-T Cambridge, UK). The shoot and root dry weights were determined after drying at 80°C for 48 hours. Destructive sampling was performed at 0, 4 and 9 weeks for determination of relative growth rate (RGR) and net assimilation rate (NAR). At each harvest, 4 plants were harvested from each treatment and RGR and NAR were calculated using formulae given by Hunt (1982).

Measurements of the stomatal conductance (gs) and net photosynthetic rate (Pn) for intact leaves were determined using an infrared gas analyser IRGA (LCA-2 Portable Photosynthesis System, ADC Hoddesdon, UK). The measurements were made 4-5 h after sunrise on clear days on the abaxial surface of young fully expanded leaves (3^{rd} - 5^{th} leaf from shoot apex). All measurements were carried out in the differential mode at IRGA with Emax set at 1.0 and boundary layer resistance at 0.3 mmol m^{-2} s^{-1} predetermined by placing the chamber on a mock leaf (of moist filter paper).

Fruits were harvested from each plant at maturity when signs of cracks appeared at the basal part of the fruit. Fruit diameter was measured at harvest using a Vernier caliper and their fresh weight was deter-

TABLE 1
Effect of irradiance and cultivar on leaf length, breadth, area and dry weight of leaf, root and stem at day 56. Data are means of the main effect as interaction between irradiance × cultivar is not significant except for leaf area

Treatments	Mean Leaf Length (cm)	Mean Leaf Width (cm)	Leaf Area (cm^2)	Mean Dry Weight		
				Leaf	Root	Stem
				(g/plant)		
Irradiance (MJ m^{-2} day^{-1})						
11.4	16.24 a	21.24 a	6190 a	34.28 a	7.66 a	17.40 a
8.2	13.56 b	18.27 b	4774 b	27.26 b	5.90 b	16.23 a
6.1	12.92 b	16.84 c	3959 c	12.22 c	3.58 c	12.24 b
3.0	7.40 c	9.93 d	965 d	5.63 d	1.13 d	2.22 c
Cultivar						
Birdie	12.50 a	16.60 a	4644 a	21.86 a	5.21 a	13.19 a
Charity Ball	12.37 a	16.51 a	3659 b	20.26 a	4.55 a	12.03 a
Jade Dew	12.72 a	16.68 a	3613 b	17.42 b	3.94 b	10.84 a
Interaction ($P < 0.05$)						
Irradiance × Cultivar	ns	ns	**	ns	ns	ns

Mean values in each column with the same letter are not significantly different at $P < 0.05$ according to DMRT. For the interaction effects; ** = significant at $P < 0.05$.

mined. A fresh sample weighing 20g was placed in a weighed glass petri dish and oven dried at 80°C for 60 h, and total fruit dry matter was estimated. Data were obtained on soluble solids content with a hand refractometer (Currence and Larsen 1941) on all fruit harvested from each plant.

RESULTS AND DISCUSSION

Table 1 shows the growth responses of melon cultivars to different levels of irradiance. There was no significant interaction ($P > 0.05$) between irradiance and cultivar on the leaf length and width and dry weight of leaf, stem and root. Leaf length and width were reduced significantly ($P < 0.05$) with irradiance below 6.1 MJ $m^{-2}d^{-1}$. Similarly, low irradiance resulted in a significant reduction ($P < 0.05$) in leaf dry weight. This is consistent with the fact that intercepted radiant energy determines the dry matter production in plant species (Lawlor 1992). Root dry weight was reduced to 22, 53 and 88% in plants grown under 8.2, 6.1 and 3.0 MJ $m^{-2}d^{-1}$ respectively, relative to 11.4 MJ $m^{-2}d^{-1}$. The reduction in leaf growth with decreased irradiance was reported to inhibit root growth and subsequently

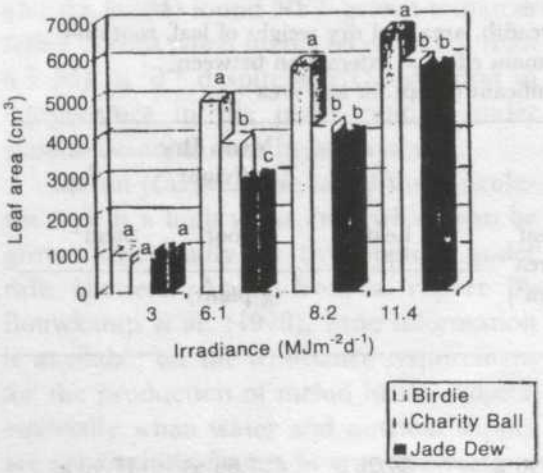

Fig. 1: The effect of irradiance and cultivar on lead area of melon plant. Means separation by DMRT ($p < 0.05$)

water uptake (Smith *et al.* 1984). The reduction in plant growth with decreasing irradiance involves many physiological and biochemical attributes which have been reported elsewhere (Blackman and Wilson 1951; Lawlor 1992). Between cultivars, Birdie produced greater root dry weight than either Charity Ball or Jade Dew. A significant irradiance and cultivar interaction ($P < 0.01$) was observed for leaf area. Cultivar Birdie produced a greater leaf area when grown under irradiance levels above 6.1 MJ m^{-2} day^{-1} (*Fig. 1*).

In general, the RGR and NAR were affected by different irradiance levels (Table 2). In the first four weeks, RGR and NAR decreased proportionately with reduction in irradiance levels. During weeks 4 - 9, no significant difference in RGR and NAR between plants grown under 11.4 and 8.2 MJ $m^{-2}d^{-1}$ was observed. RGR and NAR were significantly reduced ($P < 0.05$) with irradiance levels below 8.2 MJ $m^{-2}d^{-1}$. A similar trend of increased NAR and RGR with increased irradiance had been reported for tomatoes (Hurd and Thornley 1974; Logendra *et al.* 1990), and tomatoes, sweet pepper and cucumber (Bruggink and Heuvelink 1987). There was no significant interaction ($P > 0.05$) observed between irradiance and cultivar for NAR and RGR.

TABLE 2

Effects of irradiance on relative growth rate and net assimilation rate of melon plants. Data on cultivar are not presented as no significants were observed within cultivars. Interaction irradiance and cultivar are also not significant.

Interval/Irradiance Treatments	Relative Growth Rate ($g\ g^{-1}\ week^{-1}$)	Net Assimilation Rate ($g\ cm^{-2}\ week^{-1} \times 10^{-3}$)
0-4 weeks		
11.4 MJ $m^{-2}d^{-1}$	0.26 a	1.6 a
8.2 :	0.20 b	1.3 b
6.1 :	0.15 c	1.1 c
3.0 :	0.06 d	0.7 d
4-9 weeks		
11.4 MJ $m^{-2}d^{-1}$	0.30 a	3.6 a
8.2 :	0.32 a	3.7 a
6.1 :	0.24 b	2.6 b
3.0 :	0.20 c	1.4 c

Means separation by DMRT ($P < 0.05$), Mean values in each column with the same letter are not significantly different.

TABLE 3
Effects of irradiance and cultivar on photosynthesis rate (Pn) and stomatal conductance (gs) measured at day 24 and 40 after treatments (DAT) on melon plants. Data presented as mean from main effect as the interaction irradiance × cultivar is not significant.

Treatments	24 DAT		40 DAT	
	Pn (μmol $m^{-2}s^{-1}$)	gs (mol $m^{-2}s^{-1}$)	Pn (μmol $m^{-2}s^{-1}$)	gs (mol $m^{-2}s^{-1}$)
Irradiance MJ $m^{-2}day^{-1}$				
11.4	17.96 a	0.63 a	21.11 a	0.67 a
8.2	16.37 a	0.47 b	15.94 a	0.51 b
6.1	3.65 b	0.29 c	3.64 c	0.27 c
3.0	0.73 c	0.10 d	0.69 d	0.09 d
Cultivar				
Birdie	10.36 a	0.38 a	11.16 a	0.39 a
Charity Ball	9.94 a	0.39 a	10.31 a	0.39 a
Jade Dew	8.81 a	0.35 a	9.56 a	0.36 a
Interaction ($P < 0.05$ Irradiance × Cultivar	ns	ns	ns	ns

Mean values in each column with the same letter are not significantly different at $P < 0.05$ according to DMRT. ns = not significant.

In this study, stomatal conductance and photosynthesis rate were reduced significantly ($P < 0.01$) with decrease in irradiance (Table 2). Turcotte and Gosselin (1989) reported a similar result for glasshouse cucumber. Low dry weight values in the various plant parts indicated that less carbon was fixed in the leaves that could be translocated to other parts of the plant including fruits. For tomatoes, Ho and Hewitt (1986) showed that photosynthesis rate is mainly affected by irradiance and CO_2 concentration and that the export rate of assimilates from a leaf during the light period is proportional to the concurrent photosynthesis rate. Furthermore, leaf reserves are very low in plants grown in low light and the rate of export from such leaves can be reduced in unfavourable light conditions. This is in agreement with our study on melon where plants grown under low irradiance showed a significant decrease in fresh and dry fruit weights (Table 4). With decreased irradiance from 8.2 to 6.1 MJ $m^{-2}d^{-1}$, yield was reduced by 20-60% relative to the plants grown under 14 MJ $m^{-2}d^{-1}$. The proportional yield and intercepted radiant energy have already been established in tomatoes. Cockshull *et al.* (1992) showed that 2 kg m^{-2} fruits were produced for every 100 MJ m^{-2} of solar radiation received by the crop. Their study also showed that average fruit size was reduced with decrease in intercepted irradiance, which was also observed in this study. Within cultivars, cv. Birdie

TABLE 4
Fruit diameter, fresh weight and dry matter and total soluble solids as influenced by irradiance and cultivar. Data presented are the mean from the main effect as interaction irradiance x cultivar is not significant

Treatments	Fruit Diameter (cm)	Fruit Fresh Weight (g/plant)	Fruit Dry Weight (g/plant)	Total Soluble Solids (% Brix)
Irradiance MJ $m^{-2}day^{-1}$				
11.4	10.52 a	0.90 a	45.13 a	10.40 a
8.2	9.38 a	0.73 b	36.41 b	7.16 b
6.1	7.13 b	0.39 c	21.88 c	5.21 c
3.0	–	–	–	–
Cultivar				
Birdie	10.05 a	0.68 a	30.27 a	8.03 a
Charity Ball	9.20 a	0.63 a	26.10 b	7.32 a
Jade Dew	8.78 b	0.56 b	22.20 b	7.41 a
Interaction Irradiance × Cultivar	ns	ns	ns	ns

Mean values in each columm with the same latter are not significantly different at $P < 0.05$ according to DMRT.

and Charity Ball produced greater fruit fresh weight than Jade Dew. No significant interaction ($P > 0.05$) was found between cultivar and irradiance levels.

All melon cultivars failed to fruit at the lowest irradiance level. The disturbance in the photosynthetic activities might have inhibited assimilate partitioning which subsequently resulted in a failure in reproductive processes. The benefit of high irradiance to the reproductive processes has been reported for a wide range of crops (tomatoes: Boivin *et al.* 1987; Cockshull *et al.* 1992; strawberry: Ceulemans *et al.* 1986; rose: Zieslin and Mor 1990).

Total soluble solids (TSS) is a good measure of sweetness of melon. The relative degree of irradiance reduction was well reflected in decreased TSS. Table 4 shows TSS was reduced by approximately 3 and 5% with a reduction in irradiance interception from 11.4 to 8.2 and 6.1 MJ $m^{-2}d^{-1}$, respectively. Winsor and Adams (1976) showed a similar trend of increased TSS with high irradiance in tomatoes. Our results, however, disagree with those of Bouwkamp *et al.* (1978) who found soluble solids content decreased with increased light intensity in most of the melon cultivars they studied. This discrepancy may be due to the amount of intercepted irradiance. In their study, soluble solids decreased when irradiance increased from approximately 19 to 25 MJ $m^{-2}d^{-1}$ for 6 days prior to harvesting. This high light intensity may cause fruits to accumulate heat and attain temperatures exceeding air temperature; this subsequently results in higher respiration rates, thus lowering soluble solid content. Throughout the duration of the experiment, the maximum irradiance recorded in the present study

was only approximately 16.2 MJ $m^{-2}d^{-1}$. We suggest that when plants are grown under unlimited water and nutrient supply, environmental factors that inhibit photosynthesis rate and limit the distribution of assimilate to various plant parts including the fruit play a significant role in yield and quality.

CONCLUSION

The response of melon to the amount of irradiance varies. Irradiance lower than 8.2 MJ $m^{-2}d^{-1}$ reduced dry weight accumulation and yield. None of the cultivars was tolerant of the lowest irradiance level (3.0 MJ $m^{-2}d^{-1}$). The reduction in net photosynthesis may have contributed to reduction in yield. This result has practical applications in showing the need to maximize light transmission under protected environment.

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A New Egg Parasitoid for Possible Biological Control of the Asiatic Maize Borer in Malaysia

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ABSTRAK

Satu spesis parasitoid telur yang telah dikenalpasti sebagai Trichogramma papilionis *Nag. menyerang kelompok telur* Ostrinia furnacalis *Guenee. Ianya merupakan parasitoid telur tunggal yang ditemui sepanjang masa kajian. Edaran hidup parasitoid ini disempurnakan dalam masa 9 hari dengan 2, 4 dan 3 hari diperingkat telur, larva dan pupa. Nisbah seks (betina:jantan) ialah 3:1 yang mana didapati tinggi daripada nisbah 2:1 yang biasanya diperolehi bagi* Trichogramma *spp. Superparasitisme telah dapat diperhatikan dimana 3 individu telah dihasilkan dari sebiji telur perumah. Semua telur boleh diserang dalam masa 4 hari pada nisbah 2:1 (hos:parasitoid). Parasitoid betina menunjukkan kecekapan memburu pada tahap yang tinggi dimana ianya akan menyerang kesemua telur dalam sesuatu kelompok sebelum menyambung pemburuan. Parasitoid betina menggunakan 40% daripada keseluruhan masa untuk memburu dipermukaan atas daun jagung, 30% dipermukaan bawah daun dan 30% dipinggir daun. Kelakuan parasitoid ketika memburu dan menyerang hos menuruti geraf respon "functional" jenis III. Parasitoid betina menunjukkan respon positif terhadap bauan daun jagung menandakan kemungkinan adanya penglibatan sejenis kairomon atau kimia komunikasi kontak.* T. papilionis *adalah dianggap sejenis parasitoid telur* O. furnacalis *yang amat berkesan dan calon baik untuk program kawalan biologi perosak.*

ABSTRACT

A species of egg parasitoid tentatively identified as Trichogramma papilionis *Nag. was found attacking egg masses of* Ostrinia furnacalis *Guenée. The life cycle of the parasitoid was completed within 9 days; the egg, larval and pupal stages lasted for 2, 4 and 3 days, respectively. The female: male sex ratio was 3:1, higher than the usual 2:1 sex ratio for* Trichogramma *spp. Superparasitism was observed whereby 3 individuals were produced from one host egg. All eggs were parasitized in 4 days at 2:1 (host: parasitoid) ratio. The female parasitoid showed a high degree of searching efficiency and normally parasitized all eggs in a batch before continuing her search for the next egg batch. The female spent 40% of the time searching on the upper surface of a maize leaf, 30% on the lower surface and 30% on the leaf edges. The female searching and parasitization behaviour followed the Type III functional response curve. The female parasitoid showed positive response to the odour of the maize leaf, indicating a possibility that a kairomone or contact communication chemical is involved.* T. papilionis *appeared to be a very efficient egg parasitoid of* O. furnacalis *and a good candidate for a biological control programme of the pest.*

INTRODUCTION

The Asiatic maize stem borer, *Ostrinia furnacalis* Guenee (Lepidoptera: Pyralidae) generally can be controlled by the application of insecticides (Hussein and Kameldeer 1988). With the growing interest in the important role of biological agents (predators, parasitoids and pathogens) in integrated pest management (IPM) programmes, in 1988 Universiti Pertanian Malaysia (UPM) started research on the natural enemies of *O. furnacalis* to evaluate the possibility of using parasitoids as a major component in its management.

Among its parasitoids are a larval-pupal parasitoid, *Brachymeria lasus* Walker (Hymenoptera: Chalcididae) and a larval parasitoid, *Xanthopimpla stemmator* Thunberg (Hymenoptera: Ichneumonidae) (Hussein *et al.* 1983). An egg parasitoid, identified as *Trichogramma papilionis* Nag. (Hymenoptera: Trichogrammatidae), was recently found parasitizing eggs of *O. furnacalis* in the maize field at Serdang, Selangor (Hussein and Ibrahim 1992). It was considered that the parasitoid might be a successful control agent. This paper reports various biological studies carried out on the parasitoid.

MATERIALS AND METHODS

Life History

A study was carried out to determine the developmental period of the egg, larval and pupal stage of the parasitoid. Twenty-four hours after oviposition, eggs of *O. furnacalis* were individually reared and exposed to the parasitoid. Four days after oviposition, 100 eggs of the parasitoid were observed through the larval and pupal period of development in the laboratory at 25 ± 3°C and 60 ± 10% R.H.

Longevity

Longevity and mortality rates of the parasitoids were determined, comparing adults fed with (1) sucrose and water, and (2) water only. Fifty adult parasitoids were included in the test. The numbers of live and dead parasitoids were recorded daily.

Sex Ratio

The sex ratio of the parasitoid was determined by rearing individuals on eggs of *O. furnacalis* until the adults emerged. A total of 700 eggs were parasitized and kept for observation. The adults emerging from the host were immediately sexed, based on antennal morphology (Pak and Oatman 1982).

Superparasitism

A total of 100 24-h-old eggs of the host were exposed to allow maximum parasitism by *T. papilionis* inside a large closed petri dish (15 cm diameter) for a period of 4-5 days. The eggs were left in the laboratory until pupation of the parasitoid occurred. Each host egg was then dissected and the number of parasitoids present was counted.

Maximum Parasitization

Twenty different sized batches of host eggs were each exposed to a single newly mated female parasitoid in a closed plastic petri dish. The number of eggs parasitized was recorded daily for 5 days until all the eggs had been parasitized. The sequence of parasitizing individual eggs was recorded.

Host: Parasitoid Ratio and Parasitization Rate

The relationship of host:parasitoid ratio to the rate of parasitization was determined using a factitious host egg of *Corcyra cephalonica* under laboratory conditions. The number of host eggs was 5, 10, 14, 20 and 25. Regression analysis was performed on the data.

Functional Response

Eggs of the factitious host, *C. cephalonica*, were glued on pieces of 3 × 3 cm paper card in a regular pattern. Seven different host densities were tested: 1, 5, 9, 22, 36, 72 and 108 eggs per 9 cm^2 with 10 replications. The eggs were then placed in a closed plastic petri dish (5 cm diam). A single newly mated *T. papilionis* was released into each dish for 5 h. The parasitoid was then removed and the dish left for another 4 days, after which the number of parasitized eggs was counted. The data were fitted to Holling's Type III model of functional response (Holling 1965).

Searching Behaviour

The preference of the parasitoid to search

for its host on either the upper or lower surface of the maize leaf was measured. A freshly picked maize leaf blade was stretched horizontally between two vertical wooden sticks and illuminated from above with strong fluorescent light. One female parasitoid was released at the centre of the leaf. Several aspects of behaviour and positioning were recorded and timed using a stopwatch.

Kairomones

Four extracts were assessed for attractiveness to adult parasitoids. Two were prepared by soaking eggs of *O. furnacalis* in hexane and methanol respectively for at least 6 hours. The other two from scales of the moth, similarly soaked in hexane and methanol, were then filtered. Bioassay tests using a petri dish were run following the method described by Jones *et al.* (1973). Olfactometery tests were also carried out using a glass Y-tube (Schilthuizen 1989).

RESULTS AND DISCUSSION

The developmental period of *T. papilionis* from egg stage to adult emergence was 9 days. The egg, larval and pupal stages lasted for 2, 4 and 3 days, respectively. These periods are quite close to those of *T. australicum* (8 days) but differ from those of *T. minutum* (6 days) and *T. fasciatum* (6 days) (Metcalf and Breniere 1969). The development period of *Trichogramma* species in general is very similar (Metcalf and Breniere 1969).

In this study, the sex ratio varied around 3:1 (female:male). The females are always predominant. Usually, the sex ratio for *Trichogramma* species is 2:1. *T. papilionis*, in this study, follows the biparental and arrhenotokous mode of reproduction. The longevity of unmated female *T. papilionis* was 9-10 days when fed on sucrose and water. Females lived for only 3 days when fed on water alone.

Superparasitization was observed in *T. papilionis* parasitizing eggs of *O. furnacalis*; as many as three individuals were produced from one host egg. The phenomenon is common to all *Trichogramma* species. In general, the number of both hosts parasitized and the number of progeny increase initially with increasing host density but level off after reaching a threshold (Pak and Oatman 1982).

In the laboratory the parasitoid was able to parasitize all the maize stem borer eggs presented in a batch as it often does in the field. In this study, the maximum parasitization rate was achieved in 4 days regardless of the size of egg batch as long as the ratio was kept at 2:1 (host:parasitoid). The rate of parasitization rapidly increased at lower host ratios (*Fig. 1*). No parasitization was observed at 5:1 ratio if only 5 eggs/batch were presented. At 10 eggs/batch or more and at 3:1 ratio only 10-20% parasitization occurred. Pak and Oatman (1982) found the rate to be lower if older (> 36-h-old) host eggs are used. In this study, the host eggs used were < 36-h-old.

Not all egg parasitoids are adapted to parasitizing host eggs that are laid in batches. *T. papilionis*, as shown in this study, is very efficient in parasitizing batches of *O. furnacalis* eggs in the laboratory as well as in the maize field. When the oviposition behaviour was traced egg by egg in different batch sizes, a regular pattern was discerned (*Fig. 2*). Upon encountering an egg batch, the female parasitoid first parasitizes all the eggs before continuing her search for another batch. Burbutis *et al.* (1983) also observed the same behaviour when *T. nubilale* parasitized eggs of the European corn borer, *Ostrinia nubilais*.

The female parasitoid spent 40% of her searching time on the upper surface of the maize leaf, 30% on the lower surface and 30% on the edges of the leaf. Most

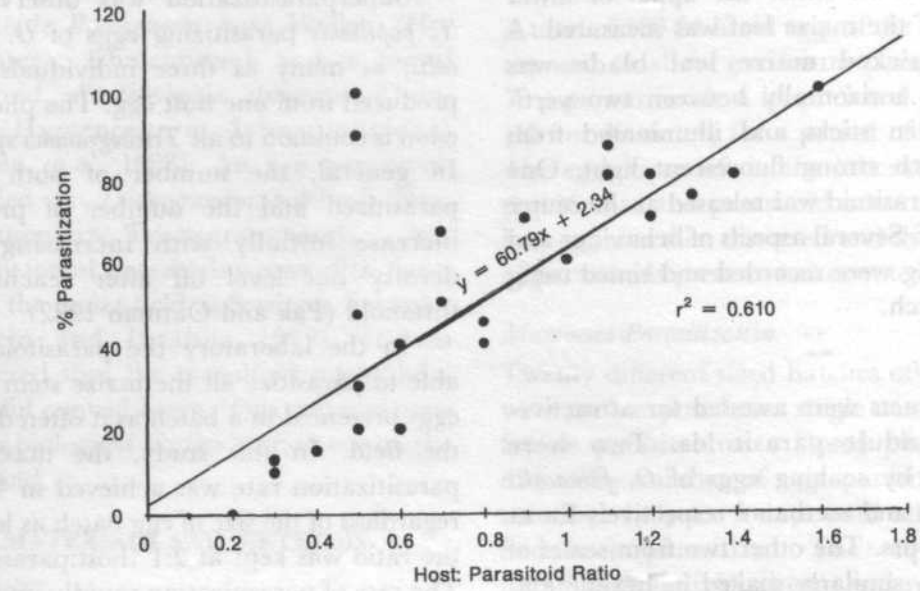

Fig 1. Effect of host-parasitoid ratio on percentage parasitization of Ostrinia furnacalis *by* Trichogramma papilionis

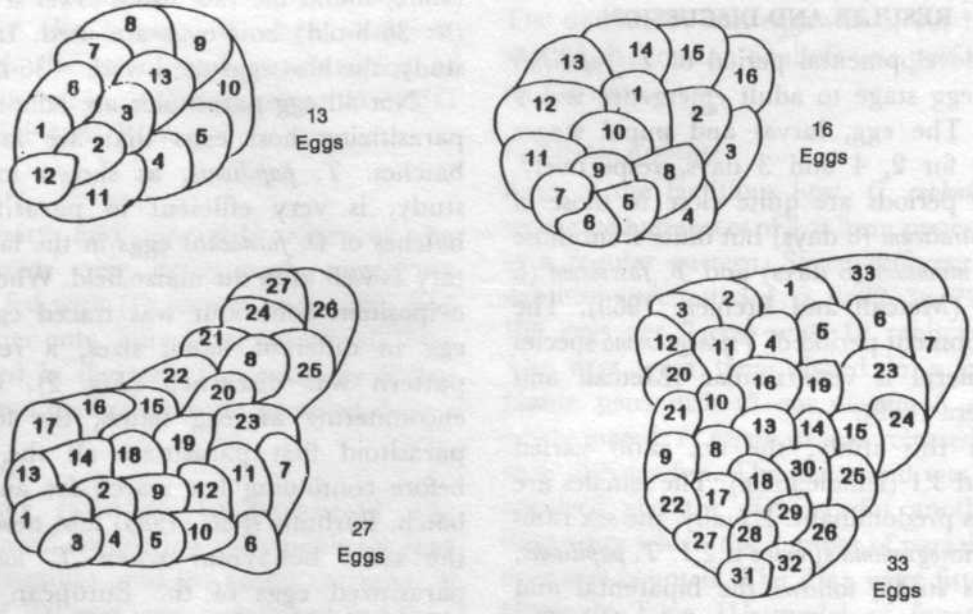

Fig 2. Sequence of egg parasitization by Trichogramma papilionis *on the different sized clusters of* Ostrinia furnacalis *eggs*

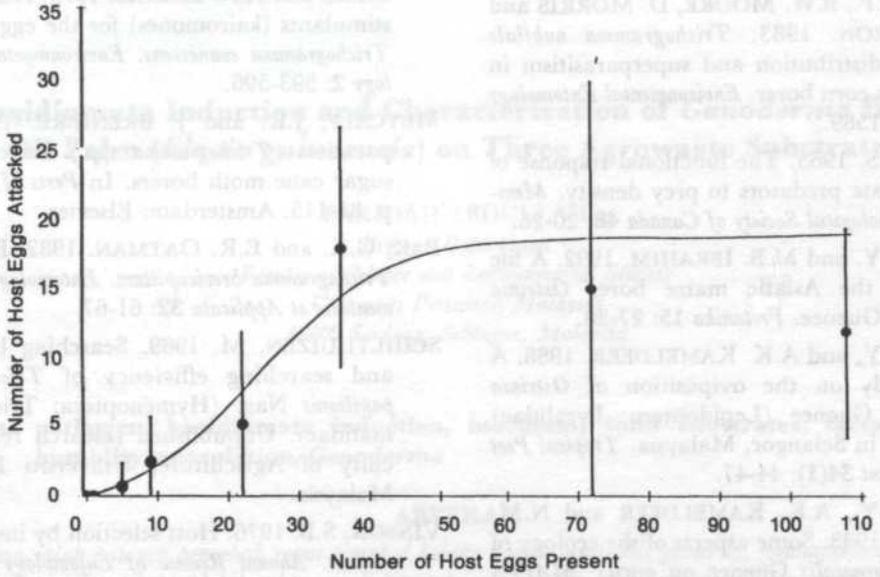

Fig 3. Functional response of Trichogramma papilionis

Trichogramma species generally prefer to search the more exposed parts of the plant (Metalf and Breniere 1969; Vinson 1976). Parasitoid searching behaviour was further elucidated by the Type III functional response curve (*Fig. 3*). The sigmoid response curve confirmed the observation mentioned earlier that at low host density some female parasitoids may find it difficult to encounter any eggs after a certain period of time and react by stopping their search.

Several egg parasitoids use kairomones to guide them while searching for host eggs in the field. In this study, attempts to demonstrate the presence of semiochemicals on the egg surface or moth scales did not produce a positive result from adult *T. papilionis* that could be detected using the petri dish method. However, using the Y-tube olfactometer, the female parasitoid showed some response to the odour of maize leaf. Whether a kairomone or a contact chemical was involved remains to be investigated.

CONCLUSION

Trichogramma papillionis can be reared successfully on eggs of *C. cephalonica*, in the absence of eggs of *O. furnacalis*. In Serdang, it parasitizes high numbers of egg masses, and almost no egg escaped parasitization. The parasitoid is well adapted to a host that produces egg masses instead of single eggs. A type III functional response indicates that relatively more hosts are attacked at high host densities. The chemical communication aspect of the parasitoid searching behaviour needs further investigation and is crucial in developing techniques to increase the success of parasitization in the field.

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Basidiomata Induction and Characterization of *Ganoderma* from Oil Palm (*Elaeis guineensis*) on Three Agrowaste Substrates

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Keywords: pathogen, basidiomata induction, inoculum, solid substrates, laccate, relative humidity, sporulation, *Ganoderma*

ABSTRAK

Ganoderma *ialah patogen penyebab reput pangkal kelapa sawit. Ahli-ahli genus ini menunjukkan kepelbagaian yang tinggi di tropika; tetapi adalah sukar untuk ditentukan pengertian kepelbagaian ini dari segi taksonomi kerana kestabilan ciri-ciri tertentu kulat ini masih belum diketahui. Kertaskerja ini merangkakan kaedah pengkulturan yang mengaruhkan pembentukan basidiomata* Ganoderma *dibawah keadaan terkawal, dan seterusnya membolehkan kestabilan ciri-ciri tertentu dinilaikan. Dengan menggunakan basidiomata aruhan, ciri-ciri terpilih dapat dihasilkan berulangkali tanpa perubahan diatas tiga jenis substrat, iaitu serabut kelapa sawit, serat kapas, dan habuk kayu getah. Dari segi kadar kolonisasi oleh miselia, kulat ini menunjukkan perbezaan bererti antara pertumbuhan diatas serat kelapa sawit, serat kapas dan habuk kayu getah; tetapi setelah basidioma mula terbentuk, kadar pertumbuhan dan perkembangan basidioma adalah sama, dan tidak bergantung pada jenis substrat. Selain daripada kadar pertumbuhan* Ganoderma, *kajian ini juga memberi kefahaman keatas status warna basidioma matang, potensi ciri perlekatan stip digunakan sebagai nilai taksonomi, dan keperluan kelembapan bandingan persekitaran yang tinggi untuk pengeluaran spora daripada basidiomata aruhan.*

ABSTRACT

Ganoderma *is a causal pathogen of basal stem rot of oil palm. Members of this genus are very diverse in the tropics but the significance of this diversity is difficult to relate to taxonomic levels in the genus, largely because of the lack of knowledge about the stability of particular features of the fungus. This paper outlines a culture method that induces the formation of* Ganoderma *basidiomata under controlled conditions, and thus enables the stability of characters to be evaluated. Using induced basidiomata, selected characteristics were found to be reproducible on 3 solid substrates, palm press fibres (PPF), cotton fibres (CF) and rubberwood sawdust (RSD). The rate of mycelial colonization varied significantly with different substrates but, once formed, the rates of basidioma growth and development were comparable irrespective of substrate type. Besides the growth rates of* Ganoderma, *this study also offers insight into the status of colour in mature basidioma, the potential of stipe attachment as a taxonomic character, and the requirement for high ambient RH values for spore production of induced basidiomata.*

INTRODUCTION

Species of *Ganoderma* are found worldwide as saprophytes on logs and stumps, and occasionally as parasites on trees. In Malaysia and Sumatra, species of this fungus cause serious root and basal stem rot of oil palm (*Elaeis guineensis*), for which an effective means of control is still not available. The bright and 'varnished' appearance of the fruiting body makes it easily recognizable in the field, but identification to species level is difficult.

