Plant Cell, Tissue and Organ Culture **31**: 105–110, 1992.

Slow growth *in vitro* conservation of coffee (*Coffea* spp.)

2: Influences of reduced concentrations of sucrose and low temperature

Anna Bertrand-Desbrunais, Michel Noirot & André Charrier
Orstom, Genetic Resources and Tropical Plant Breeding Laboratory, B.P. 4045, F 34032 Montpellier cedex 01, France

Received 5 July 1991; accepted in revised form 6 February 1992

Key words: Coffea, in vitro storage, low temperature storage, shoot culture, slow growth, sucrose concentration

Abstract

The effects of reduced sucrose concentrations and low temperature on a collection of coffee microcuttings have been examined. Sucrose concentrations of 0.5 g l^{-1} and 20 g l^{-1} and temperatures of 20°C and 27°C were compared in three accessions: the Arabusta (interspecific hybrid) and *Coffea arabica* L. cv. 'Caturra amarillo' and cv. 'Mokka de Tahiti'. After six months, low sucrose concentrations reduced microcutting growth, rooting and survival rate. At 20°C, microcutting growth was also reduced, but leaf loss and survival rate were promoted. The genotypic differences at six months were minor. After one year without subculture, survival rate was influenced by sucrose concentration and by genotype. These two species can be cold-stored six months at 20°C on a medium containing at least 20 g l^{-1} sucrose.

Abbreviations: BA – 6-benzylaminopurine, MS – Murashige & Skoog

Introduction

In vitro micropropagation is currently used for the two cultivated coffee species *Coffea arabica* L. and *C. canephora* Pierre (Söndahl 1985). Techniques have been perfected for fast growth and high multiplication rate of selected genotypes.

However, *in vitro* culture may also be used for conservation of plant genetic resources (Henshaw & O'Hara 1983; Withers 1989; Dodds 1991). In the present study, a collection of coffee vitroplants, permanently available for laboratory experiments and plant material transfers, was maintained by increasing the subculture time to 6 or 12 months. Previous experiments showed that numerous species can be cultured without 6-benzylaminopurine (BA) (Bertrand-Desbrunais et al. 1991).

The effects of low sucrose concentrations on metabolic activity have been studied in coffee germplasm preservation (Kartha et al. 1981) and in storage of grapevine (Galzy & Compan 1988). The influence of low concentrations of nutrient salts on growth limitation have been demonstrated by Schnapp & Preece (1986) for tomato and carnation microplants. Decreasing the growth temperature also successfully reduces the growth rate of cultures (Wanas et al. 1986; Westscott 1981), even in the case of tropical plants (Banerjee & de Langhe 1985; Staritsky et al. 1986).

The present work involved investigations of slow growth conditions for the storage of coffee shoots cultured *in vitro*. Reduced concentrations of sucrose in medium containing half-strength Murashige & Skoog (1962) nutrient salts, decreased culture temperature (20°C), and genotypic effects have been examined.

Materials and methods

Experimental conditions

The basal medium consisted of MS nutrient salts supplemented with Morel and Wetmore vitamins (Morel & Wetmore 1951). The pH was adjusted to 5.6 and 0.1 M KOH prior to addition of agar (7 g l^{-1}) and autoclaving (20 min, 10 KPa, 110°C). All cultures were exposed to a 12-h photoperiod under warm white fluorescent tubes providing a light intensity of 50 μmol $m^{-2} \cdot s^{-1}$.

Before the experiment, the plant material was maintained in growth phase at 27°C on multiplication medium consisting of basal medium supplemented with 30 g l^{-1} sucrose and 0.3 mg l^{-1} BA. These conditions are termed 'normal' in the experiments described below.

