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CALLUS GROWTH AND ROOTING OF CASSAVA (MANIHOT ESCULENTA CRANTZ) STEM SEGMENTS CULTURED IN VITRO

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SUMMARY

With the in vitro culture of cassava tissue the optimum sucrose level for callus growth is lower than that for the initiation and growth of roots. Auxin and cytokinin both promote callus growth, the latter by inducing cell division, the former also by stimulating cell elongation. In subcultures cytokinins are obligatory for callus growth, the natural cytokinins, zeatin and 2iP, yielding green tissues. Organ development never occurred in these subcultures.

Auxin (NAA) is required for the initiation and growth of roots on the callus. Different cytokinins reduce this rooting effect in various degrees without, however, inducing the initiation of shoot primordia.

1. INTRODUCTION

In vitro culture of the tropical root crop, cassava (*Manihot esculenta* Crantz), may be helpful in rapid vegetative propagation and for obtaining virus-free material. Up to now, however, attempts have been little successful. SADIK (1972) obtained globular embryoids in cell suspension culture, but failed to achieve their further development. In the present study the effects of growth regulators and other factors in the culture of stem segments were analysed. Callus development and rooting were readily obtained but sprout formation did not occur.

2. MATERIALS AND METHODS

The clonal cultivars Mangi, São Paulo, Bogor, and Mohede were grown in a tropical glasshouse at high relative humidity. Internodal stem segments from the upper 30 cm of young shoots were used, giving better responses in the in vitro culture than segments from older stem parts, leaves, petioles, or tubers.

The segments were superficially sterilized by a 15 sec. dip in 70% ethanol, followed by rinsing in sterile water and soaking in a 1.3% NaClO solution, containing 5 ppm (v/v) Triton X -100, during 15 minutes. After thoroughly rinsing with sterile water, transverse sections of 0.5–1.0 cm length were cut and placed with their basal ends on 15 ml agar medium in glass tubes, 23 mm internal diameter. The tubes were closed by plugs of non-absorbent paper, covered with aluminium foil to prevent drying up, and incubated at 29°C, 85% relative air humidity, with 16 hrs light per day from fluorescent and incandescent lamps (red light 72, blue light 85 mW. $m^{-2}.nm^{-1}$).

The basal medium was after MURASHIGE & SKOOG (1962), containing 3% sucrose, 0.1% casein hydrolysate, and 0.8% Difco Bacto agar (purified). The

growth-regulating substances used were 1-naphthylacetic acid (NAA), 6-benzylaminopurine (BA), 6-furfurylaminopurine (kinetin), 6-γ, γ-dimethylallylaminopurine (2iP), and zeatin, all from Sigma Chem. Comp., St. Louis, Mo., USA.

Per treatment 12 segments were incubated for 6–12 weeks until growth had ceased. The number and length of the roots were determined and the fresh weights of callus and roots measured.

3. RESULTS

3.1. Callus development

Callus growth hardly occurred in the absence of growth regulators, the auxin, NAA, considerably promoting callus development: *table 1.* Callus formation could be observed after 4 to 7 days already, about 3 g per explant being produced at the optimum concentration, 10^{-4} M NAA. This NAA-induced callus was whitish and friable, and turned brown within three weeks. The cytokinin, zeatin, also stimulated callus growth, optimum at 10^{-5} M. These calluses were smaller, greenish, and very firm. The difference resembles that between auxin- and cytokinin-induced growth of disks of parenchymous tissues, in which auxin mainly enhanced cell expansion and cytokinin cell division (BRUINSMA 1967). Consequently, the combined addition of the two regulators gave an additive or even synergistic effect (*table 1*).

Table 1. Effects of NAA and zeatin on callus growth.
Average fresh weight of callus, in mg, after 10 weeks culture of cv. Mangi stem segments.

	zeatin		
NAA	0	10^{-6} M	10^{-5} M
0	30	88	293
10^{-7} M	69	233	315
10^{-6} M	238	588	709
10^{-5} M	1354	1565	1718
10^{-4} M	3271	–	–
10^{-3} M	2185	–	–

3.2. Callus growth in subculture

From calluses of stem segments grown in the presence of 10^{-4} M NAA for two weeks, pieces of 100 to 200 mg were removed and cultured on different media. On White's medium (WHITE 1962) growth was always very poor at all combinations of regulators tried. On the Murashige-Skoog medium, growth of the first subculture at 10^{-4} M NAA was optimum at 1 per cent sucrose. Unlike the calluses grown at 3 per cent sucrose, the white calluses did not turn brownish (see also 3.4).

