Aluminium Effects on Embryo Suspensor Polytene Chromosomes of *Phaseolus coccineus* L.

G. BARTOLI, L. SANITÀ DI TOPPI, A. ANDREUCCI & M. RUFFINI CASTIGLIONE

Department of Biology, University of Pisa, Via L. Ghini 13, 56126 Pisa, Italy

Correspondence: Monica Ruffini Castiglione, Department of Biology, University of Pisa, Via L. Ghini 13, 56126 Pisa, Italy, tel +390502211317, email: monica.ruffini.castiglione@unipi.it

Abstract:

Aluminium (Al) represents a widespread environmental pollutant, with severe toxic impacts on plants. In this study we documented for the first time the structural and functional responses induced by two concentrations of $AlCl_3$ (10^{-2} M and 10^{-1} M) in the polytene chromosomes that characterize the chromatin organization in the embryo suspensor cells of *Phaseolus coccineus*.

Polytene chromosomes showed signs of dose-dependent genotoxicity following $AlCl_3$ treatments with a significant increase of both chromatin stickiness and chromatin fragmentation. Polytene chromosomes specifically reacted to $AlCl_3$ also in terms of DNA and RNA puffing activity: with respect to the control, the treatments promoted *ex-novo* and/or inhibited puff formation along chromosome arms, suggesting a fine modulation of the differential genome activity in response to the treatments. The nuclei of suspensors from control and treated seeds showed nucleoli mainly arranged by more than one NOR-bearing chromosome. In addition, $AlCl_3$ treatments affected the frequency of nucleoli organized by singular organizer chromosomes, with an increase in the frequencies of nucleoli organized by chromosome II and a reduction in the frequencies of those organized by chromosomes I or V. These results confirm that, also in our system, nucleolus may react as stress response organelle.

Key words: Aluminium, DNA/RNA puffs, nucleolus, *Phaseolus coccineus*, polytene chromosomes, toxicity.

Introduction

One of the most relevant problems for aquatic and terrestrial organisms, as well as for crop production, is represented by toxic metals. Aluminium (Al), a so called “light metal”, constitutes just over 8% of the earth's crust, representing the most abundant metal, but it is poorly bio-available in neutral and weakly acidic soil; when released and solubilised into soil solutions in acid conditions, Al toxicity has long been documented (Rout et al. 2001; Kochian et al. 2015).

The toxic effects of Al have been recognized at different levels of plant organization. Al-sensitive higher plants show a marked root growth inhibition, as the primary effect, due to tissue injury and modifications to the root apex caused by the inorganic Al monomers and polycations, that can directly explicate their toxicity by binding components of the cell wall and of the plasma membranes (Liu et al. 2008; Li et al. 2015).

Al causes a rapid decrease in both cell wall viscosity and elasticity, particularly in the root apex, partially changing chemical structure of the cell wall and inhibiting cell expansion (Ma et al. 2004). Negatively charged pectin component may be one of the most important Al binding sites (Horst et al. 2010). Notwithstanding this, in *Arabidopsis* it seems that hemicelluloses are the major cell wall component able to interact with Al (Yang et al. 2011). This interaction may inhibit the activity of xyloglucan endotransglucosylase, the enzyme involved in the cell wall loosening (Fry et al. 1992). Al symplastic interaction with the plasma membrane mainly occurs with phospholipids and provokes structural and functional changes leading to a severe cytotoxicity, inducing oxidative stress and lipid peroxidation (Yamamoto et al. 2001, 2003; Ahn & Matsumoto 2006). Other effects of soluble Al, especially those observable on the aerial part of the plant, seem to be mainly ascribed to the indirect effects of

Al, interfering with water absorption and with the uptake and transfer of some essential nutrients within the plant body (Rout et al. 2001; Ozyigit et al. 2013).

Many reports have described other specific Al effects in a large number of cellular processes (Panda & Matsumoto 2007), influencing the dynamics of the cytoskeleton (Frantzios et al. 2005) with effects on vesicle movement and chromosome segregation, growth and cell division (Doncheva et al. 2005) and synthesis of callose (Lian et al. 1998). Additionally, changes on chromosome morphology, aberrations and disturbance in the nucleolar cycle during mitosis have been also reported (Yi et al. 2010; Zangh et al. 2014). As a result of Al-induced stress, changes in gene expression have been described (Sivaguru et al. 2003; Eticha et al. 2010) with the identification of several genes involved in the response of tolerant plants.

In this work, we studied the cytological effects resulting from $AlCl_3$ treatments in a highly differentiated system, the embryo suspensor of *Phaseolus coccineus*. The *P. coccineus* suspensor, structured in about two hundred cells, persists until the cotyledonary embryo stage, after having undergone autolysis, considered to be a typical example of the so-called developmental programmed cell death (PCD) (Lombardi et al. 2007). As in other plant species, *P. coccineus* suspensor cells are involved in endoreduplication phoenomena, especially at the micropilar end of the organ, where about 20 cells become giant, in the end reaching a DNA content of 8192C (Brady 1973). Endoreduplication, amplifying the whole cell genome, provides a mechanism to increase the level of gene expression per nucleus (Larkins et al. 2001). This activity is related to the formation of unpaired polythene chromosomes (2n = 22) in a permanent prophase stage.

In *P. coccineus* suspensor cells, some polytene chromosome regions may be engaged in "DNA puffing", which is considered to be the result of disproportionate localized DNA replication (DNA amplification). In addition, massive transcription processes may be cytologically detectable as regions in which the chromatin is decondensed and expands in

"RNA puffs" (Frediani & Simonini 1980). DNA and RNA puff frequency differently characterize the embryo developmental stages and may variate depending on metabolic demand during seed development and on environmental factors (Tagliasacchi et al. 1993).

