



Original Research Paper

Optimisation of Plant Growth Regulator Mediated In Vitro Shoot Culture of *Solanum nigrum* by Managing Endophytic Bacterial Infection

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Key Words	Abstract
<i>In Vitro Shoot Culture, Plant Growth Regulators, Endophytic Bacteria, Latent Contamination, Solanum Nigrum, 6-BAP, Indole-3-Butyric Acid, Levofloxacin, Antibiotic Phytotoxicity, Micropropagation</i>	Micropropagation of field-collected medicinal plants is frequently defeated by endophytic bacteria that occupy internal tissue, survive surface disinfection and emerge only after the culture appears to have been established. This study optimised plant growth regulator (PGR) mediated in vitro shoot culture of <i>Solanum nigrum</i> L. while simultaneously managing the endophytic bacterial infection that limited it. Nodal explants were surface-sterilised with bavistin (250 µg mL ⁻¹) at four immersion durations and cultured on Gamborg B5 medium supplemented with 6-benzylaminopurine (6-BAP) and indole-3-butyric acid (IBA) in three combinations against a hormone-free control. Ten minutes in bavistin gave the most workable balance between asepsis (88%) and explant survival (59%); longer exposure raised asepsis to 97% on tissue of which only 4% survived. All PGR treatments produced significantly higher regeneration than the control ($\chi^2 = 27.94$, df = 3, $p < 0.001$), with 6-BAP 0.5 mg L ⁻¹ inducing leaves (68.75%), 6-BAP 0.5 + IBA 1.0 mg L ⁻¹ inducing roots (81.25%; mean 12 roots per explant, maximum length 5.2 cm) and 6-BAP 2.0 + IBA 2.0 mg L ⁻¹ inducing callus whose texture depended on explant origin (friable from nodal segments, 75%; soft and chunky from in vitro-derived roots, 41.70%). The PGR treatments did not differ from one another in response frequency ($p \geq 0.685$), indicating that the auxin-to-cytokinin ratio governs the morphogenic pathway rather than the probability of response. Liquid medium supported no growth. Bacterial clouding appeared at the explant-medium interface only after 25 days, confirming an endogenous rather than an environmental origin; the isolate was a motile, cluster-forming, short Gram-negative rod. Agar-well diffusion across six antibiotic classes showed greatest susceptibility to levofloxacin (38 mm at 100 mg L ⁻¹ ; 30 mm at 50 mg L ⁻¹), followed by amikacin and cefotaxime sodium. In culture (n = 100 per treatment), however, the ranking inverted: levofloxacin at 50 mg L ⁻¹ gave the highest yield of viable, colony-free shoots (49% against 2% untreated; $\chi^2 = 70.38$, df = 4, $p < 0.001$) at a phytotoxicity index of 3.9%, whereas levofloxacin at 100 mg L ⁻¹ cleared more cultures (66%) but weakened 43.9% of those it cleared ($p < 0.001$). Amikacin under-performed cefotaxime despite a larger inhibition zone. The study concludes that antibiotic selection for tissue culture must be based on usable yield rather than susceptibility ranking, and that endophyte indexing should precede rather than follow PGR optimisation in field-collected medicinal species.

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1. Introduction

Solanum nigrum L. (Solanaceae), known as black nightshade or kakamachi, is a short-lived herb of disturbed ground, roadsides and field margins across the semi-arid and sub-humid tracts of India. It occupies an unusual position in the medicinal flora: it is simultaneously a common weed, a leafy vegetable in several regional cuisines, and a recognised drug in Ayurvedic and folk practice. Preparations of the leaves and berries are used for hepatic disorders and jaundice, root extracts for whooping cough and asthma, and whole-plant infusions for dysentery, fever and inflammatory skin conditions.

Modern pharmacological investigation has largely borne out this traditional record. A recent comprehensive review identified 188 chemical constituents from the species, with steroidal saponins, alkaloids, phenolics and polysaccharides as the principal bioactive classes, and documented antitumour, anti-inflammatory, antioxidant, antibacterial and neuroprotective activity in both *in vitro* and *in vivo* systems (Chen et al., 2022). The most current synthesis, covering literature to 2025, confirms alkaloids as the dominant chemical class and adds antimalarial, hepatoprotective and gut-flora-regulating activities, while explicitly identifying quality control as the field's outstanding weakness (Wang et al., 2025).

That weakness is where plant tissue culture becomes relevant. Supply of *S. nigrum* raw drug still depends largely on wild collection, which produces material of highly variable composition: alkaloid content shifts with phenology, habitat and chemotype, and substitution or adulteration with related *Solanum* species is a recurring problem in the Ayurvedic raw-drug trade. Clonal micropropagation addresses these difficulties simultaneously. It fixes a selected genotype, decouples supply from season and collection site, and provides a controlled platform on which secondary metabolite production can be studied and manipulated. Several regeneration protocols for the species have been published using leaf, nodal, petiole, hypocotyl and root explants across a range of cytokinin–auxin combinations (Hassanein & Soltan, 2000; Bhat et al., 2010; Zou et al., 2017).

What these protocols share is an implicit assumption that surface disinfection yields an axenic culture. For explants taken from field-grown donor plants this assumption is unreliable. Every plant in nature carries a resident community of endophytic bacteria within its intercellular spaces and vascular tissue. In the field these organisms are frequently beneficial, contributing fixed nitrogen, phytohormones, siderophores and competitive exclusion of pathogens (Semenzato & Fani, 2024). Inside a culture vessel their role inverts. A nutrient-rich medium, the absence of microbial competitors and the absence of host defence allow populations that were latent *in planta* to expand rapidly, drawing on the same carbon source as the explant and releasing metabolites that alter the hormonal balance of the medium.

Because these bacteria occupy internal tissue, and in some cases the cell interior itself (Esposito-Polesi et al., 2017), they are untouched by ethanol, mercuric chloride or fungicidal dips, which act only at the explant surface. Leifert and Cassells (2001) emphasise that many such organisms escape initial sterilisation, remain latent through numerous subcultures and appear only after later transfer. The consequence is a characteristic delayed contamination profile. Contamination arising within the first three to ten days after inoculation generally reflects an environmental or handling failure; contamination arising after that window, often as clouding confined to the explant–medium interface, is diagnostic of endogenous origin (Ray & Ali, 2017). In many cases the bacterium produces no visible turbidity at all, and its presence is inferred only from arrested growth, tissue exudation, chlorosis or progressive browning. Thomas and Kumari (2010) described exactly this in papaya, where basal medium cloudiness closely resembling latex exudate proved on indexing to be endophytic bacteria.

