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Biological Activity and the Production of Azadirachtin in Neem Callus

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ABSTRACT. *The biological activity of pure azadirachtin and callus extracts was evaluated with no-choice antifeedant bioassays using the desert locust (Schistocerca gregaria). The doses to produce 50% full effect (ED_{50}) for azadirachtin and callus extracts were found to be 0.0039 μg azadirachtin ml^{-1} of ethanol and 139.82 μg callus ml^{-1} of ethanol, respectively. Calli initiated from 13 wild neem trees growing in Dry and Intermediate Zones of Sri Lanka were analysed by High Performance Liquid Chromatography (HPLC). Azadirachtin content varied from 4.2 μg g^{-1} to 189.3 μg g^{-1} of freeze dried calli. The variability observed between single trees within a site was greater than that of between sites.*

INTRODUCTION

Neem (*Azadirachta indica*) seeds contain a large number of biologically active compounds including azadirachtin, which shows insect antifeedant as well as growth regulatory properties [Mordue (Luntz) and Blackwell, 1993]. Despite the toxicity towards certain insects, azadirachtin is not apparently harmful to higher forms of life and exhibits no observable phytotoxicity (Ley and Toogood, 1990). However, the problems of obtaining a supply of good quality seeds and the cost of purification make it difficult to obtain pure azadirachtin in significant quantities (Ley and Toogood, 1990). Tissue culture of neem may provide an alternative source of producing azadirachtin and may also improve the understanding of pathways for the biosynthesis of neem metabolites and their regulation.

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In the present study, the production of azadirachtin by neem callus of different origin was investigated. Calli of Ghanaian origin were analysed by *Schistocerca gregaria* bioassays and those from Sri Lanka by HPLC.

MATERIALS AND METHODS

Callus initiation and maintenance

Neem callus was derived from leaf explants of Ghanaian trees (Kearney *et al.*, 1994) and calli were initiated from 16 wild neem trees from two different agro-ecological regions (Dry Zone and Intermediate Zone) of Sri Lanka.

Very tender and young (slightly reddish) leaves were hand picked from wild neem trees in Sri Lanka to initiate callus. Water was sprayed on to the leaves after picking and they were placed in polythene bags and transported to the University of Peradeniya. Leaves were thoroughly washed with tap water and a mild detergent (Teepol, CIC Ltd., Sri Lanka) as soon as they were taken into the laboratory. Then the leaves were washed with 0.1% $HgCl_2$ for 5 min and thoroughly rinsed with sterile distilled water. They were then treated with 10% Bleach (Care Products, Sri Lanka) and thoroughly washed with sterile distilled water. Surface sterilised leaf explants were cultured in sampling tubes (2.5 cm diameter) containing 15 ml maintenance medium (MM) (Kearney *et al.*, 1994), *i.e.* Murashige and Skoog (1962) medium supplemented with 3% (w/v) sucrose, 1 mg l^{-1} benzyl amino purine (BAP) and 4 mg l^{-1} indole butyric acid (IBA) and solidified with 0.175% Phytagel (Sigma, UK).

Calli initiated from wild Sri Lankan neem trees, and Ghanaian callus (Kearney *et al.*, 1994) were maintained on the same medium by subculturing at 6 week intervals. All the cultures were maintained at 25°C in the dark. Calli initiated from Sri Lankan trees were multiplied until the eighth subculture (approx. 1 year old). All these lines were harvested at the end of the eighth subculture, freeze dried, and stored at -20°C prior to extraction for HPLC.

Extraction of neem callus

Freeze dried calli were extracted with ethanol under reflux using the method of Kearney *et al.* (1994). Thus 0.5 g freeze dried callus was ground using a pestle and mortar and extracted twice with fresh 10 ml of 95% (v/v) ethanol under reflux for 30 min each. The residue was filtered through

Whatman number 1 filter paper. Both extracts were combined and evaporated to dryness using a vacuum oven (Gallenkamp) at 35°C and stored at -20° C.

