WANDERLEY DANTAS DOS SANTOS

ASPECTOS ANATÔMICOS E BIOQUÍMICOS DE RAÍZES DE SOJA (*Glycine max* L. Merrill) SUBMETIDAS AO ÁCIDO FERÚLICO

Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de concentração em Biologia Celular) da Universidade Estadual de Maringá, para obtenção do grau de Doutor em Ciências Biológicas.

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APRESENTAÇÃO

Esta tese é composta de uma revisão e de dois artigos científicos. Inicia revisando o papel do ácido ferúlico como aleloquímico no artigo "Ferulic Acid: An Allelochemica Troblemaker". Tem continuidade com o artigo "High Performance Liquid Cromatography Method for the Determination of Cinamyl Alcohol Dehydrogenase Activity in Soybean Roots" que descreve os procedimentos adotados para se determinar a atividade de uma das enzimas da via de fenilpropanóides, a cinamil álcool desidrogenase (CAD). Encerra com o artigo "Soybean Root Lignification Induced by Ferulic Acid: The Possible Mode of Action" que sugere um possível mecanismo para a ação deste aleloquímico. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, este artigo foi redigido de acordo com as normas da revista *Journal of Chemical Ecology.*

dos Santos, W.D. and Ferrarese-Filho, O. Ferulic acid: an allelochemical troublemaker. *Revisão.*

dos Santos, W.D., Ferrarese, M.L.L., and Ferrarese-Filho, O. High performance liquid chromatography method for the determination of cinnamyl alcohol dehydrogenase in soybean roots. *Plant Physiol. Biochem.* 44:511-515, 2006.

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PREFÁCIO

Alelopatia pode ser definida como a interação química entre plantas e comunidade local. As moléculas envolvidas em eventos alelopáticos são denominadas aleloquímicos. Em geral os aleloquímicos são compostos secundários, isto é, produtos do metabolismo vegetal que não são comuns a todas as plantas. Os compostos secundários formam assim, um grupo bastante heterogêneo que totaliza centenas de milhares de moléculas. Consideradas as interações entre os aleloquímicos e a imensa diversidade de plantas obtém-se a amplitude do desafio que a alelopatia impõe aos seus perscrutadores. Não obstante, a informação levantada sobre a ação de um determinado aleloquímico em uma determinada espécie, não constitui um dado isolado. Ao contrário, possui valor universal, uma vez que cada composto representa uma classe cujos efeitos são semelhantes – embora raramente idênticos. As informações coletadas sobre ação de um dado composto em uma planta, somadas às de outras moléculas do grupo naquela e em outras plantas, poderão, eventualmente, ser extrapoladas para toda uma classe de compostos semelhantes em uma gama de grupos vegetais. Estas informações, além de enriquecerem nosso conhecimento sobre a fisiologia vegetal básica, podem ser importantes para o melhoramento genético e o desenvolvimento de métodos de manejo agrícola progressivamente menos agressivos.

Uma das linhas de pesquisa do grupo coordenado pelo professor Osvaldo Ferrarese, vem efetuando uma varredura sobre as regiões do metabolismo afetadas pelo ácido ferúlico (FA), um dos aleloquímicos mais conhecidos (veja estrutura molecular na p. 73). De um modo geral, ele representa a ampla classe dos compostos fenólicos e mais especificamente, os fenilpropanóides. A soja e a canola foram escolhidas como plantas-modelo, devido à sua susceptibilidade ao FA e interesse agrícola. Fui incorporado a este projeto com a incumbência de estudar a relação entre a lignificação e a redução na taxa de crescimento induzida pelo aleloquímico. Ao analisarmos os dados que resultaram em minha dissertação de mestrado e compará-los com os dados disponíveis na literatura, percebemos que parecia haver um *modus operandi* padrão na alelopatia provocada pelo ácido ferúlico. Tínhamos em mãos um grande volume de dados e uma das tarefas foi organizá-los. Ao fazê-lo, íamos delineando um modelo do mecanismo de ação que não entrasse em contradição com os dados. Exames microscópicos preliminares mostravam que o FA promove uma intensa descamação da epiderme apical e da coifa, talvez devido ao seu contato mais direto (Fig. 3C-F) Houve uma visível redução nas reservas de amido, mais evidente nos amiloplastos das células apicais (Fig. 3A-D, p. 68). A atividade nuclear foi aumentada nas

células da região quiescente, indicando uma elevada atividade síntese que pode ser associada ou a um processo de detoxificação ou de divisão celular (Fig. 3B-E). No entanto, ocorreu uma redução na distância entre o centro quiescente e as primeiras células xilemáticas diferenciadas (Fig. 2C-F, p. 67). A prematura lignificação dos vasos xilemáticos (Fig. 2A-B) sugeria que esta poderia ser a causa primária da redução no crescimento (Fig. 1A-B, p. 66). Havíamos verificado haver uma correlação entre a lignificação induzida pelo FA e a redução no crescimento, mas era incerto se essa lignificação era uma resposta primária (causa da redução no crescimento) ou secundária (uma resposta da planta ao estresse alelopático). Por um lado, como já havia sido verificado pelo grupo, o composto é de fato captado pelas células radiculares. Isso sugeria que a lignificação fosse uma resposta *secundária* da planta ao estresse promovido pelo aleloquímico. Por outro lado, a presença do isótopo radioativo na lignina após o tratamento com FA marcado (com ^{14}C no anel aromático) sugeria o inverso: a lignificação poderia ser resultante *da incorporação da molécula à parede*. Portanto, um efeito primário.

Formado na via dos fenilpropanóides que leva à síntese dos monolignóis que compõem a lignina, o FA livre é esterificado a carboidratos de parede, onde sofre condensação com outros FA esterificados e age como uma ponte que interliga glicanos e linignina (Fig. 2, p. 17). Tanto as dimerizações do FA quanto a polimerização dos monolignóis são promovidas por um mecanismo de oxidação creditado a peroxidases. Os compostos fenilpropanóicos são convertidos em radicais livres e polimerizam-se espontaneamente. Produzido em excesso por gramíneas, onde parece ter ação como fotoprotetor, o FA é eventualmente liberado por lixiviação ou mesmo pela decomposição da planta. Sendo um composto comum no solo, as plantas têm de lidar com ele cotidianamente. A hipótese corrente sugere que a planta promove a lignificação excessiva como *um mecanismo de defesa ao aleloquímico*, o que de fato prejudica o seu crescimento. Assim, pareceu-nos mais plausível conjeturar que o FA provoca a lignificação excessiva dos tecidos vasculares por um mecanismo que foge ao controle da célula. Aparando nossa hipótese com a navalha de Occan, construímos o modelo mais simples possível. Conjeturamos que o ácido ferúlico exógeno ao adentrar a matriz extracelular era diretamente oxidado e incorporado à parede pelas peroxidases. Uma vez que as peroxidases apoplásticas estão do lado de fora da célula, poderiam ser menos acessíveis ao controle celular. Além disso, elas são muito resistentes e têm muitas funções, o que poderia dificultar ainda mais o controle da célula.

Para investigar esta hipótese, decidimos medir a atividade da cinamil álcool desidrogenase (CAD). Sendo a última enzima na via da síntese dos monolignóis, ela teria que

estar ativada para justificar a lignificação. Caso ela não estivesse ativada ou estivesse inibida, a hipótese da incorporação direta seria ratificada (Fig. 2, p. 17). A CAD merece uma seção a parte. Passamos *vários meses* tentando dominar as técnicas de leitura da atividade da CAD disponíveis na literatura, sem obter êxito. Decidimos então desenvolver um protocolo experimental mais rigoroso utilizando cromatografia líquida de alta eficiência (HPLC). As técnicas convencionais utilizam o coniferil álcool como substrato e medem sua oxidação. No entanto, além de caro, ele é extremamente sensível à luz e à umidade e já chegava do fornecedor (Sigma, EUA) como um líquido viscoso difícil de pesar. Além disso, o álcool absorve luz em 340 nm, o mesmo comprimento de onda do NADPH, que é produto do seu par redox na reação enzimática (enquanto um é consumido o outro é produzido) o que, ao lado da baixa atividade da enzima justificava, de certo modo, nossa incapacidade de medir a reação espectrofotometricamente. Mesmo em HPLC a reação da CAD *in vitro* permaneceu difícil de se controlar. Ela atua sobre três substratos. Nas angiospermas como a soja, os dois principais são o sinapil e o coniferil. Na planta ela reduz a forma aldeído a álcool, mas *in vitro* a reação contrária, a oxidação do álcool a aldeído, era preferida. No entanto, depois de dissolvidos, os álcoois rapidamente se oxidam mudando a concentração e a respectiva absorbância em questão de minutos. Desde até a efetiva publicação da técnica, lá se foram *três anos* de germinações, incubações e reações (cinco dias para cada experimento) e frustrantes injeções (em média, 30 min cada; no total mais de 500 injeções). Os desafios envolveram a dificuldade inerente em se definir critérios ótimos de concentração, pH, tempo de reação, fase móvel; as tentativas de pré-purificação do extrato bruto; o estabelecimento das curvas-padrão nos dois sentidos da reação (oxidação e redução), medindo tanto o desaparecimento dos substratos quanto o aparecimento dos dois álcoois e dois aldeídos (oito curvas com quatro a oito repetições); o estabelecimento dos protocolos de validação da técnica; além dos lentos e ineficazes processos de importação dos quatro padrões (álcoois e aldeídos coniferílico e sinapílico); as idas e vindas da editoração e revisão por pares, etc.

Uma vez concluída, a técnica nos concedeu a precisão necessária para constatar com rigor que a CAD *não* era ativada pelo ácido ferúlico como era de se esperar dado o aumento na lignificação que ele induz (Fig. 4, p. 69). Ao contrário, ela foi significativamente *inibida* pelo tratamento com ácido ferúlico, o que respaldava as previsões teóricas do nosso modelo de incorporação direta. De fato, a microscopia mostrava que o acúmulo da lignina era evidente apenas nas células do protoxilema, não caracterizando uma impregnação indistinta do ácido ferúlico nas paredes celulares como poderia ser pela nossa hipótese. Entretanto, o processo de

redução no crescimento se estende a todo o cilindro radicular (Fig. 2D) o que poderia ser atribuído a incorporação do FA exógeno nas paredes. Já trabalhávamos na tentativa de identificar as isoperoxidases ativadas pelo FA quando nos deparamos com a descoberta surpreendente de que mesmo a inibição completa da CAD (por silenciamento ou mutação) é incapaz de fazer cessar a lignificação, que prossegue praticamente inabalada com os aldeídos. Em outras palavras, o fato de a CAD estar inibida, poderia até indicar que a célula não estava respondendo coerentemente com relação à lignificação induzida pelo ácido, mas, não servia como evidência da incorporação direta do ácido ferúlico.

Para definir a questão decidimos usar inibidores específicos de enzimas da via. Se nosso modelo estivesse correto a lignificação prosseguiria indiferente à inibição da via. Caso contrário, se elas impedissem a lignificação, poderíamos investigar em qual passo enzimático o ácido ferúlico exógeno era admitido na via. Caso a molécula fosse de fato admitida na via dos monolignóis, o passo enzimático realizado pela enzima 4-cafeoil coenzima A ligase (4CL) era o mais provável. Ela é tida como capaz de esterificar alguns ácidos fenilpropanóicos livres à coenzima A, uma etapa obrigatória no processo de redução que leva aos monolignóis. Descobrimos então, que inibição da 4CL durante o tratamento com o ácido ferúlico realmente impedia o aumento da lignificação pelo tratamento com ácido ferúlico. Em um experimento subseqüente, inibimos a ação da cinamil 4-hidroxilase (C4H), que realiza o passo enzimático imediatamente anterior ao da 4CL, mas ele foi incapaz de prevenir a lignificação induzida pelo aleloquímico. Juntos estes experimentos indicavam que o ácido ferúlico não era incorporado diretamente, como havíamos proposto em nosso modelo de trabalho, mas introduzido na via pela reação catalisada pela 4CL.

Para incluir esses fenômenos, modificamos nosso modelo utilizando como base os modelos de resposta ao estresse biológico e abiótico. 1) O ácido ferúlico ataca as ATPases envolvidas no transporte causando um desbalanço iônico que dispara a NADPH-desidrogenase geradora de superóxido. 2) Uma peroxidase da parede transfere um elétron do ácido ferúlico para o superóxido produzido pela NADPH-desidrogenase e formando H_2O_2 e feruloil radical; 3) O ácido ferúlico captado pela célula é ou β-glicosilado ou introduzido na via dos fenilpropanóides, onde é convertido em um aldeído monolignol, extrusado e oxidado por peroxidases que utilizam o H_2O_2 formado pela oxidação do próprio ácido ferúlico (Fig. 8, p. 74).