This creates a specific methodological problem for PGR optimisation studies. If a cytokinin–auxin combination is scored as ineffective when the true cause of poor performance is a latent endophyte, the resulting protocol is an artefact. Thomas (2004b) demonstrated the general form of this error directly, showing that the progressive decline of long-maintained triploid watermelon cultures was caused

by a hidden load of covert bacteria rather than by genetic or nutritional factors. Conversely, an antibiotic added to suppress the endophyte may itself alter organogenesis. Many antibiotics used in plant tissue culture are phytotoxic at working concentrations, and the mechanism is well understood: plastids and mitochondria are of endosymbiotic prokaryotic origin, and Ellis (1969) showed that chloroplast ribosomes exhibit the same stereospecific sensitivity to chloramphenicol as bacterial ribosomes while cytoplasmic ribosomes do not.

Antibiotic selection therefore cannot rest on a susceptibility test alone. An agent producing a large inhibition zone on an agar plate may fail in culture because of poor uptake into plant tissue, degradation in warm or autoclaved medium, or unacceptable phytotoxicity at the dose required for suppression. Nauerby et al. (1997) demonstrated the breadth of this variation, finding that timentin enhanced tobacco shoot regeneration to 127% of control while carbenicillin reduced shooting frequency to 51–56%; Qin et al. (2017) found timentin essentially harmless to *Anthurium* across 50–500 mg L⁻¹ while four other cephalosporins caused chlorosis and leaf death. Effects are agent-, dose-, explant- and species-specific, and must be established empirically in the system of interest.

The present study was designed to address both problems together rather than sequentially. Its objectives were: (i) to optimise surface sterilisation and PGR combinations for *in vitro* shoot culture of field-grown *S. nigrum* on Gamborg B5 medium; (ii) to isolate and characterise the endophytic bacterium responsible for late-onset contamination and determine its susceptibility across six antibiotic classes; and (iii) to evaluate the performance of the most active agents within the culture system itself, measuring not only how effectively each clears the culture but what it costs the explant. Treating decontamination efficacy and phytotoxicity as two axes of a single decision, rather than optimising the first in isolation, is the central methodological contribution of this work.

2. Review of Literature

2.1 In vitro regeneration of *Solanum nigrum*

S. nigrum has been described as a model system for plant tissue and protoplast culture because of the ease with which it responds across media and explant types (Hassanein & Soltan, 2000). That study compared B5, MS and Schenk–Hildebrandt media and found the best shoot formation from stem internode segments on B5 medium supplemented with 0.5 mg dm⁻³ 6-BAP under a 16-hour photoperiod — a result that directly informs the medium and cytokinin concentration adopted here.

Bhat et al. (2010) achieved high-frequency direct shoot formation from leaves without an intervening callus phase, reporting 2.0 mg dm⁻³ 6-BAP with 1.5 mg dm⁻³ kinetin as optimal, 0.25 mg dm⁻³ IBA giving a maximum of 15.2 roots per plant, 100% field survival, and elevated solasodine content in regenerated shoots relative to *ex vitro* material. Zou et al. (2017) extended the comparison across five explant types, obtaining the highest shoot numbers from petioles (31.54 ± 5.76) and rooted hypocotyls (44.00 ± 1.51) at 100% induction on MS with 0.4 mg L⁻¹ IAA and 3.0 mg L⁻¹ zeatin. Kavitha et al. (2012) reported 100% shoot induction with a mean of 9.2 shoots per shoot tip on MS with 1 mg L⁻¹ 6-BA, while Padmapriya et al. (2011) found kinetin to outperform 6-BAP on nodal explants. Sridhar and Naidu (2011) established a callus-mediated regeneration route, and Park et al. (2020) demonstrated efficient regeneration and transformation from cotyledon explants.

Two points emerge from this body of work. First, reported optima differ substantially between studies, which is expected given differences in explant origin, donor-plant physiological state and basal medium, and which underlines that protocols cannot simply be transferred. Second, and more importantly for the present study, none of these reports addresses endophytic contamination, even though all used field- or glasshouse-derived starting material.

2.2 Auxin–cytokinin balance and the morphogenic pathway

The theoretical basis for PGR optimisation remains the framework established by Skoog and Miller (1957), who demonstrated that it is the quantitative balance between auxin and cytokinin,

rather than the absolute concentration of either, that directs organogenesis: a high auxin-to-cytokinin ratio favours root formation, the reverse favours shoot formation, and balanced high concentrations of both favour unorganised callus. Melnyk (2023) reappraised this framework in modern terms, codifying the root-, shoot- and callus-inducing media concepts and confirming that high auxin represses shoot formation while high cytokinin represses rooting.

Recent work has refined the mechanism. Pasternak and Steinmacher (2024) argue that cytokinin action depends on the competence of the target cell to generate endogenous auxin, and that medium composition together with the exogenous auxin-to-cytokinin ratio jointly determines the morphogenic outcome. Critically for the present study, they also emphasise that the developmental origin of the explant determines callus type — soft and friable versus compact and nodular — and therefore the downstream regeneration route available. Asghar et al. (2023) reach similar conclusions regarding dedifferentiation and competence. Friable callus is generally preferred for suspension culture and secondary metabolite work, which makes explant selection a decision with consequences well beyond the induction step itself.

2.3 Endophytic bacteria as covert contaminants

The systematic study of covert contamination in micropropagation rests on a small number of foundational contributions. Leifert et al. (1991) catalogued the range of contaminant organisms, their effects and their sources. Thomas (2004a) then established the three-step screening procedure that underlies most subsequent indexing work: visual inspection for clouding or exudate mimics, transfer of medium traces or tissue macerates to enriched bacteriological media, and microscopy or PCR for organisms that will not grow on standard media.

The scale of the hidden reservoir is substantial. Thomas et al. (2008) found that 10–60% of field banana shoot-tips yielded cultivable endophytes, at 10^2 – 10^5 colony-forming units per gram of fresh tissue, with the remainder producing no colonies yet testing positive by PCR with bacterial primers — evidence of a large population of normally non-

culturable organisms that gradually activate under *in vitro* conditions. Thomas (2004c, 2006) isolated ethanol-tolerant covert endophytes from grape, including a spore-forming Gram-negative *Brevibacillus* capable of surviving disinfection and, through residual spores, occasionally even autoclaving.

