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Responses of *Nicotiana tabacum* L. Wild-Type to Selection and Elimination Antibiotic Concentrations During Genetic Transformations

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ABSTRACT

Agrobacterium-mediated plant transformation requires antibiotics to screen transgenic cells and eradicate residual bacteria after co-cultivation. Both treatments induced phytotoxic effects that impaired the regeneration of the explants. This study determined the optimal minimum concentrations of antibiotics for the selection and elimination of wild-type *Nicotiana tabacum* L. (West Java accession) using the pBI121 vector containing the kanamycin-resistance gene (*nptII*). Kanamycin antibiotic sensitivity testing identified 100 ppm as the minimum effective selection concentration, which induced necrosis in all non-transformant explants. A comparative assessment of two broad-spectrum β-lactam antibiotics, augmentin and cefotaxime, was conducted based on three parameters: bacterial contamination rate, shoot height, and root induction percentage. The results indicated that 300 ppm of augmentin demonstrated the best performance. The results demonstrated a 0% contamination rate, accompanied by notably lower phytotoxicity than cefotaxime. These findings establish an optimized antibiotic protocol for efficient *N. tabacum* transformation with minimal tissue damage.

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1. INTRODUCTION

Genetic manipulation in plants can be achieved through both conventional and modern approaches by inserting DNA into plant tissues (transformation). This enables the transfer of genes between plant species or from cross-kingdom organisms, such as viruses, bacteria, and animals into a host genome to obtain desired traits or products (Wang *et al*., 2025). In general, the main goals of genetic transformation are to study gene activity and expression regulation, regenerate identical transgenic plants, and engineer new agronomic traits. Plant transformation techniques are categorized into direct methods, including particle bombardment and electroporation, and indirect methods that employ biological intermediaries, such as the *Agrobacterium* genus or viruses (Wang *et al*., 2025).

Nicotiana tabacum L. has historically served as a fundamental model organism for studying plant transformations. It was among the first plant species to be successfully transformed through *Agrobacterium*-mediated methods, and its highly competent regenerative capacity from leaf disc explants has established it as a canonical benchmark for protocol development in numerous subsequent studies (Niedbała *et al*., 2021). Furthermore, *N. tabacum* has been broadly adopted as a positive control in comparative transformation experiments across diverse plant families, reinforcing its indispensable role in the standardization of transformation protocols with direct applicability to economically important crops (Hwang *et al*., 2017).

Indirect transformation using *A. tumefaciens* has become the gold standard in plant genetic engineering. This method is highly preferred because of its superior effectiveness, stable integration of DNA into the host genome, and cost-effectiveness (Goralogia *et al*., 2025). This method utilizes the inherent mechanism of *A. tumefaciens* to facilitate the transfer of genetic material from prokaryotic to eukaryotic organisms. In nature, this ability is facilitated by the presence of a tumor-inducing (Ti) plasmid, which triggers crown gall formation at wound sites in various dicotyledonous plants. The bacterium transfers the T-DNA segment from the Ti plasmid to integrate into the host cell nucleus with a low copy number, resulting in more stable gene expression and minimizing gene silencing (Gelvin, 2021).

High transformation efficiency is essential for constructing an accurate genetic expression system, necessitating the use of standardized protocols to optimize regeneration yield. Transformation efficiency variations depend heavily on multiple factors, including the *Agrobacterium* strain, plasmid type, in vitro environmental conditions, phenolic compound exudation, wounding method, infection duration, selection markers, and cellular competence of the explant (Du *et al*., 2022; Wang *et al*., 2025). One of the most crucial stages of this protocol is the optimization of antibiotic use during the in vitro culture.

