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Effects of three allelopathic phenolics on chlorophyll accumulation of rice (*Oryza sativa*) seedlings: I. Inhibition of supply-orientation

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Abstract. The effects of three allelopathic chemicals, *o*-hydroxyphenyl acetic, ferulic, and *p*-coumaric acids, on the chlorophyll (Chl) supply-orientation in the rice leaf (*Oryza sativa* cul. TN67) were investigated. Two-week-old etiolated seedlings of rice were cultured in growth chamber in one-tenth Kimura's culture solution, with or without 25, 50 or 100 ppm of the phenolic compounds. The photoperiod, light intensity, and relative humidity were 12 h, 300 μmol/m^{2}s, and 70%, respectively. Leaves were harvested at 1, 3, 6, 12, 24 and 48 h after treatment with phenolics. The concentrations of Chl and its three biosynthetic porphyrin precursors—i.e. protoporphyrin IX (Proto), Mg-protoporphyrin IX (Mg-Proto), and protochlorophyllide (Pchlide)—were determined, and the mole percent of total porphyrins was calculated. Accumulation of Chl and porphyrin contents were more inhibited as the phenolic concentrations increased. While the mole percent of Mg-Proto affected by the three allelochemical phenolics exhibited the same pattern, those of Proto and Pchlide showed quite different patterns of change in concentration. The data strongly suggest that Mg-chelatase may be the major target of the three phenolics. The order of inhibition strength is ferulic acid>*p*-coumaric acid>*o*-hydroxyphenyl acetic acid. It is concluded that the supply-orientation of Chl is significantly inhibited by the exogenous phenolics, causing the shortage of Chl.

Keywords: Allelopathic phenolic; Chlorophyll; Ferulic acid; *o*-hydroxyphenyl acetic acid; *Oryza sativa*; *p*-coumaric acid porphyrin; Supply-orientation.

Introduction

Allelopathy is the direct influence of chemicals released from one plant on the development and growth of another plant (Olofsdotter, 1998). It has been well documented for many years (Rice, 1984), but the mechanisms through which allelochemicals impact the development and growth of targeted plants remains obscure.

Multiple physiological effects have commonly been observed from treatments with many allelochemical phenolics. These effects include decreases in plant growth, absorption of water and mineral nutrients, ion uptake, leaf water potential, shoot turgor pressure, and osmotic potential caused by ferulic acid (Patterson, 1981; Einhellig et al., 1985; Gerald et al., 1992). Scopoletin, a coumarin derivative, inhibited dry matter production, leaf area expansion and photosynthesis in tobacco, sunflower and redroot pigweed (Einhellig et al., 1970). Chou and Lin (1976) found the aqueous extracts of decomposing rice residues in soil contained five phenolics and several unknown compounds and that extracts inhibited the radicle growth of lettuce and rice seeds and the growth of rice seedlings. Chlorogenic acid reduced stomatal aperture in tobacco and sunflower (Einhellig and Kuan, 1971). Ferulic and *p*-coumaric acids reduced leaf water potential and stomatal diffusive conductance in sorghum and soybean (Einhellig et al., 1985). Einhellig (1995) proposed that a primary effect of phenolic acids is on the plasma membrane, and this perturbation contributes to a number of physiological effects causing growth reduction.

High levels of *p*-coumaric, ferulic, cinnamic and vanillic acids, and coumarin severely suppressed the photosynthesis of soybean and *Lemna minor* L. (Patterson, 1981; Einhellig, 1986). Three phenolic acids, *p*-coumaric and ferulic and vanillic acids, were also reported to severely inhibit photosynthesis and protein synthesis of isolated leaf cells of velvetleaf *Abutilon theophrasti* (Mersie and Singh, 1993). Chl reduction was observed in soybean plants treated with aqueous extract of velvetleaf (Colton and Einhellig, 1980) and in soybean and sorghum seedlings treated with *p*-coumaric and ferulic and vanillic acids (Einhellig and Rasmussen, 1979). It has also been suggested that some allelopathic compounds may interfere with the synthesis of porphyrin, precursors of Chl biosynthesis (Rice, 1984).

