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Evaluation of biochemical markers (sugar, proline, malonedialdehyde and ethylene) for cold sensitivity in microcuttings of two coffee species

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Microcuttings of coffee (*Coffea arabica* L. and *Coffea canephora* Pierre) were conserved in vitro for 0–20 weeks at various temperatures (12, 17, 22 and 27°C). Survival was generally high, except for *C. canephora* stored at 12°C. After a transitory increase in fructose and glucose concentration at the beginning of the storage period, the sugar level remained stable. During the first weeks of storage, the quantity of proline increased to twice the initial level for both species at 12°C. A drop in the proline concentration was observed with *C. canephora* at the end of the storage period at 12°C. Whereas in the case of *C. arabica*, no modification was noted in the malonedialdehyde (MDA) concentration, its level increased in microcuttings of *C. canephora* stored at 12°C. This increase was reversible after 10 days at 27°C at the beginning of the storage period, but became irreversible after a 20-week storage duration. Ethylene production started after transfer to 27°C after a 2-week storage period with *C. canephora*, but after 12 weeks only in the case of *C. arabica*. This synthesis decreased at the end of the storage period for *C. canephora* microcuttings stored at 12°C. These results indicate that among the four compounds studied, MDA appears as the most reliable indicator of cold sensitivity for the two coffee species studied.

Key words: Coffea arabica; *Coffea canephora*; in vitro conservation; temperature; sugar; proline; malondialdehyde; ethylene

Introduction

The exposure of plants to low temperatures induces biochemical and physiological changes, which allow them to withstand this stress [1,2]. 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. During storage at low temperature, accumulation of low molecular weight compounds is observed in numerous species [3,4]. Among them, carbohydrates like sucrose, sorbitol and raffinose [1] were the first protective substances described in plants. Sucrose is the most easily detectable sugar in cold-tolerant species. Its quantity can show an up to 10-fold increase during exposure to low temperature [4]. If its accumulation is impeded, cold tolerance is lost [5]. Similarly, accumulation of free proline is often associated with resistance of plants to numerous stresses and particularly low temperature [3,6]. Proline plays an important role in the metabolism of the stressed plants. It can be used as a source of carbon or nitrogen during recovery after stress [7]. Proline is also involved in cell osmoregulation and in the protection of proteins [8]. Its accumulation may also stabilize membranes during cold stress [9,10]. Moreover, proline could also be involved in the regulation of some enzymatic systems [6,11,12].

Another well-known aspect of acclimation of plants to low temperature exposure is the biochemical [13] and physical [14] restructuring of cell membranes. An increase in lipid content, particularly phospholipids, and in the unsaturation

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degree of the fatty acid chains is generally observed [15,16]. This is in accordance with the necessity to increase the fluidity of membranes at low temperatures [17]. However, the cold exposure can alter the structure of the membranes due to lipid peroxidation [18]. The degradation of the polyunsaturated fatty acids by means of peroxidation produces peroxide ions and malonedialdehyde (MDA) induces membrane rigidification and the death of cells [19]. Therefore, changes in MDA concentration can be a good indicator of the structural integrity of the membranes for plants subjected to low temperature.

After a conservation period, an increase in ethylene production by plant tissues is generally associated with the stress conditions [20]. Cold treatments can increase its production by cold-sensitive tissues. Depending on the plant material, ethylene production can take place during the cold exposure or only after the transfer to higher temperatures. The increase in ethylene production results from the accumulation and/or the stimulation of 1-aminocyclopropane 1-carboxylic acid (ACC), its immediate precursor [21]. The ethylene forming enzyme (EFE), membrane enzyme which transforms ACC into ethylene, is thermodependent. At low temperature, its activity is reduced or non-existent. After transfer to standard temperature, the burst of ethylene which can be observed results from the reactivation of the enzyme [22] and can thus allow estimation of the functional integrity of the membranes.

Previous works showed that in vitro microcuttings of *Coffea arabica*, a species originating from mountain areas were more resistant to cold storage than those of *Coffea canephora*, originating from tropical plains [23,24]. In this study, we observed the effect of the conservation of these two species at different temperatures on the physiology of their shootlets by following the evolution of their sugar, proline, MDA concentration and ethylene production. The results were related to 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 the mountain areas of Ethiopia, resistant to low temperatures, and *C. canephora* Pierre, originating from the 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 length of 10 mm.

