Bot. Bull. Acad. Sin. (1995) **36:** 53–58

EJ100199500053

Changes of endogenous indole-3-acetic acid, peroxidases, and auxin oxidases during pollen germination in maize

Zin-Huang Liu[1] and Bor-Heng Lee

Department of Biology, National Sun Yat-Sen University, Kaohsiung, Taiwan 804, Republic of China

(Received March 28, 1994; Accepted October 27, 1994)

Abstract. Quantitative analysis of indole-3-acetic acid (IAA) in *Zea mays* L. pollen was performed by high performance liquid chromatography with fluorescence. The IAA level declined during germination; it was 131.2 ng per gram pollen at the ungerminated stage, and 47.6 ng per gram after germination. The decline of endogenous IAA levels might have been due to the action of peroxidases, which synthesized lignins during pollen germination. IAA oxidases ($K_M = 9.0 \times 10^{-6}$ M, $V_{max} = 9.9$ nmol IAA mg^{-1} protein min^{-1}) and peroxidases were extracted and analyzed during the course of pollen germination. The activity of peroxidases (13–28 kDa fractions) was two-fold higher in germinated pollen.

Keywords: GC-MS; HPLC; IAA; Indole-3-acetic acid; Pollen; *Zea mays* L.

Introduction

Among the naturally occurring auxins of higher plants, indole-3-acetic acid (IAA) is by far the most important. It is involved in regulating cell elongation, division, and differentiation (Dietz et al., 1990; Ettlinger and Lehle, 1988; McClure et al., 1989). Many have reported that endogenous IAA levels change significantly during growth and differentiation of plant tissues (Golaz and Pilet, 1987; Hamdi, 1988; Sjut and Bangerth, 1981). Within the tissues, IAA can be modulated via several pathways, including synthesis and breakdown of conjugated IAA (bound IAA), active transport of IAA, and IAA metabolism. Two pathways for oxidative degradation of IAA have been identified (Grambow and Langenbeck-Schwich, 1983; Tsurumi and Wada, 1980). The key IAA degradative enzymes are peroxidases and IAA oxidases. Many investigators have reported that dual catalysis is associated with these two groups of isozymes (Grambow and Langenbeck-Schwich, 1983; Pressey, 1990; Quesada et al., 1992). Still others have reported the nonidentity of IAA oxidases with peroxidases (Beffa et al., 1990; Gordon and Henderson, 1973). The relationships is complicated by multiple forms of the enzymes. Conflicting reports suggest that the isozymic relationship of IAA oxidases to peroxidases varies from species to species. Little is known about the change of endogenous IAA during pollen germination. This paper describes a preliminary investigation of the changes of endogenous IAA and related peroxidases and oxidases in maize (*Zea mays* L.) pollen.

[1]Corresponding author.

Materials and Methods

Collection and Germination of Pollen

Tassels were cut from field grown maize (*Zea mays* L.), and the cut ends were placed in a flask of tap water in the growth chamber (25 ± 1°C) the day prior to collecting pollen. Pollen grains (0.2 gram) were collected and spread evenly in a 9 cm petri dish containing 4 ml of a solution of 15% sucrose, 0.03% calcium nitrate, and 0.01% boric acid, and incubated at 25 ± 1°C (Pfahler, 1973). Germination rates of 80% to 95% were obtained with different batches of pollen.

Extraction and Purification of Indole-3-Acetic Acid

After pollen germination, 16 ml methanol was mixed with 0.2 mg ml^{-1} butylated hydroxytoluene (BHT) and 40 mg ml^{-1} (50 ml) sodium ascorbate to produce a 80% methanol-BHT-ascorbate extracting solvent (Guinnet et al., 1986; Dunlap and Guinn, 1989). Ungerminated pollen, usually 0.8 to 1.0 gram fresh weight, and germinated pollen were homogenized in extraction buffer using a chilled mortar and pestle. The pollen extracts were suction filtered through Whatman No. 1 paper and the methanol was removed from the filtrates with rotary flash evaporation (RFE) at 35°C.