Reports of pathogenic species on oil palm in Malaysia range from 1 species (Ho and Nawawi 1985) to at least 4 (Steyaert 1976) and as many as 8 (Turner 1981).

Conventional taxonomic approaches are based on characteristics of the fruiting body, which is also referred to as basidioma, basidiocarp, carpophore or sporophore. Current taxonomic keys are mostly concerned with temperate species, whilst those for tropical species are mainly based on dried specimens.

Members of this genus are extremely diverse in the tropics and Ryvaarden (1995) has suggested, but not yet verified, that the taxonomic characters currently used in the identification of *Ganoderma* probably vary under different growing conditions. Mycelial isolations can be made from infected palm tissues or the sporophores themselves, but there is as yet no foolproof method of verifying that these agar cultures belong to *Ganoderma*, because *Ganoderma* cultures on agar do not form fruiting bodies. In addition, all local cultures are white and remain as sterile mycelia, making hyphal characterization of limited use. This study was undertaken to establish a reliable method of inducing the formation and sporulation of *Ganoderma* fruiting bodies on solid substrates to enable the reliability of basidiomata characters to be assessed.

MATERIALS AND METHODS

Fungal Inocula

Two *Ganoderma* isolates were selected based on the different morphological appearance and growth habit of their respective sporophores in the field.

The first sample was EGSP 03, isolated from a sporophore collected from a recently infected eighteen-year-old oil palm at Sri Pelangi Estate, a coastal area in Teluk Intan, in the state of Perak. At the time of collection the EGSP 03 basidioma had a blackish-brown stipe measuring 1.7 cm in length. The main body, or pileus, measured 6.3 cm wide at the broadest part and 4.2 cm long from the distal end to the base of the pileus. The dorsal surface was a bright reddish-brown with tinges of yellow, and had conspicuous concentric growth rings. The margin was light brown. The undersurface, or pore layer, was a light cream colour. The dorsal surface of the pileus was smooth and highly laccate ('varnished') with an index of '5' on a scale of 0 to 5, where 0 indicates a complete absence and 5 the highest degree of lacca deposition. EGSP 03 was identified as *G. boninense* based on *CMI Descriptions of Pathogenic Fungi and Bacteria Paper No. 444.*

The second sample, EGUJ 02, was from Ulu Jempul, an inland area in the state of Pahang, where the sporophore was collected from an oil palm stump which had been in the oil palm plantation for nearly 3 years. The sporophore of EGUJ 02 was sessile and 8.5 cm at the widest part and 5.6 cm in length from the distal margin to the base. The sporophore shape was subungulate; the basal portion was approximately eight times thicker than the distal margin. The basal end measured 3.8 cm from top to bottom and 0.5 cm at the distal end. The dorsal surface was thick, corrugated and uneven with the presence of ridges, which also made barely discernable concentric growth rings. The pileus was uniformly dull buff brown, while the ridges on it were blackish. The pileal surface was matt, with an index of '0' on a scale of 0-5, where 0 indicates a complete absence of lacca deposition. The pore layer was a light cream colour. Based on *CMI Descriptions of Pathogenic Fungi and Bacteria Paper No. 443*, it is probably *G. cf. applanatum.*

Fungal mycelia isolated from the contextual tissues of *Ganoderma* basidioma were used as the source of inoculum. Pieces of tissues measuring 2 × 2 × 4 mm each were cut out from its contextual layer and surface-sterilized in 10% chlorox (NaOCl) for 5 min. A single piece was picked with a flamed scalpel or tweezers and placed in the

centre of a 2% malt extract agar (MEA) culture plate, which had earlier been steam-sterilized at 120°C, 25 psi for 15 min. The inoculated plates were incubated at 28°C (± 1.5°C). Pure mycelia obtained from these isolations were subcultured on MEA slopes in universal bottles as stock cultures and stored at 10°C until required.

Substrate Preparation and Inoculation

The 3 substrates used were empty fruit bunches of oil palm commonly called palm-press fibres (PPF), rubber wood sawdust (RSD) and cotton fibres (CF). PPF was obtained from oil palm plantations, CF from textile industries and RSD from furniture-processing plants. PPF were soaked overnight to saturate the fibres. Rubberwood sawdust and cotton fibres were mixed with 1:1 volume of water. All 3 substrates were then separately drained on fine wire mesh so that the water content was just at saturation point, and packed separately into 15 × 33 × 0.05 mm heat-resistant polypropylene autoclavable bags. The substrate was pressed into compact blocks of approximately 20 cm in height, 12 cm in circumference and 960-1000 g in weight. The free end of the bag was put through a PVC pipe 5 cm long and 3 cm wide to secure the bag, and a cotton ball wrapped in gauze was put through the pipe to close it. These packed bags of substrate were then steam-sterilized at 121°C, 25 psi for 45 min, and were ready for inoculation when sufficiently cooled.

The inoculum starters were prepared by subculturing the isolate from slope cultures on 2% MEA culture plates, incubated at 28°C. A 7-day-old culture colony was selected and aseptically macerated with a flamed scalpel. The PVC tube was pulled off, the bag opened, and the macerated mycelia transferred on to the upper surface of the compacted substrate. The tube was put back in place and the culture bag subsequently closed with the cotton plug, ready for incubation.

Growth and Development on Solid Substrates

Both EGSP 03 and EGUJ 02 isolates were used in this experiment. Data collection was confined to EGSP 03 only, but induced basidiomata from both cultures were used for the morphological study. Mycelial inoculum was prepared as outlined above. One culture was used as inoculum starter for one bag. Ten replicates were made per isolate, per substrate type, and all were incubated at 28°C (±1.5°C). The bags were placed upright to allow maximum colonization of the surface area and inspected daily for mycelial extensions from the top to the lower portion of the bag. When fully colonized, a 2 × 4 cm slit was made in the side of the plastic bag to expose the mycelia, which was then transferred to the mushroom nursery to stimulate basidioma development.

The mushroom nursery consisted of an enclosure surrounded by fine-mesh plastic netting to keep out insects and pests. The ceiling was fitted with a fine-volume water sprinkler, which produced a mist-like spray. The sprinkler automatically turned on for 15 min at 6-hour intervals to maintain the nursery at 90-95% relative humidity (RH). A thermohygrograph was placed in the nursery to check that the ambient temperature was 27-28°C and the RH value was 90-95% throughout the day and night. Once spores were produced, the whole bag was placed in a 20 × 30 × 15 cm perspex box, in the bottom of which was placed a white paper lining to gauge spore production. The top was taped with a muslin cloth to confine spores within the box.

Data collection of EGSP 03 development included rates of fungal growth on 3 substrate types at 3 distinct developmental stages; namely, (i) mycelial establishment

on the substrate, (ii) time taken for basidioma formation and (iii) duration of spore production until senescence sets in.

Morphological Characteristics

Three morphological characteristics were examined in induced basidiomata of EGSP 03 and EGUJ 02. They were: colour of basidioma from stipe to pileus margin, degree of lacca deposition, and attachment of the stipe. Comparisons of these characteristics were made among replicates on PPF, between replicates on different substrates, and between a representative of induced EGSP 03 and EGUJ 02 basidioma and the original fruiting bodies obtained from the field. Another series of observations was made to compare these characteristics in mature basidioma (i.e. aged 3-7 weeks) with young basidioma (aged under 3 weeks, which had not yet produced spores).

RESULTS AND DISCUSSION

Growth on Solid Substrates

EGSP 03 completely colonized all 3 solid substrates at a rate of 13.8^a mm/day on PPF, 12.5^b mm/day on CF and 9.2^c mm/day on RSD (figures with different letters denote a significant difference at $p = 0.05$, using Duncan's multiple range test (DMRT) in the analysis of variance). The colonies were smooth, white and dense on all 3 substrates but on exposure turned brown and crustose and primordial buds arose. The total incubation time needed before the basidioma buds (or primordium) started to appear was fastest on PPF (with a mean of 21.5^a days), followed by CF (at 27.3^b days) and slowest on RSD (35.5^c days).

The buds appeared as a raised, dome-shaped, velvety white structure measuring 5-10 mm at the widest point. The time taken for the primordium to elongate into a slender stipe and to reach a constant length from its initial formation was 3.5^a days on PPF, 3.7^a days on CF and 3.7^a days on RSD. The stipe lengths reached a constant length of 2.5-3.1 cm, and each bore a white tip, the primordium, now reduced in size. From this point onwards the tip expanded to form a flat, bracket-like pileus. The time taken for the basidioma to reach a constant size was 19.2^a days on PPF, 19.3^a days on CF and 19.3^a days on RSD.

Spore Production

Basidioma maturity was marked by spore production, which occurred once the fruiting body reached a constant size. Spores were strongly ejected from the pore layer and fell on the box lining, as well as all over the basidioma surface. The 'spore deposits' were light brown and powder-like on the paper lining; they were also found on the upper surface of the basidioma, giving it a velvety brown appearance.

Spore discharge was light in the first 2 days, becoming very heavy from the third day onwards. Spores were produced between midnight and 3 a.m. Sporulation was profuse for an average of 7 weeks, irrespective of substrate type. By the 8th week, spore production started to decrease and had stopped completely by the 10th week.

Morphological Characteristics

Induced EGSP 03 basidiomata were stipitate for all replicates on all 3 substrates. A mean reading for 5 induced EGSP 03 mature basidiomata showed that stipe length was 1.7 (±0.02) cm. The pileus measured 5.3 (±0.13) cm at the widest part and was 3.4 (±0.08) cm long from the distal margin to the base of the pileus. The margin was slightly rounded ('flabelliform'), measuring 8 (±0.12) cm in thickness increasing to 1.75 (±0.07) cm at the pileus base.

Induced EGSP 03 had a blackish-brown stipe. The pileus was smooth, flat

and dimidiate. The pileus was reddish-orange with a very wide white margin. This white margin disappeared completely in mature basidioma. The dorsal surface of mature basidioma was a bright reddish-brown with a band of yellow on the outermost part. It had conspicuous concentric growth rings and a light red-brown margin. The pore layer was light cream. The young basidioma (pre-spore production) was more rounded in shape. The dorsal surface in both young and mature basidiomata was highly laccate with an index of 5. The colour of mature basidiomata was similar for all replicates on PPF, was reproducible between replicates on all 3 substrates and matched those collected from the field. The basidioma gradually darkened in colour once spore production had stopped. By the 12th week, the sporophore had lost its original colour and turned uniformly blackish-brown; the dorsal surface became dull, and the laccate index decreased from 5 to 1. The dead basidioma was dull, dry and brittle.

Induced EGUJ 02 basidiomata were sessile and applanate (i.e. of uniform thickness from base to pileus) on all 3 substrates. The young basidioma (pre-spore production) was a buff light brown colour from base to pileus with a distinct white margin all round. In mature basidioma, the pileus was the same buff, light-brown colour, but the white margin had disappeared. The dorsal surface was matt, scoring '0' on the laccate index in both young and mature basidiomata. EGUJ 02 was of approximately uniform thickness from base to margin in both young and mature basidiomata and measured 8.6 (±0.05) mm in thickness in the latter. The basidiomata colour of induced EG UJ 02 did not vary significantly within replicates and between replicates on different substrates, nor in young and mature basidiomata, and matched those collected from the field. However, there was a complete mismatch in shape for EGUJ 02. All induced EGUJ 02 were applanate irrespective of substrate type, but the original specimen collected from the field was subungulate with the basal portion approximately 8 times the thickness at the margin. Except for the reduction in size for EGSP 03, no such morphological differences were observed.

CONCLUSION

All 3 substrates supported the growth and development of *Ganoderma* basidiomata. The rates of mycelial colonization varied significantly from one substrate to the other but the rate of basidiomata growth and development was relatively uniform and independent of substrate type.

Morphological examinations showed that size and shape of *Ganoderma* basidiomata are not good taxonomic characters. Basidioma colour holds potential but has to be treated with caution as the character is not stable in young, pre-sporulating *Ganoderma boninense*, whilst it is homogeneously blackish in old, post-sporulating specimens.

However, in mature and actively-sporulating *Ganoderma boninense* basidioma (i.e aged 3-7 weeks), colour is a constant and reproducible characteristic, regardless of substrate type. Attachment of stipe holds potential as a taxonomic character. Steyaert (1976) observed that attachment of stipe may be dependent on point of insertion, whilst Ryvaarden (1994) observed that it seemed to be a consistent character in the *Ganoderma lucidum* group in Europe.

Spore production in induced basidiocarps occurs only once in its life span. Under normal circumstances, it shows a diurnal pattern of spore release, which continued for approximately 7 weeks. An RH value exceeding 90% at an ambient temperature of 28°C was essential to

stimulate spore production.

In conclusion, development of a culture technique to induce basidioma formation on solid substrates allows investigation into the growth habits of *Ganoderma* and provides a means of verifying whether particular characters are stable and are of taxonomic value.

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In Vitro Responses of *Dracaena fragrans* cv. Massangeana to Growth Regulators

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Keywords: *Dracaena fragrans*, Murashige and Skoog (MS) medium, BAP, NAA, 2,4-D, shoot formation, callus, rooting

ABSTRAK

Kajian in vitro *ke atas* Dracaena fragrans *cv. Massangeana mendapati segmen batang muda berupaya membentuk pucuk diatas media pepejal Murashige dan Skoog (MS) yang mengandungi berbagai kombinasi dan paras BAP dan NAA. Peratus eksplan membentuk pucuk paling tinggi diperolehi pada medium yang ditambah dengan 3.0 mg/l BAP dan 0.1 mg/l NAA. Jumlah pucuk se eksplan paling tinggi berlaku pada medium yang mengandungi 2.0 mg/l BAP sahaja. Peratus pembentukan kalus dan min berat basah kalus dari segmen batang muda adalah paling tinggi pada medium MS yang dibekalkan dengan 1.0 mg/l 2,4-D. Akar adventitius terbentuk selepas pucuk dialih ke medium MS tanpa hormon. Pengakaran adalah 100% bagi pucuk yang dialih dari medium yang mengandungi 0-2.0 mg/l BAP dan kepekatan NAA yang rendah (0.1 mg/l).*

ABSTRACT

In vitro *studies on* Dracaena fragrans *cv. Massangeana revealed that young stem segments were capable of proliferating shoots on agar-solidified Murashige and Skoog (MS) basal medium containing different combinations and concentrations of BAP and NAA. Highest percentage of explants forming shoots was obtained on medium supplemented with 3.0 mg/l BAP and 0.1 mg/l NAA. The highest number of shoots per explant occurred on medium containing 2.0 mg/l BAP only. Highest percentage of callus formation and highest mean fresh weight of callus from young stem segments were achieved on MS medium supplemented with 1.0 mg/l 2,4-D. Adventitious rooting occurred after transferring excised shoots onto a hormone-free MS medium. Rooting was 100% for shoots derived from media with 0-2.0 mg/l BAP and a relatively low concentration of NAA (0.1 mg/l).*

INTRODUCTION

Dracaenas are woody monocotyledons belonging to the family Agavaceae. They are popular foliage ornamentals in tropical and temperate regions and are highly desirable as indoor plants and for outdoor landscaping. *Dracaena fragrans* cv. Massangeana, characterized by its sword-shaped dark green leaves with a yellow stripe running along the centre, is among the dracaena cultivars fast gaining the attention of commercial growers. However, propagation by conventional methods is slow and therefore production of clonal plants by tissue culture will be useful to meet the increasing demand for planting material. Establishment of a plant regeneration system through direct organogenesis or via callus is also a prerequisite to further *in vitro* genetic manipulation of the

cultivar. Tissue culture procedures have been described for *D. godseffiana* (Miller and Murashige 1976), *D. marginata* cv. Tricolor (Chua *et al.* 1981), *D. deremensis* cv. Warneckii (Debergh 1975), *D. congesta* and *D. invisa* (Debergh and Maene 1981) and green-foliage *D. fragrans* (Dragan 1989). There is no report on *D. fragrans* cv. Massangeana. The present paper aims to assess the *in vitro* response of *D. fragrans* cv. Massangeana to various growth regulators.

MATERIALS AND METHODS

Vigorously growing shoots taken from 1-2 year-old plants, with leaves removed, were washed under running tap water for 30 min. They were surface-sterilized for 20 min in 10% v/v Clorox with a few drops of Tween 20 emulsifier, and rinsed in five changes of sterile distilled water. The material was further immersed in 5% v/v Clorox for 2 min, rinsed five times with sterile distilled water and finally cut into segments 3-4 mm thick. Explants were cultured individually on 10 ml of Murashige and Skoog (MS) (1962) medium with 3.0% sucrose, 0.7% Bacto-agar and the appropriate combinations and concentrations of auxin and/or cytokinin. For shoot induction, 0-0.3 mg/l a-napthaleneacetic acid (NAA) in combination with 0-30 mg/l benzylaminopurine (BAP) was used. For the initiation and proliferation of callus, stem explants were cultured on MS medium containing 0.5-2.0 mg/l 2,4-dichlorophenoxy-acetic acid (2,4-D), and on MS medium containing 0.5 mg/l BAP in combination with 1.0 and 2.0 mg/l indolebutyric acid (IBA). Shoots which attained 5-15 mm in height were separated and cultured on a hormone-free MS medium to promote root formation and shoot elongation. All cultures were incubated under a 16-hour photoperiod using white fluorescent tubes (Philips, TLD 36w/54) at an irradiance of 65 uE m^{-2} s^{-1} and a temperature of 27 ± 1°C. The experiments were arranged in a completely randomized design with three replications for each treatment and four explants per replicate. Each experiment was repeated three times. *In vitro* regenerated plantlets were removed from culture vessels, washed thoroughly to remove traces of nutrient medium and planted in small pots containing a mixture of vermiculite and sand (1:1). For the first week of transfer the plantlets were covered with plastic perforated with small holes to maintain a high humidity. Plantlets were subsequently transferred to larger pots and placed outdoors.

RESULTS

Effect of BAP and NAA on Shoot Formation

Within 20 days of culture, adventitious buds were induced at the cut surface of stem segments placed on various combinations of BAP and NAA. In most explants, moderate callus formation preceded bud formation. On medium supplemented with a higher level of BAP (3.0 mg/l) but without NAA, adventitious buds were induced directly from the explants without an intervening callus. Shoots developed from the adventitious buds by week 7.

Table 1 summarizes the effect of BAP and NAA on shoot formation from stem explants of *D. fragrans* cv. Massangeana after 8 weeks in culture. Shoot formation occurred in all treatments including the control. In treatments containing 0.1 mg/l NAA the percentage of shoot formation increased with increasing levels of BAP, with 3.0 mg/l BAP producing the highest percentage of explants with shoots (88.8%). The highest number of shoots per explant (1.44 ± 0.10) occurred on medium containing 2.0 mg/l BAP but without NAA. Highest mean length of shoots (3.68 ± 1.47 mm) was also attained on the same medium (Table 1).

TABLE 1
Effect of BAP and NAA on shoot formation from stem segments of *Dracaena fragrans* cv. Massangeana at week 8

BAP (mg/l)	NAA (mg/l)	Explants with shoots (%)	Number of shoots per explant	Mean length of shoots (mm)
0	0	44.4	0.77 ± 0.29	1.94 ± 0.63
1.0	0	22.2	0.22 ± 0.10	1.99 ± 1.26
2.0	0	77.7	1.44 ± 0.10	3.68 ± 1.47
3.0	0	22.2	0.22 ± 0.10	0.33 ± 0.19
0	0.1	44.4	0.44 ± 0.10	1.21 ± 0.55
1.0	0.1	55.5	0.88 ± 0.22	2.47 ± 0.67
2.0	0.1	66.6	0.66 ± 0.19	2.22 ± 0.48
3.0	0.1	88.8	0.88 ± 0.11	1.44 ± 0.48
0	0.3	22.2	0.55 ± 0.10	0.33 ± 0.19
1.0	0.3	66.6	0.66 ± 0.01	1.77 ± 0.48
2.0	0.3	55.5	0.55 ± 0.22	1.10 ± 0.61
3.0	0.3	66.6	0.66 ± 0.01	1.66 ± 0.57

Values given are ± standard errors

TABLE 2
Callus induction on stem explants of *Dracaena fragrans* cv. Massangeana at week 6

Concentration (mg/l)	Explants with callus (%)	Mean fresh weight of callus (g)	Response
0.5 2,4-D	77.7	0.075a	fc
1.0 2,4-D	88.8	0.135a	hfn
2.0 2,4-D	44.4	0.082a	fcn
0.5 BAP + 1.0 IBA	77.7	0.095a	hcn
0.5 BAP + 2.0 IBA	55.5	0.046a	hn

Values with a similar letter in a column indicate no significant difference at 5% probability level.

Key to Table: h = hard, f = friable, c = chlorophyllous, n = non-chlorophyllous

Effect of 2,4-D and Combinations of BAP and IBA on Callus Initiation and Proliferation

Response of stem explants after six weeks of culture on media with different concentrations of 2,4-D, and combinations of BAP and IBA is shown in Table 2. Callus was initiated at the cut surface of explants by week 2 and was obtained in all treatments. Highest percentage of explants with callus and the highest mean fresh weight of callus

were obtained on medium with 1.0 mg/l 2,4-D. Mean fresh weight of callus produced did not differ significantly among the treatments. The callus produced on medium with 1.0 mg/l 2,4-D were either hard or friable and non-chlorophyllous. At a higher level of 2,4-D (2.0 mg/l) callus produced were friable, mostly yellowish, slow growing and gradually turned brown after the third week of culture. MS medium with 0.5 mg/l BAP and 1.0 mg/l IBA produced callus which were hard and which turned green after 6 weeks in culture. Shoot differentiation from callus was observed in both media containing 0.5 mg/l BAP with 1.0 mg/l IBA and 0.5 mg/l BAP with 2.0 mg/l IBA by the fifth week of culture.

Rooting

In vitro proliferated shoots excised and transferred to hormone-free MS medium rooted by week 2. Table 3 shows the effect of BAP and NAA concentrations (used previously to induce the shoots) on rooting. Shoots derived from media with 0.1 mg/l NAA and BAP 0-2.0 mg/l showed 100% rooting on transfer to hormone-free MS medium. Rooting was also 100% with shoots derived from a previously hormone-free medium (control). Shoots induced on media with 1.0-2.0 mg/l BAP and a higher NAA concentration (0.3 mg/l) showed a lower rooting response in the hormone-free MS medium. It is also evident from Table 3 that roots generally tend to be shorter as their number increases.

DISCUSSION

Shoot formation from stem explants of *D. fragrans* cv. Massangeana occurred in all combinations of BAP and NAA tested, including the control. An increasing trend in shoot formation was attained when 0.1 mg/l NAA was added to the increasing BAP levels, indicating a stimulatory effect of the auxin at a relatively low concentration. In media without NAA and in media supplemented with a higher level (0.3 mg/l) of NAA, shoot formation was variable. In all treatments except in a medium with a

TABLE 3
Rooting response of shoots on hormone-free MS medium

Previous treatment		% of shoots with roots	Mean number of roots per plantlet	Mean length of roots (mm)
BAP (mg/l)	NAA (mg/l)			
0	0	100.0	1.33 ± 0.33	15.17 ± 5.29
1.0	0	33.3	2.00 ± 0.01	9.00 ± 0.01
2.0	0	50.3	0.50 ± 0.40	17.50 ± 4.28
3.0	0	66.3	1.00 ± 0.10	26.50 ± 5.30
0	0.1	100.0	1.00 ± 0.10	16.67 ± 5.69
1.0	0.1	100.0	2.00 ± 0.57	11.33 ± 3.81
2.0	0.1	100.0	1.66 ± 0.01	19.50 ± 1.89
3.0	0.1	–	–	–
0	0.3	–	–	–
1.0	0.3	50.0	1.33 ± 0.66	18.33 ± 9.27
2.0	0.3	33.3	2.00 ± 0.01	10.50 ± 0.10
3.0	0.3	–	–	–

Values given are ± standard errors
- indicates no shoots were transferred

higher BAP concentration (3.0 mg/l) and without NAA, moderate callus formation preceded shoot formation. The production of shoot and callus even on the hormone-free medium strongly implies the presence of endogenous hormones within the stem tissues of *D. fragrans* cv. Massangeana. Ease of shoot and callus formation from stem explants in comparison to leaf and shoot tip explants has been reported in *D. deremensis* cv. Warneckii (Debergh 1975).

Dracaenas are among the few monocotyledons which possess a cambium in their stems (Zimmermann and Tomlinson 1970; Esau 1977). The occurrence of the cambium may have facilitated shoot and callus induction from stem explants of *D. fragrans* cv. Massangeana.

In the study on callusing ability of stem segments of *D. fragrans* cv. Massangeana, 2,4-D alone or combinations of BAP and IBA were effective in stimulating callus formation. Mean fresh weight of callus did not differ significantly among the treatments, but the percentage of callus formation varied. The gradual browning of callus observed on 2.0 mg/l 2,4-D indicated the deleterious effect of the auxin at this concentration. Other researchers have reported a similar effect of 2,4-D at 2.0 mg/l and above on other dracaena species and cultivars investigated (Debergh 1975; Chua *et al.* 1981; Dragan 1989). Debergh (1975) showed the addition of 1.0 or 2.0 mg/l kinetin to 2.0 mg/l 2,4-D could neutralize the effect of 2,4-D, resulting in a more organized type of callus.

Rooting of *in vitro* shoots of *D. fragrans* cv. Massangeana was achieved on a hormone-free MS medium. Successful rooting of other dracaena species required the inclusion of low levels of auxins in the rooting medium such as IBA (0.1-2.0 mg/l) or NAA (0.1-1.0 mg/l) (Debergh 1975; Chua *et al.* 1981; Dragan 1989), or using a low salt MS medium (Debergh and Maene 1989). This implies a species-specific rooting behaviour of dracaena species under *in vitro* conditions. It is also evident that a relatively low supplement of auxin (0.1 mg/l NAA) to BAP (0-2.0 mg/l) in the shoot induction and proliferation medium has a stimulatory effect on rooting of *in vitro* shoots of *D. fragrans* cv. Massangeana when placed on a hormone-free medium.

The present study demonstrates the totipotent capacity of stem segments of *D. fragrans* cv. Massangeana to regenerate plantlets *in vitro*.

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Antiviral and Cytotoxic Activities of Some Plants Used in Malaysian Indigenous Medicine

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ABSTRAK

Ekstrak etanol 61 tumbuhan perubatan yang digunakan di Malaysia telah disaring untuk aktiviti antivirus and sitotoksik. Aktiviti antivirus telah diuji terhadap virus "herpes simplex"-jenis 1 (HSV-1), dan "vesicular stomatitis" (VSV), dan ujian sitotoksik dijalankan menggunakan sel-sel HeLa. Ekstrak Calotropis gigantea, Costus speciosus, Eugenia michelii, Hedyotis auricularia, Mentha arvensis, Orthosiphon aristatus, Polygonum minus *dan* Ricinus communis *menunjukkan aktiviti perencatan terhadap kedua-dua virus (MIC: 0.002-0.1 mg/ml). Aktiviti antivirus khusus terhadap virus HSV-1 telah ditunjukkan oleh ekstrak* Alternanthera sessilis, Blumea chinensis, Eleusine indica, Euphorbia hirta, Freycinetia malaccensis, Leea indica *dan* Solanum americanum *(0.001-0.1 mg/ml). Ekstrak* Acalypha indica, Bertholletia excelsa, Cerbera manghas, Codiaeum variegatum, Plectranthus amboinicus, Centella asiatica, Mirabilis jalapa, Morinda elliptica, Oenanthe javanica, Piper sarmentosum *dan* Premna odorata *menunjukkan aktiviti antivirus khusus terhadap virus VSV (MIC: 0.005-0.1 mg/ml). Aktiviti sitotoksik pula hadir dalam ekstrak* Acalypha indica, Andrographis paniculata, Centella asiatica, Cerbera manghas, Codiaeum variegatum, Cosmos caudatus, Elephantopus scaber, Etlingera elatior, Eugenia michelii, Freycinetia malaccensis, Hibiscus rosa-sinensis, Lecythis ollaria, Mentha arvensis, Mirabilis jalapa, Morinda elliptica, Ocimum tenuiflorum, Piper sarmentosum *dan* Polygonum minus *(CD_{50}: 0.001-0.1 mg/ml). Kedua-dua aktiviti antivirus and sitotoksik ditunjukkan oleh ekstrak* Eugenia michelii, Mentha arvensis *dan* Polygonum minus.

ABSTRACT

Ethanolic extracts of 61 medicinal plants used in Malaysia were screened for antiviral and cytotoxic activities. Antiviral activity was tested against the herpes simplex type-1 (HSV-1) and vesicular stomatitis (VSV) viruses, and cytotoxicity was assayed using the HeLa cell line. Antiviral activity against both viruses was present in the extracts from Calotropis gigantea, Costus speciosus, Eugenia michelii, Hedyotis auricularia, Mentha arvensis, Orthosiphon aristatus, Polygonum minus *and* Ricinus communis *(MIC: 0.002-0.1 mg/ml). The extracts from* Alternanthera sessilis, Blumea chinensis, Eleusine indica, Euphorbia hirta, Freycinetia malaccensis, Leea indica *and* Solanum americanum *were active in selectively inhibiting HSV-1 (0.001-0.1 mg/ml). Selective activity against VSV was shown by the extracts from* Acalypha indica, Bertholletia excelsa, Cerbera manghas, Codiaeum variegatum, Plectranthus amboinicus, Centella asiatica, Mirabilis jalapa, Morinda elliptica, Oenanthe javanica, Piper sarmentosum *and* Premna odorata *(MIC: 0.005-0.1 mg/ml). Cytotoxic activity was present in the extracts from* Acalypha indica, Andrographis paniculata, Cerbera manghas, Codiaeum variegatum, Cosmos caudatus, Elephantopus scaber, Etlingera elatior, E ugenia michelii, Freycinetia malaccensis, Hibiscus rosa-sinensis,

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Centella asiatica, Lecythis ollaria, Mentha arvensis, Mirabilis jalapa, Morinda elliptica, Ocimum tenuiflorum, Piper sarmentosum *and* Polygonum minus *(CD50: 0.001-0.1 mg/ml).Co-existing antiviral and cytotoxic activities were shown by* Eugenia michelii, Mentha arvensis *and* Polygonum minus.

INTRODUCTION

Plants are an important source of therapeutics from which 25% of the pharmaceuticals in current use have been derived (Farnsworth and Bingel 1977). However, of the estimated 250,000 species of higher plants existing throughout the world, only a fraction have been examined for pharmacological activities (Balick 1990). Phytotherapeutics exhibit a wide range of pharmacological activities, including anticancer and antiviral activities (Farnsworth and Kaas 1981; Hudson 1989). Anticancer drugs, such as the indole alkaloids vincristine and vinblastine, and podophyllotoxin derivatives etoposide and teniposide, are prominent chemotherapeutics of plant origin which were obtained either directly through isolation or derived from lead structures (Arcamone *et al.* 1980). Therefore, the screening of higher plants for antiviral and anticancer agents has been actively pursued on an international scale, especially by the US National Cancer Institute (Farnsworth and Kaas 1981; Hudson 1989). Furthermore, mammalian cell culture systems have greatly aided the routine screening of plant extracts and compounds for anticancer activity using cytotoxicity and antiviral activity, which previously relied upon time-consuming, expensive and cumbersome *in-vivo* models. These screening efforts have resulted in the discovery of several prospective antiviral and anticancer compounds currently undergoing clinical trials. Taxol is the most notable example of these compounds (Wiernik *et al.* 1987).

Although extensive phytochemical surveys have been carried out on the flora of Malaysia (Goh *et al.* 1993 and references cited therein), only a few reports deal with screening for pharmacological activities such as antimicrobial, antitumour, antitumour-promoting and cardiovascular-related activities (Nakanishi *et al.* 1965; Yadav *et al.* 1989; Ali *et al.* 1995; Goh *et al.* 1995). Reports by Teo *et al.* (1990) and references cited therein, Ahmad *et al.* (1992, 1993), Chan *et al.* (1992), Kashman *et al.* (1992), Mahmud *et al.* (1993), Patil *et al.* (1993), Alias *et al.* (1995), Ali *et al.* (1996), and Wong and Tan (1996) are examples of studies confined to the antiviral and cytotoxic activities of extracts and compounds from one or two plant species; consequently, these studies cannot be considered as screening reports.

