Supplementary Material: History of polyamine research and their properties in plants

1. Foreword

This supplemental part reports the general characteristics and the main steps in the discovery of the polyamines (PAs) in plants not reported in the previous manuscript to not interrupt it. Here the data are taken from general PA literature, also adding complementary data on *Helianthus t*. and other plants. This part has also the target to show a short history of the development of PA research and the scientific logic applied by these studies in the Polyamine Laboratory of the Bologna University since 1965 until now.

The figures are cited with an "S" before the number to distinguish them from those of the main manuscript.

2. Polyamine history in plants

As reported in the main manuscript, in plants the PAs role as growth factor was discovered in *Helianthus t.* in 1965 [1]. Since that time, a flowering of discoveries developed. Here we report the milestones of the research conducted not only in our lab, but also in other labs. At first PAs were studied in relation to their effect on nucleic acids, promoting protein synthesis [2], as at that time data at molecular level were reported on interactions with DNA and different RNAs. Successively, starting from the years 1985, PA binding with proteins were observed in *Helianthus t.* [3] and then this field of research was enlarged due to the possible regulations induced in protein functionality. Also PA metabolism and transport were object of several innovative researches, conducted especially on *Helianthus t.*. The multiple PA functions were confirmed by many other research summarized in some reviews [4,5,6]. More recently, in the years 1987-1989, an enzyme, transglutaminase, was identified and found to catalyze the conjugation of PAs to specific plant proteins [7, 8] and a new line of research was developed, as reported by the papers [9-13]. Transglutaminase research opened a new panorama on protein covalently bound PAs, previously neglected. In many cases this is the molecular condition by which PAs exert their multiple roles in the cell. In fact, PAs can bind to molecules, especially macromolecules, regulating their properties [5, 14, 15, 16, 7, 17, 18, 19, 20, 21, 22, 23, 24, 25, 9, 11, 26, 27, 10, 28, 13, 29, 30, 31, 32].

The plant organisms object of all these researches were mainly *Helianthus t*., either dormant or activated *in vitro*, the pollen and its germination in different Rosaceae fruit trees, the flowers of *Nicotiana sp.* and the unicellular alga *Dunaliella salina.* A tenth of other Tracheophyta plants, a virus and a bacterium a were less frequently studied. The main biological events considered were dormancy, *in vitro* or *in vivo* growth, flowering, pollen development, photosynthesis, cell cycle, programmed cell death.

3. Polyamine molecules

The biogenic polyamines, common to practically all living organisms, play widespread and vital roles evolutionary ancient. The crystals of PAs were observed in 1678 by Antony van Leeuwenhoek [33] in human semen, but were identified only in the last century [34].

Aliphatic low-molecular-weight polyamines are polycationic bases formed by a linear carbon hydrophobic backbone, having two terminals highly protonated aminic groups and eventually one or two internal iminic ones, thus being electronically reactive in the cell.

The most widespread PAs are the diamine putrescine (Put) the triamine spermidine (Spd) and the tetramine spermine (Spm) (Figure S1A) [33, 35]. Their backbone is flexible, see an example of Spd in Figure S1B, which have the capacity to adapt to the form of other molecules to which might be linked [35, 36]. Other aliphatic natural PAs exist, among which thermospermine and cadaverine, the last is the precursor of some alkaloids [33, 37, 38, 39].

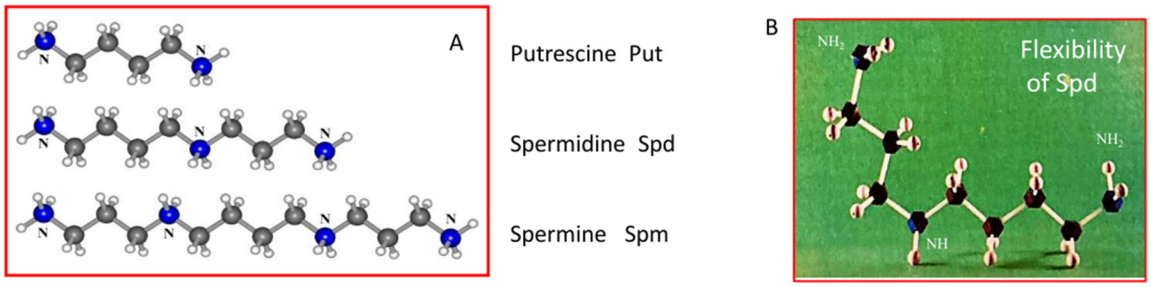

Figure S1. Structure of PAs
A. Structure of the three main aliphatic polyamines: Putrescine, Spermidine and Spermine. The N atoms of the terminal aminic or internal iminic groups are in blue color.
B. Flexibility of the aliphatic backbone, example of Spd.

