Elicitor-Enhanced Callus Culture of Catharanthus roseus: A Short Review

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***Abstract*—Catharanthus roseus (L.) G. Don is the only practical source of the anticancer dimers vinblastine and vincristine, yet these terpenoid indole alkaloids (TIAs) accumulate in the intact plant in trace amounts, and chemical or microbial synthesis is still complex and costly. Callus and cell culture offer a controllable, season-independent platform, but unmodified callus usually lacks the differentiated tissues needed for the late steps of the pathway. Elicitors, which are biotic or abiotic signals that switch on plant defence metabolism, can raise the yield of selected alkaloids and phenolics in culture. This short review brings together evidence on TIA biosynthesis and its regulation, callus induction on Murashige and Skoog medium, elicitation with methyl jasmonate, salicylic acid, fungal and chitosan-type elicitors, calcium and UV-B, the biochemical gap between callus and the mother plant, antioxidant and phenolic assays (DPPH, FRAP, Folin–Ciocalteu), chromatographic quantification, and scale-up and genetic-stability issues. Elicitation reliably raises ajmalicine, catharanthine and phenolic content in cultured cells, and in some systems vindoline, but the dimeric alkaloids have not been shown consistently in elicited callus, and direct callus-versus-mother-plant comparisons remain scarce. A comparative design with unelicited callus, five elicitor treatments and mother-plant tissue, assessed by HPLC/HPTLC and antioxidant assays, is outlined to close this gap.**

***Index Terms*—Catharanthus roseus; callus culture; elicitation; methyl jasmonate; salicylic acid; terpenoid indole alkaloids; antioxidant activity**

I. INTRODUCTION

Plants remain an irreplaceable source of anticancer drugs, and Catharanthus roseus (Madagascar periwinkle) is a textbook example. The plant makes more than 130 terpenoid indole alkaloids, among them the antitumour dimers vinblastine and vincristine, and the tiny quantities in which these accumulate make extraction expensive [1]. Total synthesis is not commercially practical either; even the most advanced alternative, an engineered yeast that makes the precursors vindoline and catharanthine de novo before chemical coupling to vinblastine, required 56 genetic edits including 34 plant-derived genes [2]. Plant cell and tissue culture therefore stays an attractive, controllable and renewable option.

Callus, an undifferentiated mass of cells grown on defined medium, is the simplest culture system. Its weakness is that unelicited callus often makes less of the target metabolites than the plant it came from. Elicitation addresses that deficit. Elicitors are classed as abiotic or biotic, with examples that include yeast extract, methyl jasmonate, salicylic acid and chitosan, and elicitation is regarded as an effective and sustainable strategy for plant cell factories [3]. Reviews of C. roseus biotechnology list culture conditions, precursor feeding, elicitation and metabolic engineering as the main levers [1].

What is missing is a single comparative framework that places elicited callus, unelicited callus and the intact mother plant side by side for both alkaloid content and antioxidant capacity. This review collates the relevant literature, highlights where evidence is strong or thin, and outlines such a framework. Throughout, claims rest on abstract-level reading of the cited sources, and numerical results are quoted only where the abstract reports them.

II. TERPENOID INDOLE ALKALOID BIOSYNTHESIS AND ITS REGULATION

TIA biosynthesis couples the iridoid (secologanin) branch with the indole branch (tryptamine) at strictosidine, from which several alkaloid classes diverge. Recent reviews add newly identified iridoid and monoterpenoid indole alkaloid steps and discuss how production differs between whole plants and cell or organ cultures [4]. More than 100 TIAs are known, transcription factors activate or repress pathway genes, transporters have been identified, and the networks that control cell-specific biosynthesis, transport and storage remain largely unresolved.

