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In Vitro Micropropagation of *Stevia rebaudiana* Using Nodal Explants and Growth Regulator Combinations

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ABSTRACT

Stevia rebaudiana is grown for the intensely sweet steviol glycosides in its leaves, yet its seeds germinate poorly and give offspring that differ from one another, which is why tissue culture is so often proposed as a route to uniform planting material. This narrative review asks how far nodal explants and combinations of growth regulators can be relied upon for in vitro micropropagation of the species, and how much weight the published protocols can carry. It draws on literature about the plant, the principles of shoot multiplication and rooting, the behaviour of cytokinins and auxins in culture, and the practical problems of contamination, browning, hyperhydricity, somaclonal variation and acclimatisation, and it reports no new data. Nodal segments are an attractive starting point because they carry axillary buds and tend to preserve the parent genotype, and a cytokinin followed by an auxin is the usual sequence for multiplication and rooting. Reports differ in genotype, medium, vessel and the way results are scored, so the best combination of regulators cannot be named from the literature as it stands, and few papers follow plants into the field or measure glycoside content afterwards. The review closes with a short list of what a comparable, decision-grade protocol study should record.

Keywords: *Stevia rebaudiana*; Micropropagation; Nodal explants; Cytokinins; Auxins; Plant tissue culture; Narrative review

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Article Class: Narrative Review

1. Introduction

Stevia rebaudiana (Bertoni) is a small perennial herb of the daisy family whose leaves hold a group of steviol glycosides, chiefly stevioside and rebaudioside A, that taste far sweeter than sugar (1, 2). Interest

in the plant has grown with the demand for natural, low-energy sweeteners, and the glycosides have a long record of discussion in food science and toxicology (3, 4). Wherever a crop is wanted at scale, the question of how to produce uniform



planting material follows.

Seed is a poor answer. The species sets seed unreliably, germination is weak, and seedlings differ from one another in vigour and in the sweet compounds they accumulate, so a field raised from seed is a mixed population (5, 6). Stem cuttings give true copies but are limited by the number of cuttings a stock plant can spare. Tissue culture offers a way around both limits, and the shoot-multiplication route from nodal segments has been the most common choice (7, 8).

What is less clear is how reliably the published protocols can be compared and reused. A reader meets many reports, each with its own medium, its own mixture of growth regulators and its own way of scoring success, and the headline claim is often a single best treatment. No local experiment is reported here. The article is a narrative review of the published literature on in vitro micropropagation of stevia from nodal explants, with attention to how growth regulator combinations are chosen and how far the results can be trusted. The aim is a fair account of what is known and what is merely repeated.

2. Literature Review

2.1 The Plant and the Case for Clonal Propagation

The commercial interest in stevia lies in the leaf, and the leaf is variable. The proportions of stevioside and rebaudioside A differ between plants, and agronomic factors such as harvest time, site and crop age shift the glycoside content further (9, 10). A grower who wants a consistent product therefore wants consistent plants, and that favours vegetative multiplication of a selected parent over seed.

Clonal propagation has a long history in horticulture and was one of the first practical applications of plant cell culture (11, 12). Standard accounts describe the

stages of establishing, multiplying, rooting and hardening shoots, each of which may need a different medium (13, 14), and the same logic is applied to medicinal plants generally (15, 16).

2.2 Explants and Their Behaviour

The choice of starting tissue shapes everything that follows (Table 1). A nodal segment carries a preformed axillary bud, and releasing that bud with a cytokinin is the most direct route to multiple shoots. Reports on stevia describe sterile nodal and shoot-tip cultures producing shoots and later roots on simple media (7, 17). Because the meristem is organised from the start, the plants that result are generally expected to resemble the parent.

Leaf and internode tissue behave differently. They usually pass through a callus stage before shoots appear, and callus formation reflects a reprogramming of differentiated cells that is not always tidy (18, 19). The older literature on somaclonal variation warned that such routes can produce plants that differ from the source (20, 21). For a crop whose value depends on leaf chemistry, that risk is a reason to prefer axillary shoots where the aim is true-to-type material.

