COLD SENSITIVITY OF *IN VITRO* MICROCUTTINGS OF *COFFEA ARABICA* AND C. *CANEPHORA* DURING STORAGE AT VARIOUS TEMPERATURES

EVOLUTION OF SUGAR, PROLINE, MDA AND ETHYLENE PRODUCTION

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INTRODUCTION

Genetic resources of coffee are generally preserved as field collections. However, plant material remains exposed to climatic hazards and to attacks by pests and pathogens. Therefore, *in vitro* collections of microcuttings have been set up, in order to ensure germplasm medium-term conservation in safe conditions. Moreover, exchanges of plant material as *in vitro* plantlets are considerably facilitated. At ORSTOM/Montpellier, the *in vitro* collection comprizes a total number of 635 accessions, representing 13 species. Intervals between subcultures can be extended to 6-12 months, depending on the species, when plants are cultivated at 27°C (Bertrand-Desbrunais *et al.*, 1992). These intervals may be significantly increased by lowering the culture temperature. However, there exists an important variability among coffee species as concerns their tolerance to low temperatures. This was illustrated recently when observing the different resistance of plantlets of two coffee species, *C. arabica* and *C. canephora*, during storage at various temperatures (Jouve *et al.*, 1991).

The exposure of plants to low temperatures induces biochemical and physiological changes which allow them to withstand this stress (Levitt, 1980; Graham and Patterson, 1982). The disorders caused by cold temperatures are particularly important in the case of tropical and subtropical species and are reversible or not depending on their sensitivity and on the storage period. Irreversible damages can occur before any visible symptom be detected on the plants. Therefore, it is of primordial importance to define marker(s) which could inform about the extent of damages caused to the plants by low temperature before they become irreversible.

During storage at low temperature, accumulation of low molecular weight compounds is observed in numerous species (Kushad and Yelenosky, 1987; Salerno and Pontis, 1989). Among them, carbohydrates like sucrose, sorbitol or raffinose were the first protective substances described with plants. Their quantity can show an up to ten-fold increase during storage at low temperature. Similarly, accumulation of free proline is often associated with resistance of plants to numerous stresses and particularly to low temperature (Kushad and Yelenosky, 1987; Krall *et al.*, 1989).

Another well known aspect of acclimation of plants to low temperature exposure is the biochemical and physical restructuration of cell membranes (Steponkus, 1984; Lynch and Steponkus, 1987). Cold exposure can alter the structure of membranes due to lipid peroxidation, thus producing malonedialdehyde. Therefore, changes in MDA concentration can be a good indicator of the structural integrity of membranes for plants submitted to low temperature. After a conservation period, an increase in ethylene production by plant tissues is generally associated with the stress conditions (Yang and Hoffman, 1984). Cold treatments can increase its production by cold-sensitive tissues. After transfer of the plant material to standard temperature, the burst of ethylene which can be observed results from the reactivation of the ethylene forming enzyme which is a thermodependent

membrane enzyme (Chen and Patterson, 1985), and can thus allow to estimate the functional integrity of membranes.
In the present study, we observed the effect of conservation at different temperatures on the physiology of shootlets of *C. arabica* and *C. canephora* by following the evolution of their sugar, proline, MDA concentration and ethylene production. The results were related with their survival rates in the various experimental conditions.

MATERIALS AND METHODS

Plant material

This study was carried out with two coffee species which differed by their ecology: *C. arabica* L, originating from mountain areas of South West Ethiopia, resistant to low temperatures, and *C. canephora* Pierre, originating from warm and humid plains of the intertropical african zone. The microcuttings of *C. arabica* belonged to the cultivar Moka de Tahiti and those of *C. canephora* to a maternal origin of clone IF 23. The explants used were orthotropic axes without terminal buds, roots or basal calluses, bearing two pairs of leaves, of an average lenght of 10 mm.

