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ACCUMULATION OF 2-PHENYLETHANOL BY CALLUS DERIVED FROM LEAF-BUD OF *ROSA DAMASCENA*

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Key Word Index—*Rosa damascena*; Rosaceae; damask rose; tissue culture; enzymes; biosynthesis; 2-phenylethanol; 2-phenylethyl-β-D-glucoside.

Abstract—Most callus lines derived from explants of axillary buds of *Rosa damascena* did not produce essential oil when maintained on standard media, but after exposure to a suitable diurnal variation of light and temperature a small proportion (5 of the 36 lines assayed) accumulated 2-phenylethanol (the main component of the natural oil) and its β-D-glucoside. The accumulation was greatest in the period of decelerated growth preceding the stationary phase and optimally was *ca* 6% of the level in petals. Addition of the allelopathic agents caffeic and ferulic acids (10^{-4} M) to the culture medium increased both the period of the growth cycle (*ca* 3-fold) and also the yield (up to 2.5-fold) from the biosynthetically active lines. The majority of callus lines derived from axillary buds and all those from leaf, petal, stem, calyx and root (totalling 205), however, did not accumulate 2-phenylethanol (either free or bonded) under any regime studied: nevertheless, samples (*ca* 10% of total) assayed all yielded cell-free extracts capable of synthesizing the alcohol from L-phenylalanine with efficiencies 30–60% those from extracts of biosynthetically active lines.

Addition of 2,4-D (*ca* 10^{-3} M) to the medium decreased the period of the growth cycle (to *ca* 0.5) but greatly reduced or abolished accumulation of 2-phenylethanol: however, in extracts of these cultures the synthetic ability was little impaired compared with controls. Fine cell suspensions prepared from active cell-lines did not produce the alcohol nor did their extracts sustain the biosynthetic pathway. The possible significance of these observations for the formation of secondary metabolites in culture is discussed.

INTRODUCTION

The most expensive rose oils (e.g., attar of roses) are extracted from petals of *Rosa damascena* Mill. cv. trigintipetala Dieck. (damask rose) and contain 2-phenylethanol as the main component together with geraniol, nerol, citronellol and traces of other volatiles [1, 2]. These alcohols also occur bonded as β-D-glucosides in the petals [3]. Callus and suspension cultures of *Rosa* species generally fail to accumulate detectable amounts of the essential oils characteristic of the parents, but we have recently shown that such biosynthetically inactive callus of the commercially used cultivar nevertheless did possess the full enzymic complement for the synthesis of geraniol and nerol from mevalonate [4, 5]. In addition, both callus and cell suspensions of this and other *Rosa* species had the capacity rapidly to degrade exogenously supplied (and perhaps endogenously produced) monoterpenoids [5, 6]. We now record the selection and establishment of callus lines from the trigintipetala cultivar that produced high levels of 2-phenylethanol, and report the effect of the synthetic auxin 2, 4-D and of two allelopathic compounds on these yields and also the importance of the nature of the explant on the biosynthetic ability of the derived cultures. We initially used callus cultures as these are usually superior to suspensions in sustaining secondary metabolism [7, 8]; and our findings supported this generalization. Our results, derived from studies on three specimens of *R. damascena* of differing provenance, differ in almost every respect from those of the only previous investigation of the production of essential oil by cultures of this species [9].

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RESULTS AND DISCUSSION

Nature of the callus cultures

Petals of our specimens of *R. damascena* yielded a solvent-extractable oil (*ca* 0.3% wt/fresh wt: in the following all % yields will refer to this comparison) composed of 2-phenylethanol (62–68%), citronellol (20–25%), geraniol (5–7%), nerol (5–6%) and 2(*E*)-farnesol (traces). A further quantity (*ca* 0.03%) of the alcohols was bonded as β-D-glucosides; mainly (>90%) as the 2-phenylethanol-derivative. These values are broadly in agreement with previous analyses [1, 3].

Callus lines from explants of stem, petal, leaf, calyx, root and axillary leaf bud were established on Murashige and Skoog (MS) medium using tissue of varying stages of development (young to mature foliage; bud to blown flowerhead etc.). Friable, yellow calli were formed (20–30% success) within three to nine months and a fraction (*ca* 10%) accumulated chlorophyll but at much lower concentrations (*ca* 5%) than found in parent tissue: this level of greening is typical in calli [10]. The slow and rather inefficient initiation was followed by an unusually

long passage time for subculture (7–12 weeks). All lines became white and translucent after three or four passages and remained friable, although browning intruded after cessation of growth. Such pigmented cultures were not necrotic and could be regenerated after several months in the stationary phase. Callus from all sources appeared stable in overall morphology and in ploidy ($2n = 56$ for parent, callus, or cell suspensions, where randomly tested) and certain lines maintained significant biosynthetic ability (see later) for up to two years. Three culture regimes were investigated: (i) isothermal—constant illumination (27°; 1000 lux); (ii) variable temperature and illumination (16 hr at 1000 lux, 27°; 8 hr dark, 20°) and (iii) isothermal—variable illumination (12 hr at 1000 lux; 12 hr dark; 27°). Although it is well known that secondary metabolism in higher plants is influenced by temperature and photoperiod, little is known of the situation *in vitro*. [cf. 11]. Under all three chosen conditions, growth rates were similar, but our successful culture lines accumulated 2-phenylethanol most efficiently under regime (ii) and levels for the other two sets of conditions rarely reached 25% of these optimum values. Consequently, most of our callus lines (from all types of explants) were maintained under regime (ii).