RESUMO GERAL

INTRODUÇÃO E OBJETIVOS – As plantas estão constantemente produzindo compostos orgânicos. Estes compostos são liberados para o solo por vários mecanismos que incluem lixiviação, exudação pelas raízes e a própria decomposição de partes da planta. Entre eles alguns estão implicados em interações entre plantas ou alelopatia. É o caso do ácido ferúlico, um aleloquímico amplamente distribuído e mencionado na literatura. Sintetizado na via dos fenilpropanóides que leva à formação da lignina, o ácido ferúlico é estruturalmente similar ao álcool coniferílico (guaiacil monolignol). Liberado no apoplasto ele pode esterificar glicanos e se ligar covalentemente a ligninas e outros resíduos feruloil esterificados, formando oligômeros (dímeros ou trimeros) que interligam os polímeros da parede, enrijecendo-a e restringindo o crescimento celular. No solo o ácido ferúlico é rapidamente absorvido por outras plantas podendo prejudicar o crescimento da raiz e da plântula. Ao ser captado, causa um desbalanço hídrico, inibe a expansão foliar e o alongamento da raiz, reduz a razão raiz/caule e causa o escurecimento das raízes. Em nível celular, ele reduz as taxas de fotossíntese, induz a peroxidação lipídica, altera a atividade de diversas enzimas do metabolismo primário e secundário, sobretudo enzimas de transporte, causando um aumento generalizado na permeabilidade da membrana plasmática e reduzindo a captação de nutrientes. Ele também reduz o crescimento da soja e induz a lignificação prematura dos tecidos radiculares, sintoma que tem sido associado com aumentos nas atividades de enzimas da via dos fenilpropanóides, como a fenilalanina amônia liase (PAL) e as peroxidases (POD).

No último passo enzimático da rota, a cinamil álcool desidrogenase (CAD) catalisa a conversão de *p*-cumaril, coniferil e sinapil aldeídos nos álcoois correspondentes. Em seguida, a POD catalisa a oxidação dos álcoois e a respectiva polimerização espontânea dos monolignóis em lignina. É sabido que a parede celular lignifica quando a célula está sob estresse ou quando se diferencia notavelmente em células do tecido xilemático. Devido ao importante papel da lignificação no crescimento vegetal, esta pesquisa teve o propósito de investigar como a lignificação se relaciona com o modo de ação do ácido ferúlico. Para tanto, foram realizados ensaios de microscopia de luz e eletrônica, determinadas as atividades da CAD e isoperoxidases, bem como o conteúdo de lignina das raízes da soja após tratamentos com ácido ferúlico e/ou inibidores da via dos fenilpropanóides.

MÉTODOS – Para descrever a metodologia, dois tópicos foram considerados. ***Determinação da atividade da CAD por HPLC.*** Para asseverar a eficiência do ensaio enzimático, dois protocolos foram seguidos. Um com raízes de plântulas crescidas por quatro dias após a embebição e outro com raízes de plântulas crescidas por três dias após e, então incubadas em solução nutritiva por 24 h. Após o crescimento, as raízes foram destacadas e o extrato bruto obtido. A atividade da CAD foi determinada cromatograficamente pela reação direta de redução do coniferaldeído e do sinapaldeído nos respectivos coniferil e sinapil álcoois. O desaparecimento dos substratos e o aparecimento dos respectivos álcoois foram monitorados após eluição isocrática com metanol:ácido acético 4% (20:80, v/v) através de uma coluna GLC-ODS (M). ***Efeitos do ácido ferúlico sobre a lignificação: aspectos anatômicos e bioquímicos.*** Plântulas de três dias foram cultivadas em solução nutritiva, contendo ou não 1 mM de ácido ferúlico. Os experimentos foram efetuados em câmara de germinação, a 25ºC, em ciclo de 12 h claro/12 h escuro e irradiação de 280 μmol m^{-2} s^{-1} por 24 h ou 48 h. Após a incubação as raízes foram medidas e preparadas para as análises microscópicas de luz e eletrônica de varredura e transmissão e para os ensaios bioquímicos. A atividade das POD foi observada por eletroforese em géis de amido e poliacrilamida. A atividade da CAD foi determinada por HPLC e o conteúdo de lignina, determinado espectrofotometricamente. O conteúdo de lignina foi determinado também após incubação das plântulas tratadas ou não com ácido ferúlico e inibidores específicos da via dos fenilpropanóides, ácidos piperonílico (PIP) ou 3,4-metilenodioxicinâmico (MDCA). ANOVA foi aplicada para verificar a significância das variações observadas. As diferenças entre os parâmetros foram avaliadas pelo teste de Scott-Knott e foram considerados significativos quando apresentaram valor de $P \leq 0,05$.

RESULTADOS E DISCUSSÃO – Dois tópicos foram considerados. ***Determinação da atividade da CAD por HPLC.*** Esta pesquisa indicou uma técnica simples e sensível para determinar a atividade da CAD baseada na obtenção do extrato bruto de raízes de soja. A atividade enzimática foi determinada por cromatografia monitorando-se o consumo do coniferil e sinapil aldeídos, substratos da reação catalisada pela CAD. Os resultados mostraram: a) que a medida do desaparecimento dos substratos é confiável e preferível; b) que a reação enzimática é especifica como demonstrado pela linearidade na formação do álcool a partir dos aldeídos, e c) que a metodologia é satisfatoriamente sensível para ser aplicada na determinação da atividade da CAD nas raízes de soja.

Efeitos do ácido ferúlico na lignificação: aspectos bioquímicos e anatômicos. O ácido ferúlico exógeno reduziu o crescimento da raiz, promoveu a desintegração da epiderme apical e da coifa, a compressão das células no centro quiescente, o aumento no diâmetro do cilindro central e a lignificação da parede celular no metaxilema. Além disso, o aleloquímico inibiu a atividade da CAD, aumentou a expressão da isoforma PODa5 e o conteúdo de lignina. Quando aplicado juntamente com o PIP, inibidor da cinamato 4-hidroxilase o ácido ferúlico induziu lignificação. Mas, quando aplicado com o inibidor da enzima 4-cumarato: Coenzima A ligase (4-CL) o ácido ferúlico não afetou significativamente o conteúdo de lignina. Os exames microscópicos combinados aos ensaios bioquímicos sugerem que o ácido ferúlico exógeno é esterificado à coenzima A em uma reação catalisada pela 4CL e subseqüentemente convertido nos coniferil e sinapil aldeídos. Estes metabólitos são secretados e incorporados à parede celular, contornando a atividade da CAD que está inibida.

CONCLUSÕES – Estes resultados indicam que o método de HPLC isocrática proposto é rápido, simples, acurado e fornece uma medida confiável para determinação da concentração de cinamaldeídos. Ele pode ser recomendado como uma ferramenta alternativa para determinar a atividade da CAD em extrato protéico não purificado de raízes de soja com potencial para aplicação em outros tecidos e espécies. A redução no crescimento das raízes induzida pelo ácido ferúlico pode ser parcialmente explicada pela introdução do ácido ferúlico na via dos fenilpropanóides e a incorporação dos respectivos monolignóis (ou aldeídos) cinâmicos formados à parede do xilema, solidificando-a e restringindo o crescimento celular.

GENERAL ABSTRACT

INTRODUCTION AND AIMS – Higher plants regularly release organic compounds into the environment. These products, often added to the soil matrix, are released in soil by rainwater, by exudation from roots, and by the natural decay of parts of plants lying above or below the ground. Some of the above have been reported to be agents of plant-plant interactions, which characterize allelopathy. It is the case of ferulic acid, a widely distributed allelochemical frequently referred in the literature. Synthesized in the phenylpropanoid pathway, involved in the lignin formation, ferulic acid is structurally similar to coniferyl alcohol (guaiacyl monolignol). However, thanks to its carbonyl group, ferulic acid is able to esterify cell wall carbohydrates. Afterward, feruloyl-esters might cross-link lignin and other feruloyl metabolites, what rigidify the cell wall and restrict cell growth. Exuded into the soil, it may be rapidly absorbed by other plants and, eventually, may cause injury to the root growth and inhibit seedling growth. After uptake, it reduces water utilization, inhibits foliar expansion and root elongation, reduces the root/stem ratio and causes browning of roots. At cellular level, ferulic acid reduces the rates of photosynthesis, induces lipid peroxidation, and alters certain enzymatic activities, especially transport enzymes what induces a generalized increase in membrane permeability and a respective reduction on nutrient and water uptake. Ferulic acid also reduces soybean growth due to the premature lignification of root tissues associated with an increase in the enzyme activities of the phenylpropanoid pathway, such as phenylalanine ammonia lyase (PAL) and peroxidases (POD).

In the last steps of this metabolic route, cinnamyl alcohol dehydrogenase (CAD) catalyzes the conversion of *p*-coumaral-, coniferal- and sinapaldehydes in their corresponding alcohols. POD, subsequently, catalyzes the monolignols polymerization leading to the lignin synthesis. It is well known that cell wall lignifies when cell expansion decreases, when cell is under stress and when it differentiates, notably to xylem tissues. Owing to the important role of lignification in plant growth, current research investigates how lignification is related to the ferulic acid´s action mode. Light and electron microscopy studies, lignin content and determinations of CAD and isoperoxidases activities were carried out after treatment of soybean roots with ferulic acid and/or inhibitors of the phenylpropanoid pathway.

METHODS – Within the methodology, two topics have been considered. ***Determination of CAD activity by HPLC***. For extraction of enzyme, the two protocols below involved: (1) roots of seedlings grown after 4 days of seed germination and (2) roots of seedlings grown after 3 days of germination followed by incubation in nutrient solution during 24 h. After growing, roots were detached and the crude enzyme extract was obtained. CAD was assayed chromatographically by the forward reaction of the reduction of coniferaldehyde or sinapaldehyde to coniferyl alcohol or sinapyl alcohol, respectively. Disappearance of cinnamaldehyde substrates and the formation of respective alcohols were monitored by isocratic elution with methanol/acetic acid 4% (20/80, v/v) through a GLC-ODS (M) column.

Effects of ferulic acid on lignification: anatomical and biochemical aspects. Three-day-old seedlings were cultivated in nutrient solution, pH 6.0, with or without 1 mM ferulic acid. Experiments were carried out in a growth chamber (25°C, 12-h light/12-h dark cycle, irradiance of 280 μmol m^{-2} s^{-1}) during 24 or 48 hours. Roots were then measured and prepared for light and electron microscopy analyses and biochemical assays. POD activities were observed by starch and polyacrylamide gel electrophoresis. CAD activity was assayed by HPLC, whereas the lignin content was determined spectrophotometrically. Subsequent determinations of lignin were made in roots growing in nutrient solution containing inhibitors of the phenylpropanoid pathway (PIP, piperonylic acid or MDCA, 3,4-methylenedioxy cinnamic acid). ANOVA was applied to test the significance of measured differences. Scott-Knott's test was applied to evaluate the difference between parameters and P values ≤ 0.05 were considered statistically significant.

RESULTS AND DISCUSSION – Two topics have been considered for results. ***Determination of CAD activity by HPLC***. Current research proposes a simple and sensitive HPLC technique to determine CAD activity. Method was based on a single enzyme extraction of soybean roots and enzyme activity was assayed chromatographically by following the consumption of coniferyl and sinapyl aldehydes, CAD reaction substrates. Results showed: a) the preference in to measure the aldehyde disappearance in forward reaction; b) the linearity in the alcohol formation from aldehyde substrates, indicating the specificity of the enzyme reaction, and c) the sensitivity of the methodology which may be applied satisfactorily to determine CAD activity in soybean roots.

Effects of ferulic acid on lignification: anatomical and biochemical aspects. Exogenously supplied ferulic acid induced premature cessation of root growth, with the disintegration of the root caps (or apical epidermis), compression of cells in the quiescent center, increase of vascular cylinder diameter, and lignification of the metaxylem. Moreover, the allelochemical decreased

CAD activity and increased the anionic isoform PODa5 activity and lignin content. When applied jointly with PIP (an inhibitor of the cinnamate 4-hydroxylase, C4H), ferulic acid increased lignin content. By contrast, the application of MDCA (an inhibitor of the 4-coumarate:CoA ligase, 4CL) with ferulic acid did not affect lignin content. Light and electron microscopy studies, combined with biochemical assays, suggested that exogenous ferulic acid was esterified with CoA, forming feruloyl-CoA, by 4CL reaction. Subsequently, it was converted into coniferal- and sinapaldehydes. These metabolites circumvented the blocked CAD reaction by polymerizing toward lignin in the cell wall.