Table 1. Explant sources used for *Stevia rebaudiana* cultures and what each offers.

Explant	Main route	Main caveat
Nodal segment	Axillary bud break and shoot multiplication (7, 17)	Needs clean, healthy stock plants (22)
Shoot tip	Direct shoot growth from the meristem region (15)	Small explant, easily damaged
Leaf or internode	Callus, then shoots (18, 19)	Greater risk of variation (20, 21)
Seedling parts	Shoots from sterile seedlings (6)	Seedlings differ genetically (5)

Note: Qualitative comparison drawn from the cited literature; no yields are given because they depend on genotype and protocol.



2.3 Media and Nutrition

Most stevia protocols start from the salt formulation of Murashige and Skoog (23), sometimes at reduced strength, with the B5 and Schenk and Hildebrandt media used less often (24, 25). The mineral supply influences how shoots grow and how they look, so a change of medium can alter the response to a growth regulator (26). Comparing two papers that used different media is therefore not the same as comparing two hormones. This is why the same medium can look excellent in one report and unremarkable in the next.

2.4 Cytokinins, Auxins and Their Balance

Growth regulators are the lever most protocols pull (Table 2). Cytokinins drive cell division and the release of axillary buds, and they are the usual choice for shoot multiplication (27, 28). Auxins work in the opposite direction at the root and are the usual choice for rooting. The interaction between the two, rather than either alone, determines whether a tissue forms shoots, roots or an undifferentiated mass (29, 30).

Table 2. Growth regulator classes and their usual roles in stevia culture.

Class	Usual role	Caution
Cytokinins (BAP, kinetin)	Shoot multiplication, release of axillary buds (27, 28)	High supply can cause abnormal shoots (31, 32)
Auxins (NAA, IBA)	Root formation; callus when high (29, 33)	Can carry over and inhibit shoots
Thidiazuron	Strong shoot induction in some species (34, 35)	Effects may persist after transfer
Regulator balance	Direction of growth depends on the ratio (13, 30)	Optimum varies with genotype (36)

Note: Roles are those described in the general plant tissue culture literature; concentrations are protocol-specific and are not given here.

This relationship is often drawn as a simple gradient (Figure 1) and it is a useful picture, but it is a picture and not a rule. The exact balance that favours shoots in one genotype or on one medium may give something else in another (13, 33). Stevia studies that compared regulator types and levels reported clear differences between treatments, though the best treatment was not the same from one study to the next (36, 37).

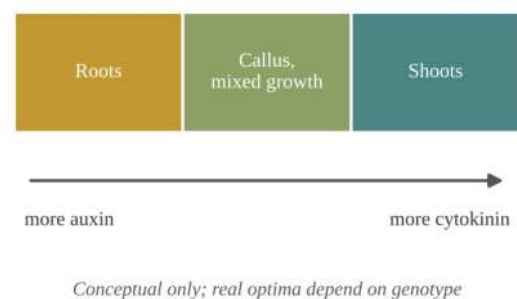


Figure 1. Direction of morphogenesis as the cytokinin-to-auxin balance shifts (conceptual schematic, not data).

2.5 Stronger Regulators and Their Costs

Thidiazuron is a synthetic compound with strong cytokinin-like activity that has been used where ordinary cytokinins fail to induce shoots (34, 35). Its strength cuts both ways: shoots can be distorted, and the effect can linger after transfer. A high cytokinin supply is also associated with the physiological disorders collected under the name hyperhydricity (31, 32).

3. Materials and Methods

3.1 Scope and Question

The review asks one question in two parts. How consistently does the published literature support nodal micropropagation of *Stevia rebaudiana*, and which features of the reported growth regulator combinations can be compared across studies? It does not attempt to recommend a concentration, because the literature does not support a single one.



3.2 Literature Assembly

The review draws on peer-reviewed work in four areas: the biology and chemistry of stevia, general principles of micropropagation, the action of plant growth regulators in culture, and the problems that arise in culture and after transfer. Items were chosen for relevance to those themes and for how clearly they described their methods, and only work published up to the date of this issue is cited.

