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Original Research

Cellular Toxicity of Aluminum in Root Tips of *Vicia faba* L.

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Abstract

In order to further understand and confirm the cytological mechanism of Al on roots of *V. faba*, the toxic effects on root growth, cell division, nucleolus and nucleoproteins (nucleophosmin, nucleolin and fibrillarin) were investigated by silver staining method and indirect immunofluorescence. The results showed that Al had an inhibitory effect on root growth at concentrations of 50 and 100 μM Al during the entire treatment (72 h) in comparison with control ($p<0.05$). The mitotic cells decreased, indicating that Al was accumulated predominantly in the meristem, where it disturbed cell division and resulted the inhibition of root growth. Al could induce c-mitosis, chromosome stickiness, lagging chromosome and chromosome bridges in root tip cells. C-mitosis and chromosome stickiness are two major types of chromosomal aberrations. Results from silver-staining indicated that Al could affect the nucleolus and induce extrusion of silver-staining nucleolar particles containing argyrophilic proteins from the nucleolus into the cytoplasm in root tip cells of *V. faba*. Evidence from indirect immunofluorescence microscopy demonstrated that silver-stained particles/material released from the nucleolus into the cytoplasm in the roots exposed to Al contained nucleophosmin, nucleolin and fibrillarin. The data obtained here can provide valuable information for monitoring and forecasting early effects of exposure to Al under realistic conditions.

Keywords: aluminum, cell division, immunofluorescent microscopy, nucleolar proteins, *Vicia faba* L.

Introduction

Aluminum (Al) is the most common metal and the third most abundant element in the earth's crust [1]. It has been reported that Al is nonessential for plants, and its toxicity is a major growth-limiting factor for plants in acid soils [2]. Al forms harmless oxides and silicates and is not toxic on plants at neutral pH. However, it is, at low pH, present as a trivalent cation (Al^{3+}) that is toxic to animals, plants, and microbes [3]. It has been known for a long time that Al is the most important soil constraint for plant growth and development in acidic soils. Due to its importance in limiting agricultural and forest productivity, there have been many reports that describe the toxic effects of Al on plant root growth and physiology [4-7].

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Plant roots are the most sensitive organ to environmental stresses. It has been well known that Al toxicity is performed primarily by inhibition of root growth, and the root meristem is one of the most sensitive sites to Al toxicity [8]. Generally, *Vicia faba* is used in many laboratories because it has many advantages, such as low cost, short test time, ease of storage and handling, large cells, and ease of observing abnormal phenomena in chromosome, nucleus, and nucleolus during mitosis [9-11].

In order to further understand and confirm the cytological mechanism of Al on roots of *V. faba*, the toxic effects of Al on root growth, cell division, nucleolus and nucleoproteins (nucleophosmin, nucleolin and fibrillarin) were investigated by silver staining methods and indirect immunofluorescence. The data obtained here can be used as early markers in cellular changes induced by Al for the evaluation of Al contamination and can be used as early warning signals for general or particular stress.

Material and Methods

Culture Conditions and Aluminum Treatment

Uniform size of *V. faba* seeds were collected and soaked in distilled water for 36 h before starting experiments. They were germinated on wet gauze in dishes at 25ºC, producing roots reaching about 1 cm in length. After that, the seedlings were grown in distilled water (pH 4.5) with added 10, 50 and 100 µM Al for 24, 48 and 72 h. Control seedlings were grown in distilled water alone. The Al was provided as aluminum sulphate ($Al_2(SO_4)_3$). The Al solutions were prepared in deionized water. The length of roots were observed, measured and recorded at the end of each time interval (24 h).

Silver-Staining Method

Twenty root tips in each treatment group and controls in the seedlings of *V. faba* treated with Al were cut every 24 h, respectively. They were fixed in 3 parts 95% ethanol: 2 parts 99.8% acetic acid for 2 h and hydrolyzed in 5 parts 1 M hydrochloric acid: 3 parts 95% ethanol: 2 parts 99.8% acetic acid for 10 min at 60ºC. Then the tips were squashed in Carbol Fuchsin solution [12] for counting the cell mitosis and observing the variation of chromosomes. In order to obtain mitotic index (MI), approximately 3,000 cells (about 1,000 cells each slide) were observed in each treatment group at the end of each time interval. For the observation of changes in nucleolus, ten root tips were cut and squashed in 45% acetic acid, dried, and after 2 days stained with silver nitrate. The root tips were squashed in 45% (v/v) acetic acid, dried, and stained with silver nitrate. The slides were also stained with Methylene Blue after the silver staining [13].