CONCLUSIONS – Results indicate that the isocratic HPLC method currently described here is simple, quick, accurate, and furnishes a reliable measure of cinnamaldehyde substrates. It may be recommended as an alternative tool to determine CAD activity in non-purified protein extracts of soybean roots and it is a promising method to apply to other plant species. Ferulic acid-induced inhibition in root growth of the soybean may be due to the entry of ferulic acid into the phenylpropanoid pathway. Further, the synthesized cinnamic aldehydes are incorporated into the cell wall, which solidifies and restricts cell growth.

Ferulic acid: an allelochemical troublemaker

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Ferulic acid: an allelochemical troublemaker

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Abstract – Ferulic acid, a cinnamic acid derivative, is a well known allelochemical widely distributed in plants. Stress of plant roots by ferulic acid affects several physiological and biochemical aspects, such as water utilization, foliar expansion, root elongation, photosynthesis, cell respiration, membrane integrity and nutrient uptake, among others. Moreover, ferulic acid may be esterified with cell wall polysaccharides, incorporated into the lignin structure or it may form bridges that connect lignin with wall polysaccharides, rigidifying the cell walls and restricting cell growth. This review describes general aspects of allelopathy and focuses on the role of ferulic acid as an allelochemical and its supposed action mode in plants.

Keywords: phenylpropanoid, allelopathy, cell wall cross-linkage, lignification.

Abbreviations:

FA, ferulic acid
CA, cinnamic acid
BA, benzoic acid
p-HCA, *p*-hydroxycoumaric acid
VA, vanillic acid,
p-HBA, *p*-hydroxybenzoic acid
PAL, phenylalanine ammonia liase
POD, peroxidases
C4H, cinnamate-4-hydroxylase
4CL, 4-coumarate:CoA ligase
HCT, hydroxiccinnamoyl CoA: quinate/ shikimate hydroxycinnamoyl transferase
C3H, coumarate-3-hydroxylase
CCoAOMT, caffeoyl coenzyme A 3-*O*-methyltransferase
CCR, cinnamoyl CoA reductase
F5H, ferulate 5-hydroxilase
COMT, caffeic acid *O*-methyltransferase
CAD, cinnamyl alcohol dehydrogenase
ALDH, aldehyde dehydrogenase

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I. Allelopathy and ferulic acid: general aspects

1. Allelopathy: a complex science. For years it has been known that plants release organic compounds into the environment from their aerial or sub-aerial parts, as exudates, volatiles, and/or decomposition residues. These compounds may accumulate in the soil environment and affect the growth and development of neighboring plants, an interaction called allelopathy (Weir et al., 2004). A main characteristic of allelopathy is the plant-plant interaction. About of the 400,000 secondary metabolites assumed to exist in plant kingdom, only about 3% of them have been studied to date and many secondary metabolites have been reported to show allelopathic properties. Secondary metabolites might be separated into three chemically distinct groups: terpenes, nitrogen-containing secondary products and phenolic compounds. The latter group is very heterogeneous, with a wide chemical diversity playing a variety of roles in the plant. While many are defense compounds against herbivores and pathogens, others act in the mechanical support, in the attraction of pollinators and fruit dispersers, in the absorption of harmful ultraviolet radiation, or in allelopathy (Taiz and Zeiger, 1998).

Although there is growing evidence on the phenomenon of allelopathy, its existence is still debated by scientists since the mechanisms from production to release and fate of allelochemicals are largely unknown. Responses to allelochemicals are also difficult to assess. This is further complicated by the wide range of chemicals of diverse molecular structures involved. Benzoic acid derivatives (e.g. *p*-hydroxybenzoic, vanillic and salicylic acids), cinnamic acid derivatives (e.g. *p*-coumaric and ferulic acids), non-protein amino acids (e.g., L-3,4-dyhydroxyphenilalanine) and flavonoids (e.g., quercetin, naringenin), have been referred as allelochemicals.

Membrane perturbations are often reported to be the primary site of action of many allelochemicals that trigger further modifications in the physiological processes of plant cell. However, a clear insight into the primary allelochemical action on plant physiology has not been obtained. Several modes of action for allelochemicals are involved in the inhibition and modification of plant growth and development (Fig. 1).

There are several factors which may influence the effects of an allelochemical. Temperature, photoperiod, mineral composition of the soil and interactions with other allelopathic compounds may affect the chemical stability, availability and toxicity of an allelochemical (Inderjit, 1996). It is thus rather difficult to examine how each factor influences plant response of plants. Another fact is that experiments with allelochemicals have been carried out in restricted conditions of

laboratory, such as temperature, light, nutrient solution and pH, among others. This is a relevant problem since laboratory conditions cannot be directly assumed for natural condition (Seigler, 1996). However, many researchers are obtaining success in their comparative studies, especially when the concentrations of allelochemicals are similar in both systems (Sène et al., 2000).

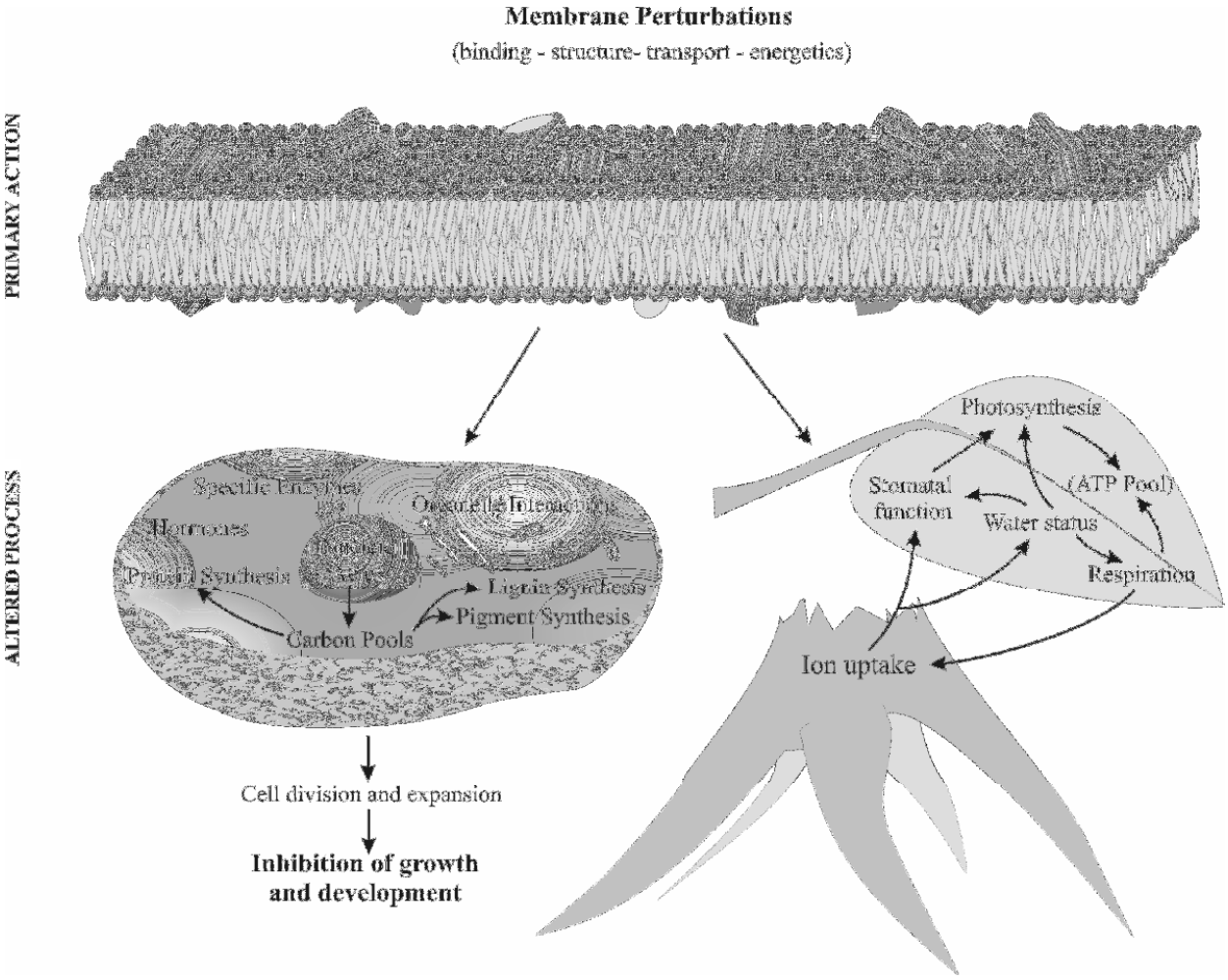

Fig. 1. Action model for allelochemicals in plants (adapted from Einhellig, 1995).

2. Ferulic acid, yesterday and today. Phenolic compounds contain aromatic substances formed via the shikimic acid pathway or the malonic acid pathway, including benzoic and cinnamic acid derivatives. These two pathways supply respectively 60% and 40% of carbon for the biosynthesis of phenolic compounds (Gross, 1981). Linked to the shikimic acid pathway, the phenylpropanoid pathway starts with deamination of phenylalanine, by phenylalanine ammonia liase (PAL) or tyrosine ammonia liase (TAL) forming cinnamic acid, its first metabolite. Cinnamate is converted in monolignols by subsequent actions of different enzymes (Fig. 2).

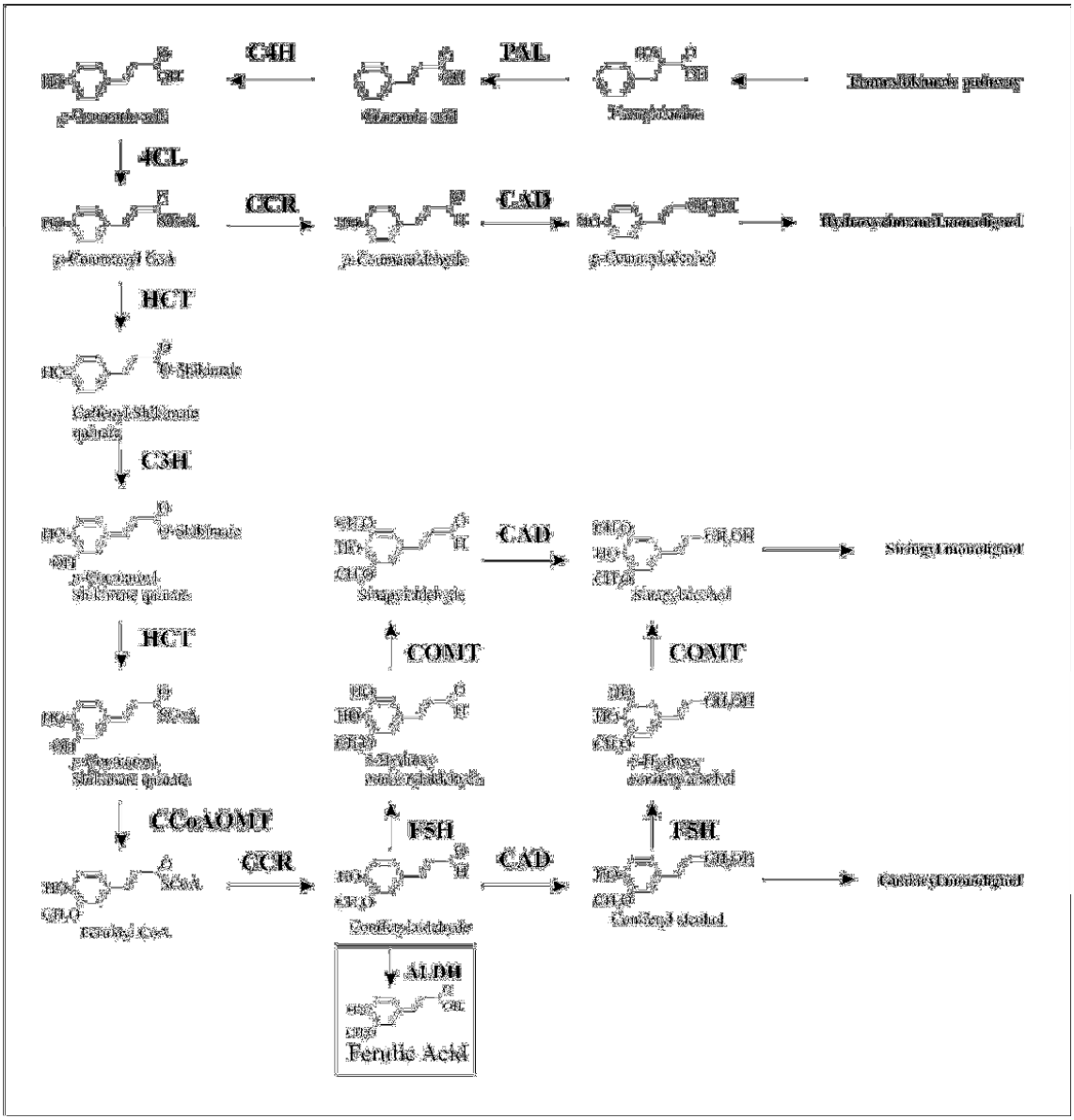

Fig. 2. Simplified phenylpropanoid pathway. PAL, phenylalanine ammonia liase, C4H, cinnamate-4-hydroxylase, 4CL, 4-coumarate:CoAligase, HCT, hydroxiccinnamoyl CoA: quinate/shikimate hydroxycinnamoyl transferase, C3H, coumarate-3-hydroxylase e, CCoAOMT, caffeoyl coenzyme A 3-*O*-methyltransferase, CCR, cinnamoyl CoA reductase, F5H, ferulate 5-hydroxilase, COMT, caffeic acid *O*-methyltransferase, CAD, cinnamyl alcohol dehydrogenase, ALDH, aldehyde dehydrogenase (Chen et al, 2006).

