

# Effects of three allelopathic phenolics on chlorophyll accumulation of rice (*Oryza sativa*) seedlings: II. Stimulation of consumption-orientation

Chi-Ming Yang<sup>1,\*</sup>, Ing-Feng Chang<sup>1</sup>, Shu-Jin Lin<sup>1</sup>, and Chang-Hung Chou<sup>2</sup>

<sup>1</sup>Institute of Botany and Research Center for Biodiversity, Academia Sinica, Nankang, Taipei, Taiwan 11529, Republic of China

<sup>2</sup>Institute of Tropical Agriculture and Research Center for Biodiversity and International Cooperation, National Pingtung University of Science and Technology, Neipu, Pingtung, Taiwan 912, Republic of China

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**Abstract.** The effects of three allelopathic phenolics, *o*-hydroxyphenyl acetic, ferulic and *p*-coumaric acids, on the chlorophyllase activity of rice leaf (*Oryza sativa* cv. TN67) were investigated. Ten-day-old green seedlings of rice were cultured in greenhouse for 16 d in Kimura's culture solution, which was changed every 4 days, with or without 50, 100 or 200 ppm of the phenolic compounds. Just before changing the culture solution, leaves were harvested to determine their chlorophyll (Chl) and chlorophyllide (Chlide) contents, and their chlorophyllase a and b activities. While the Chl and Chlide contents decreased and increased, respectively, causing the molar ratio of Chlide/Chl to increase, as the phenolic concentrations increased; the chlorophyllase a and b activities drastically increased. This suggests that the consumption-orientation of Chl was significantly stimulated by the exogenously applied phenolics. The order of inhibition of growth of the rice seedlings is: ferulic acid > *p*-coumaric acid > *o*-hydroxyphenylacetic acid. The order of inhibition effect on Chl accumulation is: *p*-coumaric acid > *o*-hydroxyphenylacetic acid > ferulic acid. The order of stimulation effect on chlorophyllase a activity is: *o*-hydroxyphenylacetic acid > ferulic acid > *p*-coumaric acid. The order of promotion effect on chlorophyllase b is: ferulic acid > *o*-hydroxyphenylacetic acid > *p*-coumaric acid. The different responses of chlorophyllase a and b activities to the same concentrations of allelochemical phenolics suggest that they may be two different enzymes. It is apparent that the three phenolics may enhance the activities of enzymes, such as chlorophyllase and Mg-dechelataase, responsible for the Chl degradative pathway. A combination of the present and the preceding data strongly suggest that the three allelopathic phenolics may comprehensively affect the biosynthetic and degradative pathways of Chl.

**Keywords:** Allelopathic phenolics; Chlorophyll; Chlorophyllase; Chlorophyllide; Consumption-orientation; Ferulic acid; *Oryza sativa*; *o*-hydroxyphenyl acetic acid; *p*-coumaric acid.

## Introduction

More than 10,000 secondary metabolites in plants, such as phenolics, terpenoids, alkaloids, fatty acids, steroids, and polyacetylenes, are known to play an important role in allelopathy, which includes positive and negative effects (Inderjit, 1996; Olofsson, 1998; Rice, 1984; Waller, 1987). Even so, it is still largely a mystery how the allelochemicals, of the targeted organelle of the targeted cell of the targeted tissue of a targeted plant, function in the targeted reaction, during replication, transcription, translation, or post-translation (Kohli et al., 1998).

The multiple effects resulting from allelopathic phenolics include decreases in plant growth, absorption of water and mineral nutrients, ion uptake, leaf water potential, shoot turgor pressure, osmotic potential, dry matter production, leaf area expansion, stomatal aperture size, stomatal diffusive conductance, and photosynthesis (Booker

et al., 1992; Chou and Lin, 1976; Einhellig et al., 1970; Einhellig and Kuan, 1971; Einhellig and Rasmussen, 1979; Einhellig et al., 1985; Gerald et al., 1992; Patterson, 1981). Allelochemicals have been reported to mediate the synthesis or degradation of some plant hormones, such as the activation of ABA synthesis by ferulic acid (Hollapa and Blum, 1991), the degradation of IAA via the stimulation of IAA oxidase by dihydroflavonone naringenin (Stenlid, 1970), and the resemblance of antiauxin and/or antigibberellin activity by sesquiterpene farnesol and sesquiterpene lactone argrophylline A and  $\beta$ -selinene (Komai et al., 1981; Watanabe et al., 1982).

