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Hyperhydricity of micropropagated shoots: a typically stress-induced change of physiological state

Claire Kevers[1,*], Thierry Franck[1], Reto J. Strasser[2], Jacques Dommes[1] & Thomas Gaspar[1]
[1]*Laboratoire de Biologie moléculaire et d'Hormonologie végétales, Université de Liège, Institut de Botanique B 22, Sart Tilman, BE-4000 Liège, Belgium;* [2]*Laboratoire de Bioénergétique, Université de Genève, Chemin des Embrouchis, 10, CH-1254 Jussy-Geneva, Switzerland (*request for offprints: Fax: +32-4-366-3872; E-mail: c.kevers@ulg.ac.be)*

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Abstract

Hyperhydricity of micropropagated shoots, formerly called vitrification, undoubtedly results from growth and culture conditions, subjectively reputated as stressing factors: wounding, infiltration of soft culture medium, generally of a high ionic strength, rich in nitrogen and in growth regulators in a special balance, in a humid and gaseous confined atmosphere. Stress is (objectively) defined as a disruption of homeostasis resulting from a constraint escaping the usual flexibility of metabolism. It induces another temporary (reversible) or definitive (irreversible) thermodynamic physiological state. The state-change concept developed by Strasser (1988) and Strasser and Tsimilli-Michael (2001) is applicable to the phenomenon of hyperhydricity. An appraisal of the redox capacities of hyperhydrated shoots together with a study of some enzymic activities that catalyse pentose phosphate and glycolytic pathways has indeed shown that such shoots have evolved towards a temporary state of lower differentiation or a juvenile state with a sufficient activity to survive and to defend themselves.

Abbreviations: HS – hyperhydric shoots; NS – normal shoots

The hyperhydricity phenomenon

Hyperhydricity has been proposed to be the right term to designate the hyperhydric malformations frequently affecting herbaceous and as well woody shoots during their *in vitro* vegetative propagation (Debergh et al., 1992). The phenomenon was previously known and described under the term vitrification (Debergh et al., 1981; Kevers et al., 1984; Gaspar, 1991; Ziv, 1991). Glassiness, translucency, glauciness, and vitrescence were other less used appellations to call this physiological disorder (Gaspar et al., 1987). The so-called vitrified, vitreous or hyperhydric shoots appear turgid (as if the cells were turgescent, at first sight), watery at their surface, and hypolignified. Their organs are somehow translucent, in some cases less green, and easily breakable. Hyperhydricity is not restricted to shoots. Some yellowish and hyperhydric callus types may be considered as vitreous (Crèvecoeur et al., 1987). Their high degree of friability therefore is related to the breakability of vitrified organs (Gaspar et al., 1991, 1992).

Vitrified shoots root poorly when they do. They do not generally survive at the acclimatisation step; when they do, they issue in malconformed plantlets. The process of vitrification leading to hyperhydricity involves problems of differentiation.

In general, stems of vitrified plantlets are broad, and thick in diameter. The lengths of the internodes are shorter than those of plants appearing normal. Leaves are thick, frequently very elongated, wrinkled and/or curled, and brittle.

Anatomically, there is hypertrophy of the cortical and pith stem parenchyma with hypertrophy of the

cells and large intercellular spaces. Increased extracellular space and air volume have been shown in vitrifying material. The vessels and tracheids in vitreous plants are not properly lignified. In vitrified leaves, palisade tissue is nonexistent or drastically reduced. The mesophyll looks spongy with large intercellular spaces. Severe structural damage is observed in the apical dome. There are no pro-cambium strands descending basipetally from the leaf primordia. Vascular bundles are few and lack the typical arrangement of a normal shoot.

The succulent mature leaf shows a thin noncontinuous cuticle, with reduced or absent deposition of epicuticular wax and fewer stomata (often plugged with an unknown material) than the mature leaf of normal plants. Most stomata of leaves from *in vitro* plantlets do not seem to have a closure mechanism. This may be the main cause of rapid water loss, and death, during acclimatisation under low relative humidity. Hyperhydric callus types are constituted of parenchymatic cells without xylem elements (Crèvecoeur et al., 1987).

