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Polyamine Patterns in Haploid and Diploid Tobacco Tissues and *in vitro* Cultures

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ABSTRACT

The aim of this work was to determine PAs levels in pith tissues and callus cultures from haploid and diploid tobacco plants, explanted from the apical and basal regions of the stem. These explants were cultured in an RM-64 medium supplied with IAA and kinetin, under light or in the dark, during successive subcultures. PAs levels followed a basipetal decrease in diploid and an increase in haploid, pith tissues. A similar pattern of total PAs (free + conjugated) was observed for the callus of diploid and haploid plants maintained in the light, and for the haploid callus in the dark, whereas the diploid callus in the dark showed a constant increase in total PAs levels until the end of culture. The PA increase in the diploid callus in the dark was related to free Put levels increase. The ploidy status of the plants could express different PA gradients together with the plant pith and in vitro callus cultures.

Key words: biotechnology, *Nicotiana tabacum*, ploidy, putrescine, spermidine, spermine

INTRODUCTION

The polyamines (PAs) putrescine (Put), spermidine (Spd) and spermine (Spm) are important modulators of biological processes, influencing the growth, various development events and stress responses in plants (Kakkar and Sawhney, 2002; dos Santos et al., 2002; Kuznetsov et al., 2006; Tun and Santa-Catarina et al., 2006; Santa-Catarina et al., 2007; Steiner et al., 2007; Baron and Stasolla, 2008; Kusano et al., 2008; Naija et al., 2009), as well as, apoptosis and programmed death in both animals and plants (Kuehn and Phillips, 2005). PAs are small, positively charged aliphatic amines at cellular pH values, and therefore bind negatively charged molecules, including nucleic acids, acid phospholipids, and proteins, consequently modulating replication, transcription, translation, membrane stabilization, cell division and expansion (Kuznetsov et al., 2006), as well as DNA-protein interactions and protein-protein interactions (Yoda et al., 2003). PAs biosynthesis and degradation, their conjugation with phenolic acids, and intracellular transport, all contribute to cellular levels of free PAs in plants, and their pathway in biosynthesis was well established and revised by various authors (Minocha and Minocha, 1995; Bouchereau et al., 1999; Bhatnagar et al., 2002; Kuznetsov et al., 2006; Kusano et al., 2008).

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The relationships among ploidy, morphogenesis and certain biochemical characteristics of tissues cultured *in vitro* have been demonstrated in several works during the last two decades (Murashige and Nakano, 1967; Lavee and Galston, 1968; De Fossard, 1974; Matthews and Vasil, 1976; Kraus et al., 1981; Floh and Handro, 1985a,b; Lederman and Floh, 1993). Morphological, physiological and biochemical gradients of several substances were found along diploid stem pith tissue of *Nicotiana tabacum* (Murashige and Nakano, 1967; Lavee peroxidase activity, IAA-oxidase, protein content and cytohistological gradients along the tobacco stem (Kraus et al., 1981; Floh and Handro, 1985a,b; Floh et al., 1989; Lederman and Floh, 1993). Although reliable evidence of interrelationships among ploidy, growth, nucleic acid, protein, peroxidase activity and tissue culture exist, there is no information about the role of PAs.

Concentration gradients of free PAs directed towards the base or the apex of the stem or root, have been reported in maize seedlings and pea and lentil epicotyl (Dumortier et al., 1983). In tobacco, free PA concentrations increased from the main axis to the floral peduncule (Tiburcio et al., 1988), and from the apical to the basal regions in pith explants (Altamura et al., 1993). Paschalidis and Roubelaskis-Angelakis (2005) demonstrated that PA titers and biosynthesis followed a basipetal decrease along the tobacco plant axis, and that they were negatively correlated to cell size.