Among so many symptoms, a decrease in photosynthesis efficiency is a common effect of allelopathic phenolics. Sorgoleone, a *p*-benzoquinone, in *Sorghum bicolor* root exudates was found to inhibit the oxygen evolution of soybean leaf disk and isolated pea chloroplast, which in turn caused growth reduction (Einhellig et al., 1993) and photosystem II electron transfer reaction (Gonzalez et al., 1997). The growth, chlorophyll content, and net photosynthesis of *Lemna minor* decrease as it is treated with juglone (Hejl et al., 1993). Benzoic and cin-

\*Corresponding author. Tel: 002-886-2-27821258 ext. 612; Fax: 002-886-2-27827954; E-mail: cmyang@gate.sinica.edu.tw

namic acids cause a reduction in chlorophyll content of soybean leaf (Baziramakenga et al., 1994). *p*-Coumaric, ferulic, cinnamic and vanillic acids, and coumarin severely suppress the photosynthesis of soybean and *Lemna minor* L. (Patterson 1981; Einhellig, 1986). Three of the above five phenolic acids, i.e. *p*-coumaric, ferulic, and vanillic acids, were also reported to severely inhibit the photosynthesis and protein synthesis of isolated leaf cells of velvetleaf (Mersie and Singh, 1993). The same three phenolic acids also caused chlorophyll (Chl) reduction in soybean and sorghum seedlings (Einhellig and Rasmussen, 1979). Chl reduction also occurred in soybean plants treated with aqueous extract of velvetleaf *Abutilon theophrasti* (Colton and Einhellig, 1980). Evernic acid, the main phenolic compound of lichen *Evernia prunastri* thalli, produced a decrease in the amount of total chlorophyll and chlorophyll a in treated spinach leaves. The chloroplast structure of spinach leaves suffered a decrease in area, number of grana, granal width, number of thylakoids per granum, and starch content (Rapsch and Ascaso, 1985). Rice (1984) has suggested that the synthesis of porphyrin precursors of chlorophyll biosynthesis may be impeded by some allelopathic compounds.

We hypothesized that the Chl reduction caused by allelochemicals may result from partial non-lethal blockage of the Chl biosynthesis pathway, by stimulation of Chl degradation mechanisms, or both (Yang et al., 2002). To test this hypothesis, a preceding study used exogenously supplied *o*-hydroxyphenyl acetic, ferulic, and *p*-coumaric acids to two-week-old etiolated rice seedlings and found that Chl and porphyrin contents decreased as the phenolic concentrations increased. The three phenolics did not affect the mole percent of Mg-protoporphyrin IX (Mg-Proto), but did slow down to various degrees the decrease and increase patterns of the mole percent of protoporphyrin IX (Proto) and of protochlorophyllide (Pchlde), respectively (Yang et al., 2002). The data strongly suggest that the enzyme Mg-chelatase, responsible for the conversion of Proto into Mg-Proto, may be the major target of the three tested phenolics, causing the shortage of Chl.

Since this hypothesis includes a supply- and consumption-orientation of Chl accumulation, i.e. Chl biosynthesis and degradation, it is needed to continue testing the other side of the hypothesis. For Chl degradation, two pathways are possible. One is dephytylation of Chl into Chlide, catalyzed by chlorophyllase, while the other is dechelation of Mg into pheophytin, catalyzed by Mg-dechelataase. Both further go through pheophorbide (Matile et al., 1996). The objective of the present study is, thus, to investigate whether the three phenolic compounds stimulate the activity of chlorophyllase, an enzyme catalyzing one of the first steps of Chl degradation.

## Methods and Materials

### Plant Material

Seeds of rice (*Oryza sativa* cultivar Tainung 67) were sterilized in 70% ethanol for 1.5 min, then in 2% sodium

hypochlorite for 30 min before being washed several times with distilled water. The sterilized seeds were soaked in the dark at 30°C for 3 d and then incubated in water in the greenhouse for 10 days. The 10-day-old green seedlings were then transferred to one-fifth fresh Kimura's culture solution in the presence or absence of 50, 100, or 200 ppm of *o*-hydroxyphenyl acetic, ferulic, and *p*-coumaric acids. The sample leaves were harvested at 4, 8, 12, and 16 d after treatment with phenolics. Plant height was measured at 16 d.

