NEEM TISSUE CULTURE AND THE PRODUCTION OF INSECT ANTIFEEDANT AND GROWTH REGULATORY COMPOUNDS.

A.J. MORDUE (LUNTZ)

Department of Zoology, University of Aberdeen, Tillydrone Avenue, Aberdeen AB9 2TN

A. ZOUNOS, I. R. WICKRAMANANDA

Departments of Agriculture and Zoology, University of Aberdeen, AB9

E.J. ALLAN

Department of Agriculture, University of Aberdeen, 581 King Street, Aberdeen AB9 1UD

ABSTRACT

Neem tissue culture methodologies have been established for both callus and cell suspension cultures. Extraction and detection methodologies for plant metabolites with biological activity have been developed and refined. The presence of antifeedant compounds has been measured using a choice bioassay for the desert locust (*Schistocerca gregaria*), with 3-4 day old fifth instar nymphs and with azadirachtin as standard. A bioassay for insect growth regulatory effects has been established using one day old fifth instar nymphs of the milkweed bug (*Oncopeltus fasciatus*), after the method of Isman *et al.*, 1990. The antifeedant activity of a callus line, from Niger, has shown to be similar to that of another callus line derived from a different geographical location (Kearney *et al.*, 1994). Cell suspension cultures from a Ghanaian line were shown to contain antifeedant compounds in both the cells and the medium and that these increased with growth.

INTRODUCTION

The Neem tree (*Azadirachta indica*) is native to the arid regions of the Indian subcontinent (Schmutterer, 1990) and has been introduced to tropical and subtropical areas of Africa, America and Australia (National Research Council, 1992). The tree has been recognised as a multiple use forest crop with important roles in afforestation, the provision of fuelwood, pesticides, oils, tannins, timber and medicinal products (Pliske, 1983). In particular, neem leaves and seeds have been exploited traditionally for insect control and the major biologically active component, azadirachtin, has been identified from seed kernel extracts (Butterworth & Morgan, 1968). This highly oxidised triterpenoid is now well known for its insect antifeedant, growth regulatory and sterilant effects (Mordue(Luntz) & Blackwell, 1994). Neem formulations have been shown to be nontoxic to man and animals (Duke & duCellier, 1993) and consequently, their potential for controlling agricultural pests in an environmentally sound manner, has recently gained impetus. This is associated with

commercial exploitation, with neem formulations being patented by Western companies (Pearce, 1993) and registered by the Environmental Protection Agency for both food and non-food crops. It is estimated that natural neem production will not meet increasing demands (Saxena, 1989) and although it is a fast growing tree, with fruit production occurring within 3-4 yrs, full productivity is not obtained until *c.* 10 years. In addition, azadirachtin itself is too complex to produce synthetically on a commercial basis and its synthesis *in vivo* is not fully understood.

Plant cell and tissue culture may provide a means of overcoming the constraints of traditional plant breeding and in the case of neem, offers an easier method for elucidating the complex synthetic pathway of azadirachtin. In addition it may also provide an alternative means for consistent and reliable production with the added potential of increasing yields in a particular cell line. Initial studies have shown that neem tissue cultures produce insect antifeedant compounds (Kearney *et al.*, 1994) and azadirachtin (Allan *et al.*, 1994). This paper will further develop the methodologies involved and will evaluate neem cell cultures, derived from trees from different geographical locations, for insect biological activity.

MATERIALS AND METHODS

Neem cell culture

Callus was derived from leaves of young trees (< 1 year old), which originated from Ghana and Niger, using the method of Kearney *et al.*, 1994. Suspension cultures were initiated by transferring friable callus (Ghana line, GH1) into 50 ml Maintenance Medium (Kearney *et al.*, 1994) in 250 ml flasks. These were incubated in the dark at 25 °C on an orbital shaker at 100 rev/min. In order to prevent the formation of large cell aggregates, subculture was achieved by discarding cells retained on a 2.83 mm Nubold mesh (Stanier & Co., UK) and subculturing the remaining cells, on a fresh weight basis, after filtering through Miracloth (Calbiochem Co., USA). Repeated subculture in this manner resulted in a more homogenous suspension. Ten flasks were thus subcultured using 4 g fresh weight material, four of these were used for monitoring growth by taking fresh and dry weight measurements (Allan, 1991) at regular intervals. Both the cells and growth medium were harvested from 3 flasks for extraction and subsequent insect bioassay at 7 and 10 d. Cells were filtered through Miracloth (Calbiochem Co., USA), immediately frozen, freeze dried and stored at -20 °C (Electrolux, UK) until extraction. The resulting medium was also frozen prior to extraction. Growth kinetics were analysed using the method of Allan (1991) using the Minitab statistics package.

