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# IRON WITHHOLDING BY PLANT POLYPHENOLS AND RESISTANCE TO PATHOGENS AND ROTS

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**Key Word Index**—plant defence; iron deprivation; bacteria; *Erwinia chrysanthemi*; polyphenols; tannins; iron complexation.

**Abstract**—Plant polyphenols (*syn.*, tannins) inhibit growth of several mutants of the bacterium *Erwinia chrysanthemi*, altered in their siderophore-mediated iron transport pathway. Growth of the mutants is restored by addition of iron(III) to the medium. Polyfunctional polyphenols, with their several chelating *o*-dihydroxyphenyl groups per molecule, also remove iron(III) from other iron/ligand complexes more efficiently than monofunctional low molecular weight phenols. Growth inhibition of the mutants is thus explained by their inability to assimilate iron in the presence of polyphenols. Polyphenols mimic animal iron-binding proteins such as transferrin and protect plants by withholding iron away from pathogens and rots.

## INTRODUCTION

Polyphenols (*syn.* tannins) are widespread in the plant kingdom (pteridophytes, gymnosperms and angiosperms) and are found in leaves, fruits, bark or wood in concentrations sometimes as high as 50% of the dry weight [1]. They contribute to the plant defence by deterring herbivores from feeding on tannin-rich plant tissues [2] and limiting growth of pathogens and rots [3]. Their toxicity towards microorganisms is relatively weak, but, when present in large concentrations, they become a defence with a wide spectrum of action. They are considered as a quantitative defence as their protective effects against predators depend on their concentration in plant tissues [4, 5].

Their role in plant defence is well established, but their mode of action has not yet been clarified. Many authors have suggested that they inhibit microorganisms growth by forming complexes with either microbial enzymes or protein nutriments, but no unambiguous proof has so far been given [3]. One of us has proposed that polyphenols could inhibit the growth of microorganisms through iron deprivation [3]. Iron is involved in the action of many enzymes and redox proteins and is essential to most living organisms. However, it is not easily available as it readily forms insoluble hydroxides. Plants, animals or microorganisms have developed elaborate mechanisms to compete for this rare resource. Bacteria and fungi often produce low molecular weight siderophores having a high affinity for iron(III) [6]. Plant polyphenols, with several *o*-dihydroxyphenyl groups in their structures, are also excellent chelators of various metal ions, and in particular of iron(III) with which they form blue–black complexes largely insoluble in water [7], which may hinder iron assimilation by microorganisms.

It is remarkable that virtually all tannins, either hydrolysable tannins or proanthocyanidins (*syn.* condensed tannins), the most ubiquitous plant polyphenols after lignins, contain *o*-dihydroxyphenyl groups. Proanthocyanidins with monohydroxylated aromatic rings instead, extremely rare in plants [8], have been synthesized and were shown to precipitate proteins as efficiently as *o*-dihydroxyphenyl group-containing proanthocyanidins [9]. The presence of *o*-dihydroxyphenyl groups in tannins may thus not necessarily serve their protein-binding properties, but it certainly serves the formation of chelates with iron(III) or other metal ions.

To determine if tannins in plant tissues render iron non-available for microorganisms, we have compared growth on polyphenol-rich media of the wild-type strain 3937 of *Erwinia chrysanthemi*, a pectinolytic bacterium causing soft rot on a wide range of plants, to that of mutants altered in their siderophore-mediated iron transport pathway.

## RESULTS AND DISCUSSION

Three mutants have been used: 3937 *cbs29* (Cbs ) is a chrysobactin (the catechol-type siderophore of *E.*