Even though a reduction of photosynthesis has been widely observed in the allelochemical-targeted plants, the component of photosynthesis which is directly or indirectly affected by the allelochemicals is still unknown. One possibility is that the allelochemicals may partially block

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the biosynthetic pathway of Chl (i.e. an inhibition of supply-orientation), or stimulate the degradative pathway of Chl (i.e. a stimulation of consumption-orientation), or both, leading to a reduction of Chl accumulation, in turn causing a reduction of photosynthesis and finally diminished total plant growth.

To test the hypothesis, the present study is aimed at gauging the inhibitory effect of three allelopathic phenolics on the Chl biosynthetic pathway. A subsequent paper will report the effect of the same compounds on stimulating the activity of chlorophyllase, an enzyme catalyzing the first step 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 and finally washed several times with distilled water. The sterilized seeds were soaked and incubated in water in the dark at 30ºC for 2 weeks. The above culture was carried out in three 20 cm diameter pots (triplicate). Each pot grew more than 200 seedlings. The etiolated two-week-old seedlings were then transferred to fresh one-tenth Kimura's culture solution containing 0, 25, 50, or 100 ppm of either *o*-hydroxyphenyl acetic, ferulic, or *p*-coumaric acids. The photoperiod, light intensity, and relative humidity were 12 h, 300 µmol/m^{2}s, and 70%, respectively. The sample leaves of 25 seedlings from each pot were harvested at 1, 3, 6, 12, 24 and 48 h after treatment. Plant height was measured at 1 week after treatment with phenolics.

Chl Determination

Following extraction of liquid-nitrogen frozen leaf with 80% acetone, the concentration of Chl was determined according to the spectrophotometric method of Porra et al. (1989). Absorbance was measured with a Hitachi U2000 UV-visible spectrophotometer.

Porphyrins Determination

The contents of three porphyrin precursors of Chl biosynthesis-i.e. protoporphyrin IX (Proto), magnesium-protoporphyrin (Mg-Proto), and protochlorophyllide (Pchlide)—were determined by the method of Kahn et al. (1976). The mole percent of porphyrin was calculated in the following manner: (%) = [Proto (or Mg-Proto or Pchlide)/(Proto+Mg-Proto+Pchlide)] × 100%.

Results and Discussion

Plant Height

The three phenolics tested retarded the growth of etiolated rice seedlings, as described in much of the literature (Rice, 1984). While *o*-hydroxyphenyl acetic acid showed the least inhibition, ferulic and *p*-coumaric acids inhibited plant height to a similar degree (Figure 1). High levels of phenolic resulted, not only in growth retardation, but also leaf dehydration, causing shrinkage and a decrease in leaf width (data not shown). A similar effect was observed in an experiment using 10-day-old green rice seedlings as material (data not shown).

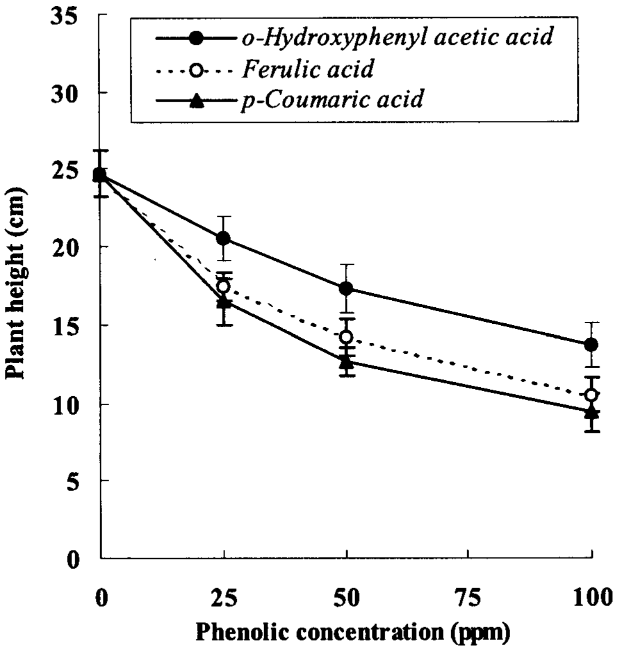

Figure 1. The effect of three phenolics on the plant height of two-week-old rice seedlings. (A) *o*-hydroxyphenyl acetic acid; (B) ferulic acid; and (C) *p*-coumaric acid. The data is the average of 20 seedlings, and the bar indicates the standard deviation.