In *A. tumefaciens*-mediated transformation, plant explants must be selected on a solid medium containing specific antibiotics corresponding to their resistance genes. Furthermore, explants require antibiotic treatment to eliminate residual *A. tumefaciens* cells after the co-cultivation phase. Consequently, optimizing the type and concentration of antibiotics, for both selection and elimination agents, is highly critical. The effectiveness and phytotoxicity levels of antibiotics vary widely depending on the bacterial strain, binary vector, host species, explant tissue type, nutrient media composition, and selection agent itself (Du *et al*., 2022). This variability necessitates species-specific and often genotype-specific calibration of antibiotic regimens, as concentrations proven effective and non-toxic in one host system may either fail to suppress bacterial overgrowth or impose excessive phytotoxic pressure in another (Azizi-Dargahlou & Pouresmaeil, 2024). While the phytotoxic

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effects of selection and elimination antibiotics are well-documented across various species, systematic comparative data for wild-type *N. tabacum* accessions derived from Indonesian landraces remain absent from the literature. This gap is particularly consequential, given that minor genotypic and ecotypic differences within a species can substantially alter explant sensitivity to antibiotic stress, ultimately influencing organogenesis potential and overall transformation yield (Varlamova *et al*., 2021). Addressing this gap, the present study aimed to determine the optimal and minimal concentrations of both selection and elimination antibiotics, specifically evaluating their physiological impacts on wild-type *N. tabacum* L. explants sourced from West Java, Indonesia.

2. METHODS

2.1. Plant Material and Bacterial Strains

This study utilized leaf explants from 4–5-week-old *in vitro* cultures of wild-type tobacco (*Nicotiana tabacum* L.) sourced from a local plantation in West Java, Indonesia. *Agrobacterium tumefaciens* strain GV3101, harboring the pBI121 plasmid, was used as the transformation vector. The plasmid contains a T-DNA sequence that encompasses the neomycin phosphotransferase (*nptII*) gene, which serves as a selectable marker that provides resistance to kanamycin. Optimization of the post-cocultivation elimination stage used two broad-spectrum β-Lactam antibiotics: augmentin and cefotaxime.

2.2 Surface Sterilization and Micropropagation

Tobacco plantlet multiplication and surface sterilization were performed by adapting the standardized micropropagation protocols described by Bakhsh (2020) and Hesami and Jones (2020). Leaves from healthy donor plants were washed under running water for 30 min. Surface sterilization was conducted inside a laminar airflow cabinet by submerging the leaves in a commercial bleach solution (containing 2.6% active NaOCl) at a 50% v/v concentration, enriched with 1–3 drops of Tween 20. The leaves were immersed in an Erlenmeyer flask for 5–7 min until the leaf margins appeared white. The sterilant residue was then removed through 3–4 rinses using sterile deionized water until no foam remained. The leaves were air-dried on sterile filter paper in petri dishes. Once dried, the leaves were cut into 1 × 1 cm pieces using a sterile scalpel and subcultured onto solid Murashige and Skoog (MS) basal growth medium supplemented with 1 ppm BAP and 0.1 ppm IAA for shoot induction. The cultures were maintained in an incubation room at 25°C under a 16-h light/8-h dark photoperiod.

2.3 Preparation of *A. tumefaciens* GV3101 Culture

A single colony of *A. tumefaciens* GV3101 carrying the pBI121 plasmid (CaMV35S construct) was inoculated into solid YEP medium supplemented with 50 ppm rifampicin and 50 ppm kanamycin. As a control, the GV3101 strain without the plasmid was grown on a medium containing only 50 ppm rifampicin. All bacterial cultures were incubated at 25°C in the dark for 48 hours prior to infection (Piao *et al*., 2025).

2.4. Explant Sensitivity Test to Selection Antibiotics

A kanamycin sensitivity assay was conducted to determine the minimum tolerance threshold of non-transformed tobacco explants to kanamycin, thereby preventing the survival of "escape" plants during selection. Following a modified selection optimization protocol, 1 × 1 cm leaf explants were inoculated onto solid MS medium containing shoot-inducing hormones, as described in Section 2.2. Kanamycin was added to the medium at

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concentrations of 0 ppm (control), 50 ppm, 100 ppm, 150 ppm, and 200 ppm (Bhatt *et al*., 2021). Explant viability and necrosis rates were observed for one month to determine the optimum concentration.

