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Autoclaved L-Glutamine Enhances *Agrobacterium*-Mediated Genetic Transformation of Recalcitrant High-Polyphenol Plant Explants

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Abstract

Plant polyphenols act as major defense compounds and are known to interfere with Agrobacterium tumefaciens–mediated genetic transformation, particularly in recalcitrant plant species. The present study investigated the effect of leaf polyphenols and autoclaved L-glutamine on the growth of A. tumefaciens and evaluated their combined influence on bacterial proliferation. Leaf polyphenols extracted from different high-polyphenol plant species and tea cultivars significantly suppressed bacterial growth in a concentration-dependent manner. Un-autoclaved L-glutamine showed no stimulatory effect on A. tumefaciens growth. However, L-glutamine autoclaved for 40 minutes markedly enhanced bacterial growth compared to 20-minute autoclaving. When combined with leaf polyphenols, autoclaved L-glutamine significantly alleviated polyphenol-induced growth inhibition, resulting in a substantial fold increase in bacterial growth after 18 hours of incubation. Maximum stimulation was observed at intermediate phenol concentrations, beyond which no further enhancement occurred. The findings demonstrate that autoclaved L-glutamine effectively counteracts the inhibitory effects of leaf polyphenols and may serve as a useful additive to improve Agrobacterium performance during genetic transformation of recalcitrant, high-polyphenol plant species.

Keywords: *Agrobacterium tumefaciens, polyphenols, L-glutamine, genetic transformation, vir genes, PPO activity recalcitrant explants*

1. Introduction

Agrobacterium mediated genetic transformation is a commonly acceptable method employed for horizontal gene transfer as it provides several like lower degree of transgene rearrangement, cost effectiveness, and simplicity of usage (Hamilton *et al,* 1997). However, the *Agrobacterium* method of gene transfer is highly constrained by its limited range of specific hosts (Potrykus 1990). Thus, while most dicots are successfully transformed by *Agrobacterium*, monocots remained largely resistant until the use of Acetosyringone were adopted for Agrobacterium mediated genetic transformation. Furthermore, a limited host range specificity and the differential ability of *Agrobacterium* to infect cultivars or genotypes within a particular species was also reported (Hawes *et al*., 1989; Biao *et al*. 1998). The major reasons for this resistance were attributed to plants' natural defense mechanisms, including 'surface characteristics' and 'wound response' (Kumar *et al*., 2004).

Exudation of polyphenols and their oxidation is a most common practice of defence mechanism employed by plants to ward off pathogen and pest attacks. Polyphenols interfere with the biophysiochemical properties of invading pathogens (Haslam, 1989; Huang *et al*., 1992). Therefore, it was reported that even dicot explants are recalcitrant to Agrobacterium infection because of the presence of toxic amounts of plant polyphenols (Kumar *et al*., 2003; Sandal *et al*., 2006). Polyphenols constitute a large and heterogeneous group of compounds often present at high concentrations in leaves, bark, and fruits and plants of woody species (Kolodziej, *et al*., 2005). Such explants are considered as poor candidates when subjected to Agrobacterium mediated transformation. The polyphenols exudates from the explants were oxidised to quinone or quinic acid a brown colour compound by activity of PPO enzyme resides in the vacuole of the plants. This brown colour compound leads to the tissue necrosis and not only inhibit in vitro regeneration of the explants but render pathogens infection, including Agrobacterium and it became a major constraint to develop genetically modified plants in such species.

In 2007, Saini and her coworkers proposed a hypothesis that the recalcitrancy generated by toxic plant polyphenols can be alleviated by addition of 2-amino glutarimide a compound generated during the autoclaving of L-glutamine. 2-amino glutarimide have a quenching property that enables *Agrobacterium* to successfully infect otherwise recalcitrant and resistant explants of tea cultivars. This approach could potentially facilitate effective gene transfer in a broad range of polyphenol-rich, recalcitrant plant species. Therefore, it is important to investigate the proposed hypotheses using explants from multiple plant species characterized by high polyphenol content. The present study aimed to examine the association between polyphenol content and *Agrobacterium* infection, develop effective strategies to overcome the recalcitrance of polyphenol-rich explants to *Agrobacterium*-mediated genetic transformation, and elucidate the mechanisms underlying recalcitrance and transformation efficiency in explants containing elevated levels of polyphenols.