In this work, pith tissue of haploid and doubled haploid (DH) plants obtained via androgenesis was used. This process is a way of producing genetically true-breeding DH plants of significant interest for developmental and genetic research, as well as for plant breeding and biotechnology (Liu et al., 2002). Pith tissue is a suitable system because it contains few conjugated PAs contrasts with whole plants and plant organs, in which uptake of the precursors by different tissues and their intercellular transport, complicate the calculation of flux rates of metabolites. The lack of fully developed chloroplasts also eliminates the possibility of compartmentation of PAs in this organelle (Paschalidis and Roubelaskis-Angelakis, 2005).

The aim of this study was to evaluate the content and pattern of PAs, in haploid and DH plants and calli culture under light contrasting conditions.

and Galston, 1968). A crescent ploidy gradient from the apical to basal region of the stem, was associated to similar gradients of protein and nucleic acids and, to an inverse gradient of morphogenetic responses (Lavee and Galston, 1968). These gradients were correlated with IAA (indol 3-acetic acid) contents (Lavee and Galston, 1968) and peroxidase/IAA oxidase activity gradients along pith tissue (Kraus et al., 1981; Floh and Handro, 1985a). Haploid and poliploid pith tissues also exhibit the same pattern of

MATERIAL AND METHODS

Plant material

Nicotiana tabacum L. cv. W-38 haploid plants (2x=24) were first obtained from a single diploid plant (2n=4x=48), through anther culture (Nitsch, 1969). Pith tissue from different heights in the stem were explanted from one of these haploids, and cultured in a bud- inducing medium – RM-64 (Linsmaier and Skoog, 1965) with 2.0 $mg.L^{-1}$ IAA plus 0.2 $mg.L^{-1}$ kinetin. The regenerated shoots were excised and rooted in the same medium without growth regulators. A single diploid plant was grown for seed production from the population of the regenerated plants. Seeds of the diploid plants were sown with the objective of obtaining two populations of a different ploidy level and of comparable age. The populations (ca. 50 plants each) were homogeneous in appearance. The ploidy of each plant was estimated by counting the number of chloroplasts in the guard cells, which, in tobacco, was closely related to the ploidy level, especially in the case of haploid and diploid classes (Floh and Handro, 1985a).

Plant tissue culture

Stem segments (10 cm long) from five plants, with 60 days old, of each class of ploidy, and from the apical region (4 cm from the shoot apex) were surface sterilized and pith cylinders were obtained with a 5mm – diameter cork borer. Three explants, each 3 mm long, were cultured in 125 mL Erlenmeyer flasks. For sub-culturing at 40-days intervals, during 120 of days culture, small fragments (ca. 20 mg) from callus surface were inoculated under the same conditions as described above, in a medium supplemented with 2.0 $mg.L^{-1}$ IAA and 0.2 $mg.L^{-1}$ kinetin. These conditions were

chosen since pith tissues grow very differently, depending on their ploidy (Floh and Handro, 1985a). The cultures were maintained either in the dark at 27±1 °C, or under a 16 h photoperiod (27±1°C light, and 22±1°C dark), with a photon lux density of 150 $\mu molm^{-2}s^{-1}$ (day-light fluorescent lamp).

Polyamines determinations

Pith tissues cylinders (160 mg FM) of each ploidy level, from two regions, apical (4 cm from the shoot apex) and basal (ca. 30 cm from the apical segment), and 40 and 120 days old calluses (250 mg FM) of each treatment cultured, were stored at –80 °C for PAs determinations. All manipulations were carried out at 4^o C and the analyses were performed in triplicate, with three determinations for each aliquot.

PA determination was performed according to Silveira et al. (2004). The samples were ground in 3 mL of 5% (v/v) perchloric acid, and after 1 h, the samples were centrifuged for 20 min at 20,000 g at 4 °C. Free PAs were determined directly from the supernatant. Conjugated PAs were extracted by hydrolyzing 200 µL of the supernatant with 200 µL of 12 N HCl for 18 h at 110 °C. The samples were dried under nitrogen and then solubilized in 200 µL of 5% perchloric acid. Free and conjugated PAs were derived by dansyl chloride and identified by high performance liquid chromatography (HPLC), using a 5-µm reverse-phase column (Shimadzu Shin-pack CLC ODS). The gradient was developed by mixing increasing proportions of absolute acetronitrile to 10% acetonitrile in water (pH 3.5). The gradient of absolute acetonitrile was programmed to 65% over the first 10 min, from 65 to 100% for between 10 and 13 min, and 100% for between 13 and 21 min, at 1 $mL.min^{-1}$ flow at 40 °C. PA concentration was determined using a fluorescence detector at 340 nm (excitation) and 510 nm (emission). Peak areas and retention times were measured by comparison with standard PAs: Put, Spd and Spm.

