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Induction of Abnormal Mitosis and Changes in Protein Compositions after Treatment of Root Meristems with Phosphoric Amide Herbicide APM in *Allium*

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Abstract The effect of Amiprophose-methyl (APM) on mitotic cells of the root meristems of *Allium cepa* was studied at a range of concentrations (4—6 μmol/L), length of treatments (4—16 h). The results showed that the mitosis metaphase index (Met. I) could be obviously improved from 0.8 (in control) to 5.0 or 5.2 respectively when the root meristems were treated with APM at a range of concentrations 5—6 μmol/L for 16 h. The result also indicated that APM treatment (more than 4 μmol/L) severely affected the cell division. The polypolar divided cells (especially 3—4 polar), metaphase chromosome condensed and micronuclei, were revealed in the cells of the root meristems. In 2D SDS-PAGE, 5 new protein spots with molecular weight of 24—90 kD, showing p*I* ranging from 5.0—7.3, were detected and 2 protein spots with molecular weight of 24 kD, 40 kD with p*I* of 4.8, 5.5 were lost in the root meristems treated with APM. Judging from the relationship between accumulation lost of these protein spots and treatment of APM, these proteins may be related to APM treatment.

Key words Abnormal mitosis; Multipolar spindle; APM; 2D PAGE; *Allium cepa*

磷酰胺除草剂 APM 诱导洋葱根尖分生组织细胞异常有丝分裂和蛋白质组分变化

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摘 要 研究了不同浓度 APM(4～6 μmol/L)和不同处理时间(4～6 h)对洋葱根尖细胞有丝分裂的影响。结果表明,当用 5.6 μmol/L APM 处理洋葱分生组织 16 h 后,中期细胞有丝分裂指数(Met. I)从 0.8(对照)分别提高至 5.0、5.2。结果也同时表明,当 APM 浓度超过 4 μmol/L 时,严重影响细胞有丝分裂。在根尖细胞中,多极分裂细胞(尤其是 3～4 级),中期染色体凝集和微核被检测到。用 2D SDS-PAGE 分析,5 种分子量处于 24～90 kD、p*I* 在 5.0～7.3 的新的蛋白质被检测到,而分子量为 24 kD、40 kD,p*I* 为 4.8、5.5 的蛋白组分消失。从 APM 处理后蛋白质组分消失来分析,这些蛋白质变化可能与 APM 处理有关。

关键词 异常有丝分裂;多级纺锤丝;APM;2D-PAGE;洋葱
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Organophosphorus herbicide is still used in agriculture of China because of its rapidly degraded and little residual problem to the environment. Amiprophose-Methyl (APM) is the phosphoric amide herbicide. Many experiments demonstrated that it could block cell division at the metaphase after root meristems were treated with APM[1,2,3]. In the past decade, APM had been widely used in induction mitotic metaphase synchronization in order to isolate mass metaphase chromosomes for further analysis of biochemical composition[2,4], morphological

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structure[6] and in situ hybridization. In the early 1990s, although considerable progress had been made concerning the metaphase chromosome isolation and chromosome morphological structure after treatment with APM, up to now, little information has been obtained regarding the changes in protein compositions and abnormal mitosis after treatment of root meristems with APM. In this paper, the effect of APM on chromosome structure variation, micronuclei formation and protein composition change of root meristems of *Allium cepa*, were investigated at a range of concentrations (4—6 μmol/L), length of treatment (4—16 h). The main experiment results were reported and discussed.

1 Materials and Methods

1.1 Plant materials and culture

The common onion *Allium cepa* L. ($2n = 16$) was used as experiment material, equal-sized bulbs were surface sterilized with a solution of 0.1 % $HgCl_2$ for 5 min and washed two times with tap water. The bulbs were grown in beakers with distilled water for 24 h, then the bulbs were again placed in beakers with solution of amiprophos-methyl (APM) ranging from 4—6 μmol/L, treatment time from 4—16 h. All treatments were performed at 23 ℃ in darkness; at the end of the treatment, root tips of 0.1—0.2 cm in size were excised from the bulbs. After rinsing three times with distilled water, part of the roots were kept in ice water for 24 h in order to segregate chromosomes and decrease their stickiness, then fixed in 70 % ethanol for cytological analysis[4], other fresh root tips (without the fixation) were used direct for biochemical analysis.