Indirect Immunofluorescent Microscopy

Meristematic zones of root tips from control and seedlings of *V. faba* treated with Al were cut and fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS, pH 7.0) for 1.5 h in darkness at room temperature and then were washed with the same buffer. Meristematic cells were digested with a mixture of 2.5% cellulose and 2.5% pectolase at 37ºC and then washed in PBS three times. They were squashed on slides and extracted in freshly prepared 1% (v/v) Triton X-100 in PBS when slides dried. After three washings in PBS, the cells were subsequently incubated with mouse primary antibodies respectively against nucleophosmin, nucleolin and fibrillarin for 1 h at 37ºC or at 4ºC overnight in a moist, sealed chamber. After washing (3 × 10 min) in PBS, the cells were incubated with secondary antibodies for detection of the primary antibodies for 45 min in darkness at 37ºC. After repeated washing in PBS, nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma) at a final concentration of 1µg per 1 ml for 15 min at room temperature. After washing (3 × 10 min) in PBS, the cells were mounted in antifade mounting medium. The slides were stored at 4ºC in the dark until observed. The immunofluorescent specimens were examined under a fluorescence microscope (Nikon, HB-0101AF) with a digital camera (Pixera Pro 600CL), using violet (355-425 nm) and blue (450-490 nm) specific filters for proteins and nuclei, respectively. Photographs were taken and images were processed with Adobe Photoshop 7.0.

Antibodies used in this study were as follows:

- Nucleophosmin: primary antibody: a mouse monoclonal antibody to nucleophosmin (Sigma, B0556) at dilution 1:100; secondary antibody: FITC-conjugated goat antimouse IgGs (Sigma, F9137) at dilution 1:50. FITC was used for the detection of signal.
- Nucleolin: primary antibody: a mouse monoclonal antibody to nucleolin (Santa, SC-8031) at dilution 1:100; secondary antibody: TRITC-conjugated goat antimouse IgGs (Sigma, T5393) at dilution 1:50. TRITC was used for the detection of signal.
- Fibrillarin: primary antibody: a mouse monoclonal antibody to fibrillarin (Santa, SC-81273) at dilution 1:100; secondary antibody: TRITC-conjugated goat anti-mouse IgGs (Sigma, T5393) at dilution 1:50. TRITC was used for the detection of signal.

Statistical Analysis

Each treatment was replicated 3 times for statistical validity. Data from the present investigation were analyzed with standard statistical software (Sigma Plot 10.0) using means±standard error (SE). For equality of averages the *t*-test was applied. Results were considered statistically significant at $P<0.05$.

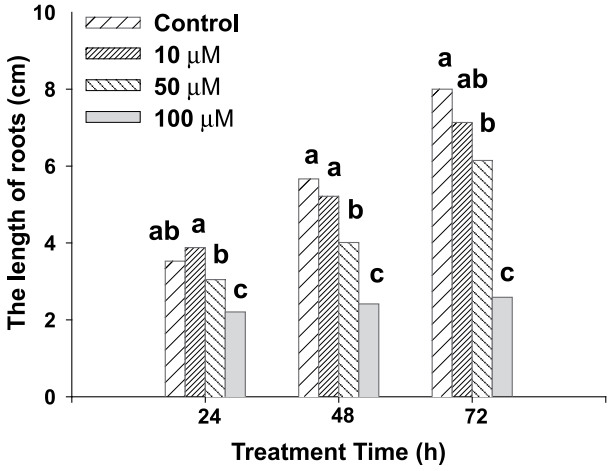

Fig. 1. Effects of different concentrations of Al on root length of *V. faba* stressed for 24, 48 and 72 h; vertical bars denote SE, n = 10; values with different letters differ significantly from each other (P<0.05, t-test).

