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Shemin pathway and peroxidase deficiency in a fully habituated and fully heterotrophic non-organogenic sugarbeet callus: an adaptative strategy or the consequence of modified hormonal balances and sensitivities in these cancerous cells? A review and reassessment

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Abstract. There are many arguments for considering a specific fully habituated (auxin and cytokinin-independent) and fully heterotrophic non-organogenic (HNO) sugarbeet callus cell line as terminating a neoplastic progression, and thus to be made of cancerous cells. The similarities with animal tumour and cancer cells are recalled. All types of habituated tissues examined in the literature share at least three common biochemical characteristics: low apparent peroxidase activity, high content of polyamines (PAs) and low production of ethylene. However, results concerning their auxin and cytokinin levels are not consistent. Peroxidase synthesis in the achlorophyllous HNO callus appears to arise from aminolevulinic acid (ALA) synthesis through the Shemin pathway, commonly used by animals and fungi. This pathway is limited by disturbed nitrogen metabolism that diverts glutamate (directly used for ALA synthesis in green higher plants) from the Kreb's cycle into PA synthesis. There is no argument to suggest that the low ethylene production is caused by a competition with PAs for their common precursor, *S*-adenosylmethionine. The results we report here indicate modified anabolic and catabolic pathways of auxins and cytokinins but also the possibilities of unusual compounds playing similar roles (dehydrodiconiferyl alcohol glucosides, for instance). A higher turnover of PAs is shown in the HNO callus, which could suggest a role for H_2O_2 and γ-aminobutyric acid, products or intermediates in the

Abbreviations ACC, 1-aminocyclopropane-1-carboxylic acid; AIB, α-aminoisobutyric acid; α-KG, α-ketoglutarate; ALA, aminolevulinic acid; AOA, aminooxyacetic acid; DCGs, dehydrodiconiferyl alcohol glucosides; GABA, γ-aminobutyric acid; GC-ECD, gas chromatography–electron capture detection; HNO, habituated non-organogenic; HPLC, high-performance liquid chromatography; IAA, indoleacetic acid; IAAasp, IAA aspartate; PAs, polyamines; SAM, *S*-adenosylmethionine.

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PA catabolic pathway, as secondary messengers. The habituated cells retain some sensitivity towards exogenous auxins and cytokinins. Their increased sensitivity to PAs and ethylene suggests modified hormonal balances for the control of these actively dividing cells.

FULLY HABITUATED AND FULLY HETEROTROPHIC NON-ORGANOGENIC CALLI AS PLANT CANCERS IN THE ABSENCE OF INTRODUCED PATHOGENS

The concept of primary and secondary tumours in plants is relatively well described. The occurrence of such tumours may be attributed to the systematic spread of an oncogenic pathogen such as a virus or a bacterium but the possible transfer of oncogenic potential from cell to cell in the absence of the pathogen has not been excluded (White & Braun 1942, Meins 1973, Braun 1978, Pengelly 1989).The so-called genetic tumours of interspecies hybrids do not depend on the introduction of a pathogen (Bayer 1982). The notion of plant cancers has existed (Bednar & Linsmaier-Bednar 1989, Kaiser 1989) but it had never been well characterized. Indeed the notions of migratory invasive cancer cells and malignancy as seen in animals hardly seems applicable to plant cells and organisms (Doonan & Hunt 1996). Might cancer simply occur in plants in the absence of oncogenic pathogens and might it result in whole plant death at its final stage? In the past seven years, we have reported observations and results that suggest this is indeed the case (Gaspar *et al.* 1991, 1994, 1995, Gasper 1995, 1988). These are summarized briefly below.

Habituation is the term commonly used for the acquired and hereditary capacity for autonomous growth, that is to say in the absence of exogenously supplied auxins and/or cytokinins, for tissues (most often calli) in *in vitro* cultures (Meins 1989). Habituated calli have long been classified as neoplasms similar to tumours of different origins, and are considered one of the four neoplastic diseases of plants (Braun 1978). The general opinion has been that habituation is a reversible process with an epigenetic basis. This probably is true in most cases examined. We have shown, however, that there might be several degrees of habituation which could be considered steps of a neoplastic progression leading to the onset of cancer in the absence of an introduced oncogenic pathogen. Cell rejuvenation with deficient differentiation, loss of the capacity to organize meristematic centres and the loss of totipotency are among the main characteristics used to define plant cancer through callus neoplastic progression (Table 1). A unique, very friable (reduced cellcell adhesion), fully habituated and fully heterotrophic (totally achlorophyllous) sugarbeet callus with undifferentiated (no lignin) cells having irreversibly lost organogenic totipotency has been recognized as composed of true cancerous cells. These cells present many similarities with animal tumour and cancer cells (Table 1)

Habituated tissues share many morphological and biochemical similarities with so-called vitreous shoots from micropropagation. Vitrification and hyperhydric malformations of shoots raised *in vitro* might also be considered steps of a neoplastic progression and the onset of cancer in the absence of an introduced oncogenic pathogen. With vitrification, cancer results in the death of the whole organism either directly, through necrosis of all stem and bud apices (the root regenerating capacity having been lost earlier), or indirectly from the loss of the capacity for primary meristems to function normally, leading to completely anarchic structures (Gaspar 1995).

Finally, carcinogenesis in plants, as in the animal kingdom, results from a multi-step process involving the irreversible conversion of a stem cell to a terminal differentiation

Table 1. Characteristics that constitute a fully habituated non-organogenic sugarbeet callus consisting of true cancerous cells, in the absence of introduced pathogens (according to Gaspar 1998)

Biological characteristics
Monoclonal origin
Full hormonal independence *in vitro*
High rate of cell division
Polyploidy and aneuploidy
Reduced cellcell adhesion (friability)
Susceptibility to necrosis
Morphological characteristics
Deficient cell wall differentiation
Deficient chloroplast and mitochondria differentiation
Big nuclei with irregular shape, with many nucleoli + micronuclei
Apoptotic bodies
Biochemical characteristics
A programmed cell death?
Hyperhydricity
Deficiency of tetrapyrrole-containing compounds
Permanent oxidative stress
Low ethylene production
Accumulation of polyamines
Typical plant cancer trait
Irreversible loss of organogenic totipotency, i.e. the capacity for such cells to reorganize primary organogenic meristems, at the end of a neoplastic progression.

resistant cell (Gaspar *et al.* 1991). Such a definition of plant cancer in the absence of pathogens is relatively new (see Anonymous 1995). There is no evidence to date that plant cancer results from one or more mutations or DNA rearrangements as in animals (Alberts *et al.* 1989).

