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

1. Influence of low concentrations of 6-benzyladenine

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Abstract

We have studied the influence of low concentrations of 6-benzyladenine on growth limitation, in order to preserve coffee germplasm through a microcutting collection. Concentrations of 0 μM, 1.3 μM and 4.4 μM were compared in four species: *Coffea congensis*, *C. canephora*, *C. liberica* and *C. racemosa*. After six months, microcutting behaviour varied between the different treatments, and a species effect was observed. The slow growing species (*C. liberica* and *C. congensis*) needed 1.3 μM; the others coffee species (*C. canephora* and *C. racemosa*) exhibited moderate caulogenesis on 6-benzyladenine-free medium. Zero and low concentrations did not affect survival rates. In conclusion 1.3 μM seems most appropriate for conserving all four species.

Abbreviation: BA – 6-benzyladenine

Introduction

More than 30 different coffee species have been collected in Africa and Madagascar (Berthaud & Charrier 1988). Seed viability does not exceed 1 or 2 years under the best conditions: partial dehydration, followed by storage at 20°C in water-saturated atmosphere (Van der Vossen 1979; Couturon 1980). This germplasm cannot be artificially conserved, and it is therefore stored in field collections, which are costly and exposed to environmental risks.

The different techniques used for *in vitro* conservation of plants producing recalcitrant seeds (small shoot-tip microcuttings, embryo rescuing, cryopreservation), have been reviewed (de Langue 1984; Withers 1989). We have investigated coffee germplasm conservation using this approach, by zygotic embryos germination and low cytokinin concentrations for the microcutting storage medium.

Microcutting and embryogenesis are currently used for *in vitro* multiplication of the two cultivated species *C. arabica* and *C. canephora* (Söndahl et al. 1984). Most media have been perfected for an intensive multiplication rate and contain BA concentrations from 2.2 μM to 44 μM (Zok 1985; Berthouly et al. 1988). With 44 μM BA, Custers (1981) obtained shoots which developed a new pair of leaves every three weeks. Currently cytokinin concentrations used may change genomic expression as observed by physiological disorders *in vitro* and abnormal physiology of micropropagated plants in the field (Debergh 1987).

In vitro conservation requires:

– preservation of genetic information,
– a maximal survival rate,
– the reduction of subculturing frequency.

Increasing the subculture period to every 6 or 12 months saves money and reduces the contamination rate and the accidental substitutions of genotypes (Withers 1989).

The aim of the present work was to study the effect of zero or low BA concentrations on *in vitro* limited growth in four coffee species of different ecological origins. In a second paper we will discuss the influence of temperature and sucrose.

Materials and methods

Experimental conditions

The basal medium consisted of Murashige & Skoog (1962) salts supplemented with Morel & Wetmore vitamins (Morel & Wetmore 1951) and sucrose (90 mM). The pH was adjusted to 5.6 with 0.1 M KOH prior to adding agar (7 g l^{-1}) and autoclaving (20 min, 110°C). All cultures were exposed to a 12-h photoperiod under warm white fluorescent tubes giving a light intensity of 50 $\mu mol.m^{-2}.s^{-1}$.

Before the experiment, the plant material was maintained in growth phase on basal medium supplemented with 1.3 μM BA.

Microcuttings of three maternal descents of *C. canephora*, one of *C. congensis*, one of *C. liberica* and one of *C. racemosa* were obtained by *in vitro* germination of zygotic embryos (Bertrand-Desbrunais & Charrier 1990). The experiment was initiated with terminal shoot tips (1–1.5 cm long, 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.

Growth on BA-free basal medium was used as a reference and was compared with growth on two levels of BA: 1.3 μM and 4.4 μM. The experiment was performed with 8 shoots per treatment for each strain. Each sample is formed by different genotypes sampled from one population. All tubes were distributed according to a random experimental design.

Parameters and data analysis

All data were collected after a six-month culture period. The following parameters were evaluated: survival, microplant height (length of the main shoot), axillary shoot development, adventitious budding, rooting, callussing and basal leaf loss. These evaluations were made in a qualitative manner as presence/absence, except for microplant height, which was scored as no growth, height between 2 and 4 cm, height greater than 4 cm.

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 influence of BA on this process and, in terms of causality, on the depending parameters, and
– to describe the behaviour difference between species according to these factors and in relation to the BA supply.