The pH of the aqueous residues was adjusted to 8.0 with 0.2 N KOH and partitioned twice with an equal volume of ethyl acetate to remove phenolic compounds and other impurities. The ethyl acetate fraction was discarded. Residual ethyl acetate in the aqueous phase was removed by RFE (Crozier et al., 1980; Guinnet et al., 1986). The pH of the aqueous fraction was adjusted to 2.5 with 0.2 N HCl,

and the fraction partitioned twice with an equal volume of ethyl acetate. The residual water in the ethyl acetate fraction was removed by adding anhydrous sodium sulfate (Na_2SO_4), and the ethyl acetate fraction was evaporated to dryness by RFE. The residue was immediately dissolved in 1.0 ml methanol.

Thin Layer Chromatography Purification

The methanol fraction was purified by thin layer chromatography (TLC), which was performed on analytical grade Kieselgel 60 (20 x 20 cm, Schichtdicke, 0.2 mm, Merck). Up to 1.0-ml volumes of extracts were loaded, and chromatograms were developed for about 1 hr with benzene:acetone:acetic acid (13:6:1, v/v). Identification of unknowns was achieved by comparing with R_fs of standard IAA that was chromatographed at the same time. The IAA bands were scraped from TLC plates immediately, dissolved in 100% methanol, and analyzed quantitatively by HPLC.

High Performance Liquid Chromatography

Samples obtained from TLC, were introduced onto a reverse phase C_{18} column (5 ´x 250 mm, TSK gel column, ODS-80TM, Tosoh, Japan). Eluates were monitored with a fluorescence spectrophotometer (Model F-1050, Hitachi). The mobile phase was composed of 35% methanol in 20 mM ammonium acetate buffer (pH 3.5). The flow rate was maintained at 0.7 ml min^{-1}, and an excitation wavelength of 280 nm with an emission wavelength of 360 nm was used. Eluant peaks, with their retention time and areas, were recorded by an attached integrator (Model D-2500, Hitachi). Because deuteriated IAA standards were not available, the quantitation of endogenous IAA levels was estimated by the HPLC-fluorescence methods developed by Crozier et al. (1980); its detection limit was less than 10 pg.

Combined Gas Chromatography-Mass Spectrometry

Electron-impact spectra of methylated samples were obtained at 70 eV with a Hewlett Packard Model 5890 combined gas chromatograph-mass spectrometer (GC-MS) filled with a 30 m x 0.2 mm OV-1 column. The carrier gas, helium, was introduced at 30 ml min^{-1}, and the injector was operated at 220°C; the column oven temperature was 200°C.

Extraction and Purification of Enzymes

After pollen germination, the extraction buffer, 1 ml of 500 mM ammonium acetate, was added to the plate (100 mM ammonium acetate, pH 6.8). The pollen-buffer mixture was ground with a pestle in a chilled mortar. The homogenate was centrifuged at 20,000 *g* for 20 min and the pellet was discarded. Ammonium sulfate was added to the supernatant liquid to obtain 60% precipitated protein. The precipitated protein was dissolved in 2 ml of 20 mM ammonium acetate buffer (pH 6.8). For the G-25 extract activities assay, the protein mixture was passed through a Sephadex G-25 (Bio-Rad) column (2.5 x 30 cm) previously equilibrated with ammonium acetate buffer. Fractions corresponding to the protein peak were pooled for the enzyme assays. The protein precipitated the 60% ammonium sulfate was also applied to a column (1.5 x 80 cm) packed with a Sephacryl S-300 gel (Pharmacia, Sweden). The flow rate was kept at 0.5 ml min^{-1}. Fractions were collected for protein, peroxidase, and IAA oxidase activities analyses.

