行政院國家科學委員會補助專題研究計畫成果報告

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※　　碳水化合物及氨基酸含量變化與　　※
※　　水稻切離葉片老化間關係之研究　　※
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計畫類別：☑個別型計畫　　□整合型計畫

計畫編號：NSC－89－2313－B－002－222

執行期間：89 年 8 月 1 日至 90 年 7 月 31 日

計畫主持人：高景輝

共同主持人：

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

☑出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：台灣大學農藝系

中　華　民　國　90　年　8　月　1　日

行政院國家科學委員會專題研究計畫成果報告

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主持人：高景輝　執行機構及單位名稱：台大農藝系

一、中文摘要

本研究主要探討黑暗下 methyl jasmonate (MJ) 及 abscisic acid (ABA) 對水稻切離葉片糖與胺基酸含量變化的影響。MJ 與 ABA 均能加速葉片之老化，但對糖含量變化之型式則明顯不同。MJ 降低蔗糖與葡萄糖之含量，然而 ABA 增加葡萄糖與果糖含量。MJ 與 ABA 都不會影響澱粉之含量。MJ 處理之葉片，其蔗糖與葡萄糖含量降低的原因，很可能被呼吸作用所利用。試驗之結果似乎認為糖不可能參與水稻葉片老化之控制。

MJ 處理降低蛋白質含量之程度大於 ABA 處理。然而 MJ 處理所增加胺基酸總量與氨含量的程度分別是小於與大於 ABA 處理。ABA 處理可增加大部分胺基酸之含量，但 MJ 處理只能增加少數幾種胺基酸之含量。結果顯示 MJ 似乎可促進胺基酸之脫氨作用。ABA 與 MJ 均可使 asparagine (Asn)，typtophan (Trp)，histidine (His) 與 GABA 含量增加，顯示這幾種胺基酸可能與水稻葉片老化之調控有關。

關鍵詞： 碳水化合物、胺基酸、水稻、葉片老化

Abstract

Changes in sugar and amino acid contents in detached rice leaves treated with methyl jasmonate (MJ) and abscisic acid (ABA) under dark condition were examined. Both MJ and ABA effectively promoted the senescence of detached rice leaves, but showed different patterns in the changes of sugar content. MJ decreased sucrose and glucose contents, when compared with the control leaves. However, glucose and fructose contents in ABA-treated leaves were higher than those in the control leaves. Both MJ and ABA had no effect on starch content in detached rice leaves. The lower content of sucrose and glucose in MJ-treated leaves is most likely due to higher respiration rate in detached rice leaves. It appears that sugars do not participate in regulating senescence of detached rice leaves.

The decrease in protein content by MJ was more pronounced than ABA. However, the contents of total amino acids and ammonia in MJ-treated leaves were much lower and higher, respectively, than those in ABA-treated leaves. ABA enhanced the release of most of the amino acids . However, only a few amino acids were found to be accumulated in MJ-treated leaves. Results appear to indicate that MJ promoted the deamination of amino acids during senescence. Both ABA and MJ were able to accumulate asparagine (ASN), tryptophan (TRP), histidine (HIS) and GABA, suggesting that these amino acids ma play some role in regulating rice leaf senescence.

Keywords: Carbohydrate, Amino Acids, *Oryza sativa*, Leaf Senescence

二、緣由與目的

許多資料顯示葉片老化過程中，可溶性糖含量增加，而且認為糖含量之增加可能導致葉片老化，或促進老化。一些試驗也顯示，澱粉的累積常伴隨著葉片老化。

然而也有證據顯示，葉片老化過程中澱粉不會累積，反而減少。水稻切離葉片老化過程中，碳水化合物如何變化，目前並不瞭解。本研究即在瞭解水稻切離葉片老化過程中蔗糖、葡萄糖、果糖與澱粉含量之變化，及其與老化間之關係。

外加胺基酸處理可加速水稻與燕麥切離葉片之老化，但葉片老化過程中各種胺基酸含量的變化瞭解的很少。Kao (1981) 與 Wang et al. (1982) 提出水稻葉片老化過程中，腺胺酸含量累積。Lahdesmaki (1968) 則發現 γ-aminobutyric acid (GABA) 含量隨著葉片之老化而增加。本計畫也希望瞭解各種胺基酸含量在水稻葉片老化過程中如何變化，以及此種變化之生理意義。

三、研究報告應含的內容

如以蛋白質含量做為水稻切離葉片老化指標，則水稻切離葉片於黑暗處理 24 小時即開始老化 (圖 1)，methyl jasmonate (MJ) 與 ABA 處理均可加速葉片老化，但 MJ 處理並不會誘導切離葉片 ABA 含量之增加 (圖 1)，顯示 MJ 所加速之老化並不是由於 ABA 所造成。水稻切離葉片在黑暗下老化過程中，蔗糖、葡萄糖、果糖與澱粉含量降低 (圖 2)。MJ 與 ABA 處理對碳水化合物的影響型式不同。MJ 處理葉片其蔗糖與葡萄糖含量低於對照處理，但果糖含量則與對照處理無明顯差異，而 ABA 處理葉片之蔗糖含量與對照處理無明顯差異，但葡萄糖與果糖含量高於對照處理 (圖 2)。MJ 或 ABA 處理不會改變澱粉含量 (圖 3)。MJ 處理之葉片呼吸速率增加 (圖 3)，顯示 MJ 處理所降低的蔗糖與葡萄糖含量之降低可能是被呼吸作用所消耗掉。KCN 處理可降低 MJ 所增加之呼吸作用速率，同時可增加 MJ 處理所降低的蔗糖與葡萄糖含量 (圖 4)。外加蔗糖處理，可增加 MJ 所降低之蔗糖及葡萄糖含量，但並不影響葉片老化 (圖 5)。

MJ 與 ABA 處理均可降低蛋白質的含量 (圖 6)。MJ 處理所降低之蛋白質含量大於 ABA 處理所降低之蛋白質含量。如果蛋白質含量之降低是由於蛋白質的分解所造成，則 MJ 處理葉片之胺基酸總量應大於 ABA 處理。但圖 6 之結果顯示 MJ 處理之胺基酸總量小於 ABA 處理。由於 MJ 處理之葉片其氨之含量大於 ABA 處理 (圖 6)，顯示 MJ 處理使得胺基酸進行脫氨作用。MJ 與 ABA 加速老化過程中，各種胺基酸含量變化之型式不同 (表 1 與表 2)，ABA 處理幾乎所有胺基酸含量均會增加，而 MJ 處理大部分胺基酸含量均降低，僅少數胺基酸會增加。ABA 與 MJ 處理均可使 asparagine (ASN)、tryptophan (TRP)、histidine (HIS) 與 GABA 含量增加，顯示這些胺基酸在老化過程中可能扮演重要之角色。

四、計畫成果自評

整個計畫執行順利，達到預期之成果。本計畫成果對水稻葉片老化過程中碳水化合物與胺基酸變化之重要性有相當之瞭解。

五、參考文獻

Kao CH (1981) Senescence of rice leaves VI. Comparative study of the metabolic changes of senescing and water-stressed excised leaves. Plant Cell Physiol 22: 683-688

Ladhdesmaki P (1968) The amount of γ-aminobutyric acid and the activity of glutamate decarboxylase in aging leaves. Physiol Plant 21: 1322-1327