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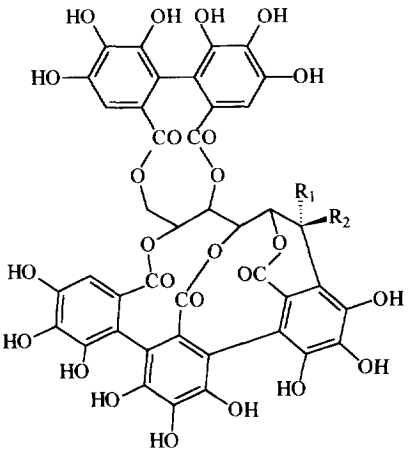
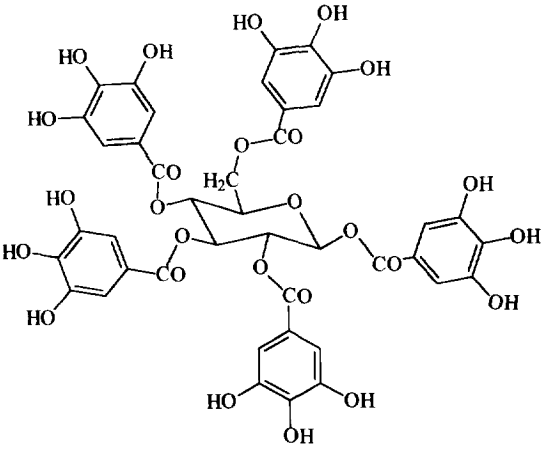
*chrysanthemi*) biosynthetic mutant; 3937 *fct18* (Fct$^-$Cbs$^+$) in addition lacks the chrysobactin receptor [10]; the mutant 3937 *fct34 acsA12* (Fct$^-$Acs$^-$) carries a transposon insertion in the *fct* gene and a second one in the *acs* gene coding for the biosynthesis of achromobactin (the second siderophore of *E. chrysanthemi* of yet unknown structure) [11]. The mutants and the wild-type strain were grown in the presence of a commercial polyphenol extract of European chestnut (*Castanea sativa* L.) heartwood. This extract contains a mixture of hexahydroxydiphenoyl esters (ellagitannins), principally vescalagin (**1**), castalagin (**2**), roburin A and roburin D [12]. Growth of the three mutants, and more particularly of the Fct$^-$Acs$^-$ mutant was more inhibited by chestnut polyphenols than that of the wild-type strain (Fig. 1). This suggests that polyphenols induce iron deprivation, which is in part counteracted by siderophores.

Similar experiments were carried out with the Cbs$^-$ mutant and pure **1**, **2**, $\beta$-penta-*O*-galloyl-D-glucose (**3**) and $\beta$-1,2,3,6-tetra-*O*-galloyl-D-glucose. Similar growth inhibition curves were obtained for the four polyphenols with no inhibition at a 6.2 $\mu$M concentration (*ca* 6 mg l$^{-1}$) and a 50% inhibition at a 33 $\mu$M concentration (*ca* 30 mg l$^{-1}$).

The Cbs$^-$ mutant was then grown in agar plates containing the same pure polyphenols (500 $\mu$M), and filter paper discs containing iron(III) chloride were added on top of the agar to see if an excess of iron could restore growth of the mutant. Bacterial colonies were observed in the vicinity of the paper discs containing 100 and 1000 nmol of iron(III) chloride for plates containing $\beta$-penta-*O*-galloyl-D-glucose or $\beta$-1,2,3,6-tetra-*O*-galloyl-D-glucose and around discs containing 1000 nmol of iron(III) chloride for plates