Chl and Porphyrin Accumulation

All three tested phenolics also exhibited inhibitory effects on the Chl accumulation of rice seedlings and demonstrated similar inhibitory patterns (Figure 2). Among the three phenolics, *p*-coumaric acid caused the least inhibition effect. Inhibition could be detected within 1 h after treatment of each phenolic, and it was extended by increasing either the concentration of phenolic or the incubation time. In addition to affecting Chl, the three phenolics also inhibited the accumulation of three porphyrins (Proto+Mg-Proto+Pchlide) and demonstrated very similar inhibitory patterns (Figure 3). Among them, ferulic acid was the most inhibitory, but the other two were close. However, the mole percentages of individual porphyrins were extremely different.

Mole Percent of Proto

Under normal conditions, that is, with no phenolics added to the rice seedlings, as the porphyrin accumulation increased (Figure 3), the mole percent of Proto plunged from about 81% to 61% within 6 h. It then gradually reached the minimal level, about 46%, within the next 18 h (Figure 4). Apparently, the mole percent of the total porphyrins can be divided into two phases: the first is a fast phase, happening within 6 h, and the second is a slow phase, taking place thereafter. The three tested phenolics at all concentrations slow the normal rate of decrease during each phase. During the early period of treatment, *o*-hydroxyphenyl acetic and *p*-coumaric acids caused similar inhibitory effects on the change in Proto; that is, the mole percent of Proto decreased from 81% to about 68%, and then to 65%, but finally to the same level as under normal conditions. However, during the same period with ferulic acid, the mole percent of Proto fell from about 81% to 75%, then to 70% and finally to about 65%. That is, ferulic acid resulted in the most inhibitory effect on the decrease of the mole percent of Proto, and it never regained the normal level during the experiment period.

Mole Percent of Mg-Proto

During the 48-hour period, the mole percent of Mg-Proto drastically increased from about 20% to 25% within 6 h, afterward gradually peaking at about 30% (Figure 5). Apparently, the alteration of mole percent of Mg-Proto and Pchlide, like that of Proto, is a biphasic reaction. For the mole percent of Mg-Proto, the three phenolics exhibited no different effects during the whole incubation period, and they all reached a similar saturation plateau within about 12 h.

Mole Percent of Pchlide

The most interesting finding was that the three phenolics caused extremely different inhibitory patterns of the mole percent of Pchlide (Figure 6). Under normal conditions, the mole percent of Pchlide soared from about 0% to 13%, then gradually reached 25%. Among the three phenolics, ferulic acid exhibited the most severe inhibition effect, *p*-coumaric acid the second most severe, and *o*-hydroxyphenyl acetic acid the least. During the same period, *o*-hydroxyphenyl acetic acid boosted the mole percent of Pchlide from 0% to only 4%, then gradually to 7%,