Wang CY, Cheng SH, Kao CH (1982) Senescence of rice leaves VII. Proline accumulation in senescing excised leaves. Plant Physiol 69:1348-1349

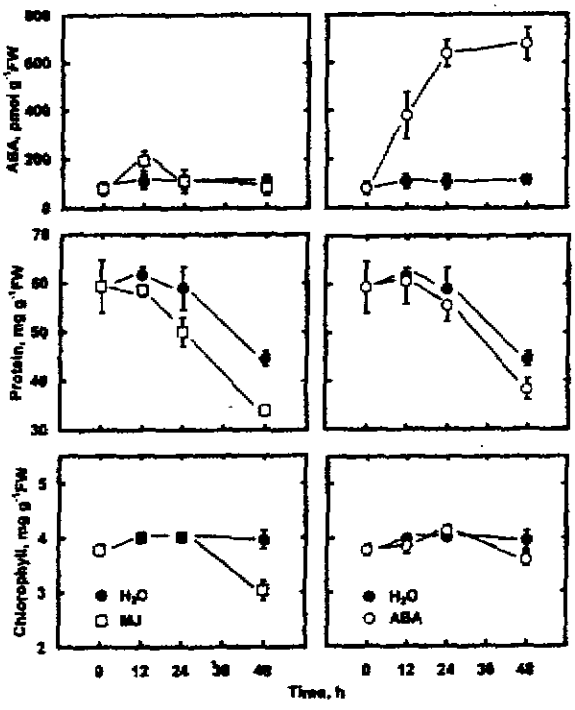

圖 1. MJ 與 ABA 處理對 ABA、蛋白質與葉綠素含量之影響。

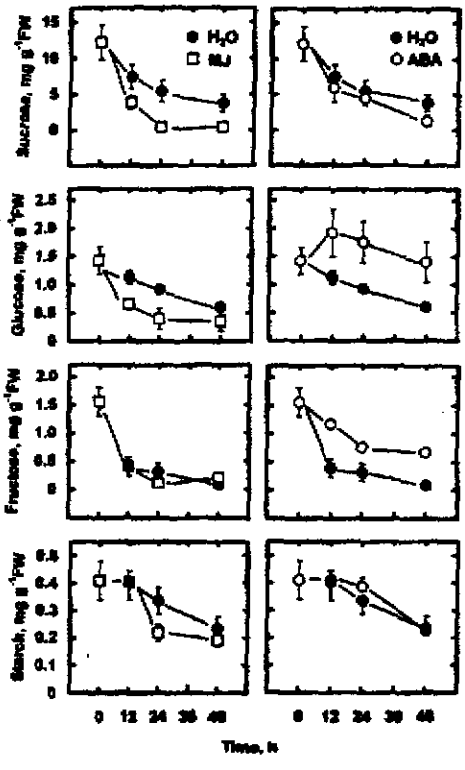

圖 2. MJ 與 ABA 處理對蔗糖、葡萄糖、果糖與澱粉含量變化之影響。

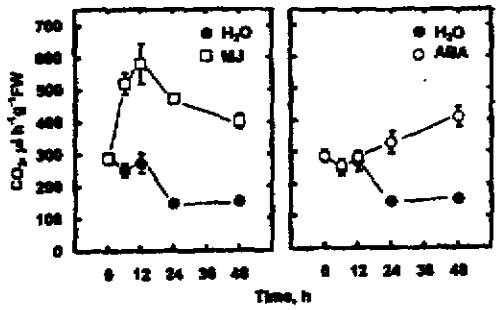

圖 3. MJ 與 ABA 處理對呼吸速率變化之影響。

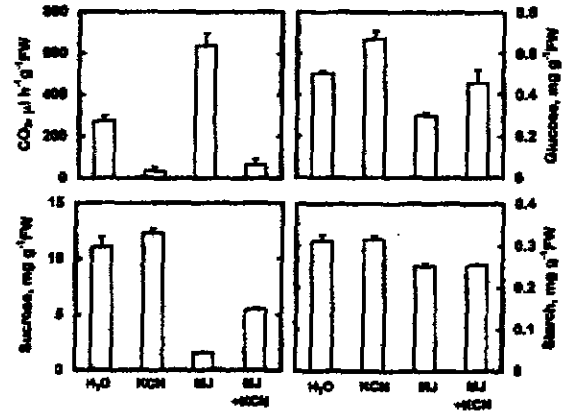

圖 4. KCN 與 MJ 處理對呼吸速率、蔗糖、葡萄糖與澱粉含量變化之影響。

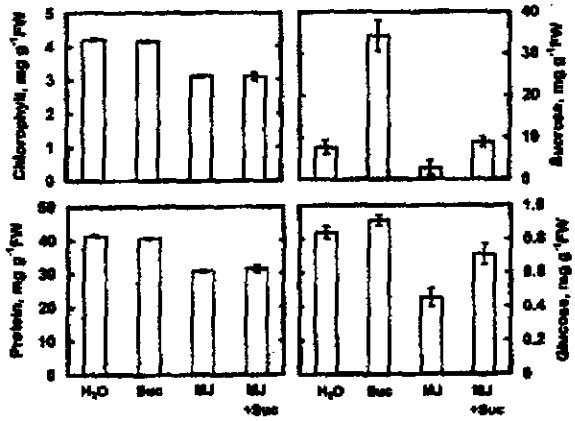

圖 5. 蔗糖與 MJ 處理對呼吸速率變化之影響。

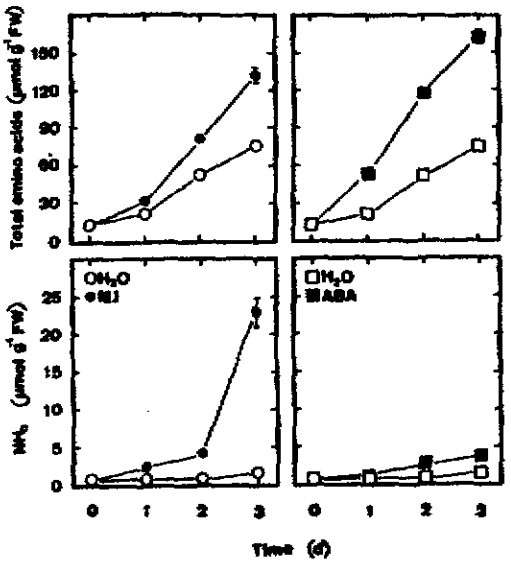

圖 6. MJ 與 ABA 處理對胺基酸總量與氨含量之影響。

表 1. MJ 與 ABA 處理一天對各種胺基酸含量之影響。

	H_2O	ABA, 45 μM		MJ, 45 μM	
Amino acids	nmol g^{-1} FW	nmol g^{-1} FW	Fold increase	nmol g^{-1} FW	Fold increase
ASN	564 ± 60	3862 ± 161	6.85	13479 ± 702	23.91
PRO	123 ± 4	782 ± 26	6.35	163 ± 9	1.32
GLN	2034 ± 95	10907 ± 519	5.36	2402 ± 125	1.18
TYR	169 ± 9	767 ± 24	4.54	123 ± 9	0.72
ARG	321 ± 16	1190 ± 42	3.71	118 ± 6	0.37
LEU	572 ± 32	2093 ± 78	3.66	366 ± 23	0.64
ILE	567 ± 27	2063 ± 60	3.64	791 ± 52	1.39
VAL	911 ± 48	3244 ± 92	3.56	1567 ± 86	1.72
PHE	317 ± 17	1109 ± 35	3.49	283 ± 19	0.89
THR	791 ± 33	2202 ± 61	2.78	996 ± 47	1.26
GLY	140 ± 5	349 ± 12	2.49	116 ± 5	0.83
TRP	272 ± 14	660 ± 27	2.43	1111 ± 43	4.08
HIS	606 ± 21	1413 ± 33	2.33	1193 ± 40	1.97
SER	2569 ± 134	5665 ± 155	2.21	1886 ± 60	0.73
ALA	1996 ± 74	3810 ± 181	1.91	377 ± 17	0.19
ORN	21 ± 2	36 ± 4	1.78	29 ± 2	1.41
LYS	515 ± 25	855 ± 29	1.66	250 ± 5	0.49
ASP	2906 ± 58	4102 ± 89	1.41	1416 ± 68	0.49
GABA	95 ± 9	122 ± 17	1.29	112 ± 4	1.18
GLU	5163 ± 81	5937 ± 179	1.15	2028 ± 72	0.39
MET	1119 ± 32	1053 ± 28	0.94	836 ± 17	0.75

表 2. MJ 與 ABA 處理二天對各種胺基酸含量之影響。

	H_2O	ABA, 45 μM		MJ, 45 μM	
Amino acids	nmol g^{-1} FW	nmol g^{-1} FW	Fold increase	nmol g^{-1} FW	Fold increase
ASN	7394 ± 552	22364 ± 702	3.02	49388 ± 961	6.68
PRO	500 ± 16	2320 ± 93	4.64	251 ± 22	0.50
GLN	3389 ± 143	19782 ± 714	5.84	3768 ± 124	1.11
TYR	657 ± 24	1691 ± 89	2.57	943 ± 68	1.43
ARG	1348 ± 31	4878 ± 250	3.62	2129 ± 110	1.58
LEU	2224 ± 52	3861 ± 184	1.74	436 ± 44	0.20
ILE	2179 ± 64	5107 ± 164	2.34	1139 ± 113	0.52
VAL	3339 ± 110	8623 ± 225	2.58	3157 ± 246	0.95
PHE	1118 ± 44	3444 ± 151	3.08	3260 ± 94	2.92
THR	2425 ± 73	6148 ± 200	2.54	1488 ± 60	0.61
GLY	465 ± 13	774 ± 23	1.67	312 ± 18	0.67
TRP	789 ± 27	1880 ± 46	2.38	2285 ± 40	2.89
HIS	1661 ± 50	3542 ± 85	2.13	3666 ± 64	1.58
