

Stage-Specific Evaluation of Plant Growth Regulators for In Vitro Regeneration of Four Himalayan Medicinal Plants: Explant Effects and Trade-offs Between Multiplication, Elongation and Rooting

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Abstract

In vitro propagation can reduce wild harvesting of medicinal plants, but optimisation is often reduced to naming a single “best” medium. This study evaluates the regeneration of *Bergenia ligulata*, *Thalictrum foliolosum*, *Selinum tenuifolium* and *Wulfenia amherstiana* on Murashige and Skoog medium with cytokinins (BAP, kinetin, TDZ) and auxins (IAA, NAA, IBA), alone and in combination, using leaf, nodal, rhizome and root explants. Shoot number, shoot length, the percentage of responding explants and rooting were analysed as separate stages. The treatment giving the most shoots rarely gave the longest shoots or the highest response. For *B. ligulata* leaf explants, BAP 0.8 + NAA 0.2 mg/l gave the most shoots (8.5) but only a 30% response, whereas BAP 1.0 + NAA 0.2 mg/l gave 8.0 shoots, 7.1-cm shoots and a 70% response, so the expected yield per inoculated explant was more than twice as high (5.6 versus 2.6 shoots). Auxin alone supported multiple shoots in *T. foliolosum* (NAA 0.5 mg/l: 11.25 shoots, 10.9 cm) and *S. tenuifolium*. Explant origin altered outcomes: root-derived *T. foliolosum* cultures reached an 89.6% response compared with 62.5% for leaf cultures. *B. ligulata* shoots rooted best without growth regulators (87.2%, 12.8 roots), and added auxins reduced rooting. Candidate two-stage protocols are proposed for each species, together with the acclimatisation, genetic-fidelity and chemical-consistency tests needed before they can support conservation or supply.

Index Terms—Micropropagation, plant growth regulators, BAP, NAA, IAA, explant, rooting, Himalayan medicinal plants, conservation, Murashige and Skoog medium

1. Introduction

Many Himalayan medicinal plants are collected from the wild, frequently for their rhizomes and roots, which removes the whole plant and threatens local populations [1]. Cultivation and in vitro propagation are recognised routes to reduce this pressure while maintaining supply [2], and tissue culture has been used to conserve many threatened species [3], [4].

This study concerns four such plants. *Bergenia ligulata* rhizomes are used in traditional medicine for urinary and inflammatory conditions [5], and *Thalictrum* species are valued for their alkaloids [6]. *Selinum tenuifolium* and *Wulfenia amherstiana* are harvested for their underground organs. Micropropagation protocols have been reported for *B. ligulata* [7] and *T. foliolosum* [8], but comparable work on the other two species is limited.

Propagation studies often report a single “optimal” medium chosen for the largest shoot number. However, regeneration is a sequence of stages—initiation, multiplication, elongation, rooting and acclimatisation—and each stage has a different goal [9]. A medium that produces many short shoots may be poor if those shoots cannot be rooted, and a high shoot count among few responding explants yields fewer plants than a moderate count among many. This paper, derived from a doctoral thesis, contributes:

- 1) A stage-specific comparison of growth-regulator treatments across four species and several explant types.
- 2) An analysis of the trade-offs between shoot number, shoot length and response percentage, including a simple yield indicator.
- 3) Rooting comparisons that identify where auxins help and where they are unnecessary.
- 4) Candidate two-stage protocols and the validation steps needed before they can be used for conservation or supply.

2. Related Work

2.1 Growth Regulators in Regeneration

Most regeneration media are based on the Murashige and Skoog formulation [10]. Cytokinins such as 6-benzylaminopurine (BAP), kinetin and thidiazuron (TDZ) promote shoot formation and axillary branching, while auxins such as indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) promote rooting and, at suitable ratios with cytokinins, shoot development [9]. The response depends on the species, the explant and its endogenous hormone status, so ratios must be established experimentally.

2.2 Propagation of the Study Species

B. ligulata has been regenerated from leaf explants on cytokinin–auxin media [7], and an in vitro protocol has been developed for *T. foliolosum* [8]. Reviews of in vitro conservation emphasise that protocols must lead to plants that survive transfer and retain genetic diversity, and that physiological disorders such as hyperhydricity can limit success [3], [11].

