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Research article

Helianthus tuberosus and polyamine research: Past and recent applications of a classical growth model

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ABSTRACT

The earliest studies concerning polyamines (PAs) in plants were performed by using *in vitro* cultured explants of *Helianthus tuberosus* dormant tuber. This parenchyma tissue was particularly useful due to its susceptibility to several growth substances, including PAs. During tuber dormancy, PA levels are too low to sustain cell division; thus *Helianthus* represents a natural PA-deficient model. When cultivated *in vitro* in the presence of auxins, *Helianthus* tuber dormant parenchyma cells at the G_0 stage start to divide synchronously acquiring meristematic characteristics. The requirement for auxins to induce cell division can be substituted by aliphatic PAs such as putrescine, spermidine or spermine. Cylinders or slices of explanted homogeneous tuber parenchyma were cultured in liquid medium for short-term studies on the cell cycle, or on solid agar medium for long-term experiments. Morphological and physiological modifications of synchronously dividing cells were studied during the different phases of the cell cycle in relation to PAs biosynthesis and oxidation. Long-term experiments led to the identification of the PAs as plant growth regulators, as the sole nitrogen source, as tuber storage substances and as essential factors for morphogenetic processes and cell homeostasis. More recently this system was used to study the effects on plant cell proliferation of platinum- or palladium-derived drugs (cisplatin and platinum or palladium bi-substituted spermine) that are used in human cancer cell lines as antiproliferative and cytotoxic agents. Cisplatin was the most active both in cell proliferation inhibition and on PA metabolism. Similar experiments were performed using three agmatine analogous. Different effects of these compounds were observed on cell proliferation, free PA levels and enzyme activities, leading to a hypothesis of a correlation between their chemical structure and the agmatine metabolism in plants.

1. The *Helianthus tuberosus* tuber model system

H. tuberosus plant, an Angiosperm of the Compositae family, originates from North America and is characterized by the production of tubers containing inulin as main storage substance. These tubers were used for many studies on morphology, function and polyamine metabolism in Nello Bagni's laboratory, on account of their physiological characteristics during winter dormancy and dormancy breaking [8,15]. The research was initiated in 1965 and continued for forty-six years.

1.1. Tuber formation

In a temperate climate of the northern hemisphere, vegetative shoot growth occurs during the summer months and the tubers begin to grow in September. At first cell division occurs in subterranean stem branches for a short period during which the biosynthesis of putrescine (Put), via ornithine decarboxylase (ODC, EC 4.1.1.17), is active (Fig. 1A). Successively, cell enlargement takes place for a longer time, when the amount of PAs, and the activities of arginine decarboxylase (ADC, EC 4.1.1.19) and S-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50), increase dramatically [3]. In this period flowering also occurs, giving rise to inflorescences

Abbreviations: ADC, arginine decarboxylase; ARGase, arginase; AO-Agm, *N*-(3-aminooxypropyl)-guanidine; CAN, canaline; CAV, canavanine; Cad, cadaverine; CHA or DCHA, dicyclohexylamine; cDDP, cisplatin; DAO, diamine oxidase; Dap, diaminopropane; 2,4-D, 2,4-dichlorophenoxyacetic acid; DFMA, α-difluoromethylarginine; DFMO, α-difluoromethylornithine; GAPA, *N*-(3-aminopropoxy) guanidine; IAA, indol-3-acetic acid; MGBG, methylglyoxal *bis*-guanylhydrazone; NGPG, *N*-(3-guanidino-propoxy)guanidine; ODC, ornithine decarboxylase; PAO, polyamine oxidase; PAs, polyamines; Put, putrescine; SAMDC, S-adenosylmethionine decarboxylase; Spd, spermidine; Spm, spermine; TGase, transglutaminase.

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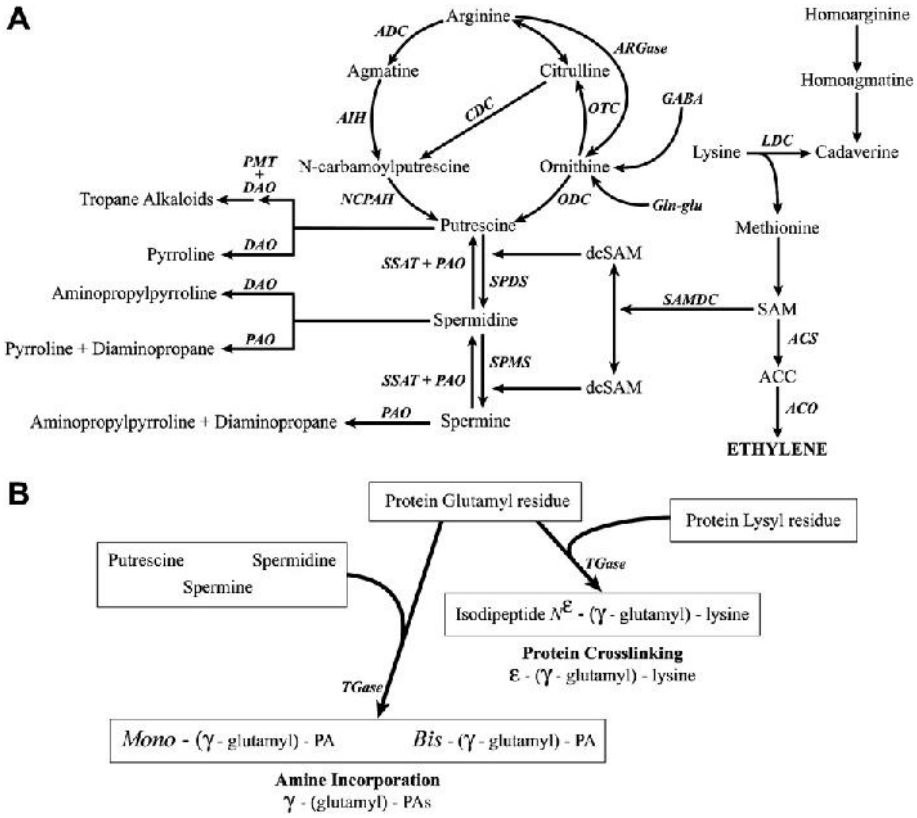

Fig. 1. Polyamine biosynthetic and catabolic pathways in plants (A). Abbreviations: ACC, aminocyclopropane-carboxylic acid; ACO, aminocyclopropane-carboxylic acid oxidase; ACS, aminocyclopropane-carboxylic acid synthase; ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; ARGase, arginase; CDC, citrulline decarboxylase; DAO, diamine oxidase; dcSAM, decarboxylated S-adenosylmethionine; GABA, γ-aminobutyric acid; LDC, lysine decarboxylase; NCPAH, N-carbamoylputrescine amidohydrolase; ODC, ornithine decarboxylase; OTC, ornithine transcarbamoylase; PAO, polyamine oxidase; PMT, putrescine methyltransferase; SAMDC, S-adenosylmethionine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase; SSAT, spermidine/spermine aminopropyltransferase. (B) Transglutaminase (TGase) catalyzed protein cross-linking between glutamyl- and lysyl-residues or PAs to produce *mono*-γ-glutamyl or *bis*-γ-glutamyl-PA derivatives.

of relatively small yellow daisies (Fig. 2A) at the apex of the stems which can be up to 3 m high. This causes the translocation of nutrients and storage substances into the flowers with a consequent transient stop of the tuber growth. At the end of November to early December, after accumulating several aminic and glucosidic substances, tubers enter into dormancy, concomitant with a decrease of hormone and PA levels. Depending on the climatic conditions, the dormancy period can last 2–3 months, during which many primary metabolic processes, such as respiration and protein synthesis, are relatively low.