In the present work we screened 61 local and introduced plant species widely used as anti-infective and anticancer agents in Malaysian indigenous medicine (traditional, ethno- and folk-medicine) for antiviral and cytotoxic activities. We adopted an ethnopharmacological approach to screening because it is more likely to yield a higher number of plants with significant biological activity than screening by random selection (Balick 1990). The ethanolic extracts of these medicinal plants were tested for antiviral activities against both herpes simplex type-1 (DNA type) and vesicular stomatitis (RNA type) viruses using Vero cells, and the cytotoxicity assay was done using the HeLa (human cervical carcinoma) cell line.

MATERIALS AND METHODS

Plants

Plant parts were collected from the Medicinal Plant Garden, Universiti Pertanian Malaysia, and identified by A. Ghani Yunus.

Plant Extracts

Samples (20 mg) of leaves from each plant (and fruits from *Cerbera manghas*) were sliced into small pieces (*ca.* 1 cm × 1 cm) and macerated in 60 ml of 80% (v/v) ethanol. After being left for one week at room temperature, the extracts were filtered using Whatman No. 1 filter paper and then evaporated at 40°C under vacuum. The residues were then stored as stock solutions of 10 mg/ml in 90% (v/v) ethanol at 4°C.

Cultivation of Cells

Vero and HeLa cell lines were obtained from the RIKEN Cell Bank, Tsukuba, Japan and cultured in RPMI-1640 medium supplemented with 5% (v/v) foetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin as a complete growth medium (CGM). Cells were maintained in 25 cm^2 flask with 10 ml of CGM at 37°C with 5% (v/v) CO_2 until attaining confluence. Confluent cells were removed from the surface of the flask by treatment with 1 ml of 0.025% (w/v) trypsin prepared in phosphate-buffered saline (PBS) solution. CGM was then added to the trypsin-treated cells to achieve a cell concentration of 1-2 × 10^4 cells/ml.

Virus Stocks

Herpes simplex virus type-1 (HSV-1) and vesicular stomatitis virus (VSV), which are DNA and RNA virus type respectively, were obtained from the Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minnesota, USA. Virus stocks were prepared as aliquots of culture medium from Vero cells infected at a multiplicity of infection of 0.1 and cultured for 3 days at 37°C. These aliquots were subsequently stored at -70°C. Working stocks of virus were prepared by serially diluting in culture medium (RPMI-1640) virus stocks to the end-points required for each virus. Serial dilutions of virus stocks in RPMI-1640 medium were assayed to their end-points using Vero monolayers in microtitre plates. These virus working stocks were stored at 4°C until further use.

Antiviral Assay

The antiviral test was performed according to the simplified plaque reduction assay (Abou-Karam and Shier 1990). Microtitre plates with confluent monolayer cultures of Vero cells were inverted to remove spent medium. In triplicate, each well was filled with 100 µl of plant extract serially diluted in RPMI-1640 medium. This was followed by the addition of 100 µl of medium containing *ca.* 30 plaque forming units (pfu) of HSV-1 or 10 pfu of VSV, per well of confluent Vero cells. In each plate, wells in the last row were used for controls, which consisted of two treatments: (1) cells not treated with plant extracts and virus, and (2) cells treated only with virus. The plates were incubated for 66 h (HSV-1) and 36 h (VSV) at 37°C, with care taken not to disturb the culture during incubation. Antiviral activity was then scored using an inverted microscope (low power) as the non-cytotoxic minimum inhibitory concentration (MIC, mg/ml) which totally prevented cytopathic effects (CPE).

Cytotoxicity Assay

The assay used was the microtitration cytotoxicity assay (Shier 1983). Varying concentrations of the plant extracts were prepared from the stock solutions by serial dilution in RPMI-1640 medium to give a total volume of 100 µl in each well. Each well was filled with 100 µl of HeLa cell suspension in CGM at 1-2 × 10^4 cells/ml. Controls containing only HeLa cells were included for each sample. The assay for each concentration of plant extract was performed in triplicate and the culture plates were kept at 37°C with 5% (v/v)

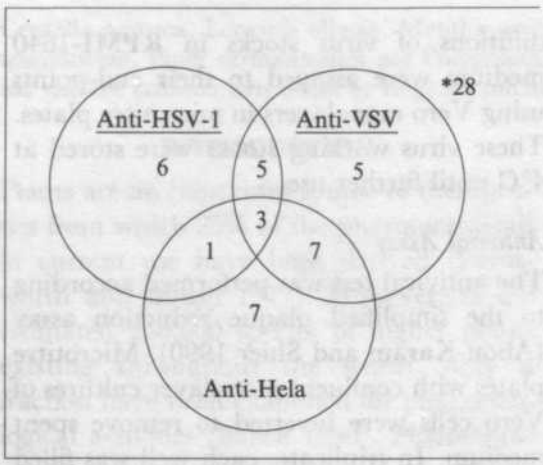

*Fig. 1. The number of plant species showing antiviral and cytotoxic activities are in the circles. Overlapping circles indicate co-existing activities. (*Species that did not show any activity)*

CO_2 for 4 days. Using an inverted microscope (low power), cytotoxicity was determined as the concentration of plant extract which reduced cell number by *ca.* 50% with reference to the control (CD_{50}, mg/ml).

RESULTS AND DISCUSSION

The overall results of the 61 plants from 33 families screened for antiviral and cytotoxic activities are summarized in Fig. 1. Table 1 lists the 28 species (46%) that gave negative results for all three tests. Table 2 lists the 26 species (43%) that exhibited antiviral activity and the 18 species (30%) which showed cytotoxicity.

Eight species (13%) (*Calotropis gigantea, Costus speciosus, Eugenia michelii, Hedyotis auricularia, Mentha arvensis, Orthosiphon aristatus, Polygonum minus* and *Ricinus communis*) showed antiviral activity against both HSV-1 and VSV. The extracts of *Calotropis gigantea, Eugenia michelii* and *Ricinus communis* showed a similar MIC value of 0.01 mg/ml against both viruses, but only *Eugenia michelii* demonstrated cytotoxicity (CD_{50}: 0.05 mg/ml). In the case of *Polygonum minus*, a similar MIC value against HSV-1 was obtained but lesser activity was shown against VSV (MIC: 0.02 mg/ml) and HeLa cells (CD_{50}: 0.1 mg/ml). Very strong anti HSV-1 but weak anti-VSV and cytotoxic activities were observed in the *Mentha arvensis* extract (MIC: 0.002 mg/ml & 0.1 mg/ml; and CD_{50}: 0.1 mg/ml, respectively). On the other hand, strong anti-VSV but weak anti HSV-1 activities were displayed by the extracts of *Costus specious* and *Hedyotis auricularia*, while weak antiviral activity against both viruses was observed in the extract of *Orthosiphon aristatus* (MIC: 0.1 mg/ml).

Selective antiviral activity towards only

TABLE 1
Species failing to show either antiviral or cytotoxic activity

Family	Species
Amaranthaceae	*Aerva lanata* (L.) Juss.
Anacardiaceae	*Spondias cytherea* Sonnerat
Apocynaceae	*Hunteria zeylanica* (Retz.) Gardn. & Thw.
	Plumeria rubra L.
Bixaceae	*Bixa orellana* L.
Bombaceae	*Ceiba pentandra* Gaertn.
Commelinaceae	*Tradescantia spathacea* Sw.
Euphorbiaceae	*Euphorbia neriifolia* L.
Gramineae	*Cymbopogon citratus* (DC.) Stapf
Labiatae	*Plectranthus scutellaroides* (L.) R. Br.
Leguminosae	*Caesalpinia pulcherrima* (L.) Sw.
	Cassia alata L.
	Cassia fistula L.
Liliaceae	*Allium schoenoprasum* L.
Lythraceae	*Lawsonia inermis* L.
Menispermaceae	*Tinospora crispa* (L.) Hook. *f.* & Th.
Myrtaceae	*Eugenia polyantha* Wight
Oxalidaceae	*Averrhoa carambola* L.
Piperaceae	*Peperomia pellucida* Kunth
	Piper nigrum L.
Plantaginaceae	*Plantago major* L. s.l.
Rubiaceae	*Gardenia augusta* (L.) Merr.
Sapotaceae	*Mimusops elengi* L.
Scrophulariaceae	*Picria fel-terrae* Lour.
Solanaceae	*Datura metel* L
	Datura inoxia Mill.
Zingiberaceae	*Alpinia galanga* (L.) Sw.
	Curcuma mangga Val. & Van Zyp,

TABLE 2
The effect of plant extracts on cells as shown by minimum inhibitory concentration values against herpes simplex virus-type 1 and vesicular stomatitis virus, and CD_{50} values towards HeLa cells

Plant	HSV-1	VSV	Cytotoxicity
	MIC[a] (mg/ml)		CD_{50}[b] (mg/ml)
Acanthaceae			
Andrographis paniculata Nees	-ve	-ve	0.1
Amaranthaceae			
Alternanthera sessilis (L.) DC.	0.001	-ve	-ve
Apocynaceae			
Cerbera manghas L.			
Fruits	-ve	0.05	0.001
Leaves	-ve	0.1	0.02
Asclepiadaceae			
Calotropis gigantea R. Br.	0.01	0.01	-ve
Compositae			
Blumea chinensis DC.	0.005	-ve	-ve
Cosmos caudatus Kunth	-ve	-ve	0.1
Elephantopus scaber L.	-ve	-ve	0.05
Euphorbiaceae			
Acalypha indica L.	-ve	0.01	0.01
Codiaeum variegatum (L.) Bl.	-ve	0.1	0.1
Euphorbia hirta L.	0.1	-ve	-ve
Ricinus communis L.	0.01	0.01	-ve
Gramineae			
Eleusine indica (L.) Gaertn.	0.1	-ve	-ve
Labiatae			
Plectranthus amboinicus (Lour.) Spreng.	-ve	0.1	-ve
Mentha arvensis L.	0.002	0.1	0.1
Ocimum tenuiflorum L.	-ve	-ve	0.1
Orthosiphon aristatus (Bl.) Miq.	0.1	0.1	-ve
Leeaceae			
Leea indica (Burm. f.) Merr.	0.05	-ve	-ve
Malvaceae			
Hibiscus rosa-sinensis L.	-ve	-ve	0.1
Myrtaceae			
Eugenia michelii Lamk.	0.01	0.01	0.05
Lecythidaceae			
Bertholletia excelsa Hump. & Bonpl.	-ve	0.005	-ve
Lecythis ollaria L.	-ve	-ve	0.1

Nyctaginaceae			
Mirabilis jalapa L.	-ve	0.05	0.1
Pandanaceae			
Freycinetia malaccensis Ridl.	0.05	-ve	0.1
Piperaceae			
Piper sarmentosum Roxb.	-ve	0.02	0.1
Polygonaceae			
Polygonum minus Huds.	0.01	0.02	0.1
Rubiaceae			
Hedyotis auricularia L.	0.1	0.05	-ve
Morinda elliptica Ridl.	-ve	0.1	0.003
Solanaceae			
Solanum americanum Mill.	0.1	-ve	-ve
Umbelliferae			
Centella asiatica (L.) Urb.	-ve	0.1	0.1
Oenanthe javanica DC.	-ve	0.02	-ve
Verbenaceae			
Premna odorata Blanco	-ve	0.05	-ve
Zingiberaceae			
Costus speciosus (Koenig) Smith	0.1	0.02	-ve
Etlingera elatior (Jack) R. M. Smith	-ve	-ve	0.1

[A]MIC = minimum inhibitory concentration, i.e. the lowest concentration of plant extract which completely inhibited virus replication.

[B]CD_{50} = cytotoxic dose at 50%, i.e. the concentration of plant extract which reduced the number of HeLa cells by 50%.

HSV-1 was seen in extracts from 7 plants (11%) (in order of decreasing activity, *Alternanthera sessilis, Blumea chinensis, Freycinetia malaccensis, Leea indica, Euphorbia hirta, Eleusine indica*, and *Solanum americanum*) with MIC values within the range of 0.001-0.1 mg/ml. Conversely, 11 plant (18%) extracts possessed selective antiviral activity against VSV (in order of decreasing activity, *Bertholletia excelsa, Acalypha indica, Piper sarmentosum, Oenanthe javanica, Mirabilis jalapa, Premna odorata, Cerbera manghas, Codiaeum variegatum, Plectranthus amboinicus, Centella asiatica* and *Morinda elliptica*) with MIC values from 0.005 - 0.1 mg/ml. More plant extracts were active against VSV than HSV-1. The selective antiviral activity of some plant extracts against either HSV-1 or VSV implicates the involvement of different mechanisms of action exploiting the difference in nucleic acid composition of the viruses.

In the case of the anti HSV-1 species, only *Freycinetia malaccensis* showed cytotoxicity (CD_{50}: 0.1 mg/ml) whereas 7 of the anti-VSV extracts (in order of decreasing activity, *Cerbera manghas, Morinda elliptica, Acalpyha indica, Centella asiatica, Codiaeum variegatum, Mirabilis jalapa* and *Piper sarmentosum*) showed cytotoxicity ranging from CD_{50} 0.001-0.1 mg/ml. Since VSV is a RNA-type virus, the concomitant anti-VSV and cytotoxic activities may involve a

related mode of action, most probably via protein interaction. Co-existing antiviral and cytotoxic activities were found in the extracts of three species (5%), i.e. *Eugenia michelii, Mentha arvensis* and *Polygonum minus*.

Of the 18 plants showing cytotoxicity, only 3 species (*Acalypha indica, Cerbera manghas* and *Morinda elliptica*) showed significant activity below the cut-off value of 0.02 mg/ml suggested by Wall *et al.* (1987) and all three species exhibited anti-VSV activity. The strongest cytotoxic activity was shown by the fruits of *Cerbera manghas* (CD_{50}: 0.001 mg/ml). The fruits of *Cerbera manghas* always exhibited stronger cytotoxic (20 times) and anti-VSV activities (twice the activity) than its leaves. This suggests that a higher concentration of the bioactive compound(s) is present in the fruits of *Cerbera manghas* than the leaves.

The *in vitro* cytotoxicity displayed by the plant extracts tested is an initial indicator of *in vivo* antitumour activity. However, since a wide range of phytocompounds are capable of exhibiting non-specific cytotoxicity, plant extracts with significant cytotoxic activity should be further assayed using animal models to confirm antitumour activity, and/or a battery of various cell lines to detect specific-cytotoxicity. This step is necessary to eliminate cytotoxic compounds with little value for further investigation as anticancer agents.

CONCLUSION

The results of this preliminary study scientifically substantiate to a certain extent the pharmacological activities of 33 plants used in Malaysian indigenous medicine and point out some plants with potential for further investigation. In addition, these results may also contribute towards the documentation of pharmacological profiles of Malaysian plants for conservation efforts and protection of biodiversity rights. Inadequate recording of the pharmacological activities of Malaysian plants may lead to the commercial exploitation of traditional knowledge by foreign parties without any benefit to the country as experienced by India in the case of the Neem tree and turmeric (Agarwal and Narain 1996).

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Modification of Soil Structure of Sand Tailings: 2. Effect of Silt, Sand and Clay Contents on Aggregate Development Using Organic Amendments

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Keywords: soil structure, sand tailings, aggregate development, amendments, skeletal materials, clay

ABSTRAK

Kesan bahan rangka (kelodak dan pasir sangat halus) dan lempung ke atas pembentukan agregat dan kestabilan agregat bagi tanah pasir (kandungan pasir, 99%) yang dirawat dengan bahan organik telah diselidiki. Dalam eksperimen ini, tanah pasir bekas lombong telah dicampurkan dengan slim (mengandungi 37% kelodak + pasir sangat halus dan 33% lempung) pada kadar yang berlainan. Kemudian campuran-campuran itu dirawat dengan efluen kilang kelapa sawit (POME) kering pada kadar 10.5 g bagi 1200 g campuran pasir-slim. Campuran-campuran itu diperam selama dua minggu dan selepas itu dikering-udarakan. Peratus pengagregatan ditentukan dengan ayakan kering dan kestabilan agregat dengan ayakan basah. Keputusan menunjukkan, penambahan kelodak + pasir sangat halus dan lempung meningkatkan pengagregatan dan kestabilan agregat tanah pasir. Nilai optimum lempung yang diperlukan untuk mendapatkan pengagregatan yang baik bagi banyaknya bahan organik yang ditambah ialah 25%. Ini memberikan peningkatan kestabilan agregat sebanyak tujuh kali ganda. Dengan menggunakan slim, yang mengandungi 33% lempung, nilai optimum lempung ini boleh dicapai daripada campuran 75% slim + 25% pasir lombong. Campuran in mengandungi 32% bahan rangka.

ABSTRACT

The effect of skeletal materials (silt and very fine sand) and clay on aggregate formation and stabilization of organically amended sand tailings (99% sand) was investigated. In this experiment, sand tailings were mixed with different proportions of slime (slime contains 37% silt + very fine sand and 33% clay) and then treated with palm oil mill effluent (POME) cake at the rate of 10.5 g of POME cake per 1200 g of sand-slime mixture. The mixtures were incubated for 2 weeks and then air-dried. The extent of aggregation of the samples was determined by dry sieving, and the aggregate stability by wet sieving. The addition of silt + very fine sand and clay improved aggregation and aggregate stability of the sandy soils. The optimum amount of clay required to achieve a good aggregation and aggregate stability for the amount of organic matter added is 25%, where the increase in stability was seven-fold over the control. With slime, which contains 33% clay, this amount of clay can be achieved from a mixture of 75% slime + 25% sand tailings. This mixture contains 32% skeletal materials.

INTRODUCTION

Organic matter is the main binding agent in soil aggregate formation and stabilization (Tisdall and Oades 1982; Chaney and Swift 1984; Bartoli *et al.* 1988). Field experiments have shown that organic amendments such as sewage sludge improve the structural stability of soils on which they are applied (Kladivko and Nelson 1979; Pagliai *et al.* 1981). While this is true for mineral soils, Lim *et al.* (1983) and Othman *et al.* (1990) have shown that the addition of organic amendments to sand tailings fail to give a positive

response on the aggregation and stability of these soils. Recently, Mokhtaruddin and Norhayati (1995) suggested that the lack of aggregation on sand tailings with the application of organic amendment is due to an insufficient amount of very fine sand- and silt-size fractions and clay in the soils. According to Emerson (1959) the first two are important skeletal materials for aggregate formation. Mokhtaruddin and Norhayati (1995) concluded that the development of soil aggregation in sand tailings using organic amendments needs the introduction of sufficient amounts of these materials and clay.

The objectives of this paper are, first, to evaluate the effect of different amounts of silt, very fine sand and clay on aggregation and stability of sand tailings and, second, to determine the minimum amount of these materials need to obtain a substantial degree of aggregation and stability of these soils. The results will lead to a better understanding of the mechanism of aggregate development in these very marginal soils following organic matter application.

MATERIALS AND METHODS

Soil

Sand tailings (Table 1) have a high sand content. The silt and very fine sand content is 5.8% and clay is found in trace amounts. The sand is acidic, has low carbon content and trace amounts of free iron oxides. The sand tailings were air-dried and sieved through a 2-mm screen.

Slime is the suspended fine fraction of the slurry pumped during tin-mining operations into a retention area for settlement. The content of silt, clay and very fine sand can be as high as 71% (Table 1). It can therefore be used to supply the fine materials required for promoting aggregation in the sandy portion of the tin tailings. Slime was obtained from the top layer of the slime retention area. It was air-dried and sieved through a 2-mm screen.

Palm Oil Mill Effluent (POME) for Organic Amendment

The type of POME used was decanter-dried raw POME cake. The nutrients and carbon contents of the cake are given in Table 2. The cake has a high content of N, K, Ca and carbon.

Experimentation

The sand tailings were mixed with various amounts of slime and then treated with the POME cake at the rate of 10.5 g POME cake per 1020 g of sand–slime mixture. The amount of slime added was calculated to give equivalent percentages of the clay fraction of 0 (control), 3, 7, 10, 15, 20 and

TABLE 1
Texture and chemical properties of sand tailings and slime

Fraction	Size (mm)	Sand tailings	Slima
Course sand (%)	> 0.50	17.4	5.4
Medium sand (%)	0.25–0.50	35.1	9.3
Fine sand (%)	0.10–0.25	40.9	14.7
Very fine sand (%)	0.05–0.10	5.6	4.4
Silt (%)	0.002–0.05	0.2	32.7
Clay (%)	< 0.002	Tr[a]	33.4
Carbon (%)		0.15	1.66
Free iron oxides (%)		Tr[a]	0.22
pH		5.42	5.77

[a]Tr = Trace

TABLE 2
Chemical properties of palm oil mill effluent cake

Element	N	P	K	Ca	Mg	Fe	C
Content (%)	1.14	0.17	0.99	1.99	0.24	0.14	14.4

25%. The total weight of each mixture was made up to 1200 g. The mixtures were thoroughly mixed and sprayed with 0.1% iron solution (ferrous sulphate) until field capacity, the moisture content at which maximum aggregate formation is attained in many soils. Iron solution at 0.1% concentration was found to enhance the aggregate stabilization in sand soils (Mokhtaruddin and Norhayati 1995). The field capacity (moisture content at 100 cm water tension) was determined using the pressure plate apparatus. Samples were placed in plastic bags and incubated for one week at ambient temperature. After incubation they were air-dried. The extent of aggregation was evaluated by dry sieving. A 100-g air-dried sample was placed on a nest of sieves with 2.0, 1.0, 0.5 and 0.3 mm openings. The nest of sieves was shaken manually 10 times with a circular motion. Aggregates remaining on each sieve were weighed. The extent of aggregation was expressed as the proportion of air-dried aggregates >2 mm (% aggregation >2 mm). The stability of soil aggregates was expressed as a percentage of water-stable aggregates >0.5 mm (% WSA >0.5 mm) (Bryan 1968). Percentage WSA >0.5 mm was determined by the wet sieving technique of Yoder (1936). A 100-g air-dried sample (<2 mm) was placed on a 0.5 mm screen. The screen was shaken up and down mechanically in water at a frequency of 40 oscillations per minute for 30 minutes. The height of oscillation was 4 cm. The aggregates remaining on the sieve were dried and weighed. The % WSA >0.5 mm was calculated as the proportion of water-stable aggregates >0.5 mm over the whole sample. To determine the particle size distribution, the samples were analysed mechanically by the pipette method using calgon as the dispersing agent (Day 1965). Each treatment was repeated on three samples for each mixture.

RESULTS AND DISCUSSION

Extent of Aggregation

In its natural state, sand tailings have a single grain structure. The poor development of aggregation in the sandy soils was thought to be due to the very low content of very fine sand and silt fractions, absence of clay and low content of organic matter (Mokhtaruddin and Norhayati 1995). Emerson (1959) suggested that the combination of these materials formed stable soil aggregates. The addition of these materials to sandy soils should therefore improve soil aggregation substantially. *Fig. 1* shows that

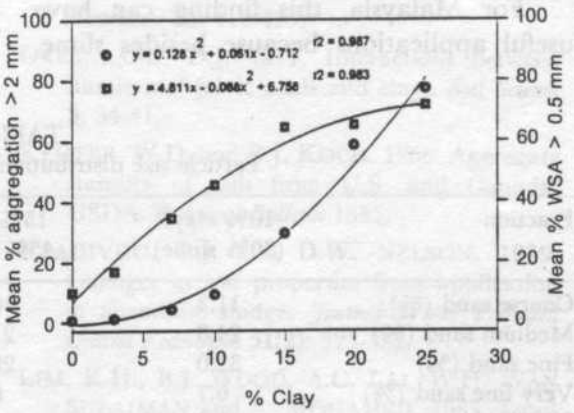

Fig 1. Regression curves of % aggregation >2 mm [o] and % WSA >0.5 mm [□] against % Clay

the mean % aggregation >2 mm increased with increase in clay content. The increase is significant compared with the control. The greatest increase occurred when the clay content was above 10%. At 10% clay, the increase was 13-fold over the control and at 15% clay, the increase was 40-fold. An addition of 20% clay caused an 82-fold increase in aggregation over the control, and by extrapolation the addition of 30% clay gave a 164-fold increase in aggregation. However, aggregate stability is more important in aggregate formation.

Aggregate Stability

Fig. 1 shows that not only the aggregation status of sand tailings was improved; the addition of clay also produced water-stable aggregates. All levels of clay caused a significant increase in % WSA > 0.5 mm ($P < 0.05$). However, the rate of increase in stability decreased with increase in clay content. The results also suggest that there is an optimum amount of clay for aggregate formation and stabilization in sand tailings. This optimum amount was found to be 25%, where the increase in stability was 7-fold over the control. The 25% clay is obtained from a mixture of 75% slime + 25% sand tailings (Table 3). The amount of very fine sand + silt in this mixture is 32%.

For Malaysia, this finding can have useful applications because besides slime, "fly ash" (solid waste from coal burned for energy production), which contains 96% silt + very fine sand particles, can be used as a source of the skeletal materials.

As mentioned above, silt + very fine sand fractions are important skeletal materials for aggregate formation. It is well known that clay particles are cementing material, which bind the skeletal materials together into stable aggregates (Peterson 1946; Kemper and Koch 1966; Dixon 1991). The presence of an organic colloid results in stronger binding by forming strong clay-organic matter complexes (Cailer and Visser 1988; Greenland 1965; Theng 1979). Emerson (1959) suggested another mechanism by which clay-organic matter interaction influences the stability of aggregates. Organic matter is bonded to the exterior surfaces of the clay domains leaving the clay still free to shrink and swell. When the clay swells, the stresses are transmitted via the organic matter bonds and the bridging between quartz (skeletal) particles remains intact and slaking is thus prevented.

Greenland (1971) has discussed the various ways in which organic ions are adsorbed by clay surfaces through the influence of aluminium and iron hydroxides on the clay surfaces. Firstly, the organic ions are adsorbed to the positive sites on the aluminium and iron hydroxides

TABLE 3
Particle size distribution of clay-sand mixtures

Fraction	10% clay[a] (30% slime)	15% clay[a] 45% slime)	20% clay[a] (60% slime)	25% clay (75% slime)
Course sand (%)	11.3	10.1	8.8	7.5
Medium sand (%)	24.8	21.5	18.2	14.9
Fine sand (%)	32.0	28.2	24.6	20.9
Very fine sand (%)	6.7	6.3	5.8	5.3
Silt (%)	15.0	18.8	22.5	26.3
Clay (%)	10.0	15.0	20.0	25.0

[a] Figure in brackets is % of slime which contains the respective amount of clay

by simple coulombic attraction. Secondly, organic ions can be bonded to aluminium and iron hydroxides through 'ligand exchange' reactions or specific adsorption. Another way by which organic substances can be bonded to clay surfaces is through precipitation by iron and aluminium hydroxides followed by dehydration. Thus it is expected that the role of clay and organic matter in aggregate stabilization will depend on the amount of clay and organic matter present in the sample. Either one can be the limiting factor. This could explain why in this experiment, further addition of clay (> 25%) with the same amount of organic matter did not produce a further increase in aggregate stability.

CONCLUSION

Addition of clay and skeletal materials (silt and very fine sand) to organically amended sandy soils (> 99% sand) not only significantly improved the aggregation status but also the aggregate stability of the soils. The optimum amount of clay required to achieve a good aggregation and aggregate stability is 25%. If slime, which contains 33% clay, is used to supply the required clay, this condition can be achieved by preparing a mixture of 75% slime and 25% sand. This mixture will contain 32% skeletal materials. The addition of these amounts of clay and skeletal materials, and POME cake at the rate of 10.5 g per 1200 g mixture of these materials resulted in a 7-fold increase in aggregate stability.

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Impact of Edapho-climatic Factors on the Dynamics of VAM Root Colonization and Spore Density in Three Forest Tree Species of Western Ghats, India

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Keywords: VAM fungi, root colonization, spore density, tree species, Western Ghats, *Eucalyptus grandis*, *Grevillea robusta*, *Tectona grandis*

ABSTRAK

Tinjauan telah dijalankan untuk menilai kepelbagaian kulat VAM yang disekutukan dengan tiga spesies pokok hutan eksotik, Eucalyptus grandis *Hill ex. Maid.,* Grevillea robusta *A. cunn dan* Tectona grandis *L. f. di ekosistem Ghat Barat, India Selatan. Contoh-contoh dikumpul daripada dua lokasi, Kodaikanal (1500 m A.S.L.) dan Siruvani (700 m A.S.L.) daripada Januari 1992 hingga Jun 1993. Faktor-faktor beriklim, harta kimia-psiko tanah rizosfera dan bilangan spota VAMF serta pengkolonian akar telah direkodkan. Pemencilan spesies adalah* Acaulospora, Gigaspora, Glomus, Sclerocystis *dan* Scutellospora *bersama* Glomus *menjadi 73% daripada jumlah spesies direkodkan. Densiti spora mikoriza adalah maksimum daripada Januari hingga Jun dalam semua contoh yang dikumpul melalui kesemua tiga spesies pokok pada kedua-dua bahagian. Pengkolonian akar yang tinggi direkodkan antara bulan September dan November. Bagi kedua-dua bahagian, densiti spota adalah berkaitan secara positif dengan suhu dan negatif dengan hujah dan lembapan tanah. Pembalikan direkodkan bagi pengkolonian akar. Wujud hubungan positif antara bilangan vesikal dan spora.*

ABSTRACT

A survey was carried out to evaluate the diversity of VAM fungi associated with three exotic forest tree species, Eucalyptus grandis *Hill ex. Maid,* Grevillea robusta *A. Cunn. and* Tectona grandis *L.f. in the Western Ghats ecosystem, South India. The samples were collected from two locations, Kodaikanal (1500 m A.S.L.) and Siruvani (700 m A.S.L.) from January 1992 to June 1993. Climatic factors, physico-chemical properties of rhizosphere soils and VAMF spore counts and root colonization were recorded. Species isolated were* Acaulospora, Gigaspora, Glomus, Sclerocystis *and* Scutellospora *with* Glomus *constituting 73% of the total species recorded. The mycorrhiza spore density was maximum from January to June in all samples collected from all three tree species at both sites. High root colonization was recorded between the months of September and November. In both sites, the spore density was positively correlated with temperature and negatively with rainfall and soil moisture. The reverse was recorded for root colonization. There was positive correlation between vesicle number and spore number.*

INTRODUCTION

A thorough understanding of the ecology of vesicular-arbuscular mycorrhizal (VAM) fungal species is needed to enable maximum manipulation of VAMF symbiosis for the benefit of minimum-input agricultural and forestry systems. Previous studies have assessed changes in total spore population under different ecosystems such as sand dunes (Koske and Halvorson 1981), tropical rain forest (Louis and Lim 1987), savannahs (Saif 1986). In nature VAMF multiply and survive by the formation of spores in and around the rhizosphere.

Spores of more than one species of VAMF may occur in the rhizosphere soil (Abbott and Robson 1977). The population composition and the activity of VAMF are affected by many factors; those affecting the symbiotic relationships between host and VAMF are well documented (Hayman and Tavares 1985). Inoculum and soil related factors are considered of primary importance. Other factors include the influence of soil pH (Hayman and Tavares 1985), soil moisture (Redhead 1975), soil fertility (Hayman 1982), organic matter (Hepper and Warner 1983), soil aeration (Saif 1981), soil clay content (Black and Tinker 1979), soil physical and chemical characteristics (Muthukumar *et al.* 1994), pesticides (Sugavanam *et al.* 1994; Udaiyan *et al.* 1995), season (Louis and Lim 1987) and biotic factors (Azcon-Aguilar and Barea 1985). The importance of these edaphic factors led Mosse (1972) to suggest that specificity may be strongly determined by interactions between fungal strain and soil rather than between fungus and its host plant. Changes in inoculum potential in soils have also been assessed using the 'most probable number' (MNP) techniques (Baltruschat and Dehne 1988). However, information on the number of VAM fungal species associated with respective tree species under natural ecosystems, and the influence of environmental factors on VAMF spore density, their distribution, establishment and survival over time is lacking. Such information is of prime importance in identifying and utilizing the most suitable mycorrhizal species for large-scale inoculation programmes. The present study was therefore conducted to (i) identify the mycorrhizal fungal species associated with three forest tree species from two different ecosystems in the Western Ghats, Tamil Nadu, India and (ii) evaluate the impact of edapho-climatic factors on the distribution and abundance of these fungi.