Table 2 shows the effect of cytokinins on callus development in the second subculture, in the presence of 1 per cent sucrose. With only 10^{-4} M NAA as

Table 2. Effects of cytokinins on callus growth in second subculture of cv. Mangi. Initial fresh weights about 150 mg; average values of 10 measurements after 11 weeks culture.

cytokinin (10^{-5} M)	NAA (M)	fresh wt. per explant (mg)	dry wt. per explant (mg.)	colour
no	10^{-4}	263	14	brown
2iP	10^{-4}	1980	52	light brown
2iP	10^{-5}	2220	86	green
2iP	10^{-6}	1930	77	green
zeatin	10^{-5}	2040	79	green
BA	10^{-5}	960	35	light brown
kinetin	10^{-5}	1620	54	brown

in the first subculture growth was completely absent, whereas the addition of 10^{-5} M 2iP caused abundant growth. As compared with the NAA effect in *table 1* the auxin concentration hardly affected growth in subculture anymore. The natural cytokinins, zeatin and 2iP, were more effective in promoting a healthy callus development than the synthetic ones, particularly striking being the green colour which turned up after five weeks in all cultures containing zeatin or 2iP at lower auxin concentration. With BA only one culture turned green after 12 weeks, kinetin induced a fragile and rapidly browning tissue. Organized growth remained absent in all cultures.

3.3. Initiation and growth of roots

Root initiation was never observed in the absence of growth-regulating substances, NAA permitting root initiation on the callus immediately after its formation on the stem segments. As can be seen in *fig. 1*, 10^{-5} M NAA was optimum for root production (41 roots per explant), higher doses being strongly inhibitory, allowing for callus growth only.

The cytokinin, BA, not only was unable to induce rooting, but progressively

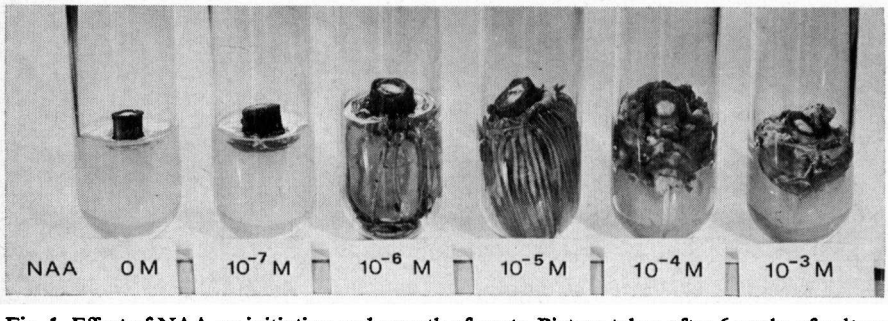

Fig. 1. Effect of NAA on initiation and growth of roots. Picture taken after 6 weeks of culture of cv. Mangi stem segments.

Table 3. Effects of BA on initiation and growth of roots at 10^{-5} M NAA. Twelve weeks culture of cv. Mangi stem segments.

BA concn. (M)	rooting explants (%)	No. of roots per explant	fresh wt. of roots per explant (mg)
0	100	34.2	923
3.10^{-7}	90	26.0	520
10^{-6}	89	9.4	404
3.10^{-6}	14	0.1	6
10^{-5}	0	0	0

inhibited root initiation (*table 3*). At 10^{-5} M root formation was completely suppressed, but shoot initials were never observed. Other cytokinins were less active than BA, but they all inhibited rooting without inducing sprouting (*table 4*). At the combination of 10^{-5} M NAA and 10^{-5} M zeatin all cultures showed inversed geotropy of roots.

The four cultivars showed large quantitative but no qualitative differences in their rooting responses to the various growth regulators.

Table 4. Interactions of NAA and different cytokinins on rooting. Ten weeks culture of cv. Mangi stem segments.