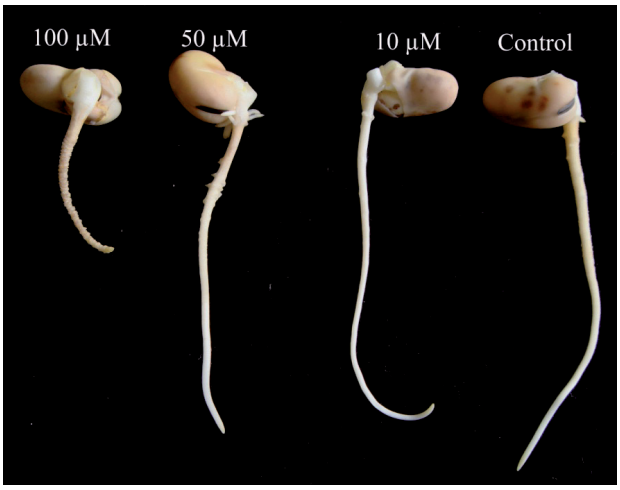

Fig. 2. Effects of different concentrations of Al on seedling growth of *V. faba* during the whole treatment (72 h).

Results

Effects of Al on Root Growth and MI

The effects of Al on the root growth of *V. faba* varied with the different Al concentrations used (Figs 1 and 2). Al had an inhibitory effect on the root growth at concentrations of and 50 and 100 μM Al during the entire treatment (72 h) in comparison with control (p<0.05). At 100 μM Al, the root length was strongly inhibited and there was no obvious growth.

As could be seen from Table 1, the MI decreased progressively with increasing Al concentration and duration of treatment except for the group exposed to 10 μM Al during the 24 h treatment. Similarly, the MI values had the same trend in the rate of root growth.

Table 1. Cytogenetic analysis of *V. faba* root tips exposed to different concentrations of Al for different periods.

Time (h)	Concentration (μM)	Mitotic index (%)	Normal dividing cell (%)		Anomalous dividing cell (%)				Anomalous mitoses (%)
			Metaphase	Anaphase	C-mitosis	Chromosome bridge	Chromosome stickiness	Lagging chromosome	
24	Control	44.3	36.4	43.0	6.3	1.6	2.4	1.2	11.5
	10	46.8	37.4	44.2	9.2	1.4	4.0	2.8	17.4
	50	39.3	35.8	38.2	10.2	3.0	8.2	5.6	27.0
	100	37.1	32.31	35.64	8.72	4.4	14.6	4.4	32.12
48	Control	43.6	40.4	37.4	8.6	0.4	6.2	3.0	18.2
	10	41.5	35.0	35.6	12.0	2.0	8.8	2.4	25.2
	50	31.6	31.4	37.0	12.6	4.0	11.8	3.4	31.8
	100	18.3	40.0	28.0	8.0	0	20.0	4.0	32.0
72	Control	39.2	34.4	39.4	9.2	1.8	7.0	2.2	20.2
	10	36.5	31.6	38.0	12.8	3.4	9.4	4.8	30.4
	50	25.1	35.0	31.4	11.4	3.0	15.8	3.4	33.6
	100	9.8	–	–	–	–	–	–	–

* Data obtained from 3,000 cells

Effects of Al on Cell Division and Nucleoli

Effects of Al on Chromosome Morphology

Fig. 3(a-d) shows normal cell division in root tip cells of *V. faba*. Excessive Al ions can disturb cell division and induce chromosome aberration. The aberrant chromosomes were observed in the root tip cells of *V. faba* after treatment with Al (Fig. 3e-h). C-mitosis induced by Al in the present investigation was the major type of chromosomal aberration (Table 1), and the highly condensed chromosomes were scattered randomly in root tip cells (Fig. 3e). Metaphase sticky chromosomes (Fig. 3f) were observed, which is also a major type of chromosomal aberration at high concentration of Al (Table 1). This type of toxic effect is most likely irreversible, which probably led to cell death. The frequency of cells with chromosome stickiness increased with increasing Al concentration and prolonged treatment time. Anaphase bridges involving one or more chromosomes (Fig. 3g) were found after the treatment with Al. Lagging chromosomes in some root tip cells were also observed (Fig. 3h). Besides, interphase cells with micronuclei (Fig. 3i) were rarely noted. In *V. faba*, the percentages of total abnormalities increased as the concentrations of applied Al increased (Table 1).

