

Callus Cultures of *Azadirachta indica* and Their Potential for the Production of Azadirachtin

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The content of azadirachtin, an insecticidally active ingredient in neem tree [*Azadirachta indica* (A. Juss.)] seeds, varies considerably due to environmental factors and for genetic reasons. Furthermore, the tree does not grow in moderate climates. It seems appropriate, therefore, to employ tissue culture techniques for the production of azadirachtin in order to obtain constant amounts of standardized quality. We investigated if and how azadirachtin contents of callus cultures derived from various cell lines are affected by different nutrient media and carbohydrate concentrations. The azadirachtin contents of the calli were analyzed by TLC and HPLC. Azadirachtin contents of callus cultures varied depending on the cell line, the nutrient medium and the carbohydrate source employed.

KEY WORDS: *Azadirachta indica*; tissue culture; azadirachtin; nutrient media; sucrose concentration.

INTRODUCTION

Alternative pesticides are becoming increasingly important. One requirement for their utilization in agriculture is constant availability, with standardized quality. In the case of complex chemical structures, production may be feasible by *in vitro* cultures. The neem tree, *Azadirachta indica* (A. Juss.) (Meliaceae), possesses insecticidally active ingredients, among them the triterpenoid azadirachtin, which to date can not be synthesized chemically. The effects of azadirachtin on insects have been reviewed in numerous publications in recent years (2,12,21-24). Due to environmental and genetic factors, the content of azadirachtin – found only in seeds – varies considerably. Furthermore, the tree does not grow in moderate climates and is not frost-tolerant. Therefore, in order to obtain constant amounts of standardized quality azadirachtin, it seems appropriate to employ tissue culture techniques for its production.

In vitro cultivation of woody species was not successful until the 1970s, when also tissue culture of neem was first reported (14,18). Since then several papers have been published on the effect of various growth factors on differentiation processes in neem tissue cultures and about secondary metabolites, notably nimbin (13,15,19,20,25). The connection between differentiation and secondary metabolism is generally accepted. It was not until the 1990s, however, that efforts were initiated to produce azadirachtin in tissue culture (1,9,26).

It is well known that *in vitro* cultures are able to produce secondary metabolites, sometimes even in quantities that allow economically feasible production (5,6,8). With

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this goal in mind, the present project aimed to elucidate if and how azadirachtin contents of 8-week-old *A. indica* callus cultures derived from four different cell lines were affected by different nutrient media and two sucrose concentrations.

MATERIALS AND METHODS

Surface-sterilized leaf and bark segments were taken from 2–3-year-old *A. indica* plants from Nicaragua and Togo and incubated in the dark at $26 \pm 1^\circ\text{C}$ on a Murashige and Skoog medium (MS) (Sigma M 5524) supplemented with 30 g/l sucrose (Serva 35580), 3 g/l Gelrite (Merck & Co., NY), 100 g/l myo-inositol (Serva 26310), 0.4 mg/l thiamine-HCl (Serva 36020), 0.2 mg/l IAA (Sigma I 2886), 0.1 mg/l BAP (Sigma B 9395); pH was adjusted to 5.8. Friable, yellow/light brown calli were subcultured after 4 weeks on four different nutrient media containing the same ingredients as the basal medium except for the sucrose concentration: MS, Nitsch's and Nitsch's (NN) (Sigma N 5639), White's (Sigma W 0876) and McCown's (Sigma M 6774) media, with 15 g/l and with 30 g/l sucrose. Calli, again friable and yellow/light brown and still undifferentiated, were once more subcultured after 4 weeks, allowed to grow on fresh medium for another 4 weeks and then evaluated as regards state of differentiation, and analyzed as to their azadirachtin content. Sample preparation involved freeze-drying the callus material, followed by petroleum ether and water extraction procedures (24 h each, 1:0.04 mg dry weight/ml) and a solid phase extraction on a C_{18} column. Finally, samples were concentrated by rotary evaporation, redissolved in methanol and stored at -20°C until analysis. Analyses were carried out using TLC (one-dimensional on silica plates 60 F 254, 5% sulfuric acid in 96% ethanol as spray reagent, heating of the plate for 15 min at 110°C after spraying) and HPLC (isocratic conditions, acetonitrile/water 40/60, v/v, flow 1 ml/min, 210 nm, Lichrospher 100 C_{18} column 250/4). Azadirachtin as external standard as well as for co-elution was purchased from Sigma (A 7430).