SER	6885 ± 173	11510 ± 258	1.89	3754 ± 218	0.62
ALA	4955 ± 156	4728 ± 206	0.95	372 ± 71	0.08
ORN	29 ± 3	116 ± 7	4.02	45 ± 5	1.57
LYS	1541 ± 31	2365 ± 117	1.53	978 ± 61	0.63
ASP	3703 ± 68	4446 ± 153	1.20	1135 ± 34	0.31
GABA	136 ± 11	192 ± 7	1.41	210 ± 18	1.54
GLU	7312 ± 92	8408 ± 230	1.15	2356 ± 180	0.32
MET	961 ± 15	956 ± 34	0.99	231 ± 19	0.24

行政院國家科學委員會補助國內專家學者出席國際學術會議報告

年　月　日

附件三

報告人姓名	高景輝	服務機構及職稱	台大農藝系教授
會議時間 會議地點	8月17日~8月22日 (89年) 德國漢堡	本會核定補助文號	NSC89-2313-B-002-222
會議名稱	(中文) 第三屆國際作物學會議 (英文) 3rd International Crop Science Congress		
發表論文題目	(中文) 細胞壁過氧化酵素活性、過氧化氫含量與氯化鈉所抑制水稻幼苗根生長間之關係 (英文) Cell wall peroxidase activity, hydrogen peroxide level and NaCl-inhibited root growth of rice seedlings		

報告內容應包括下列各項：

一、參加會議經過

二、與會心得

三、考察參觀活動(無是項活動者省略)

四、建議

五、攜回資料名稱及內容

六、其他

一、前言

第三屆國際作物學會議（3rd International Crop Science Congress）係由歐洲農藝學會（European Society for Agronomy）所籌辦之會議。來自全世界各地，對農藝學與作物科學有興趣的學者，參與該會議。國際作物學會議每四年舉辦一次，第一屆在美國舉行，第二屆在印度舉行。本屆會議期自 8 月 17 日至 8 月 22 日，為期六天。會議地點為德國漢堡市（Hamburg, Germany），參與代表約 700 人，提出之論文近 900 篇。

二、會議內容

會議期間每天上午 8 時至下午 6 時，安排演講與壁報展示。此次會議主題標榜為 " Meeting Future Human Needs "（達到未來人類之需求），其中包括五場主議題（theme），七場 workshop 以及十六場小型議題。

五場主議題分別為：

Facing growing needs of mankind.

Stress in crops and cropping systems.

Diversity in agro-ecosystems.

Designing crops and cropping systems for future.

Stress in crops and cropping systems.

七場 workshop 則包括：

The role of genetic engineering for the developing world.

Crop production in Germany-markets, marketing, competitiveness.

Strengthening of information and communication-information

technology in the developing world.

Present and future use of oil plants-production and use.

Industrial uses of oil crops.

Future of transgenetic crops.

Will precision agriculture revolutionize crop science.

十六場小型議題則包括：

Importance of natural resources.

Future food security at the level of the world, region and household.

Nutritional crop quality and human health.

Grasslands, rangelands and forage crops.

Optimizing water use.

Abiotic stress in crops.

Biotic stress in crops.

Management of complex stresses.

Management of diversity in cropping systems.

Optimizing crop diversification.

Conservation of biodiversity.

Utilisation of biodiversity.

Production potential.

New crops and cropping technologies.

Biotechnology and crop improvement.

Transgenic plants for sustainable crop production.