1.2 Cytological observation

The fixative solution was washed away with distilled water before collecting root tips. The digestion of meristems was carried out using an enzyme mixture (2.5 % cellulase RS and 2.5 % pectolyase Y—23 diluted in 75 mmol/L KCl and 7.5 mmol/L EDTA; pH 4.0) at 23 ℃. After 30 min digestion a single root tip was placed in a drop of Caebel fuchsin-stained solution on a slide kept for 2—3 min at room temperature and squashed. Mitotic division, chromosome behavior and micronucleus formation were observed by light microscopy. Metaphase index (Met. I) was expressed as the percentage of nuclei undergoing mitosis among the total number of nuclei scored in a sample. Data are based on a scoring of at least 2000 cells per treatment.

1.3 SDS-polyacrylamide gel electrophoresis

One-dimension PAGE was performed according to Laemmli (1970)[15], using a 12.5 % polyacrylamid separating gel (3 % stacking gel) in the discontinuous Tris-glycine buffer system (pH 8.3). Sample preparations were dissolved in sample buffer (500mmol/L Tris—HCl, pH 6.8; 10 % SDS, 100mmol/L, β-mercaptoethanol, 10 % glycerol, 0.1 % bromophenol blue). Electrophoresis was run for 7—8 h at 120 V.

Two-dimensional polyacrylamide gel electrophoresis was performed according to the method of O'Farrell *et al*.[16], with the use of a modified procedure: the one dimensional 3 % polyacrymide gel, containing 9 mol/L urea 0.98g; 2 % nonionic detergent p40 40 μL, 30 % polyacrylamide 340μL, ampholytes 30μL (pH 3—10) and 60 μL (pH 5—7); ddH_2O 1.09 mL, TEMED 2 μL, 10 % APS 10 μL, were each cast in glass tubes (120 mm × 3 mm) and were respectively pre-electrophoresed for 15 min at 200 V, 30 min at 300 V and 60 min at 400 V. The protein samples were dissolved in the sample buffer which contained ddH_2O 4.0 mL, 500 mmol/L Tris-HCl (pH 6.8) 1.0 mL, glycerol 0.8 mL, 10 % SDS 1.6 mL, β-mercaptothanol 0.4 mL, 10 % (w/v) bromophenol blue 0.2 mL. The first dimension was isoelectrofocusing, 60 μL protein samples (4—6 mg/mL) were located in each glass tube; electrophoresis was run for 14—16 h at 400 V. After eletrofocusing, the gels were removed from the tubes by shattering the glass and placing in equilibration buffer which contained 6 mmol/L Tris—HCl (pH 6.8), β-mercaptoethanol, 10 % glycerol, 2 % (w/v) SDS for 20 min. The second dimension was a 12.5 % SDS-PAGE that was performed according to the method of Laemmli[15].

The tube gels were placed on top of second dimension gels, 1 % agarose was overlayered and allowed to polymerize. Cylindrical gels were run at a constant voltage of 80 V for 5.5 h in a Bio-Rad unit. Gels were stained with 0.4 % $AgNO_3$ solution.[17]

2 Result

2.1 Metaphase cell arrest

The data on Met. I was given in Table 1 after treatment with APM (4—6 μmol/L) for various periods. Met. I reached a maximum (5.0 and 5.2 respectively) after treatment with 5—6 μmol/L APM for 6 h, but it was only 0.8 in the con-

trol. However, the Met. I was decreased to 0.8 —0.9 when the root meristems were treated with 6 μmol/L APM for 16 h (Table 1), even though many metaphase cells were not scored because of the losing of typical phase due to spindle function disturbance. These abnormal metaphase cells would discussed below.

Table 1 Metaphase index (Met. I) after treatment of the root meristems with APM

Content of APM (μmol/L)	Treatment times (h)				
	4	5	6	10	16
Control	0.7	0.9	0.8	1.0	0.8
4	2.1	2.2	2.3	2.2	0.9
5	2.9	3.1	5.2	1.7	0.9
6	2.7	3.0	5.0	0.9	0.8

2.2 Abnormal metaphase cells

Many abnormal metaphase cells were induced in the root meristems after treated with APM. These abnormal metaphase cells could be divided into two kinds as follows.

2.2.1 Multipolar spindle cells

APM induced spindle disturbance in metaphase cell and manifested aberrant anaphase with multipolar spindle in root meristems and this situation. It was observed very easily, more detailed data on the concentration course of the induction of multipolar spindle cells listed in Table 2. It was apparent from Table 2 that multipolar spindle cells index showed a slightly increasing trend with the increase of treatment concentration. 3-polar, 4-polar spindle cells appeared when root meristems were treatment with 4 μmol/L APM. When the concentration increased to 5 —6 μmol/L, the frequency of the cells with spindle disturbance increased very fast and 5-polar, 6-polar spindle cells could also be seen easily (Table 2).