Kinetics Study of IAA Oxidase

The reaction mixture was contained in a final volume of 1 ml, and was composed of 6.66 mM phosphate buffer (NaH_2PO_4/Na_2HPO_4) at pH 6.00 ± 0.05, 100 µM $MnCl_2$, 50 µM *p*-coumaric acid, 50–100 nM IAA, and 200 µl of the extract to be tested. Mixtures were incubated at 25 ± 0.5°C for 30 min. After incubation, 20 µl of 10% trichloroacetic acid (TCA) was added to stop the reaction and adjust the pH to 2.5. After partitioning with 1000 µl ethyl acetate, 200 µl of the organic phase was pipetted out and dried in nitrogen gas. The residues were dissolved in 500 µl methanol. The quantities of degraded IAA were estimated by HPLC methods as described. The data presented in this report were obtained from representative experiments that were repeated several times in triplicates.

IAA Oxidases Assay

The reaction mixture (final volume of 1 ml) was composed of 6.66 mM phosphate buffer (NaH_2PO_4/Na_2HPO_4) at pH 6.00 ± 0.05, 100 µM $MnCl_2$, 50 µM *p*-coumaric acid, 50 µg IAA (285.4 µM), and 200 µl of the extract to be tested. Mixtures were incubated at 25 ± 0.5°C for 30 min. Two milliliters of Salkowski reagent (Pilet and Lavanchy, 1969) was then added and the destruction of IAA determined by measuring absorbence at 535 nm after 30 min. One unit of IAA oxidase activity was equivalent to a ΔA_{535} of 1.0 for 1 mg of protein in 30 min.

Peroxidases Assay

Peroxidase activity (EC 1.11.1.7) was determined colorimetrically using guaiacol and H_2O_2, both at 5 mM in 10 mM 3,3-dimethyl-glutaric acid (3,3-DGA)-NaOH at pH 6.00 ± 0.05 (Grison and Pilet, 1978). Extracts (100 µl) were tested in final volumes of 1 ml. The oxidation of guaiacol was determined by measuring absorbences at 470 nm after 10 min incubation at 30.0 ± 0.5°C. One unit of peroxidase activity was equivalent to a ΔA_{470} of 1.0 for 1 mg of protein in 10 min.

Results

The Change of Endogenous IAA Levels During Pollen Germination

Purified extracts from pollen were quite clean and the IAA was isolable with 35% methanol in ammonium acetate buffer (pH 3.5). As shown in Figure 1, the endog-

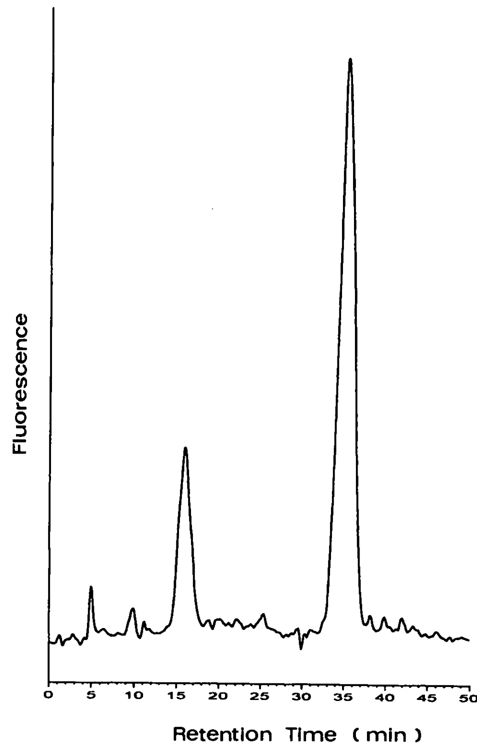

Figure 1. HPLC of purified extracts from maize pollen. Column: 5 x 250 mm, TSK gel; mobile phase: 35% methanol in 20 mM, pH 3.5 ammonium acetate buffer; flow rate: 0.7 ml min^{-1}; fluorimetric detection.