Callus from different organs and tissue of the parent consisted almost entirely of parenchymous tissue resembling that in petals although scattered meristemic centres could be recognised with cells containing prominent central nuclei and contracted vacuoles. After several sub-cultures most lines exhibited tracheid formation (4–6% of cells) and older (> 1 yr) lines had lignified cell walls. No relationship between auxin levels (see later) or other variable parameters of media or regime and tracheid formation, or between growth rate and this limited cytodifferentiation could be discerned.

Accumulation of 2-phenylethanol by callus cultures

Callus lines from various organs and tissues (20–40 from each) were assayed for total 2-phenylethanol (free and β-D-glucoside) after four to seven passages under conditions (ii). The only lines yielding detectable ($> 10^{-7}$%) accumulations of 2-phenylethanol were derived from axillary leaf buds. Only a proportion of these latter cultures were, however, biosynthetically productive (in the limited sense under discussion): thus five of a total of 36 lines assayed accumulated the alcohol, but these did so in very significant amounts (0.005–0.013%)—at maximum *ca* 6% of the levels in the petals which are the storage organs of the parent. A few cultures from all sources (19 from over 200 assayed) stored monoterpenes at the very low levels ($\leqslant 10^{-4}$%) that have been recorded and discussed previously [5].

Further experiments were confined to the productive lines from explants of axillary leaf buds. *de Novo* synthesis was never in doubt as the explants contained negligible amounts of 2-phenylethanol, but was confirmed by the incorporation into 2-phenylethanol (*ca* 0.8%) of L-[U-^{14}C]phenylalanine injected into the callus. Addition of L-phenylalanine (20–50 mg/l) to the culture medium did not, however, increase the levels of accumulation of 2-phenylethanol—in contrast to the enhancement triggered by this precursor of the yield of indole alkaloids from *Datura* cultures [12]. Maintenance of the calli in the dark led to a rapid fall in level of 2-phenylethanol (to 10% of the original) after one subculture, but this is consistent with the known activation by light of phenylalanine ammonia lyase (PAL: E.C.4.1.1.5)—the crucial enzyme controlling the funneling of the amino acid into phenylpropanoid and derived pathways [13]. Storage of 2-phenylethanol in the active culture lines plateaued after two to five passages and was then essentially constant for a further five to eight subcultures, before a slow decline set in. In the few long-term analyses made the storage levels had fallen to 20–30% of the optimum values by the 15th subculture. All 2-phenylethanol was intracellularly stored and there was no evidence for secretion into the medium.

A typical growth curve on MS medium is III, Fig. 1: this approximates to a classical profile both on account of the number of observational points (120 independent weighings) and because of the compression of the time axis (cf. curves I and IV). Assays for 2-phenylethanol were made at points A to F, corresponding to decelerated, linear and exponential growth, and lag and stationary phases respectively. These results are compared with the storage levels in the organs of the parent plant in Table 1. The maximum accumulations occurred at the entry to the stationary phase (the region where sub-culture was generally carried out), and were at least 60 to 600-fold greater than the quantities occurring in the axillary buds and leaves respectively.

2,4-Dichlorophenoxyacetic acid (2,4-D) is usually considered the most effective auxin (natural or synthetic) for induction and maintenance of growth and cell division in both callus and cell suspensions. However, its presence has generally been reported to inhibit secondary metabolism in culture: e.g. in restricting or preventing the formation of anthocyanins [14], anthraquinones [15], alkaloids [16], and terpenoids [17], although this effect is by no means universal [cf. 18, 19]. Curves I and II (Fig. 1) represent the growth profiles for callus derived from axillary leaf buds when this auxin was added to the medium *in lieu* of IAA (in these curves the experimental points are not shown to improve clarity), and Table 1

Table. 1. Accumulation of 2-phenylethanol in parent tissue and during growth cycle of callus of *R. damascena* on standard and modified MS medium

Tissue*	Assay point†	Accumulation of PEA‡
Petal	—	100
Leaf	—	< 0.01
Axillary bud	—	< 0.1
Callus I, II	A, B, C, D, E.	0.1, < 0.1, < 0.1, < 0.1, < 0.1.
Callus III	A, B, C, D, F.	6, 4, 1, 0.2, 0.8.
Callus IV	A, B, C, D, G.	15, 6, 2, 1, 13.
Callus V	A, B, C, D, G.	10, · · 0.5, 8.

* Petals at preperfect stage (8 days after splitting of calyx). Young leaf and bud tissue (*ca* 3 and 1 cm length). Callus grown on media defined in Fig. 1.

† Assay points after 3–5 sub-cultures on medium: times as in Fig. 1.

‡ Total (free + β-glucoside). Typically the latter comprised 20–25% of the yield. Assays (mean of 3 independent determinations: formal s.e. *ca* ± 30% of actual value) are expressed in terms of the accumulation in petals (100 ≡ 0.2% wt/fresh wt) < 0.1 indicates traces, if any.