Methods

The microcuttings were cultured on a medium [23] derived from that of Dublin [25]. They were placed in 150 × 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 h light/12 h dark under a photon flow of 55.5 $\mu E \cdot m^{-2} \cdot s^{-1}$. Several storage durations were experimented with: 0, 1, 2, 4, 8, 12, 16 and 20 weeks. 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 [26]. The results were expressed in $\mu g \cdot g^{-1}$ fresh wt. The free proline concentration was measured by the method of Bates et al. [27], using L-proline as a standard. Plant material was ground in 3% (w/v) sulfosalicylic 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 $mg \cdot g^{-1}$ fresh wt.

MDA content was measured by colorimetry using the method of Heath and Packer [28] as modified by Hagege et al. [29]. Specific absorbance was measured at 532 nm and non-specific absorbance at 590 nm. Results were expressed in

nmol·g^{-1} fresh wt. 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 h during 10 days at 27°C, after all storage durations and also during storage at the different storage temperatures. The microcuttings were confined for 24 h before sampling 2 ml of the test-tube atmosphere. Samples were injected into a gas chromatograph (Delsi DN 200) equipped with a flame ionisation detector and a Porapak Q column (500 × 6 mm). The results were expressed in nl·24 h^{-1}·g^{-1} fresh wt. Each point corresponded to the mean value of the maximal ethylene production of the six samples analyzed.

For data analysis, ANOVA and Newman [30] and Keuls [31] tests were used for multiple comparison of treatment means.

Results

After 20 weeks in storage, the survival rate of the microcuttings varied between 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 employed. With *C. canephora* (Fig. 1B), the results were comparable with those of *C. arabica* at 17, 22 and 27°C, whereas survival dropped to 25% after 20 weeks of storage at 12°C.

The sugar concentration varied differently during storage, depending on the storage temperature (Fig. 2). A peak of fructose (Figs. 2A and 2B) and glucose (Figs. 2C and 2D) was observed immediately after the transfer to 12°C, with a maximum after 2 weeks both for *C. arabica* (Figs. 2A and 2C) and *C. canephora* (Figs. 2B and 2D). This peak was also observed at 17°C with *C. canephora* for glucose (Fig. 2D). In the other treatments, the glucose and fructose concentrations generally remained stable over the storage duration, except for the glucose level which increased significantly for *C. canephora* stored at 27°C at the end of the storage period (Fig. 2D). The evolution of sucrose concentration (Figs. 2E and 2F) was generally

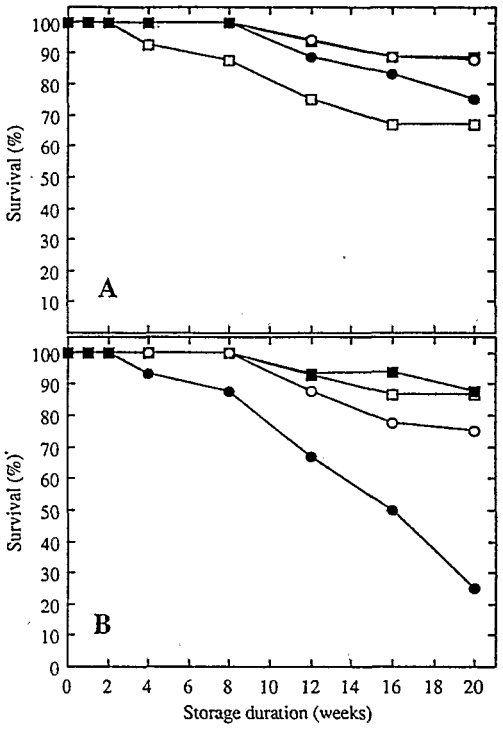

Fig. 1. Evolution of the survival rate of microcuttings of *C. arabica* (A) and *C. canephora* (B) during storage at different temperatures (27°C □, 22°C ■, 17°C ○ and 12°C ●).

similar for both species in all conditions. After 2–4 weeks in storage, the sucrose level reached 1 mg·g^{-1} fresh wt. 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 (Fig. 2E).

Proline concentration did not vary significantly in microcuttings of either species placed at 22° and 27°C (Fig. 3). At the lower temperatures, the evolution was different. At 17°C, the increase was delayed and appeared from the 8th week onwards for *C. arabica* (Fig. 3A) and from the 12th week onwards for *C. canephora* (Fig. 3B). At 12°C, proline concentration increased rapidly and reached a maximal level after 4 weeks in storage. It remained stable for *C. arabica* (Fig. 3A) but decreased progressively down to the initial value for *C. canephora* (Fig. 3B).