Feeding response of *Schistocerca gregaria* to callus extracts and pure azadirachtin

Three to four day old *S. gregaria* male fifth instars were used in all assays. In this study *S. gregaria* was selected as the test insect because it is highly sensitive to azadirachtin (Mordue and Nasiruddin, 1992; Ascher, 1993). The insects were collected daily from stock cages and were kept in 11 litre cylindrical cages with excess food. Each insect was removed, weighed and introduced into small boxes separately. They were then pretreated by a period of 16 h food deprivation followed by one complete meal of fresh lettuce before conducting the feeding experiments. The insects were observed during the lettuce meal to ensure equal satiation (Allan *et al.*, 1994). The insects were kept without food for 3 h before they were introduced into containers (20 x 7.5 x 5.0 cm) with a glass fibre disc (diameter 3.7 cm, Whatman GF/A). For each experiment, discs were pre-treated with 350 μl of 50 mM sucrose as a phagostimulant, and 300 μl test solution. For the control, discs were treated with 350 μl of 50 mM sucrose and 300 μl ethanol (Analar grade). All solvents were allowed to evaporate overnight at 35°C before use. The dry discs were weighed (Metler AE) before being placed in the container. Insects were left in the containers for 3 h. The glass fiber discs were then reweighed. The percentage antifeedancy was calculated using following equation:

$$\text{Percentage Antifeedancy} = \frac{\text{Average weight eaten by controls} - \text{Weight of test disc eaten}}{\text{Average weight eaten by control}} \times 100$$

Feeding responses of *S. gregaria* to different doses of pure azadirachtin and callus extracts were measured. Serial dilutions of azadirachtin in ethanol in the range of 0.04-0.0004 ppm ($4x10^{-5}$ - $4x10^{-7}$ mg ml^{-1}) were prepared and antifeedancy was measured using 15 insects at each dose. Freeze dried extracts from Ghanaian calli from subculture 25 (*i.e.* approx. 3 yr. old) were re-dissolved in 25 ml ethanol at a rate of 20 mg of freeze dried calli ml^{-1} of extract. This solution was used as the stock solution and serially diluted to produce concentrations in the range of 0.1 - 10 mg of freeze dried calli ml^{-1} of extract, and antifeedancy was measured using 15 insects at each dose.

Dose response curves for pure azadirachtin and the Ghanaian calli extracts were obtained by plotting the percentage probit antifeedancy *vs* log concentration and ED_{50} (50% effective dosage) and ED_{95} (95% effective dosage) values were calculated. These two curves were statistically compared and the amount of callus at each concentration was converted to azadirachtin equivalence using the value 0.0007% which was obtained from analysis of the same cell line by supercritical fluid chromatography (SFC) (Allan *et al.*, 1994). The percentage probit antifeedancies obtained for the callus extracts were plotted against azadirachtin equivalence values and compared with the dose response obtained for pure azadirachtin.

Detection of azadirachtin by high performance liquid chromatography

Calli which originated from Sri Lankan trees were extracted as described earlier. The amount of callus extracted was different for each callus line and therefore, the ethanol volume was adjusted proportionally to the freeze dried weight.

All the dried extracts were resuspended in 1 ml ethyl acetate and were cleaned using Florosil mini columns (Sundaram and Curry, 1993). The extracts were evaporated and stored at -20°C prior to HPLC analysis. Cleaned extracts were dissolved in 1 ml acetonitrile and 100 μl of this solution was diluted with 900 μl HPLC grade water. Five hundred microlitres of this was injected into the HPLC column. Conditions used for high performance liquid chromatography are described in Table 1.

Calli extracts were co-injected with azadirachtin to verify the authenticity of the peak. The amount of azadirachtin present in the calli extracts was calculated using a standard curve of authentic azadirachtin covering the range of 36 ng - 270 μg.