### Chl and Chlide Determination

Sample leaves were frozen with liquid nitrogen and then ground into powder with mortar and pestle. Following extraction of liquid nitrogen-frozen leaf with 80% acetone, the spectrophotometric method of Porra et al. (1989) was used to determine the Chl content. The acetone extract was mixed with an equal volume of hexane to remove Chls, and the absorbance of the lower phase was measured at 667 and 650 nm. Chlide content was calculated using the Beer-Lambert equation. The extinction coefficients of 74.9 mM<sup>-1</sup>cm<sup>-1</sup> at 667 nm or 47.2 mM<sup>-1</sup>cm<sup>-1</sup> at 650 nm were used for Chlide a and b, respectively. All absorbance measurements were conducted with a Hitachi U2000 UV-visible spectrophotometer.

### Preparation of the Substrates

Chl a and b were isolated and purified from fresh leaves of spinach by extraction with pre-chilled acetone (-20°C) followed by column chromatography with Pharmacia Biotech DEAE-Sepharose CL-6B (Lot No. 242436) and Sepharose CL-6B (Lot No. 230876) (Omata and Murata, 1983). The purified Chl a and b were aliquoted, dried, wrapped with aluminum foil, and stored at -20°C until use.

### Preparation of Leaf Acetone Powder

The liquid-nitrogen frozen leaf of rice seedlings was ground with a mortar and pestle and then homogenized with pre-chilled acetone (-20°C). After centrifuging the homogenate at 3,000 g and at 4°C, the precipitation was collected. The cold acetone extraction was repeated, and the sample was homogenized several times to remove all trace Chls and carotenoids. The acetone powder was dried with nitrogen and stored at -20°C until use.

### Assay of the Chlorophyllase Activity

For the determination of chlorophyllase a and b activities, 50 mg of acetone powder of rice leaf was homogenized with 5 ml extraction buffer, containing 5 mM potassium phosphate (pH 7.0), 50 mM KCl and 0.24% Triton X-100 for 60 min at 30°C. The supernatant after being centrifuged at 15,000 g for 15 min was used for the enzyme assay. The assay of chlorophyllase activity followed a modified method of McFeeters et al. (1971). The standard reaction mixture contained 0.2 ml of substrate (1 μmol/ml Chl a or Chl b), 0.3 ml of the above supernatant, and 2 ml of reaction buffer containing 100 mM sodium phos-

phate (pH 7.0) and 0.24% Triton X-100. The mixture was incubated for 30 min at 30°C and the reaction stopped with 0.5 ml of 10 mM KOH. After reaction, 1 ml of the mixture was further mixed with 5 ml of hexane/acetone (3:2, v/v) solvent to eliminate the interference of Chl. The content of Chlide a or b in the acetone phase was determined by Hitachi U-2000 spectrophotometer using an extinction coefficient of 74.9 mM<sup>-1</sup>cm<sup>-1</sup> at 667 nm or 47.2 mM<sup>-1</sup>cm<sup>-1</sup> at 650 nm. One unit of chlorophyllase was defined as the amount of enzyme necessary to catalyze the production of 1 μM Chlide a or b per min.

## Results and Discussion

### Plant Height

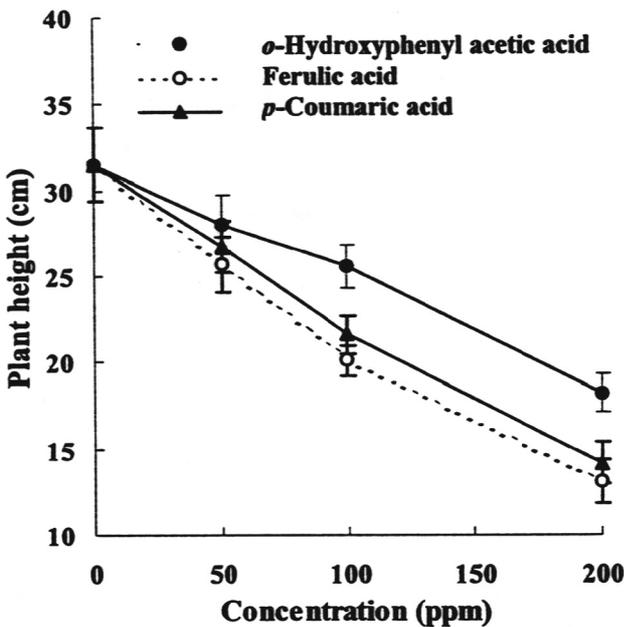
As in a preceding paper (Yang et al., 2002), all three tested phenolics retarded the growth of rice seedlings (Figure 1). Ferulic acid suppressed the growth of ten-day-old green rice seedlings most severely, *p*-coumaric acid the second most severely, and *o*-hydroxyphenyl acetic acid the least severely.