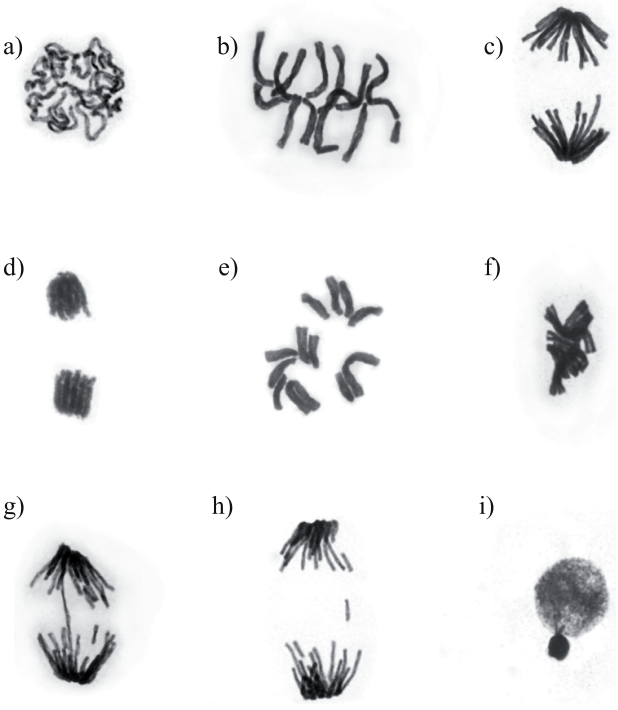

Fig. 3. Effects of Al^{3+} on root tip cells division of *V. faba* root tip cells (a-d). Normal cell division a) Prophase. b) Metaphases. c) Anaphase. d) Telophase. e) C-mitosis (50 μM Al, 24 h). f) Chromosome stickiness (100 μM Al, 48 h). g) Chromosome bridge (10 μM Al, 72 h). h) Lagging chromosome (50 μM Al, 24 h). i) Micronuclei (10 μM Al, 72 h). Scale bar = 10 μm.

Effects of Al on Nucleoli

Normally, the nucleus of *V. faba* contains one or two dark-brown nucleoli (Fig. 4a). Nucleoli appear dark brown; nuclei green and cytoplasm yellow. The toxic effects of Al on the nucleoli varied depending on the different concentrations and treatment time. Some tiny particulates containing argyrophilic proteins were scattered in the nucleus of root tip cells exposed to 10 μM Al for 24 h. More and more particles were aggregated and nearly filled the nucleus. Then they extruded from nucleolus into nucleus and cytoplasm with prolonged treatment time (10 μM Al, 72 h) (Fig. 4b). At higher concentrations of Al (50 or 100 μM Al), the amount of this particulate material increased progressively in cytoplasm (Fig. 4c) and nearly occupied the whole cytoplasm with prolonging treatment time (Fig. 4d). The nucleolar remains in the nucleus became small in size and weak in silver staining reaction (Fig. 4d).

Effects of Al on Three Major Nucleoproteins

The effects of Al on the three major nucleoproteins (nucleophosmin, nucleolin and fibrillarin) in the root tip

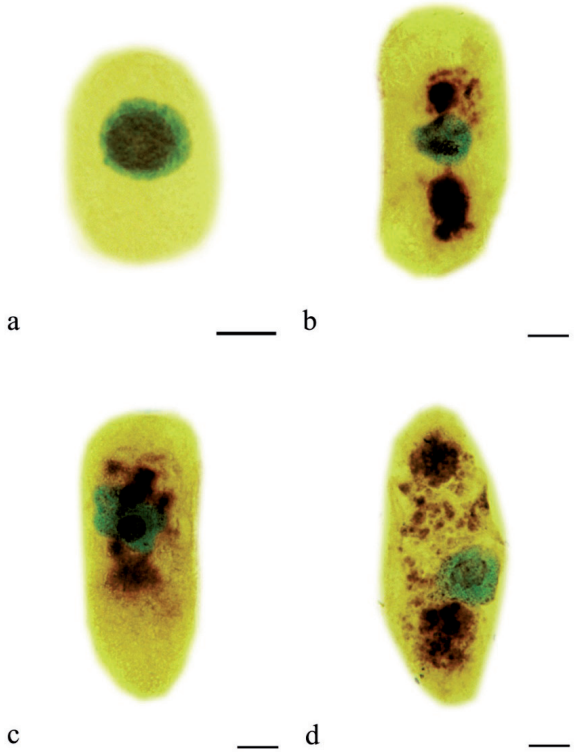

Fig. 4. Effects of different concentrations of Al on nucleoli in the root tip meristematic cells of *Vicia faba*. a) Control cell. b) Silver-stained material extruded from the nucleus into the cytoplasm (50 μM Al, 48 h). c) Silver-stained material accumulated around nucleus (100 μM Al, 24 h). d) Large amount of silver-stained material occupied nearly the whole cytoplasm (100 μM Al, 48 h). Scale bars = 10 μm. Nucleoli and nucleolar material: dark brown; Nuclei: green; cytoplasm: yellow.