The most striking demonstration that an apparently clean culture is not necessarily sterile comes from Abreu-Tarazi et al. (2010), who applied culture-independent 16S rRNA PCR with denaturing gradient gel electrophoresis, excluding chloroplast interference, to pineapple microplants maintained for five years without any visible contamination. Organ-specific communities of Actinobacteria, Alphaproteobacteria and Betaproteobacteria were recovered from roots and from young and mature leaves. Esposito-Polesi et al. (2017) subsequently combined culture-dependent isolation, PCR-DGGE and electron microscopy across three laboratories, visualising abundant intracellular endophytic bacteria in leaf tissue and identifying *Bacillus*, *Acinetobacter* and *Methylobacterium* among the culturable fraction.

2.4 Antibiotic management and its limits

The closest methodological precedent for the present study is Szewczyk-Taranek et al. (2020), who worked on *Staphylea pinnata*. Two Gram-negative endophytes emerging as medium clouding were identified as *Acinetobacter johnsonii* and *Methylobacterium rhodesianum* using VITEK®2 and 16S rRNA sequencing; disc diffusion showed susceptibility to 13 of 25 antibiotics tested, drawn from the fluoroquinolone, aminoglycoside and tetracycline groups; and gentamicin at 100 mg dm^{-3} gave the best outcome, inhibiting bacterial growth by 63% over four weeks, after which shoots transferred to antibiotic-free medium developed axillary buds without visible colonies. Nadha et al. (2012) followed a comparable workflow for *Guadua angustifolia*, identifying *Pantoea agglomerans* and *P. ananatis* by 16S rRNA and incorporating kanamycin and streptomycin into the medium at 250–1000 ppm.

An important caution comes from Ray et al. (2017), who found that ampicillin used as a surface sterilant for *Bambusa balcooa* gave 80% bud break

with only 20% contamination, yet failed to control contamination at the multiplication stage. This is the clearest published statement of a principle central to the present discussion: antibiotics suppress endophytes rather than eliminate them, and recurrence on withdrawal or at later subculture should be expected unless demonstrated otherwise.

On phytotoxicity, the literature is consistent in showing that effects cannot be predicted by drug class. Young et al. (1984) eliminated bacteria from apple and rhododendron shoot cultures using a four-antibiotic combination without phytotoxicity. Grzebelus and Skop (2014) found cefotaxime and timentin essentially non-toxic to carrot protoplasts at 100–500 mg L⁻¹, permitting high-efficiency regeneration. Yet Qin et al. (2017) found cefoxitin, ceftazidime, cefonicid and ceftriaxone — all β -lactams, like cefotaxime — to prolong callus emergence and cause chlorosis and leaf death in *Anthurium*. Reed et al. (1998) documented the same variability in micropropagated hazelnut. Dose titration within the specific culture system is therefore not optional.

2.5 The dual role of endophytes

A complete treatment must acknowledge that the organisms causing these problems are not uniformly harmful. Zawadzka et al. (2013) inoculated *Curtobacterium pusillum*, *Paenibacillus glucanolyticus* and *Methylobacterium extorquens*, all isolated as non-deleterious contaminants, onto microshoots of chrysanthemum, gerbera, hosta and rose. None caused hypersensitivity or vitropathy; *C. pusillum* stimulated axillary shoot formation in all four genotypes; and all three produced indole-3-acetic acid and assimilated atmospheric nitrogen. Thomas (2004c) similarly reported a covert grape endophyte, *Bacillus pumilus*, that induced rooting. Semenzato and Fani (2024) and Guru et al. (2026) review the deliberate use of such organisms to improve micropropagation efficiency. The distinction that matters is therefore not between beneficial and harmful organisms but between controlled and uncontrolled colonisation: an unidentified population expanding without restraint compromises reproducibility whatever its intrinsic properties.

2.6 Research gap

Although the literature on *S. nigrum* regeneration is substantial, and the literature on covert endophytes in micropropagation is well developed, the two have not been brought together. A search of the published record located no study addressing endophytic bacterial contamination specifically in *S. nigrum* tissue culture, and none in the Solanaceae that integrates PGR optimisation with endophyte isolation, identification and chemical management within a single experimental programme. The present study addresses that gap, and in doing so proposes a decision criterion for antibiotic selection — usable yield rather than inhibition zone diameter — that is applicable well beyond this species.

3. Materials and Methods

3.1 Plant material and authentication

Whole mature plants of *Solanum nigrum* L. were collected from a roadside garden site in West Midnapore district, West Bengal, India. Botanical identity was authenticated at the Botanical Survey of India, Shibpur, Howrah, and a voucher specimen was deposited under reference number BT/BBK-01. Only healthy, disease-free plants at the vegetative to early-flowering stage were selected as donors, and nodal segments were excised from actively growing shoots so as to maximise the endogenous phytohormone content and meristematic competence of the explant.

3.2 Standardisation of the source material

To document donor quality and support reproducibility, whole-plant powder was evaluated for organoleptic and physicochemical parameters by standard pharmacopoeial procedures, using a hot air oven, muffle furnace and heating mantle. Loss on drying, total ash, acid-insoluble ash, water-soluble ash, and alcohol- and water-soluble extractive values were determined. Leaf micro-morphological constants — vein-islet number, stomatal index of both surfaces and palisade ratio — were recorded under an optical microscope on sections fixed in formaldehyde : glacial acetic acid : 70% ethanol (5:5:90). A preliminary phytochemical screen of the 90% ethanolic extract, prepared by three successive three-hour reflux cycles at a 1:10 powder-to-solvent ratio, was performed using

Dragendorff's, Hager's, Mayer's, Wagner's, Barfoed's, Fehling's and Millon's reagents.

3.3 Asepsis and glassware preparation

Glassware was cleaned in chromic acid prepared by dissolving 40 g potassium dichromate in 300 mL concentrated sulphuric acid, followed by detergent washing and thorough rinsing. Distilled water and culture accessories were autoclaved at 121 °C and 15 lb in⁻² for 20 minutes. The inoculation room was fumigated overnight with formaldehyde and potassium permanganate (2:1) before each work cycle.