Data analysis

All the experiments were repeated twice with similar results, the data presented being of a single representative experiment. Mean and standard error were applied to analyze the data.

RESULTS AND DISCUSSION

It was observed that the ploidy status of a plant could express different PA gradients content throughout the plant pith tissues. PAs titers followed a basipetal decrease in diploid, and an increase in haploid, pith tissues explants. Free PAs occurred in pith tissues of both haploid and diploid plants with higher Put levels, followed by Spd and finally Spm levels (Fig.1a-b). Free Put and Spd levels were higher in haploid pith tissues in the basal region (Fig. 1a) when compared to the apical region, with an inverse situation in diploid plants (Fig. 1b). An increase in the basipetal gradient of total free PAs, especially Put, throughout diploid *N. tabacum* L., cv. Samsun stem pith explants, was demonstrated by Altamura et al. (1993), and for diploid *N. tabacum* L. cv. Xanthi plants, by Paschalidis and Roubelaskis-Angelakis (2005). In plants, free PAs, Put in particular, comprised from 70 to 98% of their total content in the cell (Bagni and Tassoni, 2001).

Paschalidis and Roubelaskis-Angelakis (2005) suggested that PA synthesis and conjugation were likely to be an essential part of the homeostatic mechanism that controlled PAs levels and their involvement in differentiation processes. PAs conjugated with hydroxycinnamic acids could regulate the intracellular PA pool, serve for PA transport, or even be a substrate for aminooxidases and peroxidases (Kuznetsov et al., 2006). The free PAs/total ratio gradually increases basipetally, but the ratio of conjugated PAs decreases, with Spd mainly determining these changes (Paschalidis and Roubelaskis-Angelakis, 2005). In the present work, except for apical diploid pith tissues, with higher conjugated Spd contents, free PA contents were higher than conjugated ones, independent of the pith tissue region and ploidy.

Altamura et al. (1993) demonstrated in tobacco diploid plants that conjugated PAs did not show any particular trend, and only Spd was present in both free and conjugated forms, whereas Spm and Put were only in a free form. Regarding pith tissues of different ploidy, conjugated PA levels were lower in haploids when compared to diploids, especially for the apical region (Fig. 1a-b). In apical haploid pith tissues, Put and Spm were not detected (Fig. 1a). The conjugated Spd content in apical diploid pith tissues (Figs. 1b) was

higher when compared with apical haploid explants (Fig. 1a). It has been shown that the potential of shoot regeneration is greater in the apical explants when compared with basal explants (Lavee and Galston, 1968; Kraus et al., 1981), and the PAs Spd and Spm are related with differentiation, while Put is involved with cell divisions (Minocha et al., 1999; Niemi et al., 2002).The amount of free Put present in plant tissue is the net result of its biosynthesis, conjugated with phenolic acids, transport to other tissues, and degradation during a given period of time (Bhatnagar et al., 2002).