1.2. Tuber dormancy and dormancy release and break

The dormant cells of the tuber medullary parenchyma are morphologically homogeneous and can be considered to be in the G_0 phase (Fig. 2B-a). They are characterized by a thin layer of cytoplasm which includes a heavily-stainable nucleus with small nucleoli, adherent to the thin cell wall, and a large vacuole [28]. After *in situ* cytometry of the Feulgen-stained nuclei and flow cytometry of the DAPI-stained nuclei [16], this hexaploid plant showed a different nuclear DNA content, as frequently occurs in storage parenchyma. Their non-green plastids, contain the tubular-like complexes [28]. These parenchyma cells have very low levels of several growth substances and a considerable amount of growth inhibitors [6]; PAs are also insufficient to sustain growth (Fig. 3). Endoplasmic reticulum and polysomes are scarce; the latter being unable to incorporate amino acids in a cell-free extract if exogenous PAs are not supplied [19]. Many biosynthetic enzyme activities [72] as well as diamine oxidase (DAO, EC 1.4.3.22) [74] and transglutaminase (TGase, EC 2.3.2.13) [59] (Fig. 1B), are low or practically absent, while in contrast arginase (ARGase, EC 3.5.3.1) [72] is active (Fig. 1A). Arginine and glutamine continue to be accumulated throughout dormancy. Tuber dormancy is a period of slow metabolism which ends, from a physiological point of view, when the utilization of reserve substances begins (mid/end of February) [6,60]. The parenchyma cells are in fact programmed to become totally depleted and die when the translocation of nutrients to the growing roots and sprouts is completed. In March storage substances decrease in conjunction with a sudden rise of free PAs.

In the laboratory, dormant tubers are maintained at 4 °C, under humid sand, in darkness and in CO_2 rich air. The natural programmed cell death can be reversed and dormancy can be broken by excising tuber-parenchyma explants and growing them *in vitro* [15] on culture media supplemented with hormones and PAs, together with mineral salts and sugars. When excised, the explants are subjected to many different environmental factors: increase of O_2 partial pressure, increase of temperature, decrease of CO_2 partial pressure, exposure to light, wounding of peripheral cells, change of mechanical pressure in internal cells, increase of water, hormones and nutrients availability and exposure to a different pH. Consequently, the cells abruptly change their fate and function allowing the identification of the metabolic and morphological parameters involved in the resumption of growth.