Hyperhydric tissues have shown several biochemical characteristics, some of which being related to the morphological abnormalities (see Gaspar, 1991):

- Reduced dry weight, that is, more water, essentially located in the apoplastic spaces. The amount of intracellular water is less important (Kevers and Gaspar, 1986).
- Less lignin, the lower lignification being associated to lower activities of enzymes involved in the synthesis of lignin precursors and in their polymerisation.
- Less cellulose, which was associated to a low C/N ratio favouring the synthesis of amino acids rather than the sugar units for cellulose.
- Higher activity of glutamate dehydrogenase, which is thought to bring about the diversion of the carbohydrate pool to amino acid synthesis.
- Low calcium content, low Ca^{2+}/uronic acids ratio, low ratio of uronic acids to neutral sugars due to higher amounts of the latters in isolated cell walls of vitrified tissues.
- Less chlorophyll, which causes translucency and presumably lower photosynthetic capacity.
- Less soluble phenols.
- Higher activity of basic peroxidases which is thought to be associated to an enhanced auxin catabolism. Polar auxin transport is also apparently reduced.
- Less soluble Ca^{2+}, Mn^{2+}, and Na^{+} and more K^{+}, at least in carnation.
- Reduced ethylene production following a burst initiated with the application of the vitrifying conditions.

The problem is among these biochemical changes to distinguish those which have engendered the underdifferentiated state and those which are the consequences.

The process of vitrification is generally considered as reversible. This does not mean that hyperhydric leaves, once fully formed and mature, reverse to normal structure and survive transplanting, but it signifies that new shoots or leaves formed by hyperhydric shoots after transfer to a nonvitrifying medium or to the greenhouse may have a morphology and anatomy approaching those of normal plants. At the opposite, subculturing hyperhydric shoots in vitrifying conditions may lead to severe damages, including death of the whole shoots as such, through apparent necrosis of all the primary meristems. Vitrification has therefore been considered as a neoplastic step to generalised cancer (Gaspar et al., 1991, 1995; Anonymous, 1995). The hyperhydric state (of newly formed shoots) can also be maintained through several subcultures without too much changes. This may mean that in this neoplastic step the shoots by themselves have found a way of survival. The 'homeostasis' of this neoplastic step is examined here as a typically stress-induced change of physiological state.

Stress definitions

Plants, particularly in temperate climates, live in an environment that changes, not only seasonally, but also from one moment to the next. These rapid environmental changes largely involve fluctuations in light and temperature. Such plants grow over a wide range of temperatures and, apart from encountering large seasonal variations in temperature (including freezing), the aerial parts of the plant may face temperature variations of tens of centigrade degrees in a single day and smaller temperature changes in a matter of minutes.

Many of the environmental factors, which fluctuate, are associated intimately with metabolic processes. Variation in light, which supplies energy for photosynthesis, has immediate effects on metabolism, while water deficit decreases stomatal conductance and therefore limits carbon dioxide supply

and changes the balance between photosynthesis and photorespiration. The source (nitrate v.s. ammonium) and concentration of nitrogen influences the location (root or shoot) and rate of nitrogen assimilation into amino acids and therefore requires a dynamic balance between photosynthesis, carbon partitioning and nitrogen assimilation (Smirnoff, 1995). Thus environment around plants fluctuates regularly and predictably under daily and seasonal cycles. The flexibility of their metabolism allows plants to deal with their constantly fluctuating environment. Co-ordination of plant metabolic activity with the surrounding environmental factors (i.e. abiotics and biotics: temperature, water, light, UV radiation, mineral nutrient, oxygen supply, bacteria, myccorhizal fungi, ...) has major effects on plant growth.

Thus, every change of an environmental factor influences plant growth and development. But every deviation of a factor from its optimum is not necessarily a stress for a flexible plant acclimatised to its environment. Stress begins with a constraint (biotic or abiotic) or with highly unpredictable fluctuations imposed on the regular metabolic pattern, that cause bodily tension. Stress, in the sense of a stressor or stress inducer (Cassells and Curry, 2001), is an unusual factor or a usual factor of the biotic and abiotic environment modified in such a way (excess or deficit) that it has the capability of causing bodily injury, disease, or aberrant physiology. The disease is considered as a condition of the living (animal or plant) body or one of its parts that impairs the performance of a vital function, or functions, as a response to environmental factors or inherent genetic defects. Stress, in the sense of the physiological state, is the condition caused by factors that tend to alter an equilibrium (Nilsen and Orcutt, 1996).

Because plants are confined to the place were they grow, they have a limited capacity to avoid unpredictable unfavourable changes in their environment (confrontation with extremes of temperature, water shortage, insufficient or excessive light or mineral nutrients, attack by pathogenic bacteria, fungi, viruses and viroids). They have developed ingenious molecular strategies to defend themselves against such biotic and abiotic stresses, most often combined with an alteration of growth and developmental patterns. This explains why the concept of stress is intimately associated with the external conditions that adversely affect growth, development or productivity (Lutts and Kinet, 1998).