3.4 Surface sterilisation

Excised shoots were washed in running tap water with detergent, then treated sequentially with amoxicillin (100 µg mL⁻¹, plant culture tested), bavistin (carbendazim, 250 µg mL⁻¹), 70% ethanol and 0.1% (w/v) mercuric chloride, with sterile distilled water rinses between steps. Because fungicide exposure time is the principal trade-off variable between asepsis and tissue injury, immersion duration in the bavistin step was varied across four treatments — T1 (1 min), T2 (5 min), T3 (10 min) and T4 (15 min) — with all other steps held constant. Explants were scored at 25 days for survival (green, turgid, non-necrotic tissue) and asepsis (absence of visible microbial growth).

3.5 Culture medium and PGR treatments

Gamborg B5 basal medium (Gamborg et al., 1968) was used throughout, prepared both with and without 2% (w/v) agar so that solid and liquid systems could be compared. pH was adjusted to 5.6–5.8 before autoclaving. Sterilised nodal segments were inoculated onto four treatments: T1, hormone-free control; T2, 6-BAP 0.5 mg L⁻¹; T3, 6-BAP 0.5 mg L⁻¹ + IBA 1.0 mg L⁻¹; and T4, 6-BAP 2.0 mg L⁻¹ + IBA 2.0 mg L⁻¹. In a parallel set, roots regenerated *in vitro* under T3 were excised and used as explants on T4 medium in order to compare callus character by explant origin. Sixteen explants were inoculated per treatment for nodal segments and twelve for *in vitro*-derived roots.

3.6 Culture conditions and growth parameters

Cultures were incubated at 25 ± 2 °C and 55–65% relative humidity under a 16-hour photoperiod of white fluorescent light at 45 µmol m⁻² s⁻¹ followed by 8 hours of darkness, and observed daily. Recorded parameters were days to first visible response, number of leaves induced per explant, number of roots induced per explant, maximum root length, and the colour and texture of any callus formed. Regeneration frequency was calculated at day 25 as the number of explants showing the response divided by the total number inoculated, expressed as a percentage.

3.7 Isolation and characterisation of the endophytic bacterium

Cultures were monitored for bacterial clouding, with particular attention to the zone where the basal cut surface of the explant contacted the medium. Material from this zone was streaked onto nutrient agar and incubated at 37 °C for 24–48 hours. A pure culture was obtained by repeated streak-plate subculture until colony morphology was uniform. The isolate was characterised by colony morphology, simple staining, Gram staining using crystal violet as primary stain with Gram's iodine as mordant, alcohol decolouriser and safranin as counterstain, and by a hanging-drop motility test.

3.8 Antibiotic susceptibility testing

Susceptibility was determined by the agar-well (cork borer) diffusion method on antibiotic assay medium no. 1 seeded with a standardised suspension of the pure isolate. Six antibiotic classes were tested, each at a high (H, 100 mg L⁻¹) and a low (L, 50 mg L⁻¹) dose: tetracycline (oxytetracycline), cephalosporin (cefotaxime sodium), β-lactam (ampicillin–cloxacillin combination), fluoroquinolone (levofloxacin), aminoglycoside (amikacin) and chloramphenicol. Wells of uniform diameter were bored with a sterile cork borer, charged with test solution, and plates incubated at 37 °C for 24 hours. Inhibition zone diameters were measured in millimetres and isolates scored as susceptible or resistant.

3.9 In vitro shoot culture with selected antibiotics

The three most active agents were carried into the culture system. Four regimens were

incorporated into the multiplication medium — levofloxacin at 100 mg L⁻¹ (L100) and 50 mg L⁻¹ (L50), amikacin at 100 mg L⁻¹ (A100) and cefotaxime sodium at 100 mg L⁻¹ (C100) — alongside an antibiotic-free control, with 100 explants per treatment. Antibiotic solutions were filter-sterilised and added to autoclaved medium after cooling. At 25 days each culture was scored independently on two axes: presence or absence of visible bacterial colonies, and shoot condition (viable, meaning green and turgid with capacity for further growth; or weakened, meaning chlorotic, necrotic or dead). This produced four mutually exclusive outcome categories per treatment, from which two derived indices were calculated:

Decontamination efficacy (%) = percentage of explants with no visible colony, irrespective of shoot condition.

Phytotoxicity index (%) = (weakened shoots with no visible colony ÷ total explants with no visible colony) × 100.

The second index isolates damage attributable to the antibiotic itself, since explants in this denominator carry no visible bacterial growth and their deterioration cannot be ascribed to infection.

3.10 Statistical analysis

All outcomes were recorded as counts of explants falling into mutually exclusive categories and were therefore analysed as categorical rather than continuous data. This choice follows the recommendation that proportional and count data from micropropagation experiments should not be forced into analysis of variance after transformation; Ibañez et al. (2003) demonstrated on a *Viburnum opulus* proliferation and rooting dataset that generalised linear models and planned contrasts outperform ANOVA on such data, and Warton and Hui (2011) showed that the arcsine transformation is inferior to logistic approaches for binomial proportions. O'Hara and Kotze (2010) reached parallel conclusions for count data.

Differences in regeneration frequency across PGR treatments were accordingly tested by

Pearson's chi-square test of independence on the 4 × 2 contingency table of nodal-explant outcomes, with all expected cell frequencies exceeding five. In the antibiotic experiment (n = 100 per treatment) the same approach was applied to three response definitions in turn: usable yield (viable and colony-free), decontamination efficacy (colony-free irrespective of shoot condition) and phytotoxicity (weakened explants as a proportion of colony-free explants only). Pairwise comparisons used Fisher's exact test, which remains exact at small expected frequencies. Significance was accepted at $p < 0.05$. Analyses were performed in Python 3 using the SciPy library.

The surface sterilisation experiment is reported descriptively. Survival and asepsis were recorded as batch percentages rather than as counts against a stated denominator, and inferential testing of proportions is not appropriate without that denominator. The magnitude and direction of the effect are nonetheless unambiguous and are interpreted as observations.