Shoots of two cultivars of *C. arabica*, 'Caturra amarillo' and 'Mokka de Tahiti', and one strain of Arabusta, interspecific hybrid (4×) (*C. arabica* × *C. canephora*) (Capot 1972) were obtained from nodal cuttings *in vitro*. The experiment was initiated with terminal shoot tips (1–1.5 cm high 2–3 nodes). Each shoot was transferred to a glass culture tube containing 20 ml of medium. The tubes were closed with glass stoppers and sealed with adhesive tape to prevent desiccation.

For the experiment, the basal medium containing half-strength MS nutrients salts, was supplemented with 0.2 mg l^{-1} indolebutyric acid and three sucrose concentrations were compared: 0 g l^{-1} (according to Kartha et al. 1981), 5 g l^{-1} and 20 g l^{-1}. All shoots were precultured for one month at 27°C before placing half of the culture at 20°C. The experiment was performed with 24 shoots per treatment (medium and temperature) for each genotype. In comparison, 24 shoots for each genotype were maintained under standard growth conditions on multiplication medium at 27°C. All tubes were distributed according to a random experimental design.

Parameters and data analysis

Data were collected after a six-month subculture. The following parameters were evaluated: survival, shoot elongation, axillary shoot development, rooting and leaf loss when the basal leaves turned yellow and fell. These qualitative observations were coded as presence/absence. The survival rate was again scored after one year of storage.

For the contingency tables analysis, the frequencies were compared using the Pearson χ^2 test. The significance level was adjusted according to Ryan (1960) to allow multiple comparisons two by two.

The frequency tables were processed by correspondence analysis (Benzecri 1973), which describes the observed variability in terms of principal uncorrelated factors and quantifies the effect of these factors. The interpretation had three aims: to establish the biological significance of these factors with the help of their correlated variates; to note the influences of temperature and sucrose on this process and, in terms of causality, on the depending parameters.

The two-dimensional graphs allow the projection of individual values and variates on this new reference system. Some simple rules help in the interpretation. The distance between an individual value and the origin expresses a difference with respect to the mean behaviour. The proximity of two individual values reflects similar behaviour. A frequent modality occurs near the origin, and two adjacent modalities indicate simultaneous presence.

Values obtained on the multiplication medium were not considered in the analysis. However, this medium was projected on the factorial plane, as a supplementary variate.

Results

Main effects of the studied factors

Variations induced by sucrose concentration

Sucrose acted on shoot elongation, rooting and survival. Shoot development was more pronounced at higher sucrose concentrations (Table 1). Rooting, weak on average (10%), was promoted only at the highest concentration. Survival declined clearly on sucrose-free medium.

Effects of temperature reduction

After six months, maintenance at 20°C improved survival in comparison with culture at 27°C and

Table 1. Effects of sucrose concentration on coffee microplant growth*.

Sucrose concentration ($g\ l^{-1}$)	Shoot elongation frequency	Rooting frequency	Survival frequency
0	8^a	3^a	71^a
5	26^b	5^a	95^b
20	68^b	10^b	95^b
χ^2	129.4	6.7	50.3
D.F. = 2	$p < 0.001$	$p < 0.05$	$p < 0.001$

* % explants exhibiting response. Each sucrose treatment was replicated 144 times, with one explant (1.5 cm long) per tube. Data were collected after six months. Results are averaged over all coffee genotypes and both temperatures (20°C and 27°C). For each variable, data followed by a different letter are significantly different ($p < 0.05$).

substantially depressed the shoot elongation rate (Table 2). In some microcuttings (5%) temperature reduction induced yellowing of basal leaves, which subsequently fell.

Genotypic effect

The average behaviour of the compared genotypes were relatively similar. The more marked differences affected the Arabusta genotype which was distinguished by better rooting and survival rate (Table 3). *C. arabica* cv. 'Caturra amarillo' presented more marked leaf loss (6%).

Temperature- and sucrose-related differences in behaviour

Only shoot elongation was influenced perceptibly by treatments (Table 4). At 27°C, Arabusta was

Table 2. Effects of temperature on coffee microplant growth*.

