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CAFFEINE AND THEOBROMINE FORMATION BY TISSUE CULTURES OF *CAMELLIA SINENSIS*

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Key Word Index—*Camellia sinensis*; Theaceae; caffeine; theobromine; purine alkaloids; plant tissue culture; callus; root.

Abstract—Callus and root suspensions from *Camellia sinensis* have been established to produce and accumulate caffeine and theobromine as secondary metabolites. Leaf fragments from a mature greenhouse tea plant, grown on MS medium supplemented with IAA (5.7 μM) produced roots, while leaf explants of the same plant cultured on MS medium supplemented with 2,4-D (4.5 μM) and BA (0.45 μM), gave rise to the formation of friable callus. Both callus and roots when transferred to MS liquid medium supplemented with 2,4-D (4.5 μM) and BA (0.45 μM), produced caffeine and theobromine, which were detected by TLC, UV and GC. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Tea is an important commercial crop that is grown in ca 30 countries and consumed worldwide. Like many other woody species, it has traditionally been propagated vegetatively, because it is highly heterozygous and the seedlings are too variable for commercial use. There have been a number of communications regarding the micropropagation of tea [1–3] and marked differences in response have been reported for different genotypes [4].

Work has been performed on the production of caffeine from suspension cultures of *Coffea arabica* (0.0–0.7% of dry weight). Usually considerable amounts of theobromine are also found (25–50% of total alkaloid) using the same culture [5–7]. There has been no work on the isolation of purine alkaloids from the tea plant using *in vitro* techniques.

The aim of the present work was to develop plant cell culture as an alternative source of secondary metabolites independent from seasonal variation and disease. We report on the accumulation and isolation of caffeine and theobromine from cultures of *Camellia sinensis*.

RESULTS AND DISCUSSION

When leaf fragments of mature tea plants were grown on MS medium supplemented with IAA (5.7 μM), roots were formed, while the addition of BA (0.45 μM) to the same media generated both roots and callus. MS media supplemented with 2,4-D (4.5 μM) and BA (0.45 μM) gave rise to the formation of friable and, sometimes, brownish callus. Callus colouring or necrosis was found to correlate with BA concentration, since reducing the quantity of BA or removing it completely, produced a colourless callus. It was also found that 2,4-D enhanced callus formation compared with IAA, while IAA enhanced multiple root formation.

Suspensions of callus and roots were screened for caffeine and theobromine using TLC, UV and GC. TLC analysis gave two spots with R_f values of 0.5 and 0.56, respectively, corresponding to theobromine and caffeine standards. The UV spectrum shared an overlapping peak at 268 nm, corresponding to both caffeine and theobromine. When caffeine and theobromine were analysed separately, the wavelengths maximums were 273 nm and 270 nm, respectively. GC analysis confirmed the presence of caffeine and theobromine, by direct comparison with the corresponding authentic compounds.

Quantitative analysis showed that the total weight of the secondary metabolites caffeine and theobromine recovered from the *in vitro* system were 0.55 and 0.09 g l^{-1}, respectively.

In conclusion, the results of the present work showed that tissue culture favours the formation of caffeine and theobromine more than any other secondary metabolite. This may be attributed to the qualitative and quantitative selection of the plant

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growth regulators used in this experiment. Further studies are being carried out using different experimental conditions to produce other constituents of this plant. Investigations are also underway to improve the yields towards pilot scale for the production of caffeine and theobromine.

EXPERIMENTAL

Plant material and tissue culture conditions

Callus and root cultures were established from *C. sinensis* leaves, obtained from the greenhouse of the University of Jordan, Amman, Jordan. Leaf fragments from mature tea plants were washed in EtOH, sterilised in 7.5% NaOCl soln, shaken for 10 min and finally rinsed × 3 with steril dist. H_2O. Leaf fragments were then placed on MS media [8] with a pH of 5.7–5.8. The media were supplemented with IAA (5.7 μM), with and without BA (0.45 μM) or 2,4-D (4.5 μM), and with and without BA (0.45 μM). The work was carried out under aseptic conditions. Cultures were incubated for 4 weeks at 35° with 16 h photoperiods. Both callus and roots were transferred to MS liquid media supplemented with 2,4-D (4.5 μM) and BA (0.45 μM). After 4 weeks, samples of the liquid medium were examined for growth by light-microscopy, while tests for secondary metabolite products were carried out using TLC, UV and GC. The method was scaled up from 50 to 250 ml suspension culture and subculturing was carried out every 4 weeks.

Isolation and identification of secondary metabolites

Roots and cell suspensions (150 ml of each) were made alkaline by the addition of NH_4OH (pH $\approx$10) and then extracted with $CHCl_3$ (3 × 30 ml). The combined $CHCl_3$ extracts were washed with H_2O (3 × 20 ml), dried (Na_2SO_4) and filtered. Solvent was removed by rotary evapn at 0°. Secondary metabolites were identified by TLC, UV and GC. The TLC solvent system used was EtOAc-MeOH-H_2O (100:13.5:10). Spots were visualised by spraying with the iodine-HCl reagent [9]. UV scans were carried out over the wavelength range 200–400 and 220–340 nm; samples were dissolved in chloroform. FID-GC analysis was carried out using a glass column (1.8 m ×, 3.4 mm ID) packed with 1.5% OV17 on Chrom WHP with N_2 at 40 ml min^{-1}. Column temp. was 230° (isothermal) and inj. and det. temps were 280°. The sample injn vol. was 1 μl.

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