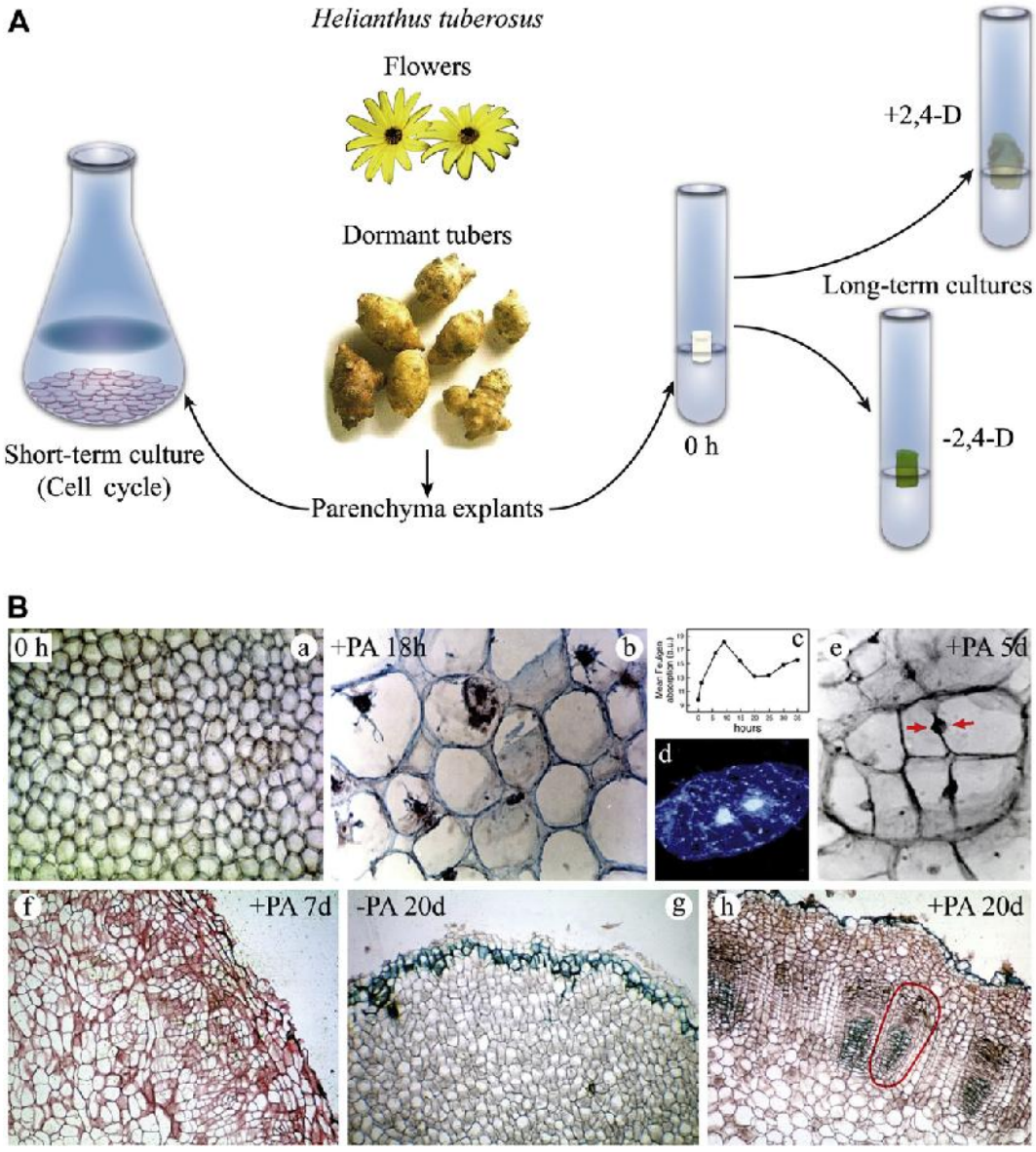

Fig. 2. (A) *Helianthus tuberosus* dormant tubers *in vitro* cultures. Short-term cultures: explants (slices of 0.9 × 0.1 cm) of medullary parenchyma cultured in liquid medium in the presence of 10 μM 2,4-D. Long-term cultures: cylinders (0.3 × 0.5 cm) of medullary parenchyma cultured in solid agar medium without or with 10 μM 2,4-D. (B) Effect of PAs on the growth of *H. tuberosus* dormant tuber explants activated with 0.1 μM Spm. Homogeneous medullary parenchyma at 0 h (a); dividing cells after 18 h from activation (b); DNA content of cultured parenchyma cells during the first synchronous cell cycle (from [16]) (c); DAPI staining during cell division soon before cytokinesis: the two newly formed nuclei and several organelles are still in the same cell (from [16]) (d); after 5 d of activation a parenchyma mother cell has produced 7 cells with the nuclei (arrows) adherent to the newly formed walls (e); adventitious cambium and cork layers formed after 7 d of treatment with 0.1 μM Spm (f); untreated explants after 20 d of culture: only a few cork layers protect the undivided parenchyma (g); Spm-treated (0.1 μM) explants after 20 d of culture: eustele collateral bundles differentiated (oval) beneath the cork layer and the parenchyma cortex (h).

1.3. *In vitro* cultures

Cylinders or slices of explanted homogeneous tuber parenchyma can be cultured in liquid medium, for short-term studies on the cell cycle, or on solid agar medium, for long-term experiments (Fig. 2A). When in contact with the medium, the cells begin to divide synchronously and after 20 days the explants are fully differentiated showing a significant increment in size (Fig. 2B).

1.3.1. Long-term cultures

The *H. tuberosus in vitro* system (Fig. 2A) provided the first demonstration in plants that PAs act as growth substances. In fact the fresh weight increment of tuber explants cultured in a culture medium containing Put, spermidine (Spd), spermine (Spm) or cadaverine (Cad), was similar to that of explants grown with indole-3-acetic acid (IAA) or natural plant extracts [1,9,15]. In addition, explants synthesised PAs very rapidly when stimulated by hormones [2]. These findings led to the initiation of the research on PAs as plant growth regulators, including studies on PAs as sole nitrogen source that showed for the first time the presence of a reverse-pathway with the formation of Put from Spd and of Spd from Spm [4,10]. These data were only recently confirmed by several other authors. PAs were also found to be prerequisite for the onset of the cell cycle [6], tuber storage substances [3], essential factors for morphogenic processes [13] and for cell homeostasis [7].

Within one day of culture, some internal cells of the explant, located in a ring 4–5 cell layers beneath the surface, start to divide with the repeated formation of internal cell walls, thus becoming

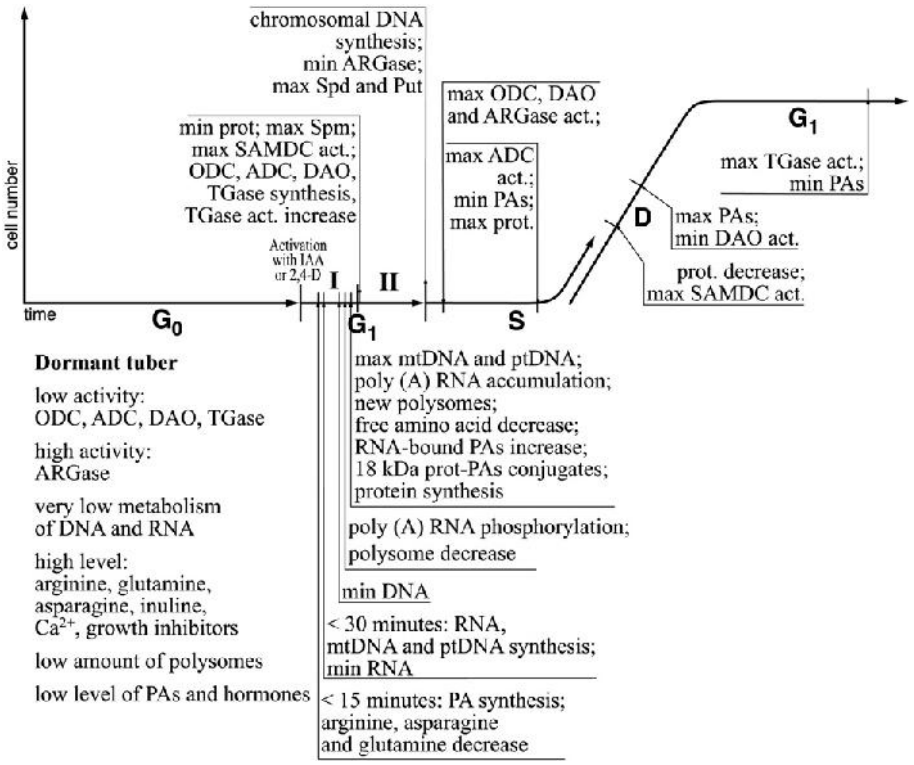

Fig. 3. Metabolism of PAs and nucleic acids during the 24 h of the first synchronous cell cycle of *Helianthus tuberosus* tuber explants activated *in vitro* with IAA or 2,4-D. G_1 is subdivided into two periods: the first is mainly related to the recovery from dormancy break and wounding, the second to the acquisition of new meristematic functions. G_2 is essentially missing. Abbreviations: act: activity; D: cell division; max: maximum; min: minimum; prot: protein. For other abbreviations see Fig. 1.

smaller and smaller [55,75] (Fig. 2B-b). Cell divisions proceed synchronously for two to three cycles (Fig. 2B-c–e). Subsequently, some of the resulting cells begin to differentiate into xylem and phloem and the walls of the superficial cells of the explant start to suberise (Fig. 2B-f). At the end of the culture period, the explants, when treated with PAs or IAA, present the typical eustelic anatomy of the intact tuber (Fig. 2B-h), whereas the untreated explants only differentiate cork layers (Fig. 2B-g). This observation supports the hypothesis that these treatments might activate the same differentiation program even though PAs, in contrast to IAA, are, in this plant system, mainly active in cell division rather than in cell extension [55]. In pea shoots PAs seemed also to be associated with cell elongation [63].

Treatment of these explants with inhibitors of PA biosynthesis, such as canavanine (CAV), canaline (CAN), α-difluoromethylornithine (DFMO) and α-difluoromethylarginine (DFMA), have confirmed the PA requirement for cell growth and differentiation [10,12].

1.3.2. H. tuberosus naturally synchronous cell cycle

Cell division is a very complex phenomena not yet completely understood. It is therefore necessary to dissect cell division into periods having predominant characteristics that can be analyzed separately. In addition it is useful to subdivide the cell itself into compartments since each of them is a microcosm with its own particular behaviour during the cell cycle.

Growth on medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) activates cell division. Tuber slices of *H. tuberosus*, during the first cell cycle after activation, are a good representation of a multicellular system of homogeneous cells dividing synchronously [56]. This model of a synchronous cell cycle was at first studied by Yeoman and co-workers [75], and successively reconsidered in connection with PAs, nucleic acids and proteins (Fig. 3).