**1** $R_1 = H, R_2 = OH$
**2** $R_1 = OH, R_2 = H$



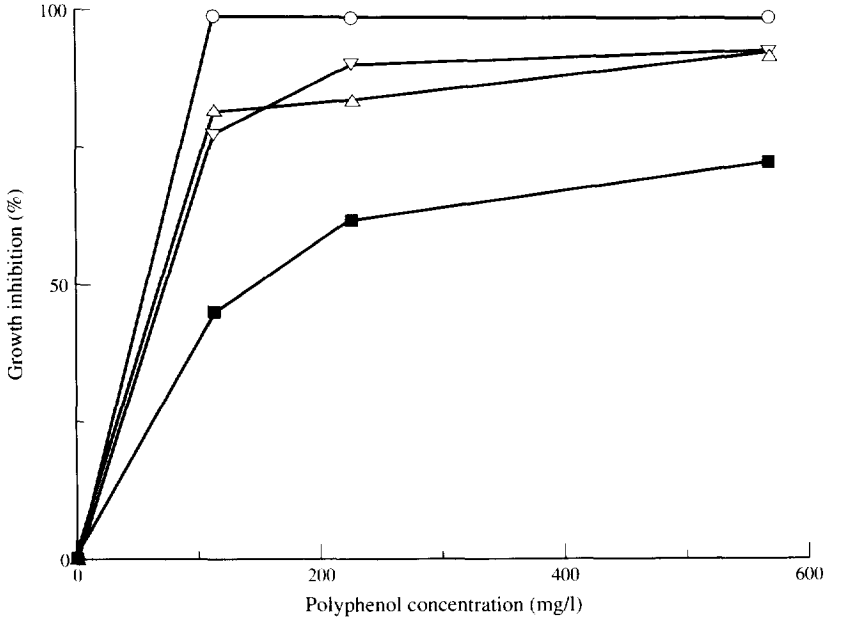



Fig. 1. Growth inhibition by chestnut polyphenols of Cbs$^+$ (△), Cbs$^-$ Fct$^-$ (▽) and Fct$^-$ Acs$^-$ (○) mutants altered in their siderophore-mediated iron transport pathway and of the wild-type strain 3937 (■) of *E. chrysanthemi*.

containing **1** or **2**. When a lower concentration of ellagitannin was used (250 $\mu$M **2**), colonies also formed around paper discs containing 100 nmol of iron. The number of colonies always increased with the amount of iron(III) chloride added. When grown in the same conditions, the wild-type strain 3937 formed colonies all over the plate. These results show that polyphenols inhibit growth of the Cbs$^-$ mutant by limiting the availability of iron(III) ions.

To appreciate better the characteristic iron-withholding properties of polyphenols, growth inhibition of the mutants by structurally related low molecular weight phenols has been studied and compared to that induced by **3**. The Cbs$^-$ and Fct$^-$ Cbs mutants and the wild-type strain 3937 were grown in the presence of varying concentrations of gallic acid, protocatechuic acid, (+)-catechin and chlorogenic acid. All these molecules possess one chelating *o*-dihydroxyphenyl group in contrast to **3**, which contains five such groups. Salicylic acid was also assayed as it is a metal chelator found in several plant species. The two mutants were 25–100 times more sensitive to the presence of **3** when calculated on a weight basis (150–500 times when calculated on a molar basis) than to the presence of the various low molecular weight phenols (Fig. 2A and B). The wild-type strain was equally sensitive to the presence of **3** and low molecular weight phenols (Fig. 2C), thus indicating that toxic effects of these compounds on the wild-type strain are unrelated to their iron-chelating properties.

The relative affinities of polyphenols and low molecular weight phenols for iron(III) were then compared by the chrome azurol S assay commonly used to detect ligands of high affinity for iron such as siderophores [13]. In this assay, the high affinity ligand displaces iron from the blue chrome azurol S/iron(III) complex. All polyphenols (**1**, **2** or **3**) removed most of the iron(III) from the chrome azurol S complex at a 25 $\mu$M concentration [iron(III) and chrome azurol S concentrations, were respectively, 7.5 and 75 $\mu$M] and formed a blue–black precipitate with iron, whereas all low molecular weight phenols only removed a minor part of iron from the chrome azurol S complex (Fig. 3). These differences in iron complexation between polyphenols and low molecular weight phenols arise from the polyfunctional status of polyphenols. Each polyphenol molecule, with its several *o*-dihydroxyphenyl chelating groups, can bind several iron(III) ions and each iron(III) can itself coordinate with up to three *o*-dihydroxyphenyl groups belonging to different polyphenol molecules. A lattice is consequently formed (Fig. 4), which eventually results in the co-precipitation of iron and polyphenols and in the removal of iron(III) from solution [14]. The lattice-forming polyphenols are precisely those which strongly inhibit growth of the siderophore deficient mutants of *E. chrysanthemi*.