Table 2 The formation of multipolar spindle cells after treatment of root meristems with APM at 4 —6 μmol/L for 16 h

Concent of APM (μmol/L)	3-polar	4-polar	5-polar	6-polar
Control	-	-	-	-
4	5	4	-	-
5	15	14	11	3
6	17	17	12	7

Typical multipolar spindle cells were observed in treated root meristems (Fig. 1), and it was clearly that the chromosomes lost regular arrangement on the spindle. The chromosome groups of 4, 5 or more chromosomes including separated single chromosome or fragments were formated in some cells (Fig. 1a), and arranged in a wide ring or in a star like shape (Fig. 1b, c, d). The existence of the multipolar spindle cells in the control was not observed. Metaphase chromosome condensation was observed in the other abnormal mitosis cells (Fig. 1e).

Metaphase chromosome condensation was showed in some cells of treated root meristems with 4 μmol/L APM for 6 h (Fig. 1e). The less efficient in chromosome condensation was observed at the low concentration (below 4 μmol/L), but the abnormal mitosis frequency of chromosome condensation increased with the increases of APM from 4 —6 μmol/L. Metaphase chromosome condensation reached a maximum of 17.5 % at the 6 μmol/L APM for 6 h. It was thought to be related to the meristems treatment with APM.

2.2.2 Micronuclei formation

The cells with micronuclei were found in the treated root meristems. The result was also thought to be directly related to the meristems treatment with APM. The existence of the cells with micronuclei has not been shown in the control, and the frequency was about 3.2 % at 16 h after APM treatment (4 μmol/L), and gradually increased with the up of APM concentration.

The maximum frequency was about 11 % when the root meristems were treated with 6 μmol/L APM for 16 h. The micronuclei per cell generally varied from 2 —9, 2 —3 micronuclei could be observed per cell in most cells (Fig. 1f), more than 9 micronuclei were only observed in a few cells.

2.3 Changes in protein species

To identify the changes of protein species existing in the treated root meristems, the protein compositions were analyzed by SDS-Polyacrylamide gel electrophoresis. One-dimensional SDS-PAGE results showed that there were 16 protein species existing in the root tips cultured on the distilled water, with a molecular weight of 12-110 kD. Out of 16 proteins, 12 protein species with the molecular weight of 14 kD, 17 kD, 21 kD, 31 kD, 37 kD, 40 kD, 55 kD, 67 kD, 74 kD, 88 kD, 95 kD, 110 kD were abound. 35 kD protein species was lost in the treated root meristems. It can be seen clearly in the scanning (Fig. 2c) indicated by arrows.

Two dimensional SDS-PAGE patterns of proteins extracted from root meristems were given on Fig. 3. The result indicated that more than 90 protein spots were detected (Fig. 3a) in the meristems cultured on the distilled water, but the com-