3. Materials and Methods

3.1 Explants and Culture Conditions

Plant material collected in Himachal Pradesh was surface-sterilised and cultured on Murashige and Skoog medium [10] supplemented with growth regulators alone or in combination. Table I lists the explants and treatment sets for each species. Observations were recorded after the stated culture interval as shoot number, shoot length (cm) and the percentage of explants responding; rooting was recorded as root number, root length and percentage of shoots rooting. Means and standard errors were reported for four replicates, with Duncan’s multiple range test letters [12].

TABLE I EXPLANTS AND GROWTH-REGULATOR TREATMENTS EVALUATED

Species	Explant	Shoot-stage treatments (mg/l)	Rooting treatments (mg/l)
<i>B. ligulata</i>	Leaf	BAP 0.5–2.0; Kn 1.0–2.5; BAP 0.2–1.0 + IAA 0.2; BAP 0.2–1.0 + NAA 0.1–0.2	IAA 1.0–2.5; IBA 1.0–2.5; IAA + IBA; IAA + NAA; none
<i>B. ligulata</i>	Rhizome	BAP 0.2–2.0 + IAA 0.1–1.5 or NAA 0.1–1.5	—
<i>T. foliolosum</i>	Leaf	BAP 0.2–1.0; NAA 0.2–0.8; BAP 0.5–1.5 + NAA 0.2–1.0	NAA + IAA; IAA + BAP; IAA + IBA
<i>T. foliolosum</i>	Root	BAP 0.2–1.0 with NAA, TDZ or Kn	—
<i>S. tenuifolium</i>	Node	BAP, IAA, NAA 0.2–1.5; BAP 0.4–1.5 + NAA or IAA 0.2–0.3	NAA + IAA; IAA + BAP; IAA + IBA
<i>S. tenuifolium</i>	Leaf	BAP 0.2–2.5; IAA 0.2–1.0; BAP 0.4–2.5 + IAA 0.2	—
<i>W. amherstiana</i>	Node	BAP, IAA 0.2–1.5; BAP 0.4–1.5 + IAA or NAA 0.2–0.3	NAA + IAA; IAA + BAP; IAA + IBA

3.2 Stage-Specific Analysis

Each stage was evaluated separately using the framework in Fig. 1. For each species and explant, the treatment giving the highest mean on each outcome was identified. To combine multiplication with the proportion of responding explants, an indicative yield was calculated as

$$Y = N_s \times (R / 100)$$
(1)

where N_s is the mean shoot number and R the percentage of explants responding. Y estimates the expected number of shoots per inoculated explant, assuming shoot number is counted per responding explant. It is a descriptive summary of reported means; no new significance tests were performed, because replicate-level data were not available.

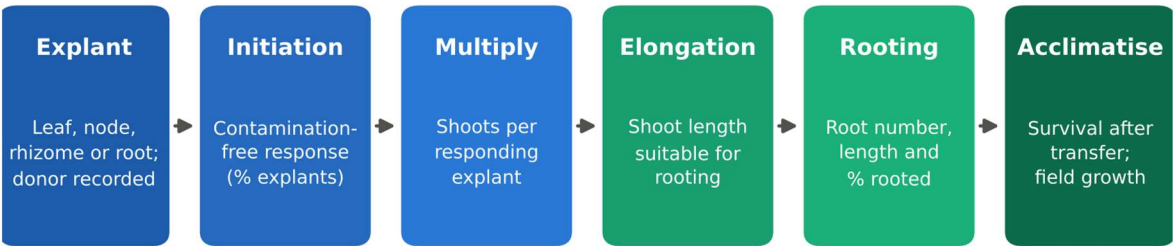


Fig. 1. Stage-specific framework for evaluating regeneration. Each stage has its own outcome, and the best treatment is selected for each stage rather than for the whole process.

4. Results

4.1 Trade-offs Between Shoot Number, Length and Response

Fig. 2 plots shoot length against shoot number for each treatment, with point size showing the response percentage. In three of the four species, the treatment that produced the most shoots differed from the one that produced the longest shoots. For *B. ligulata* leaf explants, BAP 0.8 + NAA 0.2 mg/l gave the most shoots (8.5) but only a 30% response, while BAP 0.8 +

NAA 0.1 mg/l gave the longest shoots (8.5 cm). Cytokinin alone was weak: BAP 0.5–2.0 mg/l produced 0.5–2.7 shoots and kinetin 3.5–5.2 shoots, although kinetin 2.5 mg/l gave the highest response (71%). Adding IAA 0.2 mg/l to BAP increased shoot number to 8.2 but reduced shoot length to 1.6 cm at the highest BAP level, indicating multiplication at the expense of elongation.