### Chl and Chlide Accumulation

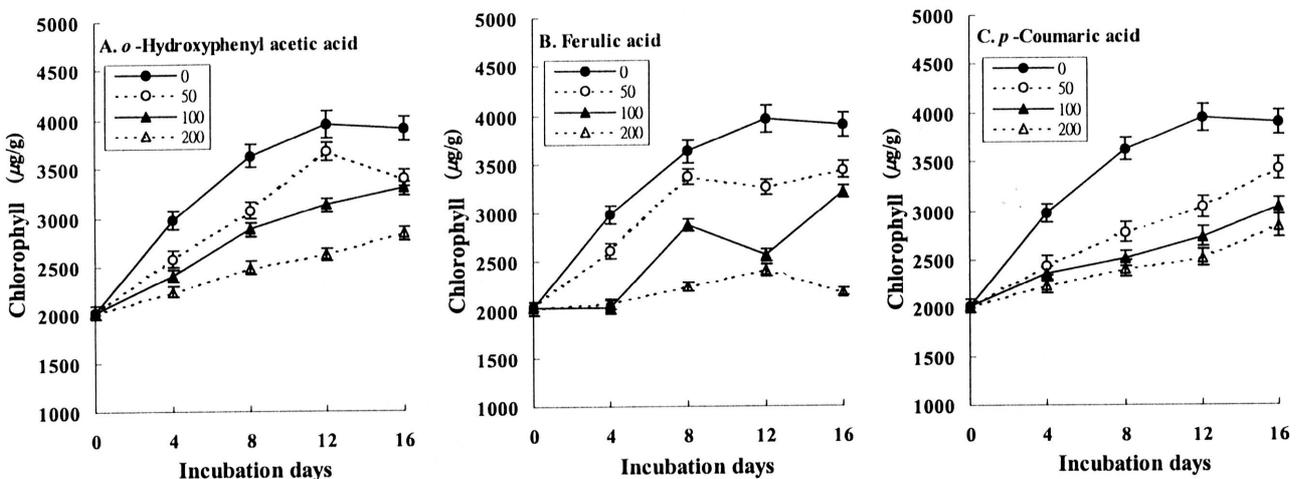
All three tested phenolics exhibited inhibitory effects on the Chl accumulation of green rice seedlings and demonstrated similar inhibitory patterns (Figure 2). The results were similar to those in a preceding paper (Yang et al., 2002). Among the three phenolics, *p*-coumaric acid exhibited the most inhibitory effect at the doses of 50 and 100 ppm, and the other two phenolics showed close inhibitory effect, with ferulic acid the next most inhibitory and *o*-hydroxyphenyl acetic acid the least. When the concentration reached 200 ppm, ferulic acid was more inhibitory than the other two compounds at 16 days after treatment. In contrast to the effects on Chl accumulation and despite very similar stimulatory patterns, the three phenolics increased Chlide accumulation within the first 8–12 days and then, in the case of ferulic and *p*-coumaric acids, declined to a level lower than the control. In the case of *o*-hydroxyphenyl acetic acid, the above decline phenomenon occurred only at the 50 ppm dose (Figure 3).