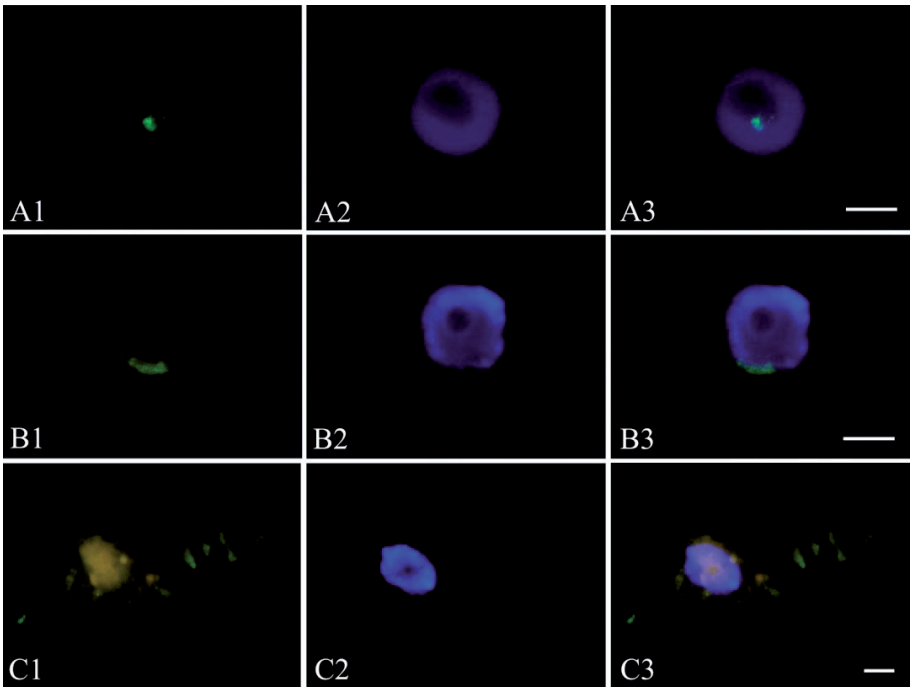

Fig. 5. Simultaneous location of nucleophosmin after reaction with antinucleophosmin antibody and secondary antibody conjugated with FITC (green) and of DNA after reaction with DAPI (blue) in the same single optical section obtained with fluorescence microscopy. A1, B1, and C1 indicate nucleophosmin detection; A2, B2, and C2 indicate DNA detection; A3, B3, and C3 indicate merged images of the above. A1-A3, Nucleophosmin was located in the nucleolus in control cells of *V. faba* (72 h). B1-B3, The migration of nucleophosmin from the nucleolus to the nucleoplasm in cells treated with 100 μM Al for 48 h; C1-C3, Nucleophosmin in the cytoplasm of cells treated with 100 μM Al for 72 h. Scale bars = 10 μm.

