

Determination of the Optimal Kanamycin Concentration for Selective Pressure in Wild-Type *Lens culinaris* Medik.: A Comparative Evaluation of In Vitro, Microinjection, and Foliar Spray Application Methods

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Abstract

Lentil (*Lens culinaris* Medik.) is a nutritionally significant grain legume, although the species resistance to both *in vitro* regeneration and *Agrobacterium-mediated* transformation hampers its genetic enhancement via transformation. A critical step for successful plant transformation is the accurate determination of the selective antibiotic concentration that effectively eliminates untransformed tissue while remaining compatible with plant recovery. In this study, we evaluated the sensitivity of wild-type lentil (Moroccan variety Hamria) to kanamycin sulfate across five concentrations (0, 60, 80, 100, and 120 mg/L) using three distinct application methods: *in vitro* tissue culture on MS-based medium, microinjection, and foliar spray. The rate of foliole/leaf yellowing, plant height, and chlorophyll content (SPAD) were assessed at two observation timepoints. One-way ANOVA followed by Tukey's HSD post-hoc test revealed highly significant differences in yellowing rate among concentrations for all three methods ($p < 0.001$).

The *in vitro* system showed a progressive, near-linear dose-dependent response ($F = 125.93$, $p < 2 \times 10^{-16}$), yielding a tissue response threshold (TR50) of approximately 101.8 mg/L, whereas foliar spray and microinjection displayed threshold-type responses that plateaued below 27% yellowing and did not reach a reliable TR50 within the tested range. These results establish 100 mg/L as the recommended kanamycin concentration for *in vitro* selection of transformed lentil tissue.

Introduction

Lens culinaris Medik. (lentil) is a cool-season food legume of critical importance to global food security, particularly in arid and semi-arid regions of South Asia, the Middle East, and North Africa (Ferguson and Erskine 2001). As a rich source of dietary protein, complex carbohydrates, iron, and folate, lentil constitutes a fundamental component of human nutrition in developing countries (Kaale et al. 2023; Montejano-Ramírez and Valencia-Cantero 2024). However, lentil production is severely threatened by a range of biotic stresses, among which parasitic weeds of the genus *Orobanche* (broomrapes) represent one of the most destructive constraints, causing yield losses of up to 100% under severe infestations (Habimana et al. 2014; Fernández-Aparicio et al. 2016; Qasem 2021). Chemical and agronomic control strategies have shown limited efficacy, and host resistance in cultivated germplasm remains rare, underscoring the need for biotechnological approaches to develop resistant varieties (Cartry et al. 2021).

Agrobacterium-mediated transformation and CRISPR/Cas9 genome editing have emerged as powerful tools for crop improvement; however, their application in lentil has been severely hindered by the species recalcitrance to tissue culture and transformation (Sarker et al. 2003; Bermejo et al. 2016). Moreover, the development of robust transformation protocols requires not only efficient *Agrobacterium* inoculation procedures but also reliable selection systems capable of discriminating transformed from non-transformed tissue with precision (Sarroukh et al. 2025).

Kanamycin sulfate, an aminoglycoside antibiotic that inhibits protein synthesis in plastids and mitochondria, is among the most widely used selectable agents in plant transformation, employed in conjunction with the *nptII* (neomycin phosphotransferase II) selectable marker gene (Carrer et al. 1993;

Scheid and Neuhaus-Url 1995). The effectiveness of kanamycin selection is strictly concentration-dependent: insufficient concentrations allow the survival of non-transformed tissue, while excessive concentrations cause indiscriminate phytotoxicity that compromises the recovery of genuine transformants (Colby and Meredith 1990; Tereso et al. 2006a). The optimal lethal concentration therefore varies considerably among species and even among tissue types and explants within the same species (Tereso et al. 2006; Mirzaee and Emadpour 2024).

For lentil specifically, very few studies have systematically characterized kanamycin sensitivity, and no published protocol has compared the effective concentration across multiple transformation delivery systems in parallel. Polowick and Yan (2023) reported that kanamycin was added to the medium over a range of concentrations of 30, 40, 50 and 60 mg/l. Surviving explants were transferred to fresh shoot elongation medium every 14 days with kanamycin increased after each round to a maximum of 60 mg/l for at least 4 rounds. The discrepancy may reflect genotypic differences, tissue type, or culture conditions, and underscores the need for systematic, multi-method characterization of kanamycin sensitivity across lentil genotypes and application systems.

With the increasing adoption of diverse transformation methods including conventional *in vitro* co-culture, simplified in planta approaches, and direct microinjection, it is essential to establish method-specific or universally applicable selection thresholds (Vinoth et al. 2013; Niazian et al. 2017). The present study was designed to determine the optimal kanamycin sensitivity concentration for three distinct application methods: *in vitro* explant culture, direct microinjection into intact seedlings, and foliar spray application. The data presented here are intended to serve as technical reference for the development of lentil transformation and the delivery of genome editing reagents, pending validation within a functional transformation system.

Materials and Methods

Kanamycin sensitivity assay

To determine the minimum inhibitory concentration of kanamycin for wild-type lentil tissue, non-transformed seedlings were exposed to five kanamycin sulfate concentrations. Kanamycin sulfate was dissolved in sterile distilled water, filter-sterilized through a 0.22µm membrane, and working solutions at concentrations of: 0, 60, 80, 100, and 120 mg/L were prepared freshly by appropriate dilution before each experiment. Three independent application methods were evaluated in parallel:

In vitro method

Seeds of lentil (*Lens culinaris* Medik., cv. Hamria, a Moroccan locally adapted variety) were surface-sterilized by immersion in 70% ethanol for 1 minute, followed by treatment with 2.5% sodium hypochlorite solution for 10 minutes under continuous agitation. Seeds were then rinsed five times with sterile distilled water and germinated on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar, pH adjusted to 5.8, at 25 ± 2°C under a

16/8 h light/dark photoperiod with a light intensity of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$. Lentil seeds were used as starting materials (Fig. 2a). Following germination, seedlings were transferred to fresh MS medium supplemented with the respective kanamycin concentrations. Cultures were maintained at 25°C under standard photoperiod conditions. Observations were recorded at two time points: observation 1 after 15 days after transfer to kanamycin supplemented medium and observation 2 after 40 days after transfer.