2.5 Optimization of Elimination Antibiotic Concentrations

Exposure to antibiotics significantly affects both bacterial overgrowth rates and tissue morphogenesis. To determine the efficacy of elimination agents without compromising plant regeneration, infected explants were tested using two types of antibiotics based on recent phytotoxicity evaluation models (Chauhan & Modgil, 2016; Du *et al*., 2022): (1) Augmentin at 200, 300, and 400 ppm; and (2) cefotaxime at 300, 400, and 500 ppm. The observed parameters included bacterial contamination percentage, root induction percentage, and average shoot height, which were evaluated periodically over 14 days after treatment.

2.6 Genetic Transformation Procedure (Leaf Disc Method)

Genetic transformation was performed using a modified leaf disc method (Hwang *et al*., 2017; Xia *et al*., 2023), comprising three main stages. (1) Inoculum Preparation: A single colony of *A. tumefaciens* GV3101 was inoculated into 5 mL of liquid YEP medium (supplemented with 50 ppm rifampicin and 50 ppm kanamycin). This precursor culture was incubated at 25°C with 200 rpm agitation in the dark for 24 hours. Subsequently, 1% of the precursor culture volume was subcultured into 50 mL of liquid YEP medium under identical antibiotic conditions and incubated until it reached an optical density (OD600) of 0.7. The culture was then centrifuged at 6,500 × g for 5 min. The supernatant was discarded, and the bacterial pellet was resuspended in 50 mL of infiltration medium (liquid ½ MS medium with 2% sucrose). (2) Infection and Co-cultivation: Leaf explant segments (1 × 1 cm) were submerged in an Erlenmeyer flask containing the bacterial inoculum suspension. The infection process lasted for 1 h at 25°C in the dark with continuous agitation at 200 rpm. Afterward, the explants were blotted dry using sterile filter paper and transferred to solid MS medium enriched with 100 µM acetosyringone. Co-cultivation was carried out for 3 days at 25°C in the dark. (3) Bacterial Elimination and Establishment: Following co-cultivation, the explants were washed repeatedly (three times) with 50 mL of liquid ½ MS medium for 5 min. The washing phase continued using liquid ½ MS medium supplemented with the elimination antibiotic (based on the optimized concentration) with gentle agitation for 15 min. Once air-dried, the explants were planted on solid MS medium containing shoot-inducing hormones along with the selection agent (kanamycin) and elimination antibiotic. Cultures were incubated at 25°C under a 16-h photoperiod.

2.7 Data analysis

All *in vitro* transformation and antibiotic optimization experiments were performed using a completely randomized design (CRD). Each treatment consisted of three biological and three technical replicates, with 15 explants per replicate. The collected data were analyzed using Analysis of Variance (ANOVA) and *Duncan's* Multiple Range Test (DMRT) at a significance level of $p < 0.05$. All statistical calculations were performed using SPSS Statistics (IBM Corp., Armonk, NY, USA).

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3. RESULTS AND DISCUSSION

3.1. Sensitivity Test of Explants to Kanamycin Selection Agent

Kanamycin sensitivity assay was conducted to establish the tolerance threshold of non-transformed tobacco explants. One-way ANOVA revealed a significant effect of kanamycin concentration on explant development percentage ($F_{4,10} = 3{,}376.00$; $p < 0.001$), confirming that antibiotic selection pressure influenced on explant viability across all tested concentrations. DMRT analysis identified three distinct homogeneous groups among the five concentration levels (Table 1).