Temperature	Shoot elongation frequency	Basal leaf loss frequency**	Survival frequency
20°C	27	5	91
27°C	41	1	83
χ^2	9.9	6.4	6.6
D.F. = 1	$p < 0.01$	$p < 0.05$	$p < 0.01$

* % explants exhibiting response. Each temperature treatment was replicated 216 times, with one explant (1.5 cm long) per tube. Data were collected after six months. Results are averaged over all coffee genotypes and all sucrose concentrations.

** The basal leaves turn yellow and fall.

Table 3. Effects of genotypes on coffee microplant growth*.

Genotypes	Rooting frequency	Basal leaf loss frequency**	Survival frequency
Arabusta	15^a	1^a	93^a
Caturra amarillo***	1^b	6^b	86^{ab}
Mokka de Tahiti***	3^b	2^a	81^b
χ^2	21.9	6.3	9.7
D.F. = 2	$p < 0.001$	$p < 0.05$	$p < 0.01$

* % explants exhibiting response. Each coffee genotype was replicated 144 times with one explant (1.5 cm long) per tube. Data were collected after six months. Results are averaged over all sucrose concentrations and both temperatures. For each variable, data followed by a different letter are significantly different ($p < 0.05$).

** The basal leaves turn yellow and fall.

*** Caturra amarillo and Mokka de Tahiti are two cultivars of the species *C. arabica*.

the least influenced by sucrose concentration, but presented the more marked differences, at 20°C.

Independence of the effects induced by sucrose concentration and temperature on growth limitation

Correspondence analysis showed that most of the observed variability (88%) was explained by two factors (Fig. 1). The first factor contrasted death and microcuttings without growth (no shoot development, no root formation) with survival and microplants with root and shoot elongation variates linked to higher sucrose concentration. This factor reflected the effect of the quantity of nutrient reserves available in the medium. In the second factor, leaf loss and the limited shoot elongation were linked to the reduced temperature of 20°C, and death and shoot development were associated with the high temperature (27°C). This factor expressed the influence of temperature and of the metabolic activity of the microcuttings. During the first six months, the effects of the sucrose concentration and temperature were independent.

Each parameter was more or less influenced by the following factors:

- Mortality was divided between the two factors. It was linked to pronounced metabolic activity and lack of sucrose.
- Shoot elongation resulted from the simulta-

Table 4. Effects of temperature, sucrose concentration and genotype on coffee microplant shoot elongation*.

	Frequency of shoot elongation					
Temperature	20°C			27°C		
Sucrose concentration	0 g l^{-1}	5 g l^{-1}	20 g l^{-1}	0 g l^{-1}	5 g l^{-1}	0 g l^{-1}
Arabusta	4	8	50	29	42	67
Caturra amarillo**	4	25	71	4	25	75
Mokka de Tahiti**	0	21	58	4	33	87

* % explants exhibiting the response. Each treatment was replicated 24 times, with one explant (1.5 cm long) per tube. Data were collected after six months.
** Caturra amarillo and Mokka de Tahiti are two cultivars of *C. arabica*.

neous presence of substantial amounts of sucrose and a high temperature.
- Rooting was correlated with sucrose concentration.
- Survival was associated with the low sucrose concentration and resulted from reduced metabolic activity.
- Leaf loss was an extreme form of metabolic deceleration dependent on reduction in temperature.
- Axillary shoot development was an infrequent phenomenon favoured by substantial nutrient reserves and metabolic activity.

Survival after one year without subculture

After one year, the overall survival rate declined substantially (Table 5). Survival was favoured by the amount of the nutrient reserves.

The Arabusta was, on average, the most tolerant strain for both the reduction of temperature and the low sucrose concentration. The 'Mokka de Tahiti', however, was clearly more fragile. There was no interaction between genotype and temperature, but the 'genotype × sucrose concentration' interaction was highly significant (Table 6). Lack of sucrose had a more depressive effect on the Arabusta, the more vigorous genotype.