Other authors define stress as changes in physiology that occur when species are exposed to extraordinary unfavourable conditions that need not represent a threat to life but will induce an alarm response (Larcher, 1980). Alarm responses are defensive or adaptive responses to the stimulus. This definition can be difficult to use. For example, if plants are placed in the sun and water is withheld, leaves may wilt. Wilting could be detrimental to the plant in the short term because carbon gain is inhibited. But wilting may be critical for survival in the long run because leaf temperature may be kept low enough to avoid permanent damage (less light energy is absorbed by a wilted leaf). Therefore, wilting is both a detrimental and an advantageous response to low water availability and high light (Nilsen and Orcutt, 1996). This grey distinction between adaptive mechanism and detrimental effect on physiology prompted the consideration of the additional term strain (Levitt, 1980).

Differentiation between stress and strain comes from applying the engineering meaning of these terms to biological systems. The biophysical definition of stress is that it is the applied force divided by the area of the force, or pressure. The term stress, in a plant physiological sense, is therefore reflective of the amount of environmental pressure for change that is placed on an organism's physiology. Strain is defined as the proportional change in a substance as a consequence of stress. Strain can be characterised as a physiological change that occurs in response to environmental stress that does not necessarily result in significant reduction of growth or reproduction (Levitt, 1982).

According to Strasser (1988), strain is any physical and/or chemical change produced by a stress, which is every established condition that forces a system away from its thermodynamic optimal state (Figures 1 and 2). The state is called optimal when the biological system is in full harmony with its environment. Harmony of a biological system with its environment is achieved when the system does not tend to change any activity or conformation whatsoever.

To summarise, although the term stress may be used for indicating the STRESSOR agent (that is the unusual factor, or a usual factor modified in such a way, excess or deficit, that it has the capability of causing bodily injury or disease), or for the alarm RESPONSE to extraordinarily unfavourable conditions (for instance wilting), it primarily concerns the altered PHYSIOLOGICAL STATE, impairing the performance of a vital function.

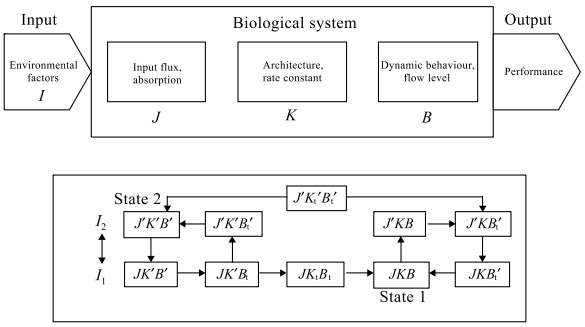

Figure 1. Top. The *JKB*-trilogy, by which any biological systems can be approached (Strasser 1988; Strasser and Tsimilli-Michael, 2001; Tsimilli-Michael et al., 1996, 2002). *J* stands for the environmental input term, *K* for the hardware extensive term and *B* for the behaviour intensive term. *I* represents the changing environmental factor(s). Bottom. The *JKB*-trilogy describing state-changes (from state 1 to state 2, and possible back to state 1) as acclimation or adaptation to a changing environment ($I_1 \leftrightarrow I_2$) (Strasser, 1988).

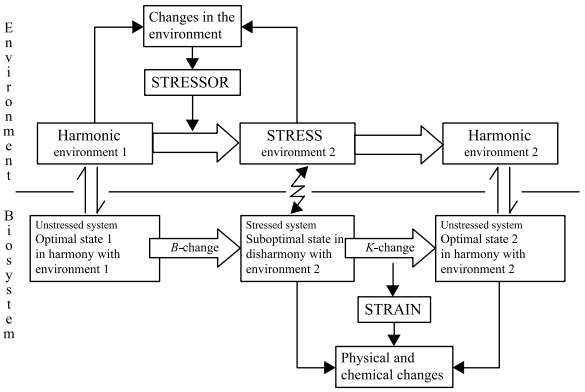

Figure 2. Strain as defined by the physical and chemical changes between the unstressed system in the harmonic environment 1 and the other unstressed system in harmony with environment 2, the latter issuing as a consequence of stressing changes in the environment (according to Strasser, 1988).

Mechanisms that permit stress survival are termed RESISTANCE mechanisms and can allow an organism to tolerate or avoid stress. Thus, physiological responses to stressors can be divided into three possibilities. In one case, TOLERANCE, in which plants have mechanisms that maintain high metabolic activity (similar to that in the absence of stress) under mild stress and reduced activity under severe stress. In contrast, mechanisms of AVOIDANCE involve a reduction of metabolic activity, resulting in a dormant state, upon exposure to extreme stress (Osmond et al., 1987). Commonly, a plant species may have several tolerance or avoidance mechanisms, or a combination of both. For instance, drought stress may induce