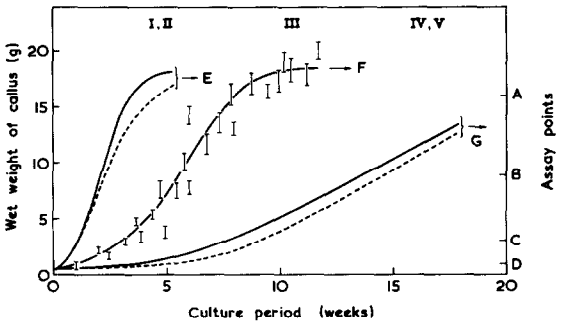

Fig. 1. Growth curves for established callus lines derived from explants of axillary buds of *R. damascena*. Curves refer to cultures on standard MS medium (III) and with modifications; I (2,4-D, 2 mg/l; no IAA); II (2,4-D, 4 mg/l; no IAA); IV (ferulic acid, 2 mg/l) and V (caffeic acid, 2 mg/l). Each bar on III represents the mean and formal s.e. of five weighings. A to D indicate periods in the growth cycles (± 1 day) when product and enzyme levels were assayed (see Tables 1, 3). E, F and G are additional assay points in the stationary phases at 8, 12 and 30 weeks respectively.

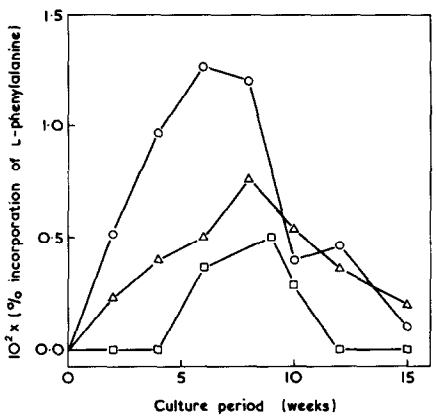

Fig. 2. Incorporations of L-[U-^{14}C]phenylalanine into 2-phenylethanol and its β-D-glucoside (—□—), phenylacetate (—△—), and (*E*)-cinnamate (—○—) in extracts from callus of *R. damascena* cultured on Nash and Davies medium containing 2,4-D (1 mg/l). Assays were made on callus entering stationary phase (*ca* 8 weeks passage time).

records the yields of 2-phenylethanol from these cultures. Addition of the synthetic auxin more than halved the passage time but reduced to extremely low levels or abolished the accumulation of the alcohol. 2,4-D had previously been shown to suppress the production of 'soluble phenolics' in suspension cultures of *R. damascena* [20] and *R. Moyesii* cv. Paul's Scarlet Climber [21]. The general repression by 2,4-D of 2-phenylethanol synthesis in our active cultures is recorded in Table 2: the choice of the last three media listed was guided by a previous study [9]; see later.

In contrast to this inhibition, Curves IV and V (Fig. 1) represent the quite different growth profiles when ferulic acid [(*E*)-3-methoxy,4-hydroxycinnamic acid; 10^{-4} M] and caffeic acid [(*E*)-3,4-dihydroxycinnamic acid; 10^{-4} M] were added to the standard (2,4-D-free) MS medium. These additives are allelopathic agents [22, 23] and were toxic to the calli at greater ($> 10^{-2}$ M) concentrations. At the lower levels they much restricted callus growth, but after three subcultures the accumulation of 2-phenylethanol had significantly increased over controls up to levels that were *ca* 15% of the amounts in petals (Table 1). Addition of (*E*)-cinnamic acid (10^{-3} to 10^{-5} M) to the growth media led to irreproducible results. Ferulic and cinnamic acids are known to stimulate 'lipid' accumulation in suspensions of *R. damascena* [24], but cinnamic acid and certain derivatives have also been reported to inhibit or reduce the activity of PAL in higher plants [25] and cultures derived therefrom [26]. Attempts were made to manipulate cultures to enhance secondary metabolism

Table 2. Accumulation of 2-phenylethanol in callus of *R. damascena* cultured in different media

Medium	Ref.	2,4-D (mg/l)	*G**	*R*†	% *PEA*‡
Murashige & Skoog	55	—	36	56	6
Murashige & Skoog	55	1.0§	35	26	<0.1
Nash & Davies	32	1.0	24	53	<0.1
Nash & Davies	32	—‖	25	65	2
White	56	2.0	9	10	<0.1
Gambourg B5	57	0.5	12	63	<0.1
Becker	58	2.0	10	75	<0.1

* Index of total growth at sub-culture; $G = m_t/m_0$ where m_o and m_t are the masses at start and end of each passage.

† Index of growth rate. R = time in days (± 5) to sub-culture at entry to stationary phase.

‡ Accumulation of 2-phenylethanol (free + β-glucoside) after 3–5 passages on medium. Expressed as a percentage of the accumulation in petals (see Table 1). <0.1 signifies traces, if any.

§ IAA replaced by 2,4-D.

‖ 2,4-D replaced by IAA (10 mg/l). 5 sub-cultures were necessary to dilute the (non-metabolized) synthetic auxin out of the system.

by addition of inhibitors of mitochrondrial formation and function and of the Krebs Cycle [cf. 27]. Such stratagems had failed to stimulate terpenoid production in cultures of *R. damascena* [5] and fared no better for 2-phenylethanol.

The variations in accumulation of 2-phenylethanol over the growth cycle of the callus (Table 2) are not unexpected. Production of secondary metabolites in whole plants freqently varies during development [28], and an inverse relationship between such production and growth rate (and cell division) which resulted in optimum accumulations in the approach to the stationary phase has been adequately documented [cf. 17] and convincingly rationalised [29, 30]. In our examples, presumably phenylalanine becomes available for shunting into phenylpropanoid metabolism only when protein synthesis slows or virtually ceases. [cf. 31].