RESULTS AND DISCUSSION

Callus initiation and maintenance of Sri Lankan material

A different and more rigorous method of sterilisation had to be used for wild trees compared to that for laboratory grown trees (Kearney *et al.*, 1994; Eeswara, 1996) and this presumably reflects the different micro-organisms present in wild grown trees. Although the surface sterilisation procedure described here was only 80% efficient this was found to be acceptable at a

Table 1. Conditions used for high performance liquid chromatography.

Instrument	**High Performance Liquid Chromatography (Gilson, France)**		
Column	C8 reverse phase column (Aquapore RP 300), 10 µm pore size 25 cm length, Brown Lee Laboratory, USA)		
Flow Rate	1 ml min - 1		
Detector	Gilson UV Detector Model 118		
Sample Loop	500 µl		
Solvent/Gradient	Time (min)	Acetonitrile (%)	H_2O (%)
	0	10	90
	10	10	90
	30	70	30
	32	100	0
	34	100	0
	37	10	90
	40	10	90

practical level. The experiments undertaken on explant sterilisation indicated that a complete success may be difficult to achieve and may depend on the individual habitat of the tree as well as seasonal variations (Eeswara, 1996).

Callus was successfully (100%) derived from every explant which could be successfully sterilised. Callus initiation occurred within one week and growth was successfully maintained on MM. It is important to note the success of this medium as the plant material was of different ecological origin than that described by Kearney *et al.*, 1993 and Mordue *et al.*, 1995. Callus lines initiated from Dambulla tree A (DA) and Kalawewa-1 (KW-1) remained hard and occasionally showed differentiation into nodular structures even one year after initiating the callus compared to other lines which became soft and friable with time.

Table 2. **Effect of different doses of pure azadirachtin and callus extracts of GhIDC(S25) on food intake by male fifth instar nymphs of *S. gregaria* in a no-choice bioassay (n=15 insects in all cases).**

Azadirachtin			Callus extracts [GhIDC (S 25)]		
Dose (ppm)	Amount eaten (mg) ± SE	AI ± SE	Dose (mg/ml)	Amount eaten (mg) ± SE	AI ± SE
Control	23.50+7.43^{a}	-	Control	24.06+15.68^{a}	-
0.0004	13.34+3.86^{b}	43.23+4.24^{a}	0.1	14.61+4.01^{b}	36.60+4.49^{a}
0.001	4.24+7.43^{c}	81.92+2.90^{b}	0.5	5.21+5.64^{a}	78.33+6.26^{b}
0.004	1.72+1.70^{cd}	92.65+1.97^{c}	1	1.32+1.62^{a}	94.50+1.79^{c}
0.01	0.23+0.21^{d}	99.04+0.23^{d}	5	0.09+0.12^{a}	99.61+0.13^{d}
0.04	0.00^{d}	100^{d}	10	0.07+0.11^{a}	99.70+0.12^{d}
LSD	2.62	0.81	LSD	5.23	1.4

Means followed by the same letters are not significantly different at 5% probability level.

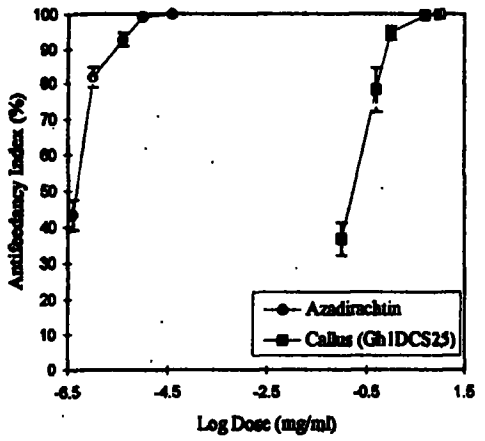
AI - Antifeedency Index

Feeding response of *Schistocerca gregaria* to callus extracts and pure azadirachtin

Figure 1a depicts the mean percentage antifeedancy indices (± SE) at various doses of pure azadirachtin and callus extracts in the no-choice bioassay. In this study 100% deterrent level was achieved with an azadirachtin concentration of 0.04 ppm ($4x10^{-5}$ mg ml^{-1}) agreeing with the results of Butterworth and Morgan (1971) and Mordue and Nasiruddin (1992).