Microinjection method

Five-day-old lentil seedlings were subjected to direct microinjection of kanamycin solutions at the specified concentrations into the meristematic zone using a micro-syringe (Fig. 2e). A single injection of 10 μl was delivered per seedling. Plants were maintained in a controlled greenhouse at 25 °C, 60% relative humidity under a 16/8h light/dark photoperiod following injection. Observations and parameter evaluation were recorded at 15 days after injection, corresponding to observation 1, and at 40 days, corresponding to observation 2, consistent with the *in vitro* evaluation schedule.

Foliar spray method

Kanamycin solutions at the specified concentrations were applied by uniform foliar spray to five-day-old seedlings until runoff (Fig. 2). Spray application was repeated every 7 days for a total of three applications. Plants were maintained under the same greenhouse conditions as described for microinjection method. Observations and parameter evaluation were recorded at the same timepoints as the microinjection group.

Each kanamycin concentration $\times$ method combination comprised 25 independent plants distributed across 5 pots, with 5 replicates per pot. The experiment was arranged in a completely randomized design (CRD).

Determination of total polyphenol contents:

Following treatment, plant tissues showing differential physiological responses were harvested, immediately frozen in liquid nitrogen, and stored at -80°C until biochemical analyses.

Extraction of Phenolic and Flavonoid Compounds

Approximately 100mg of fresh plant tissue from each treatment was ground into a fine powder using liquid nitrogen. The powdered material was transferred into 2ml microcentrifuge tubes containing 1ml of 80% methanol.

Samples were vortexed thoroughly and incubated at room temperature for 24h under gentle agitation in darkness to avoid phenolic degradation. The extracts were subsequently centrifuged at 12000 rpm for

10min at 4°C.

The supernatants were collected and used for total polyphenol determination.

Determination of total polyphenol content (TPC)

Total polyphenol content was determined using the Folin-Ciocalteu colorimetric method.

Briefly, 100 μ L of methanolic extract was mixed with 500 μ L of diluted Folin-Ciocalteu reagent (10%, V/V). After 5min of incubation at room temperature, 400 μ L of sodium carbonate solution (7.5%, W/V) was added.

The reaction mixture was incubated for 90min in darkness at room temperature. Absorbance was measured at 765nm using a UV-visible spectrophotometer (GENESYS 10S UV–VIS, Thermo Fisher).

Gallic acid was used for calibration curve preparation, and total polyphenol content was expressed as mg gallic acid equivalents (GAE) per g fresh weight (FW).

Parameters evaluated

Foliolate and leaf yellowing was assessed as a semi-quantitative visual index at each observation timepoint. For each plant, the percentage of visually yellowed leaves was calculated by counting the number of yellowed leaves relative to the total number of leaves per plant. Yellowing was defined as any leaf exhibiting more than 50% chlorotic area based on visual estimation. All assessments were performed by the same-trained observer throughout the entire experiment to ensure consistency across treatments and replicates. Chlorophyll content was measured using a portable SPAD-502 chlorophyll meter (Konica Minolta, Japan). Three SPAD readings were taken per plant on the youngest fully expanded leaf and averaged. Plant height was measured from the base of the stem to the apical meristem using a ruler.

Statistical analysis

All statistical and multivariate analyses were performed using R software (v4.4; R Core Team 2024). One-way analysis of variance (ANOVA) was performed independently for each application method, using the final observation timepoint (observation 2) as the response variable, with kanamycin concentration as the fixed factor. Model assumptions were verified prior to post-hoc testing: normality of residuals within each concentration group was assessed with the Shapiro–Wilk test, and homogeneity of variances across concentrations was assessed with Levene's test. Tukey's Honestly Significant Difference (HSD) post-hoc test was applied using the multcomp package to identify significantly different treatment pairs (Hothorn et al. 2008). Pearson's correlation coefficient (r) was calculated using the stats package to evaluate the relationship between chlorophyll content (SPAD) and foliole yellowing rate.

Dose–response relationships were characterized by linear regression of yellowing rate on kanamycin concentration. Given the approximately linear dose–response observed in the *in vitro* system, a tissue response threshold (TR50, the concentration inducing 50% yellowing) was estimated exclusively for this method, by linear interpolation of the dose–response regression at the 50% yellowing threshold using the *lm* function. For foliar spray and microinjection, yellowing did not approach 50% within the tested concentration range and plateaued at higher doses; a TR50 was therefore not estimated for these methods, to avoid extrapolating substantially beyond the observed data, and their threshold is reported as exceeding the highest concentration tested (> 120 mg/L). All results are expressed as mean $\pm$ standard error (SE). Significance levels are reported as ns (not significant), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Results

Effect of Kanamycin concentration on foliar yellowing

Kanamycin treatment significantly affected foliar yellowing in lentil seedlings across all application methods (Fig. 1; Table 1). One-way ANOVA revealed a highly significant effect of concentration for foliar spray ($p = 2.85 \times 10^{-8}$), microinjection ($p = 1.23 \times 10^{-10}$), and *in vitro* culture ($p < 2 \times 10^{-16}$), with the strongest effect observed under *in vitro* conditions.

Under foliar spray (Fig. 1A), yellowing remained low at 0 and 60 mg/L and increased significantly from at concentrations ≥ 80 mg L⁻¹. No significant differences were detected among the 80, 100, and 120 mg L⁻¹ treatments, indicating a threshold-like response. A similar trend was observed under microinjection (Fig. 1B), where yellowing increased significantly at 80 mg L⁻¹ but did not differ significantly among higher concentrations, suggesting a limited response range within the concentrations tested.