PEROXIDASES AND PLANT CANCER

We have reviewed the fate and possible role of peroxidase(s) in carcinogenesis in plants and animals (Gaspar *et al.* 1992a). The literature suggests that peroxidase may play more than one role in animal and plant carcinogenesis. One difficulty in this assessment is that, in animals as in plants, the enzyme peroxidase is present in multiple isoforms in different cell compartments. In addition, this enzyme, which can use and remove different active oxygen forms (Gaspar *et al.* 1982, Greppin, Penel & Gaspar 1986), is also able to produce these (toxic) forms (Penel 1997). The problem is further complicated by the fact that peroxidases are able to catalyse a variety of different reactions, including dehydrogenation, oxidation, peroxidation, halogenation and demethylation (Gaspar *et al.* 1982, Everse, Everse & Grisham 1991). There are solid arguments for the involvement of DNA methylation processes in animal as well as in plant neoplastic progressions (Lambé *et al.* 1997).

The active participation of peroxidase in neoplastic progression and carcinogenesis (through xenobiotics, oxidative stress, lipid peroxidation and α-oxidation of fatty acids) has been discussed previously (Gaspar *et al.* 1992a). In the present review, we would like to examine the deficiency in peroxidase synthesis (through the unusual Shemin pathway in a higher plant) in the fully habituated non-organogenic (HNO) sugarbeet callus, which is considered to be composed of true cancer cells only (Table 1), relative to a possible redistribution of the roles of hormones in their growth control.

PEROXIDASE ACTIVITY AND BIOSYNTHESIS IN NORMAL AND HABITUATED CELLS, ESPECIALLY IN AN HNO SUGARBEET CALLUS

Cells from primary normal (hormone-dependent) calli, which can be considered as teratological neoformations entering a neoplastic progression towards true cancer cells (Gaspar *et al.* 1991), are always richer in peroxidase(s) than cells of tissues or organs from which they derive (Del Groso & Alicchio 1981, Konstantinova, Aksenova & Sergeeva 1982, Phan 1983, Hirsch & Fortune 1984, Berger *et al.* 1985, Lagrimini & Rothstein 1987, Bakran-Petricioli & Krsnik-Rasol 1989, Floh, Handro & Morgante 1989). Such an increase in peroxidase activity has most often been interpreted as a result of stresses resulting from tissue excision and the culture medium. Secondary calli from the subculture of the primary callus progressively show a lower peroxidase activity (Bhattacharya & Mukherjee 1983, Bourgeade *et al.* 1989, Floh *et al.* 1989). This gradual loss of activity of total peroxidase and of specific isoperoxidases corresponds to a progressive decline in organogenic capacity in long-term cultures (Negrutiu, Jacobs & Gaspar 1979, Chawla 1991). Cells from an HNO callus, at the end of their neoplastic progression, show a very low peroxidase activity and a very low capacity to secrete isoperoxidases compared to normal auxin and cytokinin-requiring cells (Kevers *et al.* 1981, 1982, 1983, Gaspar *et al.* 1983, 1988, Penel *et al.* 1984, Crèvecoeur *et al.* 1987, Hagège *et al.* 1990, 1991a, Le Dily *et al.* 1993b). A low peroxidase activity is a general characteristic of habituated calli (Bouchet, Gaspar & Thorpe 1978, Krsnik-Rasol, Jelaska & Serman 1982, Krsnik-Rasol & Jelaska 1991, Krsnik-Rasol 1991, Hrib, Vookova & Kormutak 1997). Animal hormone-independent tumours similarly have significantly lower peroxidase levels than hormone-dependent ones (Penney & Hawkins 1981). It must be noticed, however, that certain compartments of an HNO callus (purified plasma membrane, for instance) exhibit a peroxidase activity as high as that of the normal callus (Hagège *et al.* 1991d), and that the transfer of a light-cultured HNO callus to darkness increases enzyme activity (Kevers *et al.* 1995, Bernal *et al.* 1997). The latter regulation could be mediated through soluble effectors that act as potential peroxidase inhibitors and/or by differential expression of peroxidase isoenzyme patterns (Bernal *et al.* 1997). In any case, peroxidase is not the only porphyrinic compound deficient in habituated calli. Other tetrapyrrole-containing compounds such as chlorophylls (Syono & Furuya 1974, Kaminek, Hadackova & Lustinec 1981, Crèvecoeur *et al.* 1987, Bisbis *et al.* 1994), cytochromes and catalase (Hagège *et al.* 1992) are also present in low amounts in habituated tissues.

Aminolevulinic acid (ALA) is an obligatory intermediate in the biosynthesis of tetrapyrrole-containing compounds. It is synthesized through the plastidial Beale pathway from oxoglutarate and glutamate, or through the mitochondrial Shemin pathway from succinate and glycine (Bisbis *et al.* 1997c, Figure 1). The former commonly operates in algae and higher plants while the latter is generally found in many bacteria, fungi and animals. The accumulation of ALA, haems and chlorophylls, and the activities of peroxidase and catalase were compared in normal green and in achlorophyllous white HNO sugarbeet calli, in light and under darkness, in the presence of precursors of the Beale or Shemin pathways, with or without inhibitors of the Beale pathway (Bisbis *et al.* 1997b,c). The results indicated the co-existence of both pathways in normal callus, with increased participation of the Shemin pathway under conditions that reduced the Beale pathway (darkness, inhibitors). These results confirmed the few existing results in the literature about the functioning (although at a limited rate) of the Shemin pathway in higher plants. Additional results also indicated the unique functioning of the Shemin pathway in the HNO callus (Bisbis *et al.* 1998b).This

