Bot. Bull. Academia Sinica **22**: 57-62 (1981)

CLONAL PROPAGATION OF *STEVIA REBAUDIANA* BERTONI THROUGH AXILLARY SHOOT PROLIFERATION *IN VITRO*[1]

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(Received October 2, 1980; Accepted October 30, 1980)

Abstract

Excised node segments of *Stevia rebaudiana* Bertoni were cultured on a modified Murashige and Skoog's basal nutrient medium (MS) supplemented with various cytokinins. Benzyladenine (2 mg/l), kinetin (10 mg/l) and N^6-(Δ^2-isopentenyl) adenine (10 mg/l) promoted profuse axillary shoot proliferation. Naphthaleneacetic acid stimulated rooting and callusing of shoot explants from the cytokinin-induced axillary shoots. Rooted explants were successfully established in soil where they flowered and set fruit.

Introduction

Stevia rebaudiana Bertoni, a small weed of the composite family, contains a tetracyclic diterpene derivative, stevioside, which has been proven to be some 300 times as sweet as sucrose (Wood *et al.*, 1955). Contraceptive effect of leaf decoction has been reported (Planas and Kuc, 1968). Cultivation of this species in experimental scale has been conducted recently in Taiwan in attempting to exploit the possibility of using stevioside as a natural substitute for synthetic sweeteners (Chen *et al.*, 1978; Chu and Cheng, 1976). However, since seeds are usually sterile, other propagation methods such as stem cuttings are used, these tend to be slow and impractical when carried out on a large scale for propagation of selected elite individuals. Tissue culture propagation of this species, therefore, offers a possible alternative.

We recently reported the induction of multiple shoot formation on excised leaflets with a minimum of callus formation in vitro (Yang and Chang, 1979), and proposed to use this method for large scale propagation. However, for the purpose of large scale cloning it has been assumed that proliferation by

(1) Paper No. 245 of Scientific Journal Series, Institute of Botany, Academia Sinica, Republic of China.

axillary shoot branching is the most reliable way of ensuring genetically uniform propagules (Hussey, 1977; 1978). This paper describes a method for rapid multiple propagation of *S. rebaudiana* through axillary shoot proliferation and the successful establishment of cultured plantlets in soil.

Materials and Methods

Seeds of a local accession of *Stevia rebaudiana* Bertoni were graciously supplied by Professor Kwei Chen of Department of Horticulture, National Taiwan University, Taipei, Taiwan, Republic of China. The seeds were surface disinfected with 2.5% sodium hypochlorite for about 15 minutes, washed thoroughly with sterilized distilled water, and sowed aseptically on plain agar slants. From 3-week old seedlings, one cm node segments of main shoots or laterals were excised and cultured on a MS basal medium consisting of Murashige and Skoog (1962) salts and organics, 30 g/l sucrose, 40 mg/l myo-inositol. This basal medium was supplemented with various plant growth substances according to experimental requirements. The pH of the medium was adjusted to 5.7 before jellying with Difco-Bacto agar (8 g/l) and autoclaved. Cultures were maintained at 26±1 °C with cool white fluorescent tube illumination at 2 Klx for a daily 12 hr photoperiod.

Results and Discussion

In the basal medium without hormonal adjuvants, each node produced 1 or 2 axillary shoots (Fig. 1). In the presence of cytokinins (0.1–10 mg/l) profuse axillary shoot outgrowth resulted (Fig. 1, Table 1). The effects of different cytokinins on the axillary shoot outgrowth and adventitious shoot proliferation were examined by incorporating kinetin (K), benzyladenine (BA) and N^6-($\triangle^2$-isopentenyl) adenine (2iP) into agar-gelled basal media. BA alone resulted in marked increase of shoot proliferation per node throughout the concentrations ranging from 1 to 10 mg/l. Profuse shoot proliferation occurred at 2 mg/l BA. 2iP and K at a higher concentration (10 mg/l) also favored shoot proliferation from excised node segments.

Adventitious shoot-buds generated from the leaflets of proliferated axillary shoots when touched to the agar-gelled MS media containing cytokinins (2iP, BA, or K) (Fig. 2, Table 1). Media containing BA have been demonstrated to have a far better generative effect on adventitious shoot-bud formation than 2iP or K.

Proliferated shoots about 2 cm long were excised and transferred to an agar-gelled MS medium without hormonal supplements or to moistened vermiculite to promote adventitious root formation. One or two adventitious roots formed from the basal portions of the explants over a period of 7–15

Fig. 1. Effects of three cytokinins at 2 mg/l on axillary shoot proliferation from excised node segments on an agar-gelled MS medium for 50 days at 26±1 °C under 12 hr lighting (2 Klx) per day.

