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PRODUCTION OF VERBASCOSIDE IN CALLUS TISSUE OF *EREMOPHILA* SPP.

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Key Word Index—*Eremophila denticulata*; *E. decipiens*; Myoporaceae; callus culture; hydroxyphenylethanol glycosides; verbascoside; production.

Abstract—Callus tissue cultures of *Eremophila denticulata* and *E. decipiens* accumulate up to 20% of their dry weight as a mixture of hydroxyphenylethanol glycosides of which the major component is the caffeoyl ester, verbascoside (acteoside). The production of this compound under a variety of growth conditions has been studied. Verbascoside has also been isolated from callus tissue of *E. clarkei*, *E. drummondii*, *E. glabra* and *E. viscida*.

INTRODUCTION

A number of *Eremophila* species which grow in the semi-arid regions of Australia produce abundant quantities of resin on the leaves and stems. These resins often contain oxygenated diterpenes with unique skeletons [1]. We became interested in determining whether *Eremophila* tissue cultures [2] could be induced to produce terpenes as secondary metabolites. To this end we have established callus cultures of several *Eremophila* species and have studied the growth rate of two of these under different conditions. We now report that all *Eremophila* species studied produce verbascoside (**1**) as the major extractable metabolite. Conditions in which **1** constitutes *ca* 20% of callus dry weight have been defined for both *E. denticulata* and *E. decipiens*.

RESULTS AND DISCUSSION

Following the method outlined in the Experimental, callus cultures were obtained from leaf explants of six *Eremophila* species, *E. clarkei*, *E. denticulata*, *E. decipiens*, *E. drummondii*, *E. glabra* and *E. viscida*. For the isolation of metabolites callus tissue was homogenized in dichloromethane–methanol (1:1). The lipophilic portion of the extract was methylated with diazomethane and analysed by GC-MS which showed the major components to be methyl esters of palmitic, stearic, oleic, linoleic and linolenic acids and β-sitosterol. No evidence was obtained for the presence of any terpenoid metabolites. Examination of the hydrophilic extract in each case revealed that it consisted essentially of one compound (TLC) which was identified by ^{1}H and ^{13}C NMR as the caffeoyl ester, verbascoside (**1**) (acteoside). This compound has been isolated from a number of different plant sources [3–5].

The yields of verbascoside produced for each species were *E. clarkei*, 10% of dry wt of callus tissue; *E. denticulata*, 3–9%; *E. decipiens*, 7–17%; *E. drummondi*, 1.1%; *E. glabra*, 1.5%; *E. viscida* 10%.

Lines of *E. denticulata* (A) and *E. decipiens* (B) were chosen to investigate the effect of varying levels of nutrients in the growth medium. Formation of verbascoside reached a maximum when Mg^{2+} was omitted (A 18%; B 21%) gradually decreasing with higher levels of Mg^{2+} (at 6.49 mM, A 2.2%; B 3.2%). Low levels of K^+ also favoured production of verbascoside (5 mM K^+, A 3.7%; B 17%; 40 mM K^+, A 2.5%; B 8.3%). Increasing levels of NaCl did not have any significant effect (0 mM NaCl: A 5.4%; B 18%; 150 mM NaCl: A 8.1%; B 18%). For *E. denticulata*, verbascoside production increased under conditions of low nitrogen concentration (5 mM: 15%; 120 mM: 4.5%) whereas varying phosphate levels (0.1 to 1.5 mM) had little effect. Variations in sucrose levels were well tolerated by *E. decipiens* with lower production of **1** at 10 g/l (4.2%) and 100 g/l (9.7%) compared to the normal level (12.2%).

The preliminary results presented above are interesting in a number of ways. Although verbascoside has been isolated previously from callus tissue and suspension cultures [6–8], notably from high yielding (16%) lines of *Syringa vulgaris* [6], in these cases **1** was a known metabolite of the intact plant. Prior to this study **1** had not been identified in *Eremophila* species; mannitol being the most frequently encountered hydrophilic metabolite [9]. Verbascoside (**1**) was not detected in leaves of the parent *E. denticulata* (the source of the explant). The trend towards higher production of **1** as nitrogen, potassium or magnesium levels are lowered suggests that, at least to some extent, **1** is produced in response to nutrient stress. In this context it is interesting to note the hypothesis that hydroxycinnamoyl derivatives may be produced as a result of some challenge to the integrity of the cell [10]. In addition one notes that with *Syringa vulgaris* cells, which are already specialized with respect to verbascoside production, can be induced to accumulate **1** by 2,4-D[10]. For all the *Eremophila* species studied maximum growth was achieved with 2,4-D.

Finally, interest in the production of verbascoside might be stimulated if preliminary indications of its antihypertensive and DOPA-agonistic activity [4] are confirmed and/or if, as observed for other caffeoyl derivatives [6, 11], it is shown to have significant anti-viral activity.

EXPERIMENTAL

Sterilization and culture conditions. Leaves were washed under running water for 20 min, sterilized for 20 min (*E. denticulata*, 25 min) with a 1% NaOCl soln and rinsed × 3 in sterile double de-ionized H_2O. Explants were trimmed to 10 × 5 mm segments and cultured in Murashige–Skoog medium [12] modified by supplementing with the following nutrients: thiamine HCl (5 μM), inositol (550 μM), nicotinic acid (30 μM), pyridoxine HCl (8 μM) and sucrose (30 g/l) and adjusted to pH 6.1. The cultures were maintained at 25° under continuous fluorescent lighting (Growlux). Callus initiation took 1–3 weeks and the first subculture was carried out at 6 weeks. Maximum growth of callus during the 6 weeks of initiation was achieved with hormone levels (2,4-D; kinetin) as follows: *E. denticulata* and *E. decipiens* (1 mg/l; 0), *E. clarkei*, *E. drummondii*, *E. glabra* and *E. viscida* (0.1 mg/l; 1 mg/l). Callus cultures were subcultured every four weeks onto fresh media containing the same amount of hormones.

Isolation of verbascoside (**1**). Callus tissue (10–100 g) was homogenized in a soln of CH_2Cl_2–MeOH (1:1) and left to extract overnight. If required H_2O was added until two phases formed. The upper layer was taken, the MeOH was removed under red. pres. and the aq. soln extracted with EtOAc. The crude extract was analysed by TLC (EtOAc–MeOH, 19:1) and mostly showed a single spot whose R_f varied with the amount applied to the plate. To overcome this, analysis by ^{13}C NMR was carried out for solutions in CD_3OH or $(CD_3)_3SO$. Initially the structure was deduced from analyses of the ^1H, ^1H–^1H correlation and ^{13}C NMR spectra of a sample of **1** from *E. denticulata* and by comparison with published ^1H and ^{13}C NMR data for both MeOH [13,,14] and DMSO solns. (Note that in ref. [14] some signals for quaternary carbon atoms have been reassigned.)

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