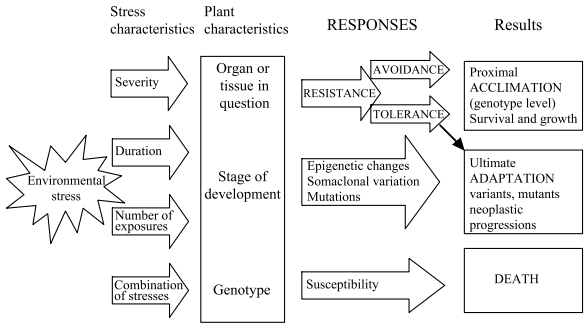

Figure 3. Plant responses to environmental stress in correspondence with stress and plant characteristics. Mechanisms that permit stress survival are termed RESISTANCE. Mechanisms of AVOIDANCE involve a reduction of metabolic activity, resulting in a dormant state. TOLERANCE involves changes of metabolic activity in dependence upon the severity of the stress. During ACCLIMATION, an organism alters its homeostasis to accommodate (further) shifts in its external environment. ADAPTATION involves microevolutionary processes changing gene frequencies of a population over time. Outside acclimation or adaptation, the only other issue is death through necrosis and/or apoptosis (see Gaspar et al., 2002).

drought tolerance that can be followed by desiccation tolerance: in the later 'dormant' state, the organism can survive the dry state for long periods, that is, years. Notice that the ability to rehydrate without damage can be considered as a part of the desiccation tolerance. The other issue is immediate or delayed DAMAGES, through somaclonal variation, mutation, neoplastic progression, ultimate death via necrosis and/or apoptosis (Figure 3) (Gaspar et al., 2002).

In plant stress physiology, an important distinction must also be made between ultimate (ADAPTATION) and proximal (ACCLIMATION) plant responses. Adaptation occurs by various mechanisms at the genetic level in populations over many generations. Microevolutionary processes change gene frequencies of a population over time. In a stressful environment, it is logical to assume that specific genotypes with appropriate gene combinations (those that confer the ability to survive and reproduce) are dominant in the population. Those particularly favourable gene combinations in plants that inhabit stressful environments are called adaptations.

Populations that have adapted through evolutionary processes acting at the genetic level to a particular climatic regime are by no means static systems. On the contrary, plants have an incredible ability to adjust physiological and structural attributes on the scale of seconds or seasons within a single genotype: this is acclimation. In other words, during acclimation, an organism alters its homeostasis, its steady-state

physiology, to accommodate (further) shifts in its external environment. For instance, prolonged exposure of chilling resistant plants to cold results in the adjustment of plant growth and metabolism to low temperature conditions and in the increased resistance of tissues to freezing temperatures. Freezing resistance is the ability of plants to survive formation of ice in tissues. Systematic acquired resistance (SAR) against pathogens is another form of acclimation.

On a long-term scale, acclimation is enhanced in plants because of the modular nature of metabolism and growth. Plant parts can be abscised and regrown in a new morphology or anatomy, specific organs can be enhanced by increasing their numbers or size. On a short-term basis (i.e. seconds or minutes), protein populations can ebb and wane, growth regulators can be released or activated, or transcription and translation can be regulated up or down.

Acclimation is a phenotypic response to different combinations of environmental characteristics. Phenotypic plasticity is an index of the amount of acclimation that is possible within one genotype (Nilsen and Orcutt, 1996).

Thus, adaptation at the population level, or acclimation at the level of the individual plant, occur through a combination of behavioural, morphological, anatomical, physiological and biochemical processes, which depend on processes at the molecular level.

What is said above means that, contrary to the general opinion, stresses must not be automatically associated with adverse detrimental effects. Stress responses include pathologies and adaptive advantages, or both successively or together.

The state change concept

The stress concept deals with a disruption of physiological homeostasis, followed by a reestablishment, in the former situation, or through another physiological state. The state change concept developed by Strasser (1988) accounts for such possibilities. It involves the interaction of environment and the biological system. Every (very complex) biological system can be reduced to the three symbols J–K–B, the so-called *JKB*-trilogy (Figure 1). J stands for all energetic inputs from the environment into the biological system. K stands for all molecules and rate constants of the biological system and so represents the architecture of the organism. B stands for the dynamic behaviour of the system. It is a measure of the overall flow level in the open system.

The biological activity is a function of B and K which both depend on J. However, J is a direct function of the environment I. With this very simple *JKB*-trilogy, it is possible to formulate a state-change of a biological system that represents an adaptation process upon a stress (Figures 1 and 2). The idea is that for any given conformation K, it is possible to adjust the relative flow level B between zero and one by changing the input parameters J which is a function of the environmental parameters I. Therefore, the efficiency of the system ($\eta(i) =$ output flux I/input fluxes) is a function of B with one or more values of B at which the efficiency is optimal. As long as K is constant, the system can only work according to its state function.

The concept of the state-change theory (Strasser, 1988) is that suboptimality is the driving force of adaptation. Suboptimality creates a force that alters the statistical distribution of the microstates what appears as a change in the conformation of the system. Every change in K to K' is called a state-change. To the new state function K' there belongs a new η versus B with a new optimum.