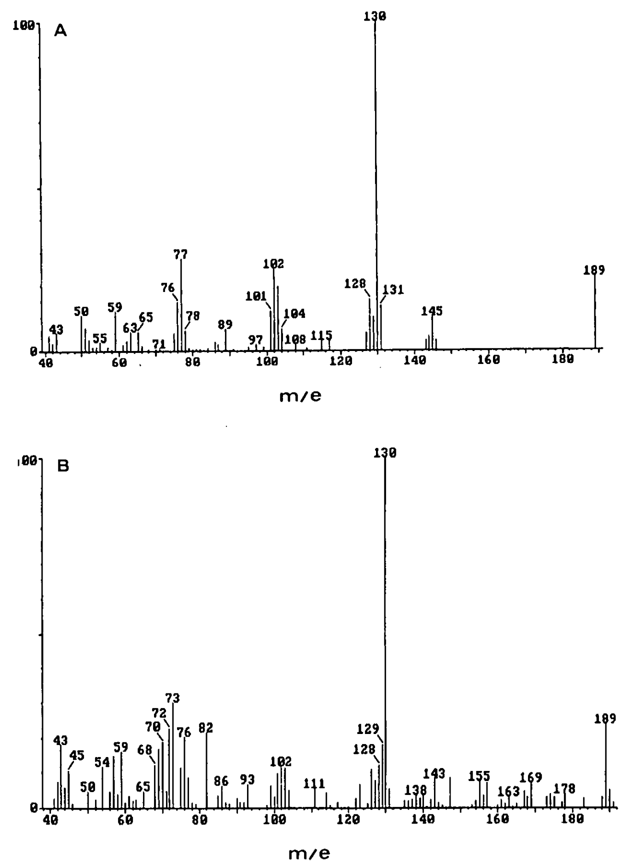

Figure 2. A) Seventy-electron-volt mass spectra of authentic IAA methyl ester; **B**) putative IAA methyl ester from maize pollen.

enous IAA was detected with a retention time of 35.3 min in the ungerminated pollen (0 min). To distinguish this peak from an impurity, another run was carried out with extracts containing standard IAA; this run also showed only one peak with an identical retention time (data not shown). Combined GC-MS of the purified, methylated HPLC peak from pollen extract confirmed the additional presence of IAA methyl ester. The mass spectrum of the pollen extract is illustrated in Figure 2, along with that of authentic IAA methyl ester. The molecular ions (M^+) of IAA methyl ester from authentic standard and of purified pollen extract were detected at m/e 189. The IAA content decreased during pollen germination (Figure 3). The IAA level was about 131.2 ng per gram of pollen in ungerminated pollen (0 min), and 47.6 ng per gram in germinated pollen (20 min).

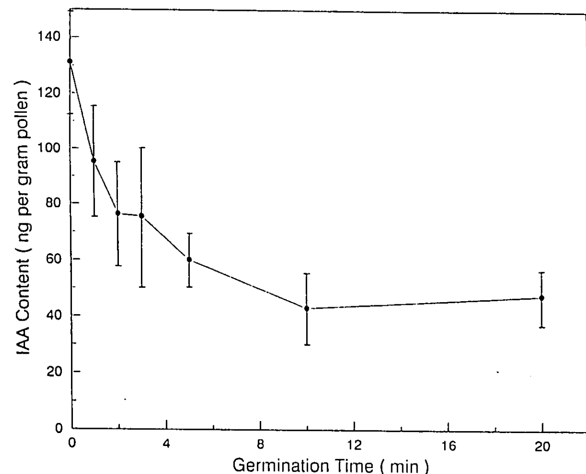

Figure 3. The change of IAA content during pollen germination in maize.

Kinetics of IAA Oxidase

Using HPLC with fluorescence procedures, it was possible to measure picogram quantities of IAA. The kinetics of IAA oxidase, purified by ammonium sulfate precipitation and Sephadex G-25 desalinization, was investigated at concentrations of IAA ranging from 50 nM to 500 nM. Double-reciprocal plotting (Figure 4) revealed a Michaelis constant (K_M) of 9.0 $\times 10^{-6}$ M, and a maximal rate (V_{max}) of 9.9 nmol IAA mg^{-1} protein min^{-1}.