Table 1. Explant viability percentage across varying kanamycin concentrations

Kanamycin Concentration (ppm)	Explant Development Percentage (%)
0	100.00 ± 0.00^{a}
50	26.67 ± 2.88^{b}
100	0.00 ± 0.00^{c}
150	0.00 ± 0.00^{c}
200	0.00 ± 0.00^{c}

In Figure 1, explants cultured on media without kanamycin (0 ppm control) remained viable and successfully differentiated to form shoots optimally (100.00 ± 0.00%). Kanamycin exposure at 50 ppm suppressed shoot formation capacity to a mere 26.67 ± 2.88%, indicating that this concentration is insufficient for complete selection of non-transformants. Increasing the concentration to 100 ppm showed no shoot growth during the observation period. Explants began to exhibit chlorosis, indicated by a whitish-green color change. More severe selection pressure at 150 and 200 ppm triggered massive cellular necrosis, in which the leaf tissue died and became transparent. The three highest concentrations (100, 150, and 200 ppm) showed statistically equivalent lethal effects, but this confirms 100 ppm as the minimum effective dose and not just a partial inhibitor.

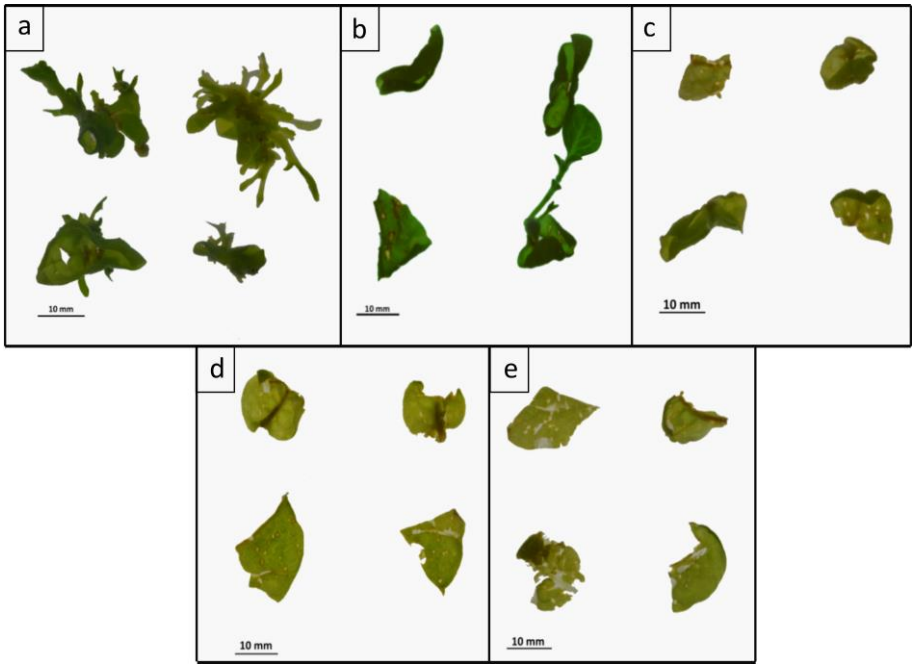

Figure 1. Phytotoxic responses of tobacco leaf explants to kanamycin. (a. 0 ppm/control; b. 50 ppm; c. 100 ppm; d. 150 ppm; e. 200 ppm).

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The severity of necrosis correlating with dose escalation is a direct effect of kanamycin. Kanamycin is an aminoglycoside antibiotic that functions by binds to the 30S ribosomal subunit, thereby blocking protein translation initiation within the plastids (Su *et al*., 2026). In genetic transformation protocols, the optimal selection agent concentration is the lowest level capable of slowly eliminating all non-transformed tissues (Du *et al*., 2022). Utilizing concentrations beyond this threshold severely suppresses the regeneration percentage of transformed cells (Poormassalehgoo *et al*., 2025). These findings indicate that a concentration of 100 ppm was identified as the most appropriate selection dose. This decision aligned with the standard *N. tabacum* transformation protocols recommended by Niedbała *et al*. (2021). The successful regeneration of transformant explants on this selective medium was confirmed by the presence of the *nptII* gene, which encodes neomycin phosphotransferase. This enzyme neutralizes the toxic effects of aminoglycosides by transferring a phosphate group from ATP to the antibiotic molecule, eliminating its affinity for the ribosome, and allowing protein synthesis to proceed normally (Su *et al*., 2026).