Moving within one state function corresponds to intensity changes of the biological activity (B-change), leading to suboptimality. Moving from one state function to another corresponds to conformational changes (K-change), leading to optimality.

Changing the environmental parameter from $I(1)$ to $I(2)$ and back to $I(1)$ (e.g. clouds–full sunshine–clouds) forces the system into suboptimality and therefore into a state-change cycle (Figure 1). That means that the way of adaptation to new conditions is different to the way of readaptation to the original conditions.

The stress concept given here is based on nonequilibrium thermodynamics and dissipative structures. It is thus regarded as a dynamic relationship between organism and environment, keeping from the physical approach the concept of action–reaction, and offers the possibility of analytical description and quantification. In this concept, no environmental factor is considered *a priori* as unfavourable and the plant has not to 'resist', but it simply reacts. As far as the system manages to adapt, which means that the attraction point is within realistic limits (Tsimilli-Michael et al., 1996, 2002), stress is not only harmless but, even more, constructive because it results in improved resistance and adaptive evolution. However, if the adaptability of the system is overtaxed then stress is destructive, leading to permanent damages or even to death (Figure 3). This

Strasser's stress concept is in full agreement with the Larcher's (1980) definition: "Every organism experiences stress, although the way in which it is expressed differs according its level of organisation. From the botanist's point of view, stress can be described as a state in which increasing demands made upon a plant lead to an initial destabilisation of functions, followed by normalisation and improved resistance. If the limits of tolerance are exceeded, permanent damage or even death may result. Stress thus contains both destructive and constructive elements: it is a selection factor as well as a driving force for improved resistance and adaptive evolution".

The stressing conditions of the *in vitro* plant cultures

Every type of plant tissue culture is an evoluating system where cell divisions, cell differentiation and developmental (organo- and morpho-genesis) processes take place during cycles on successively renewed, same or different culture media, in different types of semi-hermetically closed containers. From the beginning of subculture to the end of the cycle, the plants, more frequently 'explants', are submitted successively and/or simultaneously to unusual (as compared to greenhouse or field conditions) cultural and environmental conditions. Among them are mechanical perturbation, injuries, wounding and possible air embolism due to dissection, high osmoticity (high sucrose content of the medium, notably) plus osmotic shock due to infiltration of the culture medium in the intercellular spaces (Kevers and Gaspar, 1986; Böttcher and Göring, 1987), abnormal mineral nutrition (high ammonium concentration), unusual hormonal treatment (high cytokinin and/or auxin application), high relative humidity in the flask atmosphere, and possible accumulation of different gases (ethylene notably) in the confined atmosphere, plus a rupture of the usual cohabitation with the epiphytic micro-organisms due to previous surface disinfection. Such media are commonly used for mass cell (calli or cell suspension), organ (roots, shoots) and plantlet (through adventitious root formation on the shoots) multiplication. Some media may induce desired (mainly for experimental purposes) or accidental, undesired precocious ageing processes. Conversely, others are designed for rejuvenation purposes, or for orienting a more or less branched habit. The culture media and flasks are adapted in such ways that in most cases, cells, organs (most often shoots) and plantlets appear healthy and normal. However, it has been known for a long time that cells in culture can become habituated (i.e. independent for auxin and/or cytokinin feeding) (Gautheret, 1942) and from more recent studies that such cells may become cancerous (Gaspar et al., 1991; Gaspar, 1995, 1999) after a progressive loss of organogenic totipotency (Gaspar et al., 2000).

The relatively high frequency of somaclonal variation (De Klerk, 1990; Karp, 1993; Cassells et al., 1997, 1999a, b) and the accumulating physiological deviations (from a normal behaviour) in micropropagated plants (see Swartz, 1991; Cassells et al., 2000 for reviews; Jemmali et al., 1995 for the hyperflowering process in strawberry) leads to a reappraisal of the genetic fidelity of organised meristem-derived micropropagated plants (Philipps et al., 1994; Rani and Raina, 2000). The culture media and the confined environment are accused for the stress imposed.

The stress-induced state-change (strain) concept applied to hyperhydricity

All suggested external causes of vitrification refer exclusively to the culture conditions, medium and atmosphere (Gaspar et al., 1987). As was stated by Debergh et al. (1981), the source and physiological conditions of the mother plant at the time of explantation and inoculation do not interfere with the process. Although most of plants can adapt to the *in vitro* culture conditions, the phenomenon of hyperhydricity was often considered as a physiological response due to the simultaneous stress factors of the *in vitro* culture media (see above) plus the high relative humidity in the flask atmosphere, and the accumulation of specific gases in the confined atmosphere (Kevers et al., 1984; Ziv, 1991; Gavidia et al., 1997; Chen and Ziv, 2001). The water layer surrounding the cells in malformed hyperhydric shoots will further limit oxygen availability by these cells and may constitute an additional stress factor, hypoxia. In most plants subjected to stress, including hypoxia (Gasdaska and Baker, 1997), a variety of toxic oxygen species (e.g. oxygen superoxide anion, hydroxyl radical, singlet oxygen) and/or H_2O_2 are produced which may lead to severe damage of cell molecules, membranes and other structures (Asada, 1992). These substances are generally eliminated through a cooperative mediation of the so-called defense enzymes and antioxidants.