Bidimensional graphs allow the projection of individuals 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 frequency modality occurs near the origin, and two adjacent modalities indicate simultaneous presence.

Results

After six months, without subculturing, the survival rate remained very high (95%) for all treatments and was not dependent either on species or on BA concentration.

Variations induced by BA

Characters influenced by BA concentrations were microplant height, axillary shoot elongation, adventitious budding on scar tissue, and leaf loss. Reference medium (BA-free) reduced microplant height (98% were shorter than 4 cm), axillary shoot development, and particularly adventitious budding (Table 1). The addition of 1.3 μM BA increased microplant height, axillary shoot development (35%), and adventitious budding (6%), and reduced the rate of leaf loss. At the 4.4 μM concentration, the caulinary meristem activity clearly increased, expressed by axillary shoot development on many microcuttings (65%) and by more adventitious budding on cicatrical callus (37%).

Species effect

At all BA concentrations, each species had a different response for microcutting height, axillary shoot development, adventitious budding and rooting (Table 2). All *C. congensis* microplants exhibited a height below 4 cm and poor rooting (4% on an average). *C. liberica* also presented limited growth of the main shoot, but contrasted with *C. congensis* in terms of active rhizogenesis (54%), no adventitious budding on callus and strong apical dominance. *C. canephora* showed main shoot elongation and a high rooting frequency (83%). The three strains exhibited similar responses. Both *C. racemosa* and *C. canephora* exhibited marked main shoot elongation, but differed in terms of adventitious bud-

Table 1. Effects of BA concentrations on coffee microplant growth[a]

BA concentration (μM)	Microplant height <4 cm frequency[b]	Axillary shoot frequency	Adventitious budding frequency	Basal leaf loss frequency[c]
0	98[d]	2[d]	0[d]	25[d]
1.3	73[e]	35[e]	6[e]	6[e]
4.4	82[e]	65[f]	37[f]	10[e]
χ^2	11.7	39.6	33.9	7.8
$df = 2$	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.05$

[a] % explants exhibiting response. Each BA treatment was replicated 48 times with one explant (1.5 cm long) per tube. Data were collected after 6 months. Results are averaged over all coffee species. For each variable, data followed by a different letter are significantly different ($p < 0.05$).
[b] Microplant height represents the length of the main shoot.
[c] The basal leaves turn yellow and fall.

Table 2. Influence of *Coffea* species on coffee microplant growth[a]

Coffee species	Microplant height <4 cm frequency[b]	Axillary shoot frequency	Adventitious budding frequency	Rooting frequency
C. congensis	100[d]	46[df]	12[d]	4[d]
C. liberica	100[d]	0[e]	0[e]	54[e]
C. canephora[c]	78[e]	33[d]	12[d]	83[f]
C. racemosa	71[e]	58[f]	37[f]	83[f]
χ^2	15.2	20.1	13.5	40.8
$df = 3$	$p < 0.01$	$p < 0.001$	$p < 0.01$	$p < 0.001$

[a] % explants exhibiting response. Each coffee species was replicated 24–72 times with one explant (1.5 cm long) per tube. Data were collected after 6 months. Results are averaged over all BA treatments. For each variable, data followed by a different letter are significantly different ($p = 0.05$).
[b] Microplant height represents the length of the main shoot.
[c] The three *C. canephora* strains exhibited similar responses.

ding frequency (37% for *C. racemosa*) and apical dominance, which was very low for *C. racemosa* and induced substantial axillary shoot development (58%).

Influence of BA concentration on meristematic activity and morphogenetic orientation

Correspondence analysis of all observed characters showed that most (85%) of the observed variability was explained by two factors (Fig. 1a). The first factor contrasted microplants without growth (no shoot elongation, no root or callus formation) and exhibiting loss of leaves, with taller microplants (4 to 8 cm), with root, callus, shoot elongation, adventitious budding and little loss of leaves. The biological phenomenon likely to be at the origin of these changes is meresis (i.e. intense mitotic activity). The second factor contrasted axillary shoot development with the presence of roots, and represented morphogenetic expression reflected in caulogenesis or rhizogenesis.

The supply of 1.3 μM BA resulted in enhancement of mitotic activity. At a concentration of 4.4 μM, caulogenesis was favoured at the expense of rhizogenesis. This cytokinin therefore had two different effects depending on its concentration.