MATERIALS AND METHODS

Study Area

The study was conducted in plantation forests in Kodaikanal (Site 1) and Siruvani (Site 2) in the Western Ghats region. Kodaikanal is an offshoot of the Western Ghats located between 10° 12' and 10°15′ N latitude and 77°26′ and 77°38′ E longitude at an elevation of ca. 1500 m A.S.L. Siruvani is located at 76°37′ N latitude and 10°58′ E longitude at an elevation of ca. 700 m A.S.L. Soils at both sites were black loamy.

Climatic Data

Climatological data recorded from January 1992 to June 1993 included minimum and maximum temperature, relative humidity (RH) and rainfall (*Fig. 1* and *2*).

Sampling

Root and soil samples from three forest tree species, viz., *Eucalyptus grandis*, *Grevillea robusta* and *Tectona grandis* were collected at monthly intervals from January 1992 to June 1993. Five subsamples were collected from each species. The respective roots were carefully dug out, washed free of soil, cut into 1-cm sections, fixed in 50% formalic-acetic acid-ethanol (FAA). The rhizosphere soils from the respective tree species were mixed to form a composite soil sample, packed separately in polythene bags, and stored at 4°C for future analysis.

Analysis of Soil Physico-chemical Properties

Composite soil samples collected at monthly intervals were analysed for pH, total nitrogen, available phosphorus and potassium concentration. The total N and available P were determined respectively by the micro-Kjeldahl and molybdenum blue methods described by Jackson (1973). Exchangeable K was extracted from the soil in an ammonium acetate solution (pH 7) and measured with a digital flame photometer (Jackson 1973).

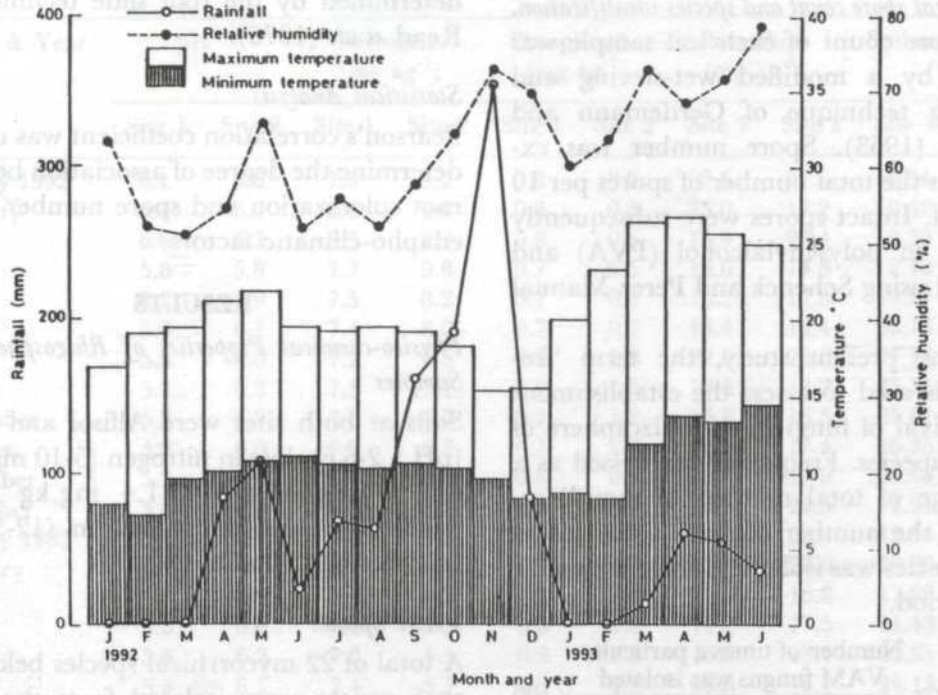

Fig. 1. Weather data at Kodaikanal (site 1) during the study period

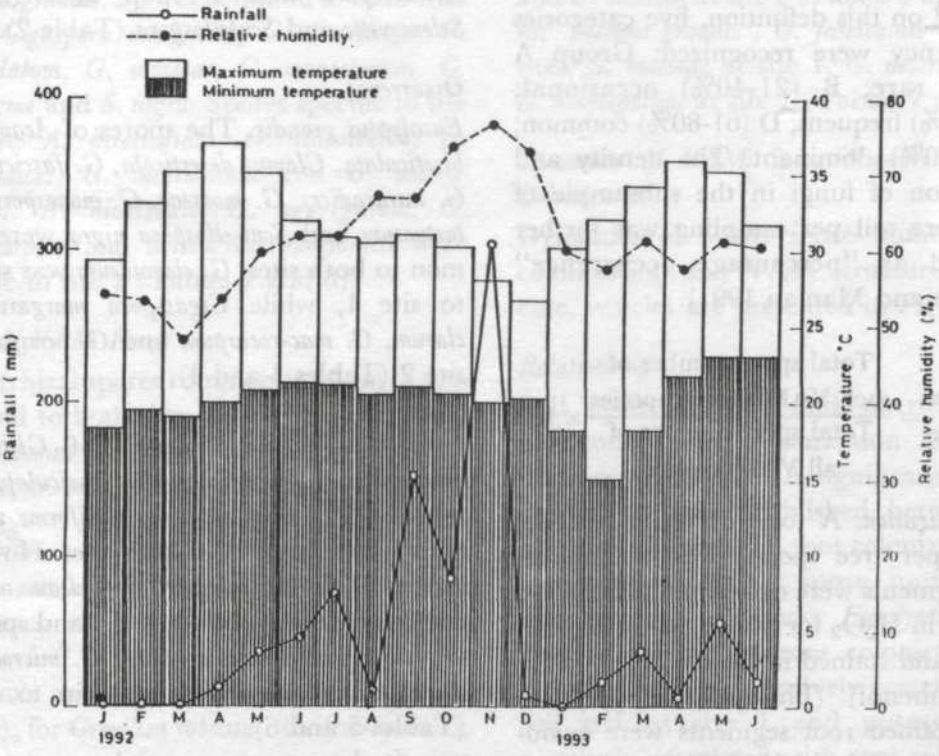

Fig. 2. Weather data at Siruvani (site 2) during the study period

Estimation of VAM

Mycorrhizal spore count and species identification. Total spore count of each soil sample was assessed by a modified wet-sieving and decanting technique of Gerdemann and Nicolson (1963). Spore number was expressed as the total number of spores per 10 g dry soil. Intact spores were subsequently mounted in polyvinylalcohol (PVA) and identified using Schenck and Perez Manual (1987).

In the present study, the term 'frequency' is used to assess the establishment and survival of fungi in the rhizosphere of the host species. Frequency, expressed as a percentage of total number of samplings, indicates the number of times a particular fungal species was isolated during the entire study period.

$$\text{Frequency} = \frac{\text{Number of times a particular VAM fungus was isolated}}{\text{Total number of samples observed (18)}} \times 100$$

Based on this definition, five categories of frequency were recognized: Group A (1-20%) rare; B (21-40%) occasional; C (41-60%) frequent; D (61-80%) common; E (81-100%) dominant. The density and distribution of fungi in the subsample of rhizosphere soil per sampling was further expressed as "percentage occurrence" (Udaiyan and Manian 1991).

$$\text{Percentage occurrence} = \frac{\text{Total spore number of each VAM fungus species}}{\text{Total spore number of all VAM species}} \times 100$$

Root colonization. A total of 100 1-cm root samples per tree species were examined. These segments were cleared in 10% KOH, bleached in H_2O_2 for 30 sec, acidified with 5N HCl and stained in trypan blue (0.05% in lactophenol) (Phillips and Hayman 1970). Stained root segments were examined for presence of VAM structures and the percentage of mycorrhizal infection determined by the root slide technique of Read *et al.* (1976).

Statistical Analysis

Pearson's correlation coefficient was used to determine the degree of association between root colonization and spore number to the edapho-climatic factors.

RESULTS

Physico-chemical Properties of Rhizosphere Soil Samples

Soils at both sites were Alfisol and acidic (pH 5.2-6.6), low in nitrogen (5-10 mg kg^{-1}) and phosphorus (0.5-1.4 mg.kg^{-1}) but medium to high in potassium (12-35 mg kg^{-1}) content (Table 1).

VAM Species

A total of 22 mycorrhizal species belonging to 5 genera were isolated from the study sites, of which 16 were *Glomus*, 3 were *Acaulospora* and 1 each of *Gigaspora*, *Sclerocystis* and *Scutellospora* (Table 2).

Occurrence

Eucalyptus grandis. The spores of *Acaulospora bireticulata, Glomus deserticola, G. fasciculatum, G. intraradices, G. mosseae, G. monosporum, G. tortuosum* and *Scut-ellospora nigra* were common to both sites. *G. claroideum* was specific to site 1, while *Gigas-pora margarita, G. clarum, G. mac-rocarpum* and *G. versiforme* to site 2 (Tables 3 and 4).

Grevillea robusta. Spores of *Gigaspora margarita, G. deserticola, G. fasciculatum, G. mosseae, G. monosporum, G. versiforme* and *S. nigra* were common to both sites. However, spores of *G. australe, G. claroideum* and *G. geosporum* were specific to site 1 and spores of *G. constrictum, G. invermaium, G. microcarpum* and *G. tortuosum* were specific to site 2 (Tables 5 and 6).

TABLE 1
Physico-chemical properties of rhizosphere soil samples from Kodaikanal (site 1) and Siruvani (site 2)

Month & Year	pH		Nitrogen (mg kg^{-1})		Phosphorus (mg kg^{-1})		Potassium (mg kg^{-1})		Soil moisture (%)	
	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2
January 1992	6.1	5.6	7.8	9.2	0.8	0.9	32.5	27.6	11.34	12.50
February	6.3	6.2	8.0	9.6	0.8	0.8	35.0	17.2	10.42	10.41
March	6.6	6.3	8.5	9.7	0.9	0.6	15.4	23.4	9.25	8.31
April	5.8	5.8	7.7	9.8	0.7	0.5	13.0	14.5	12.48	8.34
May	5.2	5.9	7.5	8.2	0.7	0.8	15.2	12.8	19.27	17.78
June	5.9	6.1	7.4	8.0	0.7	0.7	13.4	13.4	26.15	20.18
July	5.6	6.0	7.2	6.5	0.9	1.0	22.0	25.0	24.13	23.15
August	5.7	6.3	7.3	7.1	0.8	1.1	17.5	18.2	25.51	19.48
September	5.3	6.2	7.3	8.2	0.7	1.2	15.3	16.5	25.81	19.40
October	5.2	6.0	7.0	6.3	0.7	1.0	16.5	17.4	26.35	23.50
November	5.2	6.6	6.8	9.6	0.6	0.6	17.4	17.8	26.48	27.38
December	5.4	5.8	7.2	8.2	0.5	0.5	20.5	21.5	11.21	7.00
January 1993	5.8	5.3	7.8	7.3	0.6	0.7	26.0	23.6	12.34	8.52
February	6.0	6.1	8.2	6.4	0.7	1.3	15.3	27.0	10.00	10.26
March	6.2	6.3	8.3	6.3	0.8	1.4	12.4	16.8	17.28	7.27
April	6.0	6.0	8.0	5.2	0.8	1.2	14.2	17.5	21.43	15.25
May	5.3	6.2	7.8	5.5	0.9	1.0	15.3	13.4	23.25	18.75
June	5.4	6.1	7.4	5.7	0.6	1.2	13.8	16.2	24.15	26.32

Tectona grandis. Spores common to both sites were *Gigaspora margarita*, *G. deserticola*, *G. fasciculatum*, *G. mosseae*, *G. monosporum*, *G. versiforme* and *S. nigra*. Spores specific to site 1 were *A. bireticulata*, *G. claroideum*, *G. intraradices*, *G. invermaium* and *G. macrocarpum*. *A. nicolsonii*, *G. aggregatum*, *G. macrocarpum* and *Sclerocystis rubiformis* were specific to site 2 (Tables 7 and 8).

Common VAM Fungi

Mycorrhizal spores common to all three species and to both sites were: *Gigaspora margarita*, *Glomus deserticola*, *G. fasciculatum*, *G. mosseae*, *G. monosporum* and *Scutellospora nigra*.

Abundance

Dominant mycorrhizal species, i.e. with a frequency of 81-100% were for *Eucalyptus grandis* : *G. monosporum* at site 1 and *G. deserticola* and *G. mosseae* at site 2 (Tables 3 and 4); for *Grevillea robusta* : *G. deserticola*, *G. fasciculatum* and *G. monosporum* at both sites and *G. mosseae* at site 2 (Tables 5 and 6) and for *Tectona grandis* : *G. fasciculatum* at both sites, *G. mosseae* at site 1, *G. deserticola* and *G. monosporum* at site 2 (Tables 7 and 8).

Dynamics of VAM Spore Number and Root Colonization

Dynamics of VAM spore numbers, root colonization and VAM structures (arbuscule, vesicle) are presented in *Fig. 3 - 8*.

Relationship

Soil moisture, pH and nutrient levels influenced root colonization and spore number (Table 10). A significant negative correlation was established between root colonization and pH, root colonization and nitrogen at site 1; spore number and nitrogen at site 2 under *Eucalyptus grandis*. In *Grevillea robusta*, root colonization was significantly and negatively correlated with soil pH at site 1 and potassium was positively correlated with root colonization

TABLE 2
Spore types recorded from the rhizosphere of *Eucalyptus grandis*, *Grevillea robusta* and *Tectona grandis*

Sl. No.	VAM Fungi	Code
1.	*Acaulospora bireticulata* Rothwell & Trappe	ABTR
2.	*A. nicolsoni* Walker, Read & Sanders	ANCS
3.	*A. scrobiculata* Trappe	ASCB
4.	*Gigaspora margarita* Becker & Hall	GMRG
5.	*Glomus aggregatum* Schenck & Smith	LAGR
6.	*G. australe* (Berck.) Berch.	LAST
7.	*G. claroideum* Schenck & Smith	LCRD
8.	*G. clarum* Nicolson & Schenck	LCLR
9.	*G. constrictum* Trappe	LCST
10.	*G. deserticola* Trappe, Bloss & Menge	LDST
11.	*G. fasciculatum* (Thaxter Sensu Gerd.) Gerd. & Trappe	LFSC
12.	*G. geosporum* (Nicol & Gerd.) Walker	LGSP
13.	*G. intraradices* Schenk & Smith	LINR
14.	*G. invermaium* Hall	LIVM
15.	*G. macrocarpum* Tul. & Tul.	LMCC
16.	*G. microcarpum* Tul. & Tul.	LMRC
17.	*G. mosseae* (Nicol. & Herd.) Gerd. & Trappe	LMSS
18.	*G. monosporum* Gerdemann & Trappe	LMNS
19.	*G. tortuosum* Schenk & Smith	LTRT
20.	*G. versiforme* (Karsten) Berch.	LVSF
21.	*Sclerocystis rubiformis* Gerdemann & Trappe	SRBF
22.	*Scutellospora nigra* (Redhead) Walker & Sanders	CNGR

and spore number at site 2. For *Tectona grandis* pH was significantly and positively correlated with spore number at site 1 and root colonization at site 2.

DISCUSSION

Spores of VAMF were observed in the rhizospheric soil of all tree species, with the highest count for *Grevillea robusta*. However, compared to the cropland system (Abbott and Robson 1977) the spore count was comparatively lower. The viable mycorrhizal fungi persist in roots of perennial plants and are the main source of inoculum for further infection of new roots. This may be the reason for low spore production of the mycorrhizal fungi in forest land (Baylis 1969). The presence of *Gigaspora margarita*, *Glomus deserticola*, *G. fasciculatum*, *G. mosseae*, *G. monosporum* and *Scutellospora nigra* in the rhizosphere of all tree species indicates their broad host range.

Although a variety of VAMF species has been recorded in the rhizosphere of tree species from both sites, the species composition and their density, distribution and survival varied from host to host. Schenck and Kinloch (1980) observed that the incidence of VAM fungal species depend upon the plant species colonized. This influence of host plant on the incidence of VAMF has also been observed by Kruckelmann (1975) on a site where 6 crops were grown in monoculture for 16 years. It appears that the host plant can affect sporulation, and possibly the survival of VAMF.

The variations in spore number and mycorrhizal colonization were similar at both sites, i.e. when spore numbers were high, the percentage of mycorrhizal colonization was low and vice versa. A similar

TABLE 3
Percentage occurrence and frequency of VAM fungi isolated from the rhizosphere of *Eucalyptus grandis* at site 1

Sl. No.	VAM Fungi	Percentage Occurrence																		Percentage Frequency	Frequency Class
		1992												1993							
		Jan.	Feb.	Mar.	Apr.	May.	June	July	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	June		
1.	*Acaulospora bireticulata*	4.6	5.7	0.0	0.0	0.0	8.2	3.5	0.0	2.6	0.0	0.0	0.0	11.0	9.1	0.0	0.0	0.0	5.0	44	*Frequent*
2.	*Gigaspora margarita*	8.4	11.3	17.8	0.0	0.0	30.1	35.2	17.2	9.4	38.6	42.5	48.1	0.0	0.0	0.0	0.0	10.4	24.6	72	*Common*
3.	*Glomus claroideum*	3.4	0.0	5.3	7.6	7.4	0.0	0.0	0.0	11.1	4.5	0.0	0.0	13.7	6.4	20.4	15.1	3.4	0.0	61	*Common*
4.	*G. deserticola*	32.1	17.0	24.1	35.0	26.2	24.1	0.0	0.0	5.2	12.1	15.5	10.2	25.1	0.0	27.8	20.6	19.1	15.9	77	*Common*
5.	*G. fasciculatum*	0.0	9.1	38.4	23.1	22.4	0.0	16.6	14.7	8.3	22.1	12.9	9.2	18.1	21.3	0.0	0.0	0.0	18.2	77	*Common*
6.	*G. invermaium*	7.7	6.7	0.0	8.1	0.0	3.1	5.1	4.6	0.0	6.0	0.0	0.0	4.3	3.2	7.3	5.4	0.0	0.0	61	*Common*
7.	*G. mosseae*	18.4	14.7	10.9	19.8	0.0	0.0	7.5	21.1	6.6	0.0	9.8	18.1	10.8	10.5	0.0	0.0	19.0	9.2	77	*Common*
8.	*G. monosporum*	15.8	35.3	0.0	0.0	36.1	24.3	13.0	27.4	8.2	12.7	19.2	14.3	12.7	41.0	44.0	42.4	10.6	8.6	83	*Dominant*
9.	*G. tortuosum*	9.4	0.0	3.3	6.4	2.3	0.0	0.0	0.0	4.5	3.8	0.0	0.0	4.0	4.0	0.0	0.0	8.7	0.0	55	*Frequent*
10.	*Scutellospora nigra*	0.0	0.0	0.0	0.0	5.4	10.1	19.0	14.8	43.8	0.0	0.0	0.0	0.0	4.3	22.3	16.5	28.6	18.2	55	*Frequent*

TABLE 4

Percentage occurrence and frequency of VAM fungi isolated from the rhizosphere of *Eucalyptus grandis* at site 2

Sl. No.	VAM Fungi	Percentage Occurrence																		
		1992											1993						Percentage Frequency	Frequency Class
		Jan.	Feb.	Mar.	Apr.	May.	June	July	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	June		
1.	*Acaulospora bireticulata*	6.2	5.5	0.0	0.0	0.0	6.2	4.0	2.8	6.0	0.0	0.0	0.0	10.5	7.5	0.0	0.0	0.0	2.5	50	*Frequent*
2.	*Gigaspora margarita*	15.6	17.8	15.1	4.5	8.5	0.0	0.0	10.8	22.7	18.1	42.6	58.8	42.7	59.2	9.1	0.0	0.0	41.2	77	*Common*
3.	*Glomus claroideum*	0.0	0.0	3.9	3.1	4.0	4.2	3.5	0.0	0.0	0.0	5.1	1.8	0.0	0.0	2.8	2.5	2.9	4.6	61	*common*
4.	*G. deserticola*	28.9	14.6	14.1	13.9	19.8	0.0	0.0	32.7	25.4	12.1	6.8	7.9	8.5	8.4	36.0	31.2	41.4	0.0	83	*Dominant*
5.	*G. fasciculatum*	10.1	23.3	22.6	0.0	0.0	9.3	19.4	9.4	12.0	30.2	8.5	7.3	0.0	3.0	11.6	6.6	13.7	8.4	88	*Dominant*
6.	*G. intraradices*	0.0	0.0	5.9	2.4	2.7	2.5	0.0	0.0	0.0	7.4	4.5	3.1	5.2	0.0	0.0	1.7	1.8	2.1	61	*Common*
7.	*G. macrocarpum*	10.1	2.8	3.3	2.6	4.3	0.0	3.7	2.8	2.1	5.1	0.0	0.0	6.5	2.8	3.2	0.0	0.0	0.0	66	*Common*
8.	*G. mosseae*	7.8	12.5	10.5	17.0	25.5	34.2	23.1	13.9	11.2	0.0	0.0	4.3	11.8	9.1	14.2	13.3	15.2	8.0	88	*Dominant*
9.	*G. monosporum*	9.4	12.0	8.8	30.8	16.4	17.3	0.0	0.0	13.3	12.1	13.1	6.7	14.5	9.8	6.4	18.7	0.0	0.0	77	*Common*
10.	*G. versiforme*	11.7	11.2	11.5	10.1	0.0	0.0	43.9	27.2	7.0	14.8	12.5	6.1	0.0	0.0	14.4	16.7	13.6	17.5	77	*Common*
11.	*Scutellospora nigra*	0.0	0.0	4.2	15.4	20.2	16.3	2.1	0.0	0.0	0.0	6.8	3.7	0.0	0.0	1.9	9.2	11.2	15.5	61	*Common*

TABLE 5

Percentage occurrence and frequency of VAM fungi isolated from the rhizosphere of *Grevillea robusta* at site 1

Sl. No.	VAM Fungi	Percentage Occurrence																		
		1992											1993						Percentage Frequency	
		Jan.	Feb.	Mar.	Apr.	May.	June	July	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	June	Frequency	Class
1.	*Gigaspora margarita*	25.6	10.3	9.2	10.1	0.0	0.0	4.9	17.7	15.0	19.6	0.0	22.2	29.6	18.5	0.0	0.0	12.4	17.1	72	*Common*
2.	*Glomus australe*	4.5	4.7	0.0	5.3	6.1	2.2	0.0	0.0	2.5	0.0	0.0	9.9	3.0	4.2	0.0	0.0	3.6	3.3	61	*Common*
3.	*G. claroideum*	3.9	4.1	3.1	0.0	1.5	5.1	1.0	2.4	0.0	0.0	0.0	13.4	5.3	6.6	14.9	3.9	0.0	0.0	66	*Common*
4.	*G. deserticola*	20.3	16.9	31.9	9.6	25.5	0.0	0.0	31.2	15.0	13.4	52.4	0.0	10.7	13.7	39.8	13.3	13.8	22.9	83	*Dominant*
5.	*G. fasciculatum*	14.4	32.1	18.5	34.8	35.0	20.1	14.8	0.0	0.0	14.7	15.2	42.6	18.5	23.5	14.6	18.1	0.0	9.3	83	*Dominant*
6.	*G. geosporum*	6.4	5.1	0.0	5.5	1.6	6.4	7.3	2.2	9.7	0.0	0.0	0.0	3.2	2.1	0.0	4.1	12.5	4.3	72	*Common*
7.	*G. mosseae*	0.0	0.0	9.4	22.7	11.8	30.4	31.7	3.7	18.6	25.4	10.3	0.0	5.1	7.3	0.0	12.4	15.3	13.8	77	*Common*
8.	*G. monosporum*	18.2	20.1	12.6	7.6	12.3	19.3	0.0	0.0	11.3	12.0	22.0	11.7	8.8	10.2	11.9	19.4	0.0	5.2	83	*Dominant*
9.	*G. versiforme*	6.5	6.4	14.1	0.0	0.0	5.1	27.7	12.5	22.7	14.7	0.0	0.0	12.3	11.2	16.7	28.7	42.2	9.6	77	*Common*
10.	*Scutellospora nigra*	0.0	0.0	1.1	4.3	6.0	11.1	16.8	16.2	0.0	0.0	0.0	0.0	3.5	2.5	0.0	0.0	0.0	14.3	50	*Common*

TABLE 6
Percentage occurrence and frequency of VAM isolated from the rhizosphere of *Grevillea robusta* at site 2

Sl. No.	VAM Fungi	Percentage Occurrence																		
		1992												1993					Percentage Frequency	Frequency Class
		Jan.	Feb.	Mar.	Apr.	May.	June	July	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	June		
1.	*Gigaspora margarita*	22.6	44.0	12.5	0.0	0.0	0.9	16.5	40.3	28.6	13.5	34.7	14.6	25.0	8.5	0.0	0.0	2.9	8.7	77	*Common*
2.	*Glomus constrictum*	0.0	0.0	3.1	3.8	1.7	1.7	2.9	0.9	0.0	0.0	1.4	1.8	3.9	7.9	1.8	2.1	0.0	0.0	66	*Common*
3.	*G. fasciculatum*	12.6	2.1	14.1	0.0	0.0	20.7	26.9	17.9	15.3	12.2	5.9	13.7	3.4	3.9	9.4	16.6	0.0	14.6	83	*Dominant*
4.	*G. invermaium*	2.8	1.6	1.2	0.9	2.6	0.0	0.0	1.3	1.3	1.7	1.9	0.0	0.0	0.0	4.4	2.6	1.6	2.1	72	*Common*
5.	*G. deserticola*	7.1	9.7	15.6	16.6	27.7	25.9	0.0	0.0	25.1	12.8	17.5	14.15	8.6	5.2	7.3	7.9	13.1	0.0	83	*Dominant*
6.	*G. microcarpum*	4.3	0.0	0.0	1.7	2.4	3.1	2.7	2.5	5.5	5.9	3.3	7.6	8.9	9.8	0.0	0.0	2.8	1.7	77	*Common*
7.	*G. mosseae*	36.0	10.4	8.5	10.7	11.4	13.8	13.8	0.0	0.0	8.7	17.5	34.5	9.8	8.5	19.1	14.3	22.4	0.0	83	*Dominant*
8.	*G. monosporum*	10.7	8.3	29.6	35.1	21.2	0.0	0.0	6.9	8.1	29.4	11.8	6.3	10.9	10.5	7.3	12.3	35.1	14.4	88	*Dominant*
9.	*G. versiforme*	0.0	0.0	11.2	18.5	21.8	18.2	24.1	22.4	11.5	12.9	0.0	0.0	15.9	19.1	25.3	0.0	0.0	46.9	66	*Common*
10.	*G. tortuosum*	1.3	2.0	0.0	0.0	1.4	1.8	3.2	1.7	1.3	0.0	1.0	1.3	2.2	6.6	0.0	2.7	8.2	0.0	72	*Common*
11.	*Scutellospora nigra*	0.0	0.0	4.1	12.4	9.6	13.5	10.0	4.5	1.9	0.0	0.0	1.3	3.6	4.6	25.3	41.3	13.7	9.8	77	*Common*

TABLE 7

Percentage occurrence and frequency of VAM fungi isolated from the rhizosphere of *Tectona grandis* at site 1

Sl. No.	VAM Fungi	Percentage Occurrence																		Percentage Frequency	Frequency Class
		1992												1993							
		Jan.	Feb.	Mar.	Apr.	May.	June	July	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	June		
1.	*Acaulospora bireticulata*	7.5	10.6	0.0	0.0	5.6	3.6	8.8	0.0	0.0	0.0	0.0	0.0	9.4	13.8	0.0	0.0	9.5	8.8	50	*Frequent*
2.	*Gigaspora margarita*	20.8	28.5	22.7	11.1	0.0	0.0	11.7	8.1	8.8	20.0	0.0	0.0	7.3	6.8	13.3	47.7	0.0	13.7	72	*Common*
3.	*Glomus claroideum*	5.1	6.2	0.0	0.0	3.9	4.7	11.2	0.0	0.0	0.0	0.0	0.0	3.4	2.8	3.8	0.0	0.0	2.3	50	*Frequent*
4.	*G. deserticola*	11.7	8.3	10.7	14.6	25.2	0.0	0.0	12.8	0.0	0.0	38.8	54.1	5.9	10.8	28.9	4.6	0.0	5.9	72	*Common*
5.	*G. fasciculatum*	10.0	4.8	12.7	11.4	13.4	17.5	14.8	0.0	17.9	14.5	21.5	12.2	0.0	14.0	15.5	6.7	11.9	0.0	83	*Dominant*
6.	*G. intraradices*	0.0	0.0	4.8	2.5	8.8	5.0	6.2	9.3	29.9	8.6	0.0	0.0	6.9	4.2	5.9	9.4	0.0	5.6	73	*Common*
7.	*G. invermaium*	9.1	2.7	4.1	5.4	4.2	0.0	0.0	27.5	0.0	0.0	0.0	0.0	4.5	5.1	3.3	5.5	6.8	7.6	66	*Common*
8.	*G. mosseae*	14.9	16.4	9.9	24.5	16.7	32.3	5.7	0.0	11.3	9.8	19.0	22.4	33.6	0.0	0.0	4.2	18.0	4.8	83	*Dominant*
9.	*G. monosporum*	11.9	15.3	17.5	11.5	0.0	0.0	25.4	16.4	7.0	36.0	20.6	11.2	5.6	25.2	13.1	0.0	0.0	9.1	77	*Common*
10.	*G. versiforme*	0.0	0.0	9.1	12.6	8.7	14.6	11.7	9.6	10.9	10.9	0.0	0.0	7.1	9.6	6.2	6.8	31.6	28.5	77	*Common*
11.	*G. macrocarpum*	8.6	6.9	2.9	0.0	0.0	6.6	4.4	7.8	0.0	0.0	0.0	0.0	9.4	7.4	9.6	7.0	22.1	0.0	61	*Common*
12.	*Scutellospora nigra*	0.0	0.0	5.3	6.2	13.4	15.5	0.0	0.0	14.0	0.0	0.0	0.0	11.8	0.0	0.0	7.9	0.0	13.3	44	*Frequent*

TABLE 8
Percentage occurrence and frequency of VAM fungi isolated from the rhizophere of *Tectona grandis* at site 2

Sl. No.	VAM Fungi	Percentage Occurrence																	Percentage Frequency	Frequency Class
		1992											1993							
		Jan.	Feb.	Mar.	Apr.	May.	June	July	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	June		
1.	*Acaulospora bireticulata*	7.1	3.4	2.8	1.3	3.8	0.0	0.0	0.0	3.4	1.9	3.6	0.0	4.0	8.4	7.8	3.9	3.0	0.0	72	*Common*
2.	*Gigaspora margarita*	12.5	17.9	43.5	0.0	0.0	4.9	19.0	33.1	29.4	19.9	27.7	14.0	14.1	22.2	0.0	0.0	8.4	15.3	77	*Common*
3.	*Glomus aggregatum*	0.0	0.0	2.3	2.8	3.2	2.8	3.2	0.0	0.0	0.0	2.3	7.4	0.0	0.0	6.7	2.6	2.0	1.4	61	*Common*
4.	*G. deserticola*	10.7	11.2	11.2	26.7	17.6	0.0	0.0	14.2	16.8	18.8	12.6	45.6	6.4	8.4	56.5	33.8	21.8	15.4	58	*Dominant*
5.	*G. fasciculatum*	11.6	32.0	14.3	18.9	25.3	11.1	24.1	14.7	0.0	0.0	3.6	8.8	33.5	17.8	19.9	13.3	25.8	22.8	88	*Dominant*
6.	*G. microcarpum*	0.0	0.0	2.8	3.2	2.2	2.4	4.7	0.0	2.4	1.9	2.8	0.0	0.0	0.0	3.1	1.8	1.2	1.4	66	*Common*
7.	*G. mosseae*	9.8	6.0	4.6	9.5	15.5	0.0	0.0	15.1	18.6	0.4	18.3	0.0	4.0	8.7	15.4	19.1	12.3	0.0	77	*Common*
8.	*G. monosporum*	24.1	6.7	6.5	7.6	8.2	14.6	0.0	0.0	23.9	31.2	18.8	16.2	41.7	8.7	0.0	0.0	13.3	14.5	88	*Dominant*
9.	*G. versiforme*	24.1	6.7	6.5	7.6	8.2	14.6	0.0	0.0	23.9	31.2	18.8	16.2	41.7	8.7	0.0	0.0	13.3	14.5	77	*Common*
10.	*Scutellospora nigra*	0.0	0.0	1.0	13.6	13.8	20.9	11.9	7.0	4.4	3.1	2.1	0.0	0.0	5.1	3.3	11.6	0.0	15.1	72	*Common*
11.	*Sclerocystis rubiformis*	11.6	4.3	0.9	0.0	0.0	0.0	1.8	1.4	1.1	2.7	1.1	0.0	10.1	7.0	2.0	0.0	0.0	0.0	61	*Common*