The activity of protective systems against activated oxygen species, the reducing agents and the markers

Table 1. Chlorophyll fluorescence parameters of dark adapted leaves (mean ± SD; $n = 6$ and plasma membrane redox capacity (mean ± SD; $n = 3$), some enzyme activities of glycolysis and OPP, and relative differences (in % of control) between HS (on gelrite) and NS (on agar) of *P. avium* after 28 days of culture[a]

	NS	HS	%
Chlorophyll fluorescence			
Measured intensities			
F_0 initial intensity	77 ± 1	44 ± 2	−43
F_m maximal intensity	375 ± 6	178 ± 11	−53
Calculated expressions			
$F_v = F_m - F_0$ variable fluorescence	298 ± 6	134 ± 10	−55
$F_0/F_m = \varphi_{D0} = DI_0/ABS = k_n/(k_P + k_n)$			−17
$F_v/F_m = 1 - F_0/F_m = \varphi_{P0} = TR_0/ABS = k_P/(k_P + k_n)$	0.795 ± 0.003	0.753 ± 0.005	−4
$F_v/F_0 = \varphi_{P0}/\varphi_{D0} = TR_0/DI_0 = k_P/k_n$			−21
F_m/F_0	4.87 ± 0.06	4.05 ± 0.09	
Ferricyanide reduction ($\Delta OD\ min^{-1}\ g^{-1}$ DW)	7.63 ± 0.42	5.83 ± 0.36	−24
Glycolysis enzymes			
Hexokinase	18.9 ± 1.1	7.1 ± 1.8	−62
Hexose phosphate isomerase	89.3 ± 10.9	26.9 ± 6.4	−70
Glycerol-3-phosphate dehydrogenase	11.1 ± 0.8	6.5 ± 0.8	−41
Phosphofructokinase	8.9 ± 1.2	7.1 ± 1.0	−20
OPP enzymes			
6-Phosphogluconate dehydrogenase	5.6 ± 0.3	3.9 ± 0.4	−30
Glucose-6-phosphate dehydrogenase	37.0 ± 3.0	4.9 ± 0.9	−87

[a] φ_{D0} = maximum quantum yield of energy dissipation, φ_{P0} = maximum quantum yield of primary photochemistry, DI_0 = maximum dissipation, TR_0 = maximum trapping, ABS = photon absorption, k_n = deexcitation rate constant for nonphotochemical events and k_P = deexcitation rate constant for photochemical events. According to Franck et al. (2001).

of lipid peroxidation have been evaluated comparatively in normal (NS) and hyperhydrated (HS) *Prunus* shoots (Franck et al., 1995, 1998b). The authors pointed out some paradoxical results in an extensive classical analysis of stress criteria (Gaspar, 1995; Franck et al., 2000). A very simple explanation was that some of the early burst reactions to stress were missed in the analyses and that some determinations were performed during the transition from a homeostatic state 1 (normal shoots) to another homeostatic state 2 (hyperhydric shoots), according to schemes of Figures 1 and 2. Investigations during hyperhydricity in other plants also suggested the existence of an oxidative stress (Olmos et al., 1997; Chen and Ziv, 2001).

Nevertheless, after only one hyperhydric culture cycle, in spite of their modified aspect (and metabolism), *Prunus* shoots seem viable (Franck et al., 1998b, 2001), presenting few symptoms of necrosis.

A drop in the chlorophyll fluorescence parameters (F_0, F_m, F_v, F_v/F_m and F_m/F_0) was observed in hyperhydric leaves (Table 1). After 28 days of culture, the reduction of ferricyanide was lower in hyperhydric shoots (HS) than in normal shoots (NS) (Table 1). In HS, lower activities of some enzymes involved in glycolysis (hexokinase, hexose phosphate isomerase, glycerol-3-phosphate dehydrogenase, phosphofructokinase) and OPP (6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase) were observed in comparison to NS (Table 1).