The cell cycle duration is of about 30 h when utilizing slices of tuber parenchyma (diameter 0.9 cm, thickness 0.1 cm) grown in liquid medium supplied with 10 μM 2,4-D. In smaller slices the cell cycle is more rapid. The G_1 phase is rather long (12 h) and can be subdivided into two parts: the first (from 0 to 6 h) is mainly a wound response due to excision and transfer of the explant into *in vitro* culture. The cells are also acquiring new characteristics linked to their new meristematic state, that are fully expressed in the second part of G_1 phase (from 6 to 12 h) [56]. This first "two-period G_1" hypothesis is also supported by the fact that subsequent cycles have a shorter G_1 phase.

The sudden switch from a state of slow metabolic activity during dormancy to a more active one, characterized by the induction of the first cell cycle, is evident from the appearance of morphological and physiological modifications.

1.3.2.1. Morphological modifications. After 3 h of *in vitro* cultures, the nuclei already begin to enlarge and to move toward the centre of the cell; the nucleoli also enlarge and fuse together before nuclear division. In a few cells, probably those having a nurse function for the adjacent dividing cells, nucleoli are occasionally extruded [28,55]. After about 20 h an increasing number of cells contain two nuclei that stain less densely, with several nucleoli; the nuclei are separated by a thin cell wall that grows centripetally [55]. Although mitoses have been observed [56,75], it was shown, by using colchicine, that during the first cycle the nuclei divide prevalently amitotically (Fig. 2B-c, d), while in the subsequent cycles, normal mitoses take place [55]. The first cell divisions last for 27–30 h, as confirmed by autoradiographic studies showing that the S phase persists from 12 to 24–27 h. The G_2 phase is virtually non-existent [55,56]. The length of the cell cycle is constant when the cells are excised during deep dormancy, otherwise G_1 can be longer, probably because the programmed

cell death is starting and the recovery of meristematic capacity is more difficult [75].

1.3.2.2. Physiological modifications. The main metabolic features occurring in dormant tubers and in activated slices during the first cell cycle are summarized in Fig. 3.

1.3.2.2.1. The G_1 phase. PA biosynthesis is probably one of the earliest metabolic events induced by parenchyma activation, since an increase in their content can already be measured 15 min after transfer onto growth medium (an increase of 2-fold in Spm, 3-fold in Put and Spd) [56]. After 1 h, the PA level is already sufficient to sustain protein synthesis. The synthesis of RNA and non-nuclear DNA becomes detectable after 20–30 min [56] (Fig. 3). Within 1 h many storage substances are utilized: the arginine content is halved within 30 min [56] (Fig. 3), and asparagine, glutamine, and aspartic acid, as well as other free amino acids or amides, also decrease [53].

The synthesis of PAs was studied in explants by adding [^{3}H] arginine to the growth medium, which led to the appearance of [^{3}H]-labelled Put, Spd and Spm after only 6 h [56] (Fig. 3). However, at the same time, neither ODC nor ADC activities are particularly high in cell-free extracts [72] (Fig. 3), and DFMO and DFMA inhibitors did not cause a substantial reduction of Put synthesis, even though they inhibited of 49% ODC activity and of 69% ADC activity [70]. At this early stage, by using the $^{14}CO_2$ entrapment, it is not possible to clearly distinguish between ODC and ADC activities, and the third Put-biosynthetic pathway, via citrulline (Fig. 1A), that is detectable in aged *H. tuberosus* slices [65]. The enzyme SAMDC appears to be active and is considerably inhibited by dicyclohexylamine (DCHA later called CHA) after 6 h [72]. Within 1 h, Spm and Spd content is lowered to 40% by methylglyoxal *bis*-guanylhydrazone (MGBG), but the inhibition disappears by 6 h [12]. In conclusion, the very early Put synthesis does not appear to be clearly correlated with ODC and ADC activities assayed in cell-free exctracts. However caution is necessary in evaluating the enzyme activities in cell-free extracts as well as the efficiency of inhibitors in explants.

Free PAs continue to increase throughout the G_1 phase, in part as a consequence of the very low activities of enzymes that remove PAs from their pool, either by oxidization (DAO activity) or by covalently binding to proteins via transglutaminase (TGase) activity [59,74] (Fig. 1B). In addition, free PAs might be released from their bound forms, as shown mainly for Spm [60].

To try to understand the physiological role of PAs accumulation during this phase of the cell cycle, some concomitant cellular events must be taken into account.

DNA, RNA, proteins and polysomes are very rapidly degraded but also newly synthesised [24,56,71]. DNA degradation was probably the result of programmed death of scattered cells of dormant parenchyma. The enzymes involved in degradation, such as proteases and probably RNase and DNase, seem to be newly synthesised [56,71]. PAs have been found tightly-bound and non-tightly-bound to tRNA, rRNA and to a poly(A)RNA, and the molar ratio PAs/RNA increases in early G_1 phase (particularly Put/RNA); this suggests that PAs can be directly involved in the activation of RNAs or in protecting them from nucleases [61]. It was demonstrated that Spm bound to phe-tRNA, maintains tRNA biological conformation, and that PAs enhance the efficiency and fidelity of translation [48] (as recently reconfirmed by Igarashi and Kashiwagi in mammalian cells [36]). Moreover, ribosomes extracted from dormant tuber cells require the addition of PAs and Mg^{2+} to be active, whereas ribosomes extracted from dividing cells are active, since they already contain PAs in sufficient amount [19].

Another early metabolic event is the synthesis of DNA, which starts at 30 min and peaks around 3–6 h [56,71]. Mitochondria and plastids were shown to be the site of this early DNA synthesis [28,71], while the nuclear DNA synthesis peaks at after 12 h (Figs. 2B-c and 3). Thus, organelle and nuclear DNA replication are independent. DNA and protein synthesis in mitochondria are probably related to the division of these organelles or to their functionality [28]. After 3 h, all three major PAs are present in mitochondria [71], as well as their precursors and biosynthetic enzymes [73]. It has been demonstrated that there is an energy-dependent Spd uptake, driven by membrane potential, in mitochondria isolated from dormant tubers [47]. The presence of PAs in mitochondria is concomitant with an enhanced rate of respiration but also with mtDNA synthesis, which might require PAs for its stabilization. The observed respiratory increase might be necessary for the high energy requirement connected with subsequent cell divisions and with the development of other organelles, such as bioenergetically incompetent proplastids [28]. Similarly to mtDNA, ptDNA synthesis may be a preliminary event for plastid divisions that are observed some hours later. In long-term culture in the light, such plastids can differentiate thylakoids and become photosynthetically competent [55].