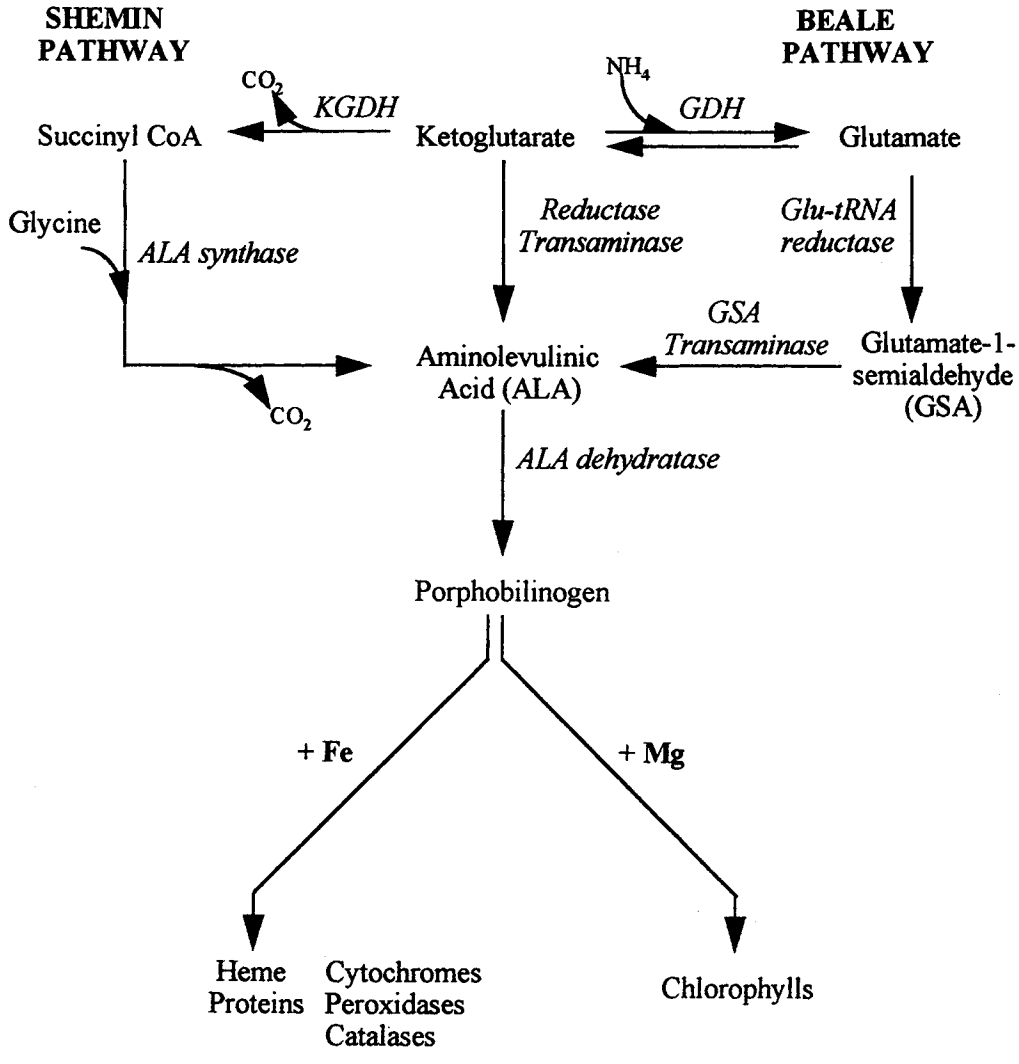

Figure 1. Beale and Shemin pathways for the biosynthesis of tetrapyrrole-containing compounds (including peroxidase) via aminolevulinic acid (see Bisbis *et al.* 1997c).

discovery confirms the ontogenetic retrogradation of these cancerous cells as very primitive cells. Furthermore, the HNO callus preferentially accumulates benzoic derivatives contrary to the normal callus, which synthesizes cinnamic derivatives (Engelmann, Macheix & Gaspar 1993). It was shown that most benzoic derivatives inhibit 5-aminolevulinate dehydratase,the enzyme which converts ALA to porphobilinogen, in contrast to most cinnamic derivatives, except ferulic and caffeic acids (Le Dily *et al.* 1993b). Thus both the disturbance in phenolic metabolism and the Shemin pathway might lead to the reduction of the porphyrin pathway and especially of haemoprotein synthesis in the HNO callus. Indeed, the HNO cells also appeared to be deficient in the α-ketoglutarate (α-KG) dehydrogenase complex that converts α-KG to succinyl-CoA and in succinyl-CoA synthetase that synthesizes succinate from succinyl-CoA. However a minimum of succinate necessary for the Shemin pathway can be

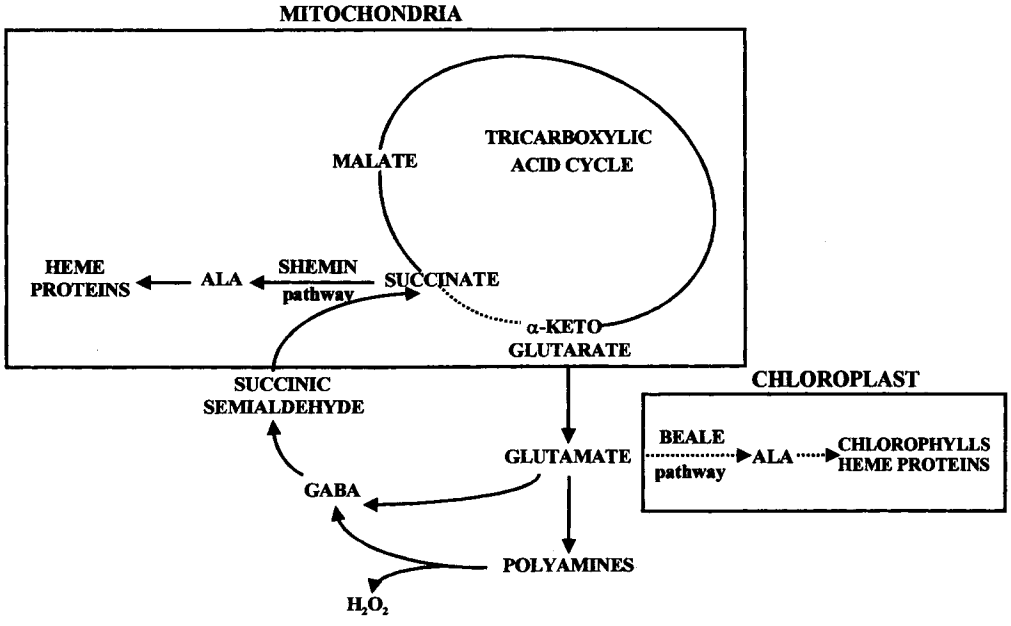

Figure 2. GABA-shunt, through glutamate and through polyamines, in the tricarboxylic acid cycle, with the indications of the Beale (from glutamate) and Shemin (from succinate) pathways of aminolevulinic acid (ALA) and porphyrin (including peroxidase) biosynthesis.

provided via a GABA (γ-aminobutyric acid) shunt (Bisbis, Kevers & Gaspar 1997a) as shown in Figure 2. As this model indicates, the Shemin pathway only supports the synthesis of heme proteins (at a limited rate), while the Beale pathway supports chlorophyll synthesis, of course when chloroplasts are present, which cannot be the case for the HNO cells.

INDOLE ACETIC ACID-OXIDASE ACTIVITIES AND INDOLE ACETIC ACID LEVELS IN HABITUATED TISSUES

Although early and some later studies have reported that habituated tissues accumulate higher amounts of auxins (Kulescha & Gautheret 1948, Kulescha 1952, Smith 1972, Tandon & Arya 1980, Wyndaele *et al.* 1988), this has never been fully confirmed (Nakajima *et al.* 1979, Everett 1981, Kutacek *et al.* 1981, Pengelly & Meins 1982, 1983, Mousdale, Fidgeon & Wilson 1985, Chen 1987, Campell & Town 1991, Campell, Su & Pengelly 1992, Szabò *et al.* 1994). A gas chromatography–electron capture detection (GC-ECD) titration of free indole acetic acid (IAA) in our normal and HNO sugarbeet calli also did not reveal any significant difference between both tissue types (Kevers *et al.* 1981). As the popular hypothesis that IAA conjugates are merely slow-release storage forms of IAA is in question (Oetiker & Aeschbacher 1997), we reinvestigated the content of free and conjugated (the aspartate form was found) IAA during the culture of the two cell lines using an high-performance liquid chromatography (HPLC) technique (Nordström & Eliasson 1991). As shown from the results presented in Figure 3, free IAA in the HNO callus was higher than in the normal callus. Except at day 7, the contrary was true for IAAasp. These results reopen the debate about discrepancies in free IAA content in habituated tissues and the role of the IAA conjugated forms. These apparent discrepancies might also arise from the different extraction and