During storage, the MDA concentration varied little with *C. arabica*, whatever the temperature (Fig. 4A). At 12 and 27°C only, the MDA level

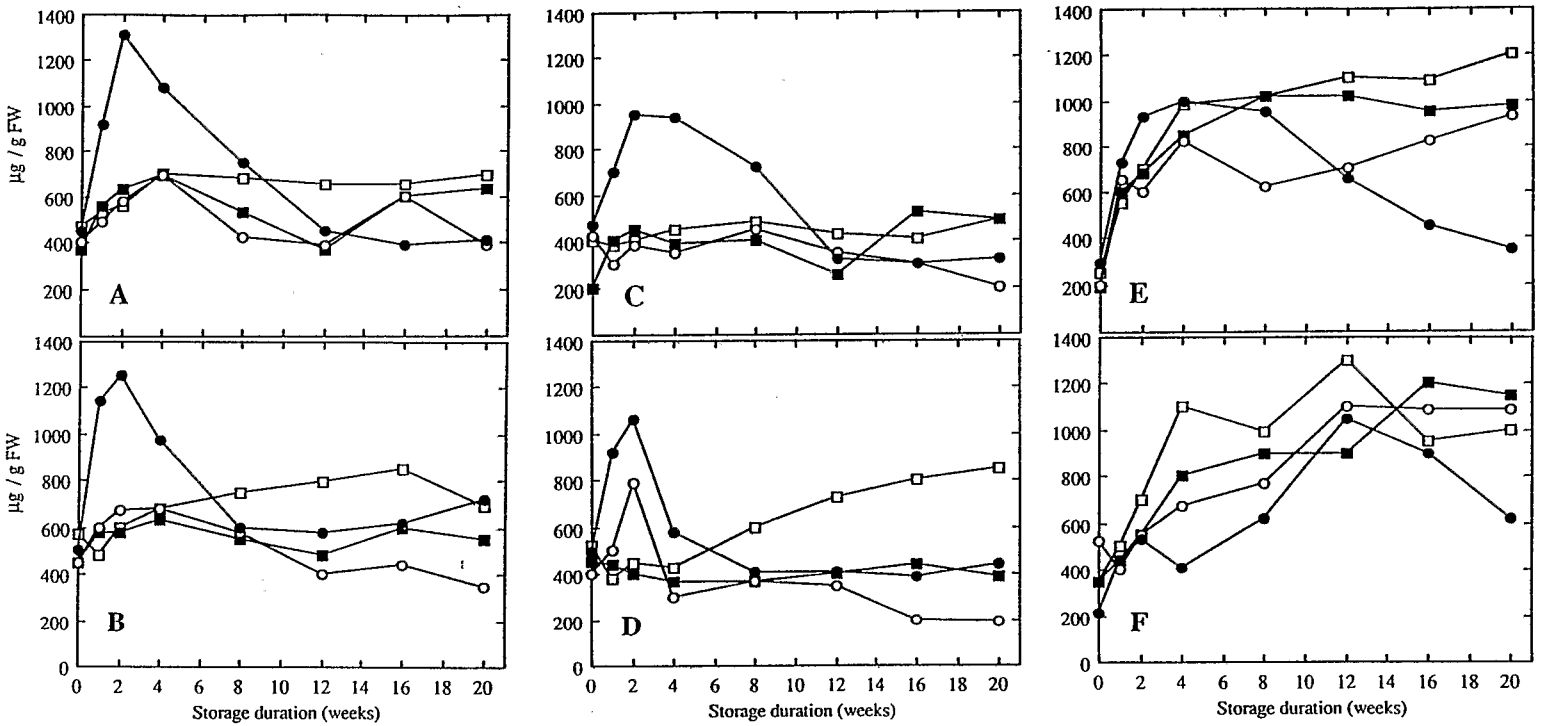

Fig. 2. Evolution of the concentration of fructose (A, B), glucose (C, D) and sucrose (E, F) of microcuttings of *C. arabica* (A, C, E) and *C. canephora* (B, D, F) after storage at different temperatures (27°C □, 22°C ■, 17°C ○ and 12°C ●).