Enzyme levels in callus

We previously found that callus of *R. damascena* which did not accumulate monoterpenes nevertheless possessed the full enzymic complement for the formation of geraniol and nerol from mevalonate [5]. Consequently, analogous assays for enzyme systems converting L-phenylalanine into 2-phenylethanol were made on extracts from both biosynthetically-active and inactive cultures. Activities in extracts from three callus lines are compared with those achieved from parent tissue in Table 3. The values from the last source were some 10-fold less than previous reports [3] but nevertheless represent our experience of the levels attainable for a variety of preparations derived from petal tissue that are capable of sustaining terpenoid or phenylpropanoid biosynthesis. Callus of types II, III and IV (growth profiles in Fig. 1) all yielded cell-free extracts that synthesized 2-phenylethanol with 30–80% the efficiency of preparations from petal, and the levels were at least 10^3-fold greater than those extractable from the axillary buds from which the culture lines were derived. In contrast, extracts from callus of *R. damascena* were capable of synthesizing the lower terpenoids at levels up to 100-fold greater than the corresponding extract from the parent tissue. The 2-phenylethanol-forming systems did not possess this level of activity (cf. with that in petals) but are none the less remarkably potent allowing for the number of steps between the precursor and the end-product and the lack of any attempt to optimise the yields (e.g. by variation of the concentration of cofactors and metal ions). Cultures of type I (Fig. 1) in which 2,4-D had repressed formation of 2-phenylethanol nevertheless contained adequate enzymic machinery (as gauged by the activities of cell free extracts) to synthesise the alcohol from its parent amino acid. Extracts from 20 randomly, selected callus lines that were derived from petal, leaf, calyx etc. and did not accumulate 2-phenylethanol also possessed the ability to convert phenylalanine into 2-phenylethanol in yields 0.5–23% of that achieved by the petals of the parent. The inference is that all the callus lines that we established probably possessed some synthetic ability to form 2-phenylethanol, although this was not usually expressed in accumulation of the metabolite.

Table 3. Formation of 2-phenylethanol in cell-free extracts from callus and parent tissue of *R. damascena*

Tissue*	Type of assay†	Yield‡
Petal	Whole tissue (control)	5.3%
Petal	Extract	0.13% (≡100)
Leaf	Extract	<0.1
Axillary bud	Extract	<0.1
Callus I	Extract: A, B, C, D, E	31, 29, 15, 6, 9
Callus III	Extract: A, B, C, D, F	80, 28, 38, 6, 4
Callus V	Extract: A, B, C, D, G	71, 70, 38, 6, 8

*Parent and callus tissue as used for data in Table 1.

†Extracts prepared as in Experimental section. A–G are assay points as defined in Fig. 1 and Table 1.

‡Conversion of L-[U-^{14}C]phenylalanine into 2-phenylethanol (free + β-glucoside). The first two rows show % conversion. The later rows are scaled in terms of the conversion in extracts from petals (≡100). All latter values are *ca* ±30% (of actual values).

Figure 2 records the variations of extractable enzymic activities for the conversion of L-phenylalanine into (*E*)-cinnamate, phenylacetate and 2-phenylethanol over the growth cycle of a callus line (derived from stem; passage time *ca* 8 weeks) which did not accumulate detectable quantities of 2-phenylethanol. The extractable activity leading to formation of 2-phenylethanol (at maximum *ca.* 4% of that from cell-free extracts of petals) peaked at the time of subculture, i.e. just before entry into the stationary phase, and incorporation into the other products followed similar profiles. (*E*)-Cinnamate may not be on the direct route to phenylacetate and 2-phenylethanol and indeed the extracts did not convert-(*E*)-[^{14}C]cinnamate into the latter, but [^{14}C]phenylacetate was efficiently (32%) thus converted by the crude preparation. The variation of extractable enzyme activities over the growth cycle which mirrors the similar profile of accumulated products (previous section) is also a well-established phenomenon for the production of certain secondary metabolites in culture and occurs *inter alia* for the enzyme systems producing phenolics in *R. damascena* [32] and for several related systems of phenylpropanoid and cinnamate metabolism [30, 33–36].

Synthetic capabilities of cell suspensions

Fine cell suspensions were readily established from both productive and non-active callus lines derived from axillary buds and from calli from other tissues of *R. damascena*. All generally comprised clusters of from 1 to 10 cells, most cells being *ca* spherical (25 μm diameter) but some being elongated giants (300 × 25 μm). Typically the biomass increased 80-fold after 14–30 days in culture and most (>95%) of the resulting cells were viable. None of the lines accumulated 2-phenylethanol or other secondary metabolites of low molecular mass; nor did cell-free extracts contain any synthetic activity similar to that derived from callus. Recently, manipulation of the photoperiod has induced non-yielding cell suspensions of *Pelargonium* species to accumulate monoterpenes [11] but this approach (using a wide range of regimes) had no success in our hands. Previous detailed studies on a variety of oil-producing plants had not resulted in the establishment of biosynthetically-active cell suspensions [37], but much more work is required before it can be assumed that suspensions from *R. damascena* lack the capacity to synthesise or accumulate 2-phenylethanol. More so than for callus, the predominating cell type in fast growing suspensions may be selected for growth at the

expense of differentiation and/or the capacity for secondary metabolism, and the predominant type may critically reflect the nature of the explant and the culture conditions.