Due to their structure and size, polytene chromosomes can be considered a cytogenetic model system, allowing the evaluation, *in vivo* and *in vitro*, of structural and functional chromatin changes following stressful conditions (Nagl 1970; Sholes & Paige 2015). On this basis, the aim of our work was to study the cytological response of the *P. coccineus* embryo suspensor following $AlCl_3$ treatment at two different concentrations. Since it has been well documented that stress perception at cellular level may influence nucleolus organization and dynamic (Boulon et al. 2010), we also focused our attention on the assessment of possible perturbations involving nucleolus and in its cytological organization.

Materials and Methods

Plant Material and $AlCl_3$ Treatments

Developing seeds of *Phaseolus coccineus* L. were collected from fruits taken from plants grown in the open air at the Botanical Garden of the University of Pisa (Italy). 11 mm-long seeds, mainly containing embryos at the early cotyledon stage, were considered in this study. The actual developmental stage of the embryo suspensor was assessed on histological sections. Briefly, 10 seeds randomly selected were dissected and the portion containing the embryo suspensor was fixed and processed according to Bartoli et al. (2016). Semi-thin sections were stained with toluidine blue O (TBO, 0.05 % in 0.1 M benzoate buffer at pH 4.4) for histological investigations. Having verified the homogeneity of the developmental stage of the suspensor in the seed pool, the seeds were kept for 24 hours in distilled water (the control samples) or in aqueous solution of $AlCl_3$ (Sigma-Aldrich) at two different concentrations (10^{-2}

M and 10^{-1} M). The Al concentrations were selected to be applied for a short time but relevant to assess the impact of the metal to our system, reproducing acute exposure.

Both the treated and the control seeds were fixed in ethanol and acetic acid (3:1, v/w) for 2 hours and the embryo suspensors were then excised under a dissecting microscope.

Cytological Chromosome Staining

Fifty embryo suspensors from the Al-treated and the control seeds were macerated with a 5% aqueous solution of pectinase (Sigma-Aldrich) at 37 °C for 40 minutes and then squashed under a cover slip in a drop of 45% acetic acid. The slides were frozen with dry ice, and the coverslips removed. The slides obtained were then stained with Giemsa (Merk) solution at 2% in stock solution in phosphate buffer at pH 8, for a general observation of the squashes, and the best slides were processed with the Feulgen method according to Giorgetti and Ruffini Castiglione (2016) for specific DNA staining. The samples were then air-dried, mounted in DPX (Fluka) and analysed using a Leitz Diaplan light microscope (Wetzlar, Germany). Images of each slide were captured using a Leica DFC 420 camera (Leica Microsystems, Germany) and polytene chromosomes were identified as described by Nagl (1967). At least 20 slides for both the control and the Al-treated suspensors were considered for the present investigation.

AgNOR Staining

AgNOR staining was used as a rapid method for visualizing ribosomal gene activities. Ten embryo suspensors, isolated from the treated and control seeds, were fixed and squashed as previously described. The squashes were air dried and then subjected to AgNOR staining, according to Howell and Black (1980) with minor modifications. Briefly, the squashes were treated with freshly prepared silver colloidal solution (1 part by volume of 2% gelatin in 1% formic acid and two parts by volume of 50% aqueous silver nitrate solution) in a closed coupling jar for 30 min at 37 °C, while ensuring that a dark environment was maintained

throughout the reaction time. The slides were washed thoroughly with distilled water, counterstained with Giemsa solution (2% stock solution in phosphate buffer, pH 6.8) and dehydrated. After drying, the slides were cleared in xylene, mounted in DPX and observed. The number of nucleoli organized by single and multiple NOR-bearing chromosomes (I, II, V) in both embryo suspensor cells from control and $AlCl_3$ treated seeds were evaluated by captured images. Twenty randomly selected polytene nuclei were analysed for each treatment and for control.

Data Analysis

The data acquired were statistically processed by analysis of variance (ANOVA) following *post-hoc* multiple comparison (Bonferroni test).

Results and Discussion

In this work we have assessed the structural and functional responses induced by 10^{-2} M and 10^{-1} M concentrations of $AlCl_3$ on a highly differentiated and high-resolution cytogenetic system: the polytene chromosomes of the embryo suspensor of *P. coccineus*.

Figure 1A shows a schematic drawing of the immature 11 mm long seed of *P. coccineus* (at the early cotyledonary stage) with the embryo proper and the suspensor. A representative image of the embryo suspensor is reported in Figure 1B: the fully developed suspensor appears to be formed by giant cells organized in a knob at the micropilar side, connected to the embryo proper by means of smaller sized cells of the neck region. Polytene chromosomes and chromosomal NORs in the embryo suspensor giant cells are shown in Figure 1 C, D, E. Figure 1F illustrates the ideogram of the polytene chromosomes of *P. coccineus*. The satellited chromosomes I and V and the submetacentric chromosome II bear ribosomal cistrons (Nagl 1967; Forino et al. 1979). These chromosomes are differently involved in the

formation of the nucleolus depending on the embryo developmental stage, because they possess functional and structural heterogeneous ribosomal cistrons (Pierotti et al. 1998).