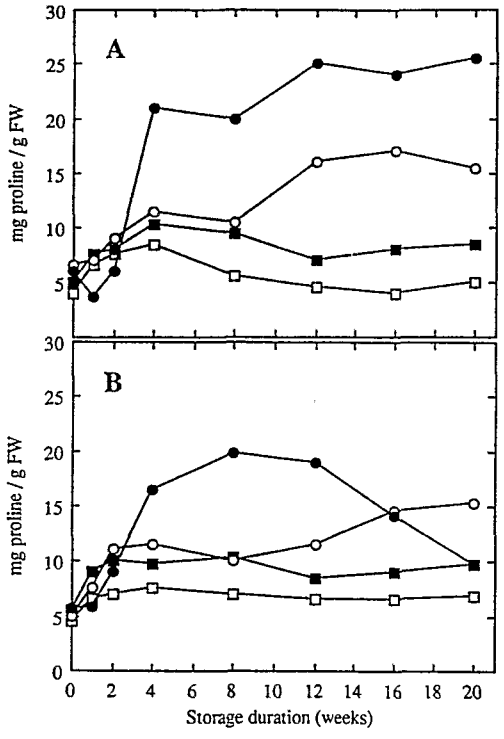

Fig. 3. Evolution of the proline concentration of microcuttings of *C. arabica* (A) and *C. canephora* (B) during storage at different temperatures (27°C □, 22°C ■, 17°C ○ and 12°C ●).

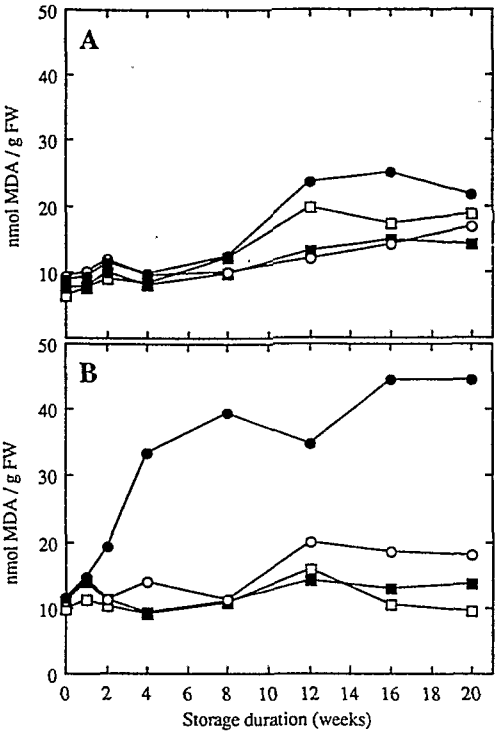

Fig. 4. Evolution of the malonedialdehyde (MDA) concentration of microcuttings of *C. arabica* (A) and *C. canephora* (B) during storage at different temperatures (27°C □, 22°C ■, 17°C ○ and 12°C ●).

increased transitorily between the 12th and 16th weeks of storage. The MDA contents did not differ between the batches subjected 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. 4B). 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 I). 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.

During storage and whatever the temperature, the ethylene production remained low and constant (between 30 and 200 nl·24 $h^{-1}\cdot g^{-1}$ fresh wt., data not shown). 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 (Figs. 5A and 5B). In the case of *C. arabica* (Fig. 5A), 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 present for plants stored at 12° and 17°C. For *C. canephora* (Fig. 5B), a peak of ethylene was

Table I. Modifications in the MDA concentration (nmol/g fresh wt.) after 4, 16 and 20 weeks of storage at different temperatures (storage) and 10 days at 27°C (storage + 10 days). The different letters indicate significantly different values ($P = 0.05$)