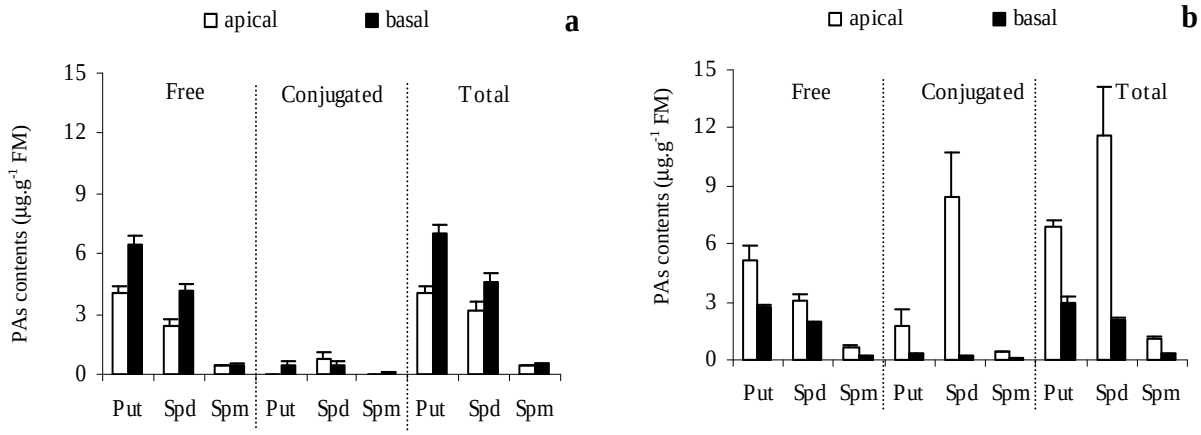

Figure 1 - PA patterns in pith tissue explants from haploid (**a**) and diploid (**b**) plants of *N. tabacum* cv. Wisconsin-38 (mean ± SD; n=3). Put = putrescine; Spd = spermidine; Spm = spermine.

Regarding PAs levels, the results demonstrated that pith tissues with initially low values, rapidly increased their content when explanted and cultured *in vitro*, independent of the original ploidy. This situation could reflect the stress condition that the material was submitted to at the time when it was explanted and cultivated *in vitro*. Similar results were obtained for peroxidase activity and protein content (Floh and Handro, 1985a). Biotic and abiotic stress signals induce similar responses in terms of polyamine metabolism, namely increases in titers and biosynthesis of PAs and/or their conjugates (Bouchereau et al., 1999). Cellular PAs can act as endogenous antioxidant molecules by inhibiting NADPH oxidase activity and O_2^- generation (Yoda et al., 2003; Papadakis and Roubelakis-Angelakis, 2005). Under conditions of stress (Bouchereau et al., 1999), it is also mostly Put that is to be found to fluctuate widely, without major changes in Spd and Spm, this suggesting that the levels of Spd and Spm in the cells are under tight homeostatic regulation. The exact mechanism of this homeostasis is not known. It could be achieved through their regulated biosynthesis, increased degradation, or both (Bhatnagar et al., 2002). In plants, the relationship between increased Put production and its catabolism is not clear. Although a number of laboratories have reported the production of transgenic cell lines and plants expressing genes for PA biosynthetic enzymes, and the resulting increases in cellular PA contents, none have investigated the effect of PA overproduction on their turnover and catabolism (Bhatnagar et al., 2002).

Although the influence of light and dark treatments on peroxidase activity in the same callus culture tissues of *N. tabacum* cv. Wisconsin-38 (Floh and Handro, 1985a) has been carried out, until now no work was undertaken to study the PA pattern in these tissue. The effects of light and dark culture conditions on endogenous levels of

PAs have been demonstrated by several authors (Bernet et al., 1999; Arena et al., 2003). In this work, a similar pattern of total PAs (free + conjugated) in diploid and haploid callus maintained in the light was observed (Fig. 2a), and in haploid calluses in the dark (Fig. 2b), increasing the values during 40 days of culture, and then decreasing until 120 days. However, diploid callus kept in dark conditions showed a constant increase until 120 days of culture (Fig. 2b). Dark conditions lead to a decrease in PA levels when compared with light treatment during rooting of *Berberis buxifolia* (Arena et al., 2003) and *Olea europaea* (Rugini, 1992), where a rapid degradation of PAs occurs in the dark.

During successive subcultures, free and conjugated Put titers were higher than Spd, with the lowest and most constant values for Spm being in the callus, independent of culture conditions and ploidy (Fig. 3 and 4). Similar results were observed in *in vitro* callus of *Vitis vinifera* (Bertoldi et al., 2004), *Prunus persica* (Liu and Moriguchi, 2007), and in *N. tabacum* cv. Wisconsin-38 leaves (Gemperlová et al., 2006).