Ferulic acid (FA) is a cinnamic acid derivative. It was firstly isolated as a yellow precipitate, by alcoholic extraction, from the commercial resin of *Ferula foetida* (Umbelliferae, Apiaceae) by Hlasiwetz and Barth (1866). They then determined its chemical composition as $C_{10}H_{10}O_4$ and named *Ferulasäure* or ferulic acid [3,(4-hydroxy-3-methoxyphenyl)-2-propenoic acid]. During 60 years no additional information was reported on this compound. Between 1925 and 1988, it was chemically synthesized; its *cis* and *trans* isomers were separated; stereochemistry was ascertained by NMR spectroscopy and unequivocally confirmed by X-ray crystallographic analysis. FA is a strong dibasic acid. The first proton dissociation generates the carboxylate anion, while the second produces a phenolate anion. The anion has a high degree of resonance stabilization, which increases its acidity in comparison to similar phenolic acids (Graf, 1992).

Several reports have evaluated its occurrence, soil levels, metabolism in plants and microorganisms, industrial applications, physiological role during lignification, and its role as allelochemical. Widely present in plant kingdom, it has been also studied for its properties as an anti-oxidant, food conservant (Walters et al, 1997; Graf, 1992), co-adjuvant, anti-inflammatory, analgesic (de Campos et al., 1998) and anti-carcinogenic (Dobhal et al., 1999). Moreover, FA may be found as feruloyl-CoA in the phenylpropanoid pathway and as a component of cross-linked polymers in cell wall (Smart and O'Brien, 1979, Sánchez et al, 1996). As a metabolite of the monolignols synthesis, FA is ester-linked to primary cell wall oligosaccharides, typically glucuronoarabinoxylans. Smith and Harris (2000) reported that FA may be ester-linked in monocotyledons (Arecales, Commelinales, Poales, Zingiberales, etc). In dicots, FA may be present in Caryophyllales (Hartley and Harris, 1981), Solanales (Keller et al, 1996); Brassicaceae (Chen et al, 1998), and Apiales (Parr et al., 1997). In soils, FA has been considered as a strong allelochemical with several effects on plants, such as reduction in water utilization, inhibition of foliar expansion and root elongation, reduction in the rates of photosynthesis and inhibition of nutrients uptake (Siqueira et al, 1991; Einhellig, 1995). Although different physiological effects are known, its primary mode of action has not been conclusively established.

2.1. Allelochemical-soil-microrganism interactions: a big hindrance. With the elucidation of certain mechanisms by which allelochemicals cause their effects, researchers have been forced to realize that it is rare for a single allelochemical to exist alone under field conditions in concentrations large enough to have significant effects (Einhellig, 1995). As described earlier, an interfering factor in allelopathy is the interaction between the allelochemical and another

chemical compound in the soil. Mixtures of non-inhibitory concentrations of individual phenolic acids may inhibit plant growth in an *additive* (equal to the sum of the effects of each allelochemical tested separately), *synergistic* (greater than the sum of the effects of each allelochemical) or *antagonistic* (lower than the sum of the effects of each allelochemical) manner (Rasmussen and Einhellig, 1977). Blum et al. (1985) demonstrated that, in nutrient solutions, the leaf expansion and dry weight were reduced by single and multiple treatments of FA, vannilic acid (VA) and *p*-coumaric acid (*p*-CA). The effects of the mixture of allelochemicals were additive (for 0.5 mM FA plus 0.5 mM *p*-CA mixture) and antagonistic (for 0.5 mM FA plus 0.5 mM VA mixture). Using soil systems, Blum et al. (1989) and Gerig and Blum (1991) reported that the effects of FA plus VA, FA plus *p*-hydroxybenzoic (*p*-HBA) and *p*-CA plus *p*-HBA acids mixtures on leaf area expansion revealed additive effects. Conducting split-root experiments, Lehman et al. (1994) verified that the simultaneous effects of FA and *p*-CA on leaf expansion were additive. The inhibition of leaf expansion was directly related to the concentrations of the acid(s) and the proportion of roots treated with the acid(s). Soybean roots cultivated in nutrient solution containing FA or VA (0.5 mM; 1.0 mM and equimolar mixtures), for 48 h, were affected (Suzuki et al., 2003). Acting by himselves, both compounds (at 0.5 or 1.0 mM) decreased root length, fresh and dry weights, and increased soluble and cell wall-bound peroxidases activities. At 1.0 mM, FA increased (but VA decreased) the phenylalanine ammonia lyase (PAL) activity. Acting simultaneously, the effects of the allelochemical interaction were lower than the sum of the effects of each compound tested separately, an example of antagonism.

Another complex fact is soil interactions. Reversible sorption of phenolic acids by soils provides short-term protection to FA and other phenolic acids from microbial degradation, affecting intensity and duration of this intensity (Blum, 1998). Introduction of microorganisms in soil indicates rapid utilization of allelochemicals. Differential soil fixation, microbial production of benzoic acids (VA, *p*-HBA) from cinnamic acids (FA and *p*-CA, respectively) and further differential utilization of cinnamic and benzoic acids by microorganisms reveals that these conditions may influence the magnitude and duration of the phytoxicity of the individual allelochemical. Furthermore, rhizosphere and bulk-soil bacteria may affect the access of phenolic acid toward the root. Phenolic acid utilizing bacteria are induced/selected by less than 0.1 µmol g^{-1} of phenolic acid. For a 0.6 µmol g^{-1} soil equimolar phenolic acid mixture composed of *p*-CA, FA, *p*-HBA and VA, modeling indicated that a 500% increase in rhizosphere phenolic acid, utilizing bacteria, would decrease by about 5% in inhibition of cucumber leaf expansion. In some

cases, there is an inverse relation between the size of the microbial rhizosphere population and the intensity of the allelochemicals' effects (Blum et al, 2000).

2.2. Uptake of FA: more complexity. One of the aims of investigating allelopathic interactions has been to develop means of predicting plant effects after its uptake. In this context, the uptake of FA (as radiotracer U-ring-^{14}C ferulic acid) from solutions (0.1 to 1.0 mM, pH 4.0 to 7.0) was monitored in intact and excised cucumber roots by Shann and Blum (1987). Results revealed that FA uptake was directly proportional to its concentration and inversely to pH of the nutrient solution. The effects were more evident in relation to the concentration of FA than its net uptake (Lehman and Blum, 1999). After uptake, the intensity of the effects depended on the constant presence of the allelochemical surrounding seedling roots. If removed from the nutrient solution, effects may be reverted. Moreover, the proportion of the root system in direct contact with FA affects directly the allelopathic responses, such as root growth, water utilization and nutrient uptake (Klein and Blum, 1990). In fact, Lehman and Blum (1999) demonstrated that the inhibition of net phosphorous uptake was rather related to the direct contact of the root system with FA than to its uptake. In general, uptake of nutrients (Mg^{2+}, Ca^{2+}, K^{2+}, PO_4^{2-}, Fe^{3+}, Mn^{2+}, NO_3^- and NH_4^+) may be reduced by FA treatments in several plant species (Glass, 1973 e 1974, Danks et al., 1975, McClure et al, 1978, Kobza and Einhellig, 1987, Lyu and Blum, 1990, Booker et al., 1992, Bergmark et al., 1992).

A significant interaction has been verified between environmental temperatures and FA treatments. At 0.4 mM, FA reduced dry weight of soybean seedlings grown at 34°C, while its effects were lower at 23°C. It may be plausible that temperature stress enhances allelochemical inhibition, indicating that interactions with the environment should be taken into account in understanding allelopathy (Einhellig and Eckrich, 1984). Similarly, exudation of some benzoic and cinnamic acids derivatives by cucumber roots also increased with the temperature and/or photoperiod (Pramanik et al, 2000). FA caused more damage in root growth, water utilization, and leaf transpiration than other cinnamic and benzoic acids derivatives (Rasmussen and Einhellig, 1977, Blum and Dalton, 1985, Gerig and Blum, 1991). In general, the main effects were associated with increase in number of secondary roots and reduction of root/stem ratio (Blum and Rebbeck, 1989, Vaughan and Ord, 1990).

II. Effects of FA on metabolism: seeking answers.

There are several proposed modes of action for allelochemicals. As pointed out by Einhellig (1995), "*the phytotoxicity of many allelochemicals may be from a generalized cellular disruption rather than a specific mechanism*". Due to the diversity of compounds, a common allelochemical does not exist. Similarly, a single mode of action for all allelochemicals is not extant. Therefore, the mode of action remains an open question. Some FA effects on plant metabolism will be related bellow.

1. Effects on carbon partition, carbohydrates and lipids. It has been verified that FA reduces the conversion of glucose to soluble amino acids, proteins and organic acids (Danks et al., 1975), as well as to the incorporation of phenylalanine in proteins (Van Sumere et al. 1971). A plausible explanation is that carbohydrate partition in plants rather drives toward growth and synthesis of secondary metabolites, during differentiation or under stress (Matsuki, 1996). Under FA´s stress, glucose may be released for the cytosol and, further, used in the shikimic and phenylpropanoid pathways, reducing the carbon flux in the primary metabolism. In addition, FA decreases CO_2 reduction by photosynthesis, which may be related to with changes in chlorophyll content (Einhellig e Rasmussen, 1979; Blum e Rebbeck 1989) and in glucose metabolism (Ferrarese, 2000). This might be associated with the conspicuous reduction in starch store of cap cells showed by ultrastructural assays (dos Santos et al., 2007).

Utilization of energy necessary for cells to grow and multiply in response to FA has been affected in plants. FA reduced lipid mobilization followed by accumulation of unsaturated fatty acids in canola (*Brassica napus*) seeds during germination (Baleroni et al., 2000). It also increased contents of saturated and unsaturated fatty acids of the polar and non-polar lipid fractions and xylose, fructose and sucrose in soybean root (Ferrarese et al., 2001), as may be seen in Table 1. Consequently, cellular structure changes appear to be, at least partially, associated to alterations in the lipid and carbohydrate metabolism (Ho, 1988; Ohlrogge and Browse, 1995; Surjus e Durand, 1996; Harwood, 1997). Another fact is that malondialdehyde content, a product of lipid peroxidation, was strongly enhanced (152%) in cucumber roots by 0.5 mM FA. Moreover, the membrane injury, which indicates the integrity of the membrane, increased by 10%, under FA action. It is well known that unsaturated fatty acids are susceptible to free radicals. Then, FA may cause lipid peroxidation and membrane disruption leading to ion

leakage, affecting nutrient uptake (Politycka, 1996). In fact, uptake of nutrients (Mg^{2+}, Ca^{2+}, K^{2+}, PO_4^{2-}, Fe^{3+}, Mn^{2+}, NO_3^- and NH_4^+) has been reduced by FA treatments in several plant species (Glass, 1973 e 1974, Danks et al., 1975, McClure et al, 1978, Kobza and Einhellig, 1987, Lyu and Blum, 1990, Booker et al., 1992, Bergmark et al., 1992).