In *T. foliolosum* leaf cultures, NAA alone gave both the most shoots (11.25) and the longest shoots (10.87 cm) at 0.5 mg/l, while BAP alone gave only 2.5–5.75 shoots. The unusually high control value (5.75 shoots) suggests a strong endogenous response and should be confirmed. In *S. tenuifolium* and *W. amherstiana* nodal cultures, auxin alone also supported multiple shoots (up to 9.5 and 8.25), comparable with BAP alone (7.9 and 8.25). Because nodes contain pre-formed axillary buds, these responses may reflect bud release rather than de novo shoot formation.

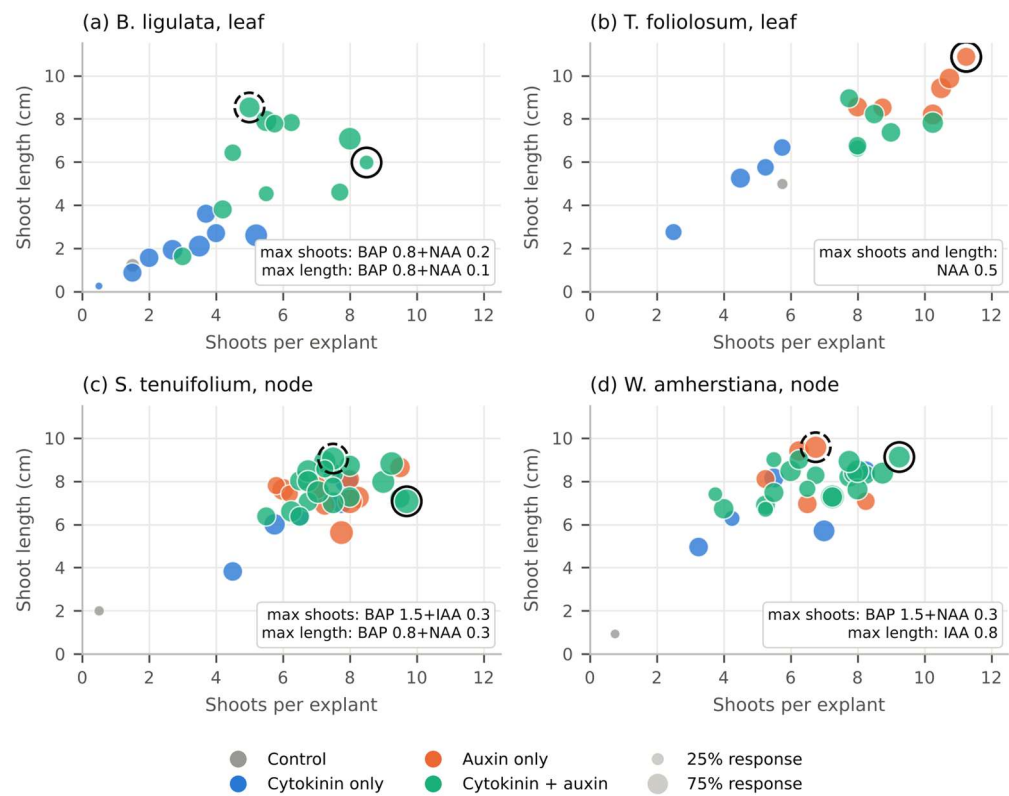


Fig. 2. Shoot length versus shoot number for each treatment. Colour shows regulator class and point size the percentage of responding explants. Solid rings mark the treatment with most shoots; dashed rings the longest shoots.

4.2 Yield per Inoculated Explant

Table II compares the treatments that maximise each outcome with the treatment that maximises the indicative yield Y (Eq. 1). For *B. ligulata* leaf explants, the highest-shoot treatment gave $Y = 2.6$, whereas BAP 1.0 + NAA 0.2 mg/l, with 8.0 shoots, 7.1-cm shoots and a 70% response, gave $Y = 5.6$. The same pattern held elsewhere: for root-derived *T. foliolosum*, BAP 0.4 + TDZ 0.5 mg/l combined 8.92 shoots with an 83.2% response ($Y = 7.4$), and for *S. tenuifolium* nodes, BAP 1.5 + IAA 0.3 mg/l combined the most shoots (9.7) with an 82.5% response ($Y = 8.0$).