4. Localization, metabolism, transport and roles of polyamines

It is almost a universal rule that all living organisms require polyamines to keep viability [4]; however, the precise biochemical functions of PAs as cytoprotective molecules are still an incompletely resolved topic. These molecules have the property to be transferred easily and rapidly among the different parts of the cell as well as among different cells. Part of these molecules can become bound and thus fixed to cell macromolecules and cell structures.

The **PAs localization** in the cell depends on the balance among biosynthesis and degradation but also by transport (efflux and uptake) [40, 41]. PAs are in fact actively transported among cellular organelles/compartments and the extracellular space [42, 43]. PA import was evidenced for the first time, in protoplasts and vacuoles [44, 45] moreover, the PA uptake was detected in mitochondria of *Helianthus t*. as well as in chloroplasts [46, 47]. The uptake is concentration-dependent, stimulated by Ca2+, energy-dependent, auxin-regulated and protein-mediated [48, 49].

PAs are transported in the entire plant also bidirectionally at long distance via xylem and phloem [50, 51, 52] with a different distribution [47, 53, 14].

In addition, being transported among different compartments and entire cell, PAs are metabolized. The **PA metabolism** is rather complex, showing that PAs can be actively interconverted. A precise optimum of PAs is necessary for each single function, and it must be finely regulated by these mechanisms.

The enzymes of the PAs metabolism are frequently compartmentalized [41, 54]. Put is the precursor of Spd directly from ornithine by the ornithine decarboxylase (ODC) [55] or indirectly from arginine by arginine decarboxylase (ADC) pathway. Spd derives from Put and Spm derives from Spd by the respective synthases. Glutamine, deriving from Krebs cycle, can act as precursor of PAs [56] .

Spd and Spm are retro-converted to Put by polyamine oxidase (PAO) in the peroxisomes and apoplast, with the generation of H_2O_2, DAP and other products, participating in plant defense and plant-bacteria communication. Diamino oxidase (DAO) oxidizes Put and also Spd. It has been shown that PAs can function as sole nitrogen source for *Helianthus t. in vitro* [56] showing that they can be efficiently converted in all primary metabolites by the complex PA interconversion. Moreover, PAs might exert different roles also when are converted into secondary metabolites [57], among which cytotoxic compounds, scavenging radicals, alkaloids [37], H_2O_2 which affects pathogen growth, induces cell-wall reinforcement and stomatal closure.

The metabolism of PA is subjected to a circadian rhythm, which strongly influence it [58]. In addition, stresses also have a very relevant effect on the metabolic pathways, as largely reported in literature, [59,60] and Spm improves stresses tolerance [61, 62].
As a consequence, not only of their transport but also of their metabolism, PAs in fact have been found compartmentalized practically in all cell organelles or structures as shown in (Figure S2A) where PAs have roles related to the function of the different parts. Their functions have effects at subcellular, cellular and organismic levels (Figure S2B,C,D) [14, 55, 63, 64].