The cytogenetic analysis of these chromosomes showed signs of dose-dependent genotoxicity following $AlCl_3$ treatments. This toxicity was basically detectable as chromatin stickiness that gives the chromatin a trabecular meshwork appearance (Figure 2 A, B) and chromatin fragmentation with microsphere extrusion (Figure 2 C, D), mainly from telomeric regions. As reported in Table I, $AlCl_3$ 10^{-2}M induced chromatin stickiness in more than 18% and chromatin extrusion in more than 17% of the analyzed chromosomes, while the treatment with $AlCl_3$ 10^{-1}M induced a significant increase of both types of genotoxic damage (40.6% and 70.3% respectively). Stickiness and chromatin microsphere extrusions are not ordinary features in suspensor cells, but they provide significant evidence of Al-induced genotoxic effects. Chromatin stickiness is characterized by severe chromosome clustering during any phase of the cell cycle (Ritambhara & Kumar 2010). As a consequence, it is a type of chromatin aberration which is easily detectable, including in such a differentiated and peculiar system such as polytene nuclei. As far as chromatin extrusion is concerned, it may be a secondary effect related to a necessary genome rearrangement following a stress condition (Aguilera & Gómez-González 2008) such as the Al treatment, or simply the result of clastogenic activity of the metal on DNA (Yi et al. 2010). Even if Al cannot directly catalyze redox reactions, however, the soluble forms Al^{3+} may trigger oxidative stress occurrence, as reported in different plant systems (Yamamoto et al. 2003), which generate hydrogen peroxide and other reactive oxygen species, which are key modulators of DNA fragmentation (Ruffini Castiglione et al. 2014). However, ROS could act as signaling molecules, stimulating or repressing specific genes involved in defence, repair and compensation processes following stress exposures (Bartoli et al. 2013). Changes in puffing activity can be considered one of the hallmarks of stress response in animal polytene chromosome systems (Singh & Singh 2015).

In *Phaseolus* too, as early as the 1970s, it was suggested that there was a correlation between structural changes in polytene chromosomes and temperature disturbance, as an expression of changes in gene activity (Nagl 1970). From our studies, we have recorded that $AlCl_3$ treatments induced significant perturbations in the puffing activity and in nucleolus-organizing polytene chromosomes features and behaviour. Figure 3 depicts the puffing activity of the polytene NOR-bearing chromosomes in the embryo suspensor cells from the control and treated immature seeds. Compared to the control, $AlCl_3$ treatments induced significant reorganization events in the chromatin of NOR-bearing chromosomes. We observed the amplification of specific chromosome regions (i.e. the splitting of the apical portions of the NOR; Figure 3C, H, I), putatively interpreted as a specific cytogenetic reaction to Al stress. We also noted different degrees of condensation of specific chromosome regions (Figure 3B, C, D, E, H), which is a sign of a differential transcriptional activity of the loci clustered on these chromosome portions.

Table II reports the puffing frequencies resulting from the detailed analysis of cytologically detectable DNA and RNA puffs in the whole set of polytene chromosomes. Several differences in the puffing activity of the DNA were detectable following the $AlCl_3$ treatments, often with heterogeneous behaviour of the single chromosomes and/or of the different regions within the same chromosome. Chromosome pair II (band A), pair IV (bands A, B) and IX (band E) displayed an increasing trend of the puffing activity while pair I exhibited a decrease in puffing activity (band B) compared to the control. In some cases, specific chromatin regions of certain chromosome pairs were induced by the treatment to *ex-novo* puffing (e.g. band E of chromosome pair I, band D of chromosome pair II, band D of chromosome VII), in parallel with other chromosome regions involved in DNA puffing in the control samples and that stopped this activity following $AlCl_3$ treatments (e.g. band B of chromosome VII, bands

B and E of chromosome VIII). On the contrary, chromosome X and XI seemed not particularly affected by the treatments concerning DNA puffing.

With reference to RNA puffing (Table II), in this case too, we have observed significant changes depending on the two $AlCl_3$ treatments. The RNA puffing frequencies varied widely among different regions, also in the same chromosome pair. In almost all the chromosomes of the complement at least one or more regions were characterized by the loss of RNA puffing activity, which was instead detected in control samples. These are: band G of chromosome I; band A, E of chromosome III; band G, I, L of chromosome IV; band C, F, G, H of chromosome VI; band A, B of chromosome VII; band E of chromosome VIII; band A, B, C, F of chromosome IX; band A of chromosome X and band A of chromosome XI. Some *ex novo* concentration-dependent RNA puffing was recordable as well in some specific chromosome regions of three different chromosomes: III (band B and C), V (band C), VIII (band C). In addition, other chromosome regions, which were already engaged in RNA puffing, were positively induced in control samples, especially at the $AlCl_3$ higher concentration (e.g. band E of chromosome I; bands A and F of chromosome II; band B of chromosome VI; bands A and F of chromosome VIII).

These results confirm the high responsiveness of the polytene chromosomes to Al and demonstrate that this metal may interfere strongly with the stage-specific chromatin activity of the embryo suspensor, disturbing chromosome puffing differently, inhibiting puffing formation and resulting in specific new puffs. We may speculate that these results partly depend on both a significant structural/functional chromatin injury and on a differential activation of specific stress-responsive genetic loci elicited by Al treatments and probably involved in the stress defence. In our highly differentiated system of study, we cannot exclude that some *ex-novo* puffing activities may be related to stress-induced copy number variation of specific genes as reported for maize. In this species a greater gene copy number of Al

tolerant gene MATE1 is the basis for higher MATE1 expression levels, resulting in increased Al tolerance (Maron et al. 2012).

We also focused our attention on the assessment of possible perturbations involving nucleolus and its cytological organization. Figure 4 shows nucleoli organized by both multiple and single NOR-bearing chromosomes, as obtained by squashing embryo suspensor basal cells. The stress perception at cytological level can be accompanied by nucleolar dysfunctions, the nucleolus being a well recognized stress sensor, able to coordinate stress response (Forino et al. 2012; Bellani et al. 2014). Little information is available about the effects of Al on plant nucleoli: in root tip cells of *Vicia faba* and *Allium cepa* the nucleolus showed an altered distribution and loss of argyrophilic material from the nucleus to the cytoplasm, as well as an abnormal nucleolar cycle during mitosis (Zhang et al. 2009; Qin et al. 2010).