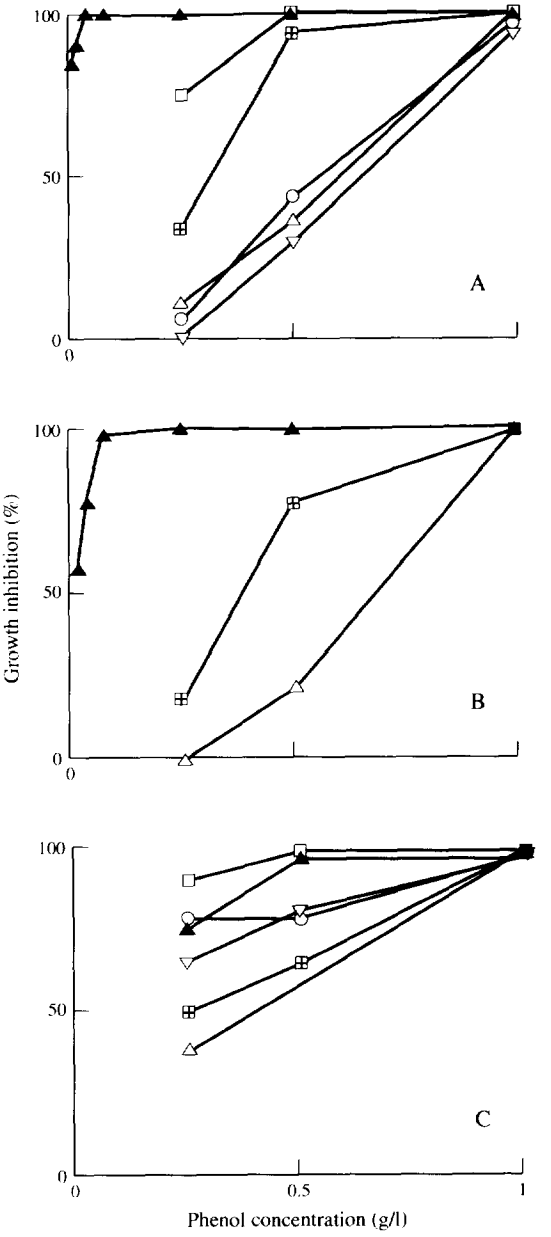



Fig. 2. Growth inhibition by $\beta$-penta-*O*-galloyl-D-glucose and low molecular weight phenols of (A) Cbs$^-$ and (B) Fct$^-$ Cbs$^-$ mutants altered in their siderophore-mediated iron transport pathway and of the (C) wild-type strain 3937 of *E. chrysanthemi*. (▲) $\beta$-Penta-*O*-galloyl-D-glucose; (△) gallic acid; (○) protocatechuic acid; (⊞) chlorogenic acid; (▽) (+)-catechin; (□) salicylic acid.

## CONCLUSIONS

The present results demonstrate for the first time that the iron-chelating properties of polyphenols contribute to limit the growth of microorganisms unless they have developed particularly efficient biochemical systems to displace iron(III) from the polyphenol/iron complex. Plant colonization by a pathogenic bacterium [15, 16] or wood-decaying fungi [17] have been reported to depend on the production of siderophores or siderophore-like compounds. Accumulation of polyphenols in plant tissues may be an evolutionary response to the harsh competition for iron. Plant polyphenols would thus play in plants the role played by iron-binding proteins such as transferrin, conalbumin or lactoferrin