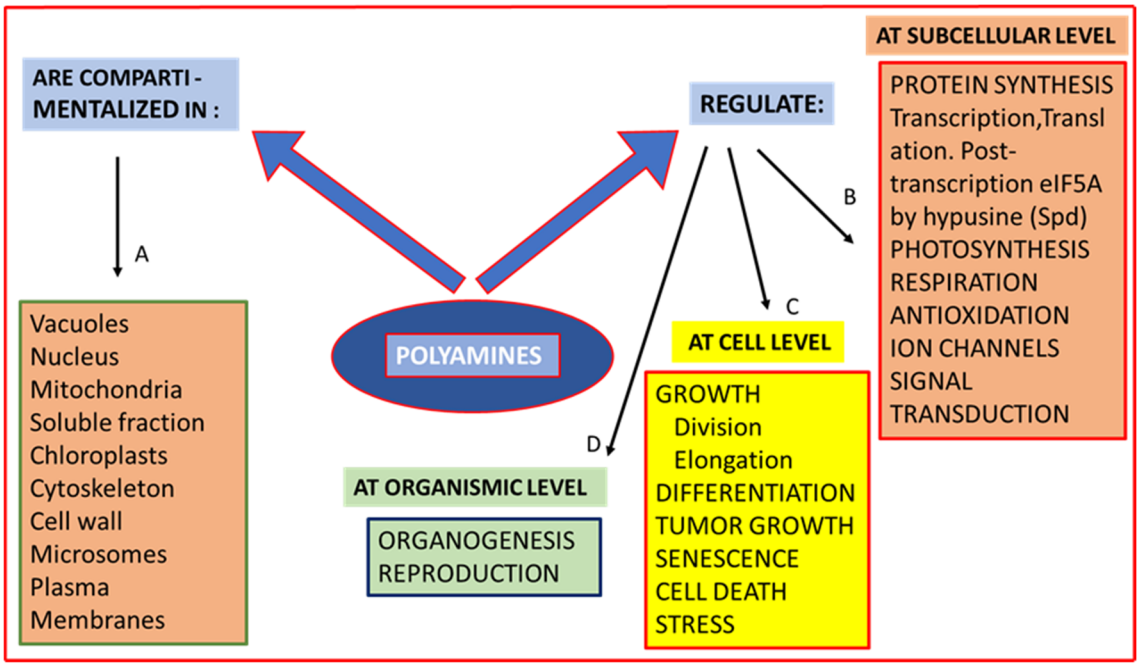

Figure S2. Localization and regulation of different functions of PA.
A. Compartmentalization of PAs in different structures or organelles of the cell.
B. - D. PA regulatory role at: B. subcellular; C. cellular; D. organismic events.

In the different compartments PAs might be involved in photosynthesis, respiration, protein synthesis, signal transduction, regulation of ion channels, membrane potential, electrolyte balance, in cell homeostasis.
About the role of PAs in cellular organelles, the chloroplast is probably the most studied organelle for the peculiar PA role in the more specific and energetically relevant function in plants, namely photosynthesis (Figure S2A,B). The presence of PAs in chloroplasts was firstly reported in the alga *Euglena g.* in 1974 [65] and then PAs were found in all plants examined. PAs regulate structure and functioning of the photosynthetic apparatus, being present in thylakoid and stroma compartments. The conversion of pro-plastids into chloroplasts, observed in *Helianthus t.* during *in vitro* culture in the light, is stimulated by the presence of PAs [66].
As a consequence of their presence in various organelles, PAs have many regulative roles at cell level (Figure S2C) and influences many fundamental cell processes, such as cell division, elongation, differentiation, senescence/PCD, stress- and external stimuli-induced, homeostatic adjustments etc. [43, 67]. These distributions in the plant cell have consequences also on the organ differentiation (Figure S2D), such as embryogenesis, organogenesis of roots, leaves, flowers, tubers and fruits [52]. PAs are also critical in

reproduction, self-incompatibility from pollen development to fertilization (Figure S2D) [12, 68].
The single PA has different roles in plant cell life. PAs act as pro-survival molecules, as rejuvenation factors [**69**], whereas in other conditions they accelerate cell death [**70**]. Put frequently contrasts Spd and Spm, emphasizing that individual biogenic amines have defined action and they differentially affect growth and development [71].
Usually, PAs are present at high concentrations in the growing organs [72], and tumors represent an important model. In animals a correlation between the level of PAs and tumor growth velocity was established. The role of PAs in cancer, and also in many other pathologies, assumed great relevance in mammal pathophysiology [72, 73].
Similarly to other growth substances, the relationship between growth rate and concentration of PAs is represented by a Gaussian curve: PA depletion or excessive accumulation may both have inhibitory effects. DNA synthesis and cell viability can be compromised by extreme levels of PAs; moreover, an increase in PA degradation involves the generation of aldehydes, some of them highly toxic.

5. Free and bound PAs.
PAs are present in the cells either in free or in bound forms, namely linked to other molecules of which could modify either their chemical charge or structure [5, 74].
The free ones (Figure S3A) could exert their roles as long polycations, being mostly charged at the physiological cell pH. Free PAs are easily metabolized, transported, exchanged and possibly could have an osmotic role and contribute to the activation of gene expression.