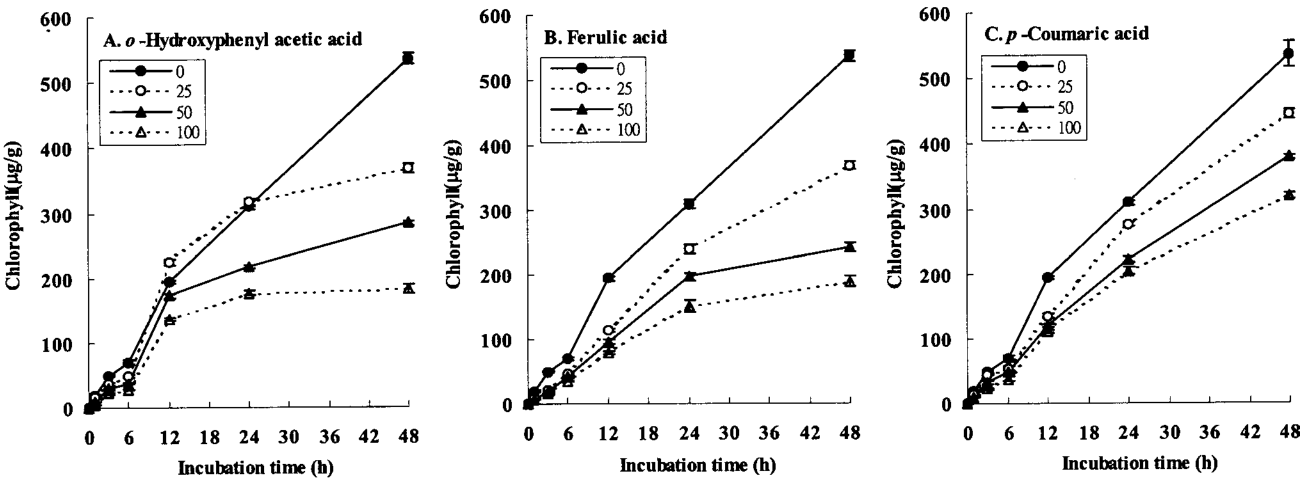

Figure 2. The effect of three phenolics on the Chl content of two-week-old rice seedling. (A) *o*-hydroxyphenyl acetic acid; (B) ferulic acid; and (C) *p*-coumaric acid. The data is the average of three determinations, and the bar indicates the standard deviation.

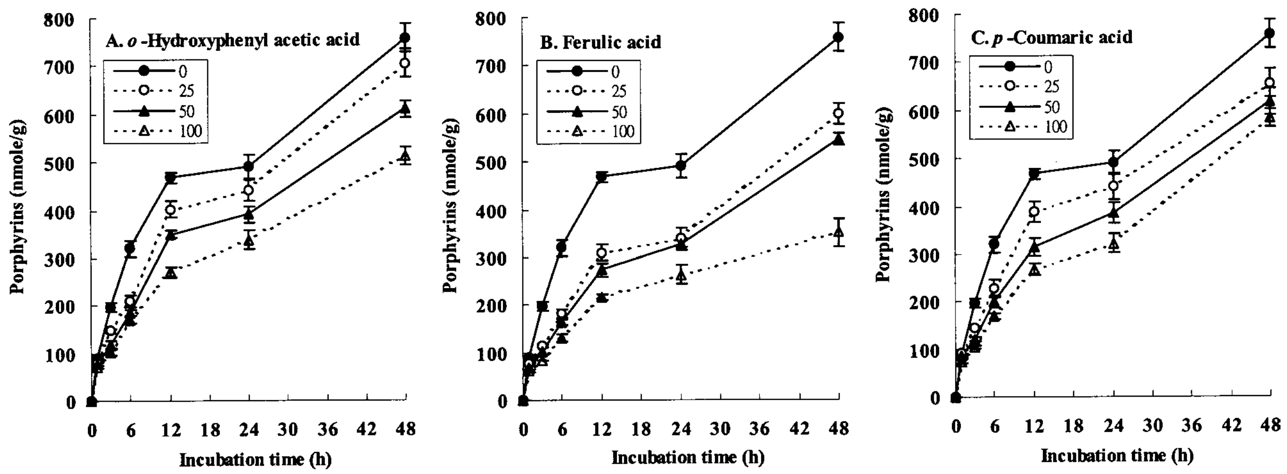

Figure 3. The effect of three phenolics on the Porphyrin content of two-week-old rice seedling. (A) *o*-hydroxyphenyl acetic acid; (B) ferulic acid; and (C) *p*-coumaric acid. The data is the average of three determinations, and the bar indicates the standard deviation.