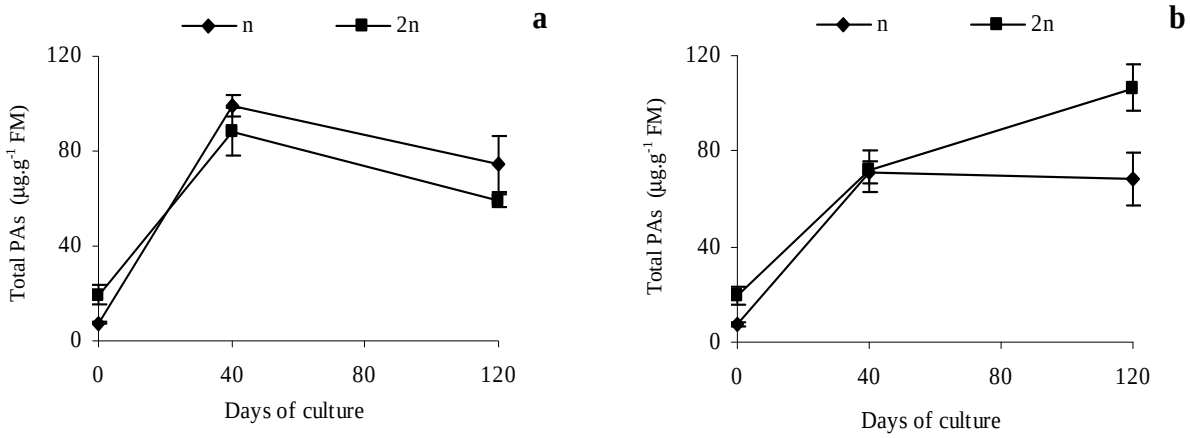

Figure 2 - Total PA levels in haploid (n) and diploid (2n) cell cultures from apical regions of *N. tabacum* cv. Wisconsin-38, cultivated in light (**a**) and dark (**b**) conditions (mean ± SD; n=3).

Higher levels of free Put, compared with Spd and Spm suggested that Put might be intimately involved in the regulation of cell growth through influencing cell division (Bouchereau et al., 1999; Liu and Moriguchi, 2007) and during active mitotic division (Gemperlová et al., 2005). On the other hand, Spd and Spm are involved in the growth inhibition and cell differentiation in different species, whereas Put is involved in culture growth (Niemi et al., 2002; Silveira et al., 2006).

In *N. tabacum*, the free Spd and Spm pattern in haploid and diploid callus, kept in light and dark treatments, showed an increase over 40 days, followed by a decrease by the end of culture at 120 days (Figs. 3a-d). However, free Put levels in diploid callus cultivated in the dark, exhibited an increase during 120 days of culture (Fig. 3d).

Similar results were observed by Bernet et al. (1999) with maize callus, which demonstrated that with cells cultured in a maintenance medium, the changes were not significant in light and dark treatments, except for free Put levels which increased in the dark period of culture. In plants, PAs often occur as free molecular bases, but they can also be associated with small molecules such as phenolic acids (conjugated forms), by the formation of an amide linkage (reviewed in

Bouchereau et al., 1999). Although the contents of conjugated PAs soluble in perchloric acid, can vary during the cell cycle (Altamura et al., 1993; Bouchereau et al., 1999), their metabolic function has not yet been elucidated. In the present work, conjugated Put and Spm levels expressed a similar pattern variation, independent of light conditions and ploidy (Figs. 4a-d), whereas conjugated Spd contents were lower in haploid and diploid callus cultivated in the dark (Figs. 4c and d). The results observed in this work suggested the possible effects of a dark condition on endogenous conjugated Spd levels. However, until now few papers have been published showing the effects of the dark on conjugated PA contents (Bernet et al., 1999). These authors showed an increase in conjugated Spd and Spm levels during the dark period of maize callus culture, whereas free Put, Spd and Spm levels decreased during the same period.