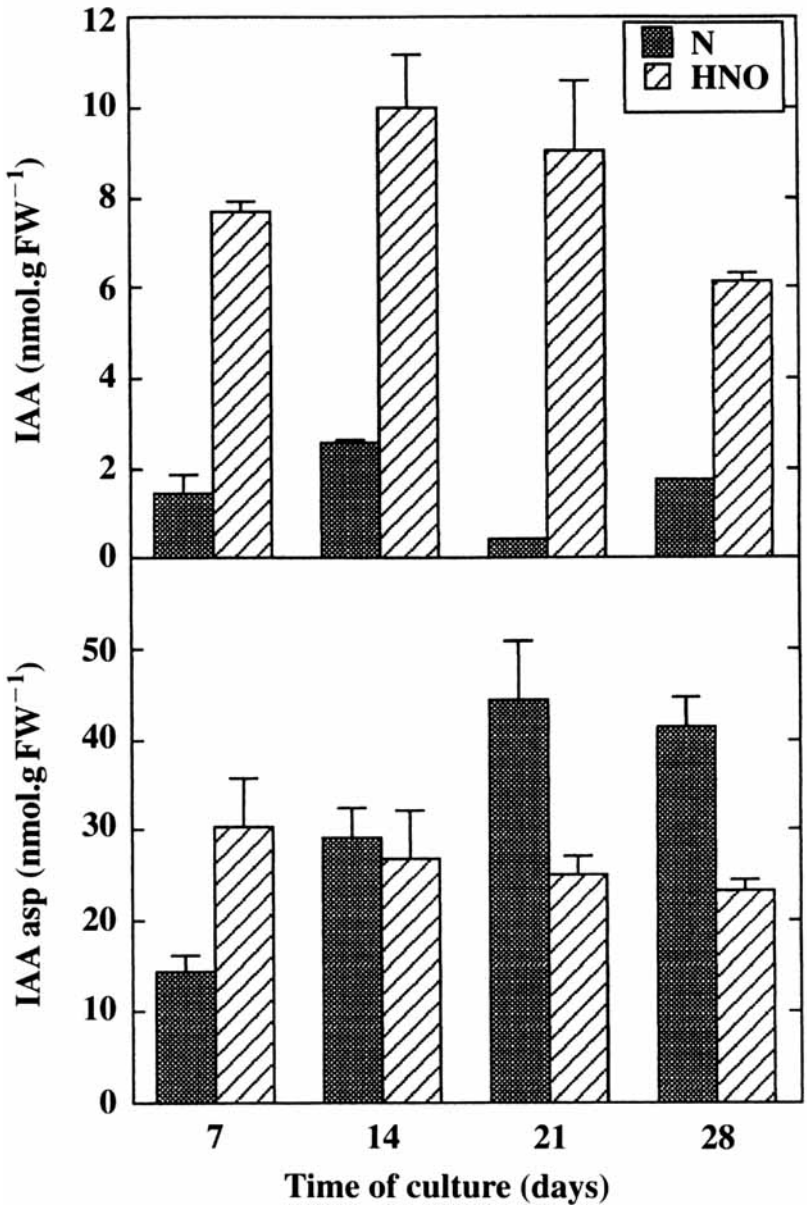

Figure 3. Content of free auxin (IAA) and conjugate (IAAasp) in normal and habituated non-organogenic sugarbeet calli (see Bisbis 1998).

purification techniques used in different studies. IAA conjugates can be effective auxins themselves or by slowly releasing IAA, and they have now been shown to be rapidly metabolized in compounds which might be biologically active (references in Oetiker & Aeschbacher 1997). On the other hand, because it has been shown that exogenous or high levels of endogenous auxins can exert different feedbacks, such as activating both IAA biosynthetic and IAA-metabolic enzymes (Oetiker & Aeschbacher 1997, Slovin 1997), the

habituation phenotype could also be due to a mutation affecting its IAA pool size. There is also the possibility of a higher turnover of free and conjugated auxins in the habituated tissues. The IAA-synthesizing capacity of habituated tissues via tryptophan transaminase has indeed been found to be higher than in heterotrophic tissues (Gaal & Köves 1981, El-Bahr, Kutacek & Opatrny 1987). The same data also do not exclude other hypotheses such as different concentrations of auxin receptors, with modified affinity for the hormone as already suggested (Mousdale *et al.* 1985, Bishop 1987, Christou 1987, Jackson & Lyndon 1990, Feutry, Poder & Hagège 1995), uncoupling from the normal hormonal controls (Campell & Town 1991) or tryptophan-independent biosynthetic pathway for IAA in auxotrophs (Wright *et al.* 1991, Normanly, Cohen & Fink 1993). At least in the case of variants with low auxin sensitivity, the friable phenotypes arose by a mechanism that was independent of changes in the auxin physiology of the cells (Campell *et al.* 1992).

A similar debate can be held about IAA-oxidases. IAA-oxidases have generally been found to have lower activities in the habituated tissues than in normal calli (Bouchet *et al.* 1978, Kevers *et al.* 1981, Szabò, Tari & Köves 1981) as in other tumorous tissues (Platt 1954, Lipetz & Galston 1959, Bouillenne & Gaspar 1970), although studies with habituated or tumorous tissues of tobacco (Weiss 1967, Kovacs & Maliga 1973) and sycamore (Lescure 1970, Maillard, Pilet & Zryd 1976) gave contradictory results. The lower IAA-oxidase activities in habituated tissues can be related to their lower peroxidase activities because peroxidases are generally considered as the enzymes mediating auxin catabolism (Gaspar *et al.* 1982, Penel, Gaspar & Greppin 1992, Pedreno *et al.* 1995). But the problem is further complicated by the presence of auxin protectors (see Stonier 1970), which are found at higher levels in normal tissues (Bouchet *et al.* 1978, Kevers *et al.* 1981), and the fact that the level of these auxin protectors can be modulated by the regulators present in the culture medium for normal tissues (Atsumi & Hayashi 1978, Syono 1979). Again, further studies on the cell compartmentation of each factor will be needed for more certainty about their involvement in the habituation process.