In contrast, the *in vitro* system (Fig. 1C) exhibited a clear and progressive dose-dependent increase in yellowing. Yellowing increased progressively with concentration, with all pairwise comparisons significant except between 0 and 60 mg/L (Tukey HSD, $p < 0.05$). This linear-like response, ranging from minimal yellowing in controls to high levels at 120 mg/L, highlights the greater sensitivity and discriminatory power of *in vitro* culture assay for evaluating kanamycin response. Consistent with this observation, absolute increases in yellowing above control levels, which were substantially greater *in vitro* than in the other application methods (Table 2).

Table 1

Yellowing rate, ANOVA F-value, p-value, and TR50 by kanamycin concentration and application method. Different letters within each column indicate significant differences (Tukey HSD, $p < 0.05$). *** $p < 0.001$. Values are Mean $\pm$ Standard Error.

Concentration (mg/L)	Foliar- Spray Mean $\pm$ SE (%)	Micro-injection Mean $\pm$ SE (%)	<i>In Vitro</i> assay Mean $\pm$ SE (%)
0	0.84 $\pm$ 0.29 a	0.84 $\pm$ 0.29 a	4.05 $\pm$ 0.41 a
60	3.85 $\pm$ 2.42 a	9.24 $\pm$ 2.58 a	23.18 $\pm$ 1.93 a
80	17.72 $\pm$ 1.77 b	22.52 $\pm$ 2.34 b	36.69 $\pm$ 2.04 b
100	20.75 $\pm$ 2.58 b	23.57 $\pm$ 2.67 b	48.25 $\pm$ 3.18 c
120	26.66 $\pm$ 5.58 b	27.61 $\pm$ 3.88 b	67.84 $\pm$ 3.07 d
F value	16.52	24.99	125.9
p value	2.85 $\times 10^{-8}$ ***	1.23 $\times 10^{-10}$ ***	2 $\times 10^{-16}$ ***
TR50	> 120 mg/L	> 120 mg/L	~ 101.8 mg/L

Table 2

Absolute increase in yellowing rate (percentage points above 0 mg/L control) by kanamycin concentration and application method.

Concentration (mg/L)	Spray (percentage points)	Microinjection (percentage points)	<i>In vitro</i> (percentage points)
60	+ 3.01	+ 8.40	+ 19.13
80	+ 16.88	+ 21.68	+ 32.64
100	+ 19.91	+ 22.74	+ 44.20
120	+ 25.82	+ 26.77	+ 63.79

Tissue Response Threshold (TR50)

The dose response curve for the *in vitro* system (Fig. 3) showed a progressive and near-linear increase in yellowing rate with increasing kanamycin concentration. Yellowing increased to 67.84 $\pm$ 3.07% at 120 mg/L, confirming a strong and continuous response to antibiotic exposure. Based on this relationship, the tissue response threshold (TR50) was estimated at approximately 101.8 mg/L, indicating the 50% foliar yellowing occurs near this concentration under *in vitro* conditions.

In contrast, no reliable TR50 could be determined for foliar spray or microinjection because yellowing plateaued at approximately 27–28%, even at the highest tested concentration (120 mg/L). Although linear extrapolation suggested a theoretical TR50 of approximately 215–240 mg/L, this estimate falls

well outside the experimental range. Therefore, TR50 was considered not reached (> 120 mg/L) for foliar spray and microinjection methods.

Relationship between Yellowing Rate and Physiological Indicators

Pearson correlation analysis revealed a significant negative relationship between chlorophyll content (SPAD) and yellowing rate under both foliar spray and microinjection methods (Table 3). Under foliar spray, a moderate but highly significant correlation was observed ($r = -0.577$, $p < 0.001$), whereas under microinjection, the correlation was weaker but remained significant ($r = -0.375$, $p = 0.010$). These results indicate that increasing yellowing is consistently associated with a reduction in chlorophyll content, with a stronger relationship observed under spray conditions.

In contrast, plant height showed no significant correlation with yellowing rate under either foliar spray ($p = 0.089$) or microinjection ($p = 0.548$), indicating that growth parameters are less sensitive indicators of kanamycin-induced stress than chlorophyll-related traits under the conditions tested.

Table 3
Pearson correlation coefficients (r), R^2 , and significance levels for the relationship between yellowing rate and chlorophyll content or plant height under foliar spray and microinjection.

Method	Variables	r	R^2	p value	Significance
Foliar Spray	Chlorophyll vs Yellowing	-0.577	0.333	< 0.001	***
Microinjection	Chlorophyll vs Yellowing	-0.375	0.140	0.010	**
Foliar Spray	Plant height vs Yellowing	-0.249	0.062	0.089	ns
Microinjection	Plant height vs Yellowing	0.090	0.008	0.548	ns

*** $p < 0.001$; ** $p < 0.01$; ns = not significant.

Total Phenolic Content (TPC) Response to kanamycin Treatments:

Total phenolic content (TPC) of *Lens culinaris* cv. Hamria explants was determined using the Folin–Ciocalteu assay following three kanamycin application methods (micro-injection, Foliar spray, and continuous in vitro exposure) across five concentrations (0, 60, 80, 100, and 120 mg L⁻¹) (Fig. 4). Two-way ANOVA revealed highly significant effects of application method ($F = 225.81$), kanamycin concentration ($F = 76.67$), and their interaction ($F = 32.86$) on TPC ($P < 0.001$ for all factors), and Levene's test confirmed homogeneity of variance across treatment groups ($F = 0.93$, $P = 0.539$), indicating that the phenolic response to kanamycin was strongly dependent on the mode of antibiotic delivery.