In our highly differentiated system, at the cotiledonary stage, the nucleolus was basically organized by more than one NOR-bearing chromosome (Figure 4 and Table III) and this functional trait did not significantly change following $AlCl_3$ treatments (Table III). In contrast, significant differences appeared when the frequencies of distinct NOR-bearing chromosomes, active in nucleoli formation, were taken into account. In the control samples, chromosome I was the most engaged in the formation of nucleoli organized by just one chromosome (Table III) while chromosomes II and V were similarly involved in the nucleolus organization. $AlCl_3$ treatments affected the activity of chromosome V in nucleolus organization, reducing its contribution by 50% in respect to the control, already at the lowest concentration. Interestingly, chromosomes I and II underwent a considerable $AlCl_3$ dose dependent disturbance in the nucleolus organization contribution, showing, however, an opposite behaviour. The percentage of chromosome I in nucleolus formation passes from 39.47% in the control to 18.66% and 4.61% at $AlCl_3$ 10^{-2} and 10^{-1} M respectively, while chromosome II goes from 29.94% in the control to 66.98% and 80.83% at $AlCl_3$ 10^{-2} and 10^{-1}

M respectively (Table III). The treatments with $AlCl_3$ therefore induced a differential behaviour of the three NOR-bearing chromosomes in terms of nucleolar organization. Chromosome II, which is generally not very active in the organization of the nucleolus during embryogenesis and which bears short ribosomal genes (Tagliasacchi et al. 1993), was rather active in the treated samples, while the chromosome I, usually the most active during the suspensor life span and bearing the longest ribosomal genes (Tagliasacchi et al. 1993), showed decreased activity. This peculiar behaviour can be correlated with the different sensitivity of ribosomal genes on the different NORs that characterizes the two chromosome pairs I and II. Moreover it cannot be excluded that genes other than ribosomal ones and associated with NORs, may be differently modulated and whose expression correlates with Al toxicity and/or tolerance.

On the whole, the results of the present study give new and interesting insights into the effects of Al on polytene chromosomes of the *P. coccineus* embryo suspensor. These peculiar chromosomes were specifically responsive to the $AlCl_3$ treatments and showed, from a cytological point of view, signs of dose-dependent genotoxicity, genome rearrangements, and changes in functional activity in terms of DNA and RNA puffing. In the *P. coccineus* embryo suspensor, the puffing activity occurs in a stage-specific way, with a recognizable cytological pattern. Consequently, the observed changes in the puffing pattern cannot be a random phenomenon but must be specifically due to Al exposure and probably related to a differential expression of Al tolerance genes. $AlCl_3$ strongly influenced the nucleolus organization in terms of number and type of chromosomes involved in its constitution. Therefore, also in *P. coccineus,* the nucleolus seems to have a central regulatory role between ribosome biosynthesis and cellular metabolism during stress condition.

Acknowledgements

The authors are grateful to Professors Laura Forino and Anna Maria Tagliasacchi for their helpful discussion and critical reading of the manuscript. The authors thank Prof. Patrick Johnson for the final linguistic revision of the manuscript.

Funding

This work was supported by local funding of the University of Pisa (ex 60 %).

References

Aguilera A, Gómez-González B. 2008. Genome instability: a mechanistic view of its causes and consequences. Nat Rev Genet 9: 204–217.

Ahn SJ, Matsumoto H. 2006. The role of the plasma membrane in the response of plant roots to aluminum toxicity. Plant Signaling Behav 1: 37–45.

Bartoli G, Forino LMC, Tagliasacchi AM, Durante M. 2013. Cell death induced by ozone stress in the leaves of *Populus deltoides* × *maximowiczii*. Biol Plant 57: 514–524.

Bartoli G, Felici C, Ruffini Castiglione M. 2017. Female gametophyte and embryo development in *Helleborus bocconei* Ten. (*Ranunculaceae*). Protoplasma 254: 491-504.

Bellani LM, Muccifora S, Giorgetti L. 2014. Response to copper bromide exposure in *Vicia sativa* L. seeds: analysis of genotoxicity, nucleolar activity and mineral profile. Ecotoxicol Environ Saf 107: 245–250.

Boulon S, Westman BJ, Hutten S, Boisvert FM, Lamond AI. 2010. The nucleolus under stress. Mol Cell 40: 216–227.

Brady T. 1973. Feulgen cytophotometric determination of the DNA content of the embryo proper and suspensor cells of *Phaseolus coccineus*. Cell Differ 2: 65–75.

Carmignani A, Andreucci A, Tagliasacchi AM, Ruberti F, Forino L. 1999. Effects of α-amanitin on the transcription of the NOR of polytene chromosomes of *Phaseolus coccineus* embryo suspensor during embryogenesis. Cytobios 98: 7–15.

Doncheva S, Amenós M, Poschenrieder C, Barceló J. 2005. Root cell patterning: a primary target for aluminium toxicity in maize. J Exp Bot 56: 1213–1220.

Durante M, Cionini PG, Avanzi S, Cremonini R, D'Amato F. 1977. Cytological localization of the genes for the four classes of ribosomal RNA (25S, 18S, 5.8S and 5S) in polytene chromosomes of *Phaseolus coccineus*. Chromosoma 60: 269-282.

Eticha D, Zahn M, Bremer M, Yang Z, Rangel AF, Rao IM, Horst WJ. 2010. Transcriptomic analysis reveals differential gene expression in response to aluminium in common bean (*Phaseolus vulgaris*) genotypes. AoB 105: 1119–1128.

Forino LMC, Tagliasacchi AM, Avanzi S. 1979. Different structure of polytene chromosomes of *Phaseolus coccineus* suspensors during early embryogenesis. 1. Nucleolus organizing chromosome pairs S_1 and S_2. Protoplasma 101: 231–246.

Forino LMC, Ruffini Castiglione M, Bartoli G, Balestri M, Andreucci A, Tagliasacchi AM. 2012. Arsenic-induced morphogenic response in roots of arsenic hyperaccumulator fern *Pteris vittata*. J Hazard Mater 235-236: 271–278.

Frantzios G, Galatis B, Apostolakos P. 2005. Aluminium causes variable responses in actin filament cytoskeleton of the root tip cells of *Triticum turgidum*. Protoplasma 225: 129–140.

