ALLELOPATHIC EFFECTS OF FOUR *EUCALYPTUS* SPECIES ON COWPEA (*VIGNA UNGUICULATA*)

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SASIKUMAR. K., VIJAYALAKSHMI, C. & PARTHIBAN, K. T. 2004. Allelopathic effects of four *Eucalyptus* species on cowpea (*Vigna unguiculata*). Allelopathic investigations into the leachates of bark, fresh leaves and leaf litter of *Eucalyptus tereticornis*, *E. camaldulensis*, *E. polycarpa* and *E. microtheca* using paper and gas chromatography showed the presence of coumaric, gallic, gentisic, hydroxybenzoic, syringic and vanillic acids, and catechol. The influence of identified phenolics as well as leachates on germination, seedling length, dry matter production, vigour index and nitrogenase activity of cowpea (CO.6) was investigated. Germination was inhibited in all the cases except catechol and coumaric acid at 2 mM concentration. Vigour index was affected by catechol, gallic and syringic acids. Bioassay with leachates revealed inhibition in germination in all the cases, seven days after sowing. Dry matter production and vigour index were reduced by *E. tereticornis*, *E. camaldulensis* and *E. microtheca*. Seedling length was affected in all cases except *E. tereticornis*, 37 days after sowing. Dry matter production was affected by *E. tereticornis* and *E. camaldulensis*. Reduction in vigour index and nitrogenase activity was also noted in all cases, compared with the control.

Key words: Dry matter production – germination – nitrogenase activity – phenolic compounds – vigour index

SASIKUMAR. K., VIJAYALAKSHMI, C. & PARTHIBAN, K. T. 2004. Kesan alelopati empat spesies *Eucalyptus* terhadap kacang duduk (*Vigna unguiculata*). Kajian alelopati terhadap bahan larut lesap kulit kayu, daun segar dan sarap daun *Eucalyptus tereticornis*, *E. camaldulensis*, *E. polycarpa* dan *E. microtheca* menggunakan kromatografi kertas dan kromatografi gas menunjukkan kehadiran asid kumarik, asid galik, asid gentisik, asid hidrobenzoik, asid siringik dan asid vanilik serta katekol. Kesan sebatian fenol dan bahan larut lesap yang dikenal pasti terhadap percambahan, panjang anak benih, penghasilan jisim kering, indeks kesuburan dan aktiviti nitrogenase kacang duduk (CO.6) dikaji. Percambahan direncat dalam semua kes kecuali katekol dan asid kumarik pada kepekatan 2 mM . Indeks kesuburan dipengaruhi oleh katekol, asid galik dan asid siringik. Bahan larut lesap semua spesies *Eucalyptus* merencat percambahan kecuali *E. tereticornis.* Ini diperhatikan 7 hari selepas penyemaian. Penghasilan jisim kering dan indeks kesuburan dikurangkan oleh *E. tereticornis*, *E. camaldulensis* dan *E. microtheca.* Panjang anak benih dipengaruhi oleh semua spesies *Eucalyptus* kecuali *E. tereticornis.* Ini diperhatikan 37 hari selepas penyemaian. Penghasilan jisim kering dipengaruhi oleh *E. tereticornis* dan *E: camaldulensis.* Semua spesies *Eucalyptus* mengurangkan indeks kesuburan dan aktiviti nitrogenase berbanding dengan kawalan.

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Introduction

The inhibition of one plant by another, through the release of allelochemicals is well known (Rice 1979). The loss in the yield of field crops due to the influence of allelochemicals released from *Eucalyptus* has been reported by Lisanework and Michelson (1993). Therefore, *Eucalyptus*, though a potential industrial crop, is not recommended as an intercrop in agroforestry systems (Suresh & Rai 1987, Bansal 1988). The release of phenolic compounds adversely affects the germination and growth of plants through their interference in energy metabolism, cell division, mineral uptake and biosynthetic processes (Rice 1984). Leachates from stem fall and litter fall were suspected contributors of this effect (Molina *et al.* 1991). Showers and monsoon rains would result in periodical release and accumulation of allelochemicals from *Eucalyptus* plantations. Hence, a detailed investigation was carried out by analysing the leachates for the compounds present initially, followed by the effects of the compounds on individual and a mixture of test crops.