The concentration-dependent suppression of explant viability recorded in the present study is consistent with analogous sensitivity assays conducted in other dicotyledonous plant systems. In kiwi fruit (*Actinidia deliciosa),* kanamycin at a concentration 50 mg L^{-1} was established as the effective selection threshold, with only kanamycin-resistant (Km+) shoots surviving eight weeks of culture on a selective medium (Piao *et al*., 2025). Similarly, a stringent lethal threshold of 100 mg L^{-1} was established for rubber tree (*Hevea brasiliensis*) somatic embryo explants, where untransformed tissues underwent complete necrosis, while transformed cells exhibited vigorous secondary growth (Udayabhanu *et al*., 2022). These comparative data reinforce the principle that kanamycin exerts species-specific lethal effects on non-transformed tissues at threshold concentrations, rendering it a broadly applicable selection agent for aminoglycoside-based screening in dicotyledonous plant transformation systems. The results also underscore the necessity of conducting sensitivity assay standardization for each new host genotype and accession prior to full-scale transformation trials, as inter-accession differences have been documented to produce substantially different lethal threshold values, even within the same species (Azizi-Dargahlou & Pouresmaeil, 2024).

3.2. Optimization of *Agrobacterium tumefaciens* Elimination Antibiotics

The successful regeneration of transgenic plants post-cocultivation relies heavily on the balance between the antibiotic's ability to suppress bacterial overgrowth (bactericidal property) and its impact on host tissue morphogenesis (phytotoxicity). A comparative evaluation between augmentin and cefotaxime over a 14-day observation period revealed highly contrasting efficacy profiles (Figure 2). Augmentin treatment exhibited superior bacterial suppression capacity. Although contamination still occurred at 200 ppm (17.78%), increasing the dose to 300 ppm and 400 ppm successfully eradicated the bacteria completely (0% contamination) and showed no statistically significant difference compared to the uninfected control. On the other hand, cefotaxime demonstrated weaker bactericidal capabilities. At 300 ppm, the contamination rate remained exceptionally high (26.11%). Cefotaxime was only able to suppress bacterial overgrowth to 2.23% when the dose was elevated to 500 ppm.

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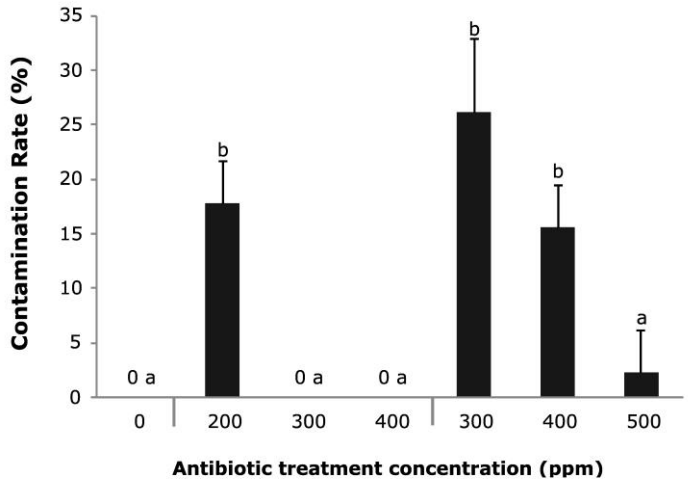

Figure 2. Contamination percentage of *A. tumefaciens* GV3101 across various antibiotic elimination treatments.

In addition to bactericidal capacity, the impact of antibiotics on explant organogenesis is a definitive parameter. Observations on nodal explants revealed that 200 ppm augmentin facilitated the best root induction (20%), which was statistically equivalent to that of the control group. Although the percentage decreased to 4.44% at 300 and 400 ppm, it remained significantly superior to the tissue responses observed with cefotaxime. The severe phytotoxic effects of cefotaxime were starkly visible at 500 ppm, where root organogenesis was completely halted (0%) throughout the observation period (Figure 3).