PAs can also bind to several molecules, such as hydroxycinnamic acids, proteins and hemicelluloses, via different types of linkages including hydrogen and ionic bonds or covalent linkages (Fig. 1B) [11,58,66]. In particular PAs can form intramolecular or cross-linked conjugates with proteins through the action of TGases, and the molecular length of the PAs involved determines the length of the bridges. In cell-free extracts of *H. tuberosus* slices, after mid-G_1, TGase activity increases significantly [57,59] and conjugates with Put, Spm and mainly Spd, are formed [23,57]. This conjugation activity is demonstrated by the identification of glutamyl-PAs and the relative released PAs after acidic hydrolysis, and by TGase immunodetection of two neo-synthesised protein bands of 90 and 58 kDa. TGase mediated PA conjugation is Ca^{2+}-dependent. This cation exerts an essential regulative role on TGase activity by modulating the switch between conjugating or deamidating activity [39]. During the cell cycle, the total endogenous Ca^{2+} content in *H. tuberosus* tuber cells decreases sharply from 8 mM (0 h) to 4.8 mM (6 h), remaining constant thereafter [59]. The rather high level of this cation could be due to the contribution of Ca^{2+} located in the cell wall and vacuole.

1.3.2.2.2. The S phase. During the S phase, the content of nuclear DNA increases due to the duplication of the entire genome (karyotype $2n = 102$) of the cells or to extra DNA synthesis (amplification). Amitoses generate pairs of similar sized nuclei having an equal DNA content [16] (Fig. 2B-c).

A decrease in total PA content takes place although the synthesis of Put, Spd and Spm still continues, as shown by the incorporation of arginine in explants [56] (Fig. 3), by the *in vitro* re-activation of ARGase, and by the increasing activities of ADC, ODC and SAMDC. ADC reaches its maximum activity in mid-S phase (Fig. 3). The decline in Put content is probably due to a considerable DAO activity (up to the early-S phase), which thereafter sharply decreases such that PAs accumulate again (at the end of the S phase) [74] (Fig. 3). DAO seems therefore to have a central role in regulating the cellular level of Put while, in contrast, PAO activity is not detectable.

The effect of inhibitors on explants, such as CHA, MGBG, CAV [12,72], on PA biosynthesis is transitory or not very marked. Instead, the combined effect of DFMO and DFMA is more evident causing a maximum Put decrease of 60%. However as cell division progresses, enzyme activities and PA levels are restored [70]. In long-term *H. tuberosus* cultures, DFMO and DFMA, alone or in combination, drastically or totally reduce growth, similarly to CAV, CAN and CHA [10,72].

PA synthesis inhibition could have some negative effect on nucleic acid metabolism, but no conclusive data were obtained due to the only partial PA depletion that was achieved. In activated

H. tuberosus slices, it was shown that Spd partially removes the *in vivo* inhibition of the antibiotic actinomycin D on DNA and RNA synthesis [5]. The direct interaction between DNA, actinomycin D and PAs was confirmed by *in vitro* studies [22]. Different models of Spd or Spm binding to DNA were proposed [29,40]. PAs are thus necessary for DNA duplication and for RNA and protein synthesis, and enhance the efficiency and fidelity of these reactions.

During the S phase, the TGase activity continues to increase becoming 7–8-fold higher than its initial value [59] (Fig. 3). Data on TGase activity during the cell cycle are also scarce in animal cells [37]. Different protein-conjugated PAs have been found during the *H. tuberosus* cell cycle: low molecular-mass conjugates appear in early G_1 phase, while very high molecular-mass conjugates were mainly formed during the S phase until cell division [23,59]. During the cell cycle, the concentration of labelled conjugates (formed after ^{14}C-Put treatment), which were bound to relatively high molecular-mass proteins, was about 0.2 μM [59]. Thus, the contribution of TGase to the decrease of the free PA pool does not seem significant in light of this low conjugate amount. In animals, similar high molecular weight aggregates of protein-PAs, due to TGase activity, were found by Beninati et al. [14]. In plants, PAs may also be involved in the formation of supramolecular cell structures. In fact, data obtained in pollen [25], demonstrated that the cytoskeletal proteins actin and tubulin are substrates of intracellular TGase. On the other hand, in the S phase many enzymes involved in DNA synthesis are active and form a multiprotein complex associated with DNA [27], in which PAs are possibly involved. In addition, TCA-soluble PAs bound to low molecular-mass compounds and peptide-PA conjugates are present in *H. tuberosus*, whereas hydroxycinnamoyl-PAs are not detectable [45].

1.3.2.2.3. The D phase. The first observed cell divisions are amitotic, lacking the formation of the mitotic spindle; instead the nucleus and its single large nucleolus divide by invagination; a thin cell wall arises centripetally from the mother cell wall [16,55] (Fig. 2B-b, d, e). The D phase, which begins at around 18–20 h, largely overlaps with the S phase. Thus, some changes in PA metabolism can be related to events of either or both phases. In the middle of the D phase, a second peak of PA accumulation is observed, with Put formation from arginine and a high level of Spd synthesis [56] (Fig. 3). The biosynthetic activities are still high though declining [72], and DFMO and DFMA become ineffective, whereas MGBG inhibits Spm accumulation, resulting in an increase in Put level [70]. CAV inhibits PA synthesis even as their content increases [12] leading us to hypothesise an effect of CAV on DAO activity (Fig. 3). The conjugation of Put via TGase increases, giving rise mainly to high molecular-mass conjugates [59]. Protein accumulation, present until early D phase, begins to decline or proteins become insoluble and more difficult to detect (Fig. 3). At the end of the D phase, PA biosynthesis is no longer detectable but PA levels seem to be sufficient to allow the further progression of cell division.

Cell wall formation, one of the main events of late D phase, probably requires PAs, thus causing a depletion of the free pool for which TGase activity is not responsible as it is quantitatively not relevant. Evidence suggests that PAs can be bound to components of the *H. tuberosus* cell walls either by ionic [21,43] and covalent linkages [26]. PAs form ionic linkages with pectic substances [21] and probably hydrogen bonds with neutral wall polysaccharides [54]. The first indication of the presence of TGase products in cell wall was provided by the digestion of cell wall polysaccharide compounds of *H. tuberosus* parenchyma, which caused the disaggregation of PA-conjugated proteins of high mass from polysaccharides, suggesting the existence of a bond between these proteins and wall polysaccharides [26]. The presence of PAs in the cell wall in other plants is well documented [43].

The sharp decline in PA content might also result from a metabolic degradation (totally or partly due to the resumption of DAO activity) as well as a slowing down of their synthesis (Fig. 3).

2. Recent applications of *H. tuberosus in vitro* growth model

Long-term *in vitro* cultures of *H. tuberosus* tuber explants can be used to evaluate the antiproliferative and/or cytotoxic effects and their possible relation with polyamine metabolism of different drugs or growth substances. Here we report two recent applications of this classical growth model.

2.1. Effect of cisplatin and other platinum- or palladium-derived anticancer drugs

Platinum-based antitumor drugs are currently used in clinic cancer treatment and have been the target of an intense drug discovery and implementation research [30,44,64,68] since Rosenberg's report on cisplatin (cDDP, *cis*-$Pt(NH_3)_2Cl_2$) in the late sixties [51]. Cisplatin and other Pt or Pd substituted drugs bind to the DNA double helix inducing structural distortions and therefore inhibiting the replication process. Since cDDP and some derived compounds present severe clinical drawbacks, such as acquired resistance and toxic side effects, the search of alternative antitumor agents has become a particularly active area of research in the last decade [46]. Some of the third-generation drugs are Pt_2 or Pd_2 complexes [46,68] that contain two or more cDDP-like centres linked by polyamine bridging units of variable length which allow a more efficient interaction with DNA (*e.g.* formation of long distance and/or DNA interstrand adducts), leading to a significant enhancement of their cytotoxic activity [34,44].