	10^{-6} M NAA		10^{-5} M NAA	
	rooting explants (%)	No. of roots per explant	rooting explants (%)	No. of roots per explant
no cytokinin	100	6.3	100	41.0
zeatin 10^{-6} M	100	6.2	100	6.2
10^{-5} M	100	4.1	100	1.9
2iP 10^{-6} M	100	4.5	100	3.0
10^{-5} M	100	4.0	20	0.2
kinetin 10^{-6} M	100	5.2	100	17.4
10^{-5} M	63	1.5	90	6.7
BA 10^{-6} M	100	10.0	89	9.4
10^{-5} M	0	0	0	0

3.4. Effects of sucrose concentration and of temperature

The requirement of sucrose is shown in *table 5*. The concentration normally used, 3 per cent, was optimum for the initiation and growth of roots, although callus weight was somewhat reduced at that level, possibly owing to competition by the roots. In subculture, however, where root initiation did not occur, 1 per cent sucrose also allowed for more callus fresh weight than 3 per cent,

Table 5. Effect of sucrose concentration in the medium on root formation and callus growth of cv. Mangi stem segments cultured during 12 weeks in the presence of 10^{-6} M NAA.

sucrose conc. (g/l)	rooting explants (%)	No. of roots per explant	root fresh wt. per explant (mg)	callus fresh wt. per explant (mg)
1	0	0	0	210
5	33	0.8	2	441
10	43	1.0	52	719
30	100	9.2	742	570

Table 6. Effect of sucrose concentration in the medium on callus growth in first subculture, cv. Mangi. Determination after 7 weeks of culture in the presence of 10^{-4} M NAA.

sucrose conc. (%)	growing cultures (%)	average callus fresh wt. (mg)	average callus dry wt. (mg)
1	30	2220	69
3	70	1260	78
5	66	460	41
7	25	380	46

higher sucrose concentrations becoming progressively inhibitory: *table 6*. Comparison of fresh and dry weights shows that the dry matter content gradually increased from 3 to 12 per cent, indicating that the water uptake was osmotically restricted at increasing sugar concentrations. At 1 per cent sucrose the dry weight production was nearly optimum, the water intake the least impaired, and the tissue did not turn brown as at the higher concentrations.

The temperature was usually kept at 29 °C, being about the optimum temperature for cassava tissue growth. Introducing an initial period at a lower temperature, however, proved to be very favourable to the rooting of, particularly, cv. São Paulo stem segments (*table 7*). This pretreatment never led to the formation of shoot initials.

Table 7. Effect of initial period of 2 weeks at lower temperature on rooting of stem segments. Kept at 29 °C during a further 7 weeks, in the presence of 10^{-5} M NAA and 10^{-5} M zeatin.

cultivar	Mangi		São Paulo	
	rooting explants (%)	No. of roots per explant	rooting explants (%)	No. of roots per explant
29° continuously	60	3.3	0	0
20° → 29°	100	15.3	0	0
15° → 29°	90	6.8	67	4.3

4. DISCUSSION

The results of the present experiments generally agree with those obtained with other plant materials (e.g., PIERIK & SEGERS 1973). The carbohydrate supply

is an important factor, both for callus growth and for root development. The optimum level for root formation, 3 per cent sucrose, is rather high for callus growth, tending to restrict the uptake of water osmotically and to cause browning of the tissue.

Auxin or cytokinin are required for callus growth, the former leading to a more brittle tissue, the latter to a firmer one. This, and their mutual reinforcement, are in accordance with the finding that auxin promotes mainly cell enlargement, and cytokinin cell division, in parenchymous tissue disks of chicory root and of Jerusalem artichoke tuber in vitro (BRUINSMA 1967). In subcultures the cytokinin requirement becomes obligatory for callus growth, the natural cytokinins then producing greening tissues under the experimental conditions. Organised growth, however, was never observed in subcultures.

Initiation and growth of adventitious roots does not occur in the absence of auxin. Cytokinins invariably counteract the rooting effect of auxin. However, they never lead to the alternative formation of sprout initials, as they do in many other plant materials in which the ratio of shoot to root initials appears to be governed by the relative amounts of cytokinins and auxin (SKOOG & MILLER 1957, PAULET & KETATA 1969, PIERIK & WOETS 1971). Possibly the cassava tissue contains a potent inhibitor of shoot induction or lacks the presence of a co-factor available in most other plant materials.

Note

During the preparation of this manuscript, KARTHA et al. (1974) reported the regeneration of cassava plants from apical meristems, cultured in the presence of BA, NAA, and gibberellic acid (GA_3).

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