CYTOKININ CONTENT AND ITS RELATION TO CYTOKININ OXIDASE ACTIVITY IN NORMAL AND HNO SUGARBEET TISSUES

Habituated plant tissues, which have lost their requirement for an exogenous supply of auxin and cytokinin, should produce and maintain a similar level of cytokinins and auxins, because both hormones are required for cell division and expansion. Habituation for cytokinins was hypothesized to result from activated cytokinin biosynthesis which, according to Meins (1989), is permanent and is transferred to daughter cells once the concentration of endogenous cytokinins reaches a threshold level. It has also been proposed that the habituation state is maintained by a positive feedback loop in which cytokinins induce their own synthesis or inhibit their degradation (Hervagault, Ortoleva & Ross 1991). Indeed, some true habituated cell lines and comparable tissues (crown galls, radiation-induced tumours, genetic tumours) have higher levels of cytokinins (Miura & Miller 1969, Dyson & Hall 1972, Einset & Skoog 1973, Wyndaele *et al.* 1988, du Plessis *et al.* 1996), but some others have not (Hansen, Meins & Milani 1985, Nandi, Palni & Parker 1990, Campell & Town 1991, Kevers *et al.* 1997b) or have even lower levels (Nakajima *et al.* 1979, Scott & Horgan 1984, Meins 1989). The discrepancies may derive from the fact that endogenous cytokinins undergo dynamic changes during growth cycles that are necessarily different for normal and habituated cells (Weiler 1981, Nandi *et al.* 1990, Peters, Füchtbauer & Beck 1995). It has also

been proposed that the cytokinin metabolic pool is different in autonomous cells (Mok *et al.* 1980, Teyssendier de la Serve, Jouanneau & Péaud-Lenoël 1982). The actual concentration of physiologically active cytokinins in plant cells is influenced by the rate of their inactivation by formation of *N*-glucosides and degradation by cytokinin oxidase. Zeatin, benzyladenine (BA), isopentenyladenine and their ribosides were found in normal and HNO sugarbeet calli at very similar concentrations if we except an excess of BA and of its riboside in the normal callus as a consequence of the BA-containing-medium. However, cytokinin oxidase from the HNO callus had a higher activity than that in normal tissue (Kevers *et al.* 1997b). Because cytokinin oxidase may well act as a substrate-inducible enzyme to maintain the cytokinins at a level suitable for stimulation of cell division, as proposed by Meins (1989), the above results may indicate that the actual rate of cytokinin biosynthesis in the HNO callus is higher than that in normal callus. Such a result was explained by Le Dily *et al.* (1993a) through a putative linkage between proline synthesis, the hexose monophosphate pathway as proposed for proliferating animal tissues (Phang 1985), and the deficiency in tetrapyrrole compounds. The same results, i.e. a difference in cytokinin oxidase activity between normal and habituated tissues, and a similarity in their cytokinin content, also suggest there is a higher cytokinin turnover in habituated tissue, as was suggested for auxins.

Another explanation for the discrepancies in the cytokinin content of autonomous cell lines may come from the discovery of dehydrodiconiferyl alcohol glucosides (DCGs) (see Gaspar *et al.* 1996b), specifically from hormone-autonomous *Catharanthus roseus* crown gall tumours (Wood *et al.* 1969). DCGs have cell division promoting activities and can replace cytokinins in cytokinin-requiring tissues (Binns *et al.* 1987, Teutonico *et al.* 1991). Their accumulation is stimulated by cytokinins, so DCGs may be a component of a cytokinin-mediated regulatory circuit controlling cell division, as suggested by Teutonico *et al.* (1991). They accumulate in habituated tissues (Binns *et al.* 1987) and even in cytokinin-autonomous transformed cell lines lacking the cytokinin-synthesizing gene (Black *et al.* 1993). The latter lines express the peroxidase that synthesizes the aglycone of DCGs.

POLYAMINE LEVEL AND TURNOVER IN HABITUATED CELLS AND GROWTH RESPONSES TO POLYAMINES

Polyamines (PAs), in their free and conjugated forms (primarily the diamine putrescine), were found at particularly high levels in the HNO sugarbeet callus as compared with the normal calli (see Bisbis *et al.* 1997a, Kevers *et al.* 1997a, 1999, Table 2). They were also high in other habituated or tumour tissues (Bagni, Serafini-Fracassini & Corsini 1972, Bagni & Serafini-Fracassini 1973, Audisio, Bagni & Serafini-Fracassini 1976, Serafini-Fracassini, Bagni & Torrigiani 1980, Kulpa *et al.* 1985). Similarly, Bajaj & Rajam (1996) showed a near loss in plant regeneration capacity in long-term callus cultures of rice, concomitant with a massive accumulation of PAs (also primarily putrescine). Animal tissues under neoplastic progression also accumulate more PAs than normal tissues (Russel 1973, Porciani *et al.* 1993, Nishioka 1996, Seiler & Moulinoux 1996).

The relationship between PAs, the Shemin pathway, and peroxidase in the HNO callus has been emphasized through two series of observations. One series was the elucidation of the pathway of PA accumulation as the result of three disturbed metabolic pathways acting cooperatively (Kevers *et al.* 1997a): firstly, a deviation of nitrogen metabolism leading to glutamate and proline accumulation that might reflect an ammonia detoxification; secondly, a favoured pentose phosphate pathway that provides the NADH surplus needed for the reduction

Table 2. Putrescine, spermidine, spermine and total free and conjugated (soluble and non-soluble) polyamine levels (nM. g^{-1} fresh weight) in normal and habituated non-organogenic sugarbeet calli after 14 days of culture (Kevers *et al.* 1999)

	Normal			HNO		
	Free	Soluble conjugated	Non-soluble conjugated	Free	Soluble conjugated	Non-soluble conjugated
Putrescine	49 ± 5	66 ± 8	74 ± 10	149 ± 13	118 ± 10	321 ± 27
Spermidine	12 ± 3	17 ± 3	22 ± 4	101 ± 8	76 ± 9	164 ± 15
Spermine	1 ± 1	0	2 ± 1	5 ± 2	4 ± 1	8 ± 1
Total	62 ± 9	83 ± 11	98 ± 15	255 ± 23	198 ± 20	493 ± 43
General total			243 ± 35			946 ± 86

Mean ± SE.

of nitrate to nitrite; and thirdly, a larger non-photosynthetic CO_2 fixation replenishing the Kreb's cycle with oxaloacetic and malic acids (Bisbis *et al.* 1995). All three metabolic pathways, as shown in Figure 4, favour the glutamate-proline pathway cycle which provides the ornithine surplus for PA synthesis. This deviation explains the abnormal Kreb's cycle with deficient α-ketoglutarate dehydrogenase between α-ketoglutarate and the succinate necessary for the Shemin pathway (see *Peroxidase activity and biosynthesis in normal and habituated cells, especially in HNO sugarbeet callus* and Figure 1). A second series of recent results (Kevers *et al.* 1999) demonstrated a particularly high turnover of putrescine in the HNO callus, compared to the normal one, as in animal neoplastic cells (Auvinen *et al.* 1992 and references therein). These results support the functioning of the GABA-shunt from PAs (particularly putrescine) to succinate as proposed by Bisbis *et al.* (1997a) (Figure 2). This pathway allows a minimal provision of succinate, a precursor of peroxidase in the Shemin pathway, through a short-circuit. The GABA-shunt must be questioned in two other respects. The hydrogen peroxide formed through PA degradation may serve peroxidase reactions but it may also inactivate them (Penel 1997). Hydrogen peroxide may also serve as a second messenger (Penel 1997) along with GABA (Ramputh & Brown 1996) in the regulation of gene expression. An earlier model (Figure 5) from Le Dily *et al.* (1993b) has shown how PAs, cytokinin and auxin metabolism might be connected with the deficiency in peroxidase, and possible hydrogen peroxide accumulation.