Methods

The microcuttings were cultured on a medium (Jouve *et al.*, 1991) derived from that of Dublin (1980). They were placed in 150 x 24 mm glass test tubes containing 20 ml of medium with an average free volume of 34 ± 1 ml. Cultures were placed at 27, 22, 17 and 12°C with a photoperiod of 12 hrs light/12 hrs dark under a photon flow of 55.5 $\mu E.m^{-2}.s^{-1}$.
At the end of each storage period, the survival rate was estimated as a function of the total number of remaining microcuttings. At each sampling date, 12 microcuttings were randomly chosen in each condition. Six of them were grouped in two batches of three and freeze-dried for the further measurement of sugar, proline and MDA concentration. The six remaining microcuttings were placed at standard temperature (27°C) for 10 days in order to measure their ethylene production. They were then grouped in two batches of three and freeze-dried for further measurement of their MDA concentration.
The concentrations in D-fructose, D-glucose and sucrose were measured enzymatically using the method of Bergmeyer and Bernt (Bergmeyer and Bernt, 1974). The results were expressed in $\mu g.g^{-1}$ fresh weight (FW).
The free proline concentration was measured by the method of Bates *et al.* (1973), using L-proline as a standard. Plant material was grounded in 3 % (w/v) sulfosalycilic acid. After filtration and reaction with ninhydric acid, the chromophore was extracted in toluene and absorbance was measured at 520 nm. The results were expressed in $mg.\ g^{-1}$ FW.
MDA content was measured by colorimetry using the method of Heath and Packer (1968) modified by Hagège *et al.* (1990). Specific absorbance was measured at 532 nm and non specific absorbance at 590 nm. Results were expressed in nmol. g^{-1} fresh weight (FW). MDA concentration was measured after each storage duration and in some cases only after a further period of 10 days at 27°C. The data correspond to the mean value of three measurements of each of the two batches for each storage duration.
Ethylene production was measured every 24 hours during 10 days at 27°C, after all storage durations and also during storage at the different storage temperatures experimented. The microcuttings were confined for 24 hours before sampling 2 ml of the test tube atmosphere. Samples were injected in a gas chromatograph (Delsi DN 200) equiped with a flame ionisation detector and a Porapak Q column (500 x 6 mm). The results were expressed in $nl.24\ hrs^{-1}.g^{-1}$ FW. Each point corresponded to the mean value of the maximal ethylene production of the 6 samples analyzed. For data analysis, ANOVA and Newman (1939) and Keuls (1952) tests were used for multiple comparison of treatment means.

RESULTS

After 20 weeks in storage, the survival rate of the microcuttings varied beetwen the two species. With *C. arabica*, the lowest survival rate (67 %) was obtained at 27°C (Fig. 1A). However, no significant differences were observed between the four storage temperatures experimented. With *C. canephora* (Fig. 1B), the results were comparable to that described previously with *C. arabica* for 17, 22 and 27°C, whereas survival dropped down to 25 % after 20 weeks of storage at 12°C.