壁報展示共有 854 篇。本人研究的興趣為環境逆境。提出參展的壁報題目為 " Cell wall peroxidase activity, hydrogen peroxide level, and NaCl-inhibited root growth of rice seedlings "，該論文係執行國科會之專題計畫。主要報導氯化鈉抑制水稻根生長係經由細胞壁過氧

化酵素活性增加以及過氧化氫含量增加而導致細胞壁變硬之結果。

三、與會心得

（一） 探討的作物包括農藝與園藝作物

傳統上作物學會議探討的作物多屬農藝作物。此次會議上傳統的農藝作物如水稻、玉米、大麥、小麥與高粱仍是討論之主題，但一般被歸屬為園藝作物的芒果、花椰菜、香瓜、大蒜、黃秋葵與胡椒亦有多篇論文提出。甚至一些國內已多年不研究的作物如棉花、亞麻與甜菜亦被許多學者作為探討的對象。或許，以後我們界定作物問題時，不應該那麼狹窄，不應只限定農藝作物。

（二） 作物的問題多元且廣泛

會議的主題是作物，研究的問題，包括相當廣。有傳統的栽培制度與遺傳種源，亦有熱門的生物技術、有機農業與精準農業；有產量問題的探討，也有品質改進的問題；有生理、栽培與土壤肥料的問題，也有生態、育種與統計的探討；有政策的說明，同時也有基因轉殖成功實例之提出。涉及問題相當多元，當然論文品質也就相當分歧。

（三） 相當多地區性的問題被涉及

作物問題有其地區性，因此探討的問題亦有其地域性。例如，日本直播水稻的問題較被重視，因此許多論文係針對如何改善水稻直播上的問題來探究，有從生理的角度切入，亦有由品種選擇來改善。作物品種的改良，端賴育種工作之努力；育種工作之成功與否，又賴種源的變異，因此多篇論文涉及

到地區性的種源歧異度。菲律賓稻米研究所所熱衷的問題是產量與植物型態問題，因此與會的菲律賓稻米研究所學者，就植物型態問題相繼提出他們的看法。

（四） 很多第三世界國家的學者參與此次會議

通常學術性的國際會議，第三世界的代表（特別是非洲或中東國家），甚少參與。令人印象深刻的是，此次會議這些國家參與的代表相當多。或許，這些國家所面臨的糧食問題嚴重，因而參與的學者較多，提出的問題也很多，這是相當不同的景象。

（五）。生物性逆境（biotic stress）與非生物性逆境亦為熱門研究課題

作物之生長常會遭受逆境（stress），逆境又分生物性與非生物性。全球氣候環境之變遷與病蟲害的問題，一直是作物生產之問題，也因此這方面的問題常受到重視。這次會議亦不例外，生物性與非生物性逆境生理研究，有多篇論文提出，論文內容也較深入。

四、建議

此次會議參與的大陸學者人數較往年會議為多，所提出之論文品質亦有極高的評價。國內對出席國際會議規定亦已較過去開放很多，不過國科會可能仍需積極鼓勵學者們參與國際會議，而達到直接交流與增廣見聞之目的。

五、攜回資料名稱

此次會議所攜回資料為 Book of Abstract for the 3rd International Crop Science Congress 2000。

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Cell wall peroxidase activity, hydrogen peroxide level and NaCl-inhibited root growth of rice seedlings

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Key words: hydrogen peroxide, NaCl, *Oryza sativa*, peroxidase, root growth

Abstract

The changes in cell-wall peroxidase (POD) activity and H_2O_2 level in roots of NaCl-stressed rice seedlings and their correlation with root growth were investigated. Increasing concentrations of NaCl from 50 to 150 m*M* progressively reduced root growth and increased ionically bound cell-wall POD activity. NaCl had no effect on covalently bound cell-wall POD activities. The reduction of root growth by NaCl is closely correlated with the increase in H_2O_2 level. Exogenous H_2O_2 was found to inhibit root growth of rice seedlings. Since ammonium and proline accumulation are associated with root growth inhibition caused by NaCl, we determined the effects of NH_4Cl or proline on root growth, cell-wall POD activity and H_2O_2level in roots. External application of NH_4Cl or proline markedly inhibited root growth, increased cell-wall POD activity and increased H_2O_2 level in roots of rice seedlings in the absence of NaCl. An increase in cell-wall POD activity and H_2O_2 level preceded inhibition of root growth caused by NaCl, NH_4Cl or proline. NaCl or proline treatment also increased NADH-POD and diamine oxidase (DAO) activities in roots of rice seedlings, suggesting that NADH-POD and DAO contribute to the H_2O_2 generation in the cell wall of NaCl- or proline-treated roots. NH_4Cl treatment increased NADH-POD activity but had no effect on DAO activity, suggesting that NADH-POD but not DAO is responsible for H_2O_2 generation in cell wall of NH_4Cl-treated roots.

Abbreviations: DAO – diamine oxidase; DW – dry weight; FW – fresh weight; POD – peroxidase

Introduction

Peroxidase (POD, EC 1.11.1.7) influences plant growth through lignin synthesis (Siegel, 1953), oxidative coupling reactions involving phenolics that are esterified to cell wall polysaccharides (Fry, 1986), formation of isodityrosine bridges that are believed to crosslink structural protein molecules (Fry, 1986). An inverse relationship between growth rate and POD activity has been reported in many plant systems (Carpita and Gilbeaut, 1993; Chen and Kao, 1995; Fry, 1986; Gardiner and Cleland, 1974; Lee and Lin, 1995;). Thus POD is generally believed to be a putative wall rigidification enzyme (Cosgrove, 1997).

The inhibition of plant growth by salinity is a widespread problem in agricultural practice. However, the mechanisms underlying this inhibition are not yet clear (Munns, 1993; Rengel, 1992). Neumann et al. (1994) demonstrated that root growth inhibition caused by salinity was associated with wall stiffening. Recently, we have demonstrated that an increase in ionically bound POD activity is associated with growth inhibition of rice seedling roots caused by NaCl (Lin and Kao, 1999). The ionically bound fraction of POD, removable from homogenized tissues with high ionic strength buffers, has been equated with the cell wall fraction. However, Mader et al. (1986) suggested that at least some ionically bound POD activity might be an artifact of homogenization. Therefore, to test the hypothesis that an increase in cell-wall POD activity is associated with growth in-

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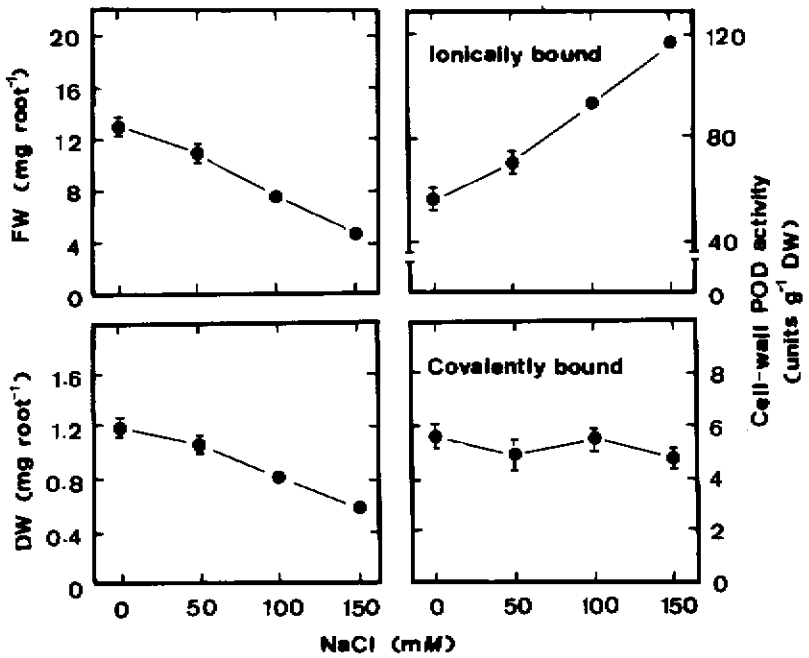

Figure 1. Effects of NaCl on root growth and cell-wall POD activities in roots of rice seedlings. Root growth and cell wall POD activities were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

hibition of seedling roots of rice, data on POD activity extracted from the cell walls are required.