Jasmonate signalling is the best-understood regulatory input. In a bHLH factor network, CrMYC2 regulates the indole branch and BIS1 and BIS2 the iridoid branch, but overexpressing either factor alone did not raise alkaloid levels; JAZ proteins repress CrMYC2 and BIS1 until jasmonate is perceived, and a repressor, RMT1, was also identified [5]. The AP2/ERF factor ORCA2 altered the first two pathway steps and four of five characterised downstream genes in hairy roots, but it also induced repressor genes, which may explain why metabolite increases are often transient and limited [6]. These observations help to explain why methyl jasmonate is the commonest abiotic elicitor and why a single treatment seldom gives unlimited gains.

Environment matters as well. Light, temperature, drought, salinity, nutrients, pathogens and insects all change TIA accumulation by acting on regulatory and enzyme genes, although these factors have been studied far less than jasmonate [7]. A second constraint is anatomical. The final acetylation step of vindoline synthesis is catalysed by an enzyme found only in specialised idioblast and laticifer cells of light-exposed tissues, so undifferentiated cells may simply lack the machinery for late pathway steps [8]. This point returns in Section 5.

III. CALLUS INDUCTION AND CULTURE ESTABLISHMENT

Callus is raised from sterilised explants on Murashige and Skoog (MS) basal medium [9] supplemented with an auxin and a cytokinin. The choice of explant and plant growth regulators shapes both callus mass and metabolite output (Table 1). With basal leaf explants carrying petioles on MS medium containing NAA or 2,4-D together with BAP or kinetin, the best combination was 3.0 mg/L BAP with 3.0 mg/L NAA, which gave the highest callus dry weight of 2.776 g [10]. In another comparison of root, hypocotyl and leaf explants, hypocotyl with 1 mg/L NAA plus 1 mg/L BAP was best for callus formation, and hypocotyl with 2,4-D gave the best somatic embryos [11]. The plant growth regulator may also steer alkaloid output: in callus raised with picloram, vindoline was the only targeted alkaloid detected (11.25–30.44 mg/kg), whereas 2,4-D and NAA gave only trace catharanthine, and the authors noted that this shift still needs experimental validation [12].

Table 1. Selected reports on callus induction and metabolite output in Catharanthus roseus

Explant / system	Medium and regulators	Key reported findings	Ref.
Basal leaf with petiole	MS; NAA or 2,4-D with BAP or kinetin	Best: 3.0 mg/L BAP + 3.0 mg/L NAA; callus dry weight 2.776 g	[10]
Root, hypocotyl, leaf	MS; 2,4-D, NAA, 2,4-D × BAP, NAA + BAP	Hypocotyl with NAA + BAP best for callus; hypocotyl with 2,4-D best for somatic embryos	[11]
Callus (picloram vs 2,4-D/NAA)	Picloram with BAP; 2,4-D; NAA	Picloram: only vindoline detected (11.25–30.44 mg/kg); 2,4-D/NAA: trace catharanthine (about 1 mg/kg)	[12]
Callus-derived suspension (PEG-stressed)	Osmotic stress applied to callus lines	Only ajmalicine detected (1.31 vs 0.41 µg/mL, stressed vs not); no vinblastine, vincristine or vindoline	[13]
Callus, SA or MJ at 0–200 µM	Elicitor for 5 or 10 days	Highest phenolics 55.1 mg/g FW (100 µM MJ, 5 d); highest flavonoids 21.5 mg/g FW (50 µM SA, 5 d); alkaloids not measured	[14]
Regenerated plantlets (reference)	Shoot tips on Nitsch and Nitsch medium	Leaf vindoline and catharanthine dominant; total alkaloids up to 2.95% (gravimetric)	[15]

Maintenance matters as much as induction. Callus is subculture at regular intervals, and the age of the culture, medium and cell line all influence elicitor response [3]. Because culture history can change genetic and epigenetic status, a callus line should be treated as a documented biological material, not a generic reagent (Section 8).