Materials and methods

Collection of samples

Bark, fresh leaves and leaf litter of *E. tereticornis*, *E. camaldulensis*, *E. polycarpa* and *E. microtheca* were collected from 10-year-old plantation growing at the Forest College and Research Institute, Tamil Nadu, India.

Preparation of samples and determination of total phenols

A quantity of 50 g of samples, i.e. bark, fresh leaves and leaf litter, of all the four species of *Eucalyptus* was soaked in 500 ml of water and leachates were collected at six hourly intervals for 36 hours. A few drops of toluene were added and the leachates were stored at 4 °C for analysis.

Preparation of total phenols

A quantity of 0.5 ml of each sample leachate was pipetted out in different test tubes and evaporated to dryness. The residue was dissolved in 5 ml of distilled water and 0.5 ml of Folin-Ciocalteau reagent was added. Then 2 ml of 20% Na_2CO_3 solution were added to each tube. After 3 min, the contents were mixed thoroughly. The tubes were placed in boiling water for 1 min, cooled and the absorbance was measured at 650 nm against a reagent blank.

Standard curve was prepared using different concentrations of pyrocatechol (AR grade). Concentration of phenols in the test sample was found out from the standard curve and expressed as mg per litre (Malik & Singh 1980).

Extraction of phenolic compounds

Five grams of sample, i.e. bark, fresh leaves and leaf litter, of four species of *Eucalyptus* were placed in a 500-ml conical flask with 100 ml water, shaken at 100 rpm for 24 hours. The leachate of each sample was filtered using filter paper and the filtrate was acidified with sulphuric acid to pH 2.0. Phenolic acids from the filtrate were extracted with an equal volume of peroxide free ether. The ether extract was then air dried and dissolved in a minimal volume (100 to 250 µl) of dioxane (Whitehead *et al.* 1983).

Identification of phenolic compounds

Ascending paper chromatography was performed using Whatman No. 1 chromatogram papers of 30 × 20 cm dimension with Isopropanol-Ammonia-Water (20:1:2 v/v) as solvent and diazotised sulphanilic acid as spraying reagent to detect the separated phenolics. Using sigma chromatographic standards, the unknowns were identified by co-chromatography (Mahadevan & Sridhar 1982).

A quantity of 100 µl of the sample was derivatised by the addition of 20 µl of pyridine and 100 µl of trimethylsilyl acetamide (TMSA) and incubated for 2 hours at 35 °C. One µl of the derivatised sample was injected into the column (DB-5; film thickness 0.25 µm; 30 m × 0.250 mm) fitted to the gas chromatograph (Shimadzu, GC-14B, Japan) connected to a flame ionisation detector. Nitrogen gas was used as the carrier (40 ml min^{-1}). Hydrogen and oxygen were used for flame (40 ml min^{-1}). Oven temperature was maintained at 200 °C, while injector and detector temperatures were maintained at 220 and 240 °C respectively.

Bioassays with identified allelopathic compounds and leachates on cowpea

The identified compounds were tested individually and as mixture at 1 mM and 2 mM concentrations for their effects on the germination and vigour index of cowpea (CO.6). Besides, leachates from different parts of *Eucalyptus* species (10% of bark, fresh leaves and leaf litter) were tested for their effects on germination, seedling length, dry matter production, vigour index and nitrogenous activity of cowpea (Abdul Baki & Anderson 1973, Agarwal 1980, Bergersen 1980). Three replications were maintained and completely randomised design was followed for statistical analysis.

Nitrogenase activity

Root of the pulse crop was detached without disturbing the nodules, thoroughly washed with water and air dried to evaporate the moisture. The root portion was put into 20 ml vials and crumpled to make them air-proof. One ml of air was sucked from the vial and one ml of acetylene was injected. Root was incubated for one hour with acetylene. One hundred ml of gas, sucked from vials were injected in a gas chromatograph fitted with poropak Q column. Acetylene and ethylene were used as standards. Nitrogenase activity (nM $g^{-1}h^{-1}$) was measured by using the formula proposed by Bergersen (1980).

$$\text{Nitrogenase activity} = \frac{\text{Area count} \times \text{gas volume of the flask} \times 0.0006}{\text{volume of gas sample injected into GC} \times \text{hours of incubation} \times \text{mg dry weight of nodules}}$$

Here, g dry weight of nodules was used instead of mg of protein in the sample (Turner & Gibson 1980).