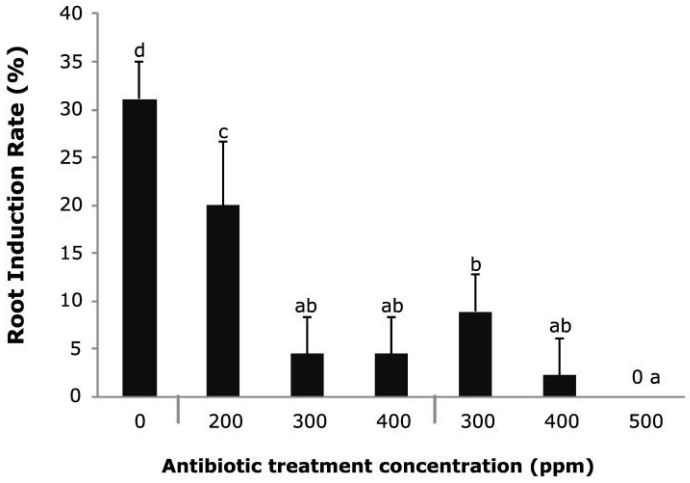

Figure 3. Root induction rates of transformant explants under elimination antibiotic pressure

Growth suppression by cefotaxime was also reflected in the average shoot elongation (Figure 4). All cefotaxime concentration variants significantly depressed shoot height compared to the control, peaking at 0.83 cm (300 ppm). Tissues exposed to high doses of cefotaxime exhibited massive browning, which significantly delayed the regeneration process and often resulted in explant death. This phytotoxicity aligns with recent experimental evidence demonstrating that cephalosporin-class antibiotics can severely inhibit in vitro morphogenetic responses and shoot organogenesis in dicotyledonous systems (Varlamova *et al*., 2021). Conversely, augmentin proved to be far less detrimental,

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maintaining a much more stable shoot elongation rate. Explants treated with 200 ppm augmentin achieved an average shoot length of 2.6 cm, showing no significant difference from the untreated control (3.3 cm). Even at a higher, more rigorous elimination dose of 300 ppm, the shoots managed to grow to 2.1 cm. This favorable growth response reinforces recent optimized transformation protocols, demonstrating that clavulanate-based antibiotics like augmentin can aggressively eradicate *A. tumefaciens* while safely preserving the explant's natural vigor (Hwang *et al*., 2017).

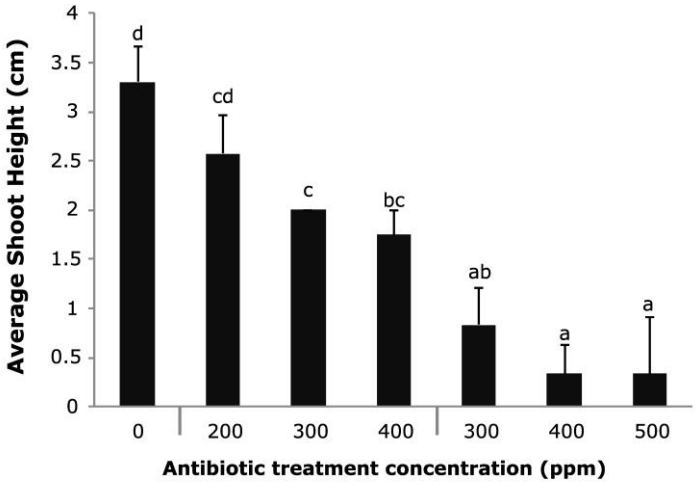

Figure 4. Shoot induction rates of transformant explants under antibiotic pressure elimination

From the standpoint of practical protocol design, the present findings advocate a clear preference for augmentin over cefotaxime in GV3101-mediated transformation of *N. tabacum*. The significantly superior performance of augmentin across all three evaluated parameters — contamination suppression, root induction, and shoot elongation — provides a comprehensive multi-parameter justification that transcends single-criterion comparisons commonly reported in older transformation literature. Indeed, recent advances in *Agrobacterium*-mediated transformation emphasize that a holistic regeneration environment, encompassing not only bacterial elimination but also the maintenance of tissue morphogenic competence throughout the post-cocultivation phase, is an equally critical determinant of final transgenic plant recovery rates (Du *et al*., 2022; Hwang *et al*., 2017). The documented ability of augmentin at 300 ppm to achieve complete bacterial clearance while sustaining meaningful shoot growth (2.0 cm) and preserving residual root organogenesis capacity (4.44%) positions it as a superior elimination agent for incorporation into standardized *N. tabacum* transformation protocols targeting international-quality transgenic plant production.