TABLE II BEST TREATMENT BY OUTCOME AND BY INDICATIVE YIELD

Species, explant	Most shoots	Longest shoots (cm)	Highest response (%)	Highest yield Y
<i>B. ligulata</i> , leaf	BAP 0.8 + NAA 0.2 (8.50)	BAP 0.8 + NAA 0.1 (8.53)	Kn 2.5 (71.0)	BAP 1.0 + NAA 0.2 (5.6)
<i>B. ligulata</i> , rhizome	BAP 2.0 + NAA 1.5 (7.00)	BAP 0.2 + NAA 0.1 (9.63)	BAP 0.6 + NAA 0.3 (62.5)	BAP 2.0 + NAA 1.5 (4.2)
<i>T. foliolosum</i> , leaf	NAA 0.5 (11.25)	NAA 0.5 (10.87)	BAP 1.0 + NAA 0.6 (62.5)	BAP 1.0 + NAA 0.6 (6.4)
<i>T. foliolosum</i> , root	BAP 0.4 + TDZ 0.5 (8.92)	BAP 0.4 + Kn 0.5 (16.20)	BAP 0.2 + TDZ 0.4 (89.6)	BAP 0.4 + TDZ 0.5 (7.4)
<i>S. tenuifolium</i> , node	BAP 1.5 + IAA 0.3 (9.70)	BAP 0.8 + NAA 0.3 (9.05)	IAA 1.5; BAP 1.5 + IAA 0.3 (82.5)	BAP 1.5 + IAA 0.3 (8.0)
<i>S. tenuifolium</i> , leaf	BAP 2.0 + IAA 0.2 (9.25)	BAP 1.0 + IAA 0.2 (9.60)	BAP 1.5 (80.8)	BAP 1.5 (7.1)
<i>W. amherstiana</i> , node	BAP 1.5 + NAA 0.3 (9.25)	IAA 0.8 (9.58)	BAP 1.0 + IAA 0.3 (77.3)	BAP 1.5 + NAA 0.3 (6.0)

Concentrations in mg/l; values in parentheses are the reported means for the outcome concerned or the indicative yield Y.

4.3 Effect of Auxin Level in Combination with BAP

Fig. 3 shows the BAP × auxin combinations tested on *S. tenuifolium* and *W. amherstiana* nodes. Raising the auxin from 0.2 to 0.3 mg/l increased shoot number at most BAP levels and consistently increased the response percentage. For *W. amherstiana* with NAA, the response rose from 30–52.5% at 0.2 mg/l to 57.3–68.8% at 0.3 mg/l, and shoot number rose at four of the five BAP levels. For *S. tenuifolium* with NAA, the response rose from 52.5–61% to 68.3–76%. Because the experiments did not include an interaction analysis, these patterns describe favourable combinations rather than demonstrating synergy between the regulators.

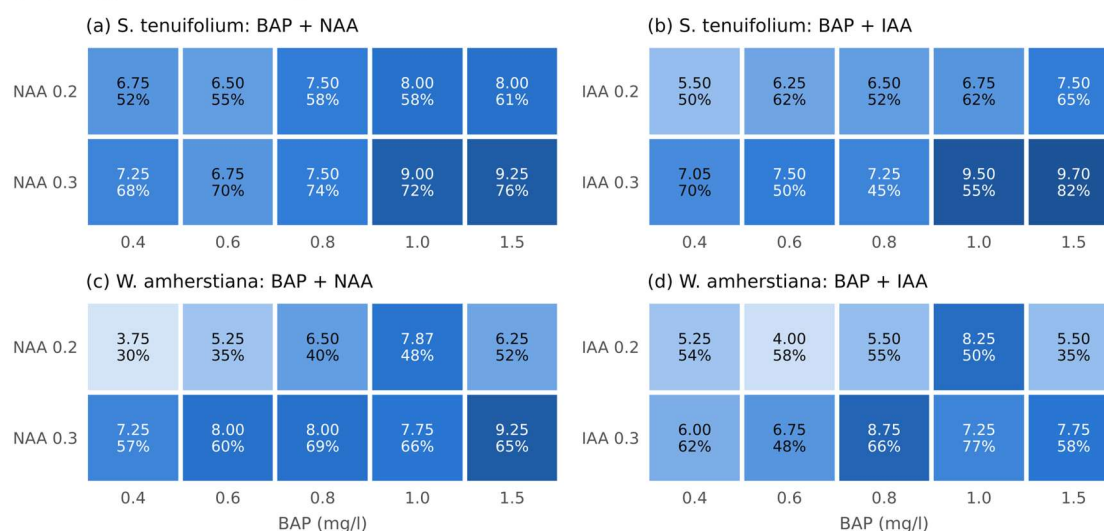


Fig. 3. Mean shoot number (upper value) and response percentage (lower value) for BAP combined with NAA or IAA at 0.2 and 0.3 mg/l on nodal explants of (a, b) *S. tenuifolium* and (c, d) *W. amherstiana*.