Extraction of cell cultures

Cell extraction methods

In order to compare extraction methods, ten week old freeze dried callus, line GH1, which had been subcultured 15 times was used. Triplicate 2 g samples were used to compare two different extraction methods viz. an ethanolic extraction (Kearney *et al.*, 1994, adapted from Govindachari *et al.*, 1990) and a water extraction (D.E. Morgan pers. comm.). Thus, 2

x 50 ml ethanol was used to extract the cells under reflux for 2 x 30 min. The resultant suspension was filtered through Whatman No. 1 paper prior to rotary evaporation and resuspended in 2 ml ethanol (Laboratory Grade). For the water extract, 2 g material was homogenised (MSE homogeniser) for 1 min in 50 ml distilled water. The resultant meal-water mixture was centrifuged at 5500 rev/min for 10 min. The meal was then homogenised and centrifuged a further 4 times using 50 ml fresh water each time. All the water extracts were then combined and an aliquot added to an equal volume of methanol. This extract was filtered through Floricil (Sigma, UK), if required, to remove any precipitate, and stored at -20 °C (Electrolux, UK). Prior to bioassay, extracts were rotary evaporated and taken-up in ethanol to 20 mg dry weight/ml. All other cell culture extractions (both callus and cells from suspension cultures) were undertaken by the water extraction method, using 0.5 g freeze dried material.

The medium was thawed prior to extraction and filtered (Whatman No. 1) to remove any precipitate and the resultant volume measured. One part medium was mixed with 0.25 parts dichloromethane in a separation flask and shaken for approximately 4 x 15 sec. After separation the lower (dichloromethane) phase was removed. This was repeated 3 times and all the dichloromethane fractions were combined. Any water was removed using approx. 10 g anhydrous magnesium sulphate which was discarded by filtration. Extracts were rotary evaporated at below 35 °C.

Bioassays

Antifeedancy assay

A choice feeding bioassay (Nasiruddin, 1993; Blaney *et al.*, 1990) was used to assay the antifeedant potency of cell culture extracts using 3-5 day old male and female fifth instar nymphs of the desert locust, *Schistocerca gregaria*. Prior to bioassay the insects were kept separately and their state of hunger was standardised. Individual insects were transferred into containers and given access to two glass fibre discs of 3.7 cm diameter (Whatman International Ltd., UK), a control and a test disc. Both discs were pre-treated with 350 µl 50 mM sucrose and allowed to oven dry at 37 °C overnight. A further 350 µl aliquot of appropriate extract or ethanol was added to the disc which was dried again. All discs were weighed before and after the experiment and results recorded as mg disc eaten. The percentage of antifeedance was calculated following the formula:

$$\frac{\text{Weight of Control disc eaten - Weight of Test disc eaten}}{\text{Weight of Control disc eaten + Weight of Test disc eaten}} \times 100$$

Results were analysed using oneway analysis of variance and Tukey's pairwise test (Minitab Statistic Package). All percentages were arcsine transformed and negative percentages indicating no antifeedant activity were given a value of 0.

Insect growth regulatory (IGR) assay

Nymphs of the large milkweed bug (*Oncopeltus fasciatus*) were obtained from a laboratory colony, maintained at 27 °C and 12:12 LD and reared on de-husked sunflower seeds. The IGR activity of azadirachtin and tissue culture extracts was assessed following topical application to newly moulted (within 24 h) fifth instar nymphs using the methods of

Isman *et al.*, (1990). Test solutions were applied to the abdominal dorsum in 1 µl acetone with a 10 µl Hamilton Syringe. Controls were treated with carrier alone. Bugs were kept in 10 cm diameter Petri dishes supplied with sunflower seeds and cotton wool soaked with water. The insects were monitored daily and assessed on day 10 by which time all the control insects had moulted (day 7). IGR activity was defined as any deviation from moulting to a morphologically normal adult, i.e. from deformity of the wings and/or legs, to death during moulting, and to a delay of the moult by 3 days or more. IGR results were corrected for control effects by Abbott's formula (Busvine, 1971).