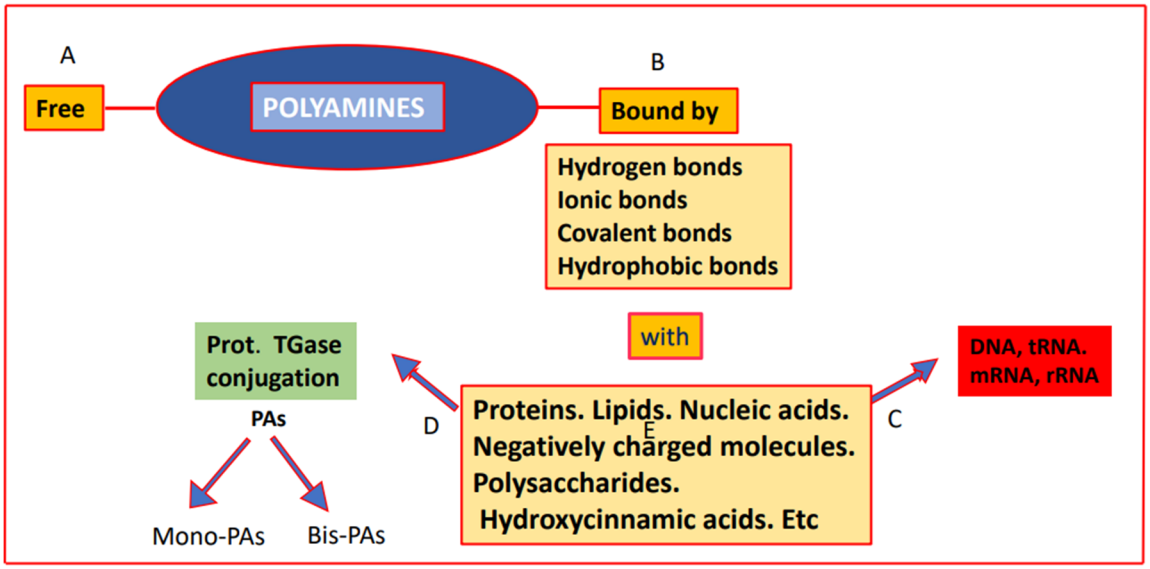

Figure S3. PAs in free and bound forms
A. Free PAs.
B. Bound PAs by different types of bonds.
C. Binding with nucleic acids.
D. Binding with proteins by means of transglutaminase (TGase). The PAs binding gives rise to: mono- (glutamyl)-PAs or, by a second transamidase reaction, to bis- (glutamyl)-PAs [**75**].

Mainly free polyamines have been studied and reviewed [71, 76, 77]. Several activities, attributed to free PAs, can instead be performed by PAs bound in a not stable form.
PAs in both free and bound forms are present in *Helianthus t*. tuber and in primary meristem of sprout apices [7,78].
PAs have high electrostatic affinity for negatively charged molecules and become bound. This term comprises PAs tightly-bound and non-tightly-bound ones.
As shown in Figure S3B, there are different types of bindings: hydrogen, ionic, covalent and hydrophobic. When aminic and iminic groups interact by ionic and hydrogen linkages with negatively charged groups of several molecules are easily reversible. Other instead are stable, like for example the covalent ones that can be disrupted only by high acid concentration hydrolysis in hot condition [16]. Conjugated PAs represent only a fraction of the total bound entities and should not be construed as an alternative definition of 'free'. Hydrophobic interactions primarily involve the lipids of membranes, proteins, nucleic acids, other lipids, cell walls, and so on, and they are reversible [79].
In addition, the PAs, when extracted, are classified in two fractions, depending on their solubility in perchloric or trichloric acids (PCA or TCA): PCA- (or TCA-) soluble and insoluble, depending on the molecular weight of their partners (below or higher than 5000 Da).
PAs can be regarded as charge donors or bridge builders. In fact, they can modify biomolecules, as nucleic acids, producing a charge effect and interacting with helical structures [77] (Figure S3C).
For example, rRNA is active in amino acid incorporation, only when it formed a complex with PA, like in *Helianthus t*. tuber cells, described in the main manuscript [80].
Another example of PA binding, a covalent one, might occur with proteins (Figure S3D), but this is catalyzed by an enzyme, transglutaminase, below described. PAs can form only a single binding (mono-PAs) cationizing the protein, or bis-PAs forming a bridge between two proteins.
Other linkages can occur with lipids, polysaccharides, hydroxycinnamic acids, etc.