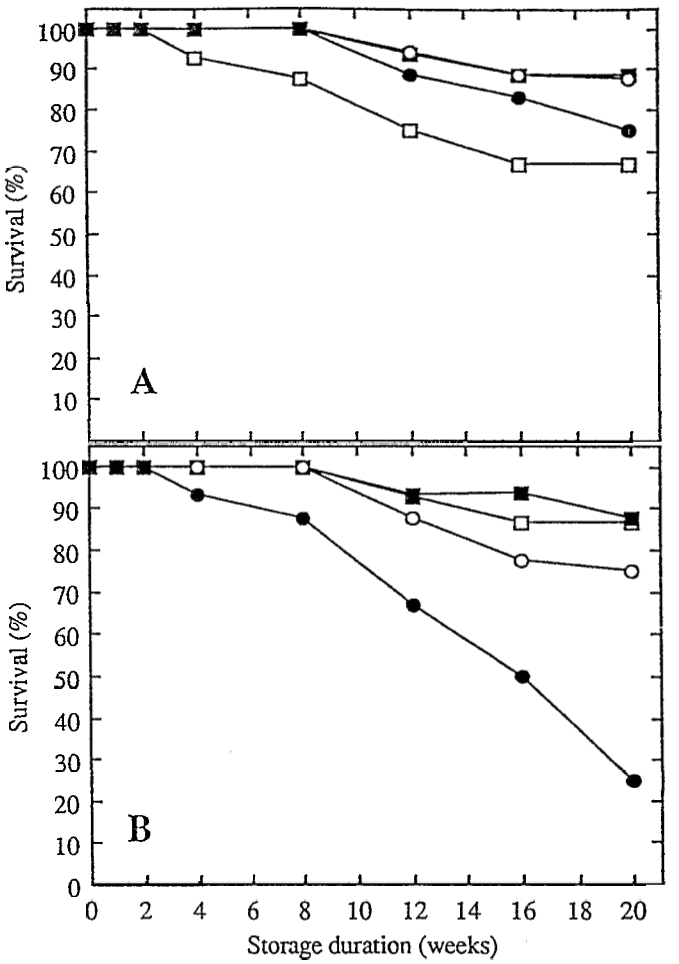

Figure 1: Evolution of the survival rate of microcuttings of *C. arabica* (A) and *C. canephora* (B) during storage at different temperatures (27°C □, 22°C ■, 17°C ○ and 12°C ●).

The sugar concentration varied differently during storage, depending on the storage temperature. A peak of fructose and glucose was observed immediately after the transfer to 12°C, with a maximum after two weeks both for *C. arabica* and *C. canephora* (1300µg/g FW for glucose and fructose). This peak was also observed at 17°C with *C. canephora* for glucose. In the other treatments, the glucose and fructose concentrations generally remained stable around 500 µg/g FW over the storage duration, but for glucose level which increased significantly for *C. canephora* stored at 27°C up to 800 µg/g FW, at the end of the storage period. The evolution of sucrose concentration was generally similar for both species in all conditions. After two to four weeks in storage, the sucrose level reached 1 $mg.g^{-1}$ FW and remained stable afterwards. The sucrose concentration decreased significantly at the end of the storage period, in *C. canephora* microcuttings kept at 12°C only.
Proline concentration did not vary significantly in microcuttings of both species placed at 22 and 27°C, remaining between 5-10 mg/g FW. At the lower temperatures, the evolution was different. At 17°C, the increase was delayed and appeared from the 8th week onwards for *C. arabic* and from the 12th week onwards for *C. canephora*. At 12°C, proline concentration increased rapidly and reached a maximal level (20 mg/g FW) after 4 weeks in storage. It remained stable for *C. arabica* but decreased progressively down to the initial value for *C. canephora*.
During storage, the MDA concentration varied little with *C. arabica*, whatever the temperature (Fig. 2A). At 12 and 27°C only, the MDA level increased transitorily between the 12th and 16th weeks of storage. The MDA contents did not differ between the batches submitted to 20 weeks of storage at different temperatures. With *C. canephora*, there was no significant increase in the MDA content during storage at 17, 22 and 27°C (Fig. 2B). On the contrary, at 12°C, an important increase in the MDA content was observed (around 4-fold that measured at the other temperatures). This value became stable and remained constant until the end of the experiment. Ten days after their transfer to 27°C, the MDA concentration was modified in the case of microcuttings stored for 4 weeks at 12, 22 and 27°C (Table 1): with *C. canephora* it decreased close to the level measured in the controls stored at 27°C. On the contrary, for *C. arabica*, the MDA concentration increased. After 20 weeks of storage, there was no further modification of the MDA level. For *C. arabica*, the levels were comparable for plants stored at 12, 22 and 27°C. Microcuttings of *C. canephora* stored at 12°C conserved a high MDA concentration which did not decrease to the level of the controls.

Table 1: Modifications in the MDA concentration ($nmol.g^{-1}$ FW) after 4, 16, and 20 weeks of storage at different temperatures (storage) and 10 days at 27°C (storage + 10 days). Different letters indicate significantly different values (p = 0.05).