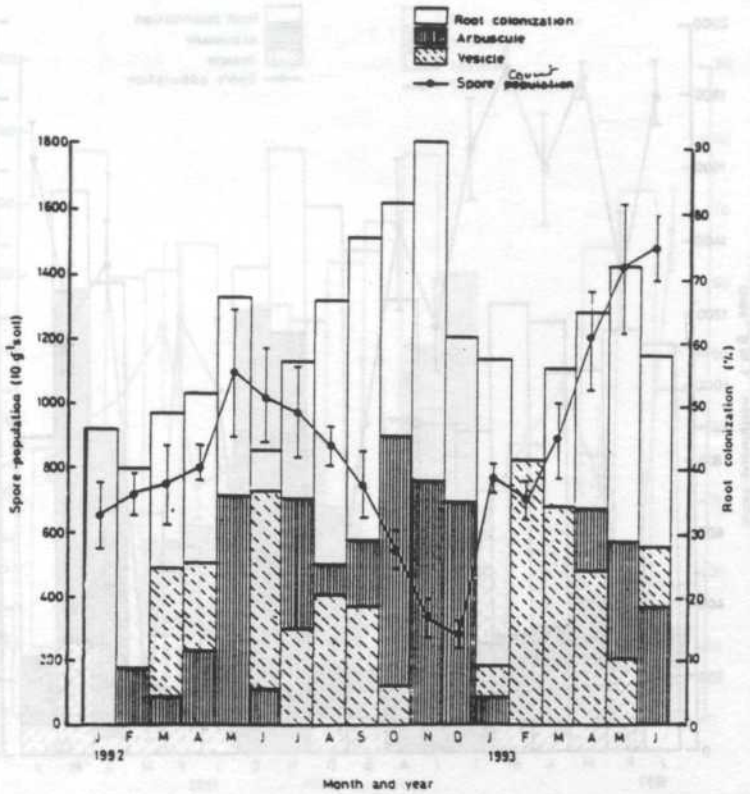

Fig. 3. Dynamics of the VAMF spore count and root colonization in Eucalyptus grandis *at site 1*

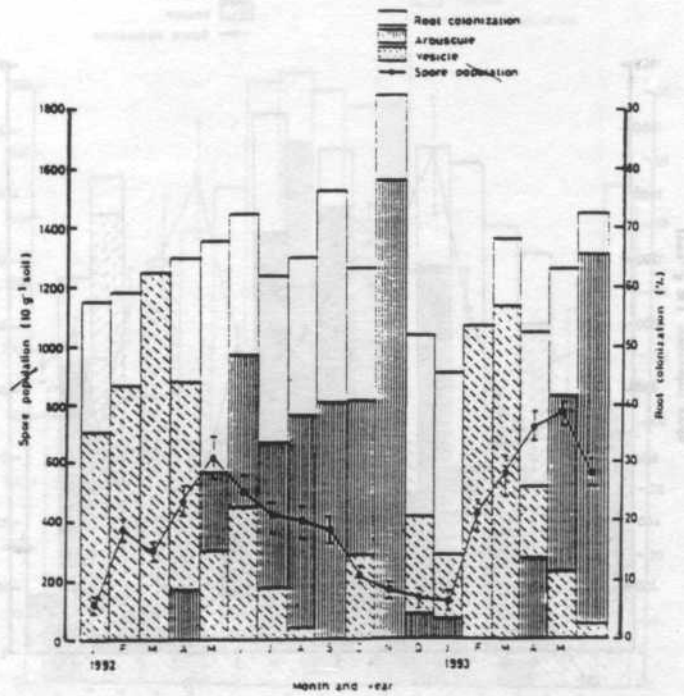

Fig. 4. Dynamics of the VAMF spore count and root colonization in Eucalyptus grandis *at site 2*

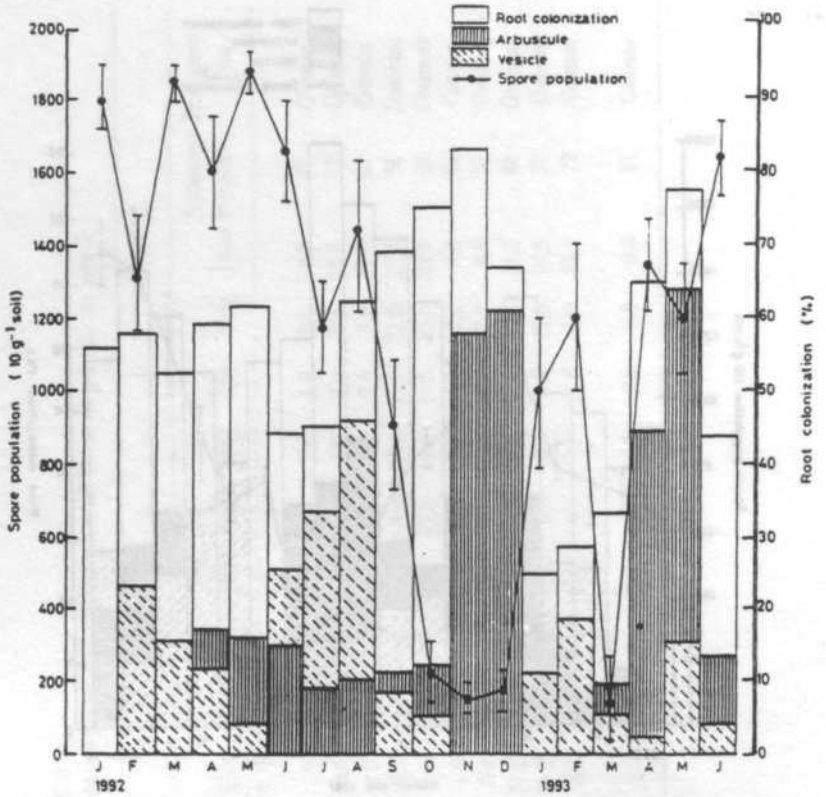

Fig. 5. Dynamics of the VAMF spore count and root colonization in Grevillea robusta *at site 1*

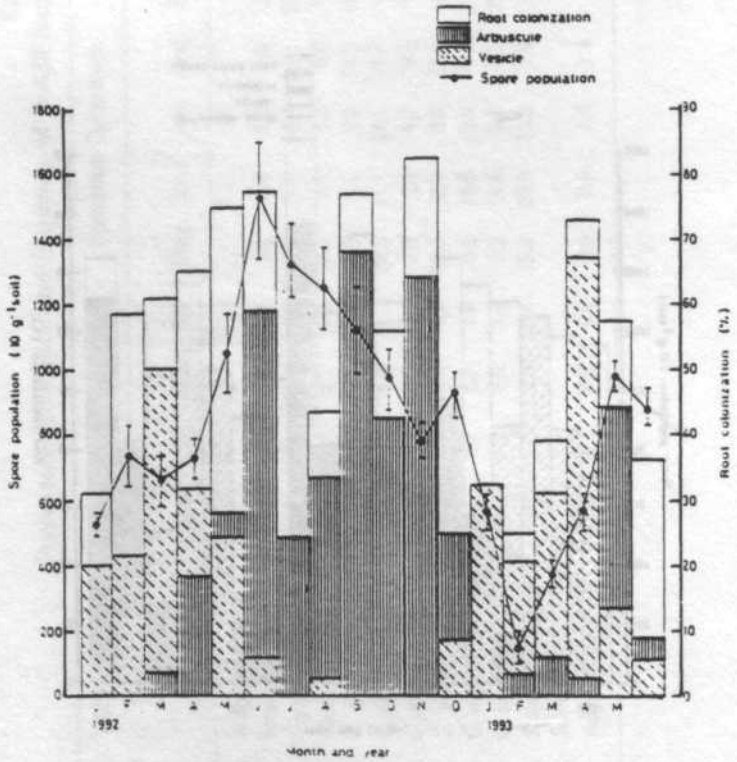

Fig. 6. Dynamics of the VAMF spore count and root colonization in Grevillea robusta *at site 2*

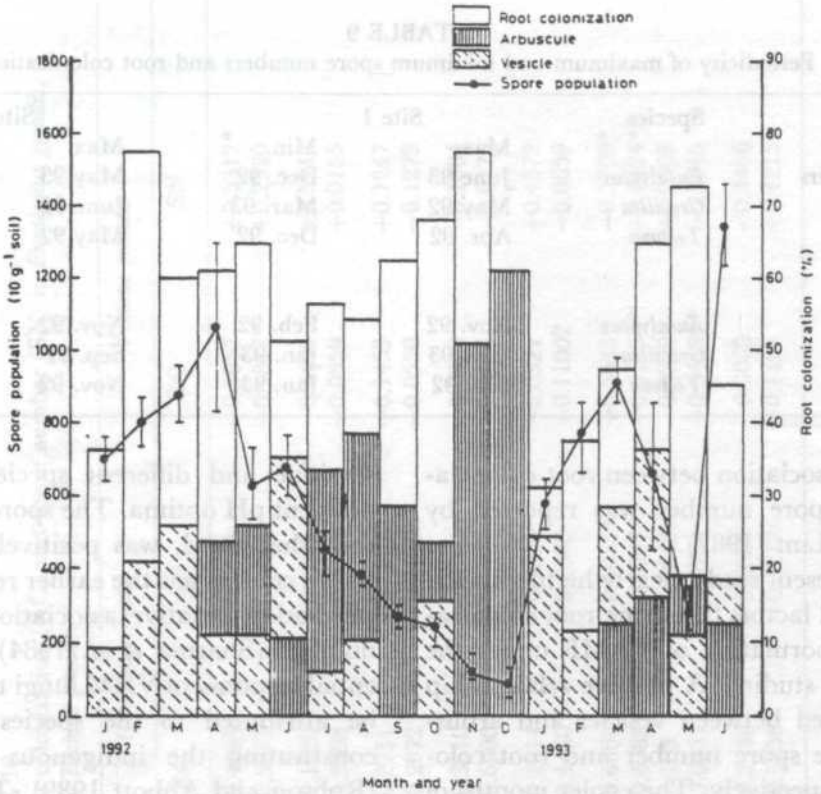

Fig. 7. Dynamics of the VAMF spore count and root colonization in Tectona grandis *at site 1*

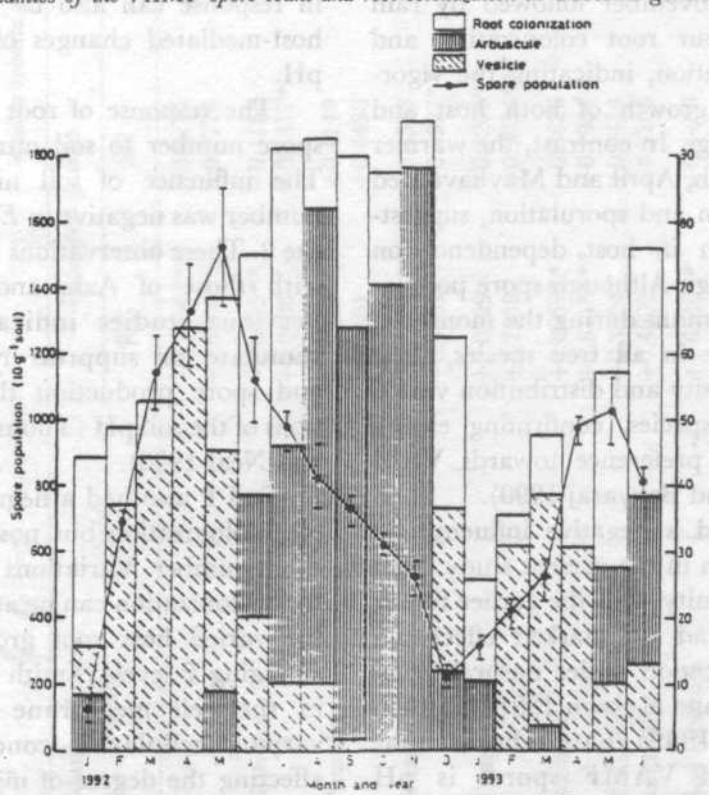

Fig. 8. Dynamics of the VAMF spore count and root colonization in Tectona grandis *at site 2*

TABLE 9
Periodicity of maximum and minimum spore numbers and root colonization

Parameters	Species	Site 1		Site 2	
		Max.	Min.	Max.	Min.
Spore numbers	*Eucalyptus*	June 93	Dec. 92	May 93	Jan. 92
	Grevillea	May 92	Mar. 93	June 92	Feb. 93
	Tectona	Apr. 92	Dec. 92	May 92	Jan. 92
Root colonization					
	Eucalyptus	Nov. 92	Feb. 92	Nov. 92	Jan. 93
	Grevillea	May 93	Jan. 93	Sep. 92	Feb. 93
	Tectona	Feb. 92	Jan. 93	Nov. 92	Jan. 93

negative association between root colonization and spore number was reported by Louis and Lim (1987).

The present study clearly highlights the periods and factors favouring root colonization and sporulation of VAMF under the tree species studied. A positive relationship was observed between vesicles and arbuscules to the spore number and root colonization respectively. The cooler months of September – November followed by rain seemed to favour root colonization and arbuscule formation, indicating the vigorous vegetative growth of both host and mycorrhizal fungi. In contrast, the warmer months of March, April and May favoured vesicle formation and sporulation, suggesting a reduction in host dependence on mycorrhizal fungi. Although spore populations were maximum during the months of January – June in all tree species, their occurrence, density and distribution varied with the host species, confirming earlier reports of host preference towards VAM fungi (Reena and Bagyaraj 1990).

Soil pH had a negative influence on root colonization in the present study. This is not in conformity with the earlier report that soil pH had no marked effects on mycorrhizal infection under natural vegetation (Abbott and Robson 1991). Robson and Abbott (1989) have shown that germination of VAMF spores is pH sensitive and different species may have different pH optima. The spore number, on the other hand, was positively correlated, which contradicts the earlier report that soil pH had a negative association with spore number (Sharma *et al.* 1984). The differential response of VAM fungi to soil pH can be attributed to the species and strains constituting the indigenous VAM flora (Robson and Abbott 1989). The variation in response can also be attributed to the host-mediated changes of the rhizosphere pH.

The response of root colonization and spore number to soil nitrogen also varies. The influence of soil nitrogen on spore number was negative in *Eucalyptus grandis* at site 2. These observations are in accordance with those of Aziz and Habte (1989). Previous studies indicate that N can stimulate or suppress root colonization and spore production through modifications of the soil pH (Thompson 1986; Sylvia and Neal 1990).

Soil P also had a negative influence on root colonization but positively influenced spore number. Variations in the response of root colonization can be attributed either to the varied host root growth response to changing P levels (Smith 1982) or changes in the cell membrane permeability of varying cellular P concentrations, thus affecting the degree of mycorrhizal coloni-

TABLE 10
Correlation matrix between edapho-climatic factors, mycorrhizal root colonization (RC) and spore number (SN) in *Eucalyptus grandis*, *Grevillea robusta* and *Tectona grandis*

Edapho-climatic Factors	Site	*Eucalyptus grandis*		*Grevillea robusta*		*Tectona grandis*	
		RC	SN	RC	SN	RC	SN
pH	1	−0.7664***	−0.0216	−0.4695*	+0.3454	−0.3057	+0.5312*
	2	+0.6906**	+0.2726	+0.3971	+0.1498	+0.4817*	+0.2320
Nitrogen	1	−0.6675**	−0.1188	−0.1020	+0.1023	−0.3449	+0.3413
	2	+0.2486	−0.5317*	+0.3398	+0.0069	+0.0859	+0.0155
Phosphorus	1	−0.2231	+0.4149	−0.3751	−0.3852	+0.1453	+0.1247
	2	−0.0101	+0.4430	−0.2972	−0.2123	−0.0550	−0.1279
Potassium	1	−0.3221	−0.3303	−0.3610	+0.0762	−0.0870	−0.1004
	2	−0.4869*	−0.5927**	−0.6900**	−0.4474	−0.3376	−0.6176
Temperature	1	+0.0329	+0.8205**	−0.1611	+0.1690	−0.0321	+0.4572
	2	+0.2558	+0.9054***	+0.4460	+0.2088	+0.11002	−0.0659
Rainfall	1	+0.8659**	−0.3826	+0.6869**	−0.5055*	+0.5633*	−0.5709*
	2	+0.8115***	−0.1934	+0.4783	+0.2097	+0.6105**	+0.8424**
Relative humidity	1	+0.3824	+0.2414	+0.103	−0.3642	−0.0299	+0.0508
	2	+0.4202	−0.3265	+0.0877	+0.3764	+0.6438**	−0.3545
Soil moisture	1	+0.6716**	+0.3160	+0.2438	−0.2036	0.3069	−0.3466
	2	−0.2161	+0.2318	+0.1256	+0.3212	−0.1361	+0.4215

*,** and *** significant $P < 0.05$, 0.01 and 0.001 respectively

zation (Ratnayake *et al.* 1978). It has also been established that soil P can reduce infections by directly inhibiting the external hyphal growth (Sanders 1975).

The role of K in root colonization and spore number of VAMF is little known compared to P and N. Potassium has previously been reported to have no effect on VAMF (Daniels and Trappe 1980), but K positively influenced spore number in all the plants at site 2 and negatively influenced root colonization in *Eucalyptus* and *Grevillea* at site 2.

Fluctuations in temperature can affect both root growth and survival and infectivity of the mycorrhizal fungi. In the present study temperature had a positive influence on *Eucalyptus*. Previous workers have shown that temperature may influence VAMF spore germination, root colonization and spore production (Tommerup 1983). The spore number was positively related to temperature in *Eucalyptus* at sites with different temperature ranges (23-30°C and 13-21°C). The occurrence of certain species common to both sites for this species indicates that the VAMF species may have multiple optimum temperatures for sporulation. Similar multiple optimum temperatures for colonization have been reported for isolates of VAMF by Schenck and Smith (1982).

The relative humidity was negatively correlated with spore number and positively with root colonization. This emphasizes that the environmental factors can strongly influence VAM fungal infection (Hayman 1974).

Percentage of root colonization was positively correlated to soil moisture which is one of the factors that determine plant growth in natural soils. An adequate moisture for plant growth may favour mycorrhizal formation due to an increase in host nutrient demand. Soil moisture optimum for plant growth has also been reported to be suitable for VAM colonization and sporulation (Redhead 1975).

CONCLUSION

The findings of this study emphasize the need for an understanding of the ecology of VAM fungi in various agroclimatic zones for the successful selection and introduction of VAM fungal species for a particular agroclimate.

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Alleviation of Cadmium Toxicity and Growth Enhancement of *Helianthus annuus* and *Triticum aestivum* Seedlings through Bacterial Inoculation

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Keywords: cadmium toxicity, plant growth promotion, bacterial inoculation

ABSTRACT

*Two Cd-resistant bacterial growth strains MA-9 (*Aeromonas*) and MA19 (affinities uncertain), which were isolated from ICI effluents, were used to inoculate seeds of* Triticum aestivum *(wheat) and* Helianthus annuus *(sunflower). Both inoculated and non-inoculated seeds were germinated and grown under different concentrations (0, 1, 2 and 3 mM) of $CdCl_2$ for 10 days. Under Cd-stress conditions, bacteria-inoculated plants had better germination and growth than non-inoculated treatments. Bacterial growth enhancement of seedlings was associated with reduced Cd uptake.*

INTRODUCTION

Cadmium, a non-essential element and an industrial pollutant, is of serious environmental and toxicological concern. It is a by-product of zinc and lead mining industries and is used in electroplating, paints, batteries (Goyer 1986). The use of phosphate fertilizers, sewage sludge, manure and lime also increases cadmium content in the soil (Anderson 1977). Cadmium is toxic to man (Goyer 1986), animals (Agrawal and Bhattacharya 1989) and plants (Page *et al.* 1981). It remains in an active state for a long time and is readily bioavailable (Goyer 1986). Plants with high cadmium content are the major source of intake, either directly or indirectly, by man (Page *et al.* 1981). In humans, its toxicity is manifested by renal dysfunction, hypertension, carcinogenic conditions, cardiovascular and chronic pulmonary diseases (Goyer 1986). Cadmium phytotoxicity is expressed by retarded growth (Greger 1989), chromosomal (Rohr and Baughinger 1976) as well as structural abnormalities (Wong *et al.* 1989), disturbed biochemical (Poschenrieder *et al.* 1989; Satakopan and Rajendran 1990) and physiological processes (Greger 1989; Poschenrieder *et al.* 1989).

It is imperative to keep the intracellular concentration of potentially noxious heavy metal ion/s at low concentrations. Some plants combat heavy metal stress by acquiring different mechanisms (Vogeli-Lange and Wanger 1990). Bacteria have developed several metabolic dependent or independent devices to counter heavy metals (Gadd 1990; Hughes and Poole 1991). These could be utilized by man for the removal and recovery of heavy metal from industrial effluents and refuse composites (Gadd 1990). Hasnain and Yasmin (1992), Sabri *et al.* (1992), Hasnain *et al.* (1993, 1995) and Saleem *et al.* (1994) have demonstrated enhanced growth of wheat seedlings under different levels of heavy metal stress through bacterial inoculation. Here, the effect of bacterial inoculation on growth of sunflower and wheat seedlings under $CdCl_2$ stress is evaluated.

MATERIALS AND METHODS

The bacterial isolates MA-9 and MA-19 are gram negative, asporogenous and facultative anaerobic rods. MA-9 was affiliated with *Aeromonas* while affinities of MA-19 remained uncertain (Sabri *et al.* 1995). Both bacterial strains were isolated from polluted waters of outlet effluents of the ICI plant (near Sheikhupura, Pakistan). Both strains tolerate up to 500 μg ml^{-1} $CdCl_2$ in the medium. Inoculum from the overnight culture (16 h), in L B (Sambrook *et al.* 1989) at 37°C (200 rpm), was replenished with fresh L broth medium and incubated at 37°C at 200 rpm. Bacterial cells from the late logarithmic growth were collected, washed and resuspended in sterile distilled water to get a final population of 10^7 cells ml^{-1}.

Certified seeds of *Triticum aestivum* var. Pak81 (wheat; Ayub Agricultural Research Institute, Faisalabad) and *Helianthus annuus* var. 256 (sunflower; Punjab Seed Corporation, Lahore) were surface sterilized by immersing in 0.1% $HgCl_2$ solution for 5-10 min. After thorough washing, seeds were soaked in bacterial suspension for 15 min, while the control seeds were drenched in sterilized distilled water for the same period. Twenty-five pretreated randomly selected seeds from each plant were spread aseptically and evenly in glass petri dishes lined with two layers of Whatman filter paper No. 1. Each plant (wheat and sunflower) was given three inoculation treatments (control, MA-9, MA-19). A total of 12 treatments were used per plant species. Fifteen ml of the respective $CdCl_2$ solutions were added to each petri dish, to ensure that the filter papers were well moistened. The petri dishes were kept in the dark at 25 ± 2°C. The dishes were regularly watered with the respective solutions. The seeds were observed daily for signs of germination. On the third day, petri dishes with germinated seeds were moved to 10 K Lux light at 25°C. Petri dishes were arranged in a completely randomized design and the position of the dishes randomized daily during the course of the experiment. An additional 15 ml of Hewitt's nutrient solution (Hewitt 1963) containing the respective $CdCl_2$ concentrations (0, 1, 2 and 3 mM) was added once to the respective treatment. The seedlings were observed daily. Growth measurements, which included length of shoot and root, fresh and dry weights of seedling, were taken 10 days after exposure to light. Presence of specific bacteria species was confirmed by isolating the bacteria from small pieces (0.5 cm) of root. The experiments were repeated four times. Data obtained were subjected to statistical analysis (means, standard error of the means, standard deviation, least significant difference, analysis of variance) adopting the method of Steel and Torrie (1981). Cadmium content in the seedlings was determined using the atomic absorption method of Rand *et al.* (1979).

RESULTS AND DISCUSSION

Seedling Germination Experiments

Hasnain and Yasmin (1992) had earlier demonstrated that Cd-resistant bacteria from the histoplane of *Suaeda fruticosa*, *Cynodon dactylon* and *Typha* could stimulate *Triticum aestivum* seedlings grown under Cd-stress conditions. Results from the present study showed that $CdCl_2$ treatments adversely affect germination of both *Triticum aestivum* and *Helianthus annuus* seeds, with a linear decrease in percentage germination as the concentration of $CdCl_2$ increased (*Fig. 1*). $CdCl_2$ at 3 mM concentration resulted in 25 and 33% decrease in percentage germination of *Triticum* and *Helianthus*, respectively, compared to control. The inhibitory effect of cadmium on germination has been reported in many plant species (Renjini and Janardhanan

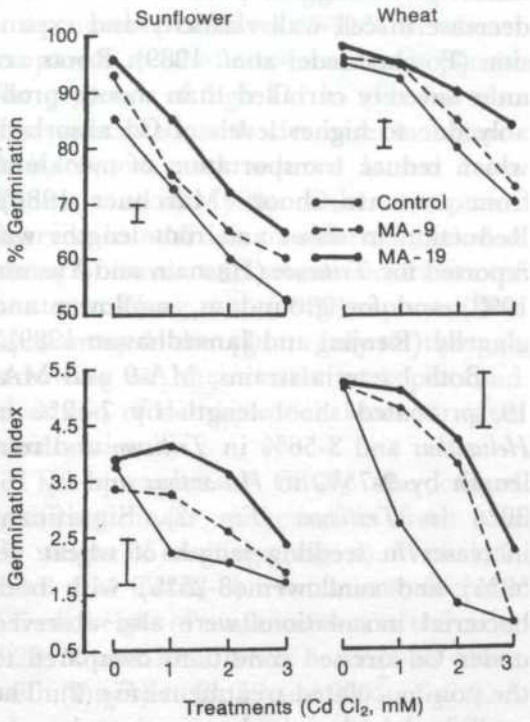

Fig. 1: Effect of bacterial inoculation on the percentage germination and germination index of sunflower and wheat seeds under different concentrations of $CdCl_2$. (Figures based on means of four replicates. Variability within means was less than 10%. Vertical bar represents least significant difference at P=0.05)

1989; Satakopan and Rajendran 1990; Hasnain and Yasmin 1992). This adverse effect may be attributed to increased uptake of cadmium which may disturb nuclear division and hinder cytokinesis (Vauline *et al.* 1978). *Fig. 1* shows inoculation of seeds with MA-9 and MA-19 enhanced and increased germination of both wheat (2-5% with MA-9; 2-14% with MA-19) and sunflower (2-8% with MA-9; 10-13% with MA-19). The stimulatory effect of the bacterial inoculum was more pronounced in the presence of cadmium.

Seedling Growth Experiments

The adverse effects of cadmium were also manifested in other growth parameters

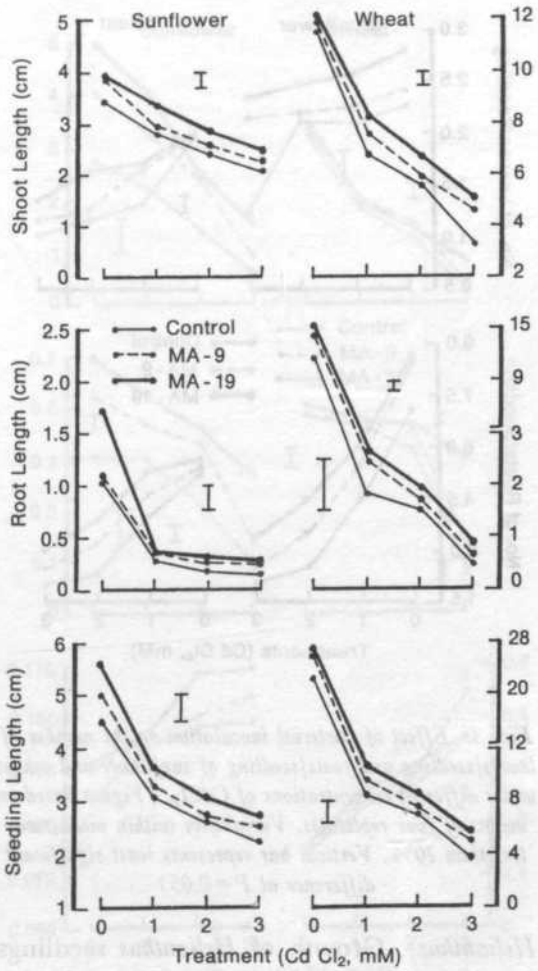

Fig. 2: Effect of bacterial inoculation on length parameters (shoot, root, seedling) of sunflower and wheat under different concentrations of $CdCl_2$. (Figure based on means of four replicates. Variability within means was less than 10%. Vertical bar represents least significant difference at P=0.05)

(shoot and root lengths, number of leaves and number of roots) of both wheat and sunflower seedlings (*Fig. 2, 3*). Presence of $CdCl_2$ also caused significant reduction in seedling lengths. At 3 mM, $CdCl_2$ shoot growth was reduced by 71% in *Triticum* and 38% in *Helianthus*. *Fig. 2* shows shoot length was relatively less curtailed compared with root growth (with 95% reduction in *Triticum* and 80% reduction in

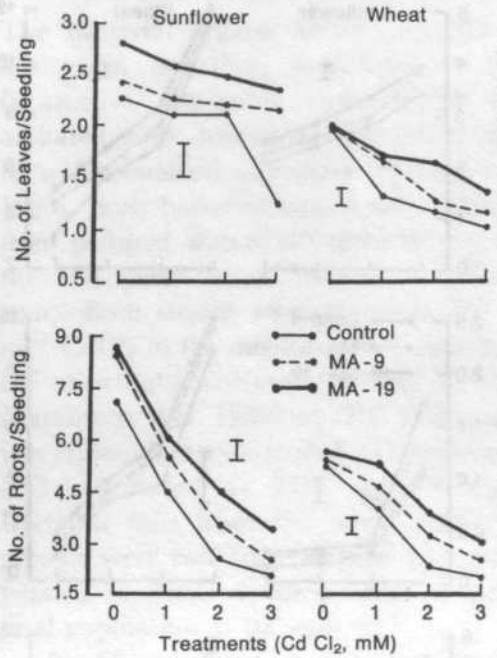

Fig. 3: Effect of bacterial inoculation on the number of leaves/seedling and roots/seedling of sunflower and wheat under different concentrations of $CdCl_2$. (Figure based on means of four replicates. Variability within means was less than 10%. Vertical bar represents least significant difference at P=0.05)

Helianthus). Growth of *Helianthus* seedlings (root and shoot) gradually decreased with increase in $CdCl_2$ concentrations (*Fig. 2*), i.e. at 1, 2 and 3mM there was a decrease of 31, 42 and 49% respectively compared with the control (0 mM). In *Triticum*, reduction in seedling length was 63, 69 and 84%, at the respective $CdCl_2$ concentrations of 1, 2, 3 mM. Affected seedlings had smaller leaves and roots, and looked unhealthy.