meristematic cells of *V. faba* were investigated using indirect immunofluorescence. The antibodies used could produce positive reactions with three nucleolar proteins above. There were obvious toxic effects on them in the root tip cells of *V. faba*-exposed 100 μM Al treatment when compared with control cells. The images in Fig. 5 obtained from fluorescence microscopy showed that nucleophosmin signals monitored by the anti-nucleophosmin antibody were all distributed in nucleolus of control cells (Fig. 5A1-A3). Under 100 μM Al stress for 48 h, the nucleophosmin migrated from the nucleolus to the nucleoplasm (Fig. 5B1-B3). The biggest alteration in the localization of nucleophosmin was seen in the group treated with 100 μM Al for 72 h. Nucleophosmin moved into the cytoplasm (Fig. 5C1-C3). Nucleolin was marked with TRITC and produced a red fluorescent signal under fluorescence microscopy. The present investigation proved that red small amounts of immunofluorescence spots of nucleolin were located in nucleolus in control cells of *V. faba* (Fig. 6A1-A3). After the treatment with 100 μM Al for 24 h, nucleolin migrated from the nucleolus to the nucleoplasm, and on the way from nucleoplasm to cytoplasm (Fig. 6B1-B3). Prolonging the treatment time, the transformation of nucleolin localization was remarkable when compared with control. More nucleolin signals in root tip cells exposed to 100 μM Al for 72 h were seen in nucleoplasm (Fig. 6C1-C3). Nucleolar protein fibrillarin was also observed using the red fluorescent signal of TRITC. The present investigation proved that fibrillarin was located exclusively in the nucleoli of control cells of *V. faba* (Fig. 7A1-A3). Fibrillarin in root tips exposed to 100 μM Al for 48 h was transferred from nucleolus to nucleoplasm when compared with control (Fig. 7B1-B3), and it was scattered in cytoplasm (Fig. 7C1-C3) with a prolonged treatment time (100 μM Al, 72 h).

Discussion

For many years excessive Al ions have been found to have caused poisoning and environmental contamination. The evaluation of action mechanisms of Al toxic to plant root tip cells and their consequences on MI, chromosomes and nucleoli, as performed in the present investigation, is very important. In the present investigation, toxic effects of Al on *V. faba* roots were observed. Al had an inhibitory effect on the root growth at concentrations of 50 and 100 μM Al during the whole treatment (72 h) in comparison with control ($p<0.05$), suggesting that roots are a primary target of Al toxicity. Some results demonstrated that in sensitive plants, cell division in the root tip meristem was quickly inhibited by Al, resulting in an immediate effect on root elongation [14-17]. The MI reflects the frequency of cell division phases and is regarded as an important parameter when determining the rate of root growth

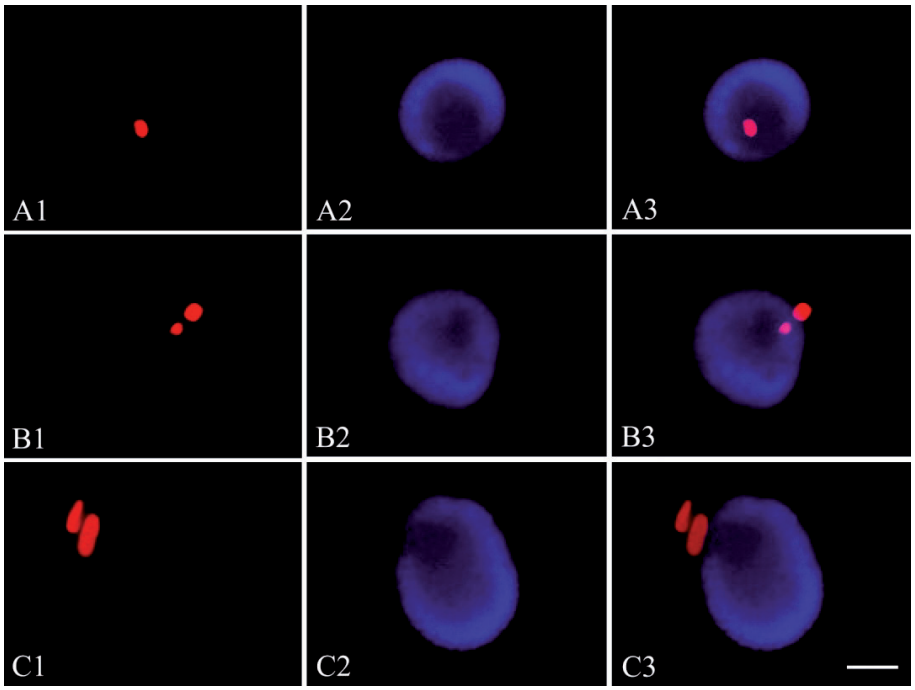

Fig. 6. Simultaneous localization of nucleolin after reaction with antinucleolin antibody and secondary antibody conjugated with TRITC (red) and of DNA after reaction with DAPI (blue) in the same single optical section obtained with fluorescence microscope. A1, B1, and C1 indicate nucleolin detection; A2, B2, and C2 indicate DNA detection; A3, B3, and C3 indicate merged images of the above. A1-A3, Nucleolin in nucleolus of control cells of *Vicia faba* (72 h). B1-B3, The migration of nucleolin from the nucleolus to the nucleoplasm in cells treated with 100 μM Al for 24 h. C1-C3, Nucleolin in the cytoplasm of cells exposed to 100 μM Al for 72 h. Scale bars = 10 μm.