Fractionation by Gel Filtration Column (Sephacryl S-300 Gel)

For the ungerminated pollen (0 min), the quantities and activities of IAA oxidase and peroxidase in fractions eluted from the Sephacryl S-300 column are shown in Figure 5. Peroxidase activities appeared in the protein fractions between 8 kDa and 28 kDa. The major peroxidases activity

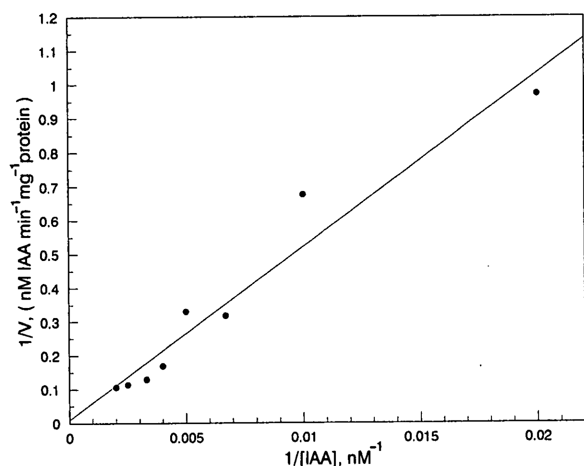

Figure 4. Double-reciprocal plot of IAA oxidases activity. Samples were incubated with IAA at concentrations ranging from 50 nM to 500 nM. $K_M = 9.0 \times 10^{-6}$ M; V_{max} = 9.9 nmol IAA mg^1 protein min^{-1}

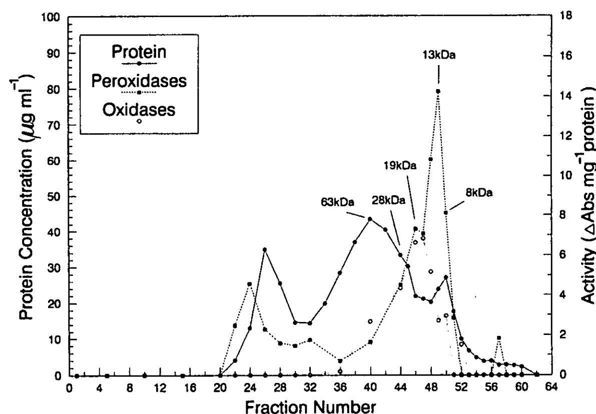

Figure 5. Elution profile of protein extract from ungerminated pollen (0 min) on Sephacryl S-300 column. Units of IAA oxidases and peroxidases activity are defined in *Materials and Methods.*

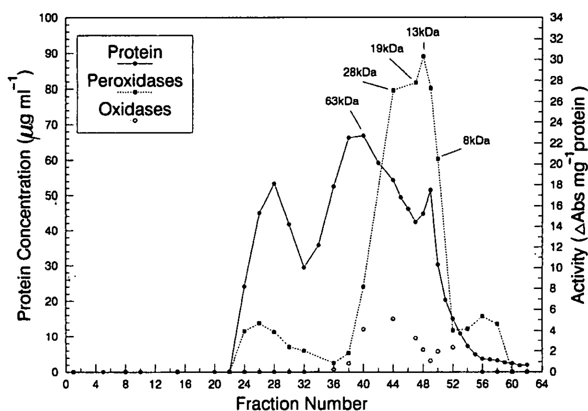

Figure 6. Elution profile of protein extract from germinated pollen (20 min) on Sephacryl S-300 column. Units of IAA oxidases and peroxidases activity are defined in *Materials and Methods.*

appeared at the peak, with an estimated molecular mass of 13 kDa. IAA oxidase activities were shown between 13 kDa and 28 kDa fractions, with the major one at 19 kDa. Similar elution profiles were obtained in germinated pollen after 20 min, (Figure 6) except the activities of peroxidase with molecular mass ranging from 13 kDa to 28 kDa were enhanced two fold. No significant changes in IAA oxidase activities were apparent after pollen germination. Thus, there was a correlation between decline of endogenous IAA levels and increasing peroxidase activities (13–28 kDa fractions) in germinated maize pollen.