Results and discussion

The total phenolic content of the leachates showed an increasing trend due to accumulation of phenolic compounds with increasing soaking time (Table 1). Leaf litter recorded comparatively higher phenolic content. During degradation of leaf litter, many secondary metabolites, which include phenolic compounds, may be formed.

Both paper and gas chromatographic analyses showed the presence of phenolic acids as the major constituent of the leachates. The phenolic acids identified were coumaric, ferulic, gallic, hydroxybenzoic, syringic and vanillic acids, apart from catechol (Tables 2 and 3). Vaughan and Ord (1990) reported that most of the phenolics released from plant parts were benzoic and cinnamic acid derivatives. Jayakumar *et al.* (1990) identified the presence of chlorogenic, coumaric, caffeic and gallic acids from *E. globulus.* The presence of gentisic, ellagic, sinapic and caffeic acids, phenolic aglycons, glycosides and terpenoides from *E. baxteri* was reported (Waller 1987a). Sivagurunathan *et al.* (1997) identified and quantified phenolics, caffeic, coumaric, ferulic, gallic, gentisic, hydroxybenzoic, syringic and vanillic acids and catechol in the bark, fresh leaves, litter, root and seed leachates of *E. citriodora, E. globulus* and *E. tereticornis,* at concentrations between 0.02 and 2.45 mM.

The mixture of allelopathic compounds (catechol, coumaric, ferulic, gallic, gentisic, hydroxybenzoic, syringic and vanillic acids) in bioassays showed pronounced inhibition on germination and vigour index of cowpea compared with the control (Table 4). Germination was inhibited in all cases except catechol and coumaric acid at 2 mM concentration. Vigour index was significantly affected by catechol, gallic and syringic acids. Vaughan and Ord (1990) also observed that caffeic, hydroxybenzoic, vanillic and syringic acids inhibited the root elongation in wheat, rye and mungbean.

Bioassay with leachates revealed significant inhibition in germination in all cases compared with the control, seven days after sowing (Table 5). *Eucalyptus tereticornis, E. camaldulensis* and *E. microtheca* significantly reduced dry matter production and vigour index. Seedling length was affected in all cases except *E. tereticornis,* 37 days after sowing (Table 6). Dry matter production was affected by *E. tereticornis* and *E. camaldulensis.* Significant reductions in vigour index and nitrogenase activity were noted in all cases compared with the control. Suresh and Rai (1987) have also observed similar inhibitions of germination, root length and dry matter production in some field crops treated with aqueous extracts of leaves of *Casuarina equisetifolia,*

Table 1 Total phenolic content (mg l^{-1}) of leachates collected from different parts of four species of *Eucalyptus* at six hourly intervals

Hours	Bark				Mean	Fresh leaves				Mean	Leaf litter				Mean
	Et	*Ec*	*Ep*	*Em*		*Et*	*Ec*	*Ep*	*Em*		*Et*	*Ec*	*Ep*	*Em*	
0	70	87	80	75	78	52	65	54	72	62	86	92	102	98	95
6	122	135	102	90	112	110	80	98	121	101	172	155	148	164	160
12	155	164	140	152	153	132	112	128	143	127	322	310	250	238	280
18	162	182	185	165	174	148	120	155	170	145	448	454	348	355	401
24	165	196	190	168	180	180	126	160	177	158	475	462	402	368	427
30	168	202	199	173	186	200	129	160	177	164	516	469	420	400	452
36	172	204	202	179	189	207	129	169	177	167	525	484	432	408	463
Mean	145	167	157	143		147	109	124	148		363	347	300	290	

p = 0.05	CD (Bark)	CD (Fresh leaves)	CD (Leaf litter)
Species	1.095	1.065	10.608
Duration	1.448	1.409	14.033
Species × Duration	2.897	1.818	28.066

Et = *Eucalyptus tereticornis*
Ec = *E. camaldulensis*
Ep = *E. polycarpa*
Em = *E. microtheca*