The reduced pyridine nucleotides (NADPH and NADH) are involved in many biochemical reactions such as oxidoreductase or dehydrogenase reactions, electron transfer, etc. A general decrease of reduced and oxidised pyridine nucleotides was observed in HS as compared to NS but the NADPH/$NADP^+$ and NADH/NAD^+ ratios were similar in the two types of shoots (Table 2). Therefore, the decrease of NADPH and NADH contents could explain the low activity of the Asada–Halliwell pathway previously shown in HS (Franck et al., 1995). However, the operation of this pathway is complex and determined by the

Table 2. Content of pyridine nucleotides (g^{-1} DW) in normal shoots (NS on agar) and in hyperhydric shoots (HS on gelrite) of *P. avium* L. after 28 days of culture (according to Franck et al., 2001)

	NS after 28 days of culture on agar	HS after 28 days of culture on gelrite
NAD^+	10.41 ± 0.08	5.80 ± 1.14
NADH	12.54 ± 2.21	7.16 ± 0.34
$NADP^+$	24.59 ± 4.10	8.52 ± 2.05
NADPH	11.15 ± 2.13	4.89 ± 0.34
$NADH/NAD^+$	1.20 ± 0.18	1.23 ± 0.21
$NADPH/NADP^+$	0.45 ± 0.01	0.57 ± 0.08

dynamic equilibrium between four redox couples, that is, ascorbic acid/dehydroascorbic acid, reduced glutathione/oxidized glutathione, $NADPH/NADP^+$ and $NADH/NAD^+$. According to Foyer et al. (1997), the preservation of the redox couples at a constant level is more important than a modification of the pool of antioxidants to ensure survival of stressed plants. The importance of redox homeostasis in the regulation of plant defence is extensively discussed in the literature (Franck et al., 2000, 2001). From a metabolic point of view, the decrease of pyridine nucleotides content without affecting $NADPH/NADP^+$ and $NADH/NAD^+$ ratios could be attributed to a general decline of metabolism with a preservation of cellular homeostasis (Gogorcena et al., 1995; Iturbe-Ormaetxe et al., 1998).

NADPH and NADH are important carriers of chemical energy in the form of reducing power. The major source of NADPH in plants is the chloroplast via the light driven electron transport chain. Chlorophyll fluorescence has been used as a tool to determine photosynthetic properties of the sample (Strasser et al., 1995; Lichtenthaler, 1996). The drop of F_0 observed in HS could be explained by the lower chloroplast number and the lower chlorophyll content that characterised these shoots (Franck et al., 1998a). Indeed, F_0 was considered as an indicator of antenna size (number of fluorescing pigment molecules of PSII) (Badiani et al., 1993; Strasser et al., 1995). Some chlorophyll fluorescence parameters are routinely used to study the physiological state of the photosynthetic apparatus and the stress level of plants (Lichtenthaler, 1996). The F_v/F_m ratio is generally used to give an estimation of the maximum quantum yield of primary photochemistry. F_v/F_m is the flux ratio of maximum energy trapped per photon absorbed (TR_0/ABS) by PS II. The F_v/F_0 ratio is often proposed subjectively as a 'vitality index' of the leaves (Björkman and Demming, 1987; Krause and Weis, 1991) even so this expression carries exactly the same information as does $F_v/F_m = (F_v/F_0)/(1 + F_v/F_0)$. In bioenergetic terms of deexcitation rate constants F_v/F_m can be written as $k_p/(k_p+k_n)$ or $1/(1+k_n/k_p)$ and therefore F_v/F_0 as k_p/k_n, where k_p and k_n relate to photochemical and nonphotochemical events respectively. Therefore when ever F_v/F_m or F_v/F_0 change then k_p and or k_n change. A slight decrease of these ratios in HS suggested a slight drop of their photochemical process in comparison to NS. Therefore, a decrease of pigment content and not a dysfunction of the photosynthetic apparatus could explain the lower photosynthetic capacity observed in HS with the consequence of a reduced production of NADPH.

Other major sources of NADPH and NADH are, respectively, OPP and glycolysis. These pathways are catabolic and convert the carbon in reduced form, which has been captured by photosynthesis, into an oxidised form, with the release of chemical energy. The OPP ensures the production of reducing equivalent (NADPH) for cytosolic biosynthetic reactions and the sugar phosphates used in the synthesis of nucleotides, lipids and cell wall polymers (Brownleader et al., 1997). Some authors underlined the straight relationship between OPP and ascorbate-glutathione (Polle et al., 1992; Iturbe-Ormaetxe et al., 1998). Glycolysis is a key metabolic feature of the respiratory process. It allows the formation of ATP, NADH, an important precursor (pyruvate) necessary for amino acids biosynthesis. In HS, the low activity of some enzymes involved in glycolysis (hexokinase, hexose phosphate isomerase, glycerol-3-phosphate dehydrogenase, phosphofructokinase) and OPP (6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase) suggests a general decrease of activity of these pathways. It is well established that *in vitro* cultured plants have a disturbed carbon metabolism (Kozai et al., 1991; Bisbis et al., 1993). Indeed, the sucrose added to the medium could inhibit chlorophyll synthesis, photosynthesis, the Calvin cycle (Kozai et al., 1991; Van Huylenbroek and Debergh, 1996) and, therefore, the endogenous sugar production. Some authors found that hyperhydricity was reduced or eliminated by replacing sucrose with reducing sugars (e.g. fructose, glucose, galactose) (Rugini et al., 1987; Druart, 1998). Druart (1988) suggested that these carbohydrates could affect the redox potential by decreasing the oxidative potential found in hyperhydric leaves.