Baseline TPC differed significantly among the three application methods, with pulverisation exhibiting the highest control value, followed by micro-injection and *in vitro* culture (Fig. 4, Table 4). Under micro-

injection, TPC remained unchanged at 60 mg L⁻¹ but declined sharply at concentrations ≥ 80 mg L⁻¹, plateauing at a maximum reduction of 46.5% relative to control at 120 mg L⁻¹ (Table 4). Foliar spray induced the strongest initial response, with TPC decreasing by 60.2% at 60 mg L⁻¹, before partially recovering at 80 mg L⁻¹ and declining again at higher concentrations; all pairwise comparisons among concentrations were statistically significant, confirming a genuine non-monotonic response rather than measurement variability (Fig. 4, Table 4). Continuous *in vitro* exposure produced smaller and less consistent changes, with significant reductions relative to control observed only at 60 and 120 mg L⁻¹, and a maximum decrease of 33.2% at 120 mg L⁻¹ (Table 4).

Cross-method comparisons confirmed significant differences in TPC among application methods at nearly every concentration tested, with the exception of foliar spray and micro-injection at 100 mg L⁻¹, which did not differ significantly from one another (Table 4). Together, these results, illustrated in Fig. 4 and detailed in Table 4, indicate that the magnitude and pattern of kanamycin-induced phenolic suppression depend on the mode of delivery rather than on concentration alone.

Table 4

Total phenolic content (TPC, mg GAE/mL, mean ± SD) of *Lens culinaris* cv. Hamria explants across three kanamycin application methods and five concentrations. Values in parentheses indicate percentage change relative to each method's own control.

Application method	0 mg L ⁻¹	60 mg L ⁻¹	80 mg L ⁻¹	100 mg L ⁻¹	120 mg L ⁻¹
Micro-injection	3.42 ± 0.00 ^{A, b}	3.27 ± 0.08 ^{AB, a}	2.51 ± 0.37 ^{B, b}	2.49 ± 0.04 ^{B, a}	1.83 ± 0.26 ^{B, b}
Foliar spray	5.17 ± 0.03 ^{A, a}	2.06 ± 0.02 ^{E, b}	3.32 ± 0.08 ^{B, a}	2.56 ± 0.03 ^{d, a}	2.94 ± 0.18 ^{c, a}
<i>In vitro</i>	2.02 ± 0.23 ^{a, c}	1.56 ± 0.16 ^{bc}	1.68 ± 0.05 ^{Ab, c}	1.83 ± 0.11 ^{ab, b}	1.35 ± 0.17 ^b

Discussion

This study provides one of the first systematic comparison of kanamycin phytotoxicity in lentil (*Lens culinaris* cv. Hamria) using three fundamentally distinct delivery strategies: foliar spray, microinjection, and continuous *in vitro* exposure, while integrating morphological, physiological, multivariate, and biochemical responses. Our findings demonstrate that the effectiveness of kanamycin as a selective agent is determined not only by antibiotic concentration but, more importantly, by the mode of delivery. Continuous *in vitro* exposure generated the greatest discriminatory power and enabled the establishment of a reproducible selection threshold, whereas the transient *in vivo* delivery methods exhibited saturation-type responses that limited their capacity to discriminate among higher antibiotic concentrations. Furthermore, visible chlorosis closely reflected chloroplast dysfunction, while secondary metabolic responses were strongly influenced by the physiological context in which antibiotic exposure occurred. Collectively, these findings establish a physiological framework for optimizing kanamycin selection in

lentil and emphasize that delivery strategy should be considered a critical experimental variable during the development of transformation protocols. The distinction between screening and true selectable marker efficacy further underscores this conclusion(Shuja et al. 2021).

The most striking finding of this study was the clear divergence in dose-response behavior between the continuous *in vitro* system and the two transient *in vivo* delivery methods. Both foliar spray and microinjection exhibited threshold-type responses characterized by a rapid increase in chlorosis up to 80 mg L⁻¹, followed by a plateau despite further increases in kanamycin concentration. Similar saturation responses have been reported during the optimization of kanamycin selection systems in several crop species, where increasing antibiotic concentrations beyond an optimal threshold reduced regeneration without proportionally improving selection efficiency(Kumar et al., 2017; Rojo et al., 2021; Ricci et al., 2023; Oey et al., 2009; Mustafa, 2024). The accumulation of the antibiotic within the tissue is evidently constrained beyond a certain point, resulting in a ceiling effect that limits the discriminatory capacity of the selection system(Acanda et al. 2017). Although the physiological basis of this saturation has not been fully resolved, it likely reflects a combination of limited tissue permeability, restricted intracellular transport, and progressive saturation of plastid ribosomal targets, together constraining the extent of additional cellular damage despite increasing external antibiotic concentrations.

Several anatomical and physiological factors probably contributed to this limitation. During foliar application, the hydrophobic cuticular wax layer, restricted stomatal penetration, and limited apoplastic diffusion reduce the entry of hydrophilic molecules such as kanamycin into leaf tissues. The cuticular barrier is particularly effective against polar solutes, with very long-chain aliphatic compounds constituting the primary permeation barrier (Staiger et al. 2019). Kanamycin, as a highly water-soluble aminoglycoside, is poorly suited to cross the lipophilic cuticle without formulation adjuvants. Likewise, microinjection delivers the antibiotic only to a localized region, where subsequent distribution is constrained by the limited injection volume, tissue architecture, and vascular connectivity. This technique is inherently limited to single or few cells per injection and produces highly localized delivery without systemic distribution. Consequently, neither delivery method ensures homogeneous exposure of photosynthetically active tissues. These physical constraints probably explain why both methods failed to achieve a reliable tissue-response threshold (TR₅₀), despite producing substantial visual injury. Importantly, these results demonstrate that tissue sensitivity cannot be accurately characterized by antibiotic concentration alone, but rather by the interaction between concentration and the delivery strategy governing antibiotic availability within plant tissues.