Results showed significant differences for the arcsin transformed antifeedancy indices between doses except for 0.01 ppm and 0.04 ppm level of pure azadirachtin (Table 2). Azadirachtin concentration at 0.0004 ppm (4×10^{-7} mg L^{-1}) showed 43.23 ± 4.24 % antifeedancy and the amount of filter paper disc eaten was significantly different from the control discs at the 5% probability level showing the high sensitivity of *S. gregaria.*

A)



B)

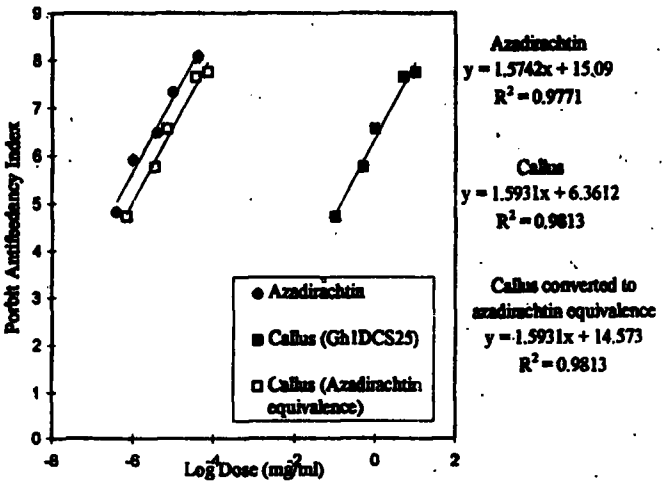


Figure 1. **a) The mean % antifeedancy index (AI±E) against log dose of pure azadirachtin (mg ml^{-1}) and Gh1DC (S25) callus extracts (mg ml^{-1}) in fifth instar *S. gregaria* (n=15) in no-choice bioassay and, b) Probit % of antifeedancy of *S. gregaria* fifth instar nymphs presented with glass fiber discs with pure azadirachtin (mg ml^{-1}), callus extracts (mg ml^{-1}), and callus extracts converted to azadirachtin equivalence based on an SFC value of 0.0007%.**

a)

b)