2. Materials and Methods

2.1 Plant Material

Leaf samples were collected from plant species known to have high polyphenol content including *Elaeocarpus sphericus* (rudraksh), *Podocarpus macrophyllus* (podocarpus, yew-pine, Japanese yew), *Podophyllum hexandrum* (Indian may apple), *Rosa* sp. (wild rose), growing in the main campus of Institute of Himalayan Bioresource Technology (IHBT), along with the, five cultivars of *Camellia sinensis* (tea) namely, T-78, TV-1, UPASI-9, UPASI-10 and Kangra Jat growing in the Tea Experimental Farm, IHBT. The leaves of *Malus domesticus* (apple) root stock MM106 and B9 served as control for all the experiments.

2.2 Extraction and Estimation of Total Polyphenols in Selected Plant Species

The influence of leaf polyphenols on *Agrobacterium* infection was investigated by quantifying the total phenolic content in the leaves of the plant species under study. Total phenols were estimated following the method described by Swain and Hillis (1959).

2.3 *Agrobacterium tumefaciens* mediated transformation

2.3.1. Strain and plasmid

A disarmed *Agrobacterium tumefaciens* strain GV3101 carrying plasmid pBI121 (Fig 1) was used in the present study to transfer the *gus* reporter gene.

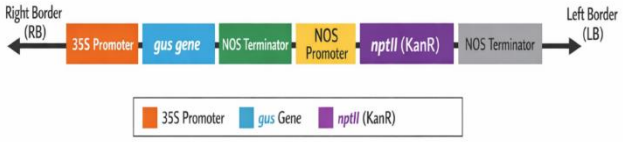


Figure 1. Schematic representation of the T-DNA region of the binary vector pBI121 used for plant transformation. The T-DNA is flanked by the right border (RB) and left border (LB) sequences. The reporter gus gene is driven by the Cauliflower mosaic virus (CaMV) 35S promoter and terminated by the nopaline synthase (NOS) terminator. The selectable marker gene nptII (conferring kanamycin resistance) is regulated by the NOS promoter and terminated by the NOS terminator.

2.3.2. Transformation procedure

Surface sterilized leaf explants were then subjected to genetic transformation using a freshly grown *Agrobacterium tumefaciens* in YMB medium with Kanamycin as a selective antibiotic. At the optical density (OD_{600}) of 0.6 the bacterial cells were pelleted by centrifugation at 6000 rpm and 25°C for 20 minutes. The supernatant containing the bacterial cell debris and antibiotics was discarded, whereas, the pellet was retained by suspending it in fresh YMB and diluted it to adjust the final cell density up to $1X10^9$ cells/ ml by using the formula.

a X OD obtained X 1 X 10Veils/ml x 3 = 1 X 10 Veils/ml x **b** ml

Where,
a = amount of bacterial suspension to be taken Irom the overnight grown bacterial culture to obtain an optical cell density of 1 X l0^{9}cells/ml
3= correction factor
b= total volume of culture required for genetic transformation

The leaf explants were submerged in diluted *Agrobacterium* suspension. Following a 15-minute infection period, the explants were blotted on sterile filter paper to remove the excess bacterial cells and placed on hormone free basal Murashige and Skoog (MS_0) medium with 0.8 per cent agar. The petriplates with infected explants were then incubated at 25^o C under dark conditions. Leaf explants of apple rootstocks B9 and MM106 were maintained as positive controls, while explants cultured on MS_0 medium without *Agrobacterium* served as negative controls. Each treatment was conducted in triplicate, with three replicates per treatment. Data were recorded as the percentage of explants exhibiting *Agrobacterium* growth at, 2, 3, 5, 10, 15, and 20 days for co-cultivation.

2.3.3. GUS Expression Analysis

Histochemical β-glucuronidase (GUS) activity was assessed according to the procedure described by Jefferson *et al.* (1987). At 1, 2, 3, 5, 10, 15, and 20 days of co-cultivation. GUS activity was evaluated based on the appearance of blue-stained spots and/or sectors on the leaf explants, and representative samples were documented using a Nikon digital camera.