Mitotic activity and morphogenetic orientation influenced some of the observed characters. Thus, meristematic activity was required for rooting, caulinary growth and adventitious budding, and was also linked to the absence of leaf loss. Of course, orientation towards rhizogenesis not only explained the presence of roots, but also indirectly the marked caulinary growth. In contrast, orientation towards caulogenesis enhanced axillary bud development and neoformation of adventitious buds. Two characters did not depend on these first two factors: mortality and the presence of calluses.

These two factors also describe species behaviour. Mitotic activity was variable: low for *C. congensis* and *C. liberica*, moderate for *C. canephora* and intense for *C. racemosa*. Moreover, *C. canephora* seemed to express a well-balanced behaviour between caulogenesis and rhizogenesis, whereas *C. congensis* and *C. racemosa* were oriented towards caulogenesis. In this respect they contrasted with *C. liberica* (Fig. 1b). *C. congensis* and *C. racemosa* exhibited similar behaviours, except in the case of meristematic activity. BA facilitated the expression of caulogenesis at low concentration, and induced meristematic activity at high concentration. *C. canephora* exhibited a different behaviour: the lowest concentration increased the mitotic activity while caulogenesis was greater at highest concentration. *C. liberica* did not exhibit any change between the concentrations of BA, which did not modify its inability to develop axillary bud into shoots. The only effect was a slight increase in mitotic activity.

Discussion

The comparison of the effects of BA on the four *Coffea* species showed that:

- low BA concentrations resulted in the growth limitation,
- the effects of BA depended on its concentration and on the species, and
- most variability was explained by two independent factors: meristematic activity and morphogenetic orientation towards caulogenesis or rhizogenesis.

In this study, the BA supply stimulated cell metabolism and high concentrations enhanced budding on microcuttings. These observations are consistent with Skoog & Miller (1957), who observed for the first time the role of the exogenous auxin/cytokinin balance in the orientation of *in vitro* organogenesis.

It was also observed that the 4.4 μM concentration inhibited shoot elongation, particularly for *C. canephora*, by reducing internode length. The same phenomenon has already been reported by Vieitez (Vieitez & Vieitez 1983) for chestnut microcuttings, using BA concentrations higher than 2.2 μM.

Our results confirmed the comparative study of Nissen (1988), who showed that cytokinin-induced effects depend on cytokinin concentration (callus growth, retention of chlorophyll in senescing leaves, expansion of cotyledons), and noted genus- and species-specific differences in