Table 5. Effects of temperature and sucrose concentration on survival rate of coffee microplants*.

Temperature	Sucrose concentration (g l^{-1})		
	0	5	20
20°C	25	46	57
27°C	7	26	60

* % surviving explants. Each treatment was replicated 72 times, with one explant (1.5 cm long) per tube. Data were collected after one year. Results are averaged over the three coffee genotypes.
The general χ^2 was: $\chi^2 = 7.4$ D.F. $= 2$ $p < 0.05$.

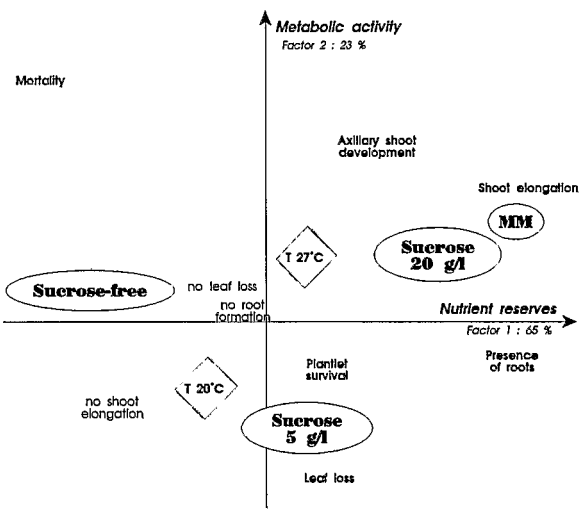

Fig. 1. Coffee microcutting behaviour.

Factorial plane 1-2
Data were collected after six months.
Media differ in sucrose concentrations:
sucrose-free 5 g.l^{-1} 20 g.l^{-1}
The growth temperatures were: 20°C and 27°C
Compared genotypes were the Arabusta interspecific hybrid and two cultivars of *C. arabica*: Caturra amarillo and Mokka de Tahiti.
The microplants on the multiplication medium (MM) containing 30 g l^{-1} sucrose and 0.3 mg l^{-1} BA were cultured at 27°C.

Table 6. Effects of the sucrose concentration and of the genotype on the survival rate of the coffee microplants*.

Genotype	Sucrose concentration (g l^{-1})		
	0	5	20
Arabusta	37	73	89
Caturra amarillo**	10	25	35
Mokka de Tahiti**	0	10	50

* % surviving explants. Each sucrose concentration was replicated 48 times, with one explant (1.5 cm long) per tube. Data were collected after one year.
** Caturra amarillo and Mokka de Tahiti are two cultivars of *C. arabica*.
The general χ^2 was: $\chi^2 = 14.2$ D.F. = 4 $p < 0.01$.

Discussion

The results obtained with multiplication medium under the normal conditions compared to those observed after the modification of the culture medium and conditions showed that:

- The use of half-strength salts medium did not affect the survival rate and the low sucrose concentrations reduced the shoot development.
- The survival rate observed on the sucrose-free medium indicated a photoautotrophic adaptation.
- Temperature reduction to 20°C slowed metabolic activity and favoured survival on media containing low sucrose concentrations.
- Genotypic effects were clearly distinguishable.

After one year, the average survival rates on media containing 20 g l^{-1} sucrose and on the multiplication medium were the same. Kartha et al. (1981) have also observed that coffee shoots maintained at 26°C on a medium similar to our sucrose-free medium exhibited root formation at high frequency (90%) and limited shoot development (after 2 years, plantlets did not exceed a height of 3–4 cm), and that the survival rate was unaffected. In our experiments, the rate of growth reduction was dependent on sucrose concentration. Similarly, lowering the sucrose concentration to 5 g l^{-1} reduced shoot and root elongation in tomato and carnation microcuttings (Schnapp & Preece 1986).