Exogenous PAs as well as inhibitors of their biosynthesis applied to the HNO callus modified both the endogenous PA level and growth (Table 3), demonstrating a great sensitivity of these habituated cells to this category of regulators.

DIFFERENTIAL GROWTH DEPENDENCY OF NORMAL AND HNO SUGARBEET CELL LINES UPON ENDOGENOUS ETHYLENE PRODUCTION AND EXOGENOUS ETHYLENE APPLICATION

The HNO sugarbeet callus emits and retains much lower ethylene quantities than the normal callus (Hagège, Kevers & Gaspar 1991b, Bisbis *et al.* 1998a, Kevers *et al.* 1985, Gaspar *et al.* 1988). A low rate of ethylene production may well be a general characteristic of habituated cell lines since it has also been observed in habituated tobacco (Köves & Szabò 1987, Szabò, Köves & Somogyi 1994) and periwinckle (J. Crèche, University of Tours, France, personal communication), and in hormone-autonomous radiation-induced tumours of *Arabidopsis*

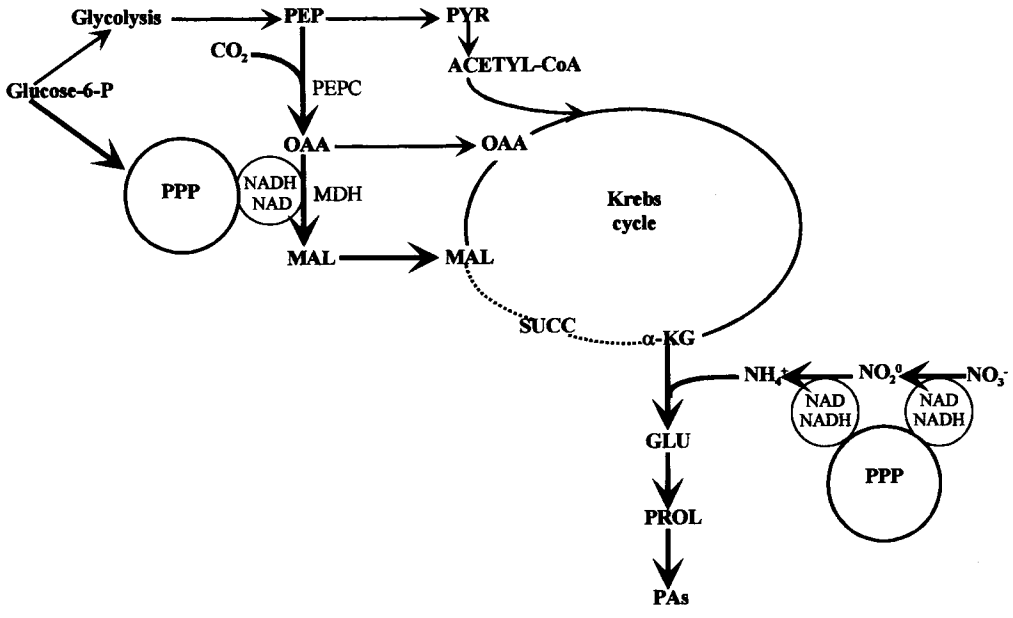

Figure 4. Scheme showing the deviated nitrogen metabolism leading to polyamine (PA) accumulation and its relationship with the privileged respiratory pathway of the habituated non-organogenic cells and their non-photosynthetic CO_2 fixation. PPP, hexose monophosphate and pentose phosphate pathway.

thaliana (Campell & Town 1991). 'Does ethylene play a role in the habituation' was the question posed by Köves & Szabò (1987). That low ethylene production resulted from the habituation process rather than causing it was examined by Bisbis *et al.* (1998a). It is difficult to provide a definitive answer. First of all, it is difficult to credit the low level of ethylene production by habituated cells to the absence of auxins and cytokinins in the culture media, even if these growth regulators are known to modulate ethylene biosynthesis (Balague & Pech 1985, Gaspar *et al.* 1989, Yahia *et al.* 1998). No treatment could enhance the ethylene production of the HNO callus to the level of the normal callus (Bisbis *et al.* 1998a). Szabò *et al.* (1994) did show this, but the difference between the tissue used in these two studies was that the former had reached an irreversible state (Gaspar *et al.* 1991, see part 1). In both cases, however, low ethylene production was related to deficient cell wall differentiation (see Kevers *et al.* 1984). Low ethylene production, resulting from a retro-inhibition of synthesis, may be responsible for hypolignification of vitreous tissues (Kevers *et al.* 1984).The HNO callus can be considered vitreous because it is hyperhydric (Crèvecoeur *et al.* 1987) and because it contains no lignin (Hagège *et al.* 1991a).

Accumulation of PAs is another characteristic of habituated and other neoplasic tissues (see *Polyamine level and turnover in habituated cells and growth responses to polyamines* above). It is known that spermidine and spermine are synthesized from *S*-adenosyl-methionine (SAM), which is also a precursor of ethylene (Figure 6). A direct relationship based on competition for SAM between PA and ethylene pathways is possible (Even-Chen, Mattoo & Goren 1982). This competition probably does not occur in the HNO callus (Biondi *et al.* 1993). However, the PAs should be able to directly inhibit ethylene synthesis, since they act as free radical scavengers and because superoxide radicals are needed for the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Apelbaum *et al.* 1981). In any