RESULTS

Differentiation With 15 g/l sucrose on MS medium, 32% of the calli (n=19) showed root differentiation. Shoot differentiation occurred on the NN medium only and was exhibited by 11% of the calli (n=18), the same results applying to root differentiation on this medium. On McCown's medium also 11% of the calli (n=18) showed root differentiation. None of the calli (n=21) grown on White's medium exhibited any differentiation.

Differentiation was less pronounced on media supplemented with 30 g/l sucrose. With this supplement root differentiation on McCown's medium was exhibited by only 5% of the calli (n=20) and on MS by 6% (n=18), and shoot differentiation on NN occurred with 8% of the calli (n=24). On White's medium differentiation was visible but it was not possible to determine at the time of evaluation whether shoots or roots would develop. Data were analyzed and were statistically sound according to the χ^2 -test; however, sample size should be increased for verification.

Azadirachtin content Azadirachtin contents from pooled samples per medium and cell line are shown in Figures 1 and 2; samples had to be pooled in order to obtain sufficient material for the preparation process.

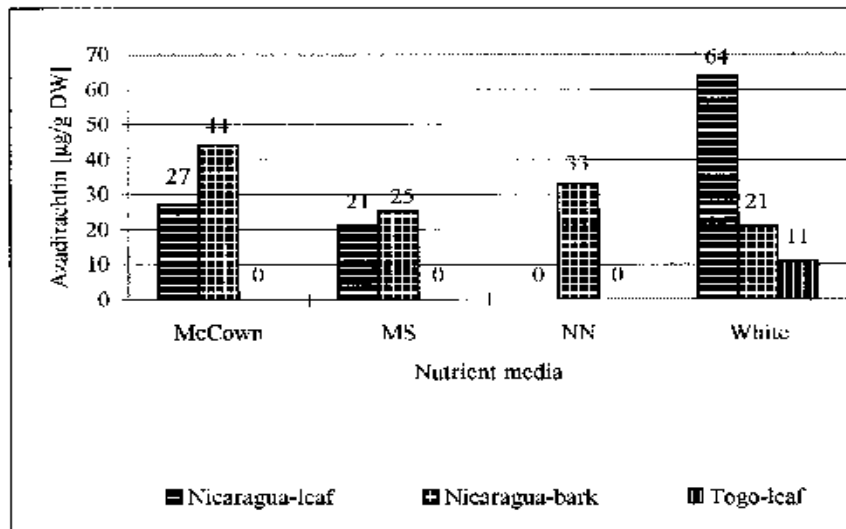


Fig. 1. Azadirachtin content ($\mu\text{g/g}$ dry weight) of callus cultures from four cell lines on four nutrient media supplemented with 15 g/l sucrose.