IV. ELICITATION OF CATHARANTHUS ROSEUS CULTURES

4.1 Concept and signalling

An elicitor is a signal that the cell interprets as stress or attack, triggering defence metabolism. Plant cell culture literature groups elicitors as biotic (yeast or fungal extracts, polysaccharides such as chitosan) and abiotic (jasmonates, salicylic acid, calcium, UV light, nitric oxide donors), and shows that outcome depends on elicitor type, dose, timing, cell line and culture age [3]. Signalling studies give a consistent outline, summarised in Figure 1B. Low-dose UV-B in C. roseus suspension cultures involved cell-surface receptors, Ca^{2+} influx, medium alkalinisation, calcium-dependent protein kinase, hydrogen peroxide and MAPK, and blocking these steps reduced catharanthine and transcription of Tdc and Str three- to four-fold [16]. In Hypericum perforatum cells, fungal-elicited hypericin required both nitric oxide and jasmonic acid, with jasmonic acid acting downstream of nitric oxide [17]. These findings justify testing combinations of elicitors and signalling modulators, but they also show that effects are species- and context-specific.

4.2 Evidence in C. roseus systems

Methyl jasmonate (MeJA) is the benchmark. In cell suspensions it roughly tripled ajmalicine, yet induced cultures remained limited by terpenoid precursors; geraniol feeding raised ajmalicine in non-induced cultures, and MeJA appeared to raise geraniol availability, possibly through increased Dxs expression [18]. An earlier study had already shown that feeding secologanin, loganin or loganic acid raised ajmalicine and strictosidine in unelicited cells and that elicitation added nothing in precursor-fed cells, so precursor supply is a real ceiling [19]. Fungal elicitors also work: an Aspergillus niger mycelium elicitor combined with tetramethyl ammonium bromide gave 48 mg/L ajmalicine in 500-mL flasks, 52 mg/L in 1000-mL flasks and 33 mg/L in a 20-L airlift bioreactor, with raised malondialdehyde indicating lipid peroxidation [20]. UV-B for five minutes on stationary-phase suspensions raised catharanthine about three-fold and vindoline about twelve-fold [21].

Differentiated or semi-differentiated systems respond differently from callus. In hairy roots, elicitors had a limited overall effect, but Aspergillus and macerozyme selectively increased ajmalicine and MeJA increased both ajmalicine and catharanthine [22]. In cambial meristematic cell (CMC) cultures, β-cyclodextrin with MeJA in a 5-L airlift bioreactor gave 7.45 mg/L vindoline, 1.76 mg/L catharanthine and 58.98 mg/L ajmalicine, higher than flask yields [23], and 25 mg/L of an Aspergillus flavus mycelium elicitor for 48 h raised vindoline about 1.45-fold and catharanthine about 3.29-fold [24]. On the whole plant, spraying leaves with chitooligosaccharides raised vindoline by about 61% and catharanthine by about 142% [25]. Table 2 collates these studies.

Table 2. Elicitor studies in Catharanthus roseus cell, root and plant systems

System	Elicitor	Key reported findings	Ref.
Cell suspension	Methyl jasmonate	About three-fold ajmalicine; precursor (geraniol) supply then limiting	[18]
Cell suspension	A. niger mycelium + tetramethyl ammonium bromide	Ajmalicine 48–52 mg/L in flasks; 33 mg/L in 20-L airlift bioreactor	[20]
Cell suspension (stationary phase)	UV-B, 5 min	Catharanthine about 3-fold and vindoline about 12-fold higher	[21]
Hairy roots	Aspergillus, macerozyme, MeJA	Elicitors selective: ajmalicine up with fungal agents; both alkaloids up with MeJA	[22]

System	Elicitor	Key reported findings	Ref.
Cambial meristematic cells	β-Cyclodextrin + MeJA	Bioreactor: vindoline 7.45, catharanthine 1.76, ajmalicine 58.98 mg/L	[23]
Cambial meristematic cells	A. flavus mycelium, 25 mg/L, 48 h	Vindoline about 1.45-fold, catharanthine about 3.29-fold, ajmaline about 2.14-fold	[24]
Whole plant leaves	Chitooligosaccharides, 0.1 µg/mL	Vindoline about +61%, catharanthine about +142%; eight alkaloid genes up	[25]