When no sucrose was provided many of the microplants of both cultivars of *C. arabica* died at 20°C and at 27°C. In contrast, the interspecific hybrid alone presented, after one year, a survival rate of 54%, for the microcutting stored at 20°C. This result was unexpected for the *C. arabica* as Kartha et al. (1981) reported that for two other cultivars, 'Catuai' and 'Caturra rojo', rooted plantlets seemed able to survive on medium lacking sucrose. This reduction of carbohydrate content also induced photosynthetic activity in some cases (Westscott 1981; Langford & Wainwright 1987; Galzy & Compan 1988). In potatoes, a sucrose concentration of 10 g l^{-1} allowed adaptation of microplants which survived afterwards on sucrose-free medium (Westscott 1981). However, although this concentration (10 g l^{-1}) also improved the photosynthetic ability of rose microcuttings, Langford & Wainwright (1987) showed that the latter were unable to survive solely by photoautotrophy.

Tropical and subtropical plants present a lesser natural cold-resistance, and temperatures of 10°C–12°C induced physical dysfunction (Lyons 1973). Banerjee & De Langhe (1985) determined the lower tolerance limits at 15°C to 18°C, whereas cassava poorly tolerated temperatures below 20°C (Roca et al. 1982). Similarly, the coffee tree, a tropical plant, is sensitive to reductions in temperature. Comparative cold (20°C) reduced metabolic activity and growth, and sometimes promoted leaf loss. As a general rule, the minimal storage temperature depended on the ecology of the geographical source of the plant. This was verified for *C. arabica*: this species, originating from tropical mountains, is naturally tolerant at 20°C. So, one-year storage on the medium containing 20 g l^{-1} sucrose did not induce a higher death rate at 20°C than at 27°C. The survival rate was improved at 20°C on medium lacking sucrose.

We observed more marked differences between Arabusta and *C. arabica* than between the two cultivars of *C. arabica*. Such intergenotypic differences in general have also been reported for grape (Galzy & Compan 1988). The better stress tolerance of Arabusta, probably due to its hybrid vigour, has also been noted under natural conditions (Capot 1972). This diversity of behaviour calls for the use of conservation methods suitable for each genotype. Further studies on other *Coffea* species are necessary in order to apply this method to the whole collection.

We are trying to reduce the metabolic activity to a minimum, while sustaining survival as longer

as possible. A high percentage survival was an imperative condition; it minimized the risks of genetic drift. The purpose was to achieve a maximal survival rate after one year. Culture storage at 20°C on the medium containing 20 g l^{-1} sucrose, subcultured yearly, seemed to be an interesting procedure for the medium-term conservation of the mountain coffee. Nevertheless, this survival rate may be improved by increasing the rooting rate: Sauer (1985) noted a relationship between cold tolerance and rooting rate in *Prunus avium*.

The sought-after medium should ensure a better survival rate, a good rooting frequency, slow growth and a lack of axillary shoot development. The best medium was that containing 20 g l^{-1} sucrose, at both storage temperatures (20°C and 27°C), since the survival rate reached about 60% after one year. On the other hand, the multiplication medium cannot be chosen because after six months it induced marked axillary shoot development (47% compared with 1% for the 20 g l^{-1} sucrose medium), greater main shoot elongation (86% versus 60%), and a lower rooting rate (3% versus 10%). Nevertheless in our laboratory we have a collection of 15 different coffee species maintained on multiplication medium in 120 ml bottles in preference to tubes (Bertrand-Desbrunais & Charrier 1990). Under these conditions subculture delay was eight months and the survival rate was greater than 90%.