Table 2 Phenolic compounds identified from different parts of four *Eucalyptus* species by paper chromatography

Compound	*E. tereticornis*			*E. camaldulensis*			*E. polycarpa*			*E. microtheca*		
	B	FL	LL	B	FL	LL	B	FL	LL	B	FL	LL
Catechol	D	D	ND	ND	ND	ND	D	D	D	ND	ND	ND
Coumaric acid	D	ND	D	D	ND	D	ND	ND	ND	ND	D	D
Ferulic acid	ND	ND	ND	ND	ND	ND	D	ND	ND	ND	ND	ND
Gallic acid	ND	D	D	ND	D	ND	ND	D	D	ND	ND	D
Gentisic acid	D	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hydroxybenzoic acid	ND	D	ND	ND	D	ND	ND	ND	ND	D	ND	ND
Vanillic acid	ND	ND	ND	D	D	ND	ND	ND	D	ND	ND	ND

ND = not detected, D = detected
B = bark, FL = fresh leaves, LL = leaf litter

Table 3 Phenolic compounds identified from different parts of four *Eucalyptus* species by gas chromatography

Compound	*E. tereticornis*			*E. camaldulensis*			*E. polycarpa*			*E. microtheca*		
	B	FL	LL	B	FL	LL	B	FL	LL	B	FL	LL
Catechol	D	D	ND	ND	ND	ND	D	D	D	ND	ND	D
Coumaric acid	D	ND	D	D	ND	D	ND	ND	D	ND	D	D
Ferulic acid	ND	ND	ND	D	ND	D	D	D	ND	ND	D	D
Gallic acid	ND	D	ND	ND	D	ND	ND	D	D	ND	ND	D
Gentisic acid	D	ND	D	ND	ND	ND	ND	ND	D	ND	ND	ND
Hydroxybenzoic acid	D	D	D	ND	D	ND	ND	ND	ND	D	ND	ND
Syringic acid	ND	ND	ND	D	ND	ND	ND	ND	ND	D	ND	ND
Vanillic acid	D	ND	ND	D	D	ND	ND	ND	D	ND	D	ND

ND = not detected, D = detected
B = bark, FL = fresh leaves, LL = leaf litter

Table 4 Effects of identified phenolic compounds on germination and vigour index of cowpea seven days after sowing

Treatment	Germination		Mean	Vigour index		Mean
	1mM	2mM		1mM	2mM	
Control	100.0	100.0	88.7	1530	1530	1530
Catechol	96.6	100.0	84.0	1385	975	1180
Coumaric acid	96.6	100.0	84.0	2315	2412	2364
Ferulic acid	93.3	93.3	75.0	1368	2198	1783
Gallic acid	93.3	90.0	73.3	1028	1780	1404
Hydroxybenzoic acid	96.6	93.3	77.2	1181	2410	1796
Syringic acid	90.0	93.3	73.3	580	1528	1054
Vanillic acid	96.6	90.0	75.5	1302	2027	1665
Mixture	93.3	86.6	67.2	412	904	658
Mean				1233	1752	

CD at 5% level	Germination	Vigour index
Concentration	0.106	9.396
Chemical	0.225	19.93
Concentration × Chemical	0.318	28.19

E. tereticornis and *Leucaena leucocephala*. Jayakumar *et al.* (1990) observed reduction in chlorophyll content of crops treated with extracts of abscised leaves of *Eucalyptus*, which hints the possibility of poor photosynthesis and in turn poor plant growth. Intervention of phenolic acids in the metabolic process and thereby inhibition of normal growth has also been reported by Moreland and Novitsky (1987). Reduction in nitrogenase activity may be due to reduction in the beneficial role played by rhizobia under allelopathic conditions (Duhan *et al.* 1994).