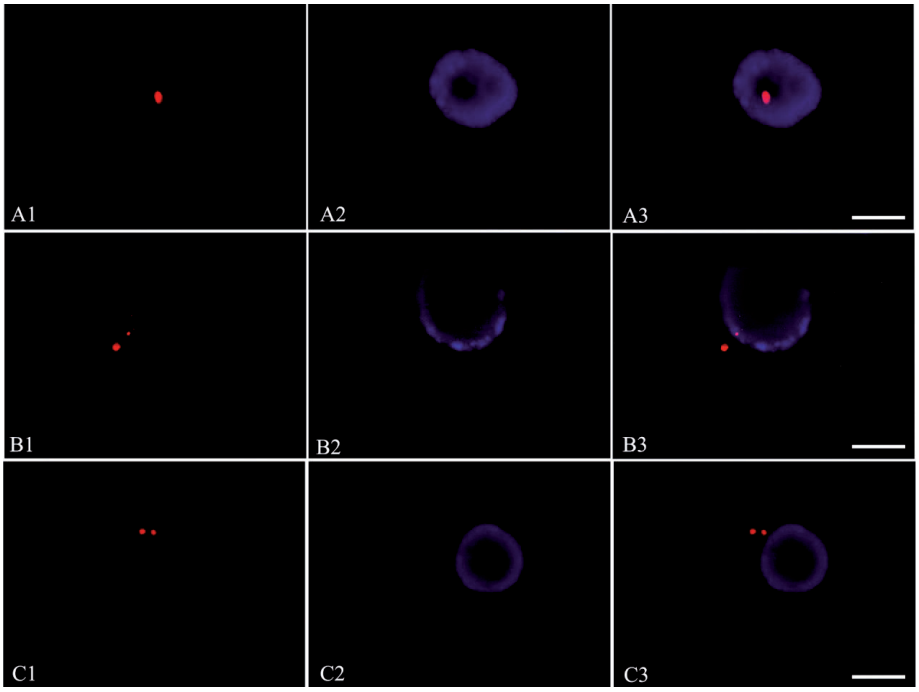

Fig. 7. Simultaneous localization of fibrillarin after reaction with primary anti-fibrillarin antibody and secondary antibody conjugated with TRITC (red) and of DNA after reaction with DAPI (blue) in the same single optical section obtained with fluorescence microscope. A1, B1 and C1, indicate fibrillarin detection. A2, B2 and C2, indicate DNA detection. A3, B3 and C3, indicate merged images of the above. A1-A3, Fibrillarin was localized in the nucleolus in control cells of *Vicia faba* (72 h). B1-B3, The migration of fibrillarin from the nucleolus to the nucleoplasm in cells treated with 100 μM Al for 48 h. C1-C3, Fibrillarin in the cytoplasm of cells exposed to 100 μM Al for 72 h. Scale bars = 10 μm.

and the effects of antimitotic agents. The results here indicated that the frequency of the MI changed in correlation with Al concentrations. The mitotic cells decreased and interphase cells increased, indicating that Al was accumulated predominantly in the meristem, where it disturbed cell division. An obvious reduction in observed MI may be due to the mitodepressive action of Al. Thus, it is clear that the inhibition of the root growth resulted from inhibiting the cell division of root tips. This fits well with the above-mentioned effects of Al on root growth.

Chromosome aberrations can be used as a measure of reproductive success and as a method for the detection of possible genetic damage by environmental agents (such as herbicides, insecticides, fungicides and metals) in plants for many years, and provide both qualitative and quantitative data on the effects [11, 18-20]. The results from the present investigation indicated that Al had toxic effects on the cell division and induced c-mitosis, chromosome stickiness, lagging chromosome and chromosome bridges, which is similar to previous reports described by Zhang et al. [15]. C-mitosis and chromosome stickiness are two major types of chromosomal aberrations in root tips exposed to Al in the present investigation. Some reports are concerned with the reasons for the formation of stickiness of chromosomes, for instance increased chromosome contraction and condensation [21], the depolymerization of DNA and partial dissolution of nucleoproteins [22]. Usually, this kind of chromosomal aberration is considered to be irreversible, which reflects highly toxic effects and probably leads to cell death. C-mitosis was first described by Levan [23] in the investigation concerning the effect of colchicine on root mistosis of *A.* cepa. In our investigation, this kind of aberration was noted in all the treated group roots. Al is thought to be extremely c-mitotically active. Generally, c-mitosis reflects slight or moderate cytological toxicity. The c-metaphase observed here suggests that Al acts on the mitotic spindle apparatus, probably interfering with the polymerization and depolymerization of microtubules [24].