The remarkable efficacy of augmentin stems from a targeted synergy between amoxicillin and clavulanic acid. While *Agrobacterium* often circumvents standard penicillins by deploying β-lactamase enzymes, clavulanic acid neutralizes this defense by binding directly to the β-lactamases, thereby preserving amoxicillin's structural integrity (Tooke *et al*., 2019). Once protected, the β-lactam antibiotic is able to safely reach and block penicillin-binding proteins (PBPs) on the bacterial plasma membrane, effectively halting cell wall synthesis and triggering rapid cell lysis (Bertonha *et al*., 2023). Because this mode of action relies entirely on disrupting prokaryotic-specific structures—namely, the peptidoglycan layer—augmentin remains highly selective. It aggressively clears the bacterial infection while inducing a

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comparatively lower phytotoxic burden on the plant's eukaryotic tissues (Varlamova *et al*., 2021).

3.3 Comparative Phytotoxicity Profile: Multivariate and Mechanistic Perspectives on Augmentin and Cefotaxime

Principal component analysis (PCA) captured 93.9% of the dataset's total variance, with the resulting biplot (Figure 5) clearly projecting the structural differences between treatments. PC3 explained only 6.1% of the remaining variance and was excluded from further interpretation. PC1 (57.5%) represents the 'tissue regeneration' gradient, driven strongly by root induction and shoot height. Meanwhile, PC2 (36.4%) captures the trade-off between bactericidal efficacy and phytotoxicity, primarily dictated by contamination rates. This inverse dynamic confirms that post-cocultivation bacterial overgrowth directly suppresses explant morphogenesis. This suggests that high contamination and high growth capacity are partially mutually exclusive within this antibiotic system. This pattern aligns with the well-documented phenomenon where post-cocultivation bacterial overgrowth suppresses explant morphogenesis (Du *et al*., 2022).

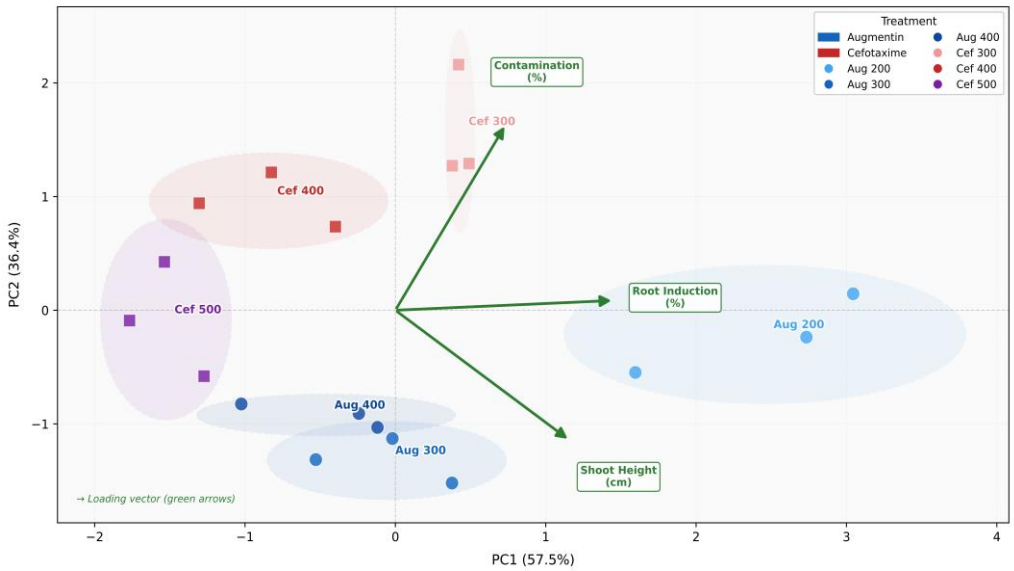

Figure 5. PCA analysis of antibiotic disinfection effect on *N. tabacum* L. Wild-type