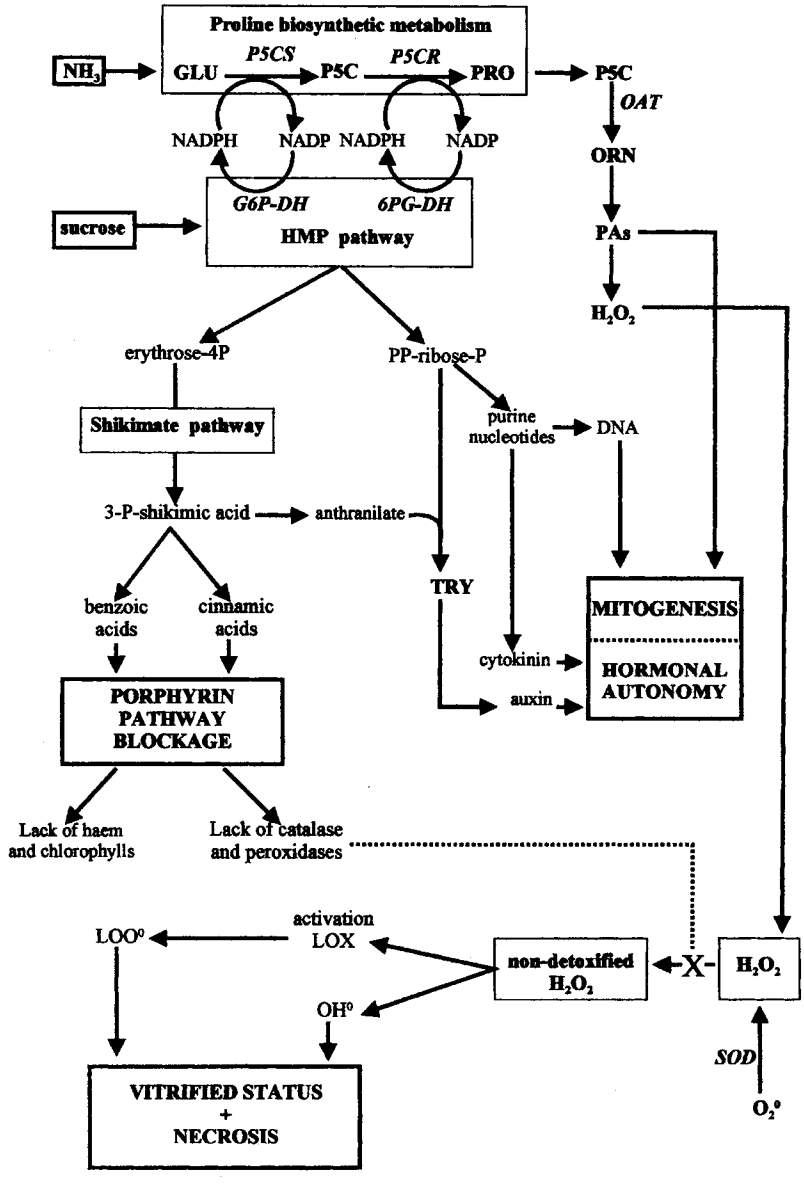

Figure 5. Relationships between porphyrin blockage and disturbed nitrogen and sugar metabolisms in the H callus of *Beta vulgaris*. Hypothetical metabolic explanation of hormonal autonomy and the hyperhydric (vitrified) status leading to necrosis through non-detoxification of H_2O_2. 6PG-DH, 6-phosphogluconate dehydrogenase; G6P-DH, glucose 6-phosphate dehydrogenase; GLU, glutamate; HMP, hexose monophosphate; OAT, ornithine aminotransferase; ORN, ornithine; P_5C, pyrroline 5-carboxylate; P_5CR, pyrroline 5-carboxylate reductase; P_5CS, pyrroline 5-carboxylate synthase; PA, polyamines; PP-ribose-P,5-phosphoribosyl 1-pyrophosphate; PRO, proline; TRY, tryptophan.

Table 3. Effects of polyamines and polyamine biosynthesis inhibitors applied during the 14 days of culture on growth index and on total free polyamines of habituated non-organogenic callus (Kevers *et al.* 1999)

	Growth index (% fresh weight)		Polyamine content (nM/g fresh weight)	
	7 days	14 days	7 days	14 days
Control	41.4 ± 4.1	126.0 ± 13.1	245 ± 41	255 ± 52
PUT (10^{-6} M)	37.5 ± 6.2	152.2 ± 20.2	138 ± 16	136 ± 9
PUT (10^{-5} M)	32.8 ± 3.5	48.5 ± 5.1	91 ± 12	134 ± 60
SPD (10^{-6} M)	47.3 ± 6.8	176.9 ± 26.5	124 ± 31	130 ± 17
SPD (10^{-5} M)	47.0 ± 2.4	59.8 ± 3.0	169 ± 21	114 ± 11
DFMA (10^{-6} M)	48.6 ± 5.6	162.2 ± 14.6	168 ± 48	153 ± 23
DFMO (10^{-6} M)	33.7 ± 4.0	192.3 ± 16.9	95 ± 14	87 ± 7
DFMO + DFMA (10^{-6} M)	32.9 ± 4.6	155.1 ± 10.9	22 ± 3	32 ± 5
MGBG (10^{-5} M)	33.3 ± 2.7	53.3 ± 4.3	106 ± 54	98 ± 26
CHA (10^{-5} M)	43.3 ± 3.9	138.0 ± 15.4	178 ± 45	142 ± 12
MGBG + CHA (10^{-5} M)	43.3 ± 3.9	138.0 ± 15.4	178 ± 45	142 ± 12

PUT, putrescine; SPD, spermidine; DFMA, difluoromethylarginine; DFMO, difluoromethylornithine; MGBG, methylglyoxal-bis-guanylhydrazone; CHA, cyclohexylamine. Mean ± SE.

case, this indicates that ethylene metabolism is probably not independent of PA metabolism, as already concluded above for the other hormones.

There is an automatic assumption that the measured release of ethylene by a tissue reflects the relative amount of active ethylene in that tissue, without considering the activity of the endogenously retained ethylene. The normal and HNO calli examined here, which produce much and little ethylene, respectively, equally retain relatively much and little ethylene, but there is no direct relationship between the amount of released and retained ethylene. This may be a result of phase displacements in sampling the calli for analysis. Unpublished data on other tissues have shown inverse diurnal rhythms of ethylene emission and retention, at least in healthy tissue. A second difficulty in investigating the growth role of ethylene is its exogenous action once emitted, particularly in a confined atmosphere, as is the case in closed tissue culture flasks (Kevers *et al.* 1992a). One must also be aware that ethylene may either autocatalytically enhance its own production and action (Bleecker & Schaller 1996) or retroinhibit them (see Kevers *et al.* 1984). A third difficulty in investigating the role of ethylene in growth is in comparing two different materials when one, the HNO callus in the present case, comprises a quite different population of small and undifferentiated cells (Crèvecoeur *et al.* 1987, 1992, Hagège *et al.* 1991c), and when, moreover, these cells are surrounded by a layer of water because they are hyperhydric like vitrified tissues (Crèvecoeur *et al.* 1987, Gaspar *et al.* 1992b). Such a surrounding layer of water may be an obstacle for the diffusion of ethylene from inside to outside and *vice versa*. This is well illustrated by the quite different reaction of the HNO callus towards ACC (soluble in the water layer) and exogenously applied ethylene, which apparently diffuses with difficulty across the water layer (Bisbis *et al.* 1998a). Results from Table 4 indicate growth dependency of both the normal and HNO calli upon the level of their endogenously biosynthesized ethylene, based on growth reactions to inhibitors of ethylene biosynthesis and ethylene action, to ACC, and to transfer from light to darkness; the dependency is also upon environmentally retained ethylene, based on growth reactions to exogenously applied ethylene and to trapped ethylene. We have noticed, however, that the growth reactions of normal callus to certain additives