Frediani M, Simonini AL. 1980. Alcune caratteristiche strutturali dei cromosomi politenici del sospensore embrionale di *Phaseolus coccineus* in due momenti dell'embriogenesi. Giorn Bot Ital 114: 251–266.

Fry SC, Smith RC, Renwick KF, Martin DJ, Hodge SK, Matthews KJ. 1992. Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. Biochem J 282: 821–828.

Giorgetti L, Ruffini Castiglione M. 2016. Oil palm in vitro regeneration: microdensitometric analysis during reproduction and development. Caryologia 69: 5–11.

Horst WJ, Wang Y, Eticha D. 2010. The role of the root apoplast in aluminium-induced inhibition of root elongation and in aluminium resistance of plants: a review. AoB 106: 185–197.

Howell WMT, Black DA. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36: 1014–1015.

Hutchinson GE. 1943. The biogeochemistry of aluminium and certain related elements. The Quarterly Review of Biology 18: 128–153.

Kawashima T, Goldberg RB. 2010. The suspensor: not just suspending the embryo. Trends Plant Sci 15: 23–30.

Kochian LV, Pineros MA, Liu J, Magalhaes JV. 2015. Plant adaptation to acid soils: the molecular basis for crop aluminum resistance. Annu Rev Plant Biol 66: 571–598.

Larkins BA, Dilkes BP, Dante RA, Coelho CM, Woo YM, Liu Y. 2001. Investigating the hows and whys of DNA endoreduplication. J Exp Bot 52: 183–192.

Li M, Qin R, Jiang W, Liu D. 2015. Cytogenetical effects of aluminium on root meristem cells of *Helianthus annuus* L.. Botanical Sciences 93: 15–22.

Lian C, Oi wake Y, Yokota H, Wang G, Konishi S. 1998. Effect of aluminum on callose synthesis in root tips of tea (*Camellia sinensis* L.) plants. Soil Sci Plant Nutr 44: 695–700.

Liu Q, Yang JL, He LS, Li YY, Zheng SJ. 2008. Effect of aluminum on cell wall, plasma membrane, antioxidants and root elongation in triticale. Biol Plant 52:87–92.

Lombardi L, Ceccarelli N, Picciarelli P, Lorenzi R. 2007. DNA degradation during programmed cell death in *Phaseolus coccineus* suspensor. Plant Physiol Biochem 45: 221-227.

Ma JF, Shen R, Nagao S, Tanimoto E. 2004. Aluminum targets elongating cells by reducing cell wall extensibility in wheat roots. Plant Cell Physiol 45: 583–589.

Maron LG, Guimarães CT, Kirst M, Albert PS, Birchler JA, Bradbury PJ, Magalhaes JV, Piñeros MA, Schatz MC, Wing RA, Kochian LV. 2013. Aluminum tolerance in maize is associated with higher MATE1 gene copy number. Proc Natl Acad Sci USA 110: 5241–5246.

Nagl W. 1967. Die Riesenchromosomen von *Phaseolus coccineus* L.: Baueigentümlichkeiten, Strukturmodifikationen, zusätzliche Nukleolen und Vergleich mit den mitotischen Chromosomen. Österreichische Botanische Zeitschrift 114: 171–182.

Nagl W. 1970. Temperature-dependent functional structure in the polytene chromosomes of *Phaseolus*, with special reference to the nucleolus organizers. J Cell Sci 6: 87–107.

Ozyigit II, Vardar F, Yasar U, Akinci S. 2013. Long-term effects of aluminum and cadmium on growth, leaf anatomy, and photosynthetic pigments of cotton. Commun Soil Sci Plant Anal 44: 3076–3091.

Panda S, Matsumoto H. 2007. Molecular physiology of aluminum toxicity and tolerance in plants. Bot Rev 73: 326–347.

Pierotti L, Andreucci AC, Giraldi E, Tagliasacchi AM. 1998. Polytene chromosomes of the embryo suspensor of *Phaseolus coccineus* L. during senescence. Cytobios 96: 31–43.

Qin R, Jiao YQ, Zhang SS, Jiang WS, Liu, DH. 2010. Effects of aluminum on nucleoli in root tip cells and selected physiological and biochemical characters in *Allium cepa* var. *agrogarum* L. BMC Plant Biol 10: 225.

Ritambhara T, Kumar G. 2010. Genetic loss through heavy metal induced chromosomal stickiness in Grass pea. Caryologia 63: 223–228.

Rout G, Samantaray S, Das P. 2001. Aluminium toxicity in plants: a review. Agronomie 21: 3–21.

Ruffini Castiglione M, Giorgetti L, Cremonini R, Bottega S, Spanò C. 2014. Impact of TiO_2 nanoparticles on *Vicia narbonensis* L.: potential toxicity effects. Protoplasma 251: 1471-1479.

Scholes DR, Paige KN. 2015. Plasticity in ploidy: a generalized response to stress. Trends in Plant Sci 20: 165–175.

Sivaguru M, Ezaki B, He Z, Tong H, Osawa H, Baluška F, Volkmann D, Matsumoto H. 2003. Aluminum-induced gene expression and protein localization of a cell wall-associated receptor kinase in *Arabidopsis*. Plant Physiol 132: 2256–2266.

Singh A, Singh R. 2015. Genotoxic effects of cadmium chloride on polythene chromosomes of *Sarcophaga ruficornis* (Fab.) (Sarcophagidae: Diptera). IJDR 5: 5458–5462.

Stockert JC, Blázquez-Castro A, Horobin RW. 2014. Identifying different types of chromatin using Giemsa staining. Methods Mol Biol 1094: 25–38.

Tagliasacchi AM, Forino LMC, Frediani M, Cremonini R, Tucci G, Maggini F, Avanzi S. 1993. Ribosomal RNA genes in *Phaseolus coccineus.* 2. Differential distribution of ribosomal cistrons and cytological localization of various replication units in polytene chromosomes of embryo suspensor. Cytobios 75: 137–147.