The score plot revealed distinct treatment clusters. Augmentin 300 ppm occupied the optimal multivariate space, achieving zero contamination while maintaining robust regeneration (2.00 cm shoot height; 4.44% root induction). Although augmentin 200 ppm showed superior growth, its residual contamination (17.78%) disqualified it as a reliable elimination agent. Conversely, all cefotaxime treatments clustered in the negative PC1 region, indicating consistently poor composite growth. The application of cefotaxime at a concentration of 300 ppm resulted in the highest level of contamination (26.11%) and significant growth inhibition. Although increasing the dosage to 400–500 ppm decreased contamination levels, it entirely eliminated the ability of the tissue to regenerate.

The severe phytotoxicity of cefotaxime is rooted in its chemical degradation during *in vitro* culture into secondary metabolites, such as desacetylcefotaxime. Although augmentin and cefotaxime belong to the β-lactam family, which eliminates bacteria by binding to transpeptidase enzymes (PBPs) to block peptidoglycan cross-linking (Bertonha *et al*., 2023), their safety profiles for plant tissues are drastically different. Cefotaxime has been shown to

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exert an inhibitory effect on callus induction and shoot organogenesis in plant tissue culture, in direct contrast to the stimulatory effects observed with amoxicillin-based antibiotics, including augmentin (Varlamova *et al*., 2021). This metabolic interference leads to massive tissue browning, stunted shoot elongation, and complete failure of root organogenesis observed at concentrations of 300 ppm and above. This explains why all cefotaxime treatments cluster densely in the low-PC1 region of the biplot, regardless of their actual contamination status.

In contrast, augmentin uses a synergistic mechanism in which clavulanic acid protects amoxicillin from bacterial enzymes, allowing complete bacterial elimination at lower, safer doses. This strategy prevents enzymes from destroying amoxicillin, significantly extending its stability and spectrum of action during tissue culture (Tooke *et al*., 2019). This combination of properties explains why 300 ppm augmentin maintained significant tissue regeneration (shoot height 2.00 cm; root induction 4.44%) while achieving zero contamination. This was also evident from the unique and favorable biplot position. Ultimately, the PCA biplot confirmed that 300 ppm augmentin was the ideal treatment, achieving the theoretically ideal PC space position: low PC2 (zero contamination) combined with a high PC1 (preserved tissue regeneration capacity). This explanation demonstrates how 300 ppm augmentin concurrently meets the criteria for both bactericidal activity and phytotoxicity (Aliu *et al*., 2024; Goralogia *et al*., 2025; Hwang *et al*., 2017).

4. CONCLUSION

Based on the research findings, the optimum minimum concentration of kanamycin for selecting post-transformation *Nicotiana tabacum* L. wild-type explants was 100 ppm. The use of augmentin at a concentration of 300 ppm during the elimination stage was significantly more effective than cefotaxime. The treatment effectively reduced bacterial contamination to 0% while exhibiting minimal phytotoxicity, although it marginally decreased the root induction rate. This was evidenced by the significantly higher average shoot elongation in regenerants and minimal risk of tissue necrosis compared to the use of cefotaxime. Principal Component Analysis (PCA) biplots confirmed that augmentin 300 ppm was the only treatment that simultaneously achieved near-zero contamination and significant tissue regeneration capacity across all three response parameters.

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6. AUTHORS' NOTE

The authors declare that there is no conflict of interest regarding the publication of this article. Authors confirmed that the paper was free of plagiarism.

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