2.4 Effect of Leaf Polyphenols on *Agrobacterium tumefaciens* Growth

Effect of total leaf polyphenols on *Agrobacterium tumefaciens* growth was evaluated by spectrophotometric and viable plate count methods. Polyphenols at concentrations of 0.125, 0.25, 0.5, and 1.0 mg mL^{-1} were added to freshly grown overnight bacterial cultures, which were incubated further to record the OD_{600} was recorded at 0, 12, 16, 18, and 24 h using a spectrophotometer (ELCOS SL-150), with data at 18 h used for comparison. To verify bacterial viability, colony forming units (CFUs) were determined by plating aliquots on YMB agar (1.5%) supplemented with corresponding polyphenol concentrations. Plates were incubated at 28°C for 36–48 h, after which CFUs were counted.

2.5 Effect of Autoclaved and Unautoclaved L-Glutamine on *Agrobacterium* Growth and Transformation

The effect of un-autoclaved and autoclaved (20 and 40 min) L-glutamine on *Agrobacterium* growth was assessed by OD_{600} measurements and CFU enumeration. Different concentrations of L-glutamine (Table 1) were added to fresh starter cultures and compared with controls lacking L-glutamine. Cultures supplemented with 200 µM acetosyringone served as positive controls. High-polyphenol leaf explants were transformed using *A. tumefaciens* and co-cultivated on media containing autoclaved L-glutamine. Apple rootstocks B9 and MM106 served as positive controls, and transformation was confirmed by histochemical GUS assay.

Table 1. Experimental combinations of L-glutamine and its autoclave duration

Autoclaving time	L-glutamine concentrations (mg mL^{-1})
No autoclaving	0.5, 1.0, 2.0, 3.0, 5.0
20 min	0.5, 1.0, 2.0, 3.0, 5.0
40 min	0.5, 1.0, 2.0, 3.0, 5.0

2.6 Combined Effect of Leaf Polyphenols and Autoclaved L-Glutamine

Leaf polyphenol extracts were added to bacterial cultures at graded concentrations (0.125-1.0 mg ml^{-1}) along with L-glutamine autoclaved for 40 minutes (2 mg ml^{-1}). Bacterial growth was measured after 18 hours, and fold increase was calculated relative to control.

2.7 Statistical Analysis

All experiments were conducted in triplicate. Mean values were calculated, and results were interpreted based on consistent trends observed across replicates.

3. Results and discussion

3.1 Total Polyphenol Content in Leaves samples

Marked variation in total polyphenol content was recorded among the analysed plant species (Fig. 2). The highest concentration was observed in *Elaeocarpus sphaericus* (75.45 mg g^{-1} fresh weight), followed by *Camellia sinensis* cultivars (50.38–70.45 mg g^{-1} fresh weight) and *Podocarpus macrophyllus* (48.58 mg g^{-1} fresh weight). The lowest polyphenol level was detected in *P. hexandrum* (23.23 mg g^{-1} fresh weight). Moderate polyphenol concentrations were observed in *Rosa* sp. and *Malus domestica* (apple rootstocks).

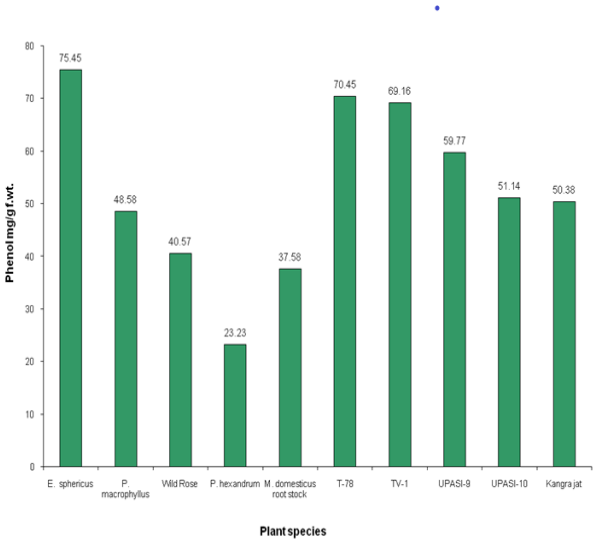


Figure 2. Total phenolic content of leaves from different plant species and tea cultivars, expressed as phenol (mg g^{-1} fresh weight). Bars represent mean phenolic content for *E. sphaericus*, *P. macrophyllus*, wild rose, *P. hexandrum*, *M. domestica* rootstock, and tea cultivars T-78, TV-1, UPASI-9, UPASI-10, and Kangra jat. Numerical values above the bars indicate mean phenolic content for each species/cultivar.