Yamamoto Y, Kobayashi Y, Matsumoto H. 2001. Lipid peroxidation is an early symptom triggered by aluminum, but not the primary cause of elongation inhibition in pea roots. Plant Physiol 125: 199–208.

Yamamoto Y, Kobayashi Y, Devi SR, Rikiishi S, Matsumoto H. 2003. Oxidative stress triggered by aluminum in plant roots. Plant Soil 255: 239–243.

Yang JL, Zhu XF, Peng YX, Zheng C, Li GX, Liu Y, Shi YZ, Zheng SJ. 2011. Cell wall hemicellulose contributes significantly to aluminum adsorption and root growth in *Arabidopsis*. Plant Physiol 155: 1885–1892.

Yi, M., Yi, H., Li, H., and Wu, L. 2010. Aluminum induces chromosome aberrations, micronuclei, and cell cycle dysfunction in root cells of *Vicia faba*. Environ. Toxicol. 25(2): 124–129.

Zhang H, Zhang S, Meng Q, Zou J, Jiang W, Liu D. 2009. Effects of aluminium on nucleoli in root tip cells, root growth and the antioxidant defense system in *Vicia faba* L. Acta Biol Cracov (Series Botanica) 51: 99-106.

Figure captions

Figure 1. The embryo suspensor of *Phaseolus coccineus*. (A) Schematic drawing of the median longitudinal section of an 11 mm long seed showing the embryo proper (ep) at cotyledonary developmental stage, its suspensor (s) and the endosperm (en); the seed micropyle (m) and the funiculus (f) are also indicated. Scale bar = 2 mm. (B) Median longitudinal section of an ovule stained with toluidine blue O, showing the embryo suspensor with the giant basal cells constituting the knob region (k) and the cells close to the embryo of the neck region (n); the embryo proper (ep) and the endosperm (en) are also indicated. Scale bar = 100 µm. (C) Histological section of the basal portion of the suspensor, stained with toluidine blue O, showing a polytene nucleus (nn) with the nucleolus organized by a NOR-bearing chromosome (arrow). Scale bar = 50 µm. (D) A squash preparation of suspensor cells showing the complete set of polyploid chromosomes: centromeres, NOR domains and other heterochromatic areas on chromosomes are heavily purple stained after Feulgen method staining. Scale bar = 50 µm. (E) A squash preparation, stained by Feulgen method, showing a distinct nucleolus organized by different NOR-bearing chromosomes. Scale bar = 25 µm. (F) Ideogram of the chromosome complement of *P. coccineus* exhibiting cytogenetic mapping of heterochromatic and euchromatic regions (according to Nagl 1967) and the distribution of NORs (according to Durante et al. 1977).

Figure 2. Genotoxic effects induced by $AlCl_3$ treatments in polytene chromosomes of *P. coccineus* embryo suspensor cells. (A, B) Chromatin with a trabecular meshwork appearance (arrows) in chromosomes of embryo suspensor cells after $AlCl_3$ 10^{-2} M (A) and $AlCl_3$ 10^{-1} M (B) treatments. (C, D) Chromatin fragmentation and microsphere extrusion from telomeric

regions (arrows) in chromosomes of embryo suspensor cells after $AlCl_3$ 10^{-2} M (C) and $AlCl_3$ 10^{-1} M (D) treatments. The chromosomes are stained by Feulgen method. Scale bars = 10 µm.

Figure 3. Polytene NOR-bearing chromosomes in *P. coccineus* embryo suspensor cells from control and treated seeds. (A, B, C) chromosome I showing the satellite (A, arrow), an inactive NOR (B, arrow) at the lowest $AlCl_3$ concentration and the doubling of the NOR with DNA puff formation, (C, arrow), at the higher $AlCl_3$ concentrations. (D, E, F) chromosome II evidencing an active NOR (D, arrow) and, in treated samples, a partially active NOR with trabecular meshwork chromatin (E, arrow) and a NOR with a well-defined RNA puff (F, arrow). (G, H, I) Chromosome V exhibiting a NOR with a RNA puff (G, arrow) and, following $AlCl_3$ treatments, a tripartite and inactive DNA puff on the NOR (H, arrow) and characterized by NOR splitting and RNA puff (I, arrow). The chromosomes are stained by Feulgen method. Scale bars = 25 µm.

Figure 4. Representative examples of AgNOR-stained squashes from embryo suspensor cells belonging to *P. coccineus* control seeds. (A) A single large nucleolus organized by multiple NOR-bearing chromosomes. (B) Small nucleoli organized by distinct NOR bearing chromosomes (chromosome II, at the top; chromosome V, bottom). The chromosomes are counterstained by Giemsa staining. Scale bars = 25 µm.

Table I. Chromatin damages (expressed as percentages) in the polytene chromosomes of embryo suspensor cells from control (C) and $AlCl_3$ treated seeds. (mean ± SE). Means followed by different letters within the same row are significantly different ($p< 0.01$).

Genotoxic effects	C	$AlCl_3$	
		10^{-2} M	10^{-1} M
Chromatin stickiness (%)	4.5±0.7 c	18.4±1.2 b	40.6±2.8 a
Chromatin fragmentation with microspheres extrusion (%)	5.2±0.5 c	17.5±2.4 b	70.3±5.2 a

Table II. DNA and RNA puff percentages in the polytene chromosomes of embryo suspensor cells from control (C) and $AlCl_3$ treated seeds. Given the difficulty to find the satellite in chromosomes I and V, the percentages of puffs in these sites have been not reported in the table (" * "). (mean ± SE). Means followed by different letters within the same row are significantly different ($p<0.01$). □: euchromatin, ▨: nucleolar organizing region (NOR), ■: eterochromatin.