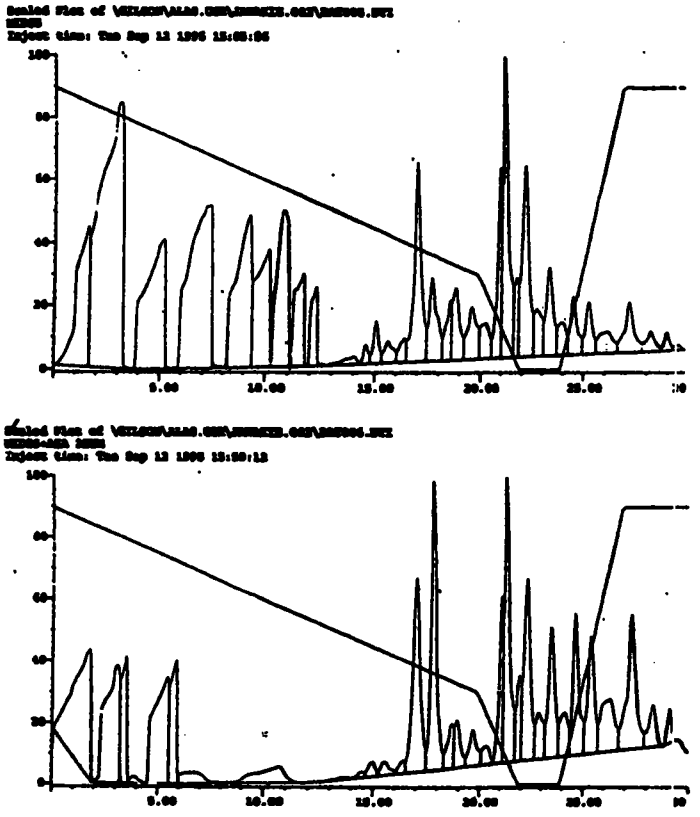


Figure 2. Traces of callus extracts of Maha-Illuppallama Tree D, (a) same extracts spiked with 18 $\mu g \cdot ml^{-1}$ of pure azadirachtin; (b) from reverse phase HPLC on C8 column under gradient conditions with acetonitrile/water as eluants, detection at 217 nm using a Gilson 118 UV detector.

The 100% deterrent level for callus was reached at 5 mg freeze dried callus ml^{-1} solvent. One way analysis of variance results for the arcsin converted antifeedancy indices showed significant differences at the 5% probability level between each dose except for 5 and 10 mg ml^{-1} concentrations. The callus extract concentration of 0.1 mg ml^{-1} showed 36.60 ± 4.49% antifeedancy. The amount of glass fiber disc eaten at this concentration was significantly different from the control discs (Table 2).

Figure 1b shows the dose response curves for callus extracts and azadirachtin. Statistical analysis showed that those were parallel indicating that the responses of insects to both callus extracts and pure azadirachtin are similar, and that some other factor is causing the callus extracts to behave as a dilution of pure azadirachtin. The ED_{50} and ED_{95} values calculated using these dose response curves for azadirachtin were 0.0039 μg ml^{-1} and 0.0043 μg ml^{-1}, respectively, and 139.82 μg ml^{-1} and 1496.25 μg ml^{-1}, respectively, for callus extracts. Based on the ED_{50} value the azadirachtin equivalence of the callus extract was 0.003 mg g^{-1} of freeze dried callus (0.0003%).

In a separate study it was found that these calli produced 0.0007% azadirachtin on dry weight basis as determined by SFC (Allan *et al.*, 1994). Therefore, each callus dose was converted to azadirachtin equivalence using this value. Probit values were plotted against log of these values and are shown in the Figure 1b. This shows a shift of the callus extract regression line very near to azadirachtin dose response line showing the dilution of activity probably due to the presence of inert material or to phagostimulants (*e.g.*, sugars). It would be possible to investigate this further by partitioning with different solvents to obtain pure extracts prior to feeding experiments.

Detection of azadirachtin in callus initiated from Sri Lankan trees by HPLC

HPLC traces obtained for MI-D callus (calli initiated from Maha Illuppallama Tree D) extracts are shown in Figure 2. The azadirachtin concentration in all the Sri Lankan lines are presented in Table 3. The amount of azadirachtin in the callus varied from 4.2 to 189.3 μg g^{-1} of freeze dried callus (0.0004 to 0.0189 %). The highest amount of azadirachtin, 189.3 μg g^{-1}, was obtained in the callus initiated from the KW-1 and the lowest amount (4.2 μg g^{-1} freeze dried material) in callus of KL-2. It was also observed that high azadirachtin producing callus lines (KW-1 and D-A) were brown in colour and partially differentiated compared to the others. These results indicated that the loss of differentiation may be associated with a decrease in azadirachtin yield

Table 3. Azadirachtin content of 13 callus lines.

	Tree	Freeze dried weight (g)	Azadirachtin ($\mu g\ g^{-1}$)
Kuliyapitiya	KL-2	0.779	4.2
	KL-3	0.667	14.0
	KL-4	0.711	45.8
Kalawewa	KW-1	0.856	189.3
	KW-2	0.766	37.1
	KW-3	0.881	46.9
Dambulla	D-A	1.098	155.5
	D-C	1.477	27.1
	D-D	0.721	45.4
Maha-Illuppallama	MI-A	1.023	15.2
	MI-B	2.288	5.7
	MI-D	2.500	26.9
	MI-E	0.980	24.0

and this preliminary observations warrants further study. Furthermore, variation between callus lines initiated from single trees within sites was noticeably greater than between sites. This results agrees with the results of Bally *et al.* (1996) who observed large deviation in the content of azadirachtin in the neem seeds collected from different trees. The HPLC profiles showed that callus extracts contained compounds other than azadirachtin. Thus, further investigation on this aspect might lead to identification of other compounds which would be important in pest control.