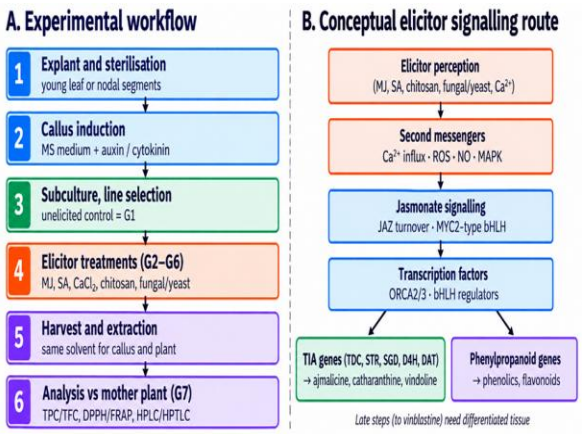


Figure 1. (A) Proposed experimental workflow for elicitor-enhanced callus culture and comparison with the mother plant. (B) Conceptual signalling route from elicitor perception to alkaloid and phenolic accumulation, assembled from the studies cited in Section 4.1 [5,6,16,17]. Schematic only; not to scale.

4.3 Phenolic and antioxidant responses in callus

Callus also answers elicitation with phenolics. In C. roseus callus treated with 0–200 µM salicylic acid or MeJA for five or ten days, the highest phenolic content (55.1 mg/g fresh weight) occurred with 100 µM MeJA at five days and the highest flavonoid content (21.5 mg/g) with 50 µM salicylic acid at five days, although alkaloids were not measured [14]. Results from other species show the same pattern with agent-specific detail. In cassava callus salicylic acid was best for growth, glutathione gave the highest phenol and flavonoid levels and MeJA the strongest DPPH scavenging [26]. In Teucrium polium callus 50 µM MeJA gave the highest phenolics, flavonoids and rosmarinic acid, with a DPPH IC_{50} of 2.066 ± 0.24 µg/mL and FRAP of 383.79 ± 33.61 mg Fe/100 g dry weight [27]. These cross-species data are suggestive only, since optimal elicitor, dose and time are not transferable between species.

V. CALLUS VERSUS THE MOTHER PLANT

The central question is whether elicited callus can match the intact plant. The available comparisons suggest that it often cannot, at least for the late-pathway alkaloids. Leaves of regenerated juvenile plants were dominated by vindoline and catharanthine, with total alkaloids reaching 2.95% by gravimetry [15], and a validated HPLC method found vindoline, vincristine, catharanthine and vinblastine in methanolic leaf extract, with flowers rich in vinblastine [28]. By contrast, suspensions from osmotically stressed callus contained only ajmalicine, and vinblastine, vincristine and vindoline were not detected [13]; in callus raised with picloram only vindoline was found [12].

The most direct antioxidant and phytochemical comparison comes from a study of callus, a somaclonal variant and normal green shoots. Green shoots had the highest phenolic and flavonoid contents and the strongest radical scavenging and reducing power, LC-MS/MS identified 39 compounds in callus, 55 in the variant and 59 in shoots, and callus showed the strongest acetylcholinesterase inhibition (IC_{50} 0.65 mg/mL) [29]. In a related study, rolB-transformed hairy roots, not callus, gave the highest phenolics and the lowest DPPH IC_{50} among the tissues compared [30]. Differentiation again appears to matter: cambial meristematic cells held more total alkaloids and more vindoline, vinblastine, catharanthine and ajmalicine than dedifferentiated cells, with about 9,500 differentially expressed genes including the alkaloid pathway [31].

Figure 2 turns these scattered observations into an evidence map. It indicates, for each tissue type,

whether a compound class was reported as detected, variable or trace, or not detected in the cited studies. The map is not a quantitative comparison; it shows that dimeric alkaloids have not been demonstrated consistently outside differentiated tissue.