As PAs bind to plant nucleic acids [29,61] and influence the cell cycle [8,56], cDDP, Pt_2-Spm ($Pt_2N_4C_{10}H_{26}Cl_4$ in the 2,2/c,c *trans* configuration) [68] and Pd_2-Spm ($Pd_2N_4C_{10}H_{26}Cl_4$ in the 2,2/c,c *trans* configuration) [20] were applied to long-term cultures of *H. tuberosus* (Fig. 2A) in an analogous fashion to tests performed on cancer cell lines [34,46,68], and the degree of cytotoxicity and the effect on cell proliferation and PA metabolism, evaluated.

Explants of dormant *H. tuberosus* tuber parenchyma were cultured for 35 d, with 1, 5, 10, 50 μM cDDP, 10, 50, 200 μM Pt_2-Spm and 10, 50, 200 μM Pd_2-Spm, in the presence of 10 μM 2,4-D to induce the breaking of dormancy. At the end of the observation period the amount of dry weight (DW), was about 20% of the fresh weight (FW) in all the treatments except for 50 μM cDDP and 200 μM Pt_2-Spm and Pd_2-Spm, where it was about 14% of FW (Fig. 4A).

The effect of cDDP both on cell proliferation (gFW, Fig. 4A) and on free PAs levels (Fig. 4B), was greater and more toxic (compared with the NT control) with increasing concentration, while Pt_2-Spm and Pd_2-Spm did not appear as effective as cDDP, having an effect only at quite high dosages (200 μM). In particular the treatment with cDDP seemed to reduce only Put levels, while Spd and Spm levels were slightly or not at all affected (Fig. 4B). These data were confirmed by ADC and ODC enzyme activities which were assayed in total cell extracts. The results showed that ADC and ODC activity were progressively reduced by increasing cDDP concentrations, whereas 50 μM of either Pt_2-Spm and Pd_2-Spm affected Put-forming activity only slightly (Fig. 5). Due to the limited amount of collected tissues, it was not possible to measure enzyme activities on samples treated with 200 μM Pt_2-Spm or Pd_2-Spm.

On the contrary to what reported for mammalian cancer cell lines [64], the Pt_2-Spm and Pd_2-Spm complexes exerted a lower effect on plant cells, even if used at a concentration 4-fold higher than cDDP (200 μM versus 50 μM) (Fig. 4). This is probably due to the interaction of these compounds to proteoglycans such as those

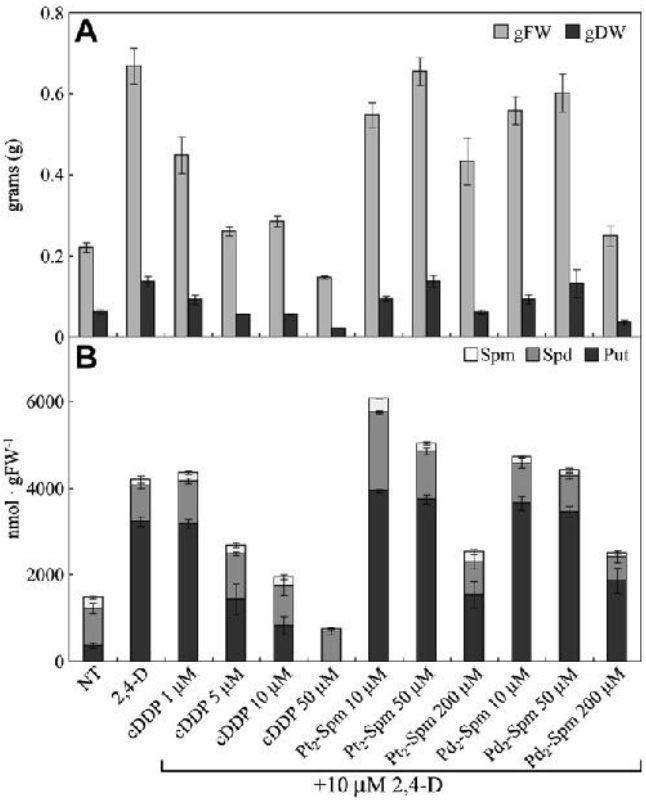

Fig. 4. Effect of cDDP, Pt_2-Spm and Pd_2-Spm compounds in the presence of 10 μM 2,4-D after 35 d of culture. (A) Cell proliferation as grams of fresh weight (gFW) and grams of dry weight (gDW). (B) Free polyamine levels. Data are the mean ± S.D.; $N = 3$.

present in the plant cell wall. This ionic interaction could lower the uptake efficiency of these compounds into the plant cells, affecting their bioavailability and consequently their activity.

Total DNA was extracted and separated through 1% (w/v) agarose gel electrophoresis showing, especially in Pt_2-treated explants, the presence of a ladder-like pattern of DNA fragmentation (data not shown) typical of apoptosis, and confirming a certain degree of toxicity of these compounds.

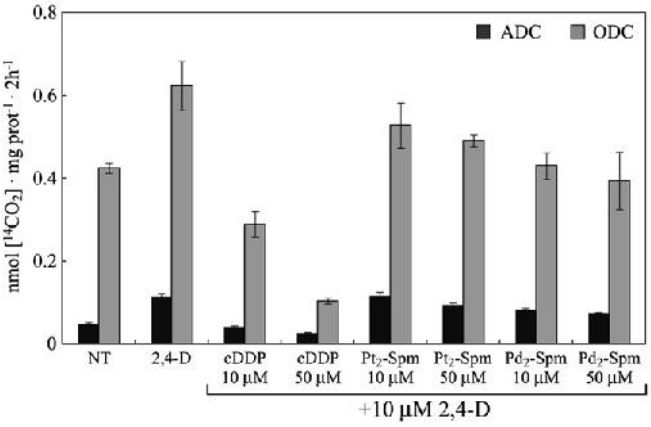

Fig. 5. ADC and ODC activities (nmol [$^{14}CO_2$] mg prot^{-1} 2 h^{-1}) in *H. tuberosus* parenchyma explants treated with cDDP, Pt_2-Spm and Pd_2-Spm compounds in the presence of 10 μM 2,4-D and collected after 35 d of culture. ADC and ODC assays were performed on whole cell extracts by using 0.2 μCi [^{14}C]arginine or [^{14}C]ornithine respectively. Data are the mean ± S.D.; $N = 3$.