CONCLUSION

Schistocerca gregaria was found to be extremely sensitive to pure azadirachtin showing 100% deterrency at 0.04 ppm concentration. Thus, antifeedant bio-assays could be employed to detect the production of azadirachtin in neem cell culture systems. However, the amount of azadirachtin present in the crude callus extracts, based on the ED_{50} value (0.0003%) was lower than the amount obtained by chemical analysis (0.0007%) suggesting the presence of phago stimulants.

The HPLC analysis of callus extracts derived from wild trees growing in Sri Lanka showed the production of azadirachtin. It was also found that amounts of azadirachtin present in the callus varied markedly between cell lines, although this could be due to different levels of differentiation between these lines.

REFERENCES

Allan, E.J., Eeswara, J.P., Johnson, S., Mordue (Luntz), A.J., Morgan, E.D. and Stuchbury, T. (1994). The Production of Azadirachtin by Tissue Culture of Neem. *Azadirachta indica*, Pesticide Science. 42: 147-152.

Ascher, K.R.S. (1993). Non-conventional Insecticidal Effects of Pesticide Available from the Neem Tree, *Azadirachta indica*. Archives of Insect Biochemistry and Physiology. 22: 433-449.

Bally, I.S.E., Ruddle, L., and Simpson, B. (1996). Azadirachtin levels in Neem Seed Grown in Northern Australia. International Neem Conference, The University of Queensland, Australia. February 4-9, 1996. p. 30 (Abstract).

Butterworth, J.H. and Morgan E.D. (1971). Investigation of the Locust Feeding Inhibition of the Seeds of the Neem Tree, *Azadirachta indica*. Journal of Insect Physiology. 17: 969-977.

Eeswara, J.P. (1996). The Production of the Insect Antifeedant Azadirachtin and Related Metabolites by Plant Cell and Tissue Culture of Neem (*Azadirachta indica* A. Juss), Ph.D. Thesis, University of Aberdeen, Aberdeen.

Kearney, M-l., Allan, E.J., Hooker, J.E. and Mordue (Luntz), A.J. (1994). Antifeedant Effects of *In vitro* Culture Extracts of the Neem tree, *Azadirachta indica*, against the Desert Locust (*Schistocerca gregaria* (Forskål). Plant Cell Tissue and Organ Culture. 37: 67-71.

Ley, S.V. and Toogood, P.L. (1990). Insect Antifeedants. Chemistry in Britain. 26(1): 31-35.

Mordue, A.J. and Nasiruddin, M. (1992). Azadirachtin Treatment and Host Plant Selection. Proceedings of the 8th International Symposium on Insect-Plant Relationships. Menken, S.B.J., Visser, J.H. and Harrewijin, P. (Eds).

Mordue (Luntz), A.J. and Blackwell, A. (1993). Azadirachtin: an Update. Journal of Insect Physiology. 39: 903-904.

Mordue (Luntz), A.J., Zounas, A., Wickramananda, I.R., and Allan, E.J. (1995). Neem Tissue Culture and the Production of Antifeedant and Growth Regulatory Compounds. pp. 187-194. *In*: McKinlay, R.G. and Atkinson, D. (Eds). Integrated Crop Protection Towards Sustainability. BCPC Symposium Proceedings, No. 63.

Murashige, T. and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bioassays with Tobacco Cultures. Physiologia Planatarum. 15: 473-497.

Sundaram, K.M.S. and Curry, J. (1993). High Performance Liquid Chromatographic Determination of Azadirachtin in Conifer and Deciduous Foliage, Forest Soils, Leaf Litter and Steam Water. Journal of Liquid Chromatography. 16: 3275-3290.