The wide interspecific variation in total leaf polyphenols observed reflects well-known species differences in constitutive chemical defenses (Haslam, 1989; Wink, 1999). Woody and evergreen taxa frequently accumulate higher phenolic loads as a defense mechanism against herbivores and pathogens (Kolodziej *et al*., 2005; Haslam, 1989). High polyphenol concentrations in *Elaeocarpus sphaericus* and several *Camellia sinensis* cultivars are therefore consistent with their ecological strategy and with previous reports that tea leaves are particularly rich in catechins and related flavanols (Ito & Yanase, 2022; Crozier *et al*., 2000). Conversely, the relatively low polyphenol level in *Podophyllum hexandrum* provides a biochemical explanation for its greater susceptibility to *Agrobacterium* colonization observed in our study (Sandal *et al*., 2011).

Polyphenol concentration also determines the potential for post-wounding oxidation mediated by polyphenol oxidase (PPO), which in turn governs the formation of toxic quinones associated with tissue browning and necrosis (Mayer, 2006; Tilley *et al*., 2023). Because PPO activity is often correlated with substrate availability (i.e., polyphenol content), the observed differences in PPO activity among species (Fig. 3) rationally explain why some explants turned brown and died rapidly during sterilization/co-cultivation while others did not (Kim *et al*., 2001; Tilley *et al*., 2023).

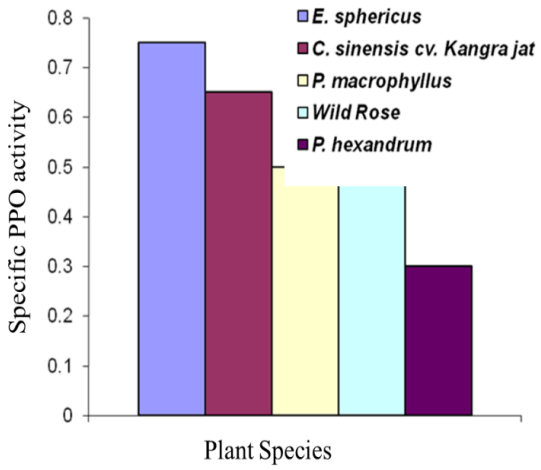


Figure 3. Specific polyphenol oxidase (PPO) activity in leaves of selected plant species. Bars represent the specific PPO activity of E. sphaericus, Camellia sinensis cv. Kangra jat, P. macrophyllus, wild rose, and P. hexandrum. Values are expressed as specific enzyme activity (units per mg protein), as indicated on the y-axis.

3.2 Effect of Leaf Polyphenols

3.2.1 On *A. tumefaciens* Growth

Leaf-extracted polyphenols significantly influenced the growth of *A. tumefaciens*, although the extent of inhibition varied with plant species and polyphenol concentration. Across all species, maximal bacterial growth was consistently observed at a polyphenol concentration of 0.25 mg mL^{-1}. Deviations from this concentration, either higher (1.0 mg mL^{-1}) or lower (0.5 mg mL^{-1}), resulted in a reduction in bacterial growth.

When bacterial growth responses to polyphenols derived from different plant species were compared, the greatest stimulation was observed with *Podophyllum hexandrum*, showing a 3.80-fold increase relative to the control. This was followed by *Rosa* sp. (2.52-fold) and *Podocarpus macrophyllus* (2.05-fold). In contrast, minimal growth enhancement was recorded in the presence of polyphenols from *Elaeocarpus sphaericus* (1.17-fold) and *Camellia sinensis* cultivars TV-1 (1.18-fold) and T-78 (1.19-fold).