The results observed in this work indicated that the endogenous PA pattern in *N. tabacum* cv. Wisconsin-38, was not only light/dark affected, but also regulated by ploidy. PAs levels showed a basipetal pattern according to the ploidy level of the pith tissues. The ploidy of *in vitro* callus under dark conditions affected the levels of total PAs (free + conjugated), thereby especially influencing free Put contents. In addition, conjugated Spd levels were affected by callus ploidy and culture conditions.

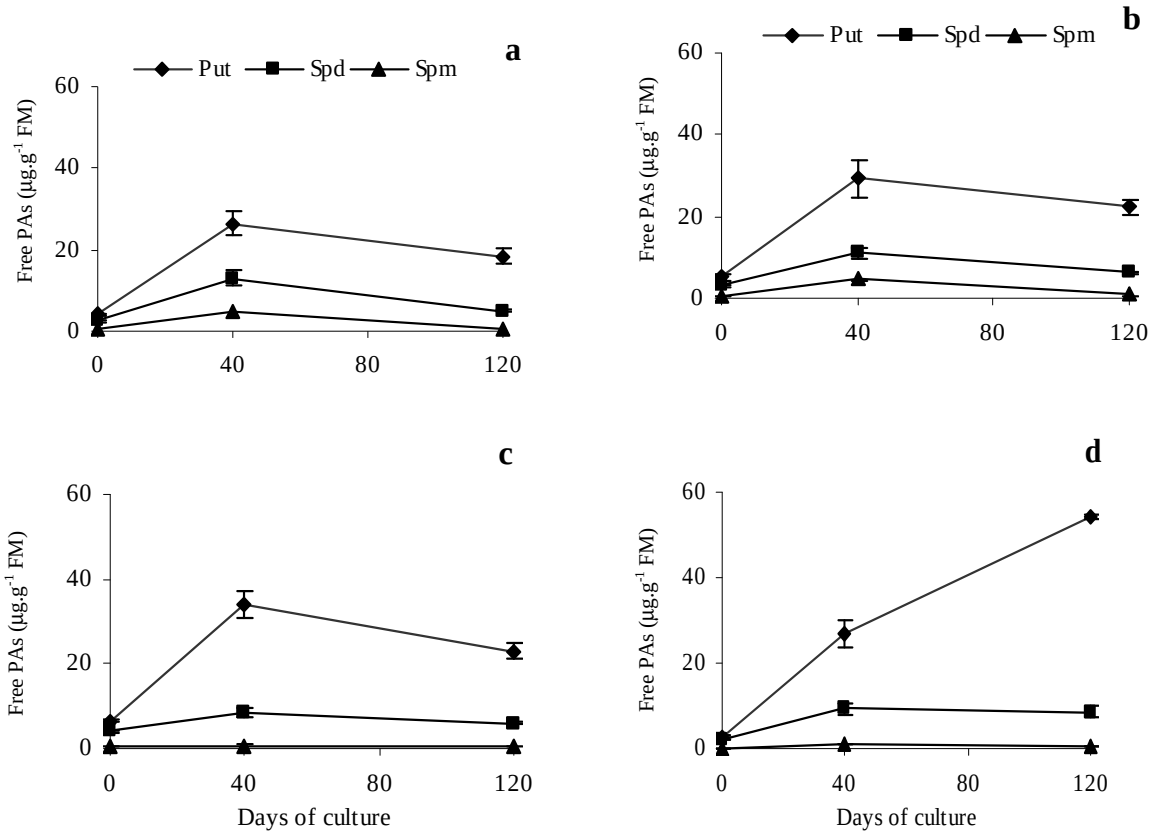

Figure 3 - Free PA levels in haploid (**a** and **c**) and diploid (**b** and **d**) cell cultures from apical regions of *N. tabacum* cv. Wisconsin-38, cultured in light (**a** and **b**) and dark (**c** and **d**) conditions (mean ± SD; n=3).