The nucleolus is thought to be a highly dynamic subnuclear domain where rRNA synthesis, rRNA processing and assembly of ribosomal subunits take place [25]. Some reports indicated that it is concerned with many fundamental cellular processes, such as cell cycle regulation, apoptosis, telomerase production, and the monitoring and response to cellular stress [26-27]. It is well known that nucleophosmin, nucleolin and fibrillarin are three major and multifunctional nucleolar proteins. Nucleophosmin and nucleolin are two major Ag-NOR (argyrophilic nucleolar organizing region) proteins [28]. Fibrillarin is distinguished from them by its lack of affinity for silver staining [29]. It has a molecular mass of 41 kDa and is present both in animal and plant cells and is required for multiple events leading to rRNA maturation and ribosome subunit assembly [30]. Nucleophosmin is involved in many cellular processes, including the regulation of cell-cycle progression [31], cell growth and proliferation [32], and response to stress stimuli [33]. Nucleolin is thought to be the most abundant nucleolar phosphoprotein, which is found in a diverse array of organisms ranging from yeast to plants to mammals. Results from Chathoth et al. [34] indicated that it is involved in fundamental processes of ribosome biogenesis, and it may interact directly to promote plant growth [35].

It was reported that nucleolar material could be extruded from nucleus into the cytoplasm in *A. cepa* cells exposed to Al using the Feulgen-light green method. The phenomenon was referred to as "Al-structure" [36]. The silver-staining technique has been widely applied in cytological studies aimed at understanding the nucleolar cycle and organization in both animals and plants. The nucleolus contains a set of acidic nonhistone proteins that have a high affinity for silver ions and can be selectively visualized by silver. Silver impregnation is considered a specific stain for the nucleolus. In the present investigation we found, by means of this method, that Al could affect the nucleolus and induce extrusion of silver-staining nucleolar particles containing argyrophilic proteins from the nucleolus into the cytoplasm in root tip cells of *V. faba*, which is similar to previous findings reported by Liu and Jiang [13] and Jiang et al. [22]. Following that finding, the toxic effects of heavy metals (Ni, Cd and Pb) on the nucleolus in the root tips of plants were investigated, indicating that these metals had a similar toxic effect on the nucleolus as Al [13, 37-39]. However, they did not indicate what kinds of proteins are involved in those reports. Therefore, we carried out the investigation concerned with the effects of Al on nucleophosmin, nucleolin and fibrillarin in root tip cells of *V. faba* exposed to different concentrations of Al using indirect immunofluorescence microscopy. New evidence obtained from the present investigation confirmed that silver-stained particles/material released from the nucleolus into the cytoplasm in roots exposed to Al contained nucleophosmin, nucleolin and fibrillarin. Here, we suggest that Al may also have toxic effects on other kinds of nucleolar proteins besides argyrophilic and acidic nucleolar proteins. More studies, however, are required in this direction.

Conclusions

From the results of this investigation, we can conclude that:

1) Excessive Al ions can decrease the mitotic index (MI) progressively with increasing of Al concentration and duration of treatment, and induce chromosome aberration, such as C-mitosis, chromosome stickiness, anaphase bridges, and lagging chromosome.

2) The toxic effects of Al on the nucleoli were as below: some tiny particulates containing argyrophilic proteins were scattered in the nucleus of root tip cells,

more and more particles were aggregated and nearly filled the nucleus, then they extruded from nucleolus into nucleus and cytoplasm with increased the Al concentrations, even occupying the whole cytoplasm with prolonged treatment time.

3) Using indirect immunofluorescence, Al caused the three major nucleoproteins (nucleophosmin, nucleolin and fibrillarin) of the root tip meristematic cells of *V. faba* to migrate from the nucleolus to the nucleoplasm, then move into the cytoplasm.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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