2.2. Effect of agmatine analogues: polyamine biosynthesis inhibitors

A similar experimental approach was used to evaluate the effect of charge-deficient agmatine analogues [62]. Agmatine [1-(4-aminobutyl)guanidine] is a bivalent amine formed by decarboxylation of arginine catalysed by ADC (Fig. 1A), and is widely distributed in bacteria, plants, animals and invertebrates [49,69]. In animal tissues it is known to act as a neurotransmitter or neuromodulator, and to display clinical properties such as neuroprotection and tumour suppression. Its biochemical properties include the induction of ornithine decarboxylase antizyme and spermidine/spermine N^1-acetyltransferase, and the inhibition of nitric oxide synthase [31,32]. Agmatine is actively transported into cells by the Put transporter and affects polyamine homoestasis [17,52]. Agmatine conversion to Put is an initial step of polyamine biosynthesis in bacteria and plants (Fig. 1A), while in animal cells Put originates from ornithine. In animals, agmatine was found, together with its metabolic enzymes ADC and agmatinase, in liver mitochondria, where its transport seemed to be differently inhibited [31] by the same agmatine analogues tested here on plant cells.

Explants of dormant *H. tuberosus* tuber parenchyma were cultured for 30 d, with 0.1 mM AO-Agm [*N*-(3-aminooxypropyl)-guanidine], GAPA [*N*-(3-aminopropoxy)guanidine] and NGPG [*N*-(3-guanidino-propoxy)guanidine] [62], with or without 10 μM 2,4-D.

None of the tested compounds exhibited a toxic effect. In the absence of 2,4-D, GAPA-treated explants were brown and showed a significant increase of cell proliferation (gFW, Fig. 6A), while not treated (NT), AO-Agm and NGPG supplemented tubers were green and showed little cell proliferation. In the presence of auxin, the samples were white/yellow and, as expected, showed a large degree of proliferation activity. Under these conditions, AO-Agm had a slight inhibitory effect on gFW, while GAPA and NGPG seem

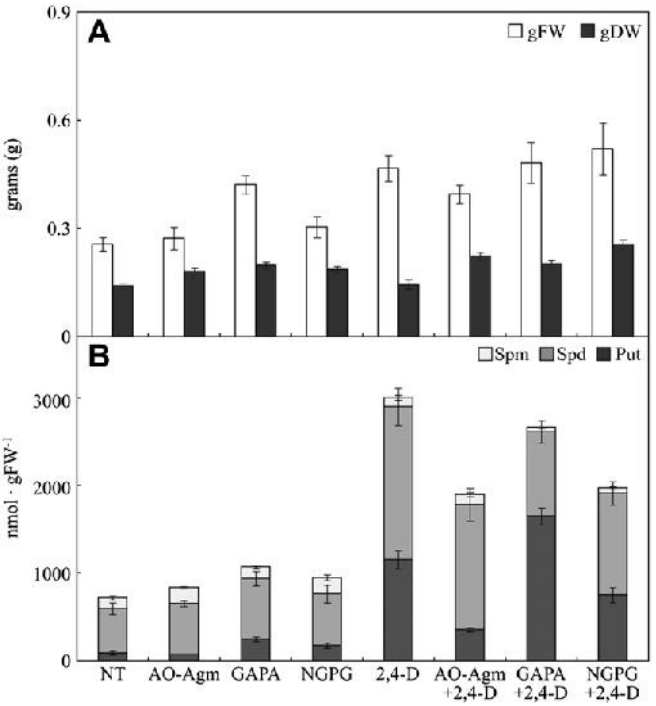

Fig. 6. Effect of 0.1 mM AO-Agm, GAPA, NGPG compounds in the presence or absence of 10 μM 2,4-D after 30 d of culture. (A) Cell proliferation as grams of fresh weight (gFW) and grams of dry weight (gDW). (B) Free polyamine levels. Data are the mean ± S.D.; $N = 3$.

not to influence cell growth (Fig. 6A). On average the DW was about 25% of the FW.

Free polyamine levels generally correlated with the proliferation data. When compared with the relative controls, a little increase in the Put level was in fact measured in the GAPA samples without and with auxin, and a Put decrease was evidenced in the AO-Agm and NGPG plus 2,4-D samples (Fig. 6B). Spd and Spm levels seemed not to correlate with cell growth. To determine whether the variation of Put levels were due to a modification of Put-forming enzyme activities, ADC and ODC enzyme assays were performed on supernatant (SN) and pellet (PT) cell fractions of tuber explants. ARGase and ornithine transcarbamoylase (OTC) (Fig. 1A) enzyme activities were also measured.

In agreement with the free polyamine results, ADC and ODC activities were higher especially in GAPA explants grown in the absence of 2,4-D (Figs. 7A and 8A). In these samples, Put seemed therefore to be synthesised following GAPA exposure. A different pattern was shown in the samples with added 2,4-D, both for ADC and ODC assays. Low levels of ADC were measured in 2,4-D-treated samples (Fig. 7A), conversely a high (on average 85% both in SN and PT fractions) ARGase activity was measured in the same samples (Fig. 7B). ODC and OTC activities were of the same order of magnitude in most of the assayed samples (without or with 2,4-D) and in particular, in the presence of 2,4-D, OTC activity (Fig. 8B) was double in the PT fraction with respect to SN fraction. In conclusion, in the absence of auxin, GAPA seemed to induce ADC, ODC and OTC activities leading to the production of Put (Figs. 7 and 8).

A different behaviour of AO-Agm and NGPG with respect to GAPA was found in the plant cells, in agreement with Grillo et al. [31] who used the same compounds to study agmatine uptake on rat liver mitochondria. In rat liver mitochondria, AO-Agm and

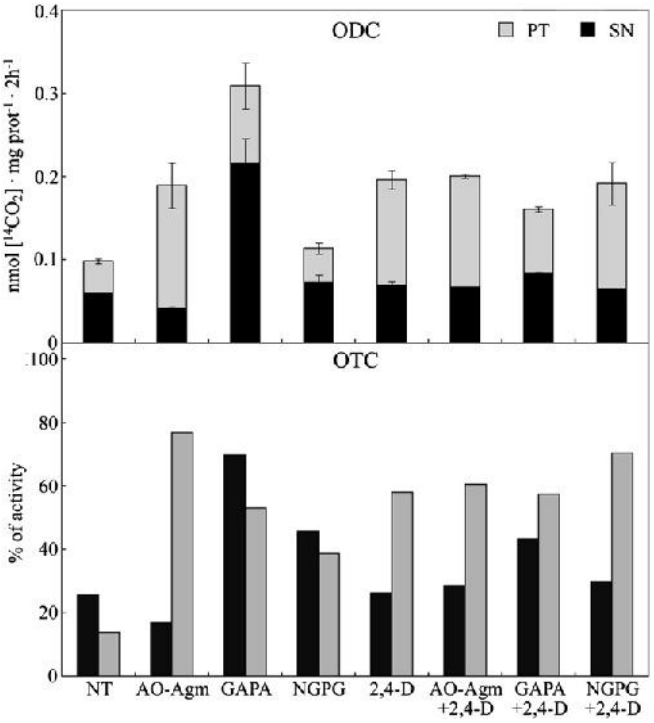

Fig. 8. ODC (nmol [$^{14}CO_2$] mg prot^{-1} 2 h^{-1}) and ornithine transcarbamoylase (OTC) activities in supernatant (SN) and pellet (PT) fractions of *H. tuberosus* parenchyma explants treated with 0.1 mM AO-Agm, GAPA, NGPG compounds in the presence or absence of 10 μM 2,4-D. The assays were performed with 0.2 μCi of [^{14}C]ornithine. The percent of OTC was calculated as the difference between the ODC activities measured without and with 20 mM unlabelled arginine. Data are the mean ± S.D.; $N = 3$.