Colony forming unit (CFU) analysis conducted after 48 h further confirmed differential effects of leaf polyphenols on *Agrobacterium* viability. The highest CFU counts, comparable to the untreated control (>500), were observed with *P. hexandrum* polyphenols at 0.125 mg mL^{-1}. However, increasing polyphenol concentrations resulted in a consistent decline in CFU numbers across all species. The lowest viable count (7 CFUs) was recorded in cultures treated with *E. sphaericus* polyphenols at 0.25 mg mL^{-1}. No CFUs were detected at higher concentrations (0.5 and 1.0 mg mL^{-1}) of polyphenols from *E. sphaericus* and *C. sinensis* cv. T-78, or at 1.0 mg mL^{-1} from cv. TV-1.

The concentration-dependent observations, stimulation of bacterial growth at low phenolic doses (0.125-0.25 mg mL^{-1}) and inhibition at higher doses are consistent with the dual nature of plant phenolics reported in the literature. Low levels of certain phenolics can act as signaling molecules and even partially mimic vir gene inducers (e.g., acetosyringone) enhancing *Agrobacterium* activity, while higher concentrations, or their oxidized products, are antimicrobial (Citovsky *et al*., 1992; Subramoni & Venturi, 2014). The peak in fold-increase at ~0.25 mg mL^{-1} across species suggests a window where phenolic compounds may promote virulence signaling without generating toxic oxidative products.

As concentration increased, the pronounced decline in CFUs is best explained by enzymatic oxidation of polyphenols to quinones via PPO, producing reactive electrophilic compounds that damage bacterial membranes and proteins (Mayer, 2006; Araji *et al*., 2014; Tilley *et al*., 2023). Species such as *E. sphaericus* and some *C. sinensis* cultivars that combined high polyphenol content with high PPO activity exerted the strongest bactericidal effect, consistent with the idea that both substrate and enzyme activity determine the level of oxidative toxicity (Kim *et al*., 2001; Gandhi & Shah, 2018). These results also align with earlier observations that phenolic composition along with quantity modulates *Agrobacterium* responses (Lee *et al*., 1995; Brencic & Winans, 2005), some phenolic structures are efficient *vir* gene inducers whereas others do not interact effectively with *VirA/VirG* and instead are toxic or inhibitory (Subramoni & Venturi, 2014).

3.2.2 On *A. tumefaciens* mediated transformation process

No successful infection of leaf explants was observed in any of the studied plant species even after 30 days of co-cultivation with *Agrobacterium tumefaciens*. Instead, progressive browning of the explants occurred, ultimately leading to tissue necrosis. An exception was observed in *Podophyllum hexandrum*, where visible bacterial growth on leaf tissues was detected within 15 days of co-cultivation.

Histochemical GUS analysis revealed no detectable β-glucuronidase activity in the leaves of most plant species examined. In contrast, *P. hexandrum* showed limited GUS expression, evidenced by a small blue-stained sector on the leaf lamina after 15 days of co-cultivation.

The near-universal failure of transformation in high-polyphenol explants manifest as browning, tissue necrosis, and absence of GUS staining confirms that polyphenol-mediated oxidative damage is a major barrier to T-DNA delivery and plant cell survival (Haslam, 1989; Mayer, 2006). Wounding during explant preparation disrupts vacuolar and plastid compartmentation, allowing PPO to oxidize phenolics and produce quinones that both kill plant cells and impair bacterial viability and attachment (Bolwell *et al*., 1995; Doke *et al*., 1996). In contrast, *P. hexandrum*, with lower polyphenol and PPO levels, showed limited GUS expression and earlier bacterial proliferation — supporting the central role of phenolic load

and oxidation in determining transformation success (Sandal *et al*., 2011).

Together, these observations indicate that mere presence of bacteria and vir gene induction is insufficient: a permissive chemical environment (i.e., limited oxidative phenolic toxicity) is essential for stable cell–cell contact, T-DNA transfer, and reporter gene expression (Subramoni & Venturi, 2014; Citovsky *et al*., 1992).