4. Results

4.1 Quality parameters of the donor material

The whole-plant powder was green, bitter and of characteristic odour. Physicochemical constants are given in Table 1. The absence of acid-insoluble ash indicates negligible siliceous contamination, and the high water-soluble extractive value relative to the alcohol-soluble value (20.93% against 4.40% w/w) indicates that most extractable matter is polar. Leaf micro-morphological constants were: vein-islet number 7–10, stomatal index 15–17 on the upper epidermis and 22–23 on the lower, and palisade ratio 2–4. Stomata were anisocytic on both surfaces and more numerous abaxially. Phytochemical screening of the ethanolic extract was positive for alkaloids, proteins, amino acids, monosaccharides, carbohydrates, tannins, resins, coumarins and phenols, and negative for steroids and terpenoids, glycosides and saponins.

Table 1. Physicochemical constants of *Solanum nigrum* whole-plant powder

S. No.	Parameter	Value (% w/w)
1	Loss on drying	17.60

2	Total ash	14.42
3	Acid-insoluble ash	Nil
4	Water-soluble ash	7.56
5	Alcohol-soluble extractive value	4.40
6	Water-soluble extractive value	20.93

4.2 Optimisation of surface sterilisation

Immersion duration in bavistin produced strongly opposed effects on the two response variables (Table 2). Asepsis rose monotonically with exposure, from 39% at one minute to 97% at fifteen minutes. Explant survival was not monotonic: it rose from 11% at one minute to a

maximum of 59% at ten minutes, then collapsed to 4% at fifteen minutes. The fifteen-minute treatment therefore delivered near-complete asepsis on tissue that was almost entirely dead, and is of no practical value. The ten-minute treatment gave the highest usable yield of clean, living explants and was adopted for all subsequent work.

Table 2. Effect of immersion duration in bavistin (250 µg mL⁻¹) on explant survival and asepsis

Treatment	Immersion time (min)	Explant survival (%)	Aseptic cultures (%)	Usable outcome
T1	1	11	39	Poor — heavy contamination
T2	5	26	74	Intermediate
T3	10	59	88	Optimum
T4	15	4	97	Aseptic but non-viable

Explants scored at 25 days. Survival denotes green, turgid, non-necrotic tissue; asepsis denotes absence of visible microbial growth. Values are batch percentages, reported descriptively (Section 3.10).

4.3 Effect of plant growth regulators on organogenesis

No growth of any kind was obtained in liquid Gamborg B5 medium, with or without PGR supplementation, indicating that a solid matrix is a requirement for explant establishment in this system. All subsequent observations refer to agar-solidified medium.

On hormone-free medium (T1) no explant responded, and progressive browning was observed. Every PGR-supplemented treatment produced a distinct and reproducible morphogenic pathway (Table 3). 6-BAP alone at 0.5 mg L⁻¹ (T2) induced leaves directly from the cut surface after 20 days, averaging four leaves per explant, at a regeneration frequency of 68.75%. Adding IBA at 1.0 mg L⁻¹ to the same cytokinin concentration (T3) shifted the response entirely to rhizogenesis: roots emerged at 16 days, elongated until day 22, and reached a mean of 12 roots per explant with a maximum root length of 5.2 cm, at the highest regeneration frequency recorded (81.25%). Raising both regulators to 2.0

mg L⁻¹ (T4) suppressed organised development in favour of callus. Explant origin determined callus character: nodal segments produced white, irregular, friable callus from day 18 at 75% frequency, whereas *in vitro*-derived roots produced brownish-yellow, soft, chunky callus from day 20 at 41.70% frequency.

Across nodal explants the four treatments differed significantly in regeneration frequency ($\chi^2 = 27.94$, df = 3, $p < 0.001$). Pairwise comparison showed that this effect was driven entirely by the contrast between the hormone-free control and each supplemented medium (T2 versus T1, $p < 0.001$; T3 versus T1, $p < 0.001$; T4 versus T1, $p < 0.001$). The supplemented treatments did not differ from one another in the proportion of explants responding (T3 versus T2, $p = 0.685$; T4 versus T3, $p = 1.000$). The three PGR regimes are therefore equally reliable at eliciting a response; what distinguishes them is the nature of that response, not its frequency. Callus induction frequency did not differ significantly between nodal and root explants on T4 medium ($p = 0.121$).

Table 3. Morphogenic response of *Solanum nigrum* explants to plant growth regulator combinations on solid Gamborg B5 medium at 25 days

Treatment (mg L ⁻¹)	Explant	Growth pattern at day 25	Responding / total	Regeneration frequency (%)
T1 (control, PGR-free)	Nodal segment	No observable growth; progressive browning	0 / 16	0
T2 (6-BAP 0.5)	Nodal segment	Direct leaf induction from day 20; mean 4 leaves per explant	11 / 16	68.75
T3 (6-BAP 0.5 + IBA 1.0)	Nodal segment	Root induction from day 16; mean 12 roots per explant, maximum length 5.2 cm	13 / 16	81.25
T4 (6-BAP 2.0 + IBA 2.0)	Nodal segment	White, irregular, friable callus from day 18	12 / 16	75.00
T4 (6-BAP 2.0 + IBA 2.0)	<i>In vitro</i> -derived root	Brownish-yellow, soft, chunky callus from day 20	5 / 12	41.70

Overall difference across nodal-explant treatments: $\chi^2 = 27.94$, $df = 3$, $p < 0.001$. Each PGR treatment differs from the control at $p < 0.001$ (Fisher's exact test); the PGR treatments do not differ from one another ($p \geq 0.685$).

4.4 Late-onset contamination and isolation of the endophyte

Bacterial clouding was first observed at approximately 25 days after inoculation, confined to the region of medium in direct contact with the basal cut surface of the explant. No contamination appeared during the conventional three-to-ten-day window in which handling or environmental failures manifest. This delayed, spatially restricted onset is characteristic of an organism released from internal explant tissue rather than introduced from outside, and confirms that the surface sterilisation protocol optimised in Section 4.2 was functioning as intended.

The appearance of the bacterium coincided with deterioration across all three morphogenic pathways: browning of induced leaves under T2, browning of the explant following successful rhizogenesis under T3, and browning and destruction of callus from day 25 under T4. In each case the PGR treatment had already produced its intended response before the culture was lost, which locates the failure at the endophyte rather than at the hormonal regime. Streaking of the affected zone onto nutrient agar yielded growth that was purified to uniform colony morphology by repeated subculture. Characteristics of the isolate are summarised in Table 4: a short, Gram-negative rod, occurring in clusters, and motile in hanging-drop preparation.