H_2O_2 is a necessary substrate for a cell wall stiffening process catalyzed by POD (Elstner and Heupel, 1976; Hohl et al., 1995; Schopfer, 1994). Recently, Schopfer (1996) demonstrated that H_2O_2 inhibited auxin-mediated growth of maize coleoptiles. The formation of H_2O_2 by isolated cell walls from horseradish has been reported (Elstner and Heupel, 1976). Using a sensitive tissue-print assay, Schopfer (1994) demonstrated that H_2O_2 is localized in the cell wall of pea epicotyls. H_2O_2 causes a rapid cross-linking of cell-wall polymers (Bradley et al., 1992; Schopfer, 1996). Therefore, a sufficient supply of H_2O_2 is required for ensuring complete stiffening of the cell wall. The present investigation was designed to study the changes in cell-wall POD activity and H_2O_2 level in roots of NaCl-stressed rice seedlings and their correlation with root growth.

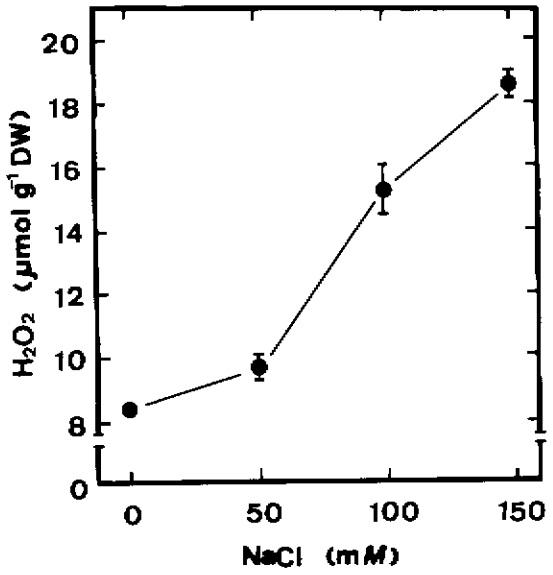

Figure 2. Effects of NaCl on H_2O_2 levels in roots of rice seedlings. H_2O_2 was determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

Materials and methods

Rice (*Oryza sativa L.*, cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in a Petri dish (20 cm) containing distilled water at 37 °C under dark condition. After 1-day incubation, uniformly germinated seeds were selected and transferred to a Petri dish (9.0 cm) containing two sheets of Whatman No.1 fil-

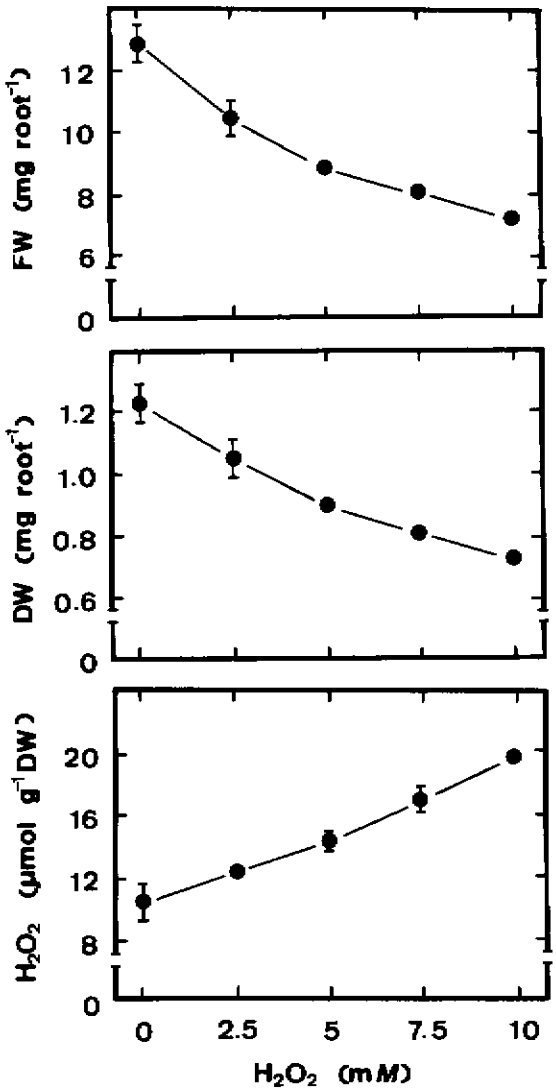

Figure 3. Effects of exogenous application of H_2O_2 on root growth and H_2O_2 levels in roots of rice seedlings. Root growth and H_2O_2 levels were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

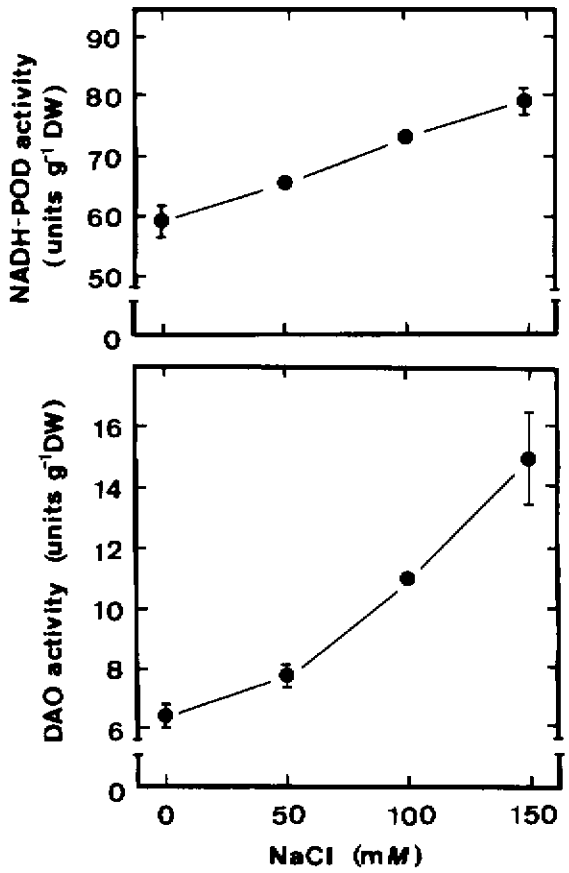

Figure 4. Effects of NaCl on NADH-POD and DAO activities in roots of rice seedlings. NADH-POD and DAO activities were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

ter paper moistened with 10 ml of distilled water or test solutions. Each Petri dish contained 20 germinated seeds. Each treatment was replicated four times. The germinated seeds were allowed to grow at 27 °C in darkness and 3 ml of distilled water or test solutions was added to each Petri dish on day 3 of the growth. Fresh weight and dry weight of roots were measured at the times indicated.

Cell walls were prepared by homogenizing roots in ice-cold phosphate buffer (50 m*M*, pH 5.8) using a pestle and mortar. The homogenate was centrifuged at 1000 *g*, and washed at least four times with 50 m*M* phosphate buffer (Lee and Lin, 1995). The pellet was collected and used as a cell wall fraction.