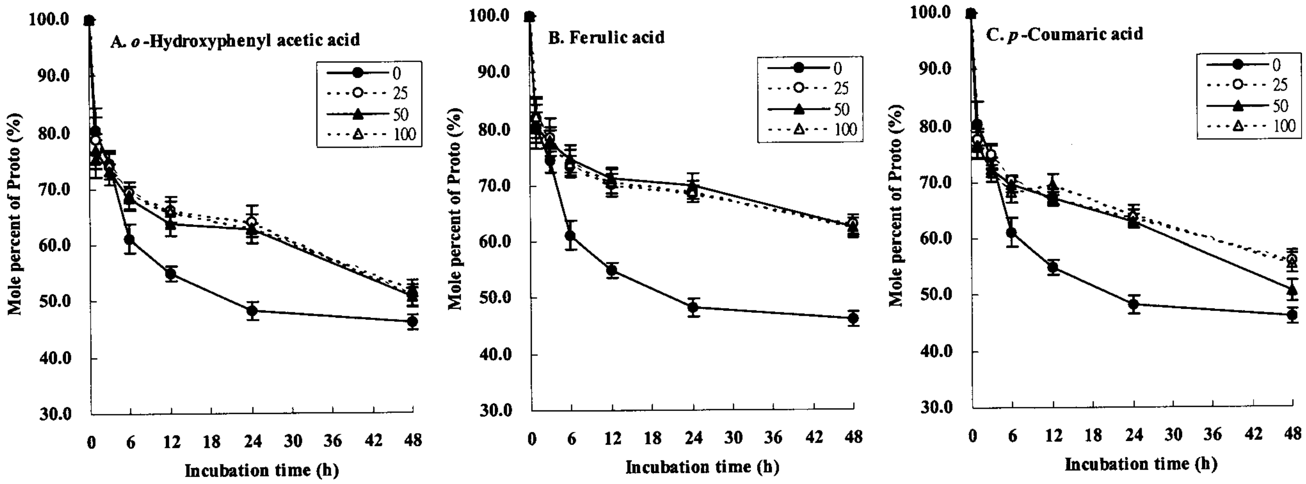

Figure 4. The effect of three phenolics on the mole percent of Proto of two-week-old rice seedling. (A) *o*-hydroxyphenyl acetic acid; (B) ferulic acid; and (C) *p*-coumaric acid. The data is the average of three determinations, and the bar indicates the standard deviation.

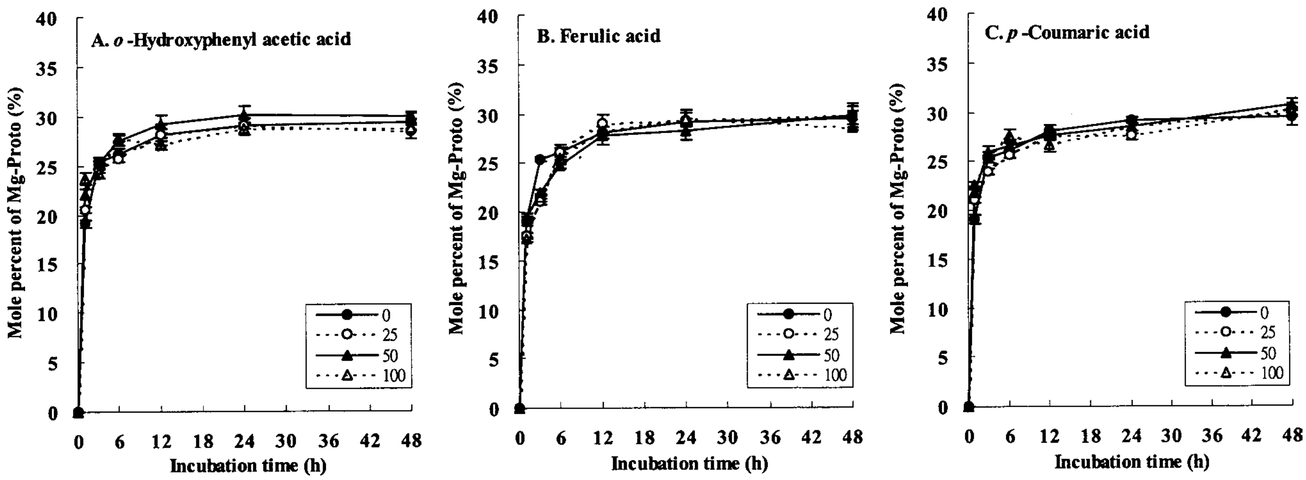

Figure 5. The effect of three phenolics on the mole percent of Mg-Proto of two-week-old rice seedling. (A) *o*-hydroxyphenyl acetic acid; (B) ferulic acid; and (C) *p*-coumaric acid. The data is the average of three determinations, and the bar indicates the standard deviation.