In contrast, continuous *in vitro* exposure produced a progressive and nearly linear increase in chlorosis across the entire concentration range, resulting in a well-defined TR₅₀ of approximately 102 mg L⁻¹. Unlike transient applications, incorporation of kanamycin into the culture medium ensures sustained exposure throughout the experimental period, allowing continuous uptake and systemic distribution as seedlings develop. The prolonged presence of the antibiotic maintains persistent inhibition of plastid protein synthesis, thereby generating a gradual accumulation of physiological damage rather than an abrupt threshold response. This continuous exposure greatly increased the discriminatory resolution of

the selection system, as each concentration between 60 and 120 mg L⁻¹ produced statistically distinct phenotypic classes. Similar observations have been reported during the optimization of selectable marker systems in legumes and other dicotyledonous species, where continuous exposure generally provides more reproducible selection than pulse or localized applications because escapes are minimized and tissue responses remain proportional to antibiotic concentration (Niazian and Shariatpanahi 2020)[7]. More broadly, these studies emphasize that optimal kanamycin concentrations are highly dependent on species, genotype, explant type, and culture conditions, reinforcing the need for species-specific optimization (Mirzaee and Emadpour 2024; Moratti et al. 2026). Furthermore, the use of liquid or semi-solid selection media with uniform antibiotic distribution reduces the frequency of escape shoots that survive on otherwise lethal concentrations due to incomplete contact (Nagashima and Koiwa 2017). Importantly, previous optimization studies have generally focused on identifying an appropriate kanamycin concentration using a single exposure strategy without evaluating whether alternative delivery methods generate comparable physiological responses. By directly comparing three fundamentally different exposure systems under identical experimental conditions, the present study demonstrates that the success of antibiotic selection depends on the combined effects of concentration and delivery strategy. This distinction represents an important conceptual advance for the optimization of selectable marker systems in lentil and potentially other recalcitrant legume species.

From a transformation perspective, the establishment of an effective selection regime requires balancing two opposing objectives: eliminating non-transformed tissues while preserving sufficient viability of transformed cells to sustain regeneration. Insufficient kanamycin concentrations permit the survival of escape tissues, whereas excessive selection pressure may impair regeneration even in transformed explants owing to delayed expression of the selectable marker or excessive phytotoxicity (Rojo et al. 2021; Ricci et al. 2023; Wang et al. 2025). The progressive dose-response generated by continuous *in vitro* exposure provides the discriminatory resolution necessary to identify this balance. Moreover, the estimated TR₅₀ offers an objective quantitative reference for initiating transformation experiments, reducing reliance on empirical trial-and-error optimization while facilitating subsequent refinement according to regeneration capacity and transformation efficiency.

A second important outcome of this study was the strong association between visual chlorosis and chlorophyll depletion. Significant negative correlations between yellowing severity and SPAD values confirmed that externally visible bleaching accurately reflects the progressive loss of photosynthetically active chlorophyll. This relationship supports the widespread use of chlorosis as a practical phenotypic marker during antibiotic selection and demonstrates that visual scoring provides a reliable surrogate for internal physiological damage. Because visual assessment is rapid, inexpensive, and non-destructive, its close agreement with SPAD measurements indicates that routine optimization of selection systems can be achieved efficiently without requiring specialized physiological instrumentation. The physiological relationship between plastid dysfunction and chlorophyll loss has been extensively documented in studies investigating kanamycin-induced chloroplast injury (Bourque et al. 1976; Stern et al. 2010). The use of SPAD measurements as a proxy for chlorophyll content is well established across diverse plant

species and stress conditions, supporting its application in kanamycin toxicity assessment (Zhang et al. 2022).

The mechanistic basis of this response is well established. Kanamycin, an aminoglycoside antibiotic, binds to the decoding A site of the 30S ribosomal subunit, thereby disrupting translational fidelity and inhibiting protein synthesis (Salian et al. 2012; Prokhorova et al. 2017; Seely et al. 2023). Because chloroplast genomes encode essential components of the photosynthetic machinery, inhibition of plastid translation progressively disrupts photosystem maintenance and chloroplast development (Stern et al. 2010). As damaged proteins can no longer be replaced efficiently, photosynthetic electron transport becomes increasingly unstable, ultimately resulting in chlorophyll degradation and the characteristic yellowing phenotype observed in antibiotic-sensitive tissues. This ultimately results in arrested chloroplast development, chlorophyll loss, and the characteristic bleaching phenotype observed in kanamycin-sensitive tissues (Bourque et al. 1976; Stern et al. 2010). The initial interaction between kanamycin and biological membranes is electrostatically driven, with the polycationic aminoglycoside preferentially targeting anionic membrane components (John et al. 2017). In plant cells, natural resistance mechanisms involving vacuolar sequestration of kanamycin by ABC transporters have also been described, highlighting the complexity of plant–antibiotic interactions at the cellular level (Rommens 2006). Similar mechanisms have been described in *Arabidopsis*, tobacco, tomato, and several legume species, where inhibition of plastid translation consistently precedes chlorophyll degradation and visible bleaching. Our findings therefore provide physiological evidence that chlorosis represents one of the earliest and most reliable indicators of kanamycin toxicity in lentil.