Table 4. Presumptive characteristics of the endophytic bacterial isolate

Characteristic	Observation
Source	Medium in contact with basal cut surface of nodal explant
Time of first appearance	~ 25 days after inoculation
Cell shape	Short rod (coccobacillary)
Gram reaction	Negative
Cellular arrangement	Cluster-forming
Motility (hanging drop)	Motile
Growth on nutrient agar	Growth at 37 °C within 24–48 h

Characterisation is presumptive and based on cultural, morphological and staining criteria. Molecular confirmation by 16S rRNA gene sequencing was not performed and is identified as a limitation in Section 5.5.

4.5 Antibiotic susceptibility of the isolate

The isolate was susceptible to five of six antibiotic classes at the high dose, but the susceptibility ranking changed markedly with concentration (Table 5). Levofloxacin was the most active agent at both doses, producing inhibition zones of 38 mm at 100 mg L⁻¹ and 30 mm at 50 mg L⁻¹. Amikacin was next (28 and 23 mm), followed by cefotaxime sodium (25 and 8 mm). Oxytetracycline and chloramphenicol produced measurable zones at the high dose only and were entirely inactive at 50 mg L⁻¹. The ampicillin–cloxacillin combination showed modest but dose-stable activity.

Two features of this profile merit attention. Levofloxacin retained 79% of its high-dose zone

diameter when halved, whereas cefotaxime retained 32% and oxytetracycline and chloramphenicol retained none. A wide margin between the effective dose and the failure dose is precisely what is required when an antibiotic must be used at the lowest concentration compatible with plant viability. Second, the overall pattern — strong fluoroquinolone and aminoglycoside activity, variable cephalosporin activity, weak chloramphenicol and tetracycline activity — is consistent with the Gram-negative morphology established in Section 4.4, and broadly parallels the fluoroquinolone and aminoglycoside susceptibility reported for the Gram-negative endophytes of *Staphylea pinnata* by Szewczyk-Taranek et al. (2020).

Table 5. Susceptibility of the endophytic isolate to six antibiotic classes by agar-well diffusion

S. No.	Class	Antibiotic	Dose	Inhibition zone (mm)	Interpretation
1	Tetracycline	Oxytetracycline	H	20	Susceptible
S. No.	Class	Antibiotic	Dose	Inhibition zone (mm)	Interpretation
2	Tetracycline	Oxytetracycline	L	0	Resistant
3	Cephalosporin	Cefotaxime sodium	H	25	Susceptible
4	Cephalosporin	Cefotaxime sodium	L	8	Susceptible
5	β-lactam	Ampicillin + cloxacillin	H	15	Susceptible
6	β-lactam	Ampicillin + cloxacillin	L	13	Susceptible
7	Fluoroquinolone	Levofloxacin	H	38	Susceptible
8	Fluoroquinolone	Levofloxacin	L	30	Susceptible
9	Aminoglycoside	Amikacin	H	28	Susceptible
10	Aminoglycoside	Amikacin	L	23	Susceptible
11	Others	Chloramphenicol	H	11	Susceptible
12	Others	Chloramphenicol	L	0	Resistant

H = 100 mg L⁻¹; *L* = 50 mg L⁻¹. Zone diameters include the well.

4.6 Performance of selected antibiotics in the culture system

Incorporation of the three most active agents into the multiplication medium produced results that diverge sharply from the susceptibility ranking (Table 6). In the antibiotic-free control the culture was effectively lost: 90% of explants were weakened and carried visible colonies, and only 2% were both viable and colony-free. Across the five

treatments (n = 100 explants each) the proportion of usable shoots differed highly significantly ($\chi^2 = 70.38$, df = 4, $p < 0.001$), as did decontamination efficacy ($\chi^2 = 98.62$, df = 4, $p < 0.001$). Every antibiotic regimen outperformed the untreated control on both measures (Fisher's exact test,

$P < 0.001$ in all cases).

Levofloxacin at 100 mg L⁻¹ gave the highest raw decontamination efficacy of any treatment

(66% of explants free of visible colonies), significantly above the 51% achieved at half that dose ($p = 0.044$). However, 29 of those 66 percentage points comprised weakened tissue, giving a phytotoxicity index of 43.9% against 3.9% at the lower dose — a highly significant difference ($p < 0.001$). The lower dose accordingly produced the highest proportion of viable, colony-free shoots of any treatment (49%), which is the outcome that matters operationally since only explants in this category are available for further multiplication. Cefotaxime sodium at 100 mg L⁻¹ was statistically indistinguishable from levofloxacin at 50 mg L⁻¹ on usable yield (44% against 49%, $p = 0.571$) and on phytotoxicity (10.2% against 3.9%, $p = 0.264$), making it a viable alternative. Amikacin at 100 mg L⁻¹ performed poorly on both axes despite its strong *in vitro* zone diameter, clearing only 33% of

cultures and weakening 39.4% of those it cleared; it was significantly inferior to both levofloxacin at 50 mg L⁻¹ ($p < 0.001$) and cefotaxime ($p < 0.001$) on usable yield, and significantly more phytotoxic than cefotaxime ($p = 0.003$).

The relationship between plate susceptibility and culture performance is therefore non-monotonic. Amikacin produced a larger inhibition zone than cefotaxime at every dose tested, yet delivered less than half the yield of usable shoots. Levofloxacin at the lower dose out-performed levofloxacin at the higher dose on usable yield despite a smaller zone diameter and lower decontamination efficacy. Selection on susceptibility alone would have identified L100 as the treatment of choice; selection on usable yield identifies L50.

Table 6. Outcome of *in vitro* shoot culture at 25 days under antibiotic-supplemented medium (% of explants)

Treatment	Viable, no colony	Weakened, no colony	Viable, with colony	Weakened, with colony	Decontamination efficacy (%)	Phytotoxicity index (%)
Control	2	0	8	90	2	0.0
L100	37	29	0	34	66	43.9
Treatment	Viable, no colony	Weakened, no colony	Viable, with colony	Weakened, with colony	Decontamination efficacy (%)	Phytotoxicity index (%)
L50	49	2	10	39	51	3.9
A100	20	13	15	52	33	39.4
C100	44	5	11	40	49	10.2

n = 100 explants per treatment. L100 / L50 = levofloxacin 100 / 50 mg L⁻¹; A100 = amikacin 100 mg L⁻¹; C100 = cefotaxime sodium 100 mg L⁻¹. Categories are mutually exclusive and sum to 100% within each treatment. Decontamination efficacy = sum of the two 'no colony' categories. Phytotoxicity index = weakened-and-colony-free as a percentage of all colony-free explants.