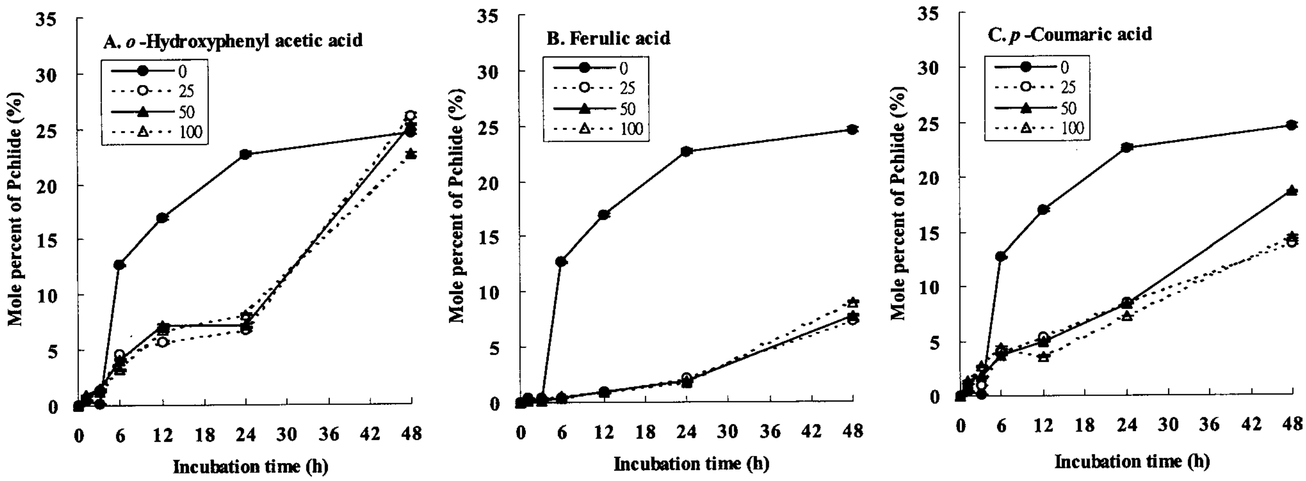

Figure 6. The effect of three phenolics on the mole percent of Pchlide of two-weeks-old rice seedling. (A) *o*-hydroxyphenyl acetic acid; (B) ferulic acid; and (C) *p*-coumaric acid. The data is the average of three determinations, and the bar indicates the standard deviation.

but the percentage finally returned to normal. The inhibition of mole percent of Pchlide caused by ferulic acid was more than 75%, and a normal level was not restored during the 48 h experiment, just as with Proto. *p*-Coumaric acid changed the increase pattern of the mole percent of Pchlide from a biphasic reaction to an almost linear reaction, as did ferulic acid. The mole percent of Pchlide, inhibited by all tested concentrations of *o*-hydroxyphenyl acetic acid, recovered almost completely within 48 h, whereas samples inhibited by ferulic and *p*-coumaric acids recovered just partially during the same period.

Possible Blockage Site

Chl molecules are the core component of pigment-protein complexes embedded in the photosynthetic membranes and play a major role in photosynthesis. A Chl reduction must result in a decrease of photosynthesis efficiency. Allelochemical phenolics may reduce Chl accumulation in three ways: the inhibition of Chl biosynthesis, the stimulation of Chl degradation, or both. The present study strongly indicates that Chl biosynthesis of rice seedlings is inhibited by the three exogenously applied allelopathic phenolics. The three phenolics exhibited apparently different degrees of inhibition on the reactions between Proto and Pchlide, and especially on the reaction between Proto and Mg-Proto (Figure 7). Since the three phenolics do not affect the mole percent of Mg-Proto, the Mg-Proto molecules are obviously transformed immediately into Mg-protoporphyrin monomethylester (Mg-Proto ME) by the catalysis of S-adenosylmethionine: Mg-protoporphyrin o-methyltransferase (Mg-Proto methylesterase) and finally converted into Pchlide. That is, these phenolics do not inhibit the Mg-Proto methylesterase, Mg-protoporphyrin IX monomethylester oxidative cyclase (Mg-proto ME cyclase), or Mg-2,4-divinyl-pheoporphyrin a5 reductase (Mg-DVP reductase). Therefore, Mg-chelatase, the enzyme responsible for the conversion of Proto into Mg-Proto, may be the major attack target of the three phenolics, causing the accumulation of Proto and thus raising its mole percent.