General

Our results show that for *R. damascena* the choice of the explant as well as selection of the correct thermo-photoperiodicity was crucial for the establishment of cultures that exhibited production of 2-phenylethanol. Presumably the axillary leaf buds must sometimes happen to be in the correct physiological state to trigger the formation of the necessary type of callus and it may be significant that regenerating buds on conventional or on submerged callus of various herbaceous species actively synthesized and accumulated essential oils [38, 39]. The low proportion of lines from the favoured source that developed the ability to accumulate 2-phenylethanol is unexpected, but it has been reported that only a limited number from apparently identical lines of cultures were active in the production of diterpenes [17] and anthocyanins [30]. Serial culture from the productive lines did not lead to an improved yield of 2-phenylethanol and some type of regulation seems to limit the accumulation of the alcohol under the particular sets of conditions. Similar results have been reported for anthocyanin-producing cultures, and if general such regulation will limit the use of selection methods to develop effective culture lines [cf. 30].

The non-productive calli of *R. damascena* nevertheless contained the enzyme systems necessary for the conversion of L-phenylalanine into 2-phenylethanol (Table 3), and a similar situation has been found for enzymes of the terpenoid pathway in non-producing cultures of *R. damascena* [5] and several other species [37]. This contradicts the view that the lack of secondary metabolism (or of a particular secondary metabolite) in culture is due to the lack of one or more of the enzymic activities necessary for the sequence [40]. The apparent ineffectiveness of the enzyme systems may result from their inhibition or compartmentation within the potentially productive cells. Another possibility is that such cells produced 2-phenylethanol but the nascent metabolite was degraded *in situ* by oxidative systems such as have been demonstrated for the breakdown of terpenoids in *R. damascena* and *Jasminum* cultures [5, 41]. Such degradation would be essential in the absence of compartmented storage (trichomes, vacuoles), detoxification via derivative formation (to glucosides?) or secretion into a two-phase medium [cf. 42, 43] in order to avoid autonecrosis. This is because 2-phenylethanol, in common with certain monoterpenes [44], is toxic to cultured cells at the levels likely to be accumulated *in vitro*: thus we have demonstrated that this additive (2 mM; 0.2 g/l) virtually destroyed (>96% mortality; 30 hr incubation) callus and suspensions of *R. damascena*. More so than for suspension cultures, callus probably contains cell types of different stages of development, differentiation and viability, and selection during the long period of incubation and subsequent slow growth could favour cell lines possessing either detoxification mechanisms or storage facilities for 2-phenylethanol: the latter cell types being present in our productive callus lines.

Our best productive callus lines gave yields of 2-phenylethanol (% wt/fresh wt) greater than most yields recorded for the formation of secondary metabolites in culture [7]. The optimum values were ca 15% of the levels of 2-phenylethanol in petals, and this is the more impressive as leaf and stem tissue of the parent contained negligible amounts of 2-phenylethanol, and comparison of the biosynthetic ability of an entire callus with that of a specialized synthetic/storage organ presumably undervalues the intrinsic capacity of the active cells of the former [cf. 45]. Some workers have discerned a correlation between secondary metabolism and differentiation in tissue cultures [46], but recently it was found that neither organogenesis nor the formation of specialised structures were a prerequisite for terpenoid formation in *Pelargonium* cultures [11]. Our productive callus lines also possessed no specific storage structures such as trichomes or modified epithelial cells as are utilized in petals. UV-fluorescence microscopy, and staining indicated that 2-phenylethanol appeared to be stored in *ca* 12% of the cell population in globules attached to the vacuolar membrane: cf. the situation for the storage of alkaloids in culture [47]. But otherwise the accumulating cells appeared to be of the parenchymous-type typical of all the calli. No obvious differentiation–save in the general but perhaps fundamental sense of a lack of meristematic activity [cf. 46] appeared present. A histological and electron-microscopical study of these cultures will be published elsewhere.

Only one previous report exists of the accumulation of essential oil by callus of *R. damascena* [9]: it was recorded that oil was found at significant levels in the leaf and stem (as well as petal) of the parent; that callus derived from a variety of organs produced 2-phenylethanol and monoterpenes in levels similar to those in explants; that such secondary metabolism was optimal at high growth rates; that maximum yields of 2-phenylethanol occurred well into the stationary phases of the growth cycles; and, most surprisingly, that high levels of 2,4-D did not reduce or abolish the yields of oil from any of the lines studied. All this is completely at variance with our experience. We have been unable to obtain the Russian cultivar used in the previous work, but we have carried out brief studies of callus derived from petal and leaf of the *R. damascena* cultivars Gloire de Guilan, and Versicolor and in no example (with or without 2,4-D in the medium) was detectable amounts of oil present. We cannot account for the discrepancies between our present study and the previous work.

EXPERIMENTAL

Materials. Potted specimens (3 to 5-yr-old) of *R. damascena* cv trigintipetala were obtained from the Royal Botanic Gardens, Kew; the UCL Botanic Garden, and Hillier and Co. (Winchester, Hants). In addition, callus lines from stem and petals (established 1–2 yrs) were donated by Dr N. Robinson (Leicester University). Oil was obtained by solvent extraction (*not* steam distillation: 2-phenylethanol is not appreciably steam volatile [1]) and β-glucosides were cleaved by acid [3] or enzymic [48] treatment using protease from *Helix pomata* (a potent source of β-glucosidases; ex Sigma Chem. Co.) with controls for incomplete cleavage in the latter assay.