4.4 Effect of Explant Origin

Explant origin changed which outcome was achievable (Fig. 4). For *T. foliolosum*, leaf explants gave more shoots (11.25 versus 8.92), but root-derived cultures gave much longer shoots (16.2 versus 10.9 cm) and a higher response (89.6% versus 62.5%). For *B. ligulata*, leaf explants gave more shoots and a higher response, but rhizome explants gave longer shoots. *S. tenuifolium* nodal and leaf explants performed similarly; in leaf cultures, all growth-regulator treatments shared the same Duncan group for shoot number, so differences among them were not distinguishable.

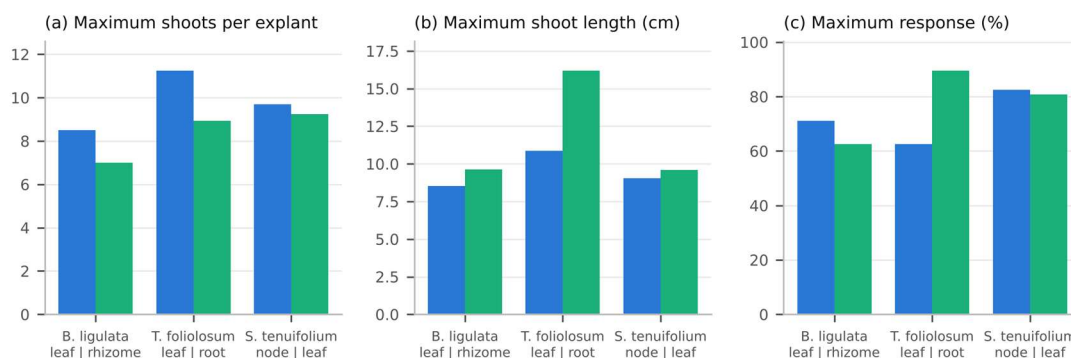


Fig. 4. Maximum reported (a) shoot number, (b) shoot length and (c) response percentage for each explant type. Blue bars show the first explant named under each species and green bars the second.

4.5 Rooting

Rooting results are shown in Fig. 5 and Table III. *B. ligulata* shoots rooted best on medium without growth regulators: 87.2% of shoots rooted, with 12.8 roots of 9.2 cm. Every auxin treatment reduced rooting. Root number declined from 12.2 to 9.7 as IAA increased from 1.0 to 2.5 mg/l, and from 8.7 to 5.0 with IBA. Combinations were poorest, with IAA 1.0 + NAA 1.5 mg/l giving only a 12.5% response. This suggests that shoots carried enough endogenous or residual auxin to root without supplementation, which would also simplify and cheapen the protocol.

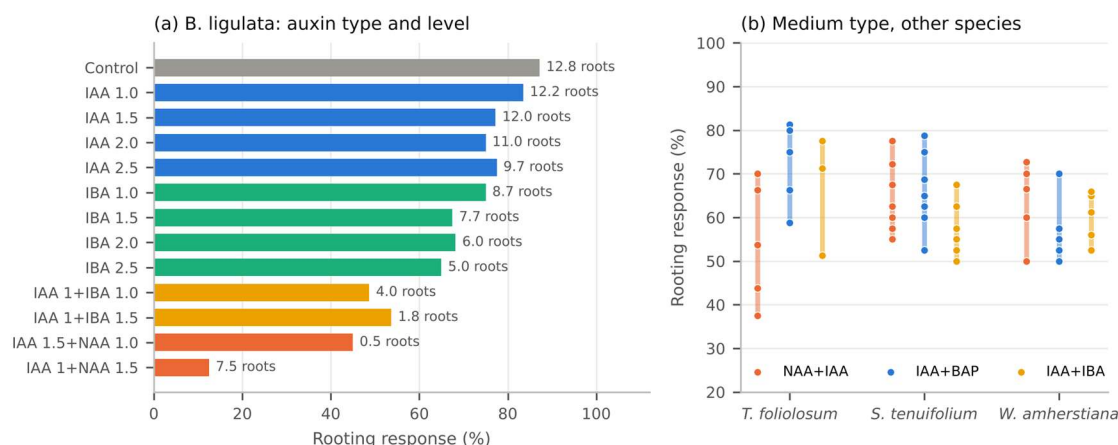


Fig. 5. (a) Rooting response and root number of *B. ligulata* shoots on hormone-free medium and with auxins. (b) Range of rooting responses on the three medium types tested for the other species; each point is one concentration combination.