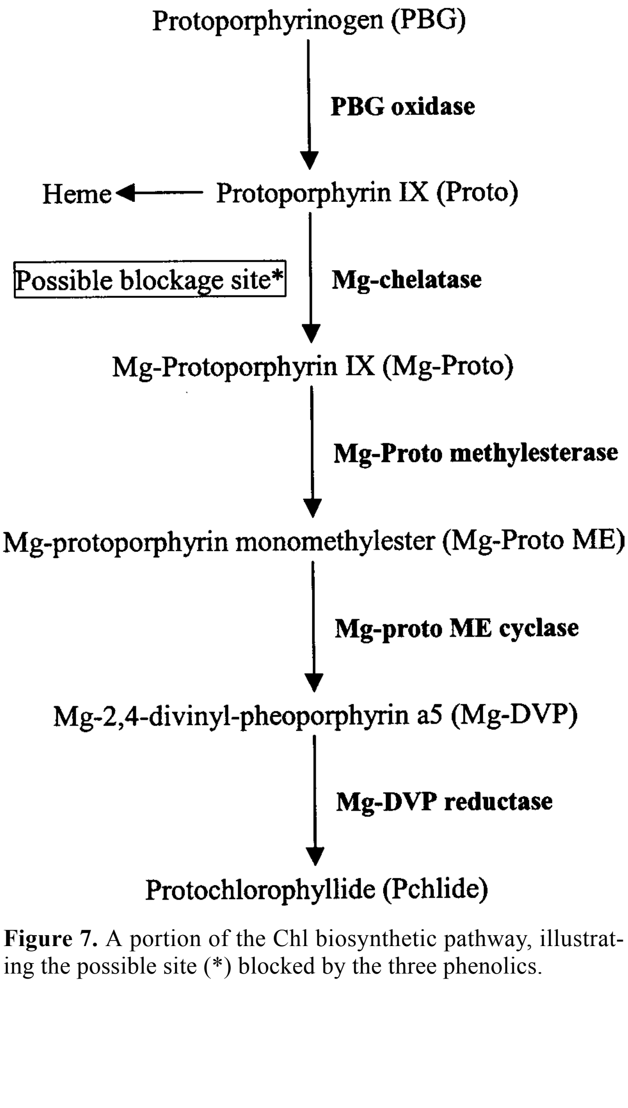

Figure 7. A portion of the Chl biosynthetic pathway, illustrating the possible site (*) blocked by the three phenolics.

Inhibition Strength

The order of inhibition strength against the decline in the mole percent of Proto and Pchlide is as follows: ferulic acid>*p*-coumaric acid>*o*-hydroxyphenyl acetic acid. A grey relational analysis also confirmed the above order (data not shown). This reflects the fact that the three phenolics attack the branch point, from where one way to heme and another to Mg-Proto (Figure 7). The latter is a magnesium insertion step catalyzed by Mg-chelatase located in the chloroplast. The three tested phenolics were exogenously applied and must be transferred from the root to the target organelle to inhibit the enzyme Mg-chelatase. It is still unknown how the exogenous phenolics are transported into chloroplast to inhibit the Mg-chelatase.

We conclude that the three phenolics alter the normal biphasic reaction in conversion of chlorophyll precursors, slowing the decrease of the mole percent of Proto and the increase of the mole percent of Pchlide, but not affecting the percent of Mg-Proto. Mg-chelatase may be the major target of phenolic interference, resulting in the shortage of the supply-orientation of Chl. The degree of inhibition of Mg-chelatase is ferulic acid>*p*-coumaric acid>*o*-hydroxyphenyl acetic acid. In combination with data presented in a following paper (Yang et al., 2002), we may further conclude that both the supply- and consumption-orientation of Chl may be affected, with the former side inhibited and the latter side stimulated.

Literature Cited

Chou, C.H. and H.J. Lin. 1976. Autointoxication mechanism of *Oryza sativa*. I. Phytotoxic effects of decomposing rice residues in soil. J. Chem. Ecol. **2:** 353-367.

Colton, C.E. and F.A. Einhellig. 1980. Allelopathic mechanisms of velvetleaf (*Abutilon theophrasti* Medic., Malvaceae) on soybean. Amer. J. Bot. **67:** 1407-1413.

Einhellig, F.A., E.L. Rice, P.G. Risser, and S.H. Wender. 1970. Effects of scopoletin on growth, CO_2 exchange rates and concentration of scopoletin, scopolin and chlorogenic acids in tobacco, sunflower and pigweed. Bull. Torrey Bot. Club **97:** 22-23.

Einhellig, F.A. and L. Kuan. 1971. Effects of scopoletin and chlorogenic acid on stomatal aperture in tobacco and sunflower.

Bull. Torrey Bot. Club **98:** 155-162.