Chromosome	Band	DNA Puffs (%)			RNA Puffs (%)		
		C	$AlCl_3$		C	$AlCl_3$	
			10^{-2} M	10^{-1} M		10^{-2} M	10^{-1} M
I	A	---*	---*	---*	---*	---*	..---*
	B	32.2±1.7 a	26±2.5 a b	19±2.0 b	60.4±2.8 a	37.5±1.8 b	25±1.4 c
	C	---	---	---	---	---	---
	D	---	---	---	---	---	---
	E	---	3.4±0.46 b	25±5 a	16.2±1.5 b	19±3.2 b	29±1.8 a
	F	---	---	---	---	---	---
	G	---	---	---	14±1.2	---	---
	H	---	---	---	---	---	---
II	A	21.7±1.8 b	28.2±2.1 b	45.5±2.8 a	33.6±1.6 b	35±1.9 b	52.5±3.1 a
	B	---	---	---	---	---	---
	C	---	---	---	---	---	---
	D	---	15.2±1.5 a	18.7±3.4 a			
	E	---	---	---	---	---	---
	F	---	---	---	21±3 b	31±6.4 a b	40±2.3 a
	G	---	---	---	---	---	---
III	A	---	---	---	12±2.1	---	---
	B	9.1±2.4	---	---	---	29±1.7 b	46±4.3 a
	C	---	16±7.5 a	18.4±6.1 a	---	21±0.9 b	30±1.6 a
	D	---	---	---	---	---	---
	E	---	---	---	13±0.8	---	---
	F	---	---	---	---	---	---
IV	A	10.1±2.9 b	18±2.1 b	29±3.4 a	17±1.1 a	15±4.1 a	24±1.8 a
	B	14.74±1 b	14±3.2 b	25±2.4 a	12±1.5 a	19±3.5 a	20±2.4 a
	C	11.6±2.4 a b	10±1.9 b	20±3 a	5.5±1 b	18±4.8 a	21±2.4 a
	D	---	---	---	---	---	---
	E	---	4±0.6 b	7±0.6 a	48±4 a	15±3.6 b	19±1.3 b
	F	---	---	---	---	---	---
	G	---	---	---	45±2	---	---
	H	---	---	---	---	---	---
	I	---	---	---	53±6	---	---
	L	---	---	---	28±6.2	---	---
V	A	---	---	---	30±4.7 b	24±6 b	50±5.2 a
	B	---	---	---	---	---	---
	C	14.7±3.2 b	7±1.8 b	30±3.1 a	---	12±4 b	45±3.1 a
	D	---	---	---	---	---	---
	E	45.05±2.1 a	25.5±1.9 b	4.25±0.1 c	46.75±2.3 a	21.2±1.2 b	1.1±0.02 c
	F	---*	---*	---*	---*	---*	---*

Table II (*continued*).

Chromosome	Band	DNA Puffs (%)			RNA Puffs (%)		
		C	$AlCl_3$		C	$AlCl_3$	
			10^{-2} M	10^{-1} M		10^{-2} M	10^{-1} M
VI	A	---	---	---	32±5.1 a	8±1.4 b	18±1.4 b
	B	12.2±1.3 a	17±4.1 a	---	9±4 b	18±3.7 b	23±2.1 a
	C	---	---	---	10±2.1	---	---
	D	---	---	---	---	---	---
	E	10±3.9 b	25±6 a	10±1.2 b	59.1±3 a	8±2.7 b	5±0.4 b
	F	---	---	---	19±5.2	---	---
	G	---	---	---	48±2.5	---	---
	H	---	---	---	33±5.1	---	---
VII	A				22±3.2	---	---
	B	10.9±1.5	---	---	30±2	---	---
	C	6±1.5 a	2±0.5 b	1±0.3 b	29±3.4 a	3±0.8 b	1±0.2 b
	D	---	6±1	4±0.3	18±4.2 a	10±2.3 ab	1±0.1 b
VIII	A	---	---	---	27±4.5 b	30±3.4 a b	41±2.4 a
	B	8.1±2.1	---	---	---	---	---
	C	8±1.2 c	22±4.3 b	37±2.4 a	---	31±4 b	47±3.2 a
	D	---	---	---	---	---	---
	E	21±3.4	---	---	49±5.2	---	---
	F	---	---	---	29±3.4 b	29±4.4 b	51±3.1 a
IX	A	---	---	---	59.1±7.2	---	---
	B	---	---	---	25±6.7	---	---
	C	---	---	---	35±3.4	---	---
	D	---	---	---	---	---	---
	E	14.6±3 b	15±3.4 b	27±3.2 a	48±7.4 a	29±3.1 b	51±2.8 a
	F	---	---	---	51.2±4.2	---	---
X	A	---	---	---	21.4±3.1	---	---
	B	10±1.1 a	11±2 a	12.1±0.5 a	15.2±4.0 a	12±1.5 a	13±2.2 a
	C	---	---	---	---	---	---
	D	---	---	---	---	---	---
XI	A	---	---	---	14.9±3.4	---	---
	B	15±4.2 a	16±1.2 a	15.6±0.3 a	15.1±3.8 a	15±0.9 a	16±1.8 a
	C	---	---	---	---	---	---
	D	---	---	---	---	---	---

Table III. Percentages of nucleoli organized by single and multiple NORs bearing chromosomes in embryo suspensor cells from control (C) and $AlCl_3$ treated seeds. (mean ± SE). Means followed by different letters within the same row are significantly different ($p<0.01$).