Phenolic compounds, namely catechol, coumaric, ferulic, gallic, gentisic, hydroxybenzoic and syringic acids were present in different plant parts of the four *Eucalyptus* species. The allelochemicals released may turn inhibitory in the field after a period of time due to accumulation of these compounds arising from poor run off (Waller 1987b). The identification of phenolic compounds in trace amounts from the root leachates and their effects on germination and vigour index clearly ruled out the possibility of the interaction of phenolics released from roots and undergrowth in *Eucalyptus* plantations (Sivagurunathan *et al.* 1997). Releases of litter and the leaf flow should be contributing towards degradation because of the complex nature and during this period more phenolics may be formed as secondary metabolites and released. Another reason for the poor establishment of understorey might be due to the tougher nature of the litter fall. Its existence in the soil may not allow seeds carried by natural element to reach the ground. They may also prevent germinated seeds from establishing itself by impeding their passage. Since the releases reach the soil, some of the beneficial organisms that promote plant growth may be affected and this also contributes to the poor undergrowth. The addition of phenolic compounds, particularly phenolic acids from the releases over a period of time may also inhibit establishment due to increase in the acidity of the soil, which is always undesirable for the establishment of plants. These may be the reasons for poor plant growth in areas where *Eucalyptus* plantation existed previously.

Table 5 Effects of leachates of different parts of four *Eucalyptus* species on germination, dry matter production and vigour index of cowpea, seven days after sowing

	Germination				Dry matter production (mg/10 seedlings)				Vigour index			
	B	FL	LL	Mean	B	FL	LL	Mean	B	FL	LL	Mean
Control	100 (88.7)	100 (88.7)	100 (88.7)	88.7	0.69	0.69	0.69	0.69	462	462	462	462
Et	92 (73.6)	96 (78.6)	80 (63.4)	71.9	–	0.36	–	0.12	–	288	–	96
Ec	80 (63.4)	92 (73.6)	80 (63.4)	66.8	0.55	–	–	0.18	290	–	–	97
Ep	80 (63.4)	88 (69.7)	96 (78.6)	70.6	0.72	0.68	0.73	0.71	376	516	586	493
Em	84 (66.4)	80 (63.4)	88 (69.8)	66.5	–	–	0.85	0.28	–	–	772	257
Mean	71.1	74.8	72.8		0.39	0.35	0.45		226	253	364	

– = Just germinated (data not recorded)
B = bark, FL = fresh leaves, LL = leaf litter
Et = *Eucalyptus tereticornis*, *Ec* = *E. camaldulensis*, *Ep* = *E. polycarpa*, *Em* = *E. microtheca*

CD at 5 % level	Germination	Dry matter production	Vigour index
Plant part	1.171	0.010	2.088
Eucalyptus	1.512	0.013	2.696
Plant part × *Eucalyptus*	2.618	0.023	4.670

Table 6 Effects of leachates of bark, fresh leaves and leaf litter on seedling length, dry matter production, vigour index and nitrogenase activity, 37 days after sowing

	Seedling length (cm plant^{-1})				Dry matter production (g/2 seedlings)				Vigour index				Nitrogenase activity (nM g^{-1} h^{-1})			
	B	FL	LL	Mean	B	FL	LL	Mean	B	FL	LL	Mean	B	FL	LL	Mean
Control	52.5	52.5	52.5	52.5	1.02	1.02	1.02	1.02	5250	5250	5250	5250	750	750	750	750
Et	51.0	45.5	57.5	51.4	0.98	1.20	0.81	0.10	4692	4368	4600	4553	907	200	390	499
Ec	33.5	40.5	39.0	37.7	1.17	0.83	0.64	0.88	2680	3726	3120	3175	145	112	207	155
Ep	43.3	53.0	51.5	48.8	1.28	2.10	1.44	1.61	3464	4614	4944	4341	194	111	169	158
Em	48.5	43.0	44.5	44.8	0.96	1.22	1.25	1.14	4074	3440	3916	3810	616	81	284	327
Mean	48.5	46.3	49.0		1.08	1.27	1.03		4032	4279	4366		522	251	360	

B = bark, FL = fresh leaves, LL = leaf litter
Et = *Eucalyptus tereticornis*, *Ec* = *E. camaldulensis*, *Ep* = *E. polycarpa*, *Em* = *E. microtheca*

CD at 5 % level	Seedling length	Dry matter production	Vigour index	Nitrogenase activity
Plant part	1.274	0.013	18.93	9.254
Eucalyptus	1.645	0.017	24.43	11.95
Plant part × *Eucalyptus*	2.849	0.030	42.32	20.70

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