As previously discussed, a lower redox potential could characterise HS (Franck et al., 1998b, 2000). Electron transfer reactions or oxido-reduction (redox) reactions are crucial for the survival of any organism, since they are required for energy transduction, the operation of many anabolic and catabolic pathways, nutrient assimilation, and for plant defense (Rubinstein and Luster, 1993). The plasma membrane of plant cells is the centre of redox activities which can transfer electrons from cytosolic donors (NAD(P)H and NADH in most cases) to extracellular electron acceptors (molecular oxygen, ferricyanide, ascorbate,...) (Asard et al., 1998). The highest redox activities of plasma membrane can be measured with NADH as electron donor and ferricyanide as electron acceptor (Berczi and Moller, 1998). According to Gonzalez-Reyes et al. (1994) and Horemans et al. (1998), plasma membrane redox reactions contribute to the cell growth and cell wall development by controlling the redox status of the apoplast. It has been demonstrated that HS are characterised by several abnormalities of the cell wall such as hypolignification and the drastic reduction of the cuticle layer. Kevers et al. (1984) showed that lignin deficiency of HS is associated to an inhibition of some enzymes involved in lignin polymerisation (e.g. acidic peroxidases). However, a relationship between the low redox activities of the plasma membrane and the modification of the cell wall composition could not be excluded (Franck et al., 2001).

Taking these considerations into account, disruption of the energetic, respiratory and structural metabolisms could explain the lower dry weight accumulation (Kevers et al., 1984; Genkov et al., 1997) and the less differentiated state or more juvenile state that characterises HS (Gaspar et al., 2000).

To summarise, transfer of *Prunus avium* shoots in hyperhydric conditions led to a destabilisation of their energetic metabolism, with the consequence that they could not transform the environmental energy (becoming excessive for HS). In order to adapt, HS must decrease the energy input to equilibrate their flow energy level. Decrease of chlorophyll and chloroplasts contents in HS and decrease of leaves area (curled leaves) are so many events that could contribute to reduce energy input (J) into the shoot. In response to a decrease of energy absorption, HS adapted their biological activity by adjusting energy output. Indeed, a slowing down of energetic pathways (glycolysis and pentose phosphate pathway) and a slowing down of some metabolic processes implicated in plant defense (Asada–Halliwell less active in HS) and plant structure (cell wall and cuticule less developed in HS) were observed in HS (compared to NS). As a conclusion, HS evolved towards another energetic state (lower than in NS) by decreasing energetic inputs (J), by minimising energy expense for plant structure (K) and by slowing down dynamic behaviour (carbon metabolism) (B). All these changes, made with a close interrelation, allowed HS, in hyperhydrating conditions, to adapt themselves and survive by preserving their redox homeostasis (no changes of the NADPH/NADP and NADH/NAD ratios) and by minimising their expense of energy to the detriment of their differentiation. This adaptation well illustrates the *JKB*-trilogy and the stress-induced state-change concept.

Conclusion

Hyperhydric shoots (HS), at least in one hyperhydrating culture cycle, adapted themselves to survive the best possible way by preserving their redox homeostasis and by minimising their expenses of energy. The stress-induced state-change allowed to the system only a restricted differentiation. However as soon as the system reaches survivability, and sustainability, it found its optimal state for the given environmental conditions. Sub-optimality and the driving force for further state changes vanish. Economically the system may produce very little in terms of biomass per unit time (quantitative aspect), but thermodynamically in terms of vitality and stability (qualitative term) the system tends to reach the most stable state, which is the best possible constellation to reach sustainability.

The stress concept by Strasser offers a theoretical approach to describe energetically the biotechnological cycle of *in vitro* cultures such as: transfer of optimal pot plants to sterile explants, followed by micropropagation and re-adaptation to nonsterile autotrophic conditions again. In addition to the biotechnological interests, *in vitro* cultures and micropropagation can be considered as model systems for the investigation of the physiological behaviour of plants under different environmental conditions. It becomes a support for the required prediction of plant behaviour due to global changes.

Thus, whether hyperhydricity may be regarded as a pathological phenomenon in terms of stress response, from a biological point of view, the state-change has been a beneficial action allowing survival and approach of sustainability.

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