3.3 Effect of Autoclaved and Un-autoclaved L-Glutamine on *Agrobacterium tumefaciens* Growth

The effect of L-glutamine on *Agrobacterium tumefaciens* growth varied markedly depending on autoclaving duration and concentration (Table 2). The negative control (without additives) showed CFU counts exceeding 500, while the positive control, acetosyringone (200 µM), exhibited profuse bacterial growth, recorded as infinite (+++). Un-autoclaved L-glutamine at concentrations ranging from 0.5 to 5.0 mg ml^{-1} did not enhance bacterial growth, the CFU counts remained comparable to the negative control (>500) across all concentrations.

L-glutamine autoclaved for 20 minutes showed a moderate stimulatory effect at intermediate concentrations. While CFU counts remained >500 at 0.5, 1.0, and 5.0 mg ml^{-1}, enhanced growth was observed at 2.0 mg ml^{-1} (infinite +) and 3.0 mg ml^{-1} (infinite), indicating partial activation of bacterial proliferation.

In contrast, L-glutamine autoclaved for 40 minutes resulted in a pronounced increase in *Agrobacterium* growth. Strong stimulation (infinite ++) was observed at 0.5 mg ml^{-1}, with consistently high growth responses across higher concentrations. The maximum bacterial proliferation was recorded at 2.0 mg ml^{-1}, beyond which no further enhancement was evident, suggesting an optimal concentration threshold.

The finding that un-autoclaved L-glutamine had no effect on *Agrobacterium* growth but that autoclaving dramatically changed its activity supports the mechanistic hypothesis proposed previously by our team: thermal cyclization of glutamine produces novel compounds with xenobiotic-detoxifying properties (Sandal *et al*., 2011). NMR and chromatographic analyses in earlier studies identified 5-oxo-proline as a major product after shorter autoclave exposure and α-amino glutarimide (2-amino glutarimide) after extended autoclaving; only the latter was effective in facilitating *Agrobacterium* growth and infection (Sandal *et al*., 2011).

The substantially greater growth stimulation observed after 40-min autoclaving (versus 20 min) likely reflects formation of 2-amino glutarimide, which can react with or "quench" oxidized phenolic species, thereby reducing their bactericidal activity (Sandal *et al*., 2011). The absence of effect with un-autoclaved glutamine confirms that intact L-glutamine is not itself the active agent; rather, it is the autoclave-derived transformation products that mediate the observed biological effect. The plateauing of response at 2.0 g L^{-1} suggests a saturable detoxification process or metabolic limitation in *Agrobacterium* (Sandal *et al*., 2011).

Table 2: CFUs in response to autoclaved and un-autoclaved L-glutamine

Samples	Conc mg/ml	CFU
Control (none)	-	>500
Acetosyringone	200 µM	Infinite (+++)
L-glutamine (un-autoclaved)	0.5	>500
	1.0	
	2.0	
	3.0	
	5.0	
L-glutamine (autoclaved for 20 min.)	0.5	>500
	1.0	>500
	2.0	Infinite +
	3.0	Infinite
	5.0	>500
L-glutamine (autoclaved for 40 min.)	0.5	Infinite ++
	1.0	
	2.0	
	3.0	
	5.0	

*CFU: Colony Forming Unit

3.4 Combined Effect of Leaf Polyphenols and Autoclaved L-Glutamine

Maximum bacterial growth was observed at 0.25 mg mL^{-1} leaf polyphenols in the presence of L-glutamine autoclaved (2 mg mL^{-1}) for 40 min, irrespective of plant species, while higher polyphenol concentrations (0.5-1.0 mg mL^{-1}) reduced growth (Fig 3). The strongest stimulation occurred with *Podophyllum hexandrum* (4.81-fold), followed by *Rosa* sp. and *Podocarpus macrophyllus*, whereas minimal enhancement was recorded with *Elaeocarpus sphaericus* and *Camellia sinensis* cultivars.

CFU counts increased markedly when 2.0 mg m L^{-1} L-glutamine autoclaved for 40 min was combined with leaf polyphenols, exceeding values obtained

with polyphenols alone (Table 2). No *Agrobacterium* growth was detected on co-cultivation media containing un-autoclaved or 20-min autoclaved L-glutamine, while growth in the presence of 40-min autoclaved L-glutamine varied among plant species.