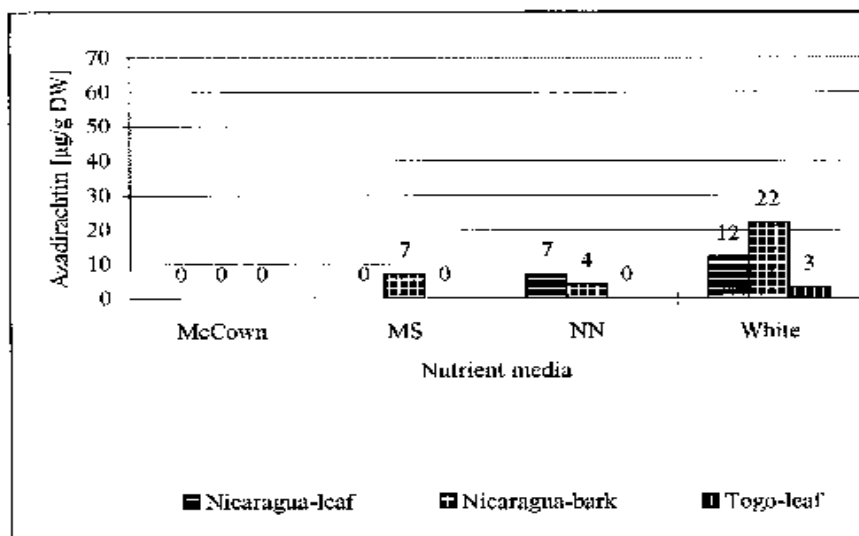


Fig. 2. Azadirachtin content ($\mu\text{g/g}$ dry weight) of callus cultures from four cell lines on four nutrient media supplemented with 30 g/l sucrose.

Nicaraguan cell lines have a significantly higher azadirachtin content ($<0.5\text{--}64\ \mu\text{g/g DW}$) than those from Togo ($<0.5\text{--}11\ \mu\text{g/g DW}$), with the cell line Togo-bark being unable to produce azadirachtin above the limit of detection. Calli derived from leaf explants from Togo yielded azadirachtin only on White's medium. The azadirachtin content was generally three times higher on media supplemented with $15\ \text{g/l}$ sucrose ($<0.5\text{--}64\ \mu\text{g/g DW}$) (Fig. 1) as compared with those with $30\ \text{g/l}$ sucrose ($<0.5\text{--}22\ \mu\text{g/g DW}$) (Fig. 2). The highest content was obtained on White's medium by the Nicaraguan leaf cell line ($64\ \mu\text{g/g DW}$). The Nicaragua-bark cell line was most successful on McCown's medium ($44\ \mu\text{g/g DW}$) with a sucrose concentration of $15\ \text{g/l}$, but produced no azadirachtin on this medium when supplemented with $30\ \mu\text{g/l}$ sucrose.

DISCUSSION AND CONCLUSIONS

It has been postulated that secondary metabolism is closely connected with differentiation processes (3,4,10,16), which depend strongly on growth regulators. These processes must be triggered also in a callus. The potential for differentiation is limited, due to a given auxin-cytokinin ratio in the nutrient medium. Therefore, substances occurring in the intact plant may not be detectable in callus cultures. Cell cultures from *Digitalis lanata* are not capable of producing glycosides of pharmaceutical value when morphologically undifferentiated. After induction of organogenesis, however, these substances could be detected again (11). Digitoxin, also of pharmaceutical importance, is synthesized in *Digitalis purpurea* cell cultures only with differentiated shoots (7). Nimbin, of pharmaceutical interest as well, occurred only in differentiated *A. indica* tissue cultures (18). In contrast, it could be shown in the present study that morphological differentiation is not a prerequisite for azadirachtin production; the highest concentrations were detected in completely undifferentiated cells. Increase in fresh weight of undifferentiated calli kept on medium with $15\ \text{g/l}$ sucrose was greater than on medium supplemented with $30\ \text{g/l}$ (data not shown). This leads to the conclusion that sucrose is abundant and obviously not a limiting factor in azadirachtin production. Growth kinetics also have to be taken into consideration as regards time of sampling. Highest levels of secondary metabolites are generally found in the late exponential and early stationary growth phase. In this study, calli were sampled in the stationary growth phase with the nutrient medium being almost exhausted. The number of subcultures (age of the callus) may also play an important role in the expression of azadirachtin in tissue cultures (27). Apparently there are superior and inferior cell lines in respect to the production of azadirachtin in callus cultures.

These results show that tissue cultures are capable of producing azadirachtin in quantities well above the limit of detection, depending on nutrient medium, sucrose concentration and cell line (plant of origin/type of explant). However, production and extraction have to be increased manyfold before large-scale production can be considered seriously.

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