Table 1 – Changes in some physiological and biochemical indicators in plants submitted to the ferulic acid treatment.

Function		Indicator	Change	
		CO_2 fixation	(–)	
Energy carbohydrate		Glucose	(–)	
		Fructose	(+)	
		Sucrose	(+)	
Structural carbohydrate		Rhamnose	(–)	
		Xylose	(+)	
Lipid metabolism	Fatty acids		Polar	Apolar
		Palmitic acid	(+)	(+)
		Stearic acid	(+)	(+)
		Behenic acid	(+)	(+)
		Oleic acid	(=)	(+)
		Linoleic acid	(+)	(+)
		Linolenic acid	(+)	(–)
	Lipid peroxidation	Malondialdehyde	(+)	(+)
Enzyme activities		PAL	(+)	
		CAD/SAD	(–)	
		POD	(+)	
		ICL	(–)	
		ATPases	(–)	
		β-GT	(+)	
		β-GL	(+)	

Symbols: (–), decreased; (+), increased; (=): unchanged. CO_2, carbon dioxide; PAL, phenylalanine ammonia liase; CAD/SAD, coniferyl/sinapyl alcohol dehydrogenase; POD, peroxidase; ICL, isocitrate liase; β-GT, β-glucosyltransferase; β-GL, β-glycosidase.

2. Effects on enzymes. FA as other allelochemicals may affect the activities of several enzymes, including amylase, β-glucosylated by phenol β-glucosyltransferase, catalase and IAA oxidase (Einhellig, 1995; Devi and Prasad, 1996; Politycka, 1996, 1998). Special mention is made on the effects of FA on phenylalanine ammonia lyase (PAL) and peroxidases, enzymes of the lignin pathway, which are involved in the synthesis of phenolic compounds in plants.

2.1. Phenylalanine ammonia-liase (PAL). As may be noted in Fig. 2, PAL is the first rate-limiting enzyme of the phenylpropenoid pathway leading to the synthesis of the phenolic acids and, later, monolignols. Similar to POD, increased activity of PAL may be a response of plants to various biotic and abiotic stresses. Few studies have been carried out on the effects of exogenous FA on PAL, and results are contradictory. For example, Sato et al. (1982) pointed out that FA was ineffective on PAL of sweet potato (*Ipomea batatas* L.) and pea (*Pisum sativus* L.). FA was unable to affect PAL activity in cucumber (*Cucumis sativus* L.) roots (Shann and Blum, 1987). In contrast, increased PAL activities were associated with a decrease in cucumber (*Cucumis sativus* L.) roots, after 24 and 48 hr of FA treatment (Politycka, 1998). In agreement with this author, dos Santos et al. (2004) demonstrated that PAL activities increase in soybean roots after 24 to 72 hr of FA treatment. In addition, Politycka (1999) reported that an increase in the PAL activity induced by the action of FA and associated with reduced root growth of cucumber depended on ethylene synthesis. Application of ethylene synthesis inhibitor (aminooxyacetic acid, AOA), cancelled out the effect of FA on PAL activity (Politycka and Mielcarz, 2007). According to these authors, ethylene participates in the retardation of cucumber root growth by FA.

2.2. Peroxidases. Some researchers have reported alterations in POD activity under FA action. For example, in cucumber root treated with FA (0.5 or 1.0 mM), the soluble and bound form of POD increased significantly (Politycka, 1996, Politycka et al., 2004; Shann and Blum, 1987). Application of 1.0 mM FA also caused a significant increase in both soluble and bound POD in maize roots and correlated with a pronounced decrease in root growth (Devi and Prasad, 1996). At 1.0 mM, FA also increased POD activity in soybean roots (dos Santos et al., 2004). Increase of soluble POD activity was accompanied by decrease in root growth. Based on these results, researchers above attributed FA effects to the production of free radicals. It is well known that soluble POD catalyzes the oxidation of diverse phenolic substrates and is often regarded as an antioxidant enzyme that protects cells from the destructive influence of oxygen radicals. However, if the cells ability the to scavenger oxygen radicals is exceeded, phenolic acid oxidation by soluble POD leads to production of quinones, which increase depolaration the cell membrane and changes in lipid composition (Politycka, 1996, 1998; Ferrarese et al., 2001; Doblinski et al., 2003). Moreover, cell wall-bound POD is associated with cell wall stiffening and growth-restriction (Passardi et al., 2005). POD is able to converted phenolic compounds

such as ferulic, *p*-coumaric, and caffeic acids into free radicals, which spontaneously polymerize. This essential role for POD in the stiffening of cell walls through the formation of biphenyl bridges between wall polymers and, thus, the reduction of the cell wall extensibility have been proposed by some researchers (Fry et al., 1992: Sanchez et al., 1996; dos Santos et al., 2004).

3. Effects on mitochondrial respiration. Several allelochemicals such as sorgoleone, juglone, quercetin, umbelliferon, gramine and cineole have been found to perturb respiratory metabolism, In general, the production of ATP in mitochondria was inhibited by a variety of flavonoids (Einhellig, 1995). Sert et al. (1998) demonstrated the effects of FA on L-malate oxidation in mitochondria isolated from soybean seedlings. FA inhibited basal and coupled respiration during L-malate oxidation, depleting the amounts of pyruvate or oxaloacetate produced. The authors suggest that the site of FA is situated at some step that precedes the respiratory chain, although this action has been occurred at high concentration of allelochemical.

III. Cell wall, FA and lignification: perfect links.

Integrated with the cytoplasm, cell wall performs a role of exoskeleton, conferring to the cell its form, mechanical resistance, pathogen protection, adherence to vicinal cells, and limiting the influx of water and macromolecules. Polysaccharides as cellulose, hemicelluloses and pectins constitute its basic structure. Lignin is the main structural component of secondarily thickened plant cell walls. The biosynthesis of lignins proceeds through a long sequence of reactions that involve the citosolic shikimate pathway. It supplies phenylalanine and tyrosine. Subsequently, general phenylpropanoid pathway converts phenylalanine (or, in lesser extent, tyrosine) into *p*-hydroxycinnamoyl-CoA esters. Lignin-specific pathway starts with *p*-hydroxycinnamoyl-CoA esters and converts them into free cinnamic acids and monolignols (Fig. 2, Boerjan et al., 2003). FA and other cinnamic acids may covalently cross-link cell wall polymers (Fry, 1986, Ramakrishna et al., 1989) hardening cell wall. This process is important to stop elongation (Fry, 1986, Iiyama et al., 1990) and block the access of pathogens (Assabgui et al., 1993) into the cytoplasm. Apoplastic peroxidases (ionic and covalently bound to the cell wall) are thought to catalyze the oxidation of both hydroxycinnamates cross-linkage and monolignol polymerization, which may be regulated by the supply of H_2O_2 and ascorbic acid, (Sánchez et al., 1996; Córdoba-Pedregosa et al., 1996; Mehlhorn et al., 1996; Vianello et al., 1997).

As reported earlier (Ferrarese et al., 2001), a decrease in rhamnose and an increase in xylose contents were verified in soybean roots treated with FA. Rhamnose is a component of pectin and related to the number of pectin gel ramifications and to reinforcement of cell wall. It is thus possible that exogenous FA, esterified to polysaccharides, decreases free rhamnose content by reducing pectin hydrolysis. On the other hand, the increased content of xylose suggests an activation of cell wall hydrolases and esterases, which release oligosaccharides (Grant Reid, 1997). In brief, FA might affect directly the structure of cell wall. During treatment, root cells may accumulate FA into the apoplast (Akin et al., 1992). Peroxidases may also catalyze the link of FA in the polysaccharides, lignin (Polyticka, 1996, Chakraborty et al., 1993, Wallace and Fry, 1999) and other FA. The dehydrodiferulic acid may form diester, ester-ether or diester-ether cross-linkage between cell wall polymers. These structures may reinforce the cell wall against cellulases, pectinases (Akin et al, 1993, Wojtaszek, 1997) and laccase (Sterjiads et al., 1993) produced by pathogens, which are involved in the cessation of cell elongation. In addition to free FA, FA-oligosaccharides also show biological activity as inhibitors of cell growth (Ishi, 1997) and are involved in signal transduction between plants and microorganism (Peters and Verma, 1990).

Exogenously applied FA incorporates in the lignin residues (Shann and Blum, 1987) while inducing lignification and related enzymes associated to the reduced root growth of treated plants (Devi and Prasad, 1996, Polyticka, 1999, dos Santos et al., 2004). Shann and Blum (1987) verified an increase in lignin contents associated to a decrease in root growth. In maize (*Zea mays* L.) roots, FA increased the activity of cell wall-bound POD correlated to a significant increase in lignin content and to a reduction in root growth (Devi and Prasad, 1996). Politycka (1999) also verified that cucumber (*Cucumis sativus* L.) seedlings treated with FA stimulated lignin production, coupled to a decrease in root growth.

IV. Is the cell wall an action site of FA?

Recent data obtained by dos Santos et al. (2007, in *this thesis*) revealed that FA affects soybean root growth due to the incorporation of FA into the phenylpropanoid pathway. Using phenylpropanoid enzyme inhibitors the above authors concluded that 4CL catalyzes the conversion of exogenously applied FA into feruloyl-CoA (Fig. 2). Feruloyl-CoA formed is then converted into coniferal- and sinapaldehydes. These metabolites must circumvent the inhibited

CAD reaction by polymerizing toward lignin in the aldehyde state. Based on this fact, and linking the information available in the literature for FA, these authors suggested a mode of action for FA (*see* Fig. 8, *pg. 73 in this Thesis*) considering an elegant model for plant response throughout biotic stress proposed by Wojtasek (1997).

In the model, the contact of exogenous FA with the root cell inactivates sulfhydryl groups of carrier proteins, causing an ionic disturbance and affecting the nutrient uptake by the cell membrane (Baziramakenga et al., 1995). In the Wojtasek's model, the pathogen infection generates a cascade of signaling events – including Ca^{2+} influx and proton efflux – that actives NADPH-oxidase complex (generating $^{\cdot}O_2^-$) and pH-sensible cell wall POD (producing H_2O_2) which produces an oxidative burst. To date, there is no data on signaling events caused by FA stress. However, the general disturbance caused by FA must be enough to increase the Ca^{2+} influx since its concentration is kept lower inside the cytoplasm by the action of ATPases. Short-time experiments revealed that the absence of Ca^{2+} in nutrient solution reduces FA effects on soybean roots (data not shown). In addition, Converso and Fernandez (1996) found evidence that Ca^{2+} modulates POD isozymes.

The reduced linoleic acid content after FA treatment (Ferrarese et al. 2001) may also be related to signaling events. Linoleic acid is a precursor of oxylipins, such as traumatin, jasmonic acid, etc (van der Selt et al., 2000), which are involved in the plant defense signaling mechanisms (Trawatha et al, 1995). The oxylipins pathway starts with the oxidation of linolenic acid by lipoxigenases, which are activated under stress/defense circumstances as, for example, increase of H_2O_2 (Fornaroli et al., 1999). On the other hand, jasmonic acid may inhibit plant growth and may be associated with the inhibition of root growth caused by FA (Creelman et al., 1992). Jasmonic acid is also related to the expression of specific POD isozymes during stress (Allison and Schultz, 2004).

The control of pH in H_2O_2 production has been demonstrated. In bean, H_2O_2 is generated from $^{\cdot}O_2^-$ by a pH-sensible cell wall POD that requires a reductant group (Wojtaszek, 1997). FA oxidation by POD might produce H_2O_2. The phenoxy radical formed may undergo dimerization. These dimers are, eventually, esterified to hemicelluloses, linking the cell wall polymers, stiffening the cell wall and decreasing the root growth (Zimmerlin et al. 1994, Bolwell et al, 1995, Blee et al., 2001, Bolwell et al., 2002, Stobiecki, 2002, Vianello et al., 1997). Furthermore, increase in H_2O_2 contents enhances activities of cell wall POD and lignification (Politycka, 2004,

Whetten et al, 1998, dos Santos et al, 2004). As cited earlier, FA cross-linkages are the initial steps of lignification (Boerjan et al, 2003).