For the other species, untreated controls rooted poorly (15–17.5%), and all supplemented media improved rooting. In *T. foliolosum*, IAA + BAP gave the highest responses (up to 81.25%) and IAA 0.4 + IBA 0.5 mg/l the longest roots (15.0 cm). For *S. tenuifolium* and *W. amherstiana*, responses on all three medium types overlapped widely (50–79%), so no medium type was clearly superior.

TABLE III ROOTING TREATMENTS GIVING THE HIGHEST RESPONSE

Species	Treatment (mg/l)	Roots per shoot	Root length (cm)	Response (%)	Control response (%)
<i>B. ligulata</i>	None (hormone-free)	12.8	9.2	87.2	87.2
<i>T. foliolosum</i>	IAA 0.2 + BAP 0.4	5.5	8.0	81.3	not reported
<i>S. tenuifolium</i>	IAA 0.4 + BAP 1.0	6.3	8.2	78.8	17.5
<i>W. amherstiana</i>	NAA 2.0 + IAA 1.0	7.0	6.5	72.8	15.0

5. Discussion

5.1 Selecting Treatments by Stage

The results show why a single optimal medium is misleading. Shoot number, shoot length and response percentage peaked under different treatments, and the yield indicator showed that a high shoot count among few responding explants can produce fewer usable shoots than a moderate count among many. A practical protocol should therefore use one medium for

multiplication and, if necessary, another for elongation before rooting, consistent with the distinct roles of cytokinins and auxins [9].

5.2 Candidate Protocols

Table IV sets out candidate two-stage protocols based on the reported means. They are starting points for validation rather than final recommendations, because acclimatisation survival, genetic fidelity and chemical consistency have not yet been measured.

TABLE IV CANDIDATE TWO-STAGE PROTOCOLS AND REQUIRED VALIDATION

Species	Multiplication stage	Rooting stage	Validation still needed
<i>B. ligulata</i>	Leaf; BAP 1.0 + NAA 0.2 mg/l (8.0 shoots, 70%)	Hormone-free MS (87.2%)	Acclimatisation survival; multiple donors
<i>T. foliolosum</i>	Root explant; BAP 0.4 + TDZ 0.5 mg/l (8.9 shoots, 83%)	IAA 0.2 + BAP 0.4 mg/l (81.3%)	Confirm treatment labels; leaf versus root donors
<i>S. tenuifolium</i>	Node; BAP 1.5 + IAA 0.3 mg/l (9.7 shoots, 82.5%)	IAA 0.4 + BAP 1.0 mg/l (78.8%)	Shoot quality before rooting; survival
<i>W. amherstiana</i>	Node; BAP 1.5 + NAA 0.3 mg/l (9.3 shoots, 9.1 cm)	NAA 2.0 + IAA 1.0 mg/l (72.8%)	Reconcile sucrose and timing records

5.3 From Regeneration to Conservation and Supply

Regeneration alone does not achieve conservation. A collection multiplied from one responsive explant represents a single genotype, so several donor plants should be maintained [4]. Genetic fidelity of regenerants should be tested, particularly where callus or TDZ is involved, and physiological disorders should be monitored [11]. If propagated plants are to replace wild-collected material, their chemical profile must also be compared with that of the material used in earlier screening, under documented growth conditions [13].

5.4 Limitations

The analysis uses reported means; replicate-level data were not available, so no new statistical comparisons were made and the yield indicator is descriptive. Some records require reconciliation, including treatment labels in the root-derived *T. foliolosum* table and sucrose concentration and timing in the *W. amherstiana* records. Acclimatisation survival was not quantified.

6. Conclusion and Future Work

Regeneration of four Himalayan medicinal plants depended on explant type and growth-regulator treatment, and the best treatment differed by stage. Balanced cytokinin–auxin combinations generally gave the highest yield per inoculated explant. Auxin alone supported shoot formation in three species. *B. ligulata* rooted best without growth regulators. Future work should test the candidate two-stage protocols with independent donor plants, follow cohorts through acclimatisation, confirm genetic fidelity and compare the chemistry of propagated and wild material, so that propagation can contribute reliably to conservation and sustainable supply [2].

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