References

Banerjee N & De Langhe E (1985) A tissue culture technique for rapid clonal propagation and storage under minimal growth condition of Musa (Banana and Plantain). Plant Cell Rep. 4: 351–354

Benzecri JP (1973) L'analyse des données. Tome 2, L'analyse des correspondances. Dunod, Paris

Bertrand-Desbrunais A & Charrier A (1990) Conservation des ressources génétiques caféières en vitrothèque. In: ASIC (Ed) XIII Intern. scientific colloquium on coffee, Païpa 1989 (pp 438–447). ASIC, Paris

Bertrand-Desbrunais A, Noirot M & Charrier A (1991) Minimal growth *in vitro* conservation of coffee (*Coffea* spp.) 1: Influence of low concentrations of 6-benzyladenine Plant Cell Tiss. Org. Cult. 27: 333–339

Capot J (1972) L'amélioration du caféier en Côte-d'Ivoire; les hybrides Arabusta. Café Cacao Thé. 16: 3–8

Dodds JH (Ed) (1991) *In vitro* Methods for Conservation of Plant Genetic Resources. Chapman and Hall, London (p 247)

Galzy R & Compan D (1988) Growth and nutrition of grapevine during *in vitro* long term storage. Plant Cell Tiss. Org. Cult. 13: 229–237

Henshaw GG & O'Hara JF (1983) *In vitro* approaches to the conservation and utilisation of global plant genetic resources. In: Mantel SH & Smith H (Eds) Plant Biotechnology, Society for Experimental Biology – Seminar series 18 (pp 219–238). Cambridge University Press, Cambridge

Kartha KK, Mroginski LA, Pahl K & Leung NL (1981) Germplasm preservation of coffee (*Coffea arabica* L.) by *in vitro* culture of shoot apical meristems. Plant Sci. Lett. 22: 301–307

Langford PJ & Wainwright H (1987) Effects of sucrose concentration on the photosynthetic ability of rose shoots *in vitro*. Ann. Bot. 60: 633–640

Lyons JM (1973) Chilling injury in plants. Ann. Rev. Plant Physiol. 24: 445–466

Morel G & Wetmore RH (1951) Fern callus tissue culture. Amer. J. Bot. 38: 141–143

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Ryan TA (1960) Significance tests for multiple comparison of proportions, variances and other statistics. Psychological Bulletin. 57: 318–328

Roca WM, Rodriguez J, Beltran J, Roa J & Mafla G (1982) Tissue culture for the conservation and international exchange of germplasm, In: Fujiwara A (Ed) Plant Tissue Culture 1982, Proc. 5th Intl. Cong. Plant Tissue & Cell Culture (pp 771–772). Jap. Ass. Plant Tissue & Cell Culture, Tokyo

Sauer A (1985) *In vitro* propagation of *Prunus avium* L. and storage of *in vitro* derived plantlets. Proc. Symp. Cherry varieties and rootstocks. Acta Hortic. 169: 351

Schnapp SR & Preece JE (1986) *In vitro* growth reduction of tomato and carnation microplants. Plant Cell Tiss. Org. Cult. 6: 3–8

Söndahl MR, Nakamura T & Sharp WR (1985) Propagation of coffee. In: Basic Life Science, Tissue Culture in Forestry and Agriculture (pp 215–232) Plenum Press, New York

Staritsky G, Dekkers AJ, Louwaars NP & Zandwoort EA (1986) *In vitro* conservation of aroid germplasm at reduced temperature and under osmotic stress. In: Withers LA & Alderson PG (Eds) Plant Tissue Culture and its Agricultural Applications (pp 277–283). Butterworths, London

Wanas WH, Callow JA & Withers LA (1986) Growth limitation for the conservation of pear genotypes. In: Withers LA & Alderson PG (Eds) Plant Tissue Culture and its Agricultural Applications (pp 285–290). Butterworths, London

Westcott RJ (1981) Tissue culture storage of potato germplasm – I – Minimal growth storage. Potato res. 24: 331–342

Withers LA (1989) *In vitro* conservation and germplasm utilization. In: Brown ADH, Frankel OH, Marschall DR & Williams JT (Eds) Use of Plant Genetic Resources. (pp 309–334). Cambridge University Press, Cambridge