Einhellig, F.A. and J.A. Rasmussen. 1979. Effects of three phenolic acids on chlorophyll content and growth of soybean and grain sorghum seedlings. J. Chem. Ecol. **5:** 815-824.

Einhellig, F.A., M.S. Muth, and M.K. Schon. 1985. Effects of allelochemicals on plant-water relationship. *In* A.C. Thompson (ed.), The Chemistry of Allelopathy, American Chemical Society, Washington, D.C., pp. 170-195.

Einhellig, F.A. 1986. Mechanisms and modes of action of allelochemicals. *In* A. P. Putnam and C. S. Teng (eds.), The Science of Allelopathy. John Wiley & Sons, New York, pp. 170-188.

Einhellig. F.A. 1995. Mechanism of action of allelochemicals in allelopathy. *In* K. Inderjit, M.M. Dakshini, and F.A. Einhellig (eds.), Allelopathy: organisms, Processes, and Applications, American Chemical Society, Washington, D. C., pp. 96-116.

Gerald, F., L. Booker, U. Blum, and E.L. Fiscus. 1992. Short-term effects of ferulic acid on ion uptake and water relations in cucumber seedlings. J. Exp. Bot. **43:** 649-655.

Kahn, V.M., N. Avivi-Bieise, and D. von Wettstein. 1976. Genetic regulation of chlorophyll synthesis analyzed with double mutants in barley. *In* T. Bhuchler (ed.), Genetics and Biogenesis of Chloroplasts and Mitochondria, Elsevier/North-Holland Biomembrane Press, Amsterdam, pp. 119-131.

Mersie, W. and M. Singh. 1993. Phenolic acids affect photosynthesis and protein synthesis by isolated leaf cells of velvet-leaf. J. Chem. Ecol. **19:** 1293-1301.

Olofsdotter, M. 1998. Allelopathy in rice. *In* M. Olofsdotter (ed.), Proceeding of the Workshop on Allelopathy in Rice, 25-27 Nov. 1996. Manila, Phillippines, International Rice Research Institute, pp. 1-5.

Patterson, D.T. 1981. Effects of allelopathic chemicals on growth and physiological responses of soybean (*Glycine max*). Weed Sci. **29:** 53-59.

Porra, R.J., A. Thompson, and P.E. Friedelman. 1989. Determination of accurate extraction and simultaneously equation for assaying chlorophyll a and b extracted with different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta. **975:** 384-394.

Rice, E.L. 1984. Allelopathy. 2nd ed. Orlando, Florida, USA, Academic Press.

Yang, C.M., I.F. Chang, S.J. Lin, and C.H. Chou. 2002. Effects of three allelopathic phenolics on chlorophyll accumulation of rice (*Oryza sativa*) seedlings: II. Stimulation of consumption-orientation. (submitted)

三種植物相剋酚酸對水稻葉綠素累積之影響：（一）供給面的抑制

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本文探討三種植物相剋酚酸 *o*-hydroxyphenyl acetic acid、 ferulic acid 和 *p*-coumaric acid 對水稻葉綠素累積供給面之影響。二週大之水稻白化幼苗水耕栽培於含或不含 25、50 或 100 ppm 酚酸之十分之一的 Kimura 培養液中，並置於生長箱中，其光週期、光強度和相對溼度分別為 12 h, 300 μmol/m^{2}s and 70%。水稻葉於處理後第 1、3、6、12、24 和 48 小時採收，並測其葉綠素及其生合成三比林種前驅物 protoporphyrin IX (Proto)、magnesium-protoporphyrin IX (Mg-Proto) 和 protochlorophyllide (Pchlide) 之含量與比林的莫耳百分比。葉綠素和比林的累積隨著酚酸濃度的增加而抑制的更多。當三種酚酸對 Mg-Proto 莫耳百分比產生的影響型態相似時，它們對 Proto 和 Pchlide 的影響型態則有很大不同。結果強烈顯示 Mg-chelatase 可能是這三種酚酸作用的主要目標，而其抑制強度則是 ferulic acid>*p*-coumaric acid>*o*-hydroxyphenyl acetic acid。我們的結論是葉綠素累積的供給面受到外來酚酸的明顯抑制，故而導致葉綠素的短缺。

關鍵詞：植物相剋酚酸；水稻；葉綠素；供給面；*o*-hydroxyphenyl acetic acid；Ferulic acid；*p*-coumaric acid；比林。