POD ionically bound to the cell walls was extracted with 1 *M* NaCl. Cell walls were prepared as described above and incubated in 1 *M* NaCl for 2 h with shaking at 30 °C and centrifuged at 1000 *g*. The supernatant was considered as the ionic cell wall fraction. POD covalently bound to the cell walls was extracted as follows: Cell walls previously subjected to the saline extraction procedure were incubated in an enzyme preparation containing 0.5% cellulase (EC 3.2.1.4, Sigma) and 2.5% pectinase (EC 3.2.1.15, Sigma), both from *Aspergillus niger*, in 0.1 *M* sodium acetate buffer, pH 5.0 (Sanchez et al., 1989). The incubation was carried out for 24 h with shaking at 25 °C. The suspension was then centrifuged at 1000 *g*

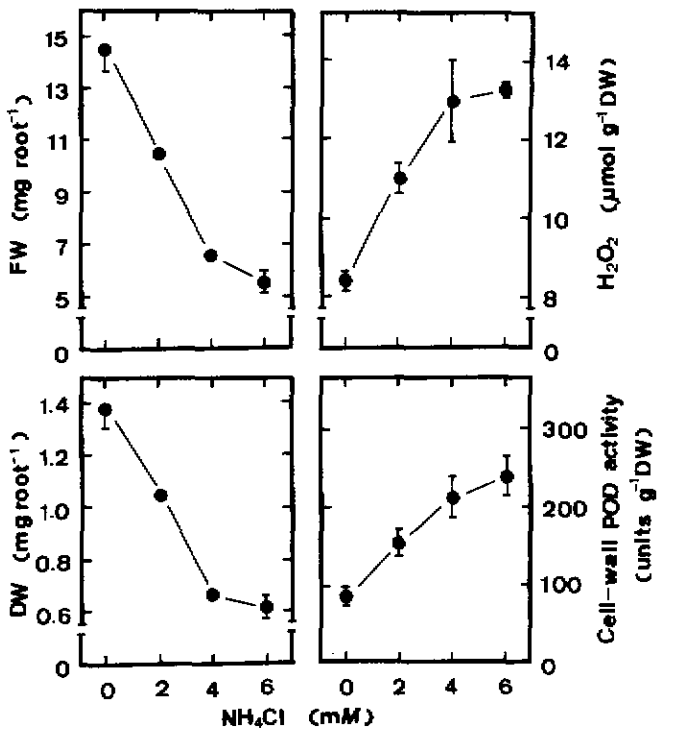

Figure 5. Effects of NH_4Cl on root growth, cell-wall POD activities and H_2O_2 levels in roots of rice seedlings. Root growth, cell-wall POD activities and H_2O_2 levels were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

for 10 min. The supernatant was considered to be the covalently bound cell wall fraction.

POD activities were measured using a modification of the procedure described by Curtis (1971). The assay medium contained 0.05 *M* phosphate buffer (pH 5.8), 7.2 m*M* guaiacol, 11.8 m*M* H_2O_2 and 0.1 ml enzyme extract in a final assay volume of 3.0 ml. The reaction was initiated by the addition of H_2O_2 and the change in absorbance at 470 nm was measured. Activity was calculated using the extinction coefficient (26.6 mM^{-1} cm^{-1} at 470 nm) for tetraguaiacol. One unit of POD was defined as the amount of enzyme that causes the formation of 1 μmol tetraguaiacol per min.

NADH-POD activities in the ionic cell wall fraction were determined according to the method of Ishida et al. (1987). The assay mixture contained 50 μM NADH in Na-acetate buffer (30 m*M*, pH 6.5), 5 m*M* $MnCl_2$ and 20 μM *p*-coumaric acid. The reaction was started by adding the enzyme, and the decrease of absorbance at 340 nm by oxidizing NADH was measured at 25 °C. One unit of NADH-POD was defined as 1 nmol NADH oxidized per min.

Diamine oxidase (DAO), which is involved in polyamine catabolism, oxidizes putrescine with the formation of Δ^1-pyrrolinetogether with H_2O_2 and ammonia (Smith, 1985). DAO activities in the ionic cell wall fraction were measured by the method of Naik et al. (1981). The incubation mixture contained 50 m*M* phosphate buffer (pH 7.8), 10 m*M* putresine, 0.1 m*M* pyridoxal phosphate and enzyme extract in a total volume of 4 ml. After incubation at 30 °C for 1 h, the reaction was terminated using 1 ml 20% (w/v) trichloroacetic acid. After 30 min, the incubation mixture was centrifuged at 5000 *g* for 15 min. One ml of ninhydrin mixture (250 mg ninhydrin in 6 ml acetic acid and 4 ml phosphoric acid) was added to the supernatant. Colour was developed at 100 °C for 30 min. After adding 1 ml of acetic acid absorbance was measured at 510 nm. In controls, trichloroacetic acid added prior to the enzyme solution. One unit of DAO was defined as an increase of 1 A_{510} per h.

The H_2O_2 level was colorimetrically measured as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing 10 roots with 3 ml of phosphate buffer (50 m*M*, pH 6.8). The homogenate was centrifuged at 6000 *g* for 25 min. To determine H_2O_2 levels, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium chloride (Aldrich) in 20%

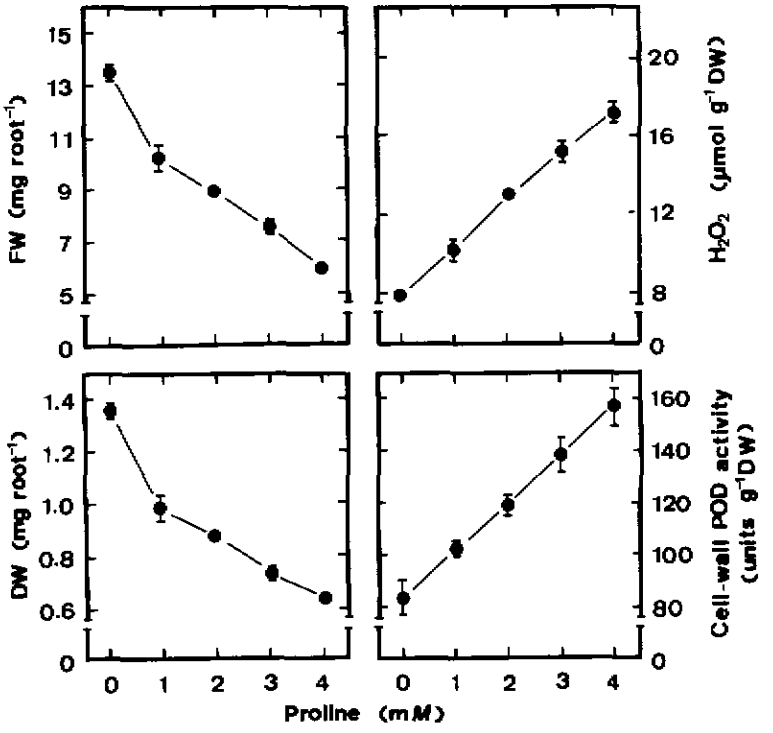

Figure 6. Effects of proline on root growth, cell-wall POD activities and H_2O_2 levels in roots of rice seedlings. Root growth, cell wall-POD activities and H_2O_2 levels were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

(v/v) H_2SO_4 and the mixture was then centrifuged at 6000 *g* for 15 min. The intensity of the yellow colour of the supernatant was measured at 410 nm. H_2O_2 level was calculated using the extinction coefficient 0.28 $\mu mol^{-1}cm^{-1}$.