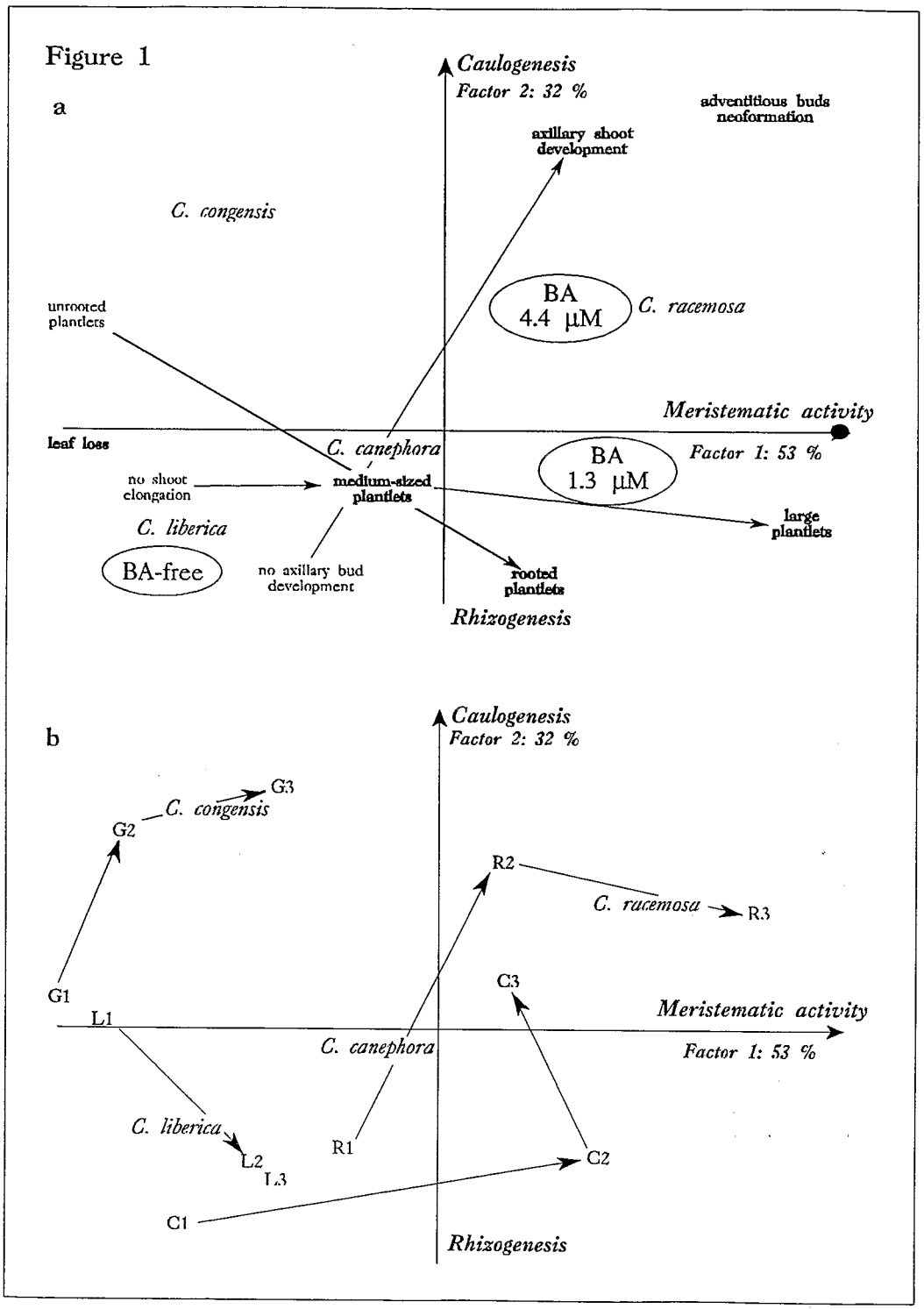

Fig. 1. Coffee microcutting behaviour. Factorial plane 1–2. Data were collected after six months. Media differ in BA concentration: (1) BA-free (2) 1.3µM (3) 4.4µM
Compared species were: (G) *C. congensis* (C) *C. canephora* (L) *C. liberica* (R) *C. racemosa*
(*a*) The parameters in bold type were the result of changes occuring between the start of culture and the observation 6 months later.
(*b*) The arrows indicate between-species differences in behaviour with respect to increasing BA concentration.

response. For *C. liberica* the BA supply did not stimulate the axillary branching.

Naturally, each coffee species requires special attention. For *C. congensis* and *C. liberica*, a low BA concentration seems to be an interesting method for germplasm maintenance in the medium-term. On the BA-free medium many microcuttings of *C. congensis* were very weak. Low BA concentration slightly enhanced growth activity and favoured the rooting response, at least for *C. liberica*. This morphological response was absolutely necessary for *in vitro* conservation of grape (Galzy 1985), and markedly improved the survival rate of *Prunus avium* stored at 2°C (Sauer 1985). For *C. canephora* and *C. racemosa*, on the other hand, the BA concentration of 4.4 μM was too high, and caused exuberant caulogenesis. In this study, the concentration of 1.3 μM appeared to be optimal for the maintenance of these four different coffee species. Reisch (1986) also chose a single BA concentration (5 μM) for the propagation of several *Vitis* species.

Behaviour differences between species, as we have observed, have also been mentioned for grape (Reisch 1986) and for different potatoes by Westcott (1981). As defined by Westcott (1981), these *in vitro* behaviour differences seem to vary as a function of the ecological origin of the sample. *C. congensis*, from the Zaïre basin, is characterized by medium-size leaves and riparian ecology (Berthaud & Guillaumet 1978). *C. racemosa* on the other hand is distinguished by small sessile leaves and comes from the dry countries of East Africa (Halle & Faria 1973). The natural populations of *C. canephora* and *C. liberica*, two coffee species with broad leaves, are distributed among the humid tropical forests of West Africa. The distribution in the Côte d'Ivoire, however, shows that *C. liberica* is adapted to more humid areas than *C. canephora* (Berthaud 1986).

In conclusion, our choice of BA concentrations well below those recommended for micropropagation (Söndahl et al. 1984) is conclusive. Maintained at limited growth under these conditions, the genetic stability of the microcutting collection will be preserved (Withers 1989).

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