### Molar Ratio of Chl/Chlide

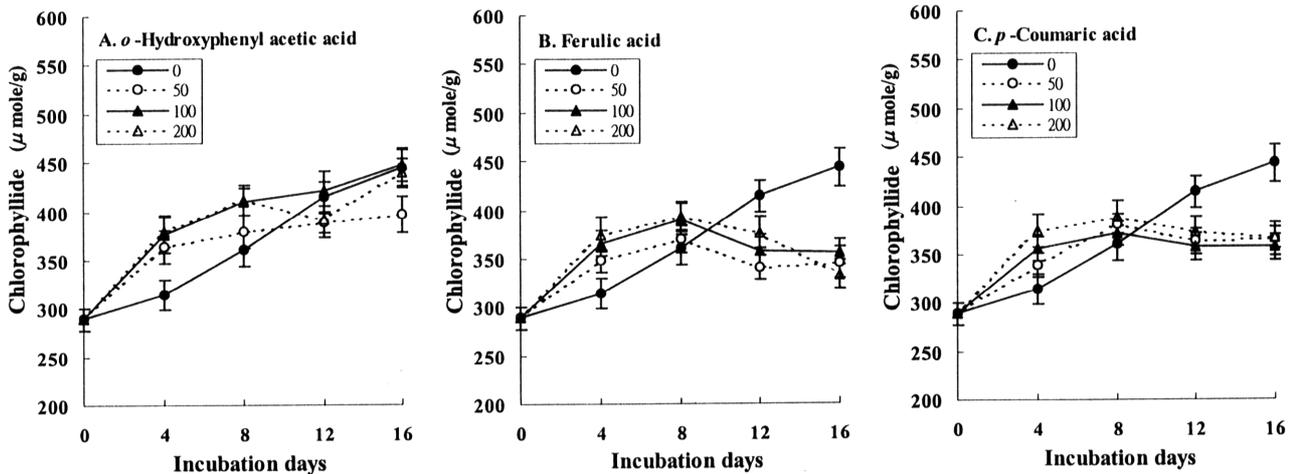
Under normal conditions, the decrease and/or increase of Chl and Chlide accumulation, respectively, lead to an increase within the first 8 d in the Chlide/Chl molar ratio, which gradually declines thereafter (Figure 4). The three phenolics all decreased the Chlide/Chl molar ratio. It seems that the conversion of Chl into Chlide, stimulated by the



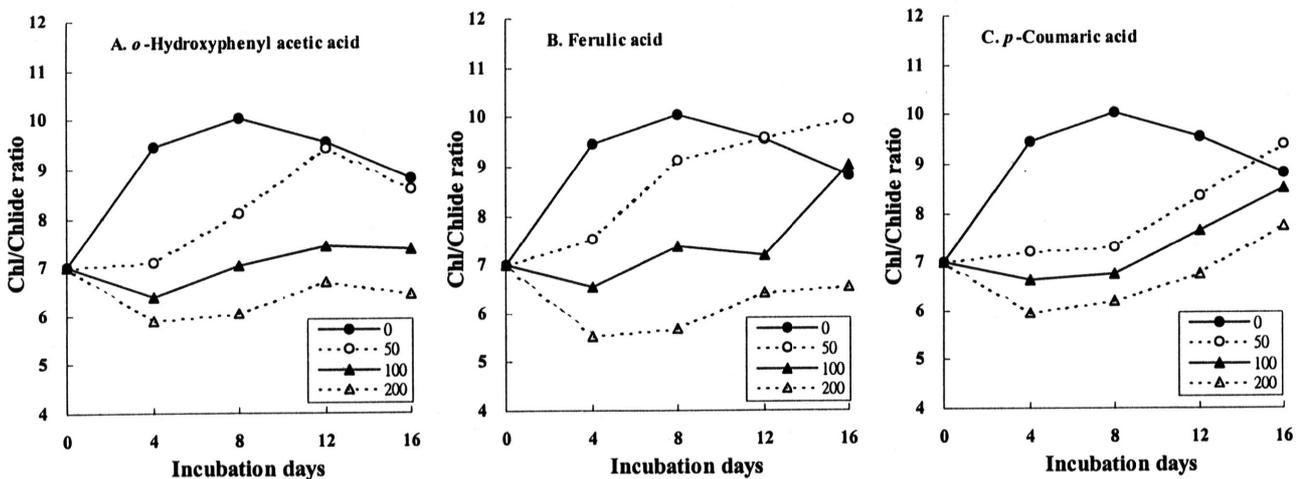
**Figure 1.** The effect of three phenolics on the plant height of ten-day-old green rice seedling. (A) *o*-hydroxyphenyl acetic acid, (B) ferulic acid, and (C) *p*-coumaric acid. Bars indicate the standard deviation.