When polyphenols were paired with 40-min autoclaved glutamine, bacterial growth markedly increased and the inhibitory effects of phenolics were largely mitigated. This observation is consistent with the proposed quenching mechanism in which α-amino glutarimide neutralizes reactive quinones or forms less toxic adducts with phenolic oxidation products (Sandal *et al*., 2011). The highest fold increases occurring at intermediate phenol concentrations indicate that detoxification is most efficient when the oxidant load is moderate; at very high phenolic

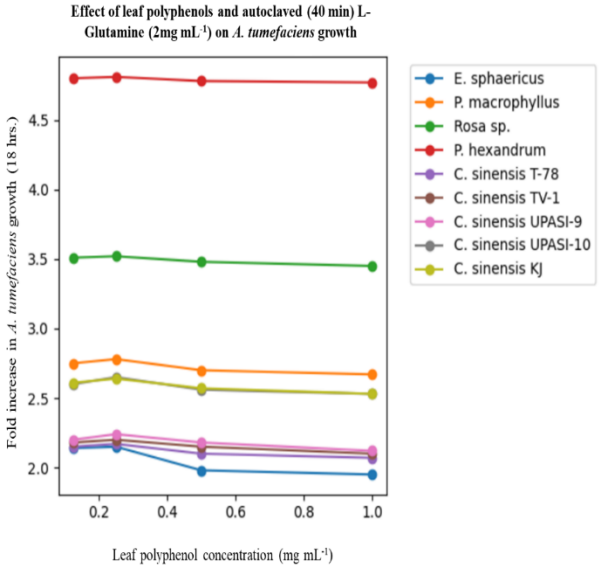
concentrations, residual toxicity may exceed the detoxifying capacity (Tilley *et al*., 2023).
Species differences in the magnitude of rescue (e.g., strong rescue in *P. hexandrum* and modest rescue in *E. sphaericus*) are likely attributable to baseline polyphenol amount, composition (which affects reactivity), and PPO activity, all of which influence the balance between detoxification and ongoing oxidative production of toxic species (Mayer, 2006; Araji *et al*., 2014). Importantly, while autoclaved glutamine does not itself induce vir genes, it creates a chemically permissive environment that allows *Agrobacterium* virulence systems and quorum-sensing networks to operate (Subramoni & Venturi, 2014; Sandal *et al*., 2011).

Figure 4. Effect of leaf polyphenols from different plant species in the presence of autoclaved L-glutamine (40 min) on the growth of *Agrobacterium tumefaciens* after 18 h of incubation. Fold increase in bacterial growth was recorded at varying polyphenol concentrations (0.125, 0.250, 0.500 and 1.000 mg ml^{-1}). Each line represents the growth response of *A. tumefaciens* to polyphenols derived from individual plant species.

Table 2: cfus in response to leaf polyphenols from different plant species and L-glutamine autoclaved (2 g mL^{-1}) for 40 min.

Plant species	Phenol content mg/g f. wt.	Phenol conc. mg/ml	cfu
Control			>500
E. sphaericus	75.45	0.125	15
		0.250	10
		0.500	3
		1.000	None
P. macrophyllus	48.58	0.125	150
		0.250	147
		0.500	120
		1.000	70
Rosa sp.	40.57	0.125	300
		0.250	290
		0.500	270
		1.000	250
P. hexandrum	23.23	0.125	>500
		0.250	>500
		0.500	450
		1.000	396
***C. sinensis* cvs:** T-78	70.45	0.125	20
		0.250	18
		0.500	9
		1.000	None
TV-1	69.16	0.125	28
		0.250	26
		0.500	19
		1.000	12
UPASI-9	59.77	0.125	75
		0.250	71
		0.500	30
		1.000	22
UPASI-10	51.14	0.125	88
		0.250	85
		0.500	70
		1.000	43
Kangra Jat	50.38	0.125	89
		0.250	87
		0.500	75
		1.000	50

3.5 Effect of Autoclaved L-Glutamine on *A. tumefaciens* mediated transformation process



Variable responses were observed among the plant species when the co-cultivation medium was supplemented with L-glutamine (2 mg mL^{-1}) autoclaved for 20 and 40 minutes. No Agrobacterium growth was detected in any of the leaf explants cultured on medium containing L-glutamine autoclaved for 20 minutes.
In contrast, supplementation with L-glutamine (2 mg mL^{-1}) autoclaved for 40 minutes markedly promoted Agrobacterium growth. Bacterial proliferation was observed within 3 days of co-cultivation in *Malus domesticus* rootstock and *Podophyllum hexandrum*. Leaf explants of wild rose and *Podocarpus macrophyllus* exhibited Agrobacterium growth after 5 and 7 days of co-cultivation, respectively (Fig 5).