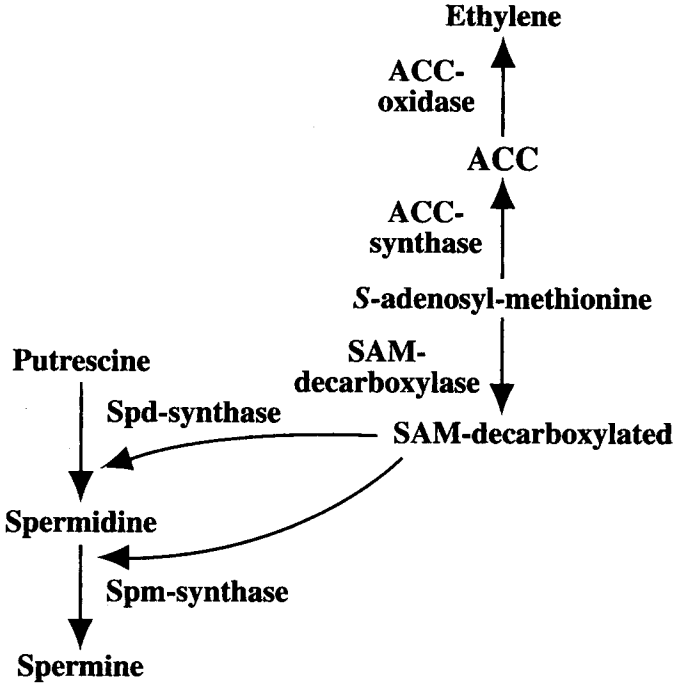

Figure 6. Biosynthetic pathway of ethylene and its relationship with polyamines (SAM, *S*-adenosyl-methionine; Spd, spermidine; Spm, spermine).

Table 4. Effects of inhibitors of ethylene synthesis (AOA and AIB), ethylene action (NRD and STS), ethylene precursor (ACC), exogenously applied ethylene, ethylene trapper and darkness on ethylene production and growth of the normal and habituated non-organogenic sugarbeet calli

	Ethylene production (% of the control)		Ethylene production (% of the control)	
	N	HNO	N	HNO
Control	100	100	100	100
AOA (100 μM)	8	65	50	55
AIB (100 μM)	33	32	104	91
NRD (4 mM)	28	4	72	11
STS (50 μM)	15	76	25	40
ACC (100 μM)	113	181	183	62
Ethylene (1 p.p.m.)	44	500	37	26
Ethylene (20 p.p.m.)	33	242	30	38
Ethylene trapper	10	461	20	28
Darkness	35	43	76	60

Values are means of at least three separate repetitions. The absolute values of the control for the ethylene production are 134.7 ± 9.8 μl. g fresh weight^{-1}. 24 h^{-1} for normal callus and 2.6 ± 0.4 for the HNO callus. For growth index, the absolute values are 261% ± 21.3 for the normal callus and 162% ± 31.8 for the HNO callus (Bisbis *et al.* 1998a). AOA, aminooxyacetic acid; AIB, α-aminoisobutyric acid; NRD, 2,5-norbornadiene; STS, silver thiosulphate; ACC, 1-aminocyclo-propane-1-carboxylic acid. Mean ± SE.

(aminooxyacetic acid (AOA) and α-aminoisobutyric acid (AIB), for instance) were not always proportional to their inhibiting effect on ethylene biosynthesis. In the same normal callus, we have even shown reduced ethylene production through an auxin-induced growth enhancement (Carrié *et al.* 1992). There have been many papers investigating relationships between callus growth and the rate of ethylene production (Huxter, Reid & Thorpe 1979, Lieberman, Wang & Owen 1979, Martin-Louçao & Rodriguez-Barrueco 1983, Köves & Szabò 1987, Mensuali Sodi, Panizza & Tognoni 1989, Vain, Flament & Soudain 1989, Hagège *et al.* 1991b, Kepczynski, McKersie & Brown 1992, Szabò *et al.* 1994), and no clearcut conclusion could be drawn. The general assessment, however, was that some of the endogenous ethylene was active as a growth regulator, although much was simply produced as a consequence of rapid growth (Huxter, Thorpe & Reid 1981). Taking into account the above considerations, the results of Bisbis *et al.* (1998a) (Table 4) also clearly demonstrated a response of the HNO callus towards exogenously applied ethylene. The callus responded to ethylene application by increasing its own ethylene production and growth was severely reduced. Bolton & Freebairn (1975) have already shown the growth reactions of habituated tissues towards exogenously applied ethylene.

The relationship of low ethylene production in the HNO callus with its low peroxidase activity and with the Shemin pathway is not directly evident, although the direct participation of peroxidases in the conversion of ACC to ethylene has been proposed (see Kevers *et al.* 1984, 1992b). However, an indirect action through control of the higher IAA level is plausible, as IAA has been shown to influence ACC synthase in the ethylene biosynthetic pathway.

HABITUATION: MODIFIED HORMONAL BALANCES AND SENSITIVITIES IN THE CANCEROUS CELLS

The first aim of the present review was to reiterate the neoplastic aspects of the habituated tissues, including the many morphological and biochemical similarities with cancerous animal tissues. The second aim was a reappraisal of the levels of the hormones, auxin and cytokinin in the cancerous plant cells. Not all types of hormone-autonomous tissues may be assumed to accumulate these hormones but a preponderance of the data in the literature indicates modified turnover with altered anabolism and/or catabolism, and alternative possibilities of hormonal control of auxinic and cytokininic types through auxin-conjugates and DCGs, for instance. Habituation of plant cells does not mean insensitivity to plant growth regulators of the auxin and cytokinin types (Kevers *et al.* 1996) Not only growth, but also protein expression patterns and secondary metabolism, can be modified by the application of auxins and cytokinins (Mérillon *et al.* 1989, 1995, Tacchini *et al.* 1995, Yahia *et al.* 1998). Another objective of the present review was to focus attention on the modified metabolism of PAs and ethylene in habituated tissues, especially on their involvement in the growth regulation of these tissues. Does this suggest that the hierarchy of hormones in growth control mechanisms is altered in habituated tissues? PAs could be expected to be important in actively dividing habituated cells since these regulators, like IAA and cytokinins, are involved in the control of the cell cycle and mitotic activities (McCann, Pegg & Sjoerdsma 1987, Del Duca *et al.* 1993). In developmental processes, such as rooting and flowering (Gaspar *et al.* 1996a, Gaspar, Penel & Greppin 1997), where new cell divisions have to be reinitiated, auxins and PAs have been shown to be indissociable effectors. The present review progresses further with data allowing the hypothesis that the metabolisms of auxins, cytokinins, PAs and ethylene might be all interdependent. The Shemin pathway may well link these four modified metabolic

pathways, the consequence being a paucity in peroxidase(s) in the fully heterotrophic and habituated cell line examined here

The conclusion may simply be the summary of the recent paper *Oncogenic alterations of metabolism* written by Dang & Semenza (1999) for animal cancerous cells: 'Over seven decades ago, classical biochemical studies showed that tumours have altered metabolic profiles and display high rates of glucose uptake and glycolysis. Although these metabolic changes are not the fundamental defects that cause cancer, they might confer a common advantage on many different types of cancers, which allows the cells to survive (and invade). Recent molecular studies have revealed that several of the multiple genetic alterations that cause tumour development directly affect glycolysis, the cellular response to hypoxia (and the ability of tumour cells to recruit new blood vessels)'. In view of the evidence presented above, there is little to be added to this summary at present.

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