5. Discussion

The central finding of this study is that a single latent endophytic bacterium was sufficient to destroy shoot cultures of *S. nigrum* that had already completed their intended morphogenic response, and that suppressing it required a decision rule different from the one a susceptibility test would supply.

5.1 Surface sterilisation is a trade-off, not an optimisation

The bavistin results illustrate a point often obscured when asepsis is reported alone. Fifteen minutes of fungicide exposure delivered 97%

aseptic cultures — the best asepsis figure in the experiment — on explants of which only 4% survived. Reporting asepsis without survival would have identified the worst treatment as the best. The mechanism is well established: prolonged exposure to carbendazim and to the mercuric chloride step causes tissue injury, and injured tissue responds with phenolic oxidation, browning and necrosis. The ten-minute treatment adopted here reflects the point at which the marginal gain in asepsis no longer compensates for the marginal loss of viable tissue.

This inverse relationship is consistently reported. Work on Pepino (*Solanum muricatum*), a

congener of the present species, found minimum contamination and maximum survival at an intermediate mercuric chloride plus bavistin exposure, with both variables shifting in opposite directions as duration increased. Studies on *Petunia hybrida* and *Lilium* hybrids similarly found that exposure durations optimal for bulb or stem tissue had to be shortened substantially for more delicate explants. The trade-off is explant- and species-specific and must be re-established for each system rather than transferred from published protocols.

5.2 PGR combination determines the pathway, not the response rate

The statistical analysis produced a result that is easy to misread. All three PGR treatments differed highly significantly from the hormone-free control, but none differed significantly from any other. It would be wrong to conclude that the combinations are interchangeable. Regeneration frequency measures whether an explant responded at all; it is blind to what the response was. The three treatments produced three entirely different developmental outcomes — caulogenesis, rhizogenesis and callogenesis — at statistically indistinguishable frequencies. The auxin-to-cytokinin ratio governed the pathway, while the presence of any exogenous cytokinin governed whether the explant escaped dormancy at all.

The specific responses conform closely to the framework of Skoog and Miller (1957) as reappraised by Melnyk (2023). 6-BAP alone released the axillary meristem and drove leaf formation. Adding IBA at twice the cytokinin concentration raised the auxin-to-cytokinin ratio and switched the explant to root initiation. Raising both to 2.0 mg L⁻¹ produced dedifferentiation to callus, as expected when high concentrations of both classes are supplied together. That leaf induction occurred at 0.5 mg L⁻¹ 6-BAP is consistent with Hassanein and Soltan (2000), who identified the same cytokinin at the same concentration on B5 medium as optimal for *S. nigrum* shoot formation.

The dependence of callus character on explant origin — white friable callus from nodal tissue, brownish-yellow chunky callus from *in vitro*-derived roots — supports the argument of Pasternak

and Steinmacher (2024) that the developmental history of the explant, and not the medium alone, conditions the callus phenotype. This distinction has practical consequences: friable callus is the more suitable of the two for suspension culture and for secondary metabolite work, which makes the nodal segment the preferred explant where alkaloid production rather than plantlet recovery is the objective. Bhat et al. (2010) reported elevated solasodine in leaf-derived callus and *in vitro* shoots relative to *ex vitro* material, indicating that callus route selection has measurable phytochemical consequences in this species.

The complete failure of liquid medium is likewise informative. Submersion of the cut surface restricts gas exchange, and oxygen limitation at the wound site is the most plausible explanation for the absence of any response. Swain et al. (2014) successfully used liquid MS for *S. nigrum* but only after establishing cultures on agar-solidified medium first, which is consistent with the interpretation that liquid systems are unsuitable for the initial establishment phase in this species even where they serve well for subsequent multiplication.

5.3 The diagnostic value of delayed contamination

Timing and location together identified the contamination as endogenous. Nothing appeared during the first three to ten days, when handling and environmental failures present; growth appeared at day 25, confined to the medium touching the basal cut surface. This pattern matches reports across unrelated systems. Abreu-Tarazi et al. (2010) recovered endophytic communities from pineapple microplants maintained five years without visible contamination, detectable only by culture-independent methods. Thomas and Kumari (2010) found that 12% of apparently clean papaya cultures showed basal clouding that indexing revealed as bacterial. Work on *Petunia* found asepsis measurably higher at two weeks than at four, precisely because endophytes emerged in the intervening period.

This has a direct consequence for how PGR studies should be read. In the present work every treatment produced its intended response before the

culture deteriorated: leaves formed at day 20 and browned afterwards, roots formed at day 16 and elongated to day 22 before the explant browned, callus formed at day 18 and was destroyed from day 25. Had cultures been scored at day 30 rather than day 25, all three PGR combinations would have appeared to fail, and the failure would have been attributed to the hormonal regime. Thomas (2004b) documented exactly this error in watermelon, where culture decline was attributed to genetic and nutritional causes until covert bacteria were identified. Late-emerging endophytes are therefore capable of generating false negatives in PGR optimisation studies, and endophyte indexing should logically precede rather than follow protocol development.

5.4 Susceptibility ranking is a poor guide to antibiotic selection

The most practically important result of this study is the dissociation between plate performance and culture performance. Amikacin generated a 28 mm inhibition zone, larger than cefotaxime's 25 mm, yet cleared only 33% of cultures against cefotaxime's 49% and weakened nearly four times as large a fraction of the explants it did clear (39.4% against 10.2%, $p = 0.003$). Levofloxacin at 100 mg L⁻¹ was the most potent agent on the plate and the most effective decontaminant in culture, yet returned fewer usable shoots than the same agent at half the dose, because 43.9% of the explants it rescued from infection it damaged itself.