We did not run a formal systematic search and we do not claim one. This is a narrative synthesis, and the selection of sources reflects judgement as well as coverage. Reviews of this kind are more useful when they say where the evidence stops than when they smooth it over.

3.3 Appraisal Framework

To judge how far a protocol report can be trusted, we asked four questions. Is the genotype or source described? Is the medium specified in full? Is the result scored in a way another laboratory could repeat? And were plants followed beyond the culture vessel?

4. Results

4.1 What the Reports Describe

Across the literature, the broad shape of a nodal protocol is familiar (Figure 2). Stock plants supply segments that are surface-sterilised and placed on a medium with a cytokinin. The shoots that emerge are multiplied by subculture, then moved to a medium with an auxin or a lower cytokinin supply to form roots, and finally hardened (7, 17, 38). Nothing in this outline is controversial. None of these steps is difficult, but each one is easy to skip.

The differences lie in the details that papers report unevenly. Explant source, number of nodes, vessel size, light, sugar

level and subculture interval all vary, and several are often missing. What the literature reveals most clearly is therefore variety rather than agreement. Such differences look small on paper, but they decide whether a protocol can be repeated elsewhere.

4.2 Growth Regulator Combinations

Reports on stevia generally find that a cytokinin alone can support shoot multiplication and that adding a low supply of an auxin can improve shoot quality, while rooting is encouraged by an auxin after multiplication (36, 37). The details, however, diverge. Different cytokinins are favoured in different papers, and the outcome is judged by shoot number in some, shoot length in others and fresh weight in a third. A grower reading a protocol needs the answer to a simple question: would this work with my plants?

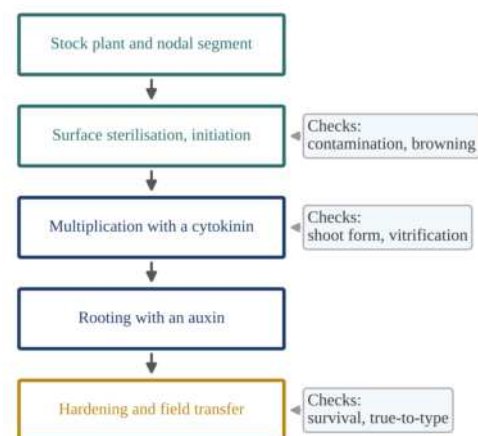


Figure 2. Stages of nodal micropropagation and the checks that surround them (schematic, not data).

These measures do not rank treatments the same way. A treatment that gives many short shoots can look best by number and worst by length, and neither says whether the shoots will root and survive. Without a shared outcome, a comparison between papers is an exercise in judgement. The point is not that a protocol is poor, only that its result travels less far than its abstract suggests.



4.3 Problems Reported in Culture

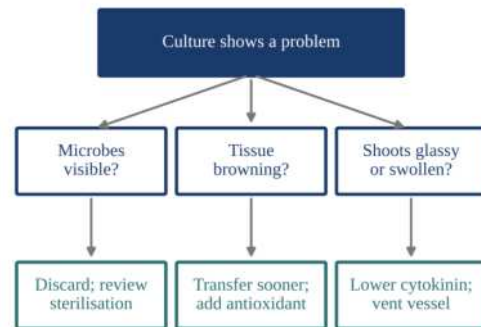
Contamination is a persistent hazard, and field-grown stock carries microbes that surface sterilisation does not always reach. Some bacteria live inside plant tissue and appear only after several subcultures (22, 39). A short decision tree for the commonest cases is sketched in Figure 3. Browning follows from the oxidation of phenolic compounds released at cut surfaces, and it is managed by prompt transfer, adsorbents such as activated charcoal, or antioxidants (40, 41). Table 3 lists the problems most often reported and the usual responses.

Table 3. Common problems in stevia micropropagation and usual responses.