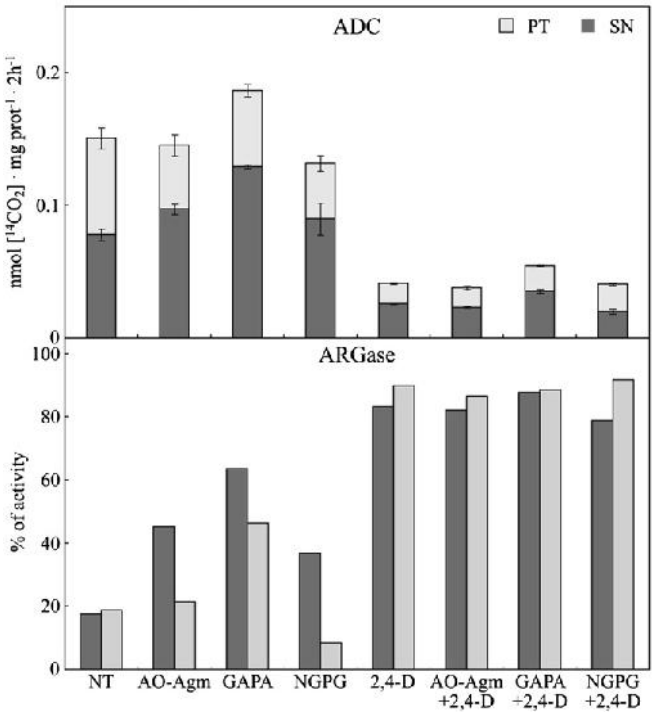

Fig. 7. ADC (nmol [$^{14}CO_2$] mg prot^{-1} 2 h^{-1}) and arginase (ARGase) activities in supernatant (SN) and pellet (PT) fractions of *H. tuberosus* parenchyma explants treated with 0.1 mM AO-Agm, GAPA, NGPG compounds in the presence or absence of 10 μM 2,4-D. The assays were performed with 0.2 μCi of [^{14}C]arginine. The percentage of ARGase activity was calculated as the difference between the ADC activity measured without or with 20 mM unlabelled ornithine. Data are the mean ± S.D.; $N = 3$.

NGPG had a competitive inhibitory activity on agmatine transport, due to the presence of a guanidine group, while GAPA was a non-competitive analogue exerting a lower effect. In our system none of the compounds inhibited Put-forming activity, and, conversely GAPA increased both ADC and ODC (Figs. 7A and 8A). Therefore these agmatine analogues did not compete for the agmatine binding site on agmatine iminohydrolase (AIH), an enzyme not present in animals [32] that in plants converts agmatine to Put via N-carbamoylputrescine (Fig. 1A). Moreover, in animal cells, it was previously reported that GAPA, once actively transported inside the cells [31,62] via the Put transport system, might be degraded to 3-aminooxy-1-aminopropane, one of the most efficient ODC inhibitors [35]. This seems not to be the case in our plant system, where on the contrary GAPA greatly stimulated ODC activity. This might be due to the presence of a different ODC regulation in plants [38] with respect to animals where a specific antizyme complex is involved [33,42].

3. Conclusions and future perspectives

It was shown in this paper that the classical model of *H. tuberosus* dormant parenchyma, which represented a formidable model system to investigate PA metabolism for the past forty-six years, could have new application in the study and evaluation of the cell toxicity and antiproliferative activity of different compounds both of natural origin or chemically synthesised. In addition, *H. tuberosus* could also be utilized as a food in the daily diet. In fact, due to its low PA levels and the presence of inulin instead of starch, it was recently suggested the preferential utilization of this tuber in the diet of people with special needs, such as patients treated by chemotherapy and patients with diabetes [50].

4. Materials and methods

4.1. Material

Tubers of *H. tuberosus* L. (Jerusalem artichoke) cv. OB1, grown in the Botanical Garden of the University of Bologna, were collected in late November, when they enter into dormancy, and stored in moist sand at 4 °C. They were utilized only the during deep dormancy period which lasts roughly until the end of February.

4.2. Culture conditions

Explants of dormant *H. tuberosus* tuber parenchyma were cultured in solid agar Bertossi medium as described by Serafini-Fracassini et al. [55].

A first set of tuber explants were cultured for 35 d with 1, 5, 10, 50 μM cDDP, 10, 50, 200 μM Pt_2-Spm and 10, 50, 200 μM Pd_2-Spm, in the presence of 10 μM 2,4-D which induces the breaking of dormancy and cell proliferation [8]. A second set of experiments was carried out for 30 d, with 0.1 mM AO-Agm [*N*-(3-amino-oxypropyl)-guanidine], GAPA [*N*-(3-aminopropoxy)guanidine] and NGPG [*N*-(3-guanidino-propoxy)guanidine] [62], with or without 10 μM 2,4-D.

At the end of the observation periods the explants were collected, weighed (fresh weight, FW), frozen with liquid N_2 and stored at −80 °C for following analyses. Some explants were placed at 100 °C for 48 h and weighted to determine the dry weight (DW).

4.3. Polyamine determination, enzyme activities and total DNA extraction

Free and conjugated (PCA-soluble and PCA-insoluble fraction) polyamine analyses on tuber explants were performed according to Tassoni et al. [67]. After analyses only free polyamines were detected; soluble- and insoluble-bound PAs were either absent or present in traces amounts.

Arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) activities were determined on tuber explants using a radiochemical method [67] by measuring the $^{14}CO_2$ evolution of 0.2 μCi of L-[U−^{14}C]arginine (specific activity 278 mCi mmol^{-1}, GE Healthcare, Little Chalfont, UK) or of 0.2 μCi of D,L-[1−^{14}C]ornithine (specific activity 56 mCi mmol^{-1}, GE Healthcare, Little Chalfont, UK) in whole cell extracts (first set of experiments), or in the supernatant (SN, soluble fraction) and pellet (PT, containing cell wall, nuclei, plastids and mitochondria) cell fractions (second set of experiments), of tuber explants cultured with and without 10 μM 2,4-D. In addition, ARGase and ornithine transcarbamoylase (OTC) enzyme activities were indirectly measured by performing the same assays in the presence of 20 mM ornithine or 20 mM arginine, for ARGase and OTC respectively [67]. Protein content of all the supernatant and pellet fractions used for the different enzyme assays was determined according to Lowry et al. [41].

Total DNA was extracted [18] and an aliquot (6 μg) separated through 1% (w/v) agarose gel electrophoresis to show the presence of DNA laddering.

Experiments were repeated twice with similar results.

Acknowledgments

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