**Figure 2.** The effect of three phenolics on the Chl content of ten-day-old rice seedling. (A) *o*-hydroxyphenyl acetic acid, (B) ferulic acid, and (C) *p*-coumaric acid. Bars indicate the standard deviation.



**Figure 3.** The effect of three phenolics on the Chlide content of ten-day-old rice seedling. (A) *o*-hydroxyphenyl acetic acid, (B) ferulic acid, and (C) *p*-coumaric acid. Bars indicate the standard deviation.



**Figure 4.** The effect of three phenolics on the Chlide/Chl ratio of ten-day-old rice seedling. (A) *o*-hydroxyphenyl acetic acid, (B) ferulic acid, and (C) *p*-coumaric acid.

three phenolics, is much faster within the first 8 d than afterward. The initial increase followed by decline suggests that the conversion of Chlide into pheophorbide (Pho) may be accelerated by the enzyme Mg-dechelataase, which also catalyzes the dechelataation of Chl into pheophytin (Phe). This further suggests that both chlorophyllase and Mg-dechelataase may be stimulated by the allelochemical phenolics.

#### Chlorophyllase a Activity

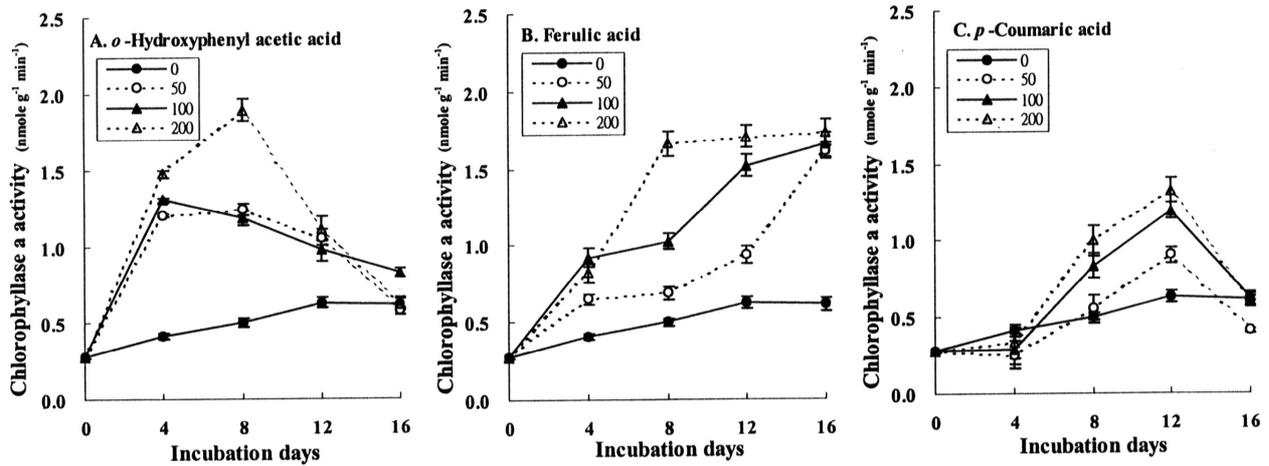
Clearly all three phenolics stimulate chlorophyllase a activity to various degrees (Figure 5). Stimulation peaked 8 and 12 days after treatment with *o*-hydroxyphenyl acetic acid and *p*-coumaric acids, respectively. Each then resumed its normal level. By contrast, ferulic acid at the level of 200 ppm reached a plateau but never returned to normal during the same period, and the 50 and 100 ppm treatments showed a continual increase in this activity over the course of the experiment, finally reaching the level of the 200 ppm treatment. Among the three phenolics, *p*-coumaric acid ex-

hibited the least stimulatory effect on chlorophyllase a activity. The other two showed similar levels of stimulation strength within 8 d, but extremely different levels thereafter.