Experiments using inhibitors of this pathway revealed that FA might be channeled into the phenylpropanoid pathway and, later on, may increase the lignin production (dos Santos et al., 2007). In addition, FA-induced lipid peroxidation (Polyticka, 1996, Doblinski et al., 2003) has been related with oxidative burst (Baziramakenga et al., 1995, Devi e Prasad, 1996). When the oxygen radicals exceed the scavenger capacity of the cells, phenolic acid oxidation by PODs will lead to accumulation of quinones (Polyticka, 1998, Appel, 1993). These compounds may act as proton carriers intensifying depolarization of the root cell membrane. In summary, excessive cross-linkages, lignification and changes in the membrane permeability may cause reduced root growth (Lee et al., 1982, Devi and Prasad, 1996).

V. Ferulic acid tomorrow: What happen next?

Available data related to the effects of FA on plants indicate plausible action sites during FA stress especially the putative starting effects on sulfhydryl groups of carrier proteins and the irreversible reduction of cell wall extensibility. Root responses to the FA action include activation of POD, cross-link between cell wall polymers and reduction in cell wall extensibility. Moreover, exogenous FA activates cell wall POD, increases apoplastic H_2O_2 content and may be incorporated into metabolic pathway which leads to lignin production.

In spite of related findings, further studies are required to elucidate some of the possibilities suggested. These include: (1) identification of reactive oxygen species (ROS), such H_2O_2 and $^{\cdot}O_2^-$**,** possibly involved in these response; (2) determination of activities of enzymes involved in H_2O_2 production, for example, NADH-oxidase and pH-dependent cell wall POD; (3) verification of the involvement other phenylpropanoid pathway enzymes (e.g. treatment of cell wall POD with specific inhibitors); (4) comparison of plants with CAD-deficient responses with non-deficient plants treated with FA; (5) identification of the aldehydes in induced lignin, etc. Further data must strengthen the role of FA and other compounds in plant allelopathy.

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High performance liquid chromatography method for the determination of cinnamyl alcohol dehydrogenase activity in soybean roots*

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High performance liquid chromatography method for the determination of cinnamyl alcohol dehydrogenase activity in soybean roots

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Abstract - This study proposes a simple, quick and reliable method for determining the cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) activity in soybean (Glycine max L. Merr.) roots using reversed-phase high–performance liquid chromatography (RP-HPLC). The method includes a single extraction of the tissue and conduction of the enzymatic reaction at 30 °C with cinnamaldehydes (coniferyl or sinapyl), substrates of CAD. Disappearance of the cinnamaldehyde substrates in the reaction mixture is monitored at 340 nm (for coniferaldehyde) or 345 nm (for sinapaldehyde) by isocratic elution with methanol/acetic acid through a GLC-ODS (M) column. This HPLC technique furnishes a rapid and reliable measure of cinnamaldehyde substrates, and may be used as an alternative tool to analyze CAD activity in enzyme preparation without previous purification.

Keywords: Cinnamyl alcohol dehydrogenase; coniferaldehyde; high performance liquid chromatography; sinapaldehyde; soybean

Abbreviations: CAD, cinnamyl alcohol dehydrogenase; HPLC, high performance liquid chromatography

1. Introduction

Lignin is the main structural component of secondarily thickened plant cell walls. It performs a relevant role in evolution, growth and resistance of the terrestrial vascular plants and in the global carbon cycle. Additionally, it is of great interest in agricultural and industrial research due its economic relevance. In plants it imparts mechanical support and efficient conduction of water and solutes over long distances within the vascular systems [1]. Structurally, lignin is a heteropolymer of hydroxylated and methoxylated phenylpropane units, derived from the oxidative polymerization of different hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl and sinapyl) connected by labile ether bonds and/or resistant carbon-carbon linkages [2]. In dicotyledonous angiosperms lignin is built from coniferyl and sinapyl alcohols, which are incorporated respectively as guaiacyl (G) and sinapyl (S) units to form heterogeneous G-S polymers. In the last step of monolignol biosynthesis, coniferaldehyde and sinapaldehyde are converted into their corresponding alcohols by cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) and probably by sinapyl alcohol dehydrogenase (SAD, a CAD homolog from aspen) in a NADPH-dependent reaction (Fig. 1). Data related to the down-regulation of CAD by genetic engineering or to the properties of CAD mutants support its role as a specific marker [3,5,6,7,8,9,11].

CAD activity has typically been assayed by spectrophotometry [5,6,8,12] and occasionally by radiometry [1]. In spectrophotometric assays, CAD activity has been measured in the reverse reaction by following the oxidation of the appropriate hydroxycinnamyl alcohol at 400 nm (formation of the respective aldehyde) or at 340 nm (formation of NADPH). Enzyme activity has also been determined in the forward reaction by following the oxidation of NADPH (at 340 nm) due to reduction of the appropriate hydroxycinnamyl aldehyde. For a long period, HPLC has been firmly established as a favorable and reliable technique for separation and determination of traces from a wide range of compounds. Efficiency, simplicity, sensitivity and reproducibility are all advantages of HPLC for the separation of extremely low quantities of complex mixtures. The current research proposes a simple and sensitive HPLC technique to determine CAD activity. The method is based on a single enzyme extraction of soybean roots. The enzyme activity is assayed chromatographically by following the consumption of coniferyl and sinapyl aldehydes, the substrates of the CAD reaction.

2. Results and discussion

As reported earlier, CAD activity has been assayed spectrophotometrically [5,6,8,12] by the oxidation of hydroxycinnamyl alcohols (at 400 nm, reverse reaction) or by the oxidation of NADPH (at 340 nm, forward reaction). In the present study, CAD was assayed chromatographically by the reduction of the corresponding aldehydes (coniferaldehyde and sinapaldehyde) in the forward reaction. In our previous experiments (data not shown), CAD activity was analyzed chromatographically by the reverse reaction (alcohols to aldehydes). Irregular resolution of the chromatographic profiles proceeded variable concentrations of coniferyl and sinapyl aldehydes were verified. By consequence, no linearity in the concentrations of aldehydes produced from alcohols was obtained. It is known that, contrary to aldehydes, coniferyl and sinapyl alcohols are not stable (air and light sensitive) and require strict storage conditions (under nitrogen and vacuum). Thus, unreliable changes during the enzymatic reaction cannot be discarded, by interfering in the aldehyde formation. In agreement, this technical protocol did not give reliable results in more than fifty independent samples. So, in a broader sense, the measure of disappearance of aldehyde substrate (in forward reaction) was more feasible.

To standardize this technical protocol, two aspects have been considered. First, the possibility of unspecific endogenous consumption of the aldehyde substrate in the crude enzyme extract inasmuch its formation (reverse reaction) was not linear. For that, controls with the enzyme extract plus the substrate (coniferaldehyde or sinapaldehyde), without NADPH were undertaken. Both aldehyde substrates were partially consumed. So, in all subsequent enzyme reactions, these values were subtracted of the aldehyde substrates (50 nmol) and considered as initial concentrations. Second, the confirmation of alcohols formation from aldehyde substrates proceeded by linear behavior of the enzymatic reaction. Figures 2A and 2C show the typical HPLC results of CAD reactions with coniferaldehyde and sinapaldehyde substrates, respectively. The HPLC chromatogram (Fig. 2A) shows the CAD reduction of coniferaldehyde (retention time [Rt] = 12.52 min) into coniferyl alcohol (Rt = 7.15 min). Similarly, the HPLC chromatogram (Fig. 2C) shows the CAD-mediated sinapaldehyde (Rt = 16.58 min) reduction into sinapyl alcohol (Rt = 8.28 min). The HPLC-profiles reveal that aldehyde substrates and alcohol products were unambiguously identified with their respective authentic standards (Fig. 2B,D). The insets show the coniferyl (Fig. 2a) and sinapyl (Fig. 2b) alcohols produced in reactions containing

different quantities of crude protein extract. Results confirm the linearity in the alcohol formation from aldehyde substrates, indicating the specificity of the enzyme reaction.

Figure 3 shows the chromatographic profiles of coniferaldehyde (left column) and sinapaldehyde (right column). Coniferaldehyde and sinapaldehyde standards (50 nmol) eluted at 12.49 min (Fig. 3A) and 16.56 min (Fig. 3B), respectively. Figures 3C and 3D show that the initial concentrations of aldehyde substrates eluted in the same retention times as standards (Fig. 3A,B). For validation of the proposed method, two different protocols were undertaken as indicated in the Methods. Figure 3 (E-H) shows the chromatographic profiles of aldehyde substrates consumed after the CAD reaction. By comparing retention times of the samples (Fig. 3E-H) and standards (Fig. 3A,B), aldehyde substrates consumed in the enzymatic reactions were identified. In 4-day-old seedling roots (protocol 1), the peak areas of coniferaldehyde (Fig. 3E) and sinapaldehyde (Fig. 3F) were smaller than those of the initial concentration of aldehyde substrates (Fig. 3C,D). Similarly, in 3-day-seedling roots incubated in nutrient solution for 24 h (protocol 2), the peak areas of coniferaldehyde (Fig. 3G) and sinapaldehyde (Fig. 3H) were also smaller than aldehyde substrates. In both cases, this confirms the disappearance of aldehyde substrate and, consequently, the CAD action with further alcohol formation (Fig. 2A,B). CAD activities were determined in 4-day-old seedlings roots (protocol 1) and in incubated seedlings roots (protocol 2). In the first condition, the enzyme activities were 7.24 nmol of coniferaldehyde consumed min^{-1} mg^{-1} protein (Fig. 4A) and 10.32 nmol of sinapaldehyde consumed min^{-1} mg^{-1} protein (Fig. 4B). In the second condition, the enzyme activities were 12.27 nmol of coniferaldehyde consumed min^{-1} mg^{-1} protein (Fig. 4A) and 24.10 nmol of sinapaldehyde consumed min^{-1} mg^{-1} protein (Fig. 4B). In brief, data presented in this communication indicate that the methodology is highly sensitive and may be applied satisfactorily for the determination of CAD activity in soybean roots.

3. Conclusion

The isocratic HPLC method described in this work is simple, quick and accurate. It can be recommended as an alternative tool to determine CAD activity in non-purified protein extracts of soybean roots and it is a promising method to apply in other plant species.

4. Methods

4.1. Experimental conditions and enzyme extraction

Soybean (*Glycine max* (L.) Merr. cv. BRS-133) seeds, previously surface-sterilized with 2% sodium hypochlorite for 2 min and rinsed extensively with deionized water, were dark-germinated (at 25 °C) on three sheets of moist filter paper. For extraction of enzyme, two protocols were followed: (1) with roots of seedlings grown after 4 days of seed germination and (2) with roots of seedlings grown after 3 days of germination followed by incubation in nutrient solution [10] during 24 h. In the first case, roots were detached from seedlings and the crude enzyme preparation was obtained as follows: fresh tissues (2 g) were ground at 4 ºC in 3 ml of an extraction medium containing 40 mM of β-mercaptoethanol and 100 mM potassium phosphate buffer (pH 7.3). The homogenate was centrifuged at 2,200 × *g* for 15 min, and the supernatant was used as enzyme preparation [12]. In the second case, twenty-five 3-day-old seedlings were supported on adjustable acrylic plate and transferred to a glass container (10 × 16 cm) filled with 200 ml of pH 6.0 half-strength Hoagland's solution [10]. The container was kept in a growth chamber (25 °C, 12-h dark/12-h light photoperiod, irradiance of 280 μmol m^{-2} s^{-1}). The nutrient solution was aerated continuously by air bubbling. Roots were detached from seedlings after 24 h and the crude enzyme extract was obtained as described earlier.

4.2 .Enzyme assay

CAD was assayed chromatographically by the forward reaction of the reduction of coniferaldehyde or sinapaldehyde to coniferyl alcohol or sinapyl alcohol, respectively. The assay was carried out, at 30 °C, in 1.0 ml of reaction mixture containing 200 μl of crude enzyme preparation (0.35 mg of protein), 104 nmol NADPH and 150 nmol Tris-HCl buffer (pH 8.0). Fifty nmol of sinapaldehyde were added at the start and stopped after 3 min of incubation by adding 50 μl of 5 N HCl. For coniferaldehyde reaction, the mixture (1.0 ml) contained 200 μl of crude enzyme preparation, 104 nmol NADPH and 150 nmol Tris-HCl buffer (pH 7.5). Fifty nmol of coniferaldehyde were added at the start and stopped after 3 min of incubation by adding 50 μl of 5 N HCl. Parallel controls with coniferaldehyde, sinapaldehyde or NADPH added in the reaction mixture without enzyme preparation were made. To exclude eventual endogenous

consumption, controls with the enzyme extract plus the substrate (coniferaldehyde or sinapaldehyde), without NADPH, were undertaken in the same experimental conditions. All samples were filtered through a 0.45 μm disposable syringe filter (Hamilton® Co., Nevada, USA) prior to chromatographic analyses.