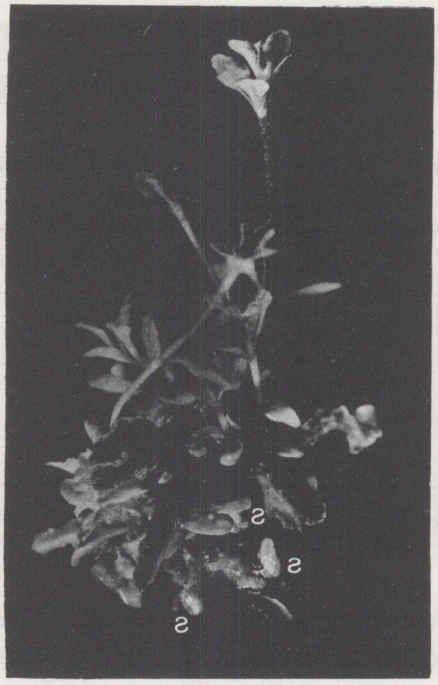

Fig. 2. Adventitious shoot (s) formed when leaflets of proliferated shoot touched the agar-gelled medium containing 2 mg/l BA.

Table 1. *Effects of cytokinins on axillary shoot proliferation, and adventitious shoot-bud formation on leaflets of axillary shoots when touched to the culture medium*

Cytokinin (mg/l)		Shoots/node	Adventitious shoot formation
	0	2.1[1]	—[2]
K[3]	0.1	1.8	—
	1	2.9	—
	2	3.6	—
	10	13.6	++
BA	0.1	3.9	—
	1	8.9	++
	2	12.5	+++
	10	13.8	++++
2iP	0,1	4.0	—
	1	6.0	+
	2	6.9	—
	10	15.0	+++

[1] Data were averaged at 10 replications per treatment at the end of 50 days culture.
[2] Signs represent the amount of adventious shoot proliferation: —, nil; +, few, ++, moderate; +++, high; ++++, highest.
[3] K represents kinetin; BA, benzladenine; 2iP, N^6-($\triangle^2$-isopentenyl) adenine.

Table 2. *Effects of NAA on the rooting and callusing of shoot explants, grown on agar-gelled medium for 2 weeks*

NAA (mg/l)	Rooting %	Roots/explants	Callusing
0	69	1.2	—[1]
0.01	54	0.8	—
0.1	70	1.4	++
1	100	5.4	+++
10	100	8.0	++++

[1] Signs represent degree of callusing: —, nil; ++, low; +++, moderate; ++++, high. Each treatment was comprised of 10 replicates.

days (Table 2). Naphthaleneacetic acid (NAA) at concentrations ranging from 0.1–10 mg/l greatly promoted both the percentage of cuttings with root formation and the number of roots formed (Table 2). However, the basal portions of cuttings tended to callus when cultured on a MS medium plus NAA at 0.1–10 mg/l. Adventitious buds occassionally formed in this callus.

Because of the well-known tendency for callus to generate genetically aberrant plants, incorporation of NAA into the medium encourages horticultural difficulties.

The rooted plantlets were then transferred to pots with sand loam soil mixture and grown into plants that flowered and set fruit normally. There was no need for particular hardening precautions. Carefully examination revealed no evidence of phenotypic changes.

Previous tissue culture work on this sweetening plant was concerned mainly with callus proliferation from various tissues (Handro *et al.*, 1977) and production of secondary metabolic products (Nabeta *et al.*, 1979; Suzuki *et al.*, 1976). We (Yang and Chang, 1979) were able to obtain plant regeneration from leaf explants and shoot formation from callus. Handro *et al.* (1977) briefly demonstrated the outgrowth of 1 or 2 axillary buds from excised node segments in a nutrient medium without hormonal supplements but did not demonstrate profuse cytokinin-induced axillary shoot proliferation as shown in our present paper. The data of this report unequivocally demonstrate that profuse axillary shoot proliferation is possible from excised node segments and plants can be successfully established from these axillary shoots in soil. Apparently the potential for cloning is immense and therefore axillary shoot proliferation may be used for rapid multiplication of this sweetening plant on a large scale.

Acknowledgements

We thank Miss Chan-Wong Huang for technical assistance. This work was support by National Science Council, Republic of China.

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用側芽培養法增殖甜菊

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含側芽之甜菊 (*Stevia rebaudiana* Bertoni) 之莖段切節用 Murashige and Skoog 基本培養基培養。Benzyladenine (2 mg/l), kinetin (10 mg/l) 和 N^6-($\triangle^2$-isopentenyl) adenine (10 mg/l) 之添加培養基可促進多量不定芽之形成。此不定芽形成之枝條，經切取成小插條在不含生長素之基本培養基或砥石中發根成活容易、成活植株生長、開花、結子正常。