Discussion

Very sensitive IAA detection is attainable by using the HPLC-fluorescence procedures developed by Crozier et al. (1980). The amount of plant material required can be reduced to sub-gram quantities. In our investigation of maize pollen, 0.5 gram of ungerminated or germinated pollen was extracted and purified by TLC, then further chromatographed on a reverse phase column operated in the isocratic mode (35% methanol in pH 3.5 ammonium acetate buffer). Rademacher and Graebe (1984) reported that IAA was destroyed in the Salkowski-like reaction when the TLC was dried. In our investigation, 85% of IAA standards was recovered (data not shown), if the chromatograms were immediately transferred to methanol. Thus, using TLC to remove impurities has been shown to be useful, necessary, and sparing of IAA. Significant amounts of IAA were detectable and estimable by HPLC and GC-MS. The data were reproducible and reliable. Pollen has unique physiological activities that are distinguishable from those of other cells. It respires, grows, and dies. As it germinates, the pollen tube elongates by expansion at the tip. In our study, germination of maize pollen began with marked decrease in IAA (Figure 3). The decrease mainly occurred during the first 5 min, and lasted throughout germination (20 min). Meanwhile, the activities of peroxidase increased two fold in germinated pollen (Figure 5 and Figure 6). The decline of endogenous IAA might be due to the action of peroxidases that is involved in the conversion of phenylpropane alcohol into macromolecular lignins during tube wall expansion. Our results confirmed several reports that suggested a positive relationship between increased activity of peroxidases and cell wall lignification (Bassiri and Carlson, 1979; Harkin and Obst, 1973). The endogenous IAA probably played no role in maize pollen germination. The destruction of endogenous IAA by peroxidases, however, may favor the synthesis of lignin and remove excess hydrogen peroxide, thus serving a detoxifying role during germination.

The IAA-oxidases were not separable from peroxidases in our investigation. The major peroxidases appeared in fractions from 13 kDa to 28 kDa. These enzymes might degrade endogenous IAA in the presence of phenolic compounds, producing indole-3- methanol or indole-3-aldehyde (Grambow and Langenbeek- Schwich, 1983; Beffa et al., 1990). In summary, our results indicated that the decline of endogenous IAA levels was related to enhanced

peroxidase activities during pollen germination. Further purification of peroxidases will be helpful in elucidating the processes associated with lignification in germinating pollen.

Acknowledgements. The authors wish to thank S.-C. Yu for her assistance with GC-MS analysis. Our discussions with Dr. H.-Y. Liu and Dr. T.-Y. Feng are also greatly appreciated.

Literature Cited

Bassiri, A. and P. S. Carlson. 1979. Isozyme patterns in tobacco plant parts and their derived calli. Crop. Sci. **19:** 909–914.

Beffa, R., H. V. Martin, and P.-E. Pilet. 1990. *In vitro* oxidation of indoleacetic acid by soluble auxin oxidases and peroxidases from maize roots. Plant Physiol. **94:** 485–491.

Crozier, A., K. Loferski, J. B. Zaeer, and R. O. Morris. 1980. Analysis of picogram quantities of indole-3-acetic acid by high performance liquid chromatography fluorescence procedures. Planta **150:** 366–370.

Dietz, A., U. Kutschera, and P. M. Ray. 1990. Auxin enhancement of mRNAs in epidermis and internal tissues of the pea stem and its significance for control of elongation. Plant Physiol. **93:** 432–438.

Dunlap, J. R. and G. Guinn. 1989. A simple purification of indole-3-acetic acid and abscisic acid for GC-SIM-MS analysis by micro-filtration of aqueous sample through nylon. Plant Physiol. **90:** 197–201.

Ettlinger, C. and L. Lehle. 1988. Auxin induces rapid changes in phosphatidylinositol metabolites. Nature **331:** 176–178.

Golaz, F. and P.-E. Pilet. 1987. Root primordia and endogenous auxin in maize roots cultured *in vitro*. Physiol. Plant. **70:** 389–393.