Growth inhibitory effects of cadmium have variously been ascribed to (i) its ability to decrease availability and transportation of calcium for various growth processes (Greger 1989) (ii) decrease in root absorption area, (iii) inhibition in cell division since Cd interferes with elongation and enlargement of cells, (iv) Cd interference with ABA metabolism and with other physiological processes, and (v) decrease in cell wall elasticity and expansion (Poschenrieder *et al.* 1989). Roots are more severely curtailed than shoots, probably due to higher levels of Cd absorbed, which reduce transportation of cytokinins from roots to shoot (Marchner 1986). Reduction in shoot and root lengths was reported for *Triticum* (Hasnain and Yasmin 1992) and for groundnut, sunflower and gingelly (Renjini and Janardhanan 1989).

Both bacteria strains, MA-9 and MA-19, promoted shoot length by 7-12% in *Helianthus* and 3-56% in *Triticum* and root length by 9-75% in *Helianthus* and by 16-80% in *Triticum* (*Fig. 2*). Significant increases in seedling length of wheat (7-59%) and sunflower (8-25%) with both bacterial inoculations were also observed under Cd stressed conditions compared to the non-inoculated treatment (*Fig. 2*). The seedling length in wheat was enhanced more than in sunflower.

Inoculation of plants with other bacterial strains has been shown to promote plant growth (Hasnain *et al.* 1993, 1995; Galiana *et al.* 1994; Saleem *et al.* 1994). Inoculating plants with specific bacterial strains increases root length, and density number, as well as the number of deformed root hairs (Bashan and Levanony 1990). Seedling growth promotion is probably the result of the bacterial cells anchoring on root surfaces (Bashan and Holguin 1993) or by changing root membrane potential for better absorption of nutrients (Bashan and Levanony 1990; Bashan 1991). The presence of various levels of $CdCl_2$ resulted in decrease in leaf number of sunflower (47%) and wheat (51%). This subsequently resulted in decrease in root number in sunflower (by 70%) and wheat (by 46%) (*Fig. 3*). This decrease in leaf number was accompanied by the appearance of brownish spots on the leaves while root tips became brown. In contrast, bacterial

inoculation resulted in significant increase in the number of leaves (5-91% in wheat) compared to the non-inoculated plants. The presence of high Cd in soils has also been associated with decrease in chlorophyll content and reduced lateral roots (Padmaja *et al.* 1990). Other symptoms observed in addition to chlorosis included wilting of leaves, and severe constriction of the stems. Wong *et al.* (1989) reported that Cd damaged the plant growth through narrowing of the vessels and pits and deposition of debris, which blocked water translocation.

The presence of $CdCl_2$ also caused significant linear reduction in fresh and dry weight of seedlings (*Fig. 4*). With 1, 2, and 3 mM $CdCl_2$ concentrations, decrease in fresh weight of sunflower was in the order of 22, 50 and 75%, while decrease in wheat was in the order 43, 59 and 90% (*Fig. 4*). A subsequent decrease in dry weights of these two species was also observed. Accumulation of dry weight, indicated by dry weight per gram fresh weight, was more pronounced with increasing concentrations of $CdCl_2$, with maximum dry weight accumulation recorded at 3 mM (*Fig. 4*). Inoculation of plants with bacteria resulted in an increase of both fresh (3-200% in wheat and 28-114% in sunflower) and dry weights (4-233% in wheat and 40-2700% in sunflower) which supersedes the weights from the respective non-inoculated treatments. Accumulation of seedling dry weight is correlated with accumulation of Cd in the seedlings (*Fig. 5*). At 3 mM concentration, Cd content in sunflower seedlings was six-fold relative to that of wheat. With both bacterial inoculations dry weight per gram fresh weight increased slightly over respective non-inoculated treatments in both plants, but significantly decreased in Cd-content of seedlings compared with respective treatments. It seems that bacterial inoculation causes a decrease

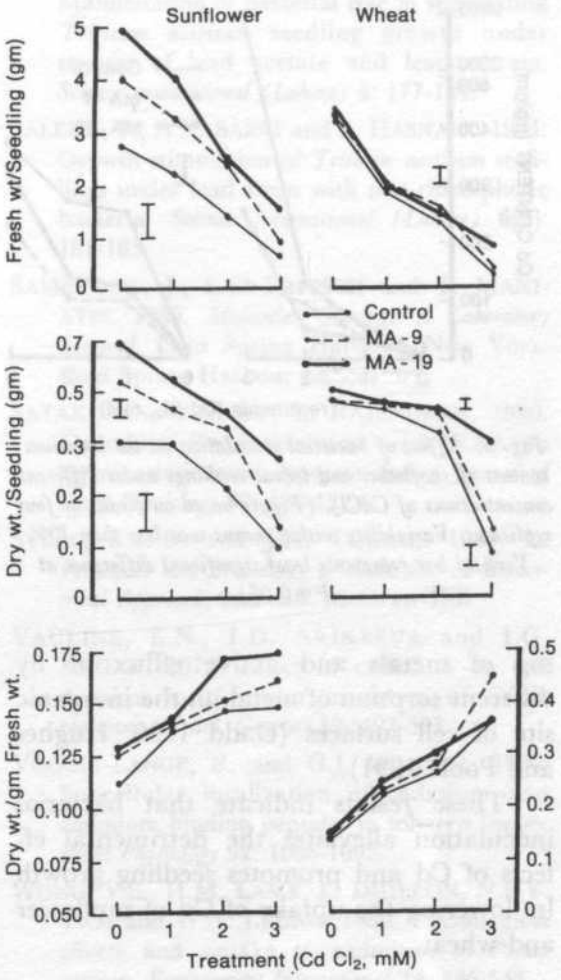

Fig. 4: Effect of bacterial inoculation on the weight parameters (fresh weight, dry weight, dry weight/gm fresh weight) of sunflower and wheat seedlings under different concentrations of $CdCl_2$. (Figure based on means of four replicates. Variability within means was less than 10%. Vertical bar represents least significant difference at $P=0.05$

in the uptake of Cd by seedlings, which ultimately induces stimulated growth, over respective non inoculated treatment, under Cd stress conditions. Bacteria exhibit detoxification mechanisms, such as metal chelating complex (Gadd 1990; Hughes and Poole 1991), Cd-peptide complexes (Konya *et al.* 1990), extracellular sequester-

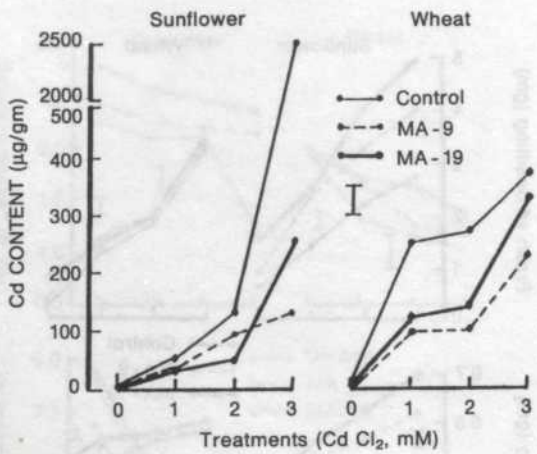

Fig. 5: Effect of bacterial inoculation on the cadmium content of sunflower and wheat seedlings under different concentrations of $CdCl_2$. (Figure based on means of four replicates. Variability within means was less than 10%. Vertical bar represents least significant difference at P=0.05)

ing of metals and active efflux or by different sorption of metal on the inorganic site of cell surfaces (Gadd 1990; Hughes and Poole 1991).

These results indicate that bacterial inoculation alleviates the detrimental effects of Cd and promotes seedling growth by lowering the uptake of Cd of sunflower and wheat.

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Mycelial Growth and Germanium Uptake by Four Species of *Ganoderma*

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Keywords: ***Ganoderma tsugae, G. subamboinense* var. *laevisporium, G. tropicum, G. lucidum*, germanium**

ABSTRAK

Miselia dari empat spesis Ganoderma *(*G. tsugae, G. subamboinense *var.* laevisporium *ATCC 52419*, G. tropicum *dan* G. lucidum*) telah ditumbuhkan dalam medium cecair yang mempunyai kepekatan germanium (Ge) yang berlainan selama 20 hari pada suhu 28°C. Didapati bahawa kadar pertumbuhan dan berat kering miselia keempat-empat spesis semakin menurun dengan penambahan kepekatan Ge dalam medium walaupun peratusan pengambilan Ge oleh miselia semakin bertambah. Tahap pertahanan miselia terhadap Ge di dalam medium pertumbuhan berbeza untuk keempat-empat spesis* Ganoderma. *Untuk setiap spesis, kepekatan optima Ge di dalam medium telah ditentukan sebagai 100mg/l untuk mendapatkan keadaan optima bagi pengambilan Ge serta pertumbuhan miselia yang optimum.*

ABSTRACT

Four Ganoderma *species (*G. tsugae, G. subamboinense *var.* laevisporium, *ATCC 52419*, G. tropicum *and* G. lucidum*) were incubated in liquid medium containing different concentrations of germanium (Ge) for up to 20 days at 28°C. Increasing the Ge concentration of the medium resulted in a gradual decrease in the growth of the fungal mycelium. However, the Ge content in the mycelium increased with increasing Ge concentration. Different species recorded different levels of tolerance towards the Ge. In each case, the optimum concentration of the incorporated Ge in the medium was established as 100 mg/l for both optimal uptake of Ge by the fungal mycelium and optimal mycelial growth.*

INTRODUCTION

Traditionally in the Orient, *Ganoderma* has been considered an "elixir of life". Today, there is a considerable body of contemporary research that shows that certain species of *Ganoderma* are highly effective medicinal agents as a popular remedy to treat hepatopathy, chronic hepatitis, nephritis, hypertension, hyperlipedemia, arthritis, neurasthenia, insomnia, bronchitis, asthma, gastric ulcer, arteriosclerosis. leukopenia, diabetes, anorexia, mushroom poisoning and debility due to prolonged illness (Willard 1990). The medicinal value of *G. lucidum* is closely linked to the presence of the following compounds: organic-Ge, polysaccharides, triterpenoids and adenosine (Tong 1995).

In Malaysia, studies on cultivation techniques of a suitable strain of *G. lucidum* well adapted to the local climatic conditions (Tong and Chen 1990), its growth characteristics and Ge uptake by the mycelium (Tong *et al.* 1994a) as well as the fruiting bodies of this fungus (Tong *et al.* 1994b) have been carried out. Because of the paucity of information on the uptake of Ge by other species of *Ganoderma* of com-

mercial value, the present study was undertaken to ascertain differences, if any, in their uptake of Ge.

MATERIALS AND METHODS

Cultures

Four species, *G. tsugae, G. subamboinense* var. *laevisporium* (ATCC 52419), *G. tropicum* and *G. lucidum* were obtained from Taiwan and maintained on potato dextrose agar (PDA) at 28°C and subcultured every 3 weeks.

Uptake of Ge by Fungal Mycelium Grown in Liquid Medium

Samples (100 ml) of potato dextrose broth containing each of the following concentrations (50, 100, 200, 300, 400 mg/l) of GeO_2 were poured into 1-l flat bottles and sterilized at 121°C, 15 psi for 15 min. Each bottle was then inoculated with three agar mycelium discs (1.3 cm diam) of a ten-day-old culture and incubated in the dark at 28°C for 20 days. The mycelium was later harvested through pre-weighed Whatman No. 1 filter paper and washed with several changes of double distilled deionised water (200 ml). The filter paper, together with the washed mycelium, was dried at 60°C for 48 h. The dry weight of the mycelium was recorded.

Analysis of Germanium

Fungal mycelium samples were ashed at 700-800°C for 1 – 3 hours. The ash was then dissolved in 5M HCl. Germanium in the solution was determined by hydride generation – inductively coupled plasma atomic emission spectrometry (ICP-AES) using the Labtest Plasmascan 710 instrument. The method of analysis was adapted from the method for arsenic analysis (Lee and Low 1987).

A spectropure (Aldrich Chemical Co. Inc.) Ge atomic absorption standard solution of 990 mg/l was used as the stock solution. The standard solutions used in the analysis were prepared by sequential dilution from the stock solution. All reagents used were analytical grade. A solution of 2% sodium borohydride in 0.1% sodium hydroxide was prepared daily from $NaBH_4$ pellets.

The rate of uptake (%) of Ge is calculated as:

$$\frac{\text{Amount of Ge in known dry weight of mycelium}}{\text{Amount of Ge in 100 ml medium}} \times 100$$

RESULTS AND DISCUSSION

Effect of Ge on Mycelial Growth in Liquid Medium

With increasing amounts of GeO_2 incorporated into the medium, there was a corresponding decrease in the dry weight of the mycelium (*Fig 1*). The effect was most obvious for *G. tsugae*, which experienced a continuous sharp drop in the mycelial dry weight at concentrations above 50 mg/l of GeO_2. Similarly for *G. lucidum*, the growth of the mycelium was

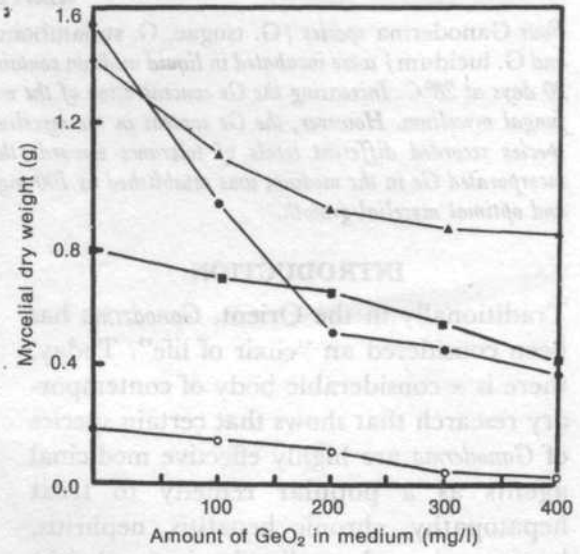

Fig 1. Mycelial growth of Ganoderma *species in liquid medium containing different concentrations of GeO_2 incubated for 20 days at 28°C. Results represent average of triplicates.*

● G. tsugae ▲ G. tropicum ○ G. lucidum
■ G. subamboinense *var.* laevisporium

TABLE 1
Effect of GeO_2 in liquid medium on the uptake of Ge by mycelium after 20 days of incubation at 28°C

Species	Conc. of GeO_2 in 100 ml liquid medium (mg/l)	Mycelial dry weight (g)	Amount of Ge in mycelium (mg/kg)	Uptake of Ge (%)
G. tsugae	50	0.79	752 + 5	11.8
	100	0.70	1892 + 15	13.3
	200	0.68	2456 + 22	8.3
	300	0.54	3855 + 127	6.9
	400	0.38	4312 + 90	4.0
G. subamboinense	50	1.43	34 + 4	1.0
var *laevisporium*	100	1.10	99 + 3	1.1
	200	0.93	175 + 11	0.8
	300	0.87	260 + 9	0.7
	400	0.82	310 + 8	0.6
G. tropicum	50	1.53	235 + 7	7.2
	100	0.94	1080 + 22	10.1
	200	0.52	2072 + 15	5.4
	300	0.46	2715 + 43	4.2
	400	0.37	4002 + 90	4.3
G. lucidum	50	0.12	687 + 18	6.4
	100	0.11	1796 + 14	8.0
	200	0.10	2845 + 31	6.0
	300	0.05	4251 + 112	3.2
	400	0.04	5431 + 87	2.6

Results represent average of triplicates

retarded considerably. This may be due to toxicity at high concentrations. Only extremely sparse growth occurred at concentrations of 250 mg/l of GeO_2 and at concentrations above 300 mg/l of GeO_2 there was no growth of the mycelium at all until the tenth day of incubation (visual observation). The limited growth which commenced after this lag period may probably be due to the induction of some detoxification mechanism.

The growth of the mycelium for *G. subamboinense* var. *laevisporum* was least affected by the amount of GeO_2 in the medium and there was a more gradual decline in the mycelial dry weight than in the other species (*Fig 1*). Of the four species tested, *G. tropicum* produced the most abundant mycelial growth at Day 20 and the effect of GeO_2 was more gradual above 200 mg/l of GeO_2 but significantly decreased between 50 and 200 mg/l.

Uptake of Ge by Fungal Mycelium in Liquid Medium

Table 1 shows that in general, Ge uptake by the mycelium increased with increasing amounts of GeO_2 in the growth medium. Of the four species tested, *G. subamboinense* var. *laevisporium* assimilated least Ge into the mycelium, which may explain why its mycelial growth is least affected by increasing quantities of GeO_2 in the medium. At 400 mg/l of the GeO_2 in the medium, *G.*

tsugae and *G. tropicum* took up almost 13 times, while *G. lucidum* took up 17 times more Ge than *G. subamboinense* var. *laevisporium*. However, it was noted that the rate of uptake (%) of Ge by the mycelium increased to a maximum at 100 mg/l of GeO_2 in all species and then decreased with increasing concentration of GeO_2 in the medium. The highest rate of uptake recorded was 13.3, 10.1, 8.0 and 1.1% for *G. tsugae*, *G. tropicum*, *G. lucidum* and *G. subamboinense* var. *laevisporium*, respectively.

Thus, under the conditions studied, it was deduced that for maximum production of fungal mycelium with optimal Ge content, the optimum concentration of GeO_2 to be added to the medium was 100 mg/l.

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Nutritional Evaluation of Full-Fat Soyabean Boiled for Three Time Periods

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ABSTRAK

Nilai nutrien kacang soya penuh-lemak yang direbus bagi tempoh masa tiga kali dikaji dalam ujian yang seimbang terhadap tikus dan percubaan memberi makan bersama khinzir penyapih. Nilai peratus protein yang belum dimasak ialah 42.43, 42.7, 43.0 dan 43.4 bagi kacang soya mentah yang masing-masing direbus 30,60 dan 90 minit. Estrak eter disusun daripada 13.2% untuk contoh mentah kepada 19.0% bagi contoh-contoh yang direbus 90 minit fon. Terdapat sedikit kenaikan dalam kandungan asid amoni dengan penambahan masa memproses sementara pembalikan merupakan kes bagi elemen-elemen mineral dan faktor-faktor antinutrien. Indeks kualiti protein menunjukkan bahawa pengambilan makanan tidak dipengaruhi oleh rebusan. Kadar keefisienan protein (PER) dan nilai penahanan protein bersih (NPR) menurun dengan penambahan masa merebus. Tambahan berat dan pemakanan khinzir bertambah baik ($P<0.05$) bersama masa merebus, manakala tidak mempunyai kesan yang signifikan terhadap pengambilan makanan.

ABSTRACT

The nutritional value of full-fat soyabean (FFSB) boiled for three time periods (30, 60, 90 min) was studied in a balanced experiment on rats and a feeding trial with weaner pigs. The crude protein percentage values were 42.34, 42.7, 43.0 and 43.4 for raw, 30, 60 and 90 min boiled soyabean respectively. Ether extract ranged from 12.3% for the raw sample to 19.0% for samples boiled for 90 min. There were slight increases in the amino acid content with increase in processing time while the reverse was the case for the mineral elements and the antinutritional factors. Protein quality indices showed that feed intake was not influenc by boiling. The protein efficiency ratio (PER) and net protein retention (NPR) values decreased with increasing boiling time. Weight gain and feed|gain of pigs were improved ($P<0.05$) with boiling time, while boiling had no significant effect on feed intake. Increasing boiling time tends to improve the nutritive value of FFSB slightly.

INTRODUCTION

The use of full-fat soyabean (FFSB) for animal feeding has increased, particularly in Nigeria, because the high oil and protein content make it useful for inclusion in diets of high energy and high nutrient concentration. Use of the raw bean is limited due to the presence of heat-labile antinutritional factors, the most important of which are trypsin inhibitors, which seriously impair protein digestibility (Balloun 1980).

Because of this increasing use of soyabean and other legumes for animal nutrition, different technologies have been introduced for feedstuff processing. Depending on the technology use, the aims of processing are to inactivate the negatively acting substances by heat and mechanical treatment to destroy the plant cell structure. The latter should guarantee

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increasing nutrient availability for the animals.

Boiling of soyabean is the most commonly used farm-adaptable processing method in Nigeria (Awosanmi 1988). The optimum duration of boiling is an outstanding question. The American Soyabean Association recommends a minimum boiling time of 30 min (Monari 1988). In this study, three durations for boiling FFSB were investigated to determine their effects on the quality and utilization of the bean.

MATERIALS AND METHODS

Processing of Full-fat Soyabean

Raw soyabean was obtained from Pfizer Livestock Feeds Limited, Lagos, Nigeria. Raw soyabean packed in jute bags was lowered into a half-drum of boiling water and allowed to boil for 30, 60, and 90 min respectively. Timing of the boiling commenced when the water reached 100°C after introducing the bags. The boiled seeds were drained of water and sundried to less than 10% moisture level before being ground and stored. Portions were withdrawn for analysis and animal feeding experiments.

Analytical Procedure

The proximate and mineral composition of the processed samples were determined according to the Official Methods of AOAC (1990). Amino acid analysis was carried out using column chromatography. The phytin and phytic phosphorus content of the samples were determined according to the method outlined by Young and Greaves (1940). Urease activity was determined according to the procedure described by McNaughton *et al.* (1981) while trypsin inhibitor activity (TIA) was obtained through the procedure outlined by AOCS (1985).

Biological Evaluation

Twenty-four male albino rats of the Wistar strain, weighing 52-55 g and appropriately 21 days old, were obtained from the Faculty of Veterinary Medicine, University of Ibadan rat colony. They were divided into 6 groups of 4 rats each on the basis of initial weight. The rats were individually housed in perforated perspex cages with facilities for separate faecal and urinary collection.

The composition of the basal diet is shown in Table 1. The soyabean samples to be evaluated were added at excess of maize starch to give 10% crude protein on a dry matter basis. Nutritional casein diet was used as the reference standard. One group of 4 rats was given the N-free basal diet, and the remaining five groups were randomly allocated to the test and standard diets.

Rats were offered water and food *adlibitum* for 14 days. The rats were weighed weekly, faecal and urinary collections were made daily for the last 7 days of the experiment. The urine from each cage

TABLE 1
Composition of the basal diet for rats

Ingredients	%
Corn starch	64.95
Glucose	5.00
Sucrose	10.00
Non-nutritive cellulose	5.00
Vegetable oil	10.00
Premix*	2.00
Oyster shell	1.00
Bone meal	2.00
Salt (NaCl)	0.25

*Supplied per kg of diet: 500,000 IU vitamin A; 100,000 IU vitamin D3; 800 mg vitamin E; 400 mg vitamin K; 1200 mg vitamin B2; 1000 mg vitamin B3; 4 mg vitamin B2; 3000 mg niacin; 4000 mg vitamin C; 11,200mg choline; 24000 mg manganese; 800 mg iron, 16,000 mg copper; 18,000 mg zinc; 500 mg iodine; 48 mg selenium; antioxidant (BHT)

was collected in small urine cups containing 3 cm^3 of 1.0M sulphuric acid as preservative. Each day's collection was stored in screw-capped bottles at -4°C. Faecal samples were collected daily, bulked for each rat, weighed, dried and stored. Duplicate samples of urine, faeces and diets were taken for nitrogen determination. From the data on nitrogen intake and excretion, and the weight gain, protein efficiency ratio (PER) and net protein retention (NPR) were calculated according to the procedure outlined by National Academy of Sciences/National Research Council (NAS/NRC) (1963) and Bender and Doell (1957) respectively, while biological value (BV) and net protein utilization (NPU) were calculated according to the procedure of Phillips *et al.* (1981). The true (nitrogen) N-digestibility was estimated according to the procedure of Dryer (1963).

Feeding Trial with Pigs

Four diets were formulated to contain raw, 30, 60, and 90 min boiled FFSB (Table 2). The diets were iso-nitrogenous and caloric, containing 20% crude protein and 3085 kcal ME/kg. A total 36 Large White × Landrace pigs weaned at 28 days, weighing initially 5.70 ± 0.09 kg, were assigned to four treatments of nine pigs each. Each treatment was replicated thrice with three pigs per replicate. Each replicate was housed in a concrete-floored pen. Feed and water were supplied *ad libitum*. The trial lasted for nine weeks.

Statistical Analysis

Data were subjected to analysis of variance, followed by the Duncan multiple range test (Steel and Torrie 1980) at 5% probability level to evaluate the difference among treatment means.

RESULTS AND DISCUSSION

Tables 3 and 4 show the proximate and mineral composition respectively of the test ingredients, while the amino acid composition is shown in Table 5. Processing has no significant effect ($P > 0.05$) on the proximate composition. The crude protein value

TABLE 2
Composition of diets for pigs

	FFSB			
	(Raw)	(30 min)	(60 min)	(90 min)
Maize	53.50	53.50	53.50	53.50
Maize offal	10.00	10.00	10.00	10.00
Fish meal	5.00	5.00	5.00	5.00
Full-fat soyabean	29.00	29.00	29.00	29.00
Bone meal	1.25	1.25	1.25	1.25
Oyster shell	0.75	0.75	0.75	0.75
Premix*	0.25	0.25	0.25	0.25
Salt	0.25	0.25	0.25	0.25
Determined (Dry matter basis)				
Crude protein (%)	21.2	21.5	21.3	20.3
Crude fibre (%)	3.8	3.9	5.3	5.9
Ether extract (%)	3.1	2.1	3.5	2.3

*Supplied per kg diet: vitamin A, 10,000 IU; vitamin D_3; 2000 IU; vitamin E, 5 IU; vitamin K, 2.24 IU; vitamin B_{12} 0.01 mg; riboflavin, 5.5 mg; pantothenic acid, 10 mg; nicotinic acid, 25 mg; choline, 35 mg; folic acid, 4 mg; manganese, 56 mg; iodine, 1 mg; iron, 20 mg; copper, 10 mg; zinc, 50 mg; cobalt, 1.25 mg

TABLE 3
Proximate composition of the test ingredients (%)

	FFSB			
	(Raw)	(30 min)	(60 min)	(90 min)
Dry matter	91.4	91.29	91.79	91.51
Crude protein	42.2	42.7	43.0	43.4
Crude fibre	5.88	6.65	6.48	6.75
Ether extract	12.3	18.6	18.5	19.0
Ash	5.14	4.54	4.54	4.48
Nitrogen-free extract	34.28	27.51	27.48	26.37

TABLE 4
Mineral composition of the test ingredients (%)

	FFSB			
	(Raw)	(30 min)	(60 min)	(90 min)
Calcium (Ca)	0.731^{a}	0.670^{a}	0.591^{b}	0.502^{b}
Phosphorus (P)	0.102^{a}	0.099^{a}	0.086^{b}	0.095^{ab}
Sodium (Na)	3.247^{a}	$2.946^{a}b$	2.667^{b}	1.765^{c}
Potassium (K)	1.380^{a}	1.100^{b}	$1.048b^{c}$	0.950^{c}
Magnesium (Mg)	0.082^{a}	$0.075^{a}b$	$0.061^{b}c$	0.059^{c}
Iron (Fe)	0.043^{a}	$0.038^{a}b$	0.033^{b}	0.025^{c}
Copper (Cu)	0.0016^{b}	0.0019^{a}	0.0020^{a}	0.0020^{a}
Zinc (Zn)	0.008^{a}	0.0010^{b}	0.0010^{b}	0.0010^{b}
Manganese (Mg)	0.0057^{a}	0.0056^{a}	0.0057^{a}	0.0058^{a}

a, b, c Means with different superscript in horizontal rows are significantly different ($P < 0.05$)

is higher than values reported by Institut National de la Recherche Agronomique (INRA) (1984) but close to the value reported by Oyenuga (1968) for soyabean grown in Nigeria. The residual oil value fell within the range reported by Balloun (1980) and Tewe (1984). The ash and fibre content agrees with the values reported by Balloun (1980) and Tewe (1984).

Soyabean has been reported to be deficient in the sulphur amino acid - methionine and cystine (Balloun 1980). However, Patrick and Schaibe (1980) reported that the deficiency was due to unavailability rather than to absence. This may be a reason for the increased level of methionine with processing of the sample (Table 5). Generally, the mineral elements of the soyabean decreased ($P > 0.05$) with processing. This may be expected since most of the minerals are volatile; hence they are lost in water and through vaporization during processing. The longer the product stays in water during processing, the greater may be the loss. The antinutritional factors in the FFSB (Table 6) decreased with increased boiling time. This is the accordance with the findings of McNaughton *et al.* (1981). None of the soybean products is over-processed, but all were properly processed (except raw soybean).

Table 7 contains a summary of the biological evaluation of protein quality. Although the animals were offered isonitrogenous diets, their protein intake dif-

TABLE 5
Amino acid composition of the test ingredients (%) of dry matter

	Arginine	Histidine	Lysine	Phenylalanine	Tyrosine	Leusine	Isoleusine	Methnionine	Valine	Cystine	Alanine	Glycine	Glutamic acid	Serine	Threonine	Aspartic acid
FFSS (Raw)	2.77	1.07	2.35	1.80	1.38	2.85	1.78	0.50	1.82	0.54	1.58	1.79	7.08	1.71	1.44	3.76
FFSB (30 min)	2.88	1.52	2.64	2.49	1.69	3.55	2.20	0.61	2.03	0.60	1.97	1.96	8.8	3.32	1.79	5.36
FFSB (60 min)	2.90	1.57	2.74	2.54	1.68	3.65	2.23	0.63	2.06	0.60	1.99	1.99	8.86	2.38	1.81	5.50
FFSB (90 min)	2.89	1.60	2.80	2.57	1.71	3.64	2.22	0.63	2.05	0.60	2.02	2.02	9.14	2.44	1.83	5.52

TABLE 6
Antinutritional factors of FFSB samples

	FFSB				
	(Raw)	(30 min)	(60 min)	(90 min)	S.E. ±
Trypsin inhibitor activity (mg/g)	8.51[a]	4.43[b]	3.20[c]	2.85[d]	0.18
Urease activity (pH)	0.25[a]	0.16[b]	0.11[c]	0.08[d]	0.05
Dye-binding capacity (Cresol red absorbed) (mg/g)	2.63[b]	4.18[a]	4.22[a]	4.32[a]	0.03
Phytin (%)	0.95[a]	0.60[b]	0.56[bc]	0.50[c]	0.20
Phytin-phosphorus (% total phosphorus)	45.13[a]	28.5[b]	26.6[bc]	23.75[c]	0.42
Phytin-phosphorus (%)	0.29[a]	0.18[b]	0.17[b]	0.15[b]	0.3

a, b, c, d Means with different superscript in horizontal rows are significantly different ($P < 0.05$)
S.E. = Standard error

TABLE 7
Biological evaluation of protein quality using rats

	FFSB				
	(Raw)	(30 min)	(60 min)	(90 min)	S.E. ±
Average initial weight (g)	55.89	55.69	55.67	56.33	0.33
Average final weight (g)	59.98	63.37	64.55	65.13	1.32
Feed intake (g)	40.12[b]	42.38[b]	48.38[a]	41.99[b]	0.49
Crude protein of diet (%)	9.89	10.09	9.99	10.14	0.02
Protein intake (%)	3.97[b]	4.01[b]	4.66[a]	4.18[b]	0.07
Weight gained (%)	5.12[c]	7.68[b]	8.88[a]	8.80[a]	0.47
Protein efficiency ratio (PER)	0.53[b]	1.95[a]	2.01[a]	2.08[a]	0.20
Net protein retention (NPR)	2.08[a]	2.17[a]	2.04[a]	1.25[b]	0.03
Biological value (BV)	41.0[a]	60.76[b]	63.97[b]	64.66[b]	0.06
True nitrogen digestibility (TND)	44.33[b]	57.59[a]	55.16[a]	55.57[a]	1.78
Net protein utilization (NPU)	30.68	35.27	35.78	36.49	0.17

a, b, c Means with different superscript in horizontal rows are significantly different ($P < 0.05$)
SEM = Standard error of mean

TABLE 8
Performance of pigs fed boiled full-fat soyabean

	FFSB				
	(Raw)	(30 min)	(60 min)	(90 min)	S.E. ±
Initial weight (kg/pig)	5.6[a]	5.6[a]	5.8[a]	5.8[a]	0.09
Final weight (kg/pig)	18.00[b]	21.66[a]	22.10[a]	23.12[a]	0.66
Feed intake (gm/day)	620[a]	625[a]	635[a]	620[a]	0.22
Weight gain (gm/day)	196.8[b]	255[a]	265[a]	275[a]	0.64
Feed/gain	3.15[a]	2.45[b]	2.40[b]	2.25[b]	1.17

a, b, c Means with different superscript in horizontal rows are significantly different ($P < 0.05$)
SEM = Standard error of mean

fered, with rats on the 60-min processed FFSB consuming the highest protein ($P < 0.05$). Weight gain reflected the same trend as protein intake. Net protein retention was higher ($P < 0.05$) in the 90-min processed sample than in the others. Biological value, true nitrogen digestibility and net protein utilization increased ($P < 0.05$) with boiling time.