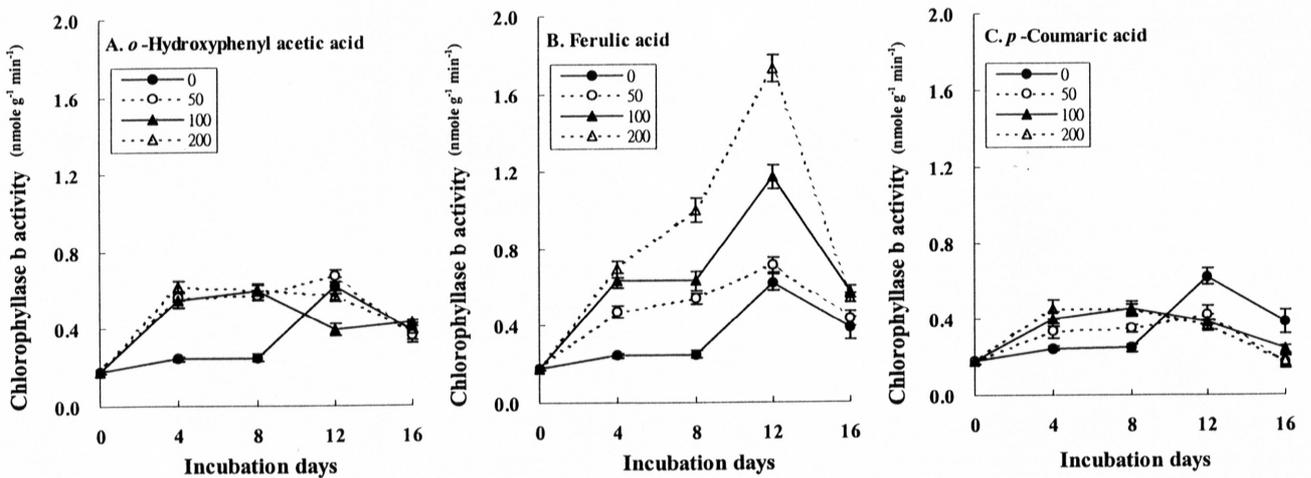
#### Chlorophyllase b Activity

In much of the literature devoted to monitoring the activity of chlorophyllase, Chl a is the exclusive substrate, with no mention of Chl b. Like chlorophyllase a activity, all three phenolics exhibited stimulatory effects on the activity of chlorophyllase b to various extents, especially in the first 8~12 days (Figure 6). Ferulic acid exhibited the highest stimulatory effect, *p*-hydroxyphenyl acetic acid the second highest, and *p*-coumaric acid the lowest.

The different responses of chlorophyllase a and b activities to the same concentrations of allelochemical phenolics imply that chlorophyllase a and b may be two different enzymes, located in the chloroplast of higher plants. These results were in line with another finding that chlorophyllase a and b activities respond differently to



**Figure 5.** The effect of three phenolics on the chlorophyllase a activity of ten-day-old rice seedling. (A) *o*-hydroxyphenyl acetic acid, (B) ferulic acid, and (C) *p*-coumaric acid. Bars indicate the standard deviation.

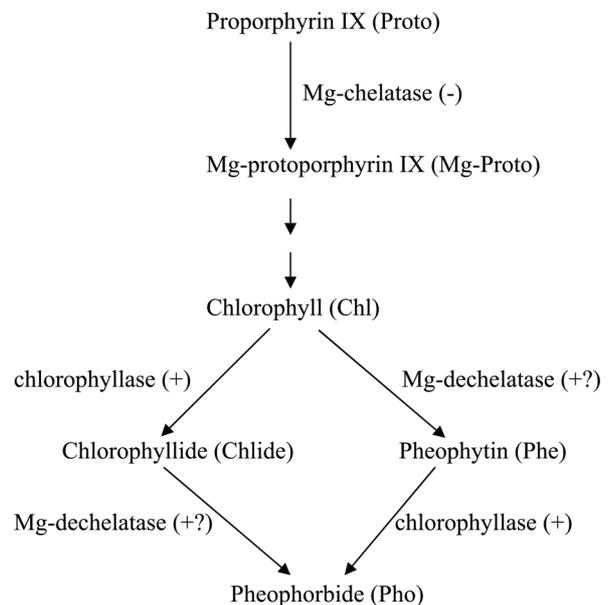


**Figure 6.** The effect of three phenolics on the chlorophyllase b activity of ten-day-old rice seedling. (A) *o*-hydroxyphenyl acetic acid, (B) ferulic acid, and (C) *p*-coumaric acid. Bars indicate the standard deviation.

humic and fulvic acids and humin isolated and purified from two forest soils in the Yuanyang Lake Nature Preserve (Yang et al., 2004). The present data demonstrate that chlorophyllase a and b, responsible for the conversion of Chl a and b into Chlide a and b, respectively, are the two enzymes directly or indirectly stimulated by the three tested allelochemical phenolics. Going further, the increase in these activities might be directly stimulated by these phenolics, or might be a part of a cascade of consequences from some fundamental alteration of plant water relations or some other effect (Figure 7).