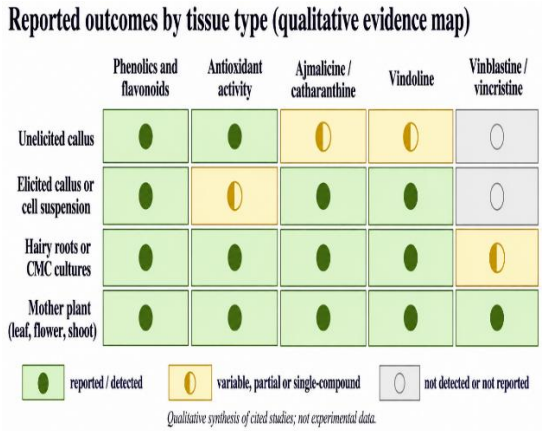


Figure 2. Evidence map of reported alkaloid and phenolic outcomes by tissue type, compiled from [12–15,21–23,28–31]. Symbols reflect abstract-level findings, not a quantitative comparison.



VI. ANTIOXIDANT AND PHENOLIC ASSAYS

Antioxidant capacity is not a single property, and no single assay captures it. Reviews classify methods as hydrogen-atom transfer assays and electron-transfer assays; DPPH, FRAP, TEAC and the Folin–Ciocalteu reaction belong to the latter group [32,33]. The FRAP assay measures the reduction of a ferric tripyridyltriazine complex to the ferrous form at low pH, read at 593 nm, and showed within-run coefficients of variation under 1% [34]. The Folin–Ciocalteu microplate protocol reads absorbance at 765 nm and its authors note that the reaction is not specific to phenolics [35], and total flavonoids are commonly estimated by aluminium chloride colorimetry [36]. The classical Folin–Ciocalteu method and an IUPAC technical report on antioxidant methods provide fuller guidance [37,38].

Table 3. Assays proposed for comparing elicited callus, unelicited callus and mother-plant tissue

Assay	Principle	Caution	Ref.
DPPH scavenging	Electron/hydrogen transfer to a stable radical; absorbance loss	Single-mechanism; results depend on solvent and time; report IC_{50}	[32,33]
FRAP	Fe^{3+}–TPTZ reduced to Fe^{2+} at low pH; read at 593 nm	Measures reducing power only; not radical scavenging	[34]
Total phenolics (Folin–Ciocalteu)	Reduction of the reagent; read at 765 nm in microplate format	Not specific to phenolics; other reducing agents interfere	[35,37]
Total flavonoids ($AlCl_3$)	Aluminium complex of flavonoids; colorimetric	Method-dependent; report standard used	[36]
Alkaloids by HPLC	RP-HPLC at 297 nm for vindoline, vincristine, catharanthine, vinblastine; RSD below 2.68%	Needs reference standards and low-level detection for dimers	[28]
Alkaloids by HPTLC	Silica gel 60 F254, toluene–methanol–diethylamine; scanned at 250 nm	Validated for vincristine only (Rf 0.76 flower, 0.80 leaf)	[39]

Several methods are therefore needed, because a callus extract may score well in one assay and poorly in another. Callus extract complexity also differs from leaf extract complexity. Methanolic leaf extract of C. roseus showed only mild antioxidant activity in one profiling study, in which 34 metabolites were annotated, 23 of them indole alkaloids [40]. In a comparison of plant parts the mixed-solvent extract

was richer in catechin hydrate, rutin hydrate, trans-ferulic acid and vanillic acid, whereas the ethanol extract had more epicatechin, kaempferol and quercetin, and both extracts were antioxidant [41]. Solvent polarity, extraction method and fraction choice thus affect assay outcomes.