Variation in performance observed with rats fed with FFSB is in agreement with the observations of Bamgbose (1988) and Awosanmi (1988) for poultry that the nutritive value of a protein supplement can be improved by processing (heat treatment) due to increased availability and digestibility of intrinsic nutrients.

Results of the utilization by weaner pigs of the differently processed FFSB (Table 8) show that average daily feed intake was not significantly influenced by processing time of FFSB. This is in accordance with the findings of Awosanmi (1988) and Bamgbose (1988) for poultry that processing of soyabean may not affect the feed intake. Haywood *et al.* (1953) reported that the failure of raw soybean meal and low temperature oilseed meals to promote good growth was not due to a lack of feed intake but due to differences in the nutritional value of their protein as a result of the methionine deficiency. Feed to gain ratio improved ($P < 0.05$) in rats and pigs with processing time of FFSB. This agrees with the findings of Veltmann *et al.* (1987) for chicks.

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Influence of Peat and Amount and Frequency of Rain on the Mobility of Alachlor and Terbuthylazine

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Keywords: alachlor, leaching, organic matter, terbuthylazine

ABSTRAK

Kajian pengaruh kandungan tanah gambut yang berbeza dan jumlah serta kekerapan hujan ke atas pergerakan alaklor dan terbutilazin telah dijalankan di rumah hijau. Pergerakan dua herbisid melalui profil tanah telah ditentukan secara bioasai dengan menggunakan Cucumis sativus. *Didapati hubungan berbalik dengan kandungan bahan organik tanah dan jumlah serta kekerapan hujan mempengaruhi larut lesap herbisid. Pada amnya, terbutilazin kurang mobil berbanding dengan alaklor dalam dua jenis tanah yang dikaji, iaitu siri Serdang dan siri Selangor. Aras fitotoksik terbutilazin terhad pada kedalaman 0-15 cm dalam kedua-dua jenis tanah apabila 640 ml air dialir sepanjang tempoh 16 hari. Sebaliknya alaklor bergerak ke kedalaman 20 dan 30 cm masing-masing di dalam tanah siri Selangor dan Serdang apabila turus tanah didedahkan kepada jumlah air yang sama.*

ABSTRACT

The influence of different levels of peat and amount and frequency of simulated rain on the mobility of alachlor and terbuthylazine in soil was investigated under greenhouse conditions. Movement of the two herbicides down the soil profile, assessed by bioassay using Cucumis sativus, *was inversely related to the organic matter content of the soil, but the amount and frequency of rain directly influenced the extent of leaching of the herbicides. In general, terbuthylazine was less mobile than alachlor in the two soils studied, namely Serdang and Selangor series. The phytotoxic levels of terbuthylazine were restricted to the 0 - 15 cm zone in both soils when 640 ml simulated rain was applied over 16 days. Alachlor, on the other hand, moved to depths of 20 and 30 cm in Selangor and Serdang series, respectively, when soil columns were exposed to the same watering treatment.*

INTRODUCTION

Leaching of herbicides within the soil profile may determine their effectiveness, explain selectivity or crop injury, or account for herbicide transpiration from the soil. Besides downward leaching with water, herbicides are also known to move laterally or upwards in the soil by capillary movement of the soil water (Ashton and Monaco 1991). Mobility of herbicide in soils is influenced by environmental and various soil factors (Aldrich 1984; Beyer *et al.* 1987). Leaching can cause poor weed control, crop injury, increased herbicide loss or herbicide accumulation in soil (Anderson 1977). High mobility may lead to contamination of ground-water in some situations (Leistra 1980).

The main factors influencing the mobility of herbicides in soil are adsorption of the herbicide into soil particles, water solubility of the herbicide, volume of water flow and soil texture (Ross and Lembi 1985; Gunther *et al.* 1993; Rahman *et al.* 1993). In general, adsorption is the most important factor affecting the leachability

of herbicides in soil. Herbicides that are adsorbed into soil particles do not leach, unless the soil particles themselves move with the flow of water. Mobility of herbicides generally decreases with increase of soil organic matter content due to increased adsorption (Nicholls *et al.* 1987; Wilson and Foy 1992). The movement of herbicides down the soil profile is also greatly influenced by the amount of rainfall (Leistra 1980; Beyer *et al.* 1987); the greater the volume the more herbicides are leached (Marriage *et al.* 1977). Leaching is also positively correlated with frequency of rainfall (Oppong and Sagar 1992).

In recent years, worldwide concern has been expressed about the potential contamination of surface waters and groundwater by pesticides in run-off and soil water from agricultural fields (Bergstrom and Jarvis 1993). However, information on mobility of herbicides under local conditions is scarce. In the present study, the influence of soil organic matter content and the amount and frequency of simulated rain on the movement of alachlor and terbuthylazine in soil columns was studied under greenhouse conditions. Alachlor and terbuthylazine are pre-emergence and early post-emergence herbicides.

MATERIALS AND METHODS

Soils

Two soils, Selangor series (silty clay) and Serdang series (sandy clay loam), were used in these mobility studies. Selangor series soil was collected from Jenderata Estate, Teluk Intan, Perak, while the Serdang series samples came from the experimental plot at Universiti Pertanian Malaysia, Serdang, Selangor. Peat soil was collected from MARDI Research Station at Kelang, Selangor. The soils were collected from the top 0-10 cm, air-dried and screened through a 1.0-cm sieve prior to use. Some characteristics of the soil samples are shown in Table 1.

Herbicides

The two herbicides used in this study were alachlor which is nonionic (Lasso®, containing 480 g a.i./l) and terbuthylazine which is weak basic (Gardoprim®, containing 500 g a.i./l) in aqueous solution.

Effect of Organic Matter (Peat) in Soil on Mobility of Herbicides

A PVC column (30 cm long and 11 cm diam.) was carefully filled to a depth of 25 cm with either sand, peat or sand-peat mixtures containing 5, 10, 20 or 50% (w/w) peat. Once the column had settled, a 5-cm thick layer of soil treated with either alachlor (0 or 4 ppm) or terbuthylazine (0 or 5 ppm) was placed on top of the peat mixture and the column was lined with one sheet of Whatman No. 3 filter paper. Five hours after adding the treated soil, the soil column was watered with 20 ml water (equivalent to 2.1 mm of rain) every day for

TABLE 1
Physico-chemical characteristics of the soils

Characteristics	Selangor Series	Serdang Series	Peat Soil
pH	4.01	4.57	3.5
silt (%)	53.5	14.6	11.7
sand (%)	3.1	50.6	43.7
clay (%)	43.4	34.8	44.6
organic matter (%)	4.33	0.78	59.3
CEC (cmol (+) kg^{-1})	23.7	4.7	145.0

16 days. On Day 17, the column was separated into 5-cm segments and the soil was bioassayed following the procedure of Akobundu and Essiet (1974). The soil was placed in individual small plastic pots (12 cm diam.) into which six seeds of the bioassay species, cucumber (*Cucumis sativus* L.), were planted at a depth of 0.5 cm. The soil was watered twice daily to maintain the moisture level at about 90% field capacity. After emergence, plants were thinned to four per pot. Seven days after emergence for alachlor and nine days for terbuthylazine, the plants were cut at soil level. The fresh weight of seedlings was recorded. The phytotoxic effect of herbicide is expressed as a percentage of the untreated control value.

Effects of Amount and Frequency of Simulated Rain on the Mobility of Herbicides

PVC columns were uniformly packed to a depth of 25 cm with 0.74 g/m^3 Selangor series or 1.05 g/m^3 Serdang series soil at 50% field capacity. In order to obtain 50% field capacity for Selangor and Serdang series, the amount of water applied was equivalent to 390 and 235 ml/kg dry soil, respectively. After equilibrating, a 5-cm layer of soil (350 g Selangor series or 500 g Serdang series) treated with either alachlor (0 or 4 ppm) or terbuthylazine (0 or 5 ppm) was placed on top of the peat mixture and the column was lined with Whatman No. 3 filter paper. Five hours after adding treated soil, the soil column was watered with either 10, 20 or 40 ml water (equivalent to 1.0, 2.1 or 4.2 mm of rain respectively) either every day or every 4 days for 16 days. On Day 17, the distribution of herbicide in each soil segment was determined by the bioassay method described above.

The experiments were arranged as a randomized complete block design with three replicates. All the data were subjected to analysis of variance followed by Duncan's multiple range test at 5% probability level.

RESULTS AND DISCUSSION

For the alachlor treatment, the highest reduction in total fresh weight of seedlings was recorded for plants grown in sand (*Fig. 1*). The phytotoxic effects on seedling fresh weight decreased with increasing levels of organic matter in the growth medium. In quarry sand, alachlor moved downward to a depth of 25-30 cm. However, in the presence of 5 and 10% peat soil, it moved only to a depth of 15-20 cm. In contrast, terbuthylazine moved only to a depth of 15 cm in the soil column containing 5, 10 or 20% peat soil (*Fig. 2*). In the soil column containing 100% peat, neither alachlor nor terbuthylazine leached below 5 cm depth. Reduction in fresh weight was greatest when alachlor and terbuthylazine persisted in the top 0-5 cm of soil.

In Serdang series soil, daily watering at 40 ml resulted in greater movement of alachlor (down to 25-30 cm depth) than at 4-day interval watering (*Fig. 3*): watering with 10 ml every 4 days moved alachlor only to the 10-15 cm depth. In Selangor series soil, the phytotoxicity of alachlor was

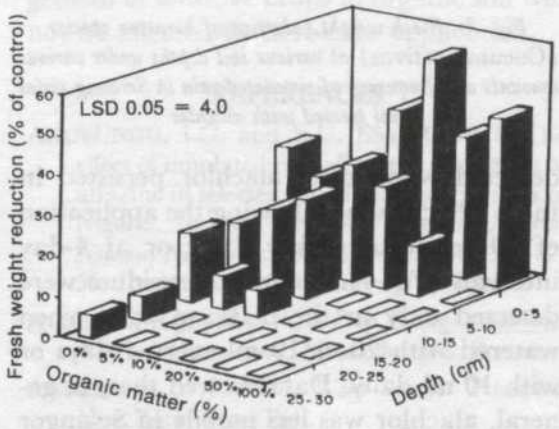

*Fig. 1: Fresh weight reduction of bioassay species (*Cucumis sativus*) at various soil depths with various organic matter contents in the presence of alachlor*

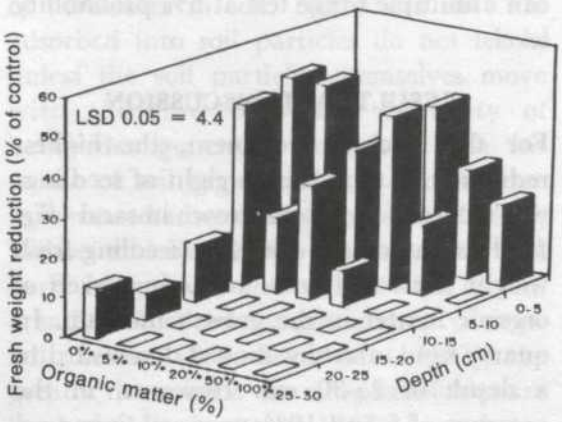

*Fig. 2: Fresh weight reduction of bioassay species (*Cucumis sativus*) at various soil depths with various organic matter contents in the presence of terbuthylazine*

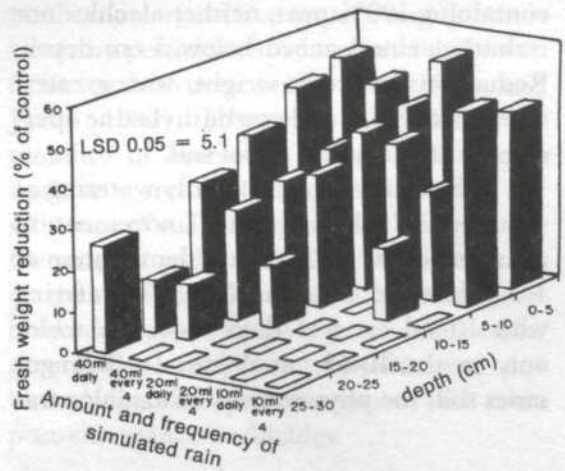

*Fig. 3: Fresh weight reduction of bioassay species (*Cucumis sativus*) at various soil depths under various amounts and frequency of simulated rain in Serdang series soil treated with alachlor*

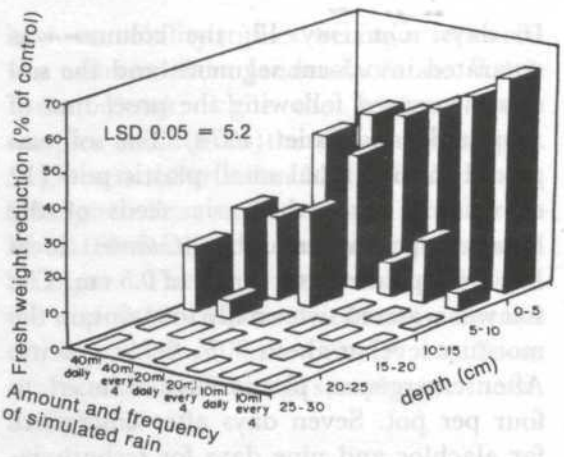

*Fig. 4: Fresh weight reduction of bioassay species (*Cucumis sativus*) at various soil depths under various amounts and frequency of simulated rain in Selangor series soil treated with alachlor.*

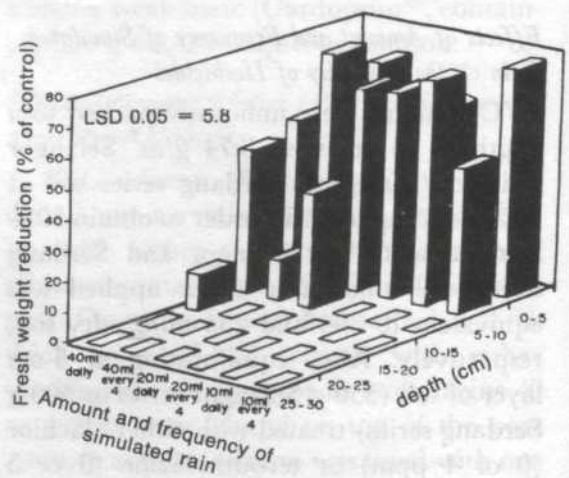

*Fig. 5: Fresh weight reduction of bioassay species (*Cucumis sativus*) at various soil depths under various amounts and frequency of simulated rain in Serdang series soil treated with terbuthylazine*

detected when most alachlor persisted in the 0 - 20 cm zone following the application of 40 ml water either daily or at 4-day intervals (*Fig. 4*). Phytotoxic residues were detected only in the 0-10 cm zone when watered with 20 or 10 ml every 4 days or with 10 ml daily. Data showed that, in general, alachlor was less mobile in Selangor series than in the Serdang series soil.

The phytotoxic effects of terbuthylazine were seen at 15-20 cm depth in treatments with daily watering at 40 ml in Serdang series soil (*Fig. 5*). Watering with 40 ml every 4 days or with 20 ml daily moved terbuthylazine only to 10-15 cm depth. Phytotoxic effects were detected mainly when terbuthylazine persisted in the 0-10 cm zone following the application of 10 ml daily or 10 and 20 ml at 4-day intervals.

In Selangor series, the phytotoxic effects of terbuthylazine were detected in the 0-15 cm zone as a result of either 20 or

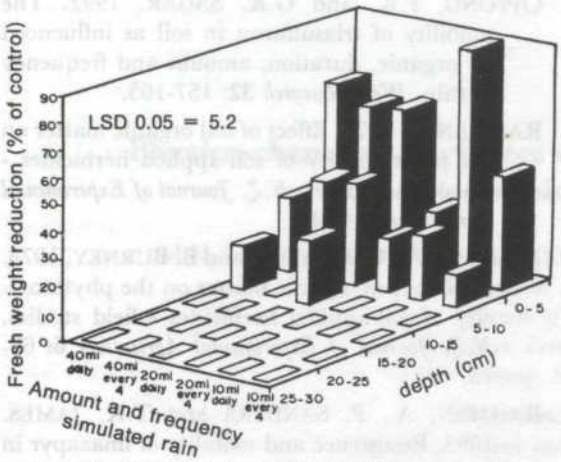

*Fig. 6: Fresh weight reduction of bioassay species (*Cucumis sativus*) at various soil depth under various amount and frequency of simulated rain in Selangor series soil treated with terbuthylazine.*

40 ml daily watering (*Fig. 6*). In this soil, the phytotoxic effect from other treatments was restricted to the 0-10 cm zone. As in the case of alachlor, terbuthylazine was less mobile in the Selangor series than in Serdang series soil.

The two soils studied have different physical and chemical properties (Table 1). The content of organic matter, clay and silt are higher in Selangor series soil, whereas Serdang series soil contains a higher percentage of sand. Soil pH was 4.01 for Selangor series and 4.57 for Serdang series. The CEC of the soils is largely a reflection of the organic matter content. The Serdang series soil (in which the herbicides are more mobile) has greater porosity, which strongly favours mobility of herbicides.

The growth of cucumber seedlings was greatly reduced by alachlor and terbuthylazine when plants were grown in quarry sand, but the presence of a high organic matter content reduced the phytotoxic effect. These results are in agreement with those of Rahman *et al.* (1978), who found an inverse relationship between the bioactivity of alachlor and atrazine (closely related to terbuthylazine) and organic matter content. Oppong and Sagar (1992) also observed high herbicide activity in gravelly sand soils. Rahman (1975) also reported that organic matter had a greater influence on the duration of bioactivity and on leaching. Increased adsorption of alachlor and terbuthylazine by the soil particles as the organic matter content increases would result in less downward movement of the herbicides in mass flow of water. It is well known that basic herbicides are usually strongly adsorbed by soil organic matter (Weber *et al.* 1969) and clay minerals (Weber 1970), whereas acidic herbicides are moderately adsorbed by organic matter and adsorbed relatively little by clay (Carringer and Weber 1974).

The present study suggests that the weak binding of alachlor in media containing organic matter leads to an increase in the concentration of herbicide available for uptake by the bioassay plants. In contrast, terbuthylazine was less mobile in media containing organic matter and in the presence of 5% or higher organic matter content the residue of terbuthylazine is restricted to the 0 - 15 cm zone. Therefore, our results have shown that herbicides were strongly bound to organic matter and not available to seedlings. Therefore the growth of sensitive crops in organic soil will not be affected by herbicide application.

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Physico-chemical Attributes of Humic Acid Extracted from Tropical Pect

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ABSTRAK

Kajian asas mengenai asid humik pada gambut tropika adalah terhad. Satu kajian mengenai ciri-ciri asid humik yang diekstrak dari gambut tropika telah dijalankan dengan menganalisis elemen dan kumpulan berfungsi, spektrum inframerah dan ujian terus pada asid humik dengan menggunakan mikroskop elektron imbasan. Berdasarkan kepada analisis elemen, jumlah C yang didapati adalah 40.26 - 53.26%, H 4.84 - 6.36%, O 38.39 - 51.62%, N 1.51 - 2.64%, dan P 0.02 - 0.06%. Kajian dalam kumpulan berfungsi menunjukkan kumpulan karboksil yang wujud dalam asid humik di gambut tropika adalah dua kali ganda lebih banyak daripada kumpulan hidroksil fenolik. Daripada spektrum inframerah, asid humik didapati mempunyai ciri-ciri aromatik. Analisis inframerah juga menunjukkan spektrum yang sama dan selaras tanpa mengira perbezaan tempat asalnya, kompaun humik yang diekstrak mempunyai struktur dan komposisi kumpulan berfungsi yang sama. Pemeriksaan menerusi mikroskop elektron pada asid humik kering menunjukkan bentuk yang berblok dan separa berliku dengan saiz yang berbeza. Semua asid humik yang diekstrak menunjukkan bentuk yang sama.

ABSTRACT

Basic research conducted on humic acid (HA) in tropical soils is limited. A study was conducted to characterize HA extracted from tropical peat by elemental and functional group analyses, infrared (IR) spectra and by direct examination on HA using scanning electron microscopy (SEM). From the elemental analysis it was found that C ranged from 40.26 - 53.26%, H 4.84 - 6.36%, O 38.39 - 51.62%, N 1.51 - 2.64%, and P 0.02 - 0.06%. Studies of functional groups indicated that the carboxyl group present in HA of tropical peat was about twice as high as that of phenolic hydroxyl groups. From IR spectra, the HA had an aromatic character. Results of IR analysis also exhibited consistently similar spectra, demonstrating that regardless of place of origin, the extracted humic compound had a similar structure and was composed of the same functional groups. Electron microscopic examination of dried HA showed a sub-angular blocky shape which varied in size. All extracted HA exhibited similar form.

INTRODUCTION

Humic acid (HA), defined as the fraction that is soluble in dilute base and coagulated upon acidification of the alkaline extract, is the main component of humic fraction in both temperate and tropical peat (Norhayati 1989; Garcia *et al.* 1991). HA is comprised of highly reactive polymeric compounds of varying molecular weight, containing C, H, O, and N, and its reactivity is influenced by the nature and amount of oxygen-containing functional groups (Stevenson and Goh 1972).

According to Cheshire *et al.* (1967),

peat humic acids have 52% C, 5.1% H, 2.2% N and 40.3% O content. A study by Visser (1987) on humic acids extracted from decomposing plant and peat core samples showed content of 34-55% C, 5-11% H and 40-55% O, different from the values of mineral soils 55-60% C, 3-5% H, 2-6% N and 30-40% O (Kononova 1961). HA extracted from Sphagnum and Carex peat showed 49.7 and 55.4% C, 6.0 and 5.0% H, 42.0 and 35.5% O, 2.3 and 3.5% N content, respectively (Garcia *et al.* 1991).

IR spectra of HA extracted from temperate peat showed aromatic character and great similarity among different samples of HA (Visser 1987; Garcia *et al.* 1991). Studies by Norhayati (1989) also indicated that the HA extracted from tropical peat exhibited very similar IR spectra.

SEM provides a non-destructive method for direct recording of the shape of tiny humic particles (Bohr and Hughes 1971; Orlov and Glebova 1972). Ghosh and Schnitzer (1982) found under SEM, humic acids to consist of small discrete sphenoid, flattened aggregates, linear chain assemblies of these aggregates and flattened filaments.

There is a dearth of information on the study of elemental composition and reactivity of HA, and its structure under SEM. This study was therefore carried out to characterize the HA extracted from tropical peat by elemental and functional group analyses, its IR spectra and its structure by SEM.

MATERIALS AND METHODS

Fourteen samples from major peat areas of Malaysia were used in this experiment, twelve from the west coast of Peninsular Malaysia and two from Sarawak (peat samples 5 and 6). Percentage of organic matter was determined as loss on ignition from sample maintained at 500°C for 4 h (Storer 1984).

Humic acid was extracted with 0.2 M NaOH using a soil to extractant ratio of 1:10. The mixture was shaken continuously on a mechanical shaker for 24 h and the soluble organic matter was separated from the peat soil by centrifugation at 10,000 rpm for 15 min. The residue was washed with distilled water in a ratio of 1:5 and washing water separated from the residue by centrifugation. The combined supernatants were acidified with concentrated H_2SO_4 to pH 1.0 and allowed to stand for 24 h. The coagulated humic acid (HA) precipitate was purified by re-precipitation and re-dissolution using H_2SO_4 and NaOH, respectively. The alkaline extract was then shaken for 48 h with excess HCl-HF mixture (0.5%, v/v). The acid mixture was removed by centrifugation and the residue thoroughly washed with distilled water until the filtrate became free of chloride. The humic acid was then dialyzed against distilled water until there was no colour change in the outer solution (distilled water) and finally frozen and dried at ambient temperature (Schnitzer 1982; Norhayati and Verloo 1984).

Carbon, H and N were determined by dry combustion using a CHN analyser (VARIO-EL), O was calculated by difference and P by dry ashing at 500°C and determined colorimetrically at 882 nm (Murphy and Riley 1962). Carboxyl groups were measured by $Ca(OAc)_2$ method (Schnitzer and Gupta 1965) and the phenolic hydroxyl groups by the colorimetric method using Folin Cicocalteu's reagent (Tsutsuki and Kuwatsuka 1978). These determinations were represented as the average result of two duplicate samples.

IR spectra were recorded on a Beckman Fourier transfer IR spectrophotometer using the KBr pellet technique. Prior to the analyses, the HA with the KBr was stored for at least 48 h in a desiccator to prevent absorption of atmospheric moisture.

For SEM examination, the dried HA obtained directly after extraction was attached to Al-stubs with the aid of colloidal silver paint. To make the specimens surface conducting, they were coated with a layer of gold-palladium. The prepared specimen were examined on a SEM (JOEL 35C) operated at 25kV.

RESULTS AND DISCUSSION

The organic matter contents per 100 g peat of the 14 peat samples studied ranged from 44 - 97% (Table 1). Percentage of HA obtained ranged from 8.30 - 39.52/100 g peat.

The data on the elemental composition of HA in terms of percentage weight are presented in Table 2. Carbon content ranged from 40.26 - 53.26%, H 4.84 - 6.3%, O 38.39 - 51.62%, N 1.51 - 2.64% and P 0.02 - 0.06% with means of 49.34% C, 5.64% H, 43.05% O, 1.99% N and 0.03% P. Comparing these with the elementary composition of HA reported by Visser (1987) and Garcia *et al.* (1991) indicated that our results fell within the reported range for temperate peat.

The analysis of the major oxygen-containing functional groups is shown in Table 3. The carboxyl group of HA extracted from tropical peat ranges from 2.50 - 3.10 meq/g of HA while phenolic hydroxyl groups range from 0.99 - 1.75 meq/g of HA. Mean values of functional groups showed 2.84 meq COOH and 1.36 meq phenolic hydroxyl/g of HA. The carboxyl groups present in HA are more than twice as numerous as in the phenolic hydroxyl groups, implying that the pH-dependent charge in peat is controlled by carboxyl groups.

IR spectra and the absorption bands of HA extracted from peat are shown in *Fig. 1* and Table 4, respectively. The broad absorption band at 3550-3200 cm^{-1} region can be attributed to H-bonded OH groups, while peaks between 2920-2860 cm^{-1} are due to aliphatic C-H stretch. According to

TABLE 1
Classification and yield of HA from peat

Peat Sample No.	[1]Classification	Depth (cm)[2] (%)	O.M.[3] (%)	HA
1	A	0-15	44	21.23
2	A	0-15	82	26.88
3	A	0-15	86	18.30
4	B	15-30	88	25.78
5	A	0-15	90	23.78
6	B	15-30	94	8.40
7	A	0-15	97	35.67
8	A	0-15	97	20.68
9	B	0-15	76	23.45
10	A	0-20	94	36.52
11	A	20-40	97	12.38
12	B	0-15	89	19.82
13	B	20-40	97	8.30
14	A	0-15	96	26.92

[1] A Typic Tropohemists, B Typic Tropofibrists
[2] Percentage of organic matter
[3] % Humic acid (HA) per 100 g peat

TABLE 2
Elemental analysis of HA extracted from tropical peat

Peat Sample No.	Elements (%)				
	C	H	O	N	P
1	51.93	5.14	40.52	2.37	0.06
2	52.01	5.62	39.88	2.45	0.04
3	45.75	6.36	45.26	2.59	0.05
4	51.25	5.23	41.33	2.16	0.04
5	53.39	5.59	38.34	2.64	0.05
6	44.39	6.23	47.72	1.63	0.03
7	51.04	5.22	41.98	1.74	0.02
8	47.80	5.77	44.68	1.73	0.02
9	49.76	4.84	43.88	1.51	0.02
10	50.52	5.14	42.14	2.18	0.03
11	49.07	5.78	43.48	1.65	0.02
12	50.00	5.67	42.16	1.71	0.02
13	53.56	5.91	38.88	1.64	0.02
14	40.26	6.32	51.60	1.84	0.02
Range	40.26-53.56	4.84-6.36	38.39-51.62	1.51-2.64	0.02-0.06
Mean	49.34	5.64	43.05	1.99	0.03

TABLE 3
Analysis of oxygen-containing functional groups of HA extracted from tropical peat

Peat Sample No.	Functional groups (meq/g of HA)	
	COOH	Phenol OH
1	2.90	1.36
2	2.75	1.07
3	2.95	1.12
4	2.75	1.39
5	2.80	0.99
6	3.10	1.56
7	2.50	1.22
8	2.80	1.44
9	2.90	1.40
10	3.00	1.75
11	3.00	1.43
12	2.85	1.54
13	2.95	1.59
14	2.50	1.22
Range	2.50-3.10	0.99-1.75
Mean	2.84	1.36

TABLE 4
Absorption bands of infrared spectra of HA from tropical peat

Bands (cm^{-1})*	3550-3200	2920-2860	1725-1705	1690-1600	1550-1500	1470-1430	1280-1200
Peat Sample No.	Relative intensity						
1#	s+	m	m	s	a	w	w
2	s	m	m	s	a	w	w
3	s	m	w	s	a	w	w
4	s	m	m	s	a	w	w
5	s	m	m	s	a	w	w
6	s	m	m	s	a	w	w
7	s	m	w	s	w	w	w
8	s	m	m	s	w	w	w
9	s	m	m	s	w	w	w
10	s	m	w	s	w	w	w
11	s	m	m	s	w	w	w
12	s	m	m	s	w	w	w
13	s	m	m	s	w	w	w
13	s	m	m	s	w	w	w
14	s	m	m	s	w	w	w

* 3550-3200 (hydrogen-bonded OH); 2920-2860 (aliphatic C-H stretch); 1725-1705 (C=O of COOH and ketones); 1690-1600 (C=O stretch of quinones, C=O and/or C=O of H conjugated ketones); 1550-1500 (aromatic ring stretch, P- and O- distributed); 1470-1430 (aromatic ring stretch, O-distributed); 1280-1200 (C-O stretch, and deformation of COOH, C-O stretch of aryl ethers)

+ (s) strong; (m) medium; (w) weak; (a) absent

Sample number for HA extracted from respective peat soil

Bellamy (1969), bands in the region of 3000-2500 cm^{-1} were produced by most carboxylic acids and arose from vibration of intermolecular OH...O band. Therefore, Schnitzer and Griffith (1975) indicated that the O-H stretch (<2800 cm^{-1}) was a test for the occurrence of H-bonded COOH groups. However, in this study only a medium peak occurred at this region similar to the results of Norhayati (1989).

Norhayati (1989) showed that higher amounts of carboxyl groups were present in HA molecules. Similar results were obtained in this study, where most of the HA spectrum exhibited a medium peak in the 1725-1705 cm^{-1} region (band for carbonyl group) with strong peaks in the 1690-1600 cm^{-1} region (band for carbonyl group).

The spectra also showed weak bands at 1550-1500 cm^{-1} and 1470-1430 cm^{-1}. Similar results were obtained by Garcia *et al.* (1991), where the above bands meant for HA extracted with alkali disappeared for HA extracted with pyrophosphate reagents in both Sphagnum and Carex peat. This indicated that HA extracted with alkali extractant exhibited stronger aromatic characteristics than that using pyrophosphate reagent.