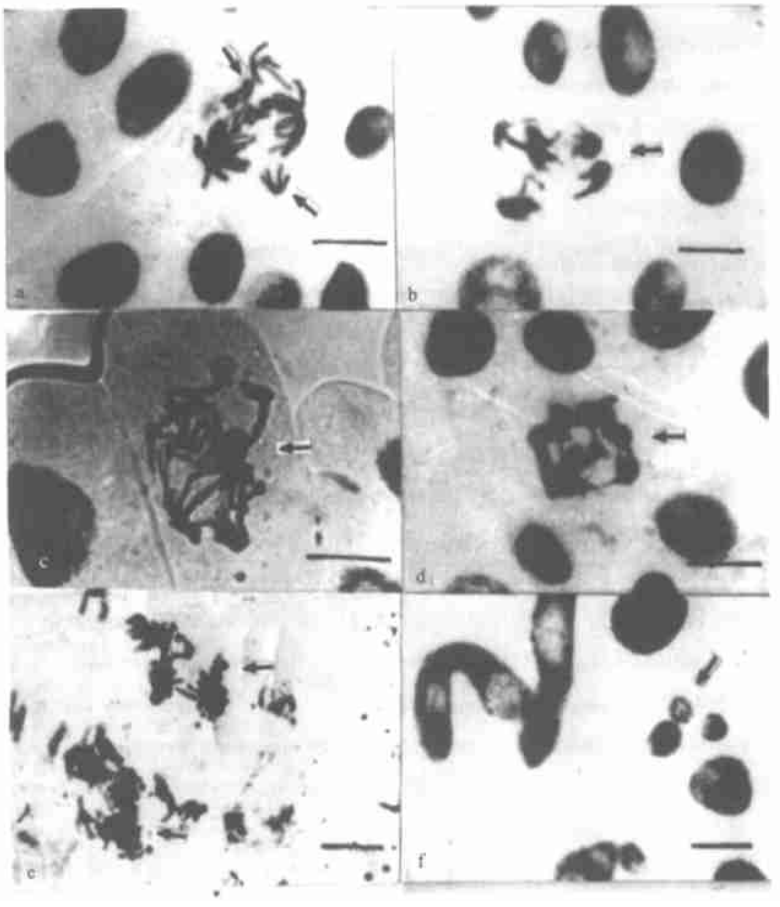

Fig. 1 The effect of APM on chromosome structure

a. The chromosome groups of 4, 5 or more chromosomes including separated single chromosome; b-d. chromosome groups in ring or a star like shape; e. chromosome condensation; f. micronuclei formation (Bar equals 10μmol/L)