RESULTS

Neem cell culture

The method of callus initiation and maintenance (Kearney *et al.*, 1994) previously shown to be successful for material of Ghanaian origin also proved successful for that derived from Niger with pale, friable, undifferentiated callus being obtained. All the callus lines used in this study had been in culture for at least 2 years. Growth of GH1 suspension culture (Fig. 1) showed an initial lag phase of 5 d followed by an exponential growth phase of 16 d at which time a maximum biomass of 25.1 g l^{-1} was obtained. On the basis of the dry weight, the specific growth rate of this line was 0.15 mg d^{-1} with a doubling time of 110 h (4.6 d).

Extraction

Comparison of antifeedancy for callus extracted using two different methods showed that the water extraction resulted in a significantly higher recovery of antifeedant compounds than did the ethanol extraction procedure (Table 1).

TABLE 1. Antifeedancy of *Azadirachta indica* callus, line GH1 (10 mg callus dry weight/ml extract) using ethanol and water extraction methods against fifth instars of *Schistocerca gregaria*, in a choice feeding bioassay (n = 8-10/ replicate). Samples are significantly different ($P < 0.05$).

Type of Extract	Number of Replicates	Percentage Antifeedancy (Mean ± SE)
Ethanol	3	46.97 ± 5.12
Water	3	84.19 ± 2.80

Bioassays

A standard curve for % probit antifeedancy to azadirachtin was set up for the choice bioassay which gave an ED_{50} of 0.00035 ppm azadirachtin. Analysis of antifeedancy of 6

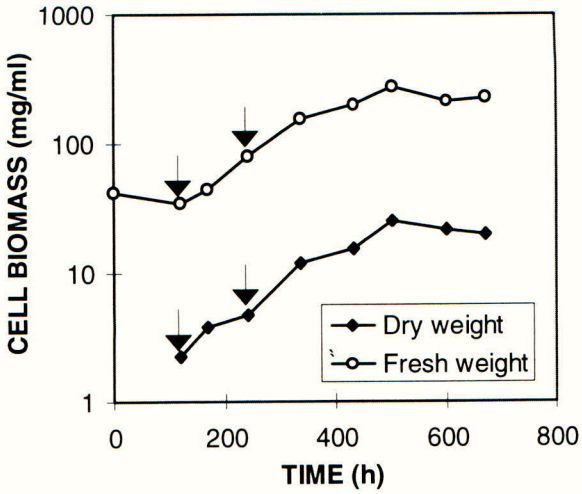

Fig. 1 Growth as measured by fresh and dry weight, of a Ghanaian *Azadirachta indica* cell suspension culture. Arrows indicate the 7 and 10 d times when cultures were harvested for insect bioassay.

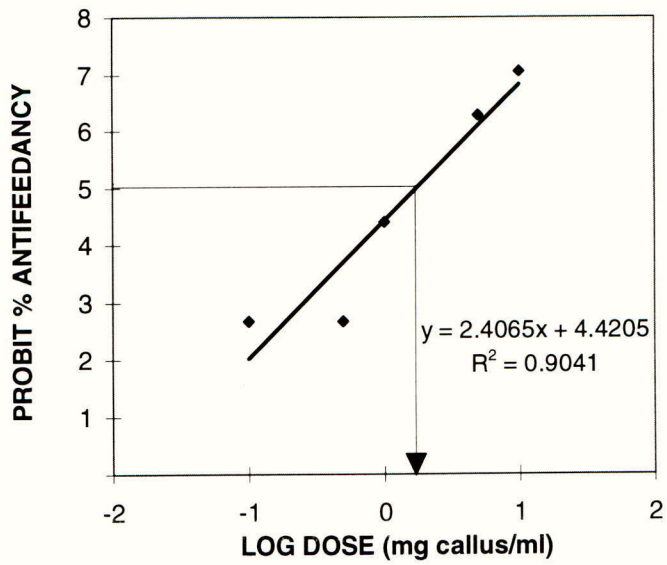

Fig. 2 Probit % antifeedancy of *S. gregaria* fifth instar nymphs presented with glass fibre discs with Niger callus extract and sucrose (50 mM) or sucrose alone in a choice bioassay, n=8-15. ED_{50}=1.74 mg callus/ml.

week old callus, line Niger 5A, showed a good dose response relationship with an ED_{50} of 1.74 mg dry weight callus/ml (Fig. 2).