VII. QUANTITATIVE ANALYSIS OF ALKALOIDS

HPLC is the most frequently used technique for C. roseus alkaloids, followed by spectrophotometry and UPLC-MS, according to a systematic review of analytical methods for Catharanthus and Rauvolfia alkaloids [42]. An isocratic RP-HPLC method at 297 nm with recoveries of about 98–108% quantified four alkaloids simultaneously and showed that water extracts held no significant levels of these markers, which argues for organic solvent extraction of callus [28]. An ICH-validated HPTLC method using silica gel and a toluene–methanol–diethylamine mobile phase has been reported for vincristine, but not for the complete alkaloid panel [39]. Dimeric alkaloids are expected at very low levels in callus, so detector sensitivity and standard availability will often decide whether a comparison is feasible at all; mass spectrometric confirmation is advisable where a peak is claimed. Group comparisons of replicated data are normally analysed by ANOVA followed by a multiple-range test; Duncan's test remains widely used in tissue culture papers [43].

VIII. SCALE-UP, VARIABILITY AND GENETIC STABILITY

A culture that works in a flask may not in a bioreactor. In one system, ajmalicine production seen in shake flasks did not occur in the bioreactor until the gas conditions were matched, and higher biomass density lowered the production rate [44]; another study found that moving from a flask to an aerated stirred bioreactor caused browning and lower ajmalicine, which recirculation of exhaust gas prevented [45]. Against that, the CMC study and the Aspergillus-elicited cultures show that airlift bioreactors can match or exceed flask yields [20,23].

The mother plant itself is variable. Across nine cultivars grown under vertical farming, total anticancer TIAs were 1.6–5.9 times higher in leaves than in flowers, and vinblastine and vincristine patterns were cultivar-dependent [46]. A mother-plant comparator should therefore be voucher-authenticated and sampled at a defined stage. Culture also changes cells. Stressful conditions, medium composition and explant type can cause genetic and epigenetic change, and genetic stability should be checked early [47]; a systematic review found that BAP and 2,4-D were the most common regulators in somaclonal variation studies and that its mechanisms remain largely unexplored [48].

IX. CRITICAL APPRAISAL

Four limitations deserve emphasis. First, most studies concern cell suspensions, CMCs or hairy roots, not callus on solid medium, so extrapolation to callus is approximate. Second, the best-documented gains are in ajmalicine and catharanthine, whereas dimer detection in unelicited cultures has been negative [12,13]. Third, studies seldom compare culture and mother plant in the same experiment, with the exception of Lee et al. [29]. Fourth, several of the reports cited here appeared in regional journals with limited peer-review visibility, so their numerical results should be read as indicative. Elicitor dose and timing also interact: the same agent that raises one alkaloid may suppress another, and effects are transient because the pathway induces its own repressors [6]. A reproducible protocol therefore needs a factorial design that treats elicitor, dose and harvest time as factors.

X. PROPOSED COMPARATIVE FRAMEWORK

The synopsis behind this review proposes a seven-group design. Group G1 is unelicited callus; G2 to G4 are abiotic treatments with methyl jasmonate, salicylic acid and calcium chloride; G5 and G6 are biotic treatments with chitosan and fungal or yeast extract; and G7 is mother-plant tissue. Nodal and leaf explants are cultured on MS medium with an optimised auxin–cytokinin combination [9,10], and elicitors are applied at doses informed by the literature [14,18]. All groups are extracted identically and analysed for total phenolics, total flavonoids and the alkaloid fraction by HPLC/HPTLC, with antioxidant activity by DPPH and FRAP [28,34–36]. Replicated data are analysed by ANOVA with a post-hoc test [43]. The key output is

the elicitor that most narrows the gap between callus and mother plant, with the caveat from Figure 2 that dimeric alkaloids may remain below the detection limit.

XI. CONCLUSION

Elicitation is an effective way to raise ajmalicine, catharanthine and phenolic content in C. roseus cultures, and in some systems vindoline, but whether elicited callus can replace the mother plant for the clinically important dimers is unproven. Success will depend on differentiation state, precursor supply, elicitor timing and verified analytical sensitivity. A comparative design that includes unelicited callus, multiple elicitors and the mother plant, measured by chromatography and several antioxidant assays, would produce the missing evidence and a reproducible protocol.

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