5. 1. Types of PA interactions
5. 1. 2. Non-tightly-bound interactions
Important examples of non-covalent linkages of PAs are those with nucleic acids.
Due to their linkages, PAs interfere with transcription [81]. The interaction between PAs and DNA has been observed also *in vitro* and *in vivo* in *Helianthus t.* [17, 18]. PAs play a molecular stabilizing role, either by interacting with the double helix or also by the covalent binding to histones [82]. Moreover, PAs are also non-tightly-bound bound to tRNA, rRNA, 5SRNA, mRNA. PAs exert several roles in the protein synthesis, acting at different levels, chromatin organization, transcription, translation [83], but PAs may also interact post-translationally with proteins. PAs interact with the membranes [5] important for the transmission of receptor-mediated signals [85].
The binding of PAs to proteins could be non-covalent or covalent. The first one is not stable. PAs have been found not covalently bound also to hemicelluloses, pectins, cellulose and lignin [86, 87]. PAs covalently bound to proteins are widespread: as an example, PAs are accumulated in the cell wall, where they interact with polysaccharides as well as by covalent linkages with proteins.

5.1.3. Tightly-bound interactions
In addition to non-tightly bound PA interactions, a large percentage of PAs can also be conjugated to other low-molecular-weight compounds [88, 89, 90], like hydroxycinnamic acids, involved in the organization of the cell wall and associated to fertility [91, 92].
PAs are also bound with high molecular mass molecules, namely proteins (Figure S3D). A widespread family of enzymes are responsible for this binding: transglutaminases (TGases) which frequently catalyzes high-order molecular complexes by PAs bridges. These bridges might have a structural role stabilizing protein structure by their flexible alkyl chains (Figure S1B), which can adapt to various conformations upon binding. Frequently high-order molecular complexes by PAs bridges are catalyzed, having a structural role in the stabilization of protein structure.

In plants TGases were found to catalyse the covalent binding of PAs to some glutamyl-residues of specific proteins. PAs can form only a single binding, mono-PAs, cationising the protein, or bis-PAs forming a bridge between two proteins if the second terminal amino group of the mono conjugated PA, is linked. When many of these bindings are formed, a protein net can be produced, eventually of high mol mass, giving rise to large structures, for example cytoskeletal ones.
The inter- or intra-protein PA bridges are of different length according to the kind of PAs and reduce the repulsive forces between negatively charged components, leading their aggregation [79, 84, 93].
In the 1980s, the presence of TGase-like activity was first detected and biochemically characterized in the sprout apices of *Helianthus tuberosus* [7], and subsequently observed during its tuber cell cycle [22] and in other plants [94]. This activity was later confirmed in *Arabidopsis thaliana* and *Zea mays* [95, 24]. Multiple forms of these enzymes were found in all tracheophyte organs tested, such as primary meristems of the tuber sprout, tubers, roots, leaves, flowers, activated tuber parenchyma, seeds, pollens, in different organelles and in algae [9, 96]. These TGases in plants have been reviewed [9, 11, 13, 26, 97].
The identification of the substrates allows to clarify the role of some of these TGases. In fact, some protein substrates are typical of certain plant cell organelle. The main TGase substrates were detected in the chloroplasts, also of *Helianthus t.* leaves and their isolated compartments, like Rubisco in the stroma and in several components of Light Harvesting Complexes of thylakoids, modifying their conformation in a light-dependent way. This influences photosynthesis and photoprotection, favoring growth and delaying senescence [24, 27, 66, 89, 98, 99, 100, 101, 102]. Since PAs act as radical scavengers, resistance to abiotic stress is conferred on the photosynthetic apparatus, affecting the photosynthetic efficiency against various stress factors [10, 28, 98]. In addition, PAs could react with atmospheric CO2 [103]. Among other cell substrates of TGase, the cytoskeleton proteins, tubulin and actin, are affected in their cytoplasmic movements with effects on mitosis, organelle position etc. [30, 31].
Other plant substrates are those of cell walls and membranes: their presence and role in regulating permeability are revealed by their rigidification during senescence and accelerating cell wall differentiation [32, 69]. An extracellular form is necessary for the growth of pollen tube possibly by rigidifying it [104,105]. Moreover, the externalised pollen TGase could be one of the mediators of pollen allergenicity [106].

Conclusion

The narrative of PAs seems infinite, making it particularly challenging to provide a comprehensive overview that elucidates all the principal roles of PAs. This narrative span various phases and objectives of research. Numerous reviews are referenced, delving into details that are merely summarized in this report. The purpose here is to offer some fundamental information to facilitate the understanding of the historical research journey concerning PCD reversal in *Helianthus t.*.

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