*Stimulation Site*

Recent evidence suggests that chlorophyllase may be located in the envelope of the chloroplast (Matile et al.,



**Figure 7.** A portion of the Chl degradation pathway illustrating the possible site inhibited or stimulated by the phenolics. (+) means stimulation; (-) is inhibition; (+?) may be stimulation.

1997). If so, Chl a and b in the pigment-protein complexes heterogeneously embedded in the thylakoid should be transported from their birthplace to this envelope to be digested by chlorophyllase a and b. Because Chl molecules are lipophilic, they can embed themselves in the lipid bilayer of thylakoid membranes. The question of how lipophilic Chl molecules are transported across the water-soluble stroma and land on or close to another lipid bilayer of envelope is an interesting one. One possibility is that before transport across the stroma, the polarity of Chl molecules is altered. But how? Obviously, more work is needed to clarify this.

Considering this data in combination with that of a preceding paper (Yang et al., 2002), we conclude that both the supply- and consumption-orientation of Chl may be affected by allelopathic phenolics, with the former inhibited and the latter stimulated. It is interesting to see a compound take such a bifunctional role, one of inhibition and another of stimulation. The role may even be trifunctional if the activity of Mg-dechelataase is also stimulated. In the case of the present study, both the partial non-lethal inhibition of Mg-chelataase of the Chl biosynthesis pathway and the stimulation of chlorophyllase a and b of the Chl degradation pathway cause the reduction of Chl, in turn leading to the decrease of photosynthesis efficiency.

With regard to the possible stimulation of Mg-dechelataase, if it is the case, the molar ratio of Phe to Chl should increase as that of Chlide to Chl. Our data confirm this (data not shown). However, the activity of Mg-dechelataase needs further investigation.

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## 三種植物相剋酚酸對水稻葉綠素累積之影響： (二) 消耗面的促進

楊棋明 張英風 林素靜 周昌弘

中央研究院植物研究所

本文探討三種植物相剋酚酸 *o*-hydroxyphenyl acetic, ferulic 和 *p*-coumaric acids 對水稻葉 chlorophyllase 活性之影響。十天齡的水稻綠幼苗水耕栽培於含或不含 50、100 或 200 ppm 酚酸之 Kimura 培養液中，並置於溫室生長。培養液每四天更換一次。每次更換前採收水稻葉，以測定其 chlorophyll (Chl) 和 chlorophyllide (Chlide) 含量，及 chlorophyllase a 和 b 之活性。隨著酚酸濃度增加，Chl 的減少與 Chlide 的增加導致 Chlide/Chl 比值增加，也造成 chlorophyllase a 和 b 活性大幅增加。此結果顯示，外加的酚酸明顯促進葉綠素的消耗面。對水稻幼苗株高之抑制順序是：*p*-coumaric acid>*o*-hydroxyphenylacetic>ferulic acid。對 chlorophyllase a 之促進順序是：*o*-hydroxyphenylacetic acid>ferulic acid>*p*-coumaric acid。對 chlorophyllase b 之促進順序是：ferulic acid>*o*-hydroxyphenylacetic acid>*p*-coumaric acid。Chlorophyllase a 和 b 之活性對酚酸之不同反應，顯示它們可能是兩個不同之酶。明顯地，這三種酚酸可能促進負責葉綠素崩解之酵素 chlorophyllase 和 Mg-dechelataase 的活性。結合先前之研究結果顯示，此三種酚酸可能同時影響葉綠素生合成與崩解途徑。

**關鍵詞：**植物相剋酚酸；葉綠素；水稻；消耗面；chlorophyllase；chlorophyllide；*p*-coumaric acid；ferulic acid；*o*-hydroxyphenylacetic acid。