Problem	Likely cause	Usual response
Contamination	Microbes on explants or in the room (22, 39)	Surface sterilisation, clean stock plants
Browning	Phenolic oxidation after cutting (40, 41)	Frequent transfer, charcoal, antioxidants
Hyperhydricity	Wet, crowded vessels, strong cytokinin (31, 32)	Drier medium, vented vessels
Poor rooting	Wrong auxin balance or weak shoots (15, 16)	Separate rooting step
Loss on transfer	Weak cuticle, humidity shock (42, 43)	Gradual hardening

Note: Responses are those discussed in the cited literature and are not ranked by effectiveness.

Hyperhydricity, once called vitrification, produces glassy, brittle shoots that root badly and die on transfer (31, 32). It is encouraged by wet, poorly ventilated vessels and strong cytokinin treatment. Where it occurs, an apparently high multiplication rate hides shoots that cannot be used. A multiplication rate that collapses once culture losses are counted is not a rate a nursery would ever see.



Schematic decision tree, not data

Figure 3. Schematic decision tree for common culture problems; not data.

4.4 Transfer to Soil and Beyond

Shoots leaving culture have thin cuticles and stomata that do not close properly, and they lose water fast in open air (42, 43). The usual remedy is gradual exposure to lower humidity. Alternative culture systems such as temporary immersion have also been described, although their use with stevia is much less documented than conventional agar-based culture (44). A shoot that has been given time to harden behaves differently from one that has not.

The reports that go further, and few do, describe plants in the field. One study of in vitro propagated stevia described enhanced stevioside accumulation in plantlets (38), but a single report cannot establish that micropropagated plants outperform conventionally raised ones in the field. Whether the chemistry of micropropagated plants matches that of the parent over several seasons is not settled by the papers reviewed here. Read this way, a poor response in one genotype is not evidence of a poor protocol.

5. Discussion

5.1 Interpretation

The main reading of this review is conditional. Nodal micropropagation of stevia works, in the sense that shoots can



be multiplied and rooted by methods that have been described repeatedly (7, 17). What does not follow is that any one combination of regulators is best. The evidence supports a general design, a cytokinin for multiplication and an auxin for rooting, and then leaves the fine settings to the laboratory and the genotype in hand. That is a modest claim, and it is the one the evidence will currently bear.

The second conditional concerns the end product. The point of the exercise is uniform plants with a desirable glycoside profile, so a protocol that produces many shoots but changes the plants or fails after transfer has missed its purpose (19, 21). Counting survivors in the field, and checking their chemistry against the parent, is the test that matters (9, 10).

5.2 Limitations

This is a narrative review and shares the limitations of that form. The selection of sources reflects judgement, and relevant work may have been missed. The reports reviewed differ so widely in genotype, medium and scoring that they cannot be pooled. Nothing in this reading argues against micropropagation; it argues for recording what actually happens in the vessel.

The review also relies on general plant tissue culture literature where stevia-specific work is thin, and these sources are imperfect proxies for a single species. Several stevia papers appear in regional journals with limited methods detail, and their findings may be more or less secure than their summaries suggest.

5.3 Directions for Future Work

Three needs stand out. First, studies should report genotype, source and medium in full, and score shoots with a shared set of outcomes. Second, comparisons of regulators should hold other factors constant and analyse results at the level of

the vessel or the plant that was actually the unit of treatment. Third, plants should be followed to the field and their glycoside profile compared with that of the parent.

6. Conclusion

Nodal micropropagation is a plausible and widely described route to uniform stevia planting material, and the general sequence of a cytokinin for shoots and an auxin for roots is well supported. The choice of a specific combination, however, rests on evidence that is hard to compare and rarely carried through to the field.

For the practitioner the message is practical rather than discouraging. A published protocol is a starting point to be tested on the genotype in hand, with losses recorded honestly and some plants taken to maturity before the method is called successful.

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9. Conflict of Interest

The authors declare no conflict of interest.

10. Author Contributions

D.S. conceived the review and wrote the sections on explants and culture problems. G.D. wrote the sections on growth regulators and media. C.P. prepared the tables and figures and contributed to the discussion. All authors read and approved the final text.

11. Ethics Statement

This is a review of published literature. No human or animal subjects were involved and no ethical approval was required.



12. Data Availability

No new data were generated. All sources are cited in the reference list.

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