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

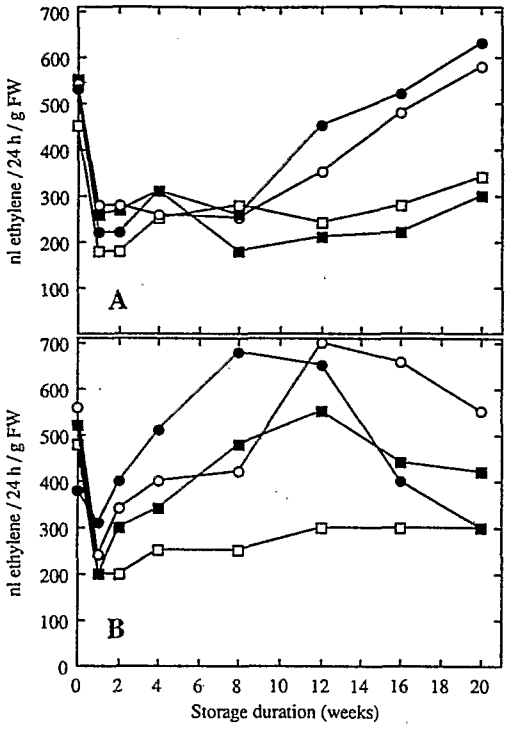

Fig. 5. Evolution of the maximal ethylene production of microcuttings of *C. arabica* (A) and *C. canephora* (B) after storage at different temperatures (27°C □, 22°C ■, 17°C ○ and 12°C ●).

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. 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

In the majority of cases, the critical storage temperature is much higher for tropical cold-sensitive species, in comparison with that of plants from temperate climates. It is generally between 8 and 15°C [32] and appears to be even higher for coffee. In this experiment, *C. canephora* microcuttings were much more cold sensitive than those of *C. arabica*, which confirms the previous results of Jouve et al. [23] 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 to the storage temperature, contrarily to what is observed in cold-tolerant plants from temperate climates during cold acclimation [4,33]. 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 with those noted in the literature [3,6]. 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 [34]. 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 for monitoring 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* [35] or tropical species such as *Carica papaya* [36] and *Elaeis guineensis* [37]. Chen and Patterson [22] 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 [37]. 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 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 examined in the present work, MDA appears to be the best indicator of cold sensitivity in the case of microcuttings of *C. arabica* and *C. canephora*. In order to complete this study, it would be interesting to compare the activity of the superoxide dismutase and catalase enzymes which are involved in the defense processes against free radicals.

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References

1 J. Levitt, Responses of Plants to Environmental Stresses, Vol. 1. Chilling, Freezing and High Temperature Stresses, 2nd ed., Academic Press, New York, 1980, 497 pp.
2 D. Graham and B.D. Patterson, Responses of plants to low, non freezing temperatures: proteins, metabolism, and acclimation. Annu. Rev. Plant Physiol., 33 (1982) 347–372.
3 N.M. Kushad and G. Yelenosky, Evaluation of polyamine and proline levels during low temperature acclimation of citrus. Plant Physiol., 84 (1987) 692–695.
4 G.L. Salerno and H.G. Pontis, Raffinose synthesis in *Chlorella vulgaris* cultures after cold shock. Plant Physiol., 89 (1989) 649–651.
5 C.L. Guy, G. Yelenosky and N.C. Sweet, Light exposure and soluble sugars in citrus frost hardiness. Fla. Sci., 43 (1980) 268–273.
6 J.P. Krall, G.E. Edwards and C.S. Andreo, Protection of pyruvate, Pi dikinase from maize against cold lability by compatible solutes. Plant Physiol., 89 (1989) 280–285.
7 A.C. Purvis and G. Yelenosky, Sugar and proline accumulation in grapefruit flavedo and leaves during cold