Gordon, W. R. and J. H. M. Henderson. 1973. Isoperoxidases of (IAA oxidase) oxidase in oat coleoptiles. Can. J. Bot. **51:** 2047–2052.

Grambow, H. J. and B. Langenbeck-Schwich. 1983. The relationship between oxidase activity, peroxidase activity, hydrogen peroxide, and phenolic compounds in the degradation of indole-3-acetic acid *in vitro*. Planta **157:** 131–137.

Grison, R. and P.-E. Pilet. 1978. Analyse critique de peroxydases de la racine de mais. Plant Sci. Lett. **13:** 213–218.

Guinnet, G., D. L. Brummett, and R. C. Beier. 1986. Purification and measurement of abscisic acid and indoleacetic acid by high performance liquid chromatography. Plant Physiol. **81:** 997–1002.

Hamdi, S. 1988. Regulation of IAA oxidase activities: Correlation with sex genes, sterility determination and IAA levels in *Mercurialis arrua*. Platn Physiol. Biochem. **26:** 639–644.

Harkin, J. M. and J. R. Obst. 1973. Lignification in trees: indication of exclusive peroxidase participation. Science **180:** 296–298.

McClure, B. A., G. Hagen, C. S. Brown, M. A. Gee, and T. J. Guilfoyle. 1989. Transcription, organization and sequence of an auxin-regulated gene cluster in soybean. Plant Cell **1:** 229–239.

Pfahler, P. L. 1973. *In vitro* germination and pollen tube growth of maize (*Zea mays* L.) pollen-VII. Effect of ultraviolet irradiation. Radiat. Bot. **13:** 13–18.

Pilet, P.-E. and P. Lavanchy. 1969. Purification d'extraits peroxydasiques (Racine de *Lens*) à activité "auxines-oxydasique." Physiol. Veg. **7:** 19–29.

Pressey, R. 1990. Anions activate the oxidation of indoleacetic acid by peroxidases from tomato and other sources. Plant Physiol. **93:** 798–804.

Quesada, M. A., C. Sànchez-Roldàn, A. Heredia, V. Valpuesta, and M. J. Bukovac. 1992. Peroxidase and IAA oxidase activities and peroxidase isozymes in the pericarp of seeded and seedless "Redhaven" peach fruit. J. Plant Growth Regul. **11:** 1–6.

Rademacher, W. and J. E. Graebe. 1984. Isolation and analysis by gas-liquid chromatography of auxins, gibberellins, cytokinins, and abscisic acid from a single sample of plant material. Ber. Deutsch. Bot. Ges. **97:** 75–85.

Sjut, V. and F. Bangerth. 1981. Effect of pollination or treatment with growth regulators on levels of extractable hormones in tomato ovaries and young fruits. Physiol. Plant. **53:** 76–78.

Tsurumi, S. and S. Wada. 1980. Metabolism of indole-3-acetic acid and natural occurrence of dioxindo-3-acetic acid derivatives in *Vicia* roots. Plant Cell Physiol. **21:** 1515–1525.

玉米 (*Zea mays* L.) 花粉萌發過程中 indole-3-acetic acid (IAA) 內含量及 peroxidases 和 IAA oxidases 活性變化

劉景煌 李柏亨

國立中山大學生物學系

玉米 (*Zea mays* L.) 花粉中內生的 indole-3-acetic acid (IAA) 可藉由高壓液相色層分析儀輔以螢光光偵測器加以定量。 IAA 的內含量隨著花粉萌發時間的延長而降低，其內含量由未萌發時的每克花粉含 131.2 ng 降低到萌發 20 分鐘後的每克花粉含 47.6 ng。 IAA oxidases 及 peroxidases 在花粉萌發過程中被萃取出來並加以分析， 花粉萌發 20 分鐘後， peroxidases 的活性 (分子量 13 kDa 到 28 kDa 間) 增強二倍。 IAA 內含量的降低可能和 peroxidases 在花粉萌發過程中合成 lignin 的反應有關。

關鍵詞 ：玉米；花粉；高壓液相色層分析。