Callus and suspension cultures. Explants of the appropriate organ or tissue were surface sterilized (3% NaOCl aq; 3 mins), well-washed (×4) with H_2O and cut into sections (*ca* 1.5 × 0.1 × 0.1 cm.) which were divided longitudinally and placed with the cut face in contact with the agar medium. MS medium (or the

other media listed in Table 2) were made-up with Oxoid agar No. 2 (0.7 to 1.0. w/w: the concentration being adjusted batchwise to ensure bedding-down of the callus). For initiation, coconut milk (10% v/v) was added as a supplement but this was weaned at the first sub-culture. After initiation at 27° and 400 lux (for periods etc. see Discussion) the first sub-culture was carried out after *ca* 5-fold increase in mass, and thence explants (0.25–0.5 g) were subcultured at the period of the growth cycle where decelerated growth heralded entry to the stationary phase (at mass 10–15 g). Regimes (i–iii), see Discussion, were employed and illumination was provided by either Thorn 'Growlux' (λ_{max} 660 nm) or (slightly less efficaciously) 'White' (λ_{max} 580 nm) fluorescent tubes. Suspension cultures were prepared from established callus on MS liquid medium at 120 rpm under regimes (i–iii) but with the upper light intensities set at 600 lux. Subculture involved inocula (10 ml) to new medium (60 ml) when densities of *ca* 10^5 cells/ml were achieved. Glutamate (1 g/l) provided a useful additive to the medium and reduced passage times by *ca* 30%. Typically, a large increase in biomass (from 0.5 to 40 g) occurred over 14–30 days.

Assay for 2-phenylethanol. Culture material (*ca* 30 g) was ground-up in liq. N_2 and extracted (Soxhlet, 3 hr) with Et_2O (150 ml), and the extract was dried (Na_2SO_4) and concentrated (to 1–2 ml) in a stream of N_2 at 0°. The plant debris was either added to 0.1 M acetate buffer (pH 5.1, 25 ml) and incubated with protease (ex *H. pomata*) and β-glucosidase (ex almonds; Sigma Chem. Co) for 3 hr at 40° or was treated with 1 M HCl (20 ml 60° for 2 hr): in each case the aliquot was extracted with n-C_6H_{14} (3 × 20 ml) to yield any 2-phenylethanol that had been bonded as β-D-glucoside (checks showed that no α-D-glucoside was present). 1-Phenylethanol (10 mg) was added as an internal standard to each sample to allow compensation for losses in extraction, concentration etc.: these losses generally were < 20% of the total alcohol present. Media to be analysed (for secreted metabolite) were liquified by addition of H_2O (*ca* 4 vols) and warmed in a sealed flask (80°/10 min), cooled to 2°, and extracted with n-C_6H_{14}: the aqueous residue was then treated to cleave glucoside as above. The concentrated extracts from the above procedures were assayed by GC (10% FFAP or 20% Carbowax 20 M on Chromosorb W, 60–80 mesh, acid washed) on a column (3.5 m × 4 mm; F.I.D., 3.8 l/hr (N_2), 90–135° programme) either alone or linked (GC/MS) to a Kratos MS-2S mass spectrometer (source 200°; interface 230°; 70 eV) with data accumulation on a Kratos 65–505 system. Once thus characterized, 2-phenylethanol was routinely separated and assayed by TLC on silica gel developed with Et_2O (R_f 0.72), followed by EtOAc–C_6H_6 (3:1, R_f 0.41). Chromatograms were sprayed with phosphomolybdic acid (10% aq.) and developed by heating (120°, 2 min).