Analysis of antifeedancy (AF) was also undertaken on the suspension culture during growth (Fig. 1). Suspension culture cells developed significant antifeedancy from day 7 to day 10 of culturing. Antifeedancy had increased significantly from day 7 to day 10 (26.7 ± 3.1 % and 55.1 ± 2.7 % AF, n = 19-21; $P < 0.05$) of culturing for 0.04 ppm dry weight of cell extract. A more detailed analysis of the cell suspension medium revealed that antifeedant products had been secreted into the medium and they too significantly increased from day 7 to day 10 of culturing (Table 2). Thus, whereas the cell biomass increased 38 %, on a dry weight basis, in that 3 day period of exponential growth, the antifeedant compounds released into the culture medium increased 20 fold.

TABLE 2. Antifeedancy of medium extracts of a cell suspension culture against fifth instars of *Schistocerca gregaria,* in a choice feeding bioassay (n = 8-10/ replicate). Medium was harvested at day 7 and day 10 of culture. (a-b, $P < 0.05$).

Sample	Dilution (ml medium/ml extract	Number of replicates	Percentage Antifeedancy (Mean ± SE)
Day 7	5.00	3	41.67 ± 3.73^{a}
Day 10	5.00	3	100^{b}
Day 10	0.50	3	91.92 ± 0.96^{b}
Day 10	0.25	3	57.29 ± 6.84^{a}

A standard curve for % probit IGR effects to azadirachtin was set-up for *O. fasciatus* nymphs. Abnormal and delayed moults occurred increasingly until there were no normal moults after a dose of 0.015 µg/insect. An ED_{50} of 0.0012 µg azadirachtin/insect was achieved.

DISCUSSION

Biomass production in the suspension culture used in this study, was much higher than that of Kearney *et al.,* (1994) with a max. biomass of 25.1 gl^{-1} compared to 14.7 $gl.^{-1}$ This presumably reflects the method used for routine subculture prior to the experiment which resulted in a more homogeneous culture. Both the max. biomass and the growth rate (as reflected by a doubling time, t_d, of 4.6 d), were also greater than has been obtained for other semi-tropical trees e.g. *Cinchona ledgeriana* max. biomass = 10 $gl,^{-1}$ t_d = 6.9 d (Scragg *et al.*, 1988) ; *Quassia amara* max. biomass = 6.2 gl^{-1} ; t_d = 5.1 d (Scragg *et al.*, 1990).

The extraction procedure of Kearney *et al.*, 1994 used an ethanolic reflux system which would expose azadirachtin to heat instability and therefore an alternative extraction

procedure was considered. Water extraction of freeze dried callus produced twice as much product, in terms of antifeedancy, and hence has been adopted as the better protocol for routine use. Tissue cultures however contain less storage material compared with seeds where the water soluble components may interfere with efficient extraction of the azadirachtin products. In this instance, ethanol extraction may be preferable.

By using the water extraction methods and constructing a dose-response curve for callus cultures of Niger 5A, an accurate ED_{50} value for antifeedancy was obtained which could be equated to the azadirachtin standard curve. The yield of antifeedant compounds, using ED_{95} values, expressed as "azadirachtin equivalents" was 0.0001 % based on dry weight of callus and 0.0003% for suspension culture medium based on dry weight cells secreting into the medium. The ED_{95} values are similar to that of Allan *et al.*, 1994 who found a yield of 0.0007 % using 100% AF and direct measurement of azadirachtin.

Antifeedancy tests of both cells and medium from cell suspension cultures revealed that antifeedant compounds are produced during growth and that substantial proportions are released into the medium. There is a marked difference in the growth kinetics found here compared to Kearney *et al.*, 1994 who used a similar cell line. This difference may reflect the greatly reduced cell aggregation in the culture used in this experiment and more detailed analysis following production of antifeedant products throughout growth would be desirable. In terms of commercial production, the fact that antifeedant compounds are secreted is advantageous as it will allow alternative systems, such as immobilisation to be undertaken. Van der Esch *et al.*, (1994) also showed that azadirachtin, as detected by HPLC, was present in both the cells and the medium of neem suspension cultures.

Our current work involves screening various neem cell lines for higher yields and the development of bioassays to measure both insect antifeedancy and growth disruption. These should yield results which will establish techniques to determine fundamental aspects concerning production of these compounds *in planta* in addition to evaluating the potential of plant tissue culture for commercial exploitation.

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