For all measurements, each treatment was repeated four times. All experiments described here were repeated at least three times. Similar results and identical trends were obtained each time. The data reported here are from a single experiment.

Results and discussion

Root growth was followed by measuring FW and DW of roots. Figure 1 shows the effect of NaCl on root growth of rice seedlings. Increasing concentrations of NaCl from 50 to 150 m*M* progressively decreased root growth.

Both ionically bound and covalently bound cell-wall PODs were present in cell walls of rice roots (Figure 1). The reduction of root growth with increasing NaCl concentrations is correlated with an increase in ionically bound cell-wall POD activity (Figure 1). The effect of different concentrations of NaCl on covalently bound cell- wall POD activities was also examined. No appreciable changes in covalently bound cell-wall POD activities occurred between 0 and 150 m*M* NaCl. Thus, ionically bound POD in the cell wall preparations was assayed in all subsequent experiments.

It has been postulated that the action of POD located in the cell walls would be to confer rigidity to the cell walls and prevent later expansion involved in growth (Carpita and Gilbeaut, 1993; Fry, 1986; Gardiner and Cleland, 1974; MacAdam et al., 1992). Thus, NaCl-induced inhibition in root growth of rice seedlings is likely due to cell wall stiffening process related to the formation of cross-linking among cell wall polymers. This process appears to involve oxidative coupling, dependent on H_2O_2 (Fry, 1986). In fact, H_2O_2 has been demonstrated to cause a rapid cross-linking of cell wall polymers (Bradley et al., 1992; Schopfer, 1996). If POD regulates cell wall stiffening by catalyzing the oxidative cross-linking of cell wall polymers, there must be a sufficient supply of H_2O_2. Thus, it is of great interest to know whether NaCl increases the level of H_2O_2 in roots of rice seedlings.

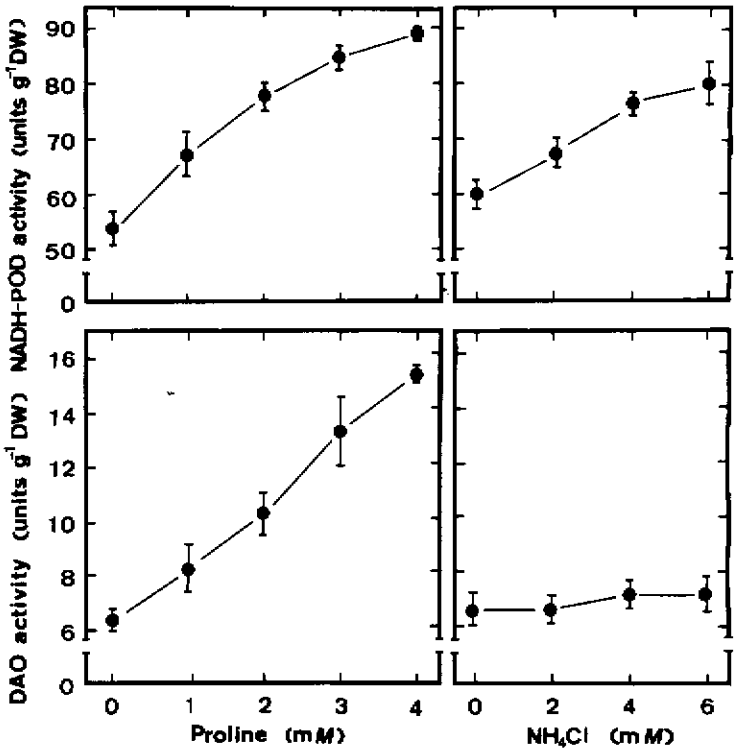

Figure 7. Effects of NH_4Cl or proline on NADH-POD and DAO activities in roots of rice seedlings. NADH-POD and DAO activities were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

Figure 2 shows the effect of NaCl concentrations on H_2O_2 levels in the roots of rice seedlings. Increasing concentrations of NaCl from 50 to 150 m*M* progressively increased H_2O_2 levels in roots. If H_2O_2 plays a role in regulating NaCl-inhibited root growth of rice seedlings, exogenous application of H_2O_2 is expected to inhibit root growth. It is indeed the case (Figure 3). Increasing concentrations of H_2O_2 from 2.5 to 10 m*M* progressively increased endogenous H_2O_2 levels in roots of rice seedlings and decreased FW and DW. Clearly, an increase in H_2O_2 levels is important in regulating NaCl-inhibited root growth of rice seedlings.

Generation of H_2O_2 in the cell walls has been previously proposed since it is a necessary substrate for the formation of cross-linking among cell wall polymers (Elstner and Heupel, 1976) and cell wall-bound malate dehydrogenase and NADH-POD activities devoted to H_2O_2 production have been detected (Goldberg et al., 1987; Gross, 1977; Mader et al., 1980). As expected, increasing NADH-POD activities were found to increase with increasing NaCl concentrations in rice seedling roots (Figure 4). Concerning the origin of the NADH required in the formation of H_2O_2, it has been postulated that a cell wall-bound malate dehydrogenase provides this electron donor (Gross, 1977). However, we have not succeeded in detecting cell wall-bound malate dehydrogenase activity in roots of rice seedlings. Frahry and Schopfer (1998) also failed to detect cell wall-bound malate dehydrogenase activity in intact soybean roots.

DAO is widespread in the Leguminosae family (Smith, 1985) and has been reported in barley (Cogoni et al., 1990) and maize (Suzuki and Hagiwara, 1993). This enzyme, which is involved in polyamine catabolism, oxidizes putrescine with the formation of Δ^1-pyrroline together with H_2O_2 and ammonia (Smith, 1985). DAO activity is mainly located in the cell wall (Angelini and Federico, 1989; Angelini et al., 1990) and perhaps plays a role in regulating putrescine levels (Smith, 1985) or providing H_2O_2 required for peroxidative reactions that occur in the cell walls for the formation of cross-linking (Angelini and Federico, 1989; Angelini et al., 1990). It is most likely that DAO is another source leading to H_2O_2 generation in NaCl-inhibited root growth of rice seedlings. To test this,

Table 1. Changes in root growth, cell-wall POD activity, H_2O_2 level, DAO activity and NADH-POD activity in roots of rice seedlings treated with NaCl. Rice seeds were germinated in distilled water for 2 days and then were transferred to distilled water and NaCl (150 mM), respectively. The data represent mean value ± standard errors, n=4

	Treatment	Time, h				
		0	4	8	12	16
FW	H_2O	7.1±0.42	7.6±0.71	8.1±0.35	8.6±0.10	9.4±0.23
(mg root^{-1})	NaCl		7.5±0.28	7.7±0.47	8.1±0.20	8.5±0.36
DW	H_2O	0.67±0.04	0.72±0.03	0.77±0.03	0.82±0.01	0.89±0.02
(mg root^{-1})	NaCl		0.71±0.03	0.73±0.05	0.77±0.02	0.80±0.03
Cell-wall POD	H_2O	38.3±0.82	36.7±1.8	38.9±2.5	38.0±1.1	39.0±1.4
(units g^{-1}DW)	NaCl		41.1±5.9	49.6±3.8	57.3±2.0	65.1±3.4
H_2O_2	H_2O	6.6±0.39	6.7±0.62	6.6±0.16	7.7±0.95	7.7±0.20
(μmol g^{-1} DW)	NaCl		7.0±0.58	8.7±0.72	9.2±0.46	10.4±0.12
DAO	H_2O	6.6±0.66	5.9±0.82	6.6±0.71	7.3±0.76	7.6±0.57
(units g^{-1} DW)	NaCl		7.0±0.68	8.2±0.47	8.6±0.20	9.5±0.36
NADH-POD	H_2O	54.3±3.2	52.3±1.7	54.0±2.4	55.6±0.52	59.8±3.7
(units g^{-1} DW)	NaCl		54.1±2.9	58.5±0.55	60.3±1.7	66.4±4.1