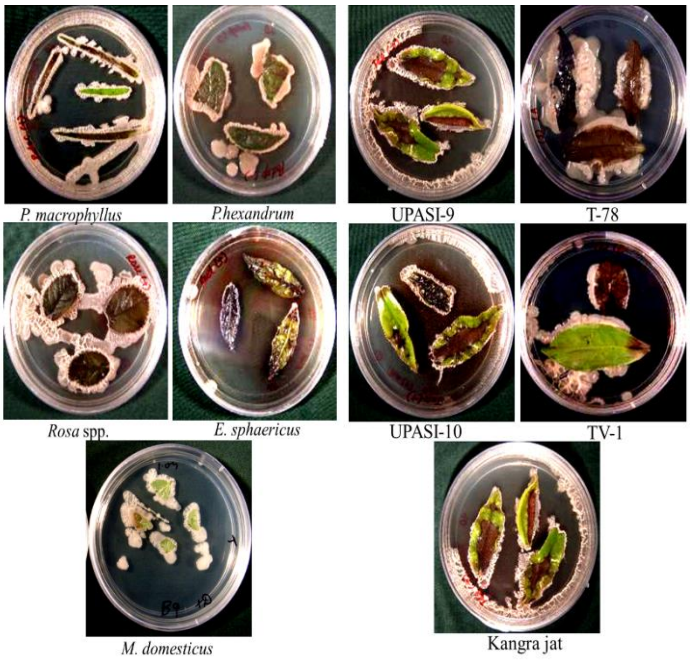


Figure 5. Effect of autoclaved L-glutamine (2 mg mL⁻¹; 40 min) on *Agrobacterium tumefaciens* proliferation during co-cultivation of leaf explants from different plant species and tea cultivars. Visible bacterial growth around the explants indicates enhanced *Agrobacterium* proliferation following supplementation with L-glutamine autoclaved for 40 minutes.

Although delayed, Agrobacterium growth was also recorded in Camellia sinensis cultivars after 10–15 days and in Elaeocarpus sphaericus after 20 days of co-cultivation. Histochemical GUS assay revealed the presence of blue-colored spots or sectors on leaf explants cultured on medium supplemented with L-glutamine autoclaved for 40 minutes, indicating successful gene expression. However, the proportion of explants exhibiting GUS expression varied among the different plant species (Fig 6).

Figure 6. Histochemical GUS assay showing transient *gus* gene expression in leaf explants of different plant species and tea cultivars following *Agrobacterium tumefaciens*–mediated transformation. Blue-colored spots or sectors indicate positive GUS expression.

The faster onset of bacterial growth and detectable GUS expression in explants cultured with 40-min autoclaved glutamine supports a model where early detoxification enables bacterial attachment, survival, and subsequent gene transfer within the crucial early co-cultivation window (first 24 h) (Sandal *et al*., 2011). Although α-amino glutarimide is chemically unstable and reverts partly to 5-oxo-proline over time, the transient detoxification it provides appears sufficient to permit initial cell–cell contact and T-DNA delivery, after which transformation can proceed (Sandal *et al*., 2011).

These data therefore reconcile two important observations: (i) phenolics suppress *Agrobacterium* growth and virulence when present or oxidized at high levels (Subramoni & Venturi, 2014; Tilley *et al*., 2023); and (ii) chemical quenchers derived from autoclaved glutamine can transiently neutralize phenolic toxicity and thereby enhance transformation success without directly acting as vir gene inducers (Sandal *et al*., 2011). This mechanistic view is consistent with the broader literature on phenolic-virulence interactions and offers a plausible, experimentally supported strategy to increase transformation efficiency of polyphenol-rich, recalcitrant explants.

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