Functional parameters	C	$AlCl_3$	
		10^{-2} M	10^{-1} M
Nucleoli organized by more NORs	82±8 a	78.4 ± 9 a	78.9±6 a
Nucleoli organized by single NOR	18±3 a	21.6 ± 2.8 a	21.1±2 a
Nucleoli organized by single NOR from chromosome I	39.47±4.7 a	18.66± 1.6 bc	4.61±0.6 c
Nucleoli organized by single NOR from chromosome II	29.94±4.4 b	66.98± 4.7 a	80.83±3.46 a
Nucleoli organized by single NOR from chromosome V	30.57±3.1 a	14.35± 2.9 b	14.54±1.8 bc

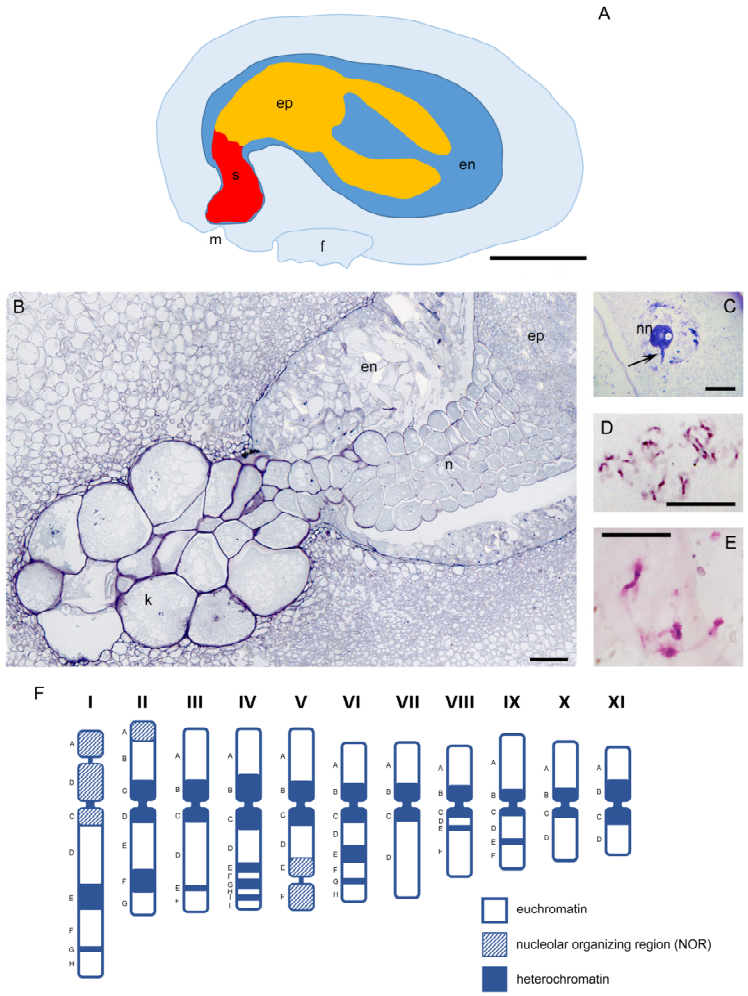
A
ep
en
s
m
f
B
ep
en
n
k
C
nn
D
E
F
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
euchromatin
nucleolar organizing region (NOR)
heterochromatin

FIGURE 1

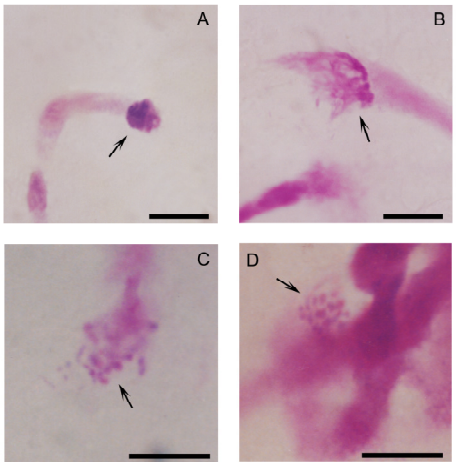

Figure 2

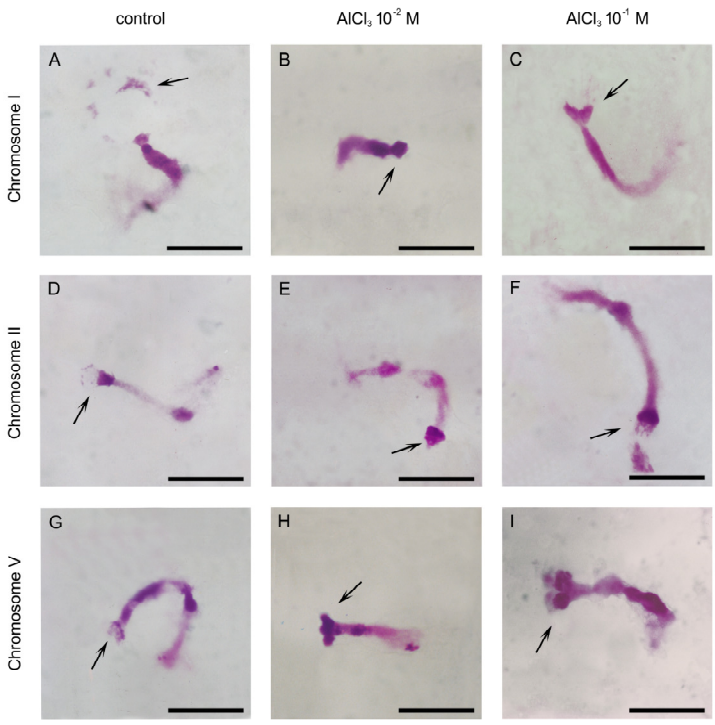

Figure 3

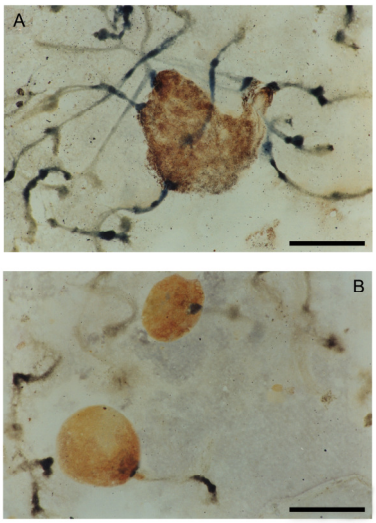

Figure 4