Table 2. Changes in root growth, cell-wall POD activity, H_2O_2 level and NADH-POD activity in roots of rice seedlings treated with NH_4Cl. Rice seeds were germinated in distilled water for 2 days and then were transferred to distilled water and NH_4Cl (4 mM), respectively. The data represent mean values ± standard errors, n=4

		Time, h				
		0	4	8	12	16
FW (mg root^{-1})	H_2O	6.8±0.60	7.1±0.31	7.6±0.16	8.9±0.36	9.7±0.32
	NH_4Cl		7.2±0.19	7.6±0.09	7.9±0.09	8.2±0.45
DW (mg root^{-1})	H_2O	0.64±0.06	0.67±0.03	0.72±0.01	0.84±0.03	0.92±0.03
	NH_4Cl		0.68±0.02	0.72±0.01	0.75±0.01	0.78±0.04
Cell-wall POD (unit g^{-1}DW)	H_2O	45.1±5.4	45.9±4.2	45.9±3.0	43.6±5.9	46.4±1.3
	NH_4Cl		47.8±1.7	53.7±2.1	60.9±3.2	68.9±3.8
H_2O_2 (μmol g^{-1} DW)	H_2O	6.7±0.49	7.0±0.72	6.9±0.12	7.5±0.73	8.3±0.49
	NH_4Cl		7.5±0.32	8.4±0.21	10.1±0.65	11.6±0.31
NADH-POD (units g^{-1} DW)	H_2O	50.3±3.4	51.3±3.8	52.3±2.1	53.1±1.8	56.1±1.4
	NH_4Cl		53.2±2.9	57.2±0.38	58.2±1.4	61.4±2.1

we determined the activities of DAO in rice seedling roots in response to various concentrations of NaCl (Figure 4). As expected, increasing concentrations of NaCl from 50 to 150 mm progressively increased DAO activities. This result is consistent with our previous work in which we showed that NaCl treatment resulted in a decline in the levels of putrescine in roots of rice seedlings (Lin and Kao, 1995).

To test the causal relationship between root growth reduction, cell-wall POD activity, H_2O_2 level, DAO activity and NADH-POD activity caused by NaCl, 2-day-old seedlings were transferred to distilled wa-

Table 3. Changes in root growth, cell-wall POD activity, H_2O_2 level, DAO activity and NADH-POD activity in roots of rice seedlings treated with proline. Rice seeds were germinated in distilled water for 2 days and then were transferred to distilled water and proline (4 mM), respectively. The data represent mean values ± standard errors, n=4

		Time, h				
		0	4	8	12	16
FW (mg root^{-1})	H_2O	6.8±0.60	7.1±0.31	7.6±0.16	8.9±0.36	9.7±0.32
	proline		7.4±0.21	7.9±0.36	7.7±0.17	7.9±0.20
DW (mg root^{-1})	H_2O	0.64±0.06	0.67±0.03	0.72±0.01	0.84±0.03	0.92±0.03
	proline		0.70±0.02	0.75±0.03	0.73±0.0.2	0.75±0.02
Cell-wall POD (units g^{-1}DW)	H_2O	45.1±5.4	45.9±4.2	45.9±3.0	43.6±5.9	46.4±1.3
	proline		52.2±5.3	60.0±2.1	69.3±2.1	80.2±3.5
H_2O_2 (μmol g^{-1} DW)	H_2O	6.7±0.49	7.0±0.72	6.9±0.12	7.5±0.73	8.3±0.49
	proline		7.2±0.18	8.2±0.35	9.2±0.35	10.8±0.66
DAO (units g^{-1} DW)	H_2O	6.2±0.55	6.3±0.49	6.7±0.68	7.2±0.56	7.7±0.37
	proline		6.4±0.59	8.0±0.23	8.3±0.37	9.0±0.44
NADH-POD (units g^{-1} DW)	H_2O	54.3±3.8	55.3±2.6	55.0±2.6	58.1±2.4	60.5±3.2
	proline		56.2±2.3	59.2±0.77	64.3±2.8	67.2±2.3

ter and NaCl, respectively, for 4, 8, 12 and 16 h. Changes in root growth, cell-wall POD activity, H_2O_2 level, DAO activity and NADH-POD activity were then monitored. As indicated in Table 1, an increase in cell-wall POD activity and H_2O_2 level preceded inhibition of root growth caused by NaCl. The observations that an increase in DAO and NADH-POD activities coincides with an increase in H_2O_2 level in roots caused by NaCl (Table 1) suggest that DAO and NADH-POD are the sources for the generation of H_2O_2 in the cell walls. Since H_2O_2 can rapidly pass from the cytoplasm to the cell wall (Allan and Fluhr 1997), a cytoplasmic origin of released H_2O_2 cannot be excluded.

It is known that ammonium strongly inhibits the growth of many plants (Haynes and Goh, 1978). Exogenous application NH_4Cl was also found to reduce root growth of rice seedlings (Lin and Kao, 1996a). It has also been shown that NaCl was effective in stimulating the accumulation of ammonium in roots of rice seedlings and that accumulation of ammonium in roots preceded inhibition of root growth caused by NaCl (Lin and Kao, 1996a). If the increases in cell-wall POD activity and H_2O_2level are important in regulating growth reduction of roots caused by NaCl, the exogenous application of NH_4Cl would be expected to increase cell-wall POD activity and H_2O_2level in roots of rice seedlings. Figure 5 shows that addition of NH_4Cl inhibits root growth, increases cell-wall POD activity and increases H_2O_2 level in roots.

In previous work, we have shown that proline accumulation is associated with root growth inhibition of rice seedlings caused by NaCl (Lin and Kao, 1996b). In the present study, we also demonstrated that proline treatment resulted in an inhibition of root growth, an increase in cell-wall POD activity and an increase in H_2O_2 level (Figure 6). The data in Figure 7 clearly show that DAO and NADH-POD are responsible for the generation of H_2O_2 in the cell wall of proline-treated roots, whereas NADH-POD but not DAO is the source for the generation of H_2O_2 in NH_4Cl-treated roots. Furthermore, we also observed that an increase in cell-wall POD activity and H_2O_2 level preceded inhibition of root growth caused by NH_4Cl or proline and an increase in H_2O_2level coincided with an increase in NADH-POD or DAO activity (Tables 2 and 3).

The observations that rice seedlings treated with NH_4Cl or proline, which resulted in an increase in cell-wall POD activity and H_2O_2 level in roots, reduced root growth in the same way that NaCl did, further support our suggestion that cell-wall POD and H_2O_2 are likely participated in the regulation of root growth reduction of rice seedlings under NaCl condition.

Acknowledgement

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