Storage duration (weeks)	Storage temperature (°C)	Species	malonedialdehyde	
			storage	storage + 10 days
4	27	*C. arabica*	8.6 a	16.3 ab
	12	"	9.6 a	20.8 b
	27	*C. canephora*	9.0 a	13.2 a
	12	"	33.2 c	22.9 b
16	27	*C. arabica*	17.3 b	19.7 b
	12	"	25.0 b	28.1 b
20	27	*C. arabica*	18.8 ab	17.5 ab
	22	"	14.1 a	12.5 a
	12	"	21.7 b	18.3 ab
	27	*C. canephora*	9.5 a	14.0 a
	22	"	44.4 c	42.3 c

During storage and whatever the temperature, the ethylene production remained low and constant (between 30 and 200 $nl.24h^{-1}.g^{-1}FW$). During the 10 days following the transfer at 27°C, differences in ethylene production were observed, depending on the species and the storage temperature. For both species, a peak was present immediately after the transfer of the microcuttings (500-600 nl/24 h/g FW). In the case of *C. arabica*, there was no significant difference in the ethylene production for microcuttings stored at 22 or 27°C, whatever the storage duration. At 12°C, a peak appeared after 12 weeks of storage only. After 16 and 20 weeks of storage, it was

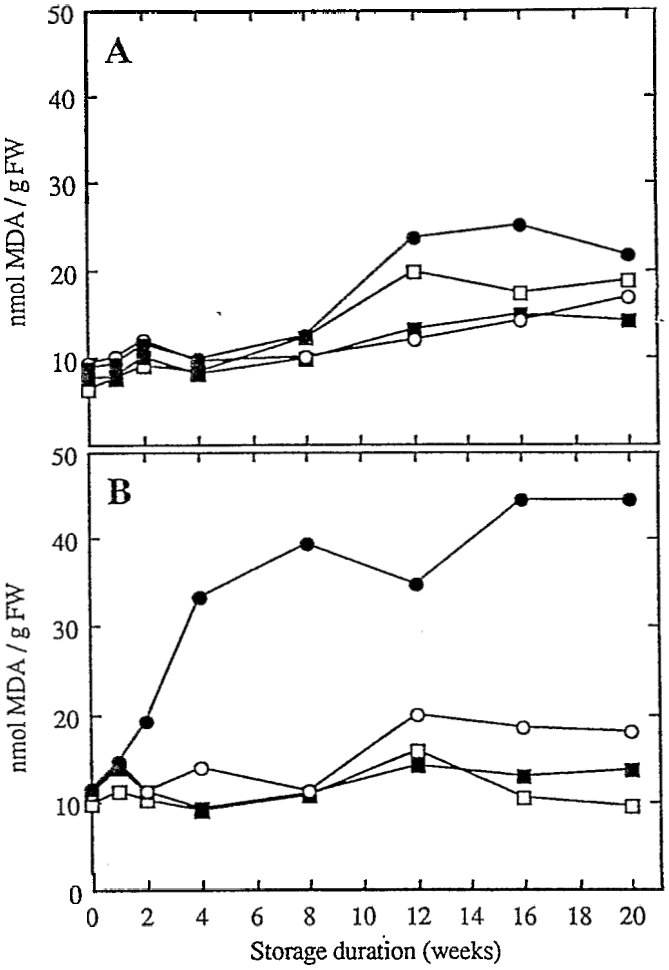

Figure 2: Evolution of the malonedialdehyde (MDA) concentration of microcuttings of *C. arabica* (A) and *C. canephora* (B) during storage at different temperatures (27°C □, 22°C ■, 17°C ○ and 12°C ●).

present for plants stored at 12 and 17°C. For *C. canephora*, a peak of ethylene was already observed after 2 weeks of storage at 12, 17 and 22°C. After 4 and 8 weeks of storage, its intensity increased for these three temperatures up to 600-700 nl/24 h/g FW. However, after 12 weeks of storage, an important reduction of the ethylene burst was observed for the microcuttings conserved at 12°C, whereas it was higher for the ones stored at 17 and 22°C.