Interestingly, plant height was not significantly correlated with chlorosis under any delivery method, indicating that growth inhibition and chloroplast damage follow distinct temporal trajectories. Chloroplast dysfunction develops rapidly following inhibition of plastid translation because photosynthetic proteins exhibit high turnover rates and require continuous replacement. By contrast, reductions in shoot elongation emerge only after prolonged impairment of carbon assimilation, energy production, and cellular metabolism. Growth therefore represents an integrated response to cumulative physiological stress rather than an immediate consequence of chloroplast injury. Similar temporal decoupling between photosynthetic impairment and growth reduction has been described in studies examining herbicide injury, oxidative stress, and plastid-targeting antibiotics, where visible chlorosis consistently precedes measurable reductions in biomass accumulation or plant height. For instance, adjuvants that impair leaf transpiration and photosynthetic activity produce chlorosis well before detectable effects on biomass accumulation (Räsch et al. 2018). From a practical perspective, these findings indicate that chlorosis constitutes a substantially more sensitive endpoint than growth measurements for optimizing antibiotic selection systems during genetic transformation.

The biochemical responses further demonstrate that kanamycin-induced stress is strongly influenced by the mode and duration of exposure. Continuous *in vitro* treatment imposed sustained physiological stress throughout seedling development, whereas foliar spray and microinjection generated localized or transient responses that allowed partial physiological adjustment. Consequently, antioxidant and

secondary metabolic responses appear to be governed not only by the severity of chloroplast dysfunction but also by the temporal dynamics and spatial distribution of antibiotic exposure. These observations emphasize that biochemical indicators should be interpreted within the context of the delivery strategy employed rather than as universal markers of antibiotic toxicity.

Although the present study focused on the lentil cultivar Hamria, genotype-dependent variation in kanamycin sensitivity has been reported in numerous transformation systems, where optimal selection concentrations differed substantially among cultivars and explant types (Ricci et al. 2023; Mirzaee and Emadpour 2024; Moratti et al. 2026). The sensitivity of plant cells to the selection agent depends upon the genotype, the explant type, the developmental stage, and the tissue culture conditions (Angenon et al. 1994). In pigeonpea, for example, different cultivars exhibit markedly different thresholds for kanamycin-induced growth inhibition, emphasizing the need for genotype-specific optimization (Baburao and Bhat 2020). Differences in regeneration capacity, chloroplast physiology, and antibiotic uptake may alter the optimal selection threshold among cultivars. Therefore, the concentration identified here should be regarded as a robust reference for Hamria, while the experimental framework established in this study provides a transferable strategy for determining genotype-specific selection regimes in another lentil germplasm.

Several limitations should also be acknowledged. The experiments were performed under controlled conditions using a single genotype and evaluated seedling responses rather than regenerating explants. Although these conditions enabled precise characterization of kanamycin phytotoxicity, future studies should validate the proposed selection threshold during *Agrobacterium-mediated* transformation and determine its applicability across diverse lentil genotypes and regeneration systems. It should be noted that the three application methods differed not only in delivery mode but also in growth environment and tissue developmental stage: in vitro explants were cultured under controlled photoperiod and light intensity, whereas foliar spray and microinjection were applied to intact seedlings under greenhouse conditions. The marked differences in baseline (0 mg/L) TPC among methods likely reflect this combination of factors rather than delivery mode alone, and cross-method comparisons of absolute TPC values should therefore be interpreted with caution; comparisons within each method across concentrations remain robust.

Overall, these findings reinforce previous recommendations that kanamycin selection must be optimized for each transformation system while demonstrating that delivery strategy represents an additional determinant that should be considered during protocol development (Ricci et al. 2023; Moratti et al. 2026). Continuous in vitro exposure generated the highest discriminatory power, enabled objective estimation of TR_{50} , and provided the most reliable basis for defining an efficient selection regime. Beyond lentil, these findings highlight the importance of integrating delivery strategy with physiological and biochemical characterization when optimizing selectable marker systems for genetic transformation in recalcitrant crop species.

Conclusion

This study demonstrates that the effectiveness of kanamycin as a selective agent in lentils is governed not only by the antibiotic concentration but also significantly by the mode of delivery. Among the strategies evaluated, continuous *in vitro* exposure provided the highest discriminatory power, enabling the establishment of a clear tissue response threshold (TR50) estimated at 101.8 mg/L. Consequently, a concentration of 100 mg/L is recommended as the optimal selection pressure for *in vitro* transformation protocols of *Lens culinaris* cv. Hamria. In contrast, transient *in vivo* methods, including foliar spray and microinjection, exhibited saturation-type responses that limited their capacity to achieve equivalent selection stringency.

The physiological analysis confirms that visual chlorosis and SPAD measurements are highly sensitive and reliable indicators of kanamycin-induced chloroplast dysfunction, whereas plant height is a less suitable marker for early selection. While these results establish a robust technical reference using wild-type tissues, it is important to emphasize that these parameters await final validation within a functional transformation system to confirm the successful recovery of transgenic lines. Overall, this research provides a vital physiological framework for optimizing selection regimes, offering practical guidance to overcome the recalcitrance of lentils and other legume species in genetic transformation and genome editing applications.

Declarations

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Ethics declaration: Not applicable.

Conflicts of Interest:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contribution

Imane Sarroukh conceived and designed the study, performed the experiments, collected and analyzed the data, interpreted the results, and wrote the first draft of the manuscript. Ahmed Mahmoudi and Boubakar Thierno Dia contributed to the experimental design, data interpretation, and critical revision of the manuscript. Mohammed Ibriz and Rabha Abdelwahd supervised the research, critically revised the manuscript, and approved the final version. Outmane Bouhlal, Ghizlane Diria, Driss Iraqi, and Fatima

Gaboun contributed to data interpretation and critically reviewed the manuscript. All authors read and approved the final manuscript.