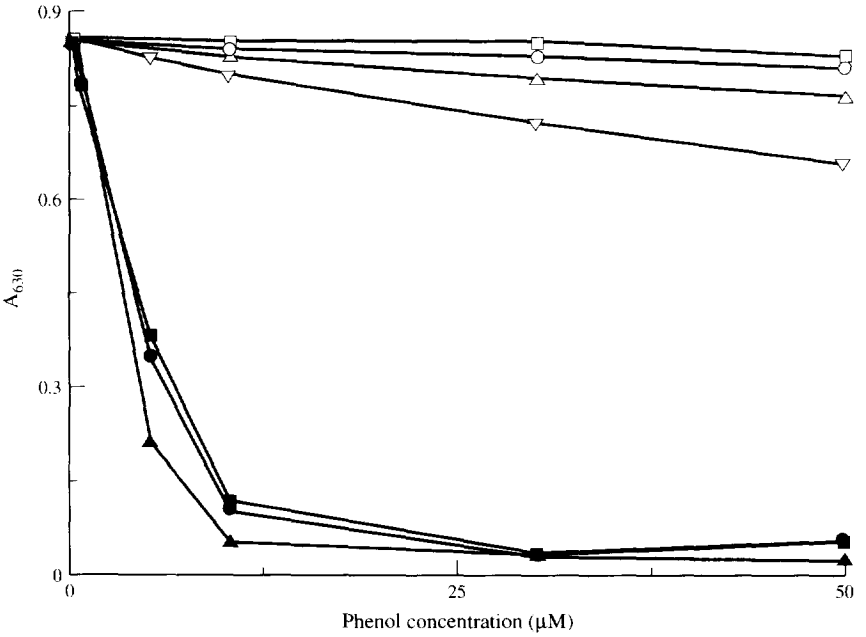



Fig. 3. Decrease of absorbance in the chrome azurol S assay for high affinity ligands of iron(III), upon addition of increasing concentrations of various polyphenols and low molecular weight phenols: (●) vescalagin; (■) castalagin; (▲) β-penta-*O*-galloyl -D-glucose; (△) gallic acid; (○) protocatechuic acid; (▽) (+)-catechin; (□), salicylic acid.

in animals [18]. Both polyphenols and iron-binding proteins accumulate in high concentrations in plant and animals: e.g. 5–50% (w/w) polyphenols in bark according to species [19] and 10% conalbumin in the egg white solids [20]. These high concentrations may be required to deprive iron efficiently.

Polyphenols may also withhold other metal ions as essential to the growth of plant pathogens such as copper(II) or zinc(II) [21] by similar mechanisms of complexation–precipitation [14, 22]. Such metal-withholding properties of polyphenols may operate in leaves or fruits where proanthocyanidins have been shown to limit infection by pathogens [23, 24]. They could be particularly important in dead tissues of plants, unable to trigger active defences upon infection. They would explain the protection against rots of many durable heartwoods (the dead inner part of wood in the trunk) by polyphenols [5] and contribute indirectly to the mechanical resistance of the trunk and branches in species of high longevity. In bark, they would constitute a chemical barrier preventing microorganisms from reaching the underlying living tissues (phloem, cambium and xylem) just as conalbumin in egg white prevents microorganisms from reaching the developing embryo in the yolk.

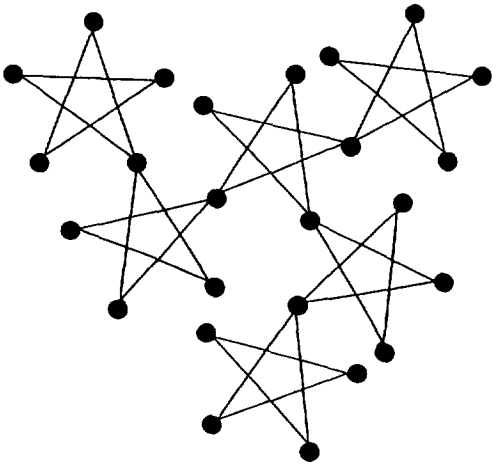


Fig. 4. Polyphenol/iron(III) lattice formed upon complexation of iron(III) (dark spot) by a polyphenol (star) containing five *o*-dihydroxyphenyl functional groups (triangles).

## EXPERIMENTAL

*General.* The extract of European chestnut heartwood ('châtaignier N') was obtained from Tanin International (Labruguière, France). Compounds **1** and **2** were purified from chestnut wood [12] and the two galloyl esters, **3** and β-1,2,3,6-*tetra*-*O*-galloyl-D-glucose, from methanolysed tannic acid (Fluka) [25, 26]. Gallic acid, protocatechuic acid, (+)-catechin originated from Fluka, chlorogenic acid from Serlabo and salicylic acid from Prolabo. The *E. chrysanthemi* mutants *cbs29*, *fct18* [10] and *fct34 acsA12* [11] of the wild-type strain 3937 have been described previously.

*Culture of bacteria.* For growth inhibition studies, the various strains ($10^5$ CFU $ml^{-1}$) were grown in L broth [27] in the presence of chestnut extract or pure phenolic compounds. Bacterial growth after one night culture in aerobiose at 30° was determined by plating on agar medium. Cross-feeding experiments with iron(III) were carried out with agar plates containing L broth, Difco agar (12 $gl^{-1}$), pure polyphenols (250 or

500 $\mu$M) and the Cbs$^-$ mutant ($10^5$ CFU ml$^{-1}$). Filter paper discs (1 cm diameter) containing 10, 100 and 1000 nmol $FeCl_3$ were added on top of the agar. Bacterial colonies were observed after a 48 hr incubation at 30° and appeared as white halos around the filter paper discs.

*Chrome azurol S assay*. This was carried out as originally described [13].

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