DISCUSSION/CONCLUSION

In the majority of cases, the critical storage temperature is much higher for tropical, cold sensitive species, in comparison to that of plants from temperate climates. It is generally comprized between 8 and 15°C (Banerjee and De Langhe, 1985) and appeared to be even higher for coffee. In this experiment, *C. canephora* microcuttings were much more cold sensitive than that of *C. arabica*, which confirms the previous results of Jouve *et al.* (1991) obtained with the same species and reflects their different ecology. The four markers used in this study to assess the cold sensitivity of coffee plantlets varied differently.

No increase in the sugar concentration was noted in relation with the storage temperature, contrarily to what is observed in cold tolerant plants from temperate climates during cold acclimation (Calderon and Pontis, 1985; Salerno and Pontis, 1989). The transitory increase in glucose and fructose contents at the beginning of the storage period could be due to a decrease in metabolic activity, leading to their accumulation and not to an active phenomenon, like in cold tolerant species.

The fluctuations in the proline level observed in the present study were relatively low in comparison to that noted in the literature (Kushad and Yelenosky, 1987; Krall *et al.*, 1989). Moreover, differences in proline level were not apparent when survival started to diminish and may be an effect rather than a cause of the decrease of the susceptibility.

For *C. arabica* conserved at 12°C, the MDA concentration did not increase during the whole storage duration, whereas that measured in *C. canephora* microcuttings stored at the same temperature significantly increased from the 4th week on. This showed an increased production of peroxide ions in this species in comparison with *C. arabica* or a less efficient detoxification by the superoxide dismutase-catalase system (Benson, 1990). This modification in the MDA concentration was reversible after short-term exposures to cold temperatures, which reflected the fact that the metabolic activity was sufficient to repair the damages induced by cold. On the contrary, after longer storage durations at low temperature, no reduction in the MDA concentration was observed after transfer to 27°C, which indicates that the structural integrity of the membranes was too severely damaged for repair mechanisms to take place. Evolution of MDA clearly preceded the decrease in survival rate of the microcuttings, thus demonstrating the usefulness of this compound to monitor early damage.After transfer to standard temperature following cold storage, an increased ethylene production is often observed for cold sensitive plants or organs like *Citrus* (Cooper *et al.*, 1969) or tropical species such as *Carica papaya* (Chan *et al.*, 1985) and *Elaeis guineensis* (Corbineau *et al.*, 1990). Chen and Patterson (1985) indicate that an important ethylene production after the transfer to high temperature corresponds to a reversible stress, whereas a more severe stress induces the irreversible incapacity to produce ethylene. A progressive decrease in ethylene production after extended storage periods at low temperatures was observed in the case of oil palm somatic embryos stored for more than 11 weeks at 12°C, in line with a progressive drop in their survival rate (Corbineau *et al.*, 1990). In the present study, for both species and at all temperatures, dissection was a stressing event, as assessed by an important peak of ethylene occurring immediately after their transfer in storage conditions. After transfer at 27°C, a slight increase of ethylene occurred after different storage durations at low temperature for the two species. However, it did not seem clearly correlated with a decrease in survival. A simple hypothesis for the occurrence of this ethylene burst would be that ACC, the precursor of ethylene, accumulates at low temperature because of the inhibition of EFE in these conditions and is converted to ethylene after transfer at higher temperature.

In conclusion, among the four potential markers experimented in the present work, MDA appears as the best indicator of cold sensitivity in the case of *C. arabica* and *C. canephora* microcuttings. In order to complete this study, it would be interesting to compare the activity of the enzymes superoxide dismutase and catalase which are involved in the defense processes against free radicals.

ISBN 2-900212-14-6

QUINZIÈME COLLOQUE SCIENTIFIQUE INTERNATIONAL SUR LE CAFÉ

Montpellier, 6-11 juin 1993

Volume I

Association Scientifique Internationale du Café
(ASIC)
42, rue Scheffer, 75116 Paris