hardening of young trees. Physiol. Plant., 80 (1982) 159–168.
8 N.I. Shevyakova, Metabolism and physiological role of proline in plants under conditions of water and salt stress. Sov. Plant Physiol., 31 (1984) 597–608.
9 U. Heber, L. Tyakova and K.A Santarius, Stabilisation and inactivation of biological membranes during freezing in the presence of amino acids. Acta Biochim. Biophys., 241 (1971) 578–592.
10 U. Heber, L. Tyankova and H.A Santarius, Effects of freezing on biological membranes in vivo and in vitro. Acta Biochim. Biophys., 291 (1973) 23–37.
11 M.S. Liu and J.A Hellebust, Regulation of proline metabolism in the marine centric diatom *Cyclotella cryptica*. Can. J. Bot., 54 (1976) 949–959.
12 C.R Steward and S.F. Boggess, The effect of wilting on the conversion of arginine, ornithine and glutamate to proline in bean leaves. Plant Sci. Lett., 8 (1977) 147–153.
13 D.V. Lynch and P.L. Steponkus, Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L. cv. Puma). Plant Physiol., 83 (1987) 761–767.
14 P.L. Steponkus, Role of plasma membrane in freezing injury and cold acclimation. Annu. Rev. Plant Physiol., 35 (1984) 543–584.
15 P.J. Quinn and W.P. Williams, Plant lipids and their role in membrane function. Prog. Mol. Biol., 34 (1978) 109–173.
16 E. Sikorska and A. Kackperska-Palacz, Phospholipid involvement in frost tolerance. Physiol. Plant., 47 (1979) 144–150.
17 J. Berry and J.K. Raison, Responses of macrophytes to temperature, in: O. Lange, C.B. Osmond and P.S. Nobel (Eds.), Encyclopedia of Plant Physiology, New Series, Vol. 12A, Springer Verlag, New York, 1982, pp. 277–338.
18 R.M. Harber and L.K. Fuchigami, The relationship of ethylene and ethane production to tissue damage in frozen rhododendron leaf disks. J. Am. Hortic. Sci., 111 (1986) 434–436.
19 I. Engelmann-Sylvestre, Evolution des lipides membranaires au cours de la sénescence de pétales d'oeillet coupé (*Dianthus caryophyllus* L. cv. Ember). Relation avec la crise éthylénique. Thèse d'Université, Université Paris 6, 150 pp.
20 S.F. Yang and N.E. Hoffman, Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol., 35 (1984) 155–189.
21 C.B.S. Tong and S.F. Yang, Chilling-induced ethylene production by beans and peas. J. Plant Growth Regul., 6 (1987) 201–208.
22 Y.Z. Chen and B.D. Patterson, Ethylene and 1-aminocyclopropane-1-carboxylic acid as indicators of chilling sensitivity in various plant species. Aust. J. Plant Physiol., 12 (1985) 377–385.
23 L. Jouve, F. Engelmann and A. Charrier, Effets de l'hypoxie et de la température sur la conservation in vitro de pousses feuillées de *Coffea arabica* L. Café Cacao Thé, 35 (1991) 205–210.
24 A. Bertrand-Desbrunais, M. Noirot and A. Charrier, Minimal growth in vitro conservation of coffee (*Coffea* spp.). 2: Influences of reduced concentration of sucrose and low temperatures. Plant Cell Tiss. Org. Cult., 31 (1992) 105–110.
25 P. Dublin, Induction de bourgeons néoformés et embryogenèse somatique. Deux voies de multiplication des caféiers cultivés. Café Cacao Thé, 24 (1980) 121–129.
26 H.U. Bergmeyer and E. Bernt, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, 2nd ed., Academic Press, New York, 1974, pp. 1176–1179.
27 L.S. Bates, R.P. Waldren and I.D. Teare, Rapid determination of free proline for water-stress studies. Plant Soil, 39 (1973) 205–207.
28 R.L. Heath and L. Packer, Photoperodixation in isolated chloroplasts I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys., 125 (1968) 189–198.
29 D. Hagège, A. Nouvelot, J. Boucaud and T. Gaspar, Malonedialdehyde titration with thiobarbiturate in plant extracts: avoidance of pigment interference. Photochem. Anal., 1 (1990) 86–89.
30 D. Newman, The distribution of range in samples from a normal population expressed in terms of an independent estimate of standard deviation. Biometrika, 31 (1939) 20–30.
31 M. Keuls, The use of studentised range in connexion with analysis of variance. Euphytica, 1 (1952) 112–122.
32 N. Banerjee and E. de Langhe, A tissue culture technique for rapid clonal propagation and storage under minimal growth conditions of *Musa* (banana and plantain). Plant Cell Rep., 4 (1985) 351–354.
33 P. Calderon and H.G. Pontis, Increase of sucrose synthase activity in wheat plants after chilling shock. Plant Sci., 42 (1985) 173–176.
34 E.E. Benson, Free Radical Damage in Stored Plant Germplasm, Int. Board for Plant Genetic Resources, Rome, 128 pp.
35 W.C. Cooper, G.K. Rasmussen and E.S. Waldon, Ethylene stimulated by chilling in *Citrus* and *Persea* spp. Plant Physiol., 44 (1969) 1194–1196.
36 H.T. Chan, S. Sanxter and H.M. Couey, Electrolyte leakage and ethylene production induced by chilling injury of papayas. HortScience, 20 (1985) 1070–1072.
37 F. Corbineau, F. Engelmann and D. Côme, Ethylene as an indicator of chilling injury in oil palm (*Elaeis guineensis* Jacq.) somatic embryos. Plant Sci., 71 (1990) 29–34.