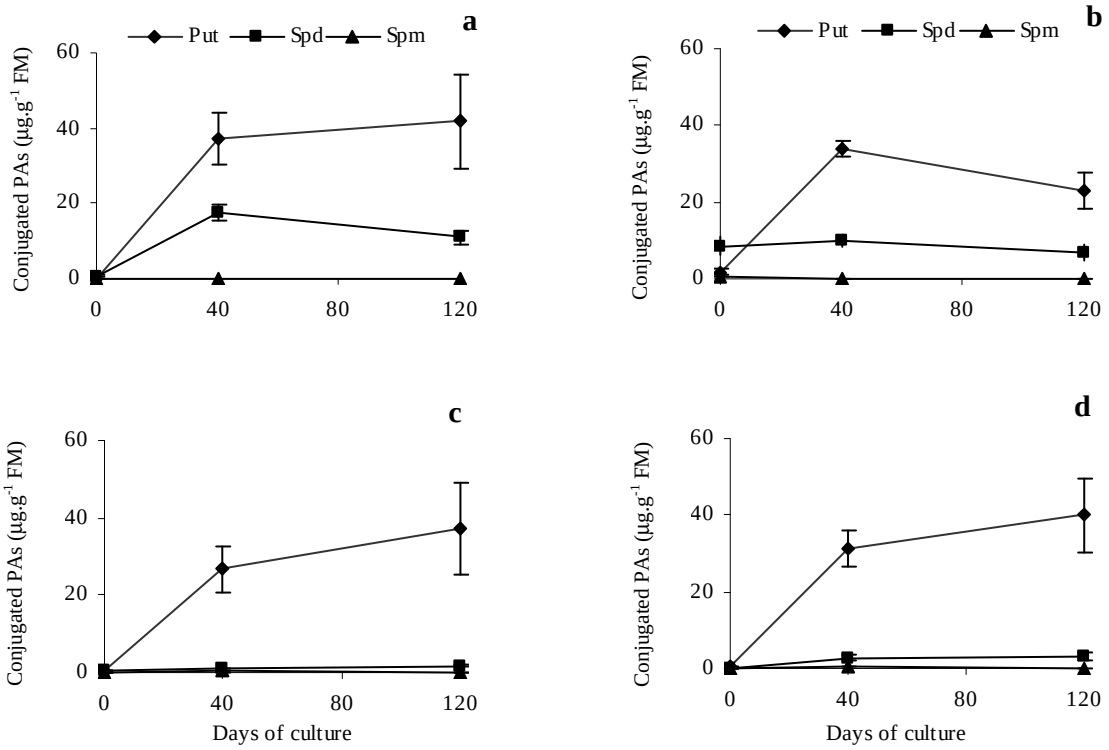

Figure 4 - Conjugated PA levels in haploid (**a** and **c**) and diploid (**b** and **d**) cell cultures from apical regions of *N. tabacum* cv. Wisconsin-38, cultured in light (**a** and **b**) and dark (**c** and **d**) conditions (mean ± SD; n=3).

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RESUMO

O objetivo deste trabalho foi determinar os níveis de PAs em tecidos de medula e cultura de calos de plantas haplóides e diplóides de tabaco, obtidas da região apical e basal do caule. Estes explantes foram cultivados em meio RM-64 suplementado com AIA e cinetina, na luz e no escuro, durante vários subcultivos. Nos tecidos medulares, os níveis de PAs apresentam um decréscimo basípeto em diplóides e um aumento em haplóides.Um padrão similar nos níveis de PAs totais (livres+ conjugadas) foi observado em calos haplóides e diplóides mantidos na luz, e haplóides no escuro, enquanto os diplóides cultivados no escuro mostraram um aumento constante até o final do cultivo. O aumento no conteúdo de PAs nos calos diplóides no escuro, foi devido ao aumento do conteúdo de Put livre. Foi observado que a ploidia da planta pode expressar diferentes gradientes de PA ao longo do tecido medular e nas culturas de calos in vitro.

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