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Figures

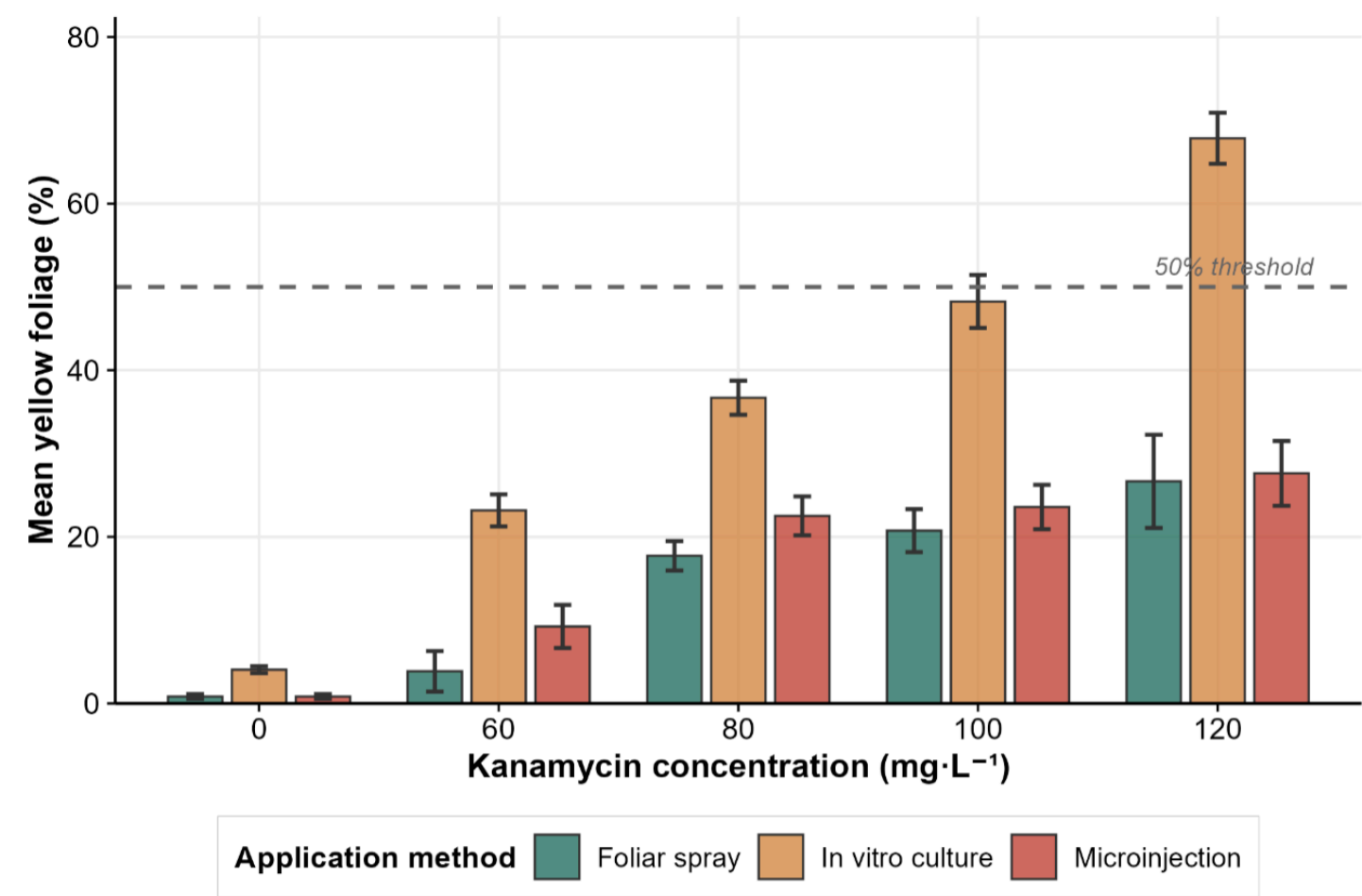


Figure 1

Effect of kanamycin sulfate concentration on yellowing rate (%) of wild-type *Lens culinaris* Medik. (cv. Hamria) under three application methods. (A) Foliar spray; (B) Microinjection; (C) In vitro culture.



Figure 2

Overview of lentil kanamycin sensitivity assays. (a) Germination of untreated control seedlings; (b) Representative CADE explants following four weeks of in vitro culture on MS medium supplemented with kanamycin. Left: explants cultured at 60 mg/L kanamycin showing active shoot regeneration and maintained green tissue. Right: explants cultured at 120 mg/L kanamycin exhibiting complete growth inhibition and extensive tissue necrosis.; (c) Foliar application assay across kanamycin concentrations;

(d) Chlorosis development in treated leaves by foliar spray; (e) Micro-injection assay; (f) Representative leaf yellowing following kanamycin exposure by micro-injection.