4.3. HPLC procedure

Samples (20 μl) were analyzed with a Shimadzu® Liquid Chromatograph (Tokyo, Japan) equipped with a LC-10AD pump, a Rheodine® injector, a SPD-10A UV detector, a CBM-101 Communications Bus Module, and a Class-CR10 workstation system. A reversed-phase Shimpack® GLC-ODS (M) column (150 × 4.6 mm, 5 μm) was used at room temperature together with the same type of pre-column (10 × 4.6 mm). The mobile phase was methanol/acetic acid 4% in water (20/80, v/v) with a flow rate of 1.2 ml min^{-1} for an isocratic run of 20 min. Absorption was detected at 340 nm (for coniferaldehyde), 345 nm (for sinapaldehyde) and 260 nm (for coniferyl and sinapyl alcohols), which were previously determined by spectrophotometric scanning of standards dissolved in the mobile phase. Data collection and integration were performed with Class-CR10 software (Shimadzu®, Tokyo, Japan). Hydroxycinnamyl aldehydes and alcohols were identified by comparing their retention times with that of standards. Concentration was calculated by dividing the peak area of each aldehyde (or alcohol) in the samples by the peak area of the initial concentration of each aldehyde substrate. Hydroxycinnamyl aldehydes and alcohols were purchased from Sigma-Aldrich® Chemical Co (St Louis, USA) and all other reagents used were of the purest grade available or chromatographic grade. CAD activity was expressed as nmol coniferaldehyde or sinapaldehyde consumed min^{-1} mg^{-1} protein. Data are expressed as means of four independent experiments ± S.E.

4.4. Protein determination

Protein in soluble enzyme extracts was determined spectrophotometrically at 595 nm [4], with bovine serum albumin as a standard.

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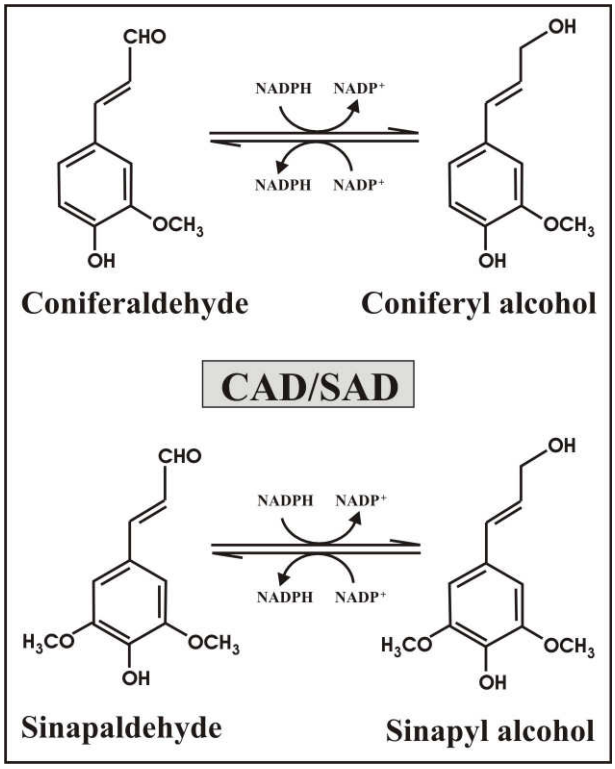

Fig. 1 - Last reaction of monolignol biosynthesis in angiosperms.

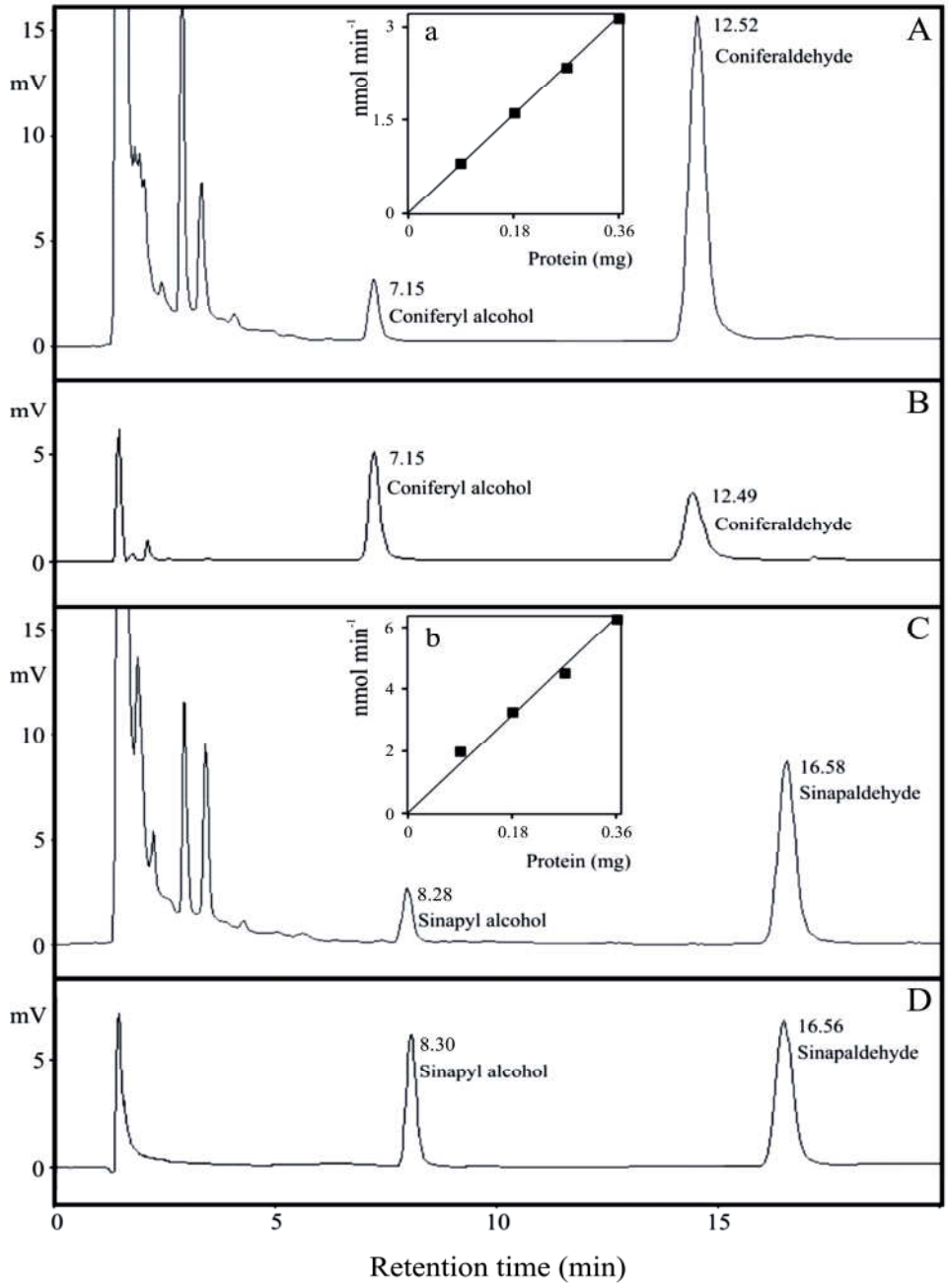

Fig. 2 - HPLC chromatograms showing the CAD reduction of coniferaldehyde into coniferyl alcohol (A) and sinapaldehyde into sinapyl alcohol (C). Coniferyl alcohol (Rt = 7.15 min) and coniferaldehyde (Rt = 12.49 min) standards (B), and sinapyl alcohol (Rt = 8.30 min) and sinapaldehyde (Rt = 16.56 min) standards (D). The insets show the coniferyl (a) and sinapyl (b) alcohols produced in reactions with different quantities of crude protein extract. Coniferaldehyde, sinapaldehyde and hydroxycinnamyl alcohols were detected at 340 nm, 345 nm and 260 nm. The experimental conditions followed protocol 2 as indicated in Methods.

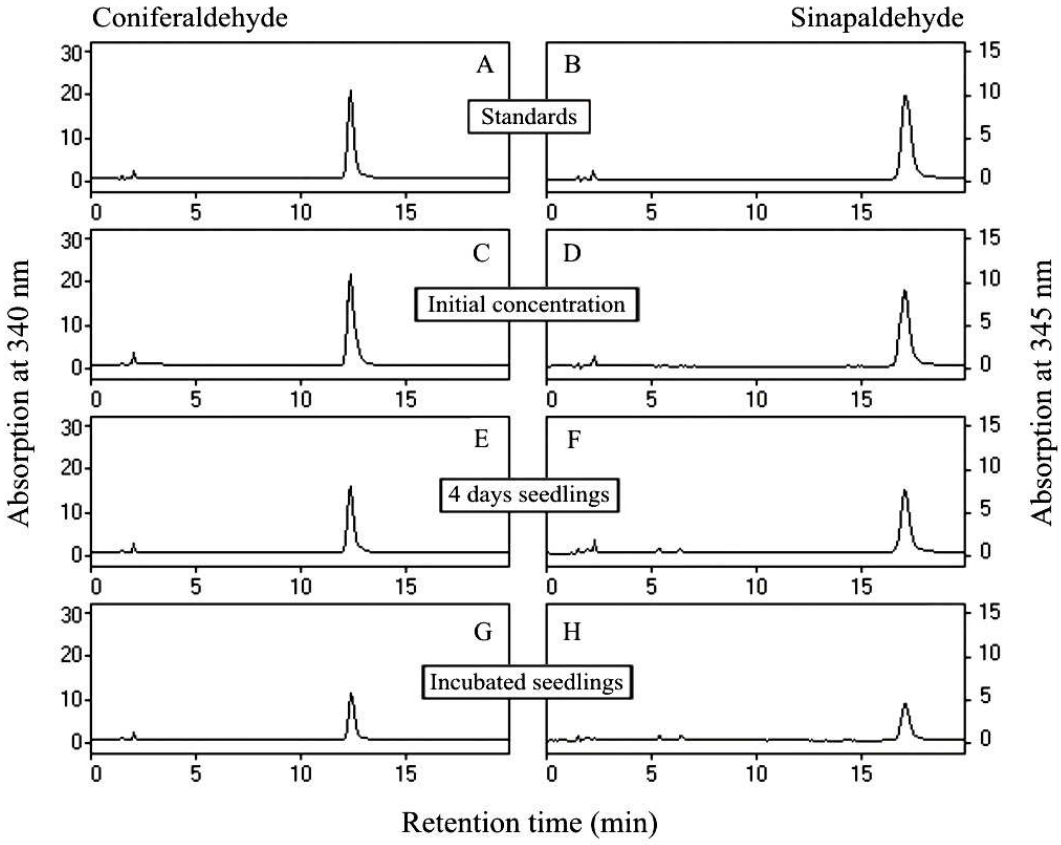

Fig. 3 - HPLC profiles of coniferaldehyde (left) and sinapaldehyde (right) in the reaction mixture to measure CAD activity. Coniferaldehyde (A) and sinapaldehyde (B) standards (50 nmol) dissolved in Tris-HCL buffer; initial concentration of coniferaldehyde (C) or sinapaldehyde (D) substrates in the reaction mixture; roots of seedlings grown after 4 days of seed germination (E, F) and roots of seedlings grown after 3 days of germination, followed by incubation in nutrient solution during 24 h (G, H).

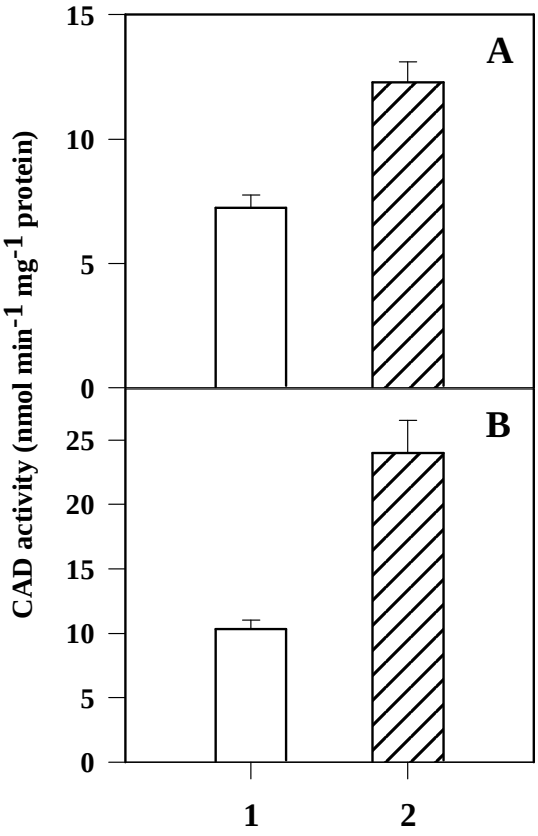

Fig. 4 - CAD activity in soybean roots. Coniferaldehyde (A) and sinapaldehyde (B) as enzyme substrates. Roots of seedlings grown after 4 days of seed germination (1) and roots of seedlings grown after 3 days of germination followed by incubation in nutrient solution during 24 h (2). Values shown are mean ± S.E. (n = 4).