Preparation of cell-free extracts. Parent plant material (for comparison purposes) was young leaves and axillary leaf buds (from mature potted specimens), and flower buds in the pre-perfect stage (8 days after initial splitting of calyx: the stage for optimum accumulation of oil, cf. [49, 50]). Callus, cell suspension and parent tissue (10–15 g) was washed with EDTA (1% aq.), well rinsed, and vacuum-infiltrated with 0.1 M Pi buffer (pH 7.0 30 ml) containing sodium diethylthiocarbamate (1 mM) and 2-mercaptoethanol (1 mM): this and all subsequent steps were carried out at 4°. The tissue was then pulverized in liq. N_2 and stirred over 10 mins into the extraction buffer comprising 0.1 M MES (pH 7.0, 30 ml) containing 0.3 M sucrose, 0.1 M ascorbate and a suspension of insoluble PVP (insoluble Polyclar-AT; ex. GAF Ltd., Manchester). The last component is essential to inhibit phenol oxidases cf. [51]. The slurry was allowed to thaw (to 4°; *ca* 45 mins), filtered through glass wool that had been well washed with aq. EDTA, and centrifuged (10^4 g; 20 min.). For preparations involving petal tissue, the endogenous monoterpenes and 2-phenylethanol were removed at this stage by stirring in Amberlite XAD-7 resin (10 g) [37]. The S_{10} supernatant was desalted (Biogel P-2 column; 2 × 30 cm) with elution (30 ml/hr) with MES (0.01 M; pH 7.0) containing 2-mercaptoethanol (1 mM). The forerun (*ca* 16 ml; 0.2 to 0.4 mg protein/ml, by Potty's assay method [52]) was collected. This soln (1 ml) in an incubation buffer comprised of 0.01 M Tris or MES (pH 8.0, 2 ml) containing 2-mercaptoethanol (1 mM), $MgSO_4$ (40 mM), ATP (2 mM) pyridoxal phosphate, α-oxoglutarate, pyruvate (all 1 mM) and NAD^+, $NADP^+$, NADH, NADPH and lipoic acid (all 5 mM) was incubated in sealed tubes with L-[U-^{14}C] phenylalanine (1.0 μCi, 10 mCi/mmol) at 27° to the plateau regions (3 hr). This exotic but not optimized cocktail was chosen in the hope of satisfying the (unknown) cofactor requirements of the several enzymic steps between L-phenylalanine and 2-phenylethanol. Four replicate assays and one boiled-enzyme control were carried out for each extract. After the incubation period the solution was cooled (to *ca* 0°) and extracted with Et_2O (2 × 2 ml). The extract was dried ($MgSO_4$), concentrated and analysed by TLC as in the preceding section to isolate and characterize (if need be). 2-phenylethanol. These simple assay methods gave results identical within the experimental error (± 5%) with those achieved by the rigorous but laborious technique of isotope dilution analysis after addition of 2-phenylethanol carrier (500 mg) and purification of the extracted alcohol as its phenylurethane (mp 80°) to constant sp. radioactivity by repeated recrystallization (ex EtOH: Me_2CO). When other metabolites of L-phenylalanine were to be assayed the initial TLC separation on silica gel H (with Et_2O) yielded cinnamate and phenylacetate (R_f 0.33) and phenylpyruvate (R_f 0.12). The first band was further separated on cellulose (Merck) with Et_2CO–Et_2NH–H_2O (115:1:9) to give cinnamate (R_f 0.29), phenylacetate (R_f 0.20) and any contaminating phenylpyruvate (R_f 0.57).

Miscellaneous. (i) Chromosome numbers were determined in the latter half of the exponential phase of growth: mitotic arrests were achieved by use of colchicine or 8-hydroxyguinoline. (ii) Quantification of tracheid formation was obtained by examination of plastic-embedded sections stained with Toluidine Blue. The total cell population was measured in at least 4 sections from each of 2 calli from a particular cell line. (iii) Cell viability was conventionally determined [53]. (iv) Total soluble phenols were analysed by extraction with MeOH and visualization with Folin–Ciocalteau reagent [20]. (v) *de Novo* synthesis in calli was proven by injection with sterile (via Millepore filter, 0.22 μ pore size) solns of L-[U-^{14}C] phenylalanine (20 μCi, 59 mCi/mmol) and assay after a 3–10 day metabolism period. (vi) Chlorophyll was extracted and assayed by measurement at λ 645 and 663 nm [54]. (vii) Radiochemical assays used LSC with Butyl-PBD (0.8% v/v) in deoxygenated PhMe as scintillant (counting efficiency, 90–5%). 4×10^4 Disintegrations were accumulated so that 2σ was ± 1%.

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REFERENCES

1. Guenther, E. (1952) *The Essential Oils* Vol. 5, p. 3. Van Nostrand, New York.
2. Gildermeister, E. and Hoffmann, F. (1959) *Die Ätherischen Öle* vol. 5, p. 228. Akademie, Berlin.
3. Bugorskii, P. S. and Zaprometov, M. N. (1978) *Biokhimiya* **43**, 2038.
4. Banthorpe, D. V. and Barrow, S. E. (1983) *Phytochemistry* **22**, 2727.