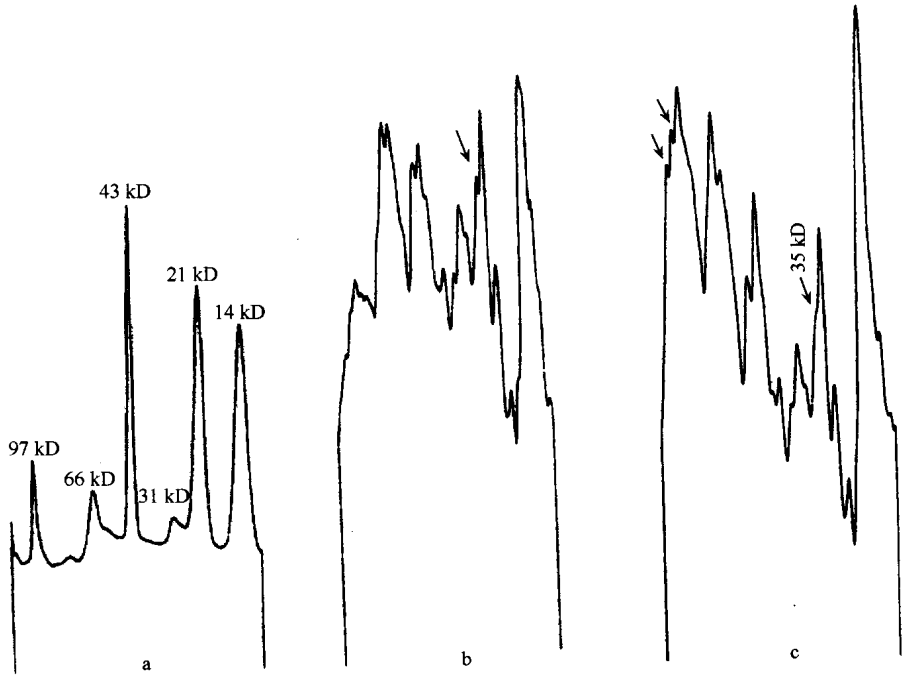

Fig. 2 SDS-PAGE analysis of protein compositions of root meristeims

a. molecular weight marker; b. control; c. treatment with APM

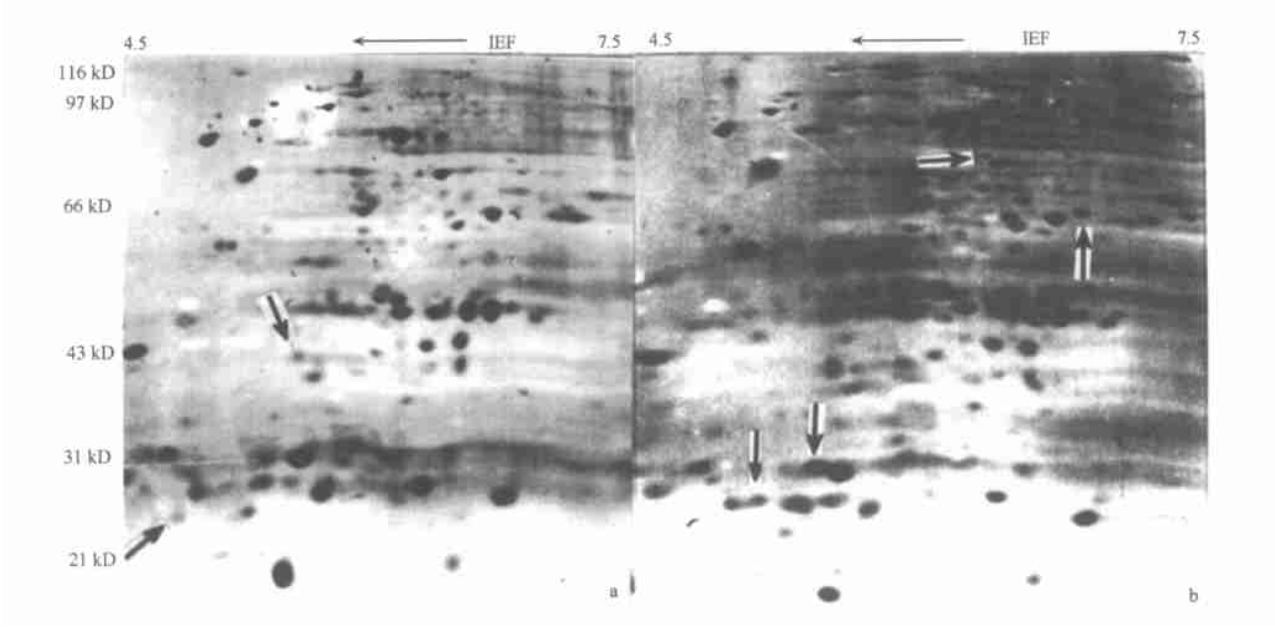

Fig. 3 2D SDS-PAGE analysis of protein composition of root meristems
a. control; b. treatment with 6 μmol/L APM for 16 h

positions obviously changed in the meristems treated with 6 μmol/L APM for 16 h. 5 new protein spot, at least, which had molecular weight and p*I* of 26 kD/p*I* 5.0, 30 kD/p*I* 5.4, 78 kD/p*I* 6.9, 76 kD/p*I* 7.3, 90 kD/p*I* 6.8, respectively, were found in the treated meristems by using 2D SDS-PAGE analysis (Fig 3b indicated by arrows). 2 protein spots, which located in low and middle molecular weight region (24 kD/p*I* 4.8, 40 kD/p*I* 5.5) disappeared in the treated meristems.

3 Discussion

APM was a phosphoric amide herbicide, also a specific drug that directly interrupts microtubule dynamics in plant cells[18]. Therefore, it could disturb the spindle function in division cells and was used widely to induce mitotic metaphase synchromization[1]. Although phosphoric amide herbicide was widely used in agriculture, little information on cytological and biochemical toxicology had been obtained when plants were treated with the herbicide. In this paper, the cytotoxicity and biochemtoxicity of APM on division cells were investigated in order to safely and suitable using herbicide in agriculture. In our experiment, root tips were treated with 4 μmol/L APM (induction mitotic metaphase synchronization concentration) and 5—6 μmol/L APM, the results showed that metaphase index (Met. I) could be obviously increased when root tips were treated with 4—6 μmol/L APM for 4—10 h (Table1). Met. I reached 5.0, 5.2 respectively at 5—6 μmol/L APM for 6h, but only 0.8—0.9 Met. I was observed when root tips were treated with 6 μmol/L APM for 16h. It was thought treatment time was more important than concentration. Although the same results were reported in culture cell lines[3, 6, 8~14], root tips was used directly as experiment materials to examine the cytotoxicity and biochemtoxicity of division cells of the root tips treated with 4—6 μmol/L APM for 4—16 h in this study. The result indicated that APM could induce spindle disturbance in metaphase cells and manifested aberrant metaphases with tri- or multipolar spindles, chromosome groups in ring or a star like shapes as well as cells with micronuclei when root tips were treated with 4—6 μmol/L APM for 16 h. The occurrence frequency of aberrant chromosome cells got up with increase of APM concentration. The changes of protein compositions was analyzed by using SDS-PAGE method in order to further study the poison mechanism of APM on plant cell division at biochemical level by using 2D PAGE analysis. More than 90 protein species at least were identified and more than 30 protein species were abundant in the control root meristems, but 5 new protein species (24 kD/p*I* 5.0, 30kD/p*I* 5.4, 78 kD/p*I* 6.9, 76 kD/p*I* 7.3, 90 kD/p*I* 6.8) were found, and 2 protein species (24 kD/p*I* 4.8, 40 kD/p*I* 5.5) were lost in the meristems treated with 6 μmol/L APM for 16 h.

Up to now, only the tubulin studies have been made concerning the effect of APM on cell division in higher plants[18], little other biochemical information exists concerning the protein composition change in plants which have been treated with APM[7]. In this paper, 5 new protein species were induced and 2 protein species were lost when root meristems were treated by APM at 6 μmol/L for 16 h, although we have not understood the exact function of these changed protein species, it may provide valuable information in relation to possible the poisen mechanism of crops.

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