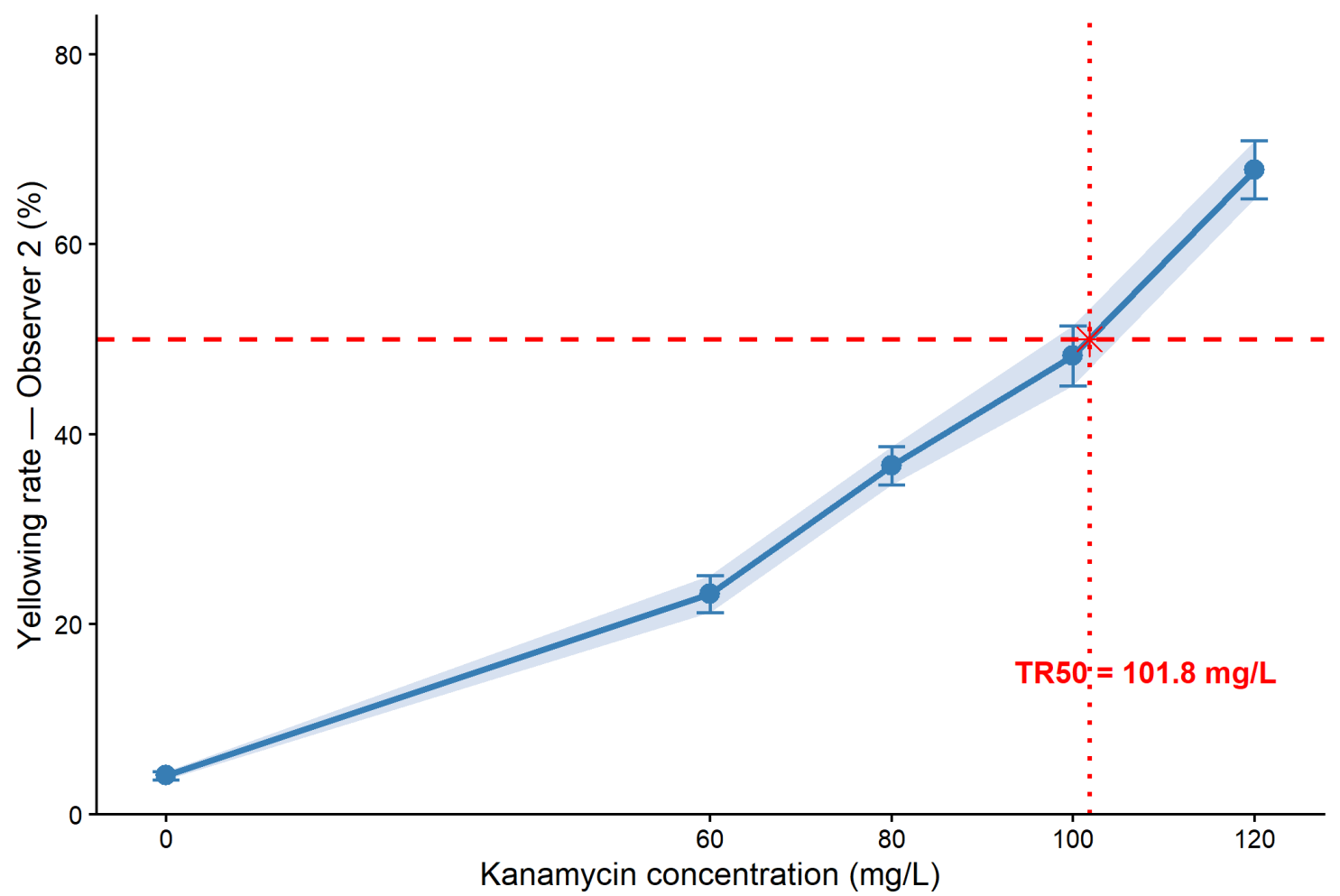


Figure 3

Dose-response curve for in vitro kanamycin sensitivity in wild-type *Lens culinaris* Medik. (cv. Hamria).

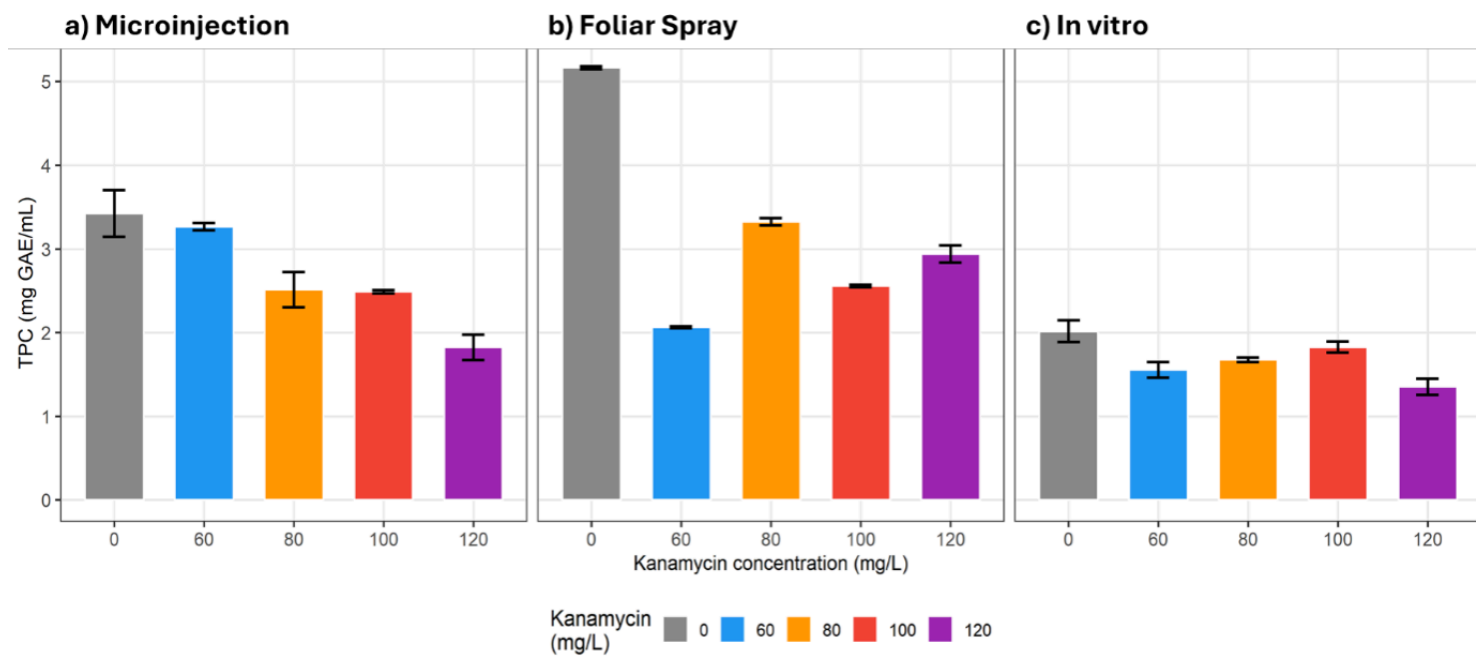


Figure 4

Effect of kanamycin concentration on TPC across three application methods (bar chart, Mean $\pm$ SE)