5. Banthorpe, D. V., Grey, T. J., Poots, I. and Fordham, W. D. (1986) *Phytochemistry* **25**, 2321.
6. Jones, L. H. (1973) in *Industrial Aspects of Biochemistry* (Spencer, B., ed.), p. 813. FEBS, London.
7. Charlwood, B. V., Hegarty, P. K. and Charlwood, K. A. (1986) in *Secondary Metabolism in Plant Cell Cultures* (Morris, P., *et al.* eds), p. 15. Cambridge University Press, Cambridge.
8. Butcher, D. N. (1977) in *Applied and Theoretical Aspects of Plant, Cell, Tissue and Organ Cultures* (Rheinhart, J. and Bajaj, Y. P. S., eds), p. 668. Springer, Berlin.
9. Kireeva, S. A., Bugorskii, P. S. and Reznikova, S. A. (1977) *Fiziol. Rast.* **24**, 824.
10. Lichtenthaler, H. K., Straub, V. and Grumbach, K. H. (1975) *Plant Sci. Letters* **4**, 61.
11. Brown, J. T. and Charlwood, B. V. (1986) *FEBS Letters* **204**, 117.
12. Sairam, V. and Khanna, P. (1971) *Lloydia* **34**, 170.
13. Dudley, K. and Northcote, D. H. (1979) *Planta* **146**, 433.
14. Ozeki, Y. and Komamine, A. (1985) *Plant Cell Physiol.* **26**, 903.
15. Zenk, M. H., El-Shagi, H. and Schulte, U. (1975) *Planta Med. Suppl.* 79.
16. Knoblich, K. H. and Berlin, J. (1980) *Z. Naturforsch.* **35**, 551.
17. Miyasaka, H., Nasu, M., Yamato, T. and Yoneda, K. (1985) *Phytochemistry* **24**, 1931.
18. Kutney, J. P. and 12 others, (1983) *Planta Med.* **48**, 158.
19. Masumoto, T., Nishida, K., Naguchi, M. and Tamani, E. (1973) *Agric. Biol. Chem.* **37**, 561.
20. Lam, T. H. and Street, H. E. (1977) *Z. Pflanzenphysiol.* **84**, 121.
21. Davies, M. E. (1972) *Planta* **104**, 50.
22. Gilmore, A. L. (1985) *J. Chem. Ecol.* **11**, 583.
23. Harborne, J. B. (1982) *Introduction to Ecological Chemistry* 2nd Edn, p. 218. Academic, London.
24. Danks, M. L., Fletcher, J. S. and Rice, E. L. (1975) *Am. J. Botany* **62**, 311.
25. Harborne, J. B. (1980) in *Secondary Plant Products* (Bell, E. A. and Charlwood, B. V. eds), p. 376. Springer, Berlin.
26. Bolwell, G. P., Cramer, C. L., Lamb, C. J., Schuch, V. and Dixon, R. A. (1986) *Planta* **169**, 97.
27. Loomis, W. D. and Croteau, R. (1973) *Rec. Adv. Phytochem.* **6**, 147.
28. Luckner, M. (1980) in *Secondary Plant Products* (Bell, E. A. and Charlwood, B. V. eds), p. 23. Springer, Berlin.
29. Yeoman, M. M., Lindsey, K., Miedzybrodzka, M. B. and McLauchlan, W. R. (1982) in *Brit. Soc. Cell Biol. Sym. No.* 4, Differentiation in Vitro, (Yeoman, M. M. and Truman, D. E. S. eds), p. 65. Cambridge University Press, Cambridge.
30. Hall, R. D. and Yeoman, M. M. (1986) *J. Exp. Botany* **37**, 48.
31. Margna, V. (1977) *Phytochemistry* **16**, 419.
32. Nash, D. T. and Davies, M. E. (1972) *J. Exp. Botany* **23**, 75.
33. Hahlbrock, K. and Wellmann, E. (1970) *Planta* **94**, 236.
34. Fritsch, H. Hahlbrock, K. and Grisebach, H. (1971) *Z. Naturforsch.* **26B**, 581.
35. Grisebach, H. and Hahlbrock, K. (1974) *Biochim. Biophys. Acta* **362**, 417.
36. Berlin, J., Knobloch, K. H., Höfle, G. and Witte, L. (1982) *J. Nat. Prod.* **45**, 83.
37. Banthorpe, D. V., Branch, S. A., Njar, V. C. O., Osborne, M. G. and Watson, D. G. (1986) *Phytochemistry* **25**, 629.
38. Webb, J. K., Banthorpe, D. V. and Watson, D. G. (1984) *Phytochemistry* **23**, 903.
39. Brown, J. T. and Charlwood, B. V. (1986) *J. Plant Physiol.* **123**, 409.
40. Böhm, H. (1982) in *Plant Tissue Culture* (Fujiwora, A. ed.) p. 327. Japanese Assoc. for Plant Tissue Culture, Tokyo.
41. Banthorpe, D. V. and Osborne, M. G. (1984) *Phytochemistry* **23**, 905.
42. Berlin, J., Witte, L., Schubert, W. and Wray, V. (1984) *Phytochemistry* **23**, 1277.
43. Forche, E., Schubert, W., Kohl, W. and Holfe, G. (1984) *Proc. Third Eur. Cong. Biotechnol.* Munich, Vol. 1, p. 189.
44. Brown, J. T., Hegarty, P. K. and Charlwood, B. V. (1987) *Plant Sci.* **48**, 195.
45. Fowler, M. (1985) *Bioessays* **3**, 172.
46. Brown, J. T. and Charlwood, B. V. (1986) in *Secondary Metabolism in Plant Cell Cultures* (Morris, P. ed.), p. 68. Cambridge University Press, Cambridge.
47. Deus-Neumann, B. and Zenk, M. H. (1986) *Planta* **167**, 44.
48. Bugorskii, B. S., Reznikova, S. A. and Zaprometov, M. N. (1976) *Biokhimiya* **44**, 1068.
49. Francis, M. J. O. and Allcock, C. (1969) *Phytochemistry* **8**, 1339.
50. Francis, M. J. O. and O'Connell, M. (1969) *Phytochemistry* **8**, 1705.
51. Loomis, W. D. (1974) *Methods Enzymol.* **31**, 528.
52. Potty, V. H. (1969) *Analyt. Biochem.* **29**, 535.
53. Widholm, J. H. (1972) *Stain Technol.* **47**, 189.
54. Sunderland, N. (1966) *Ann. Botany* **30**, 253.
55. Murashige, T. and Skoog, F. (1962) *Physiol. Plant* **15**, 473.
56. White, P. R. (1963) *Cultivation of Animal and Plant Cells* 2nd Edn, p. 246. Ronald, New York.
57. Gambourg, O. L., Murashige, T., Thorpe, T. A. and Vasil, I. K. (1976) *In Vitro* **12**, 473.
58. Becker, H. (1970) *Biochem. Biophys. Pfanzen* **161**, 425.