A weak band obtained for all samples in the 1289-1200 cm^{-1} region is attributed to OH, from COOH deformation. Other

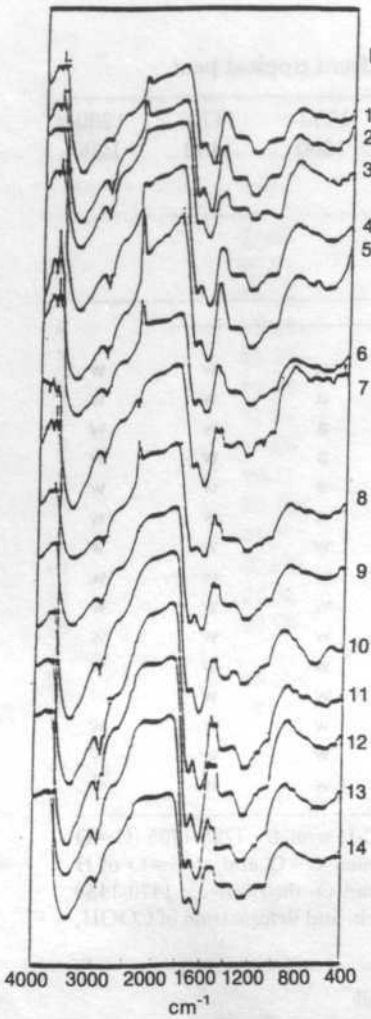

Fig. 1. Infrared spectra of HA extracted from tropical peat

bands, such as 1440, 1125 and 1034-1080 cm^{-1} were also observed. However, all these bands were of weak intensity, and were absent in some samples (*Fig. 1*). Bands occurring at 1440 cm^{-1} indicated the presence of the carboxyl anion (Schnitzer and Griffin 1975); 1125 and 1034-1080 cm^{-1} indicated aromatic ring P- and O- disubstituted, C-O-O stretch of aryl ethers and C-O stretch, aromatic ether, hydrated polyols and carbohydrates, respectively (Garcia *et al.* 1991). Thus, this study demonstrates that HA of tropical peat has a greater amount of carboxyl groups.

The IR spectra of the HA extracted from peat of diverse origin displayed similar bands and indicated the presence of similar structures, differing mainly in the number of functional groups. The results corresponded with those of Norhayati (1989).

The shape and particle arrangement of HA under SEM are shown in *Plate 1*. The HA materials are sub-angular blocky, vary in size, and are irregular in shape (*Plate 1a*). *Plate 1b* shows the plate-like structure of HA surface upon higher magnification (1 μm).

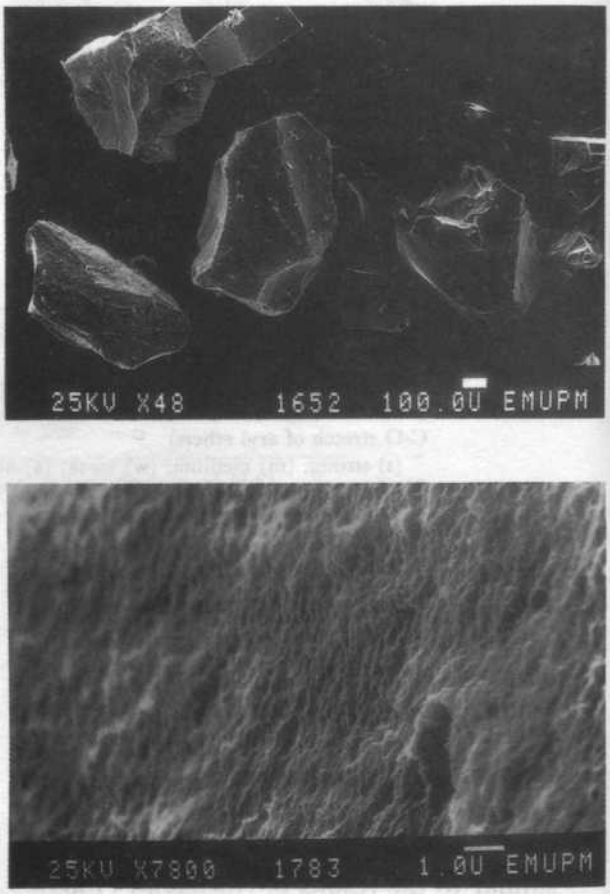

Plate 1. Scanning electron micrographs of shape and particle size humic acid; a) top: sub-angular blocky which vary in size and shape, b) bottom: plate like structure of HA surface.

CONCLUSION

The elemental analysis of HA showed C ranged from 40.26 - 53.26%, H 4.84 - 6.36%, O 38.39 - 51.62%, N 1.51 - 2.64% and P 0.02 - 0.06%. Studies of the functional groups indicated that carboxyl groups present in HA of tropical peat are about two times more numerous than phenolic hydroxyl groups. From IR spectra, the HA obtained is of aromatic character. The IR analyses also exhibited consistently similar spectra, demonstrating the possibility that regardless of sample site, the extracted HA has a similar structure and is composed of the same functional groups. SEM examination of dried HA showed sub-angular blocky shape of varying size was similar for all the HA extracted. These results support those obtained by Norhayati (1989) for Malaysian peat, and demonstrate the similarity between tropical and temperate peats regardless of plant origin or climate.

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The Effectiveness of Two Arbuscular Mycorrhiza Species on Growth of Cocoa (*Theobroma cacao* L.) Seedlings

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Keywords: arbuscular mycorrhiza (AM), cocoa, effectiveness, seedling growth

ABSTRAK

Satu kajian berpasu telah dijalankan untuk mengkaji keberkesanan dua spesies kulat mikoriza arbuskul ke atas pertumbuhan anak benih koko. Anak benih koko dari hibrid UIT1 × Na32 yang diinokulasi dengan Glomus mosseae *dan* Scutellospora calospora *sama ada sebagai inokulum tunggal, atau campuran dan tanpa inokulum (kawalan), ditanam pada 2 kg tanah disucihama daripada siri Tai Tak. Kajian menggunakan rekabentuk rawak lengkap (CRD) iaitu jenis inokulum sebagai rawatan, dengan empat replikasi. Di antara empat rawatan yang digunakan, pokok yang diinokulasi dengan inokulum campuran* Glomus mosseae *dengan* Scutellospora calospora *memberi tumbesaran yang lebih baik dan bererti dan peratus jangkitan akar lebih tinggi berbanding rawatan-rawatan yang lain. Tinggi pokok dan jumlah luas daun tertinggi (43.7 cm dan 1819.2 cm^2 masing-masing) diperolehi dari anak benih yang diberi inokulum campuran berbanding anak benih dirawat inokulum tunggal* G. mosseae *(37.9 cm dan 1007.2 cm^2), inokulum tunggal* S. calospora *(32.3 cm dan 1316.2 cm^2) dan kawalan (28.5 cm dan 736.7 cm^2). Peratus jangkitan akar tertinggi (72%) juga diperolehi dari anak benih yang dirawat dengan inokulum campuran diikuti oleh inokulum tunggal* S. calospora *(54%) dan inokulum tunggal* G. mosseae *(43%).*

ABSTRACT

A pot experiment was conducted to study the effectiveness of two arbuscular mycorrhiza (AM) species in enhancing growth of cocoa seedlings. Cocoa seedlings of hybrid UIT1 × Na32 inoculated with Glomus mosseae *and* Scutellospora calospora *either as a single inoculum or as a mixed inoculum and an uninoculated control were grown in 2 kg sterilized Tai Tak series soil. The experiment was a single factor experiment arranged in a completely randomized design, with type of inoculum as a factor with four replications. Of the four treatment used, plants inoculated with mixed inoculum* Glomus mosseae *and* Scutellospora calospora *gave more pronounced and significant vegetative growth and higher percentage of root colonization than the other treatments. Maximum plant height and total leaf area of 43.7 cm and 1819.2 cm^2 respectively were obtained from seedlings inoculated with mixed inoculum compared to seedlings given either* G. mosseae *(37.9 cm dan 1007.2 cm^2), or* S. calospora *(32.3 cm and 1316 cm^2) and control (28.5 cm and 736.7 cm^2). The highest percentage of root colonization (72%) was aslo recorded from seedlings inoculated with mixed inoculum followed by those given* S. calospora *(54%) and single inoculum* G. mosseae *(43%).*

INTRODUCTION

The importance of arbuscular mycorrhiza (AM) fungi in the improvement of plant growth under greenhouse and field conditions is now well documented (Mathew and Johri 1989; Sieverding 1991; Stanley *et al.* 1993; Azizah *et al.* 1996). It is also generally accepted that AM fungi are non-specific in their host selection, since in nature, individual species have been found to infect plant species belonging to different genera and families (Sieverding 1991). The efficiency of a particular AM fungus varies markedly between species and strains of the host

plant. The response of the host plant to AM species also varies between clones. This claim was substantiated by Anand (1993), who found that of the two cocoa clones tested, mycorrhizal PBC 139 gave more pronounced and significant vegetative growth than mycorrhizal PBC 140. Hence, a suitable host-fungal combination is of prime importance in order to obtain maximum AM effectiveness in enhancing crop productivity. This study, therefore, aimed to evaluate the effectiveness of two AM species, *Glomus mosseae* and *Scutellospora calospora*, as either single or mixed inoculum on growth of cocoa seedlings of hybrid UITI × Na32 under controlled greenhouse conditions.

MATERIALS AND METHODS

A completely randomized design (CRD) pot experiment with four replications comprising the following treatments was set up: single inoculum with *Glomus mosseae* (Gm), single inoculum with *Scutellospora calospora* (Sc), mixed inoculum comprising *G. mosseae* and *S. calospora* (GmSc) and uninoculated control.

Mycorrhizal soil inoculum of *G. mosseae* [WUM 9 (6)] and *S. calospora* [WUM 12 (3)] was originally obtained from Prof. A.D. Robson, from University of Western Australia, Perth. The inoculum was mass propagated under *Setaria anceps* var. *splendida* as the host plant in the UPM greenhouse for six months (Azizah and Omar 1986).

Soils

The soil used was a clayey Tai Tak soil series (Typic Paleudult) consisting of 50.13% clay, 6.89% silt and 42.98% sand. Soil from the top 0 - 15 cm was collected from a cocoa farm in Labu, Negeri Sembilan. The soil was passed through a 2-mm diam. mesh sieve. The chemical properties of this soil after steam-sterilization for 1 hour at 100°C are as follows: 0.19% total N (determined by Kjeldahl method), 37.92 $\mu g\ g^{-1}$ extractable P (determined by the molybdenum blue method-NH_4F and HCl), 0.18 cmol (+)/kg K, 0.87 cmol (+)/kg Ca and 0.17 cmol(+)/kg Mg (determined by the neutral ammonium acetate leaching technique) (Husni *et al.* 1990). Sixteen pots of 16-cm diameter were each filled with 2 kg of this soil. The soil was raised to 6.0 through addition of 2.8 g ground magnesium limestone (GML).

Preparation and Planting of Cocoa Seedlings

Fifty uniform-sized cocoa seeds from hybrid UIT1 × Na32 were pregerminated in sterilized sandy soil for 10 days. Sixteen uniform seedlings were then transplanted, one per pot, with 30 g AM inoculum spread as a layer 2 cm below the roots, as well as around the roots to ensure better infection. Uninoculated or control plants received 30 g of sterilized sandy soil so as to maintain similar conditions. Five grams of compound fertilizer (NPK 8:8:8) were applied as basal fertilizer at the time of transplanting. No other nutrients were added to the soil throughout the entire duration of the experiment. The plants were watered daily to field capacity.

Data Collection

The plants were harvested 12 weeks after transplanting. Plant height and stem diameter were recorded. The leaf, stem and roots were then cleaned and separated. Total leaf area per plant was recorded using a leaf area meter (Licor model 3100). The roots were carefully washed free of adhering soil particles. Three grams of roots were randomly sampled from each plant and subsequently assessed for percentage of root colonization (Giovannetti and Mosse 1980; Brundrett *et al.* 1984). The remaining root samples were then dried to constant weight at 75°C for 3 days and their dry

weights determined. The plant tissues were subsequently processed for determination of N (Kjeldahl method), P, K, Ca and Mg (dry ashing method in concentrated HCl and 20% HNO_3) (Husni *et al.* 1990).

Statistical Analysis

All the data obtained were subjected to the analysis of variance (ANOVA). Treatment means were further separated by LSD for test of significance at $P < 0.05$.

RESULTS

Plant Growth

All mycorrhiza-inoculated seedlings were taller than the controls (*Fig. 1*). However, there was no significant difference between treatments Sc and Gm or between treatments Sc and control. Maximum height of 43.7 cm was obtained from seedlings inoculated with the mixed inoculum GmSc.

Inoculation with either Sc or with mixed inoculum GmSc resulted in marked

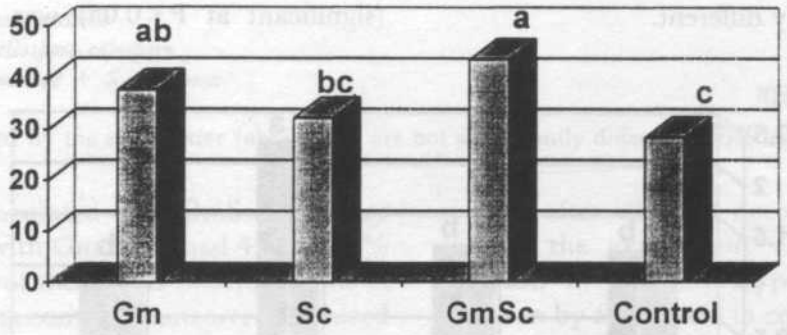

Fig. 1. Effect of Glomus mosseae *and* Scutellospora calospora *as a single or mixed inoculum on height of cocoa seedlings at week 12*

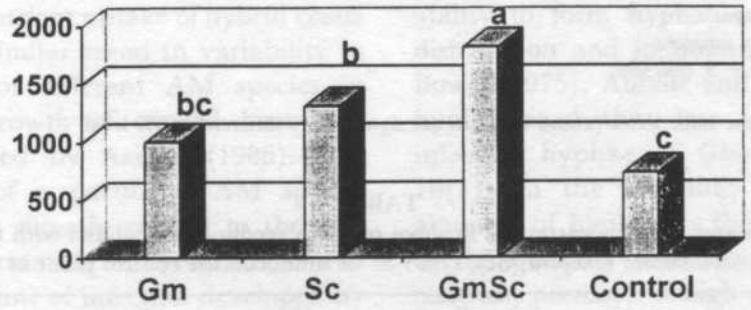

Fig. 2. Effect of Glomus mosseae *and* Scutellospora calospora *as a single or mixed inoculum on total leaf area per plant at week 12.*

Gm = *Glomus mosseae*
Sc = *Scutellospora calospora*
GmSc = *G. mosseae* + *S. calospora*

Means followed by the same letter (above bar) are not significantly different at 5% level

and significant ($P < 0.05$) increase in total leaf area per plant (1316.2 and 1819.2 cm^2, respectively) compared with 736.7 cm^2 for the uninoculated control (*Fig. 2*). Cocoa seedlings responded most positively to the mixed inoculum, as shown by the maximum and significantly greater total leaf area when compared to single inoculum inoculations.

A similar trend was noticed for root dry weight (*Fig. 3*). A significant ($P < 0.05$) increase in root dry weight per plant was observed only from plants given mixed inoculum. The rest of the treatments were not significantly different.

Nutrient Uptake

In line with the other parameters recorded, seedlings inoculated with mixed inoculum GmSc resulted in maximum and significantly higher tissue P, K, Ca and Mg concentrations (Table 1). However, tissue N, K and Mg in Gm or Sc treated plants were at par with those of the control.

Mycorrhizal Infection

The cocoa seedlings responded positively to all mycorrhizal inoculation but exhibited various degrees of mycorrhizal colonization (*Fig. 4*). The highest colonization of 72% (significant at $P < 0.05$) was observed in

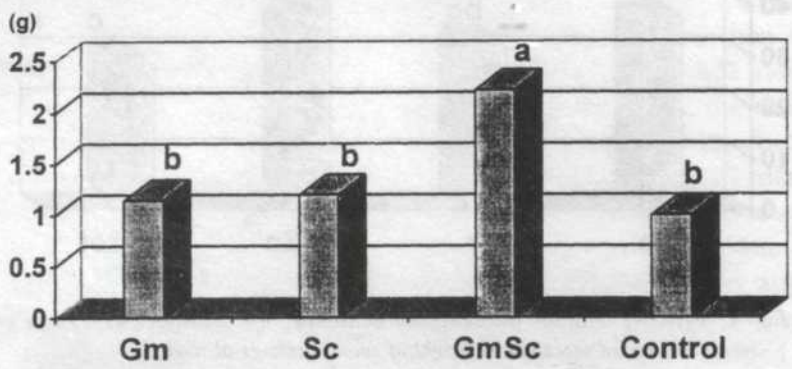

Fig. 3. Effect of Glomus mosseae *and* Scutellospora calospora *as a single or mixed inoculum on root dry weight of plant*

Gm = *Glomus mosseae*
Sc = *Scutellospora calospora*
GmSc = *G. mosseae* + *S. calospora*

Means followed by the same letter (above bar) are not significantly different at 5% level

TABLE 1

N, P, K, Ca and Mg concentrations in shoot of cocoa seedlings inoculated with *Glomus mosseae* (Gm), *Scutellospora calospora* (Sc), Gm + Sc or uninoculated control plant at week 12

AM Inoculum	N	P	K	Ca	Mg
			(%)		
G. mosseae (Gm)	1.99^{a}	0.176^{b}	1.85^{b}	0.25^{b}	0.28^{b}
S. calospora (Sc)	1.90^{a}	$0.165b^{bc}$	1.98^{b}	0.27^{b}	0.29^{b}
Gm + Sc	2.08^{a}	0.205^{a}	2.44^{a}	0.31^{a}	0.34^{a}
Control	2.11^{a}	0.155^{c}	2.10^{b}	0.17^{c}	0.27^{b}

In a column, the means followed by the same letter are not significantly different at 5% level.

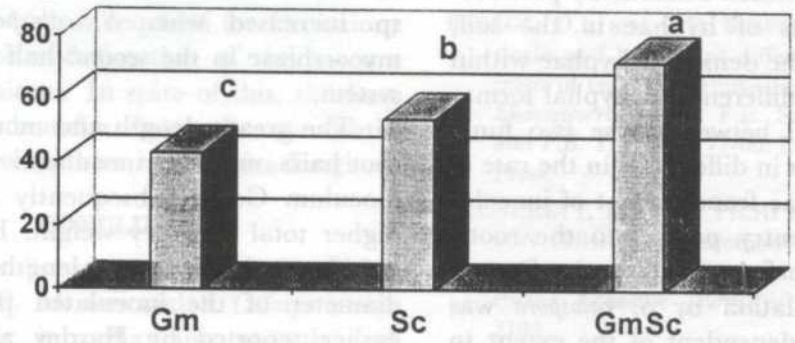

Fig. 4. Percentage root colonization of plants inoculated with Glomus mosseae *(Gm) and* Scutellospora calospora *(Sc) as a single or mixed inoculum*

Gm = *Glomus mosseae*
Sc = *Scutellospora calospora*
GmSc = *G. mosseae* + *S. calospora*

Means followed by the same letter (above bar) are not significantly different at 5% level.

seedlings inoculated with GmSc. Seedlings inoculated with Gm or Sc had 43 and 54% respectively of their roots infected. None of the roots from control plants were colonized by AM.

DISCUSSION

There was variability in the ability of *G. mosseae, S. calospora* and a mixture of *G. mosseae* and *S. calospora* to stimulate plant growth and nutrient uptake of hybrid cocoa seedlings. A similar trend in variability in effectiveness of different AM species in stimulating growth of winged bean was earlier reported by Azizah (1986). The effectiveness of a particular AM species appears to be directly related to the rate and time of formation of mycorrhiza as well as to the amount of infection developed by the fungi (Abbott and Robson 1981). Pearson and Schweiger (1994) found that the isolate *Glomus* sp. maintained a similar level of colonization on subterranean clover root at all harvests, but percentage root length colonized by the isolate *S. calospora* increased until the seventh week after sowing, after which it decreased until the end of the experiment (11 weeks). In contrast, in this study, percentage root infection by *S. calospora* in cocoa was higher than infection by *G. mosseae* even at 12 weeks after transplanting. This is probably due to the longer duration of the present experiment, different soil type, host plant and isolate of *S. calospora* used in this study.

AM species may also differ in their ability to form hyphae in soils, both in distribution and in quantity (Bevege and Bowen 1975). Abbott and Robson (1984) hypothesized that low initial levels of infective hyphae of *Glomus* sp. (WUM 10(1)) in the soil may lead to small amounts of hyphae in the soil in relation to the quantity present in the roots. In contrast, presence of high density infective hyphae in the soil could result in greater extension of the exponential phase of colonization in the roots, which subsequently stimulate massive development of hyphae in the soil. An association of this nature, i.e. between formation of hyphae in the soil and within the roots may not occur

for *S. calospora*, which consistently produces large quantities of hyphae in the soil, irrespective of the density of hyphae within the roots. The difference in hyphal formation in the soil between these two fungi probably results in differences in the rate of spread of hyphae from a point of inoculation (i.e. the entry point into the root). Hyphal spread from roots away from a point of inoculation by *S. calospora* was found to be independent of the extent to which the individual roots are colonized. However, for *Glomus* sp., the growth of hyphae in the soil and subsequent spread are strongly dependent on the rapid and extensive colonization within the plant roots (Abbott and Robson 1984).

Differences in effectiveness between VAM species have also been related to their pattern and mechanism of sporulation (Abbott and Robson 1981). Fungi which sporulate gradually may not deplete nutrient resources available for the hyphae within plant roots, whereas species which sporulate excessively or suddenly may deplete the hyphae from substances necessary for continued or renewed growth. Azizah (1986) found that the superiority of *G. mosseae* over the other inocula lies in the low spore production of *G. mosseae* for a long period of plant growth as well as its ability to produce rapid and extensive external hyphae.

In the present study, the mixed inoculation (*G. mosseae* and *S. calospora*) gave the highest plant growth increment and nutrient concentrations in cocoa shoot compared to the control. Positive growth responses as a result of inoculation with the mixed inoculum GmSc clearly indicate the probable synergistic effects between these species, even though they exhibit different growth pattern. Using a split-root technique, Pearson *et al.* (1993) reported that root weight and total root length of one half of the root system of subterranean clover inoculated with *Glomus* sp. increased when *S. calospora* formed mycorrhizae in the second half of the root system.

The greater length and more intensive root hairs on plants inoculated with mixed inoculum GmSc subsequently resulted in higher total root dry weight. Higher and significant higher root length and root diameter of the inoculated plants were earlier reported by Hardey and Leyton (1981). The greater root length and number of root branches probably indicate that the mycorrhizal plant has a higher potential for uptake and absorption of relatively mobile nutrients through exploration of a greater soil volume. This subsequently results in higher nutrient concentrations in the shoots of these plants.

Mycorrhiza treatment, either as a single or mixed inoculum, also gave significantly higher P concentration in shoots compared to the control. The highest concentration of 0.205% was obtained from plants inoculated with GmSc followed by Gm (0.176%) and Sc (0.165%). These findings are in agreement with results obtained earlier by Pearson *et al.* (1994) and Azizah *et al.* (1996). *S. calospora* has been shown to be a significant drain of host photosynthates compared to *Glomus* sp., probably in part due to its inability to supply the host with sufficient P, especially in soils with low to moderate P levels (Pearson *et al.* 1994).

High root colonization of plants inoculated with GmSc and, subsequently, greater absorption of nutrients could also result in greater production of leaf area, i.e. increases the area for photosynthesis, and hence produce more carbohydrate to support better plant growth (Sieverding 1991; Kumaran and Azizah 1995), as well as to support growth of the VAM fungi in the plants. Jakobsen and Rosendahl (1990) reported that mycorrhizal plants have to

pay a price of 10-20% of the net photosynthates, which is required for formation, maintenance and function of the mycorrhizal symbionts. In spite of this, the host plant still benefits from the symbiotic association with these mycorrhiza fungi.

CONCLUSION

Under greenhouse conditions, mixed inoculum of *G. Mosseae* and *S. Calospora* was far superior in enhancing growth and nutrient uptake in cocoa seedlings (hybrid UITI × Na32) compared to inoculation with either single inoculum of *G. mosseae* or *S. calospora*.

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COMMUNICATION

Effect of Thiobencarb Formulations on Freshwater Shrimp, *Macrobrachium lanchesteri* (De Man)

ABSTRAK

Kajian dijalankan di makmal untuk membandingkan kesan formulasi lepasan terkawal thiobenkarb kob jagung (TA1 dan TA2) dan alginat (TAL 70192) dengan formulasi granul biasapada udang air tawar, Macrobrachium lanchesteri *(De Man). Kematian udang yang dirawat dengan formulasi biasa 24 jam selepas rawatan adalah lebih tinggi ($P \leq 0.05$), dan kematian ini didapati meningkat sehingga 93.3% selepas 120 jam. Formulasi lepasan terkawal TA1, TA2 dan TAL 70192 masing-masing menyebabkan kematian 19.2, 5.8 dan 8.5% pada 120 jam selepas rawatan. Perbezaan ini disebabkan oleh lepasan thiobencarb yang perlahan melalui formulasi lepasan terkawal.*

ABSTRACT

The effect of corn-cob controlled-release formulations (CRF) of thiobencarb (TA1 and TA2) and alginate CRF (TAL 70192) to the freshwater shrimp, Macrobrachium lanchesteri *(De Man), in comparison to a conventional granular formulation was studied in the laboratory. Mortality of the shrimps 24 h after exposure to the conventional formulation was significantly higher ($P \leq 0.05$), and increased to 93.3% after 120 h. The CRF of TA1, TA2 and TAL 70192 caused mortality of 19.2, 5.8 and 8.5%, respectively, 120 h after exposure. This difference is due to the slower release of thiobencarb using CRF.*

INTRODUCTION

Herbicides are commonly used to control weeds in rice fields in Malaysia. These chemicals are used as either pre-emergent or post-emergent herbicides, and are applied as emulsifiable concentrates (EC) or granular formulations. The formulations are designed to release the active ingredient (a.i.) almost immediately after application. This feature often results in significant amounts of the herbicide not being taken up by the weeds and, therefore, remaining in the surrounding environment (Collin *et al.* 1973). The immediate release also increases the concentration of the a.i. in the environment, which can cause deleterious effects on other organisms in the ecosystem.

Recent interest in controlled-release formulation (CRF) herbicides has led to the development of thiobencarb formulations for the control of the weed, *Echinochloa crus-galli*, in rice fields (Omar and Mohamad 1994). CRF is an approach towards safer and more effective use of herbicides. The concept of CRF is to slowly release small amounts of the toxicant over an extended period of time, sufficient to control weeds while being low enough not to cause serious effects on non-target organisms. This study was conducted to evaluate the toxicity of the new CRF of thiobencarb in comparison with the conventional granular formulation on the non-target organism, *Macrobrachium lanchesteri*, a species of freshwater shrimp commonly found in irrigation systems and the rice ecosytem.

MATERIALS AND METHODS

Chemical

Three CRFs of thiobencarb used were corn-cob formulations coded as TA1 (4% thiobencarb) and TA2 (4% thiobencarb) and alginate formulation TAL 70192 (7.56% thiobencarb). These formulations

were obtained from International Atomic Energy Agency (IAEA), Vienna, Austria. The commercial granular formulation, Saturn 5G (5% thiobencarb), was obtained from Agriculture Chemical Malaysia, Butterworth, Malaysia.

Shrimps

The shrimps, *M. lanchesteri*, were collected from the freshwater ponds at Universiti Pertanian Malaysia, Selangor, Malaysia. Their average weight was 27.3 ± 4.7 mg (n = 100) and average length was 3.53 ± 0.28 cm (n = 100). The shrimps were acclimatized for 48 h in the laboratory by being kept in glass tanks half-filled with pond water.

Treatment

The experiment was conducted in glass tanks (45 × 22 × 23 cm), in the laboratory at 27 ± 3°C and 70 ± 20% relative humidity; 5 l of pond water were used in each tank. The water quality was as follows: pH 6.5, BOD 1.5 mg/l, hardness 3.4 mg/l of $CaCO_3$ and conductivity 126 µmhos. The formulations, calculated to give 2.5 mg a.i./l, were added to the water in each tank. Aeration was provided by means of an aerator. Twenty shrimps were released into each tank immediately after application of the herbicide. Six replicates were used for each treatment, including control. Mortality was recorded 24, 48, 72, 96 and 120 h after releasing the shrimps into the tanks. Results were subjected to analysis of variance and means were compared by Duncan's multiple range test using an SAS computer package (SAS Institute Inc, 1982, Cary, North Carolina, USA). Percentage mortality was subjected to arc sin transformation before analysis.

RESULTS AND DISCUSSION

Fig. 1 shows the percentage mortality of *M. lanchesteri* over a period of time following exposure to various thiobencarb formulations. Higher mortality after 24 h was recorded for Saturn 5G, a conventional commercial formulation of thiobencarb, compared with the three CRF. Calculated by probit analysis (Finney 1971), the time taken by the conventional formulation to kill 50% of the exposed shrimps was 30.8 h (lower and upper fudicial limit of 25.7 and 35.3 h, respectively) following treatment. All CRF formulations caused less than 20% mortality of the shrimps after 120 h exposure. This indicates that the immediate release of substantial amounts of thiobencarb from conventional granular formulation causes higher mortality. Earlier studies showed the mortality rate caused by conventional granular formulation of thiobencarb applied at 2.5 kg a.i./ha on $1^1/2$-month-old *Clarias batrachus* and a hybrid of *Oreochromis massambicus*/*O. niloticus* was 100 and 87% respectively, 48 h after treatment (Omar 1989).

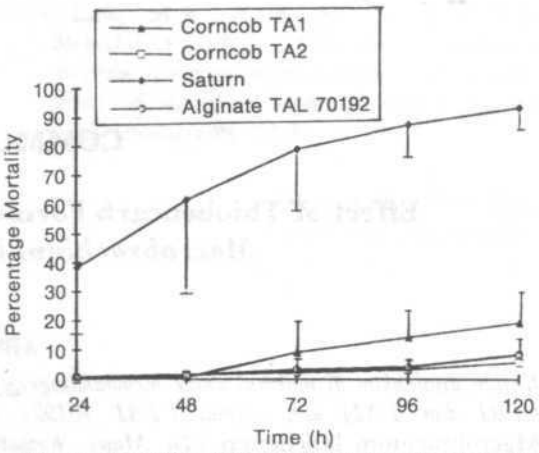

Fig. 1: Mortality of Macrobrachium lanchesteri *over time following exposure to four formulations of thiobencarb (The control showed zero mortality throughout the experiment)*

The significantly lower mortality ($P \leq 0.05$) 120 h after treatment for the CRF of TA1, TA2 and TAL 70192 indicated that these formulations reduced the biological hazard of thiobencarb to *M. lanchesteri*

TABLE 1
Effect of formulations on *Macrobrachium lanchesteri* 120 h after treatment[1]

Formulation	Mortality[2] ± S.D.
TA1	19.2 ± 11.5 a
TA2	5.8 ± 5.8 a
TAL 70192	8.5 ± 4.8 a
Saturn	93.3 ± 8.8 b

[1] No mortality was observed in non-treated control tanks
[2] Means followed by the same letter in the row are not significantly different ($P \leq 0.05$)

(Table 1). The CRF of TA2 was even less hazardous than TA1. This is ascribed to the much slower rate of release of the a.i. from the TA2 formulation, as Soerjani (1991) showed that the release rate at 24 and 48 h measured as percentage radioactivity of C-14 thiobencarb was slower for TA2 than TA1. Thus, TA2 has a less toxic effect due to its slower release and hence lower concentrations in the water.

Studies by Chen *et al.* (1981) on the fate of thiobencarb showed that 23 days after C-14 thiobencarb application, radioactivity corresponded to 2.73 and 0.31% of the initial radioactivity applied in water and biota, respectively. Li and Kang (1979) showed that the herbicide was not detected in paddy water 74 days after application. Although Chen *et al.* (1981) considered thiobencarb non-harmful to the environment because of its low ecological magnification and high biodegradability, the immediate toxicity to non-target organisms should not be ignored. The results of this study indicate that the use of CRF could substantially reduce the immediate hazard to the non-target organism, *M. lanchesteri.*

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