Several mechanisms plausibly contribute. Antibiotic uptake into plant tissue differs from diffusion through agar, so an agent may not reach the intracellular niche an endophyte occupies — a consideration made concrete by the electron-microscopic demonstration of intracellular endophytic bacteria by Esposito-Polesi et al. (2017). Some antibiotics are unstable in autoclaved or warm medium. Most directly, aminoglycosides inhibit prokaryotic ribosomes, and plastids and mitochondria are of prokaryotic origin; Ellis (1969) established that chloroplast ribosomes are stereospecifically inhibited by chloramphenicol exactly as bacterial ribosomes are, while cytoplasmic ribosomes are unaffected. This provides a direct route to phytotoxicity independent of any effect on the target organism, and accounts

for the poor showing of both amikacin and, at the high dose, levofloxacin. Fluoroquinolones target bacterial DNA gyrase, an enzyme without a close plant counterpart, which is consistent with the very low phytotoxicity index recorded for levofloxacin at 50 mg L⁻¹ and suggests that the damage at 100 mg L⁻¹ reflects a concentration-dependent off-target effect rather than the primary mechanism.

The practical rule that follows is that antibiotic selection for tissue culture should be made on usable yield — the proportion of explants simultaneously viable and colony-free — rather than on inhibition zone diameter or on decontamination efficacy alone. Reporting the phytotoxicity index alongside decontamination efficacy makes the trade-off explicit and allows protocols to be compared across studies. On this criterion levofloxacin at 50 mg L⁻¹ is the agent of choice for *S. nigrum*, with cefotaxime sodium at 100 mg L⁻¹ as a statistically equivalent alternative where fluoroquinolone use is restricted. The finding sits comfortably alongside the wider literature: Nauerby et al. (1997), Grzebelus and Skop (2014) and Qin et al. (2017) all report that phytotoxicity varies unpredictably within as well as between antibiotic classes, and cannot be inferred from antimicrobial potency.

An important qualification is that antibiotic supplementation suppresses rather than eliminates an endophyte. Ray et al. (2017) showed for *Bambusa balcooa* that ampicillin effective at the establishment stage failed at multiplication. Szewczyk-Taranek et al. (2020) achieved 63% inhibition over four weeks and reported no visible colonies after transfer to antibiotic-free medium, but visible colonies are a weak endpoint given the demonstrated existence of non-culturable and intracellular populations (Thomas et al., 2008; Esposito-Polesi et al., 2017). Whether the *S. nigrum* endophyte reported here is genuinely eliminated or merely held below the threshold of visibility cannot be determined from the present data and requires culture-independent verification.

5.5 Limitations

Three limitations should be stated plainly. First, the isolate was characterised only by cultural, morphological and staining criteria. The

identification is presumptive at the level of a motile, cluster-forming, Gram-negative short rod, and no genus or species assignment is claimed. Gram reaction, cell shape and motility are together compatible with a large number of genera, and confirmation requires 16S rRNA gene amplification and sequencing with BLAST comparison and phylogenetic placement (Weisburg et al., 1991), supported at minimum by oxidase testing and growth on King's B medium if a fluorescent pseudomonad is suspected. It should be noted that the widely used 27F/1492R primer pair can cross-amplify plant chloroplast DNA, so any such work on green tissue must be confirmed by sequencing rather than by amplicon size (Frank et al., 2008). This work is in progress and is the highest priority for extension of the study.

Second, the surface sterilisation data are descriptive. Survival and asepsis were recorded as batch percentages, and while the direction and magnitude of the immersion-time effect are unambiguous, the absence of a stated denominator precludes formal testing.

Third, in the antibiotic experiment the outcome categories were scored across 100 explants per treatment, but these were not distributed across independent biological replicates, so the analysis treats the individual explant as the unit of replication. This is common practice in contamination-control studies but is a weaker design than one with true biological replication, and the precision implied by the reported p-values should be regarded as optimistic. Related to this, no re-inoculation assay was performed to confirm that the isolate is the causal agent of the observed decline rather than a coincidental coloniser. Such an assay, in which axenic shoots are challenged with the pure isolate and compared against uninoculated controls, would establish causality directly. It would also determine whether this organism has any growth-promoting capacity of the kind reported by Zawadzka et al. (2013) for *Curtobacterium*, *Methylobacterium* and *Paenibacillus* strains in ornamental species, or by Thomas (2004c) for a rhizogenesis-inducing *Bacillus pumilus* in grape.

6. Conclusion

This study optimised plant growth regulator mediated *in vitro* shoot culture of *Solanum nigrum* while identifying and managing the endophytic bacterial infection that constrained it. Surface sterilisation with bavistin at 250 µg mL⁻¹ for ten minutes gave the best balance of asepsis (88%) and explant survival (59%); longer exposure improved asepsis at the cost of nearly all viable tissue. On solid Gamborg B5 medium, 6-BAP at 0.5 mg L⁻¹ induced leaves (68.75%), 6-BAP 0.5 with IBA 1.0 mg L⁻¹ induced roots (81.25%, mean 12 roots per explant, maximum length 5.2 cm), and 6-BAP 2.0 with IBA 2.0 mg L⁻¹ induced callus whose texture depended on explant origin. All three differed significantly from the hormone-free control ($p < 0.001$) but not from one another, establishing that the auxin-to-cytokinin ratio determines which morphogenic pathway is followed rather than how often an explant responds. Liquid medium supported no growth at any hormone concentration.

A latent, motile, cluster-forming Gram-negative short rod was isolated from the explant–medium interface at approximately 25 days, causing progressive browning and loss of cultures that had already completed leaf, root or callus induction. Its delayed and spatially restricted onset identifies it as endogenous to the explant rather than as an environmental contaminant, and confirms that surface disinfection, however well optimised, cannot address it.

The isolate was most susceptible to levofloxacin, then amikacin and cefotaxime sodium. In culture, however, the ranking inverted: levofloxacin at 50 mg L⁻¹ returned the highest proportion of viable, colony-free shoots (49% against 2% in the untreated control; $\chi^2 = 70.38$, $df = 4$, $p < 0.001$) at a phytotoxicity index of 3.9%, while levofloxacin at 100 mg L⁻¹ achieved better decontamination but damaged 43.9% of the explants it cleared ($p < 0.001$), and amikacin underperformed cefotaxime despite a larger inhibition zone. The study therefore concludes that antibiotic selection for plant tissue culture should be based on usable yield rather than on susceptibility ranking, and that endophyte indexing should precede rather than follow plant growth regulator optimisation in field-collected medicinal species. Molecular identification of the isolate, re-inoculation to

establish causality, and replicated dose–response trials with acclimatisation and genetic fidelity assessment are the immediate priorities for further work.

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