Soybean root lignification induced by ferulic acid. The possible mode of action

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Soybean root lignification induced by ferulic acid: The possible mode of action

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Abstract – Ferulic acid, in the form of feruloyl CoA, occupies a central position as an intermediate in the phenylpropanoid pathway. Due to the allelopathic function, its effects were tested on root growth, lignin content and activities of cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) and peroxidase (POD, EC 1.11.1.7) from soybean (*Glycine max* (L.) Merr.) root seedlings. Three-day-old seedlings were cultivated in half-strength Hoagland and Arnon nutrient solution (pH 6.0), with or without 1.0 mM ferulic acid in a growth chamber (25°C, 12/12 h light/dark photoperiod, irradiance of 280 μmol m^{-2} s^{-1}) for 24 or 48 hr. Exogenously supplied ferulic acid induced premature cessation of the root growth, with disintegration of the root cap, compression of cells in the quiescent center, increase of the vascular cylinder diameter, and earlier lignification of the metaxylem. Moreover, the allelochemical decreased CAD activity and increased the anionic isoform PODa5 activity and lignin content. When applied jointly with PIP (an inhibitor of the cinnamate 4-hydroxylase, C4H), ferulic acid increased lignin content. By constrast, the application of MDCA (an inhibitor of the 4-coumarate:CoA ligase, 4CL) with ferulic acid did not affect lignin content. Taken together, these results suggest that ferulic acid may be channelled into the phenylpropanoid pathway (by the 4CL reaction) and, further, may increase the lignin monomers by solidifying the cell wall and restricting the root growth.

Key Words – Allelopathy, cinamil alcool dehydrogenase, lignin, phenylpropanoid pathway, root growth, soybean.

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INTRODUCTION

The phenylpropanoid pathway is one of the most important metabolic pathways since it is involved in the synthesis of phenolic compounds and a wide range of secondary products in plants, including lignin. The first rate-limiting enzyme of this pathway is phenylalanine ammonia-lyase (PAL), which in association with cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL) and cinnamoyl-CoA reductase (CCR) among others, leads to the synthesis of *p*-coumaral-, coniferal- and sinapaldehydes. These metabolites are converted into their corresponding alcohols by cinnamyl alcohol dehydrogenase (CAD). In the last step of the pathway, peroxidase (POD) catalyzes the monolignol polymerization leading to the lignin synthesis. Thus, lignin is a complex heteropolymer of hydroxylated and methoxylated phenylpropane units, derived from the oxidative polymerization of different hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl and sinapyl) connected by labile ether bonds and/or resistant carbon-carbon linkages (Boerjan et al., 2003; Passardi et al., 2005). As the main structural component of secondarily thickened plant cell walls, lignin contributes to the compression strength of stems imparting mechanical support and to the efficient conduction of water and solutes over long distances within the vascular systems (Donaldson, 2001).

In general, cell walls are known to become lignified when cell expansion decreases, either when the cell is under stress or when it differentiates to a particular specialization, notably the xylem (Christensen et al. 1998). Ferulic acid, a cinnamic acid derivative, is an abundant allelochemical frequently mentioned in the literature. Stress of plant roots from ferulic acid reduces water utilization, inhibits foliar expansion and root elongation, reduces rates of photosynthesis and inhibits nutrient uptake. At the cellular level, the allelochemical induces lipid peroxidation, affects certain enzymatic activities and rapidly depolarizes the root cell membrane causing a generalized increase in the membrane permeability, blocking plant nutrient uptake (Weir et al., 2004). At the same time, ferulic acid may be esterified with cell wall polysaccharides, incorporated into the lignin structure or form bridges that connect lignin with wall polysaccharides, rigidifying the cell walls and restricting cell growth (Iiyama et al., 1990; Sánchez et al., 1996; Lam et al., 2001).

More recently, dos Santos et al. (2004) reported that ferulic acid reduction of soybean growth might be due to premature lignification of root tissues associated with increases in enzyme activities of the phenylpropanoid pathway, such as phenylalanine ammonia lyase (PAL) and

peroxidase (POD). The aim of the present report was to investigate how the action mode of ferulic acid is related to the lignification process. For this, light and electron microscopy studies and determinations of CAD and POD activities, and lignin content were carried out after treatment of soybean roots with ferulic acid and inhibitors of the phenylpropanoid pathway.

METHODS AND MATERIALS

General Procedures. Soybean (*Glycine max* L. Merrill) seeds, surface-sterilized with 2% sodium hypochlorite for 5 min and rinsed extensively with deionized water, were dark-germinated (at 25°C) on two sheets of moistened filter paper. Twenty-five 3-day-old seedlings of uniform size were supported on an adjustable acrylic plate and transferred into a glass container (10 × 16 cm) filled with 200 ml of half-strength Hoagland's solution (pH 6.0), with or without 1.0 mM ferulic acid. Additional experiments with 0.1 mM of piperonylic acid (PIP) or 2.0 mM of 3,4-(methylenedioxy) cinnamic acid (MDCA) were made as indicated in the figure legends. The container was kept in a growth chamber (25°C, 12/12 h light/dark photoperiod, irradiance of 280 μmol m^{-2} s^{-1}). Roots were measured at the beginning and at the end of experiments (24 or 48 hr). When indicated, the fresh root weight was determined immediately after incubation and the dry weight was estimated after oven-drying at 80°C until it reached a constant weight. CAD and POD activities and lignin contents were determined after the incubation period of 24 hr, while light and electron microscopy analyzes were carried out after 48 hr of incubation. Ferulic acid, PIP and MDCA were purchased from Sigma-Aldrich Chemical Co (St Louis, USA), and all other reagents used were of the purest grade available or chromatographic grade.

Light Microscopy Studies. Samples of material for morphological and anatomical studies were fixed in F. A. A. 50 (Johansen, 1940). The material was conserved in ethanol 70% (Jensen 1962). The anatomical description was made from the analysis of permanent slides obtained of longitudinal and transversal sections of the roots. In the preparation of permanent slides the plant material was embedded in glycol methacrylate according to the technique described by Gerrits (1991). These slides were stained with Toluidine Blue O (O'Brien et al., 1964) and mounted in Permount. Micrographs were photographed using an Olympus® photomicroscope. Scales were calculated using a decimal ruler and a micrometer under the same optical conditions used for each case.

Electron Microscopy Studies. For scanning electron microscopy, fresh root segments were fixed

in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. The tissues were postfixed in a solution containing 1% osmium tetroxide, 0.8% potassium ferrocyanide, and 5 mM calcium chloride in 0.1 M cacodylate buffer, and then in a 1% tannic acid solution. Further, the samples were dehydrated in graded ethanol solutions, critical-point-dried in CO_2, sputter-coated with gold, and examined on a Jeol-JSM-5310® field emission scanning electron microscope. For transmission electron microscopy, fresh root segments were washed in 0.01 M phosphate-buffered saline and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The tissues were postfixed in a solution containing 1% osmium tetroxide and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer, washed in the same buffer, dehydrated in acetone, and embedded in Spurr. Thin sections were stained with uranyl acetate and lead citrate, and examined in a Zeiss 900® transmission electron microscope.

Cinnamyl Alcohol Dehydrogenase Assay. CAD was extracted from fresh roots (2 g) with 3 ml of an extraction medium containing 40 mM of β-mercaptoethanol and 100 mM potassium phosphate buffer (pH 7.3). The homogenate was centrifuged at 2200 × *g* for 15 min, and the supernatant was used as enzyme preparation (dos Santos et al., 2006). CAD was assayed chromatographically by the reaction of reduction of sinapaldehyde to sinapyl alcohol. The assay was carried out, at 30 °C, in 1.0 ml of reaction mixture containing 200 µl of crude enzyme preparation (≤0.35 mg of protein), 104 nmol NADPH and 150 nmol Tris-HCl buffer (pH 8.0). Fifty nmol of sinapaldehyde was added at the start and the reaction was stopped after 3 min of incubation by adding 50 µl of 5 N HCl. Parallel controls with sinapaldehyde added in the reaction mixture (without NADPH) were made. All samples were filtered through a 0.45 µm disposable syringe filter (Hamilton® Co., Nevada, USA) and analyzed (20 µl) with a Shimadzu® Liquid Chromatograph (Tokyo, Japan) equipped with an LC-10AD pump, a Rheodine® injector, an SPD-10A UV detector, a CBM-101 Communications Bus Module, and a Class-CR10 workstation system. A reversed-phase Shimpack® GLC-ODS (M) column (150 × 4.6 mm, 5 µm) was used at room temperature together with the same type of pre-column (10 × 4.6 mm). The mobile phase was methanol/acetic acid 4% in water (20/80, v/v) with a flow rate of 1.2 ml min^{-1} for an isocratic run of 20 min. Absorption was detected at 345 nm. Data collection and integration were performed with Class-CR10 software (Shimadzu®, Tokyo, Japan). Sinapyl alcohol was identified by comparing its retention time with standard values. CAD activity was expressed as nmol sinapaldehyde consumed min^{-1} mg^{-1} protein. Protein was determined spectrophotometrically at 595 nm (Bradford, 1976), with bovine serum albumin as a standard.

Peroxidase Assay. Root (0.8 g) was homogenized in Eppendorf tubes containing 0.12 ml of cold extraction medium containing 0.886 M sodium potassium buffer pH 7.0, 1.0 mM EDTA, 1.0 mM sodium metabisulfite, 9.96 mM sodium borate, 5% PVP-40 (polyvinylpyrrolidone), 0.5% β-mercaptoethanol, 10% glycerol, 2% ascorbic acid, and 4% PEG (Pereira et al, 2001). The homogenates were centrifuged (21900 × *g*, 30 min, 4°C) and the supernatant was used as soluble POD extract. For cell wall-bound POD isolation, fresh roots (5 g) were macerated with 67 mM phosphate buffer (50 ml, pH 7.0) containing 0.5 g PVP (dos Santos et al., 2004). The extract was centrifuged (2200 × *g*, 5 min, 4 °C). The pellet was washed with deionized water until no soluble POD activity was detected in the supernatant. The pellet was then incubated in 10 ml of 1 M NaCl (prepared in 50 mM phosphate buffer, pH 7.0) for 1 hr. The homogenate was centrifuged (2200 × *g*, 5 min, 4 °C) and the supernatant obtained. Cold acetone (30 ml) was slowly added in the supernatant, under constant stirring. After centrifugation (10000 × *g*, 30 min, 4 ºC), the pellet was resuspended with 0.15 ml of 1M NaCl and considered as cell wall-(ionically)-bound POD.

For starch electrophoresis, the enzyme extracts were absorbed onto Whatman No. 3 paper strips (0.5 cm^2), which were inserted into 16% cornstarch (Penetrose-50) gels prepared in 9.0 mM Tris/3.0 mM citric acid, pH 8.3. Electrophoresis was performed for 15 hr at 4 ºC and 60 V. For polyacrylamide gel electrophoresis (PAGE), samples (50 µl) of the enzyme extract were applied in 12% gel prepared in 0.375 M Tris–HCl pH 8.8 buffer. Electrophoresis was performed for 5 hr at 200 V. The running buffer used was 0.1 M Tris-glycine pH 8.3 (Pereira et al., 2001). Both gels were incubated for 15 min in an appropriate staining solution (50 ml of 1 M sodium citrate, pH 4.7; 50 ml of methanol and 0.05 g of benzidine). The POD activities in both the gels were detected by adding 5 ml of 30% H_2O_2 (Mangolin et al., 1994).

Lignin Quantification. After the incubation period, dry roots (0.3 g) were homogenized in 50 mM potassium phosphate buffer (7 ml, pH 7.0) with a mortar and pestle and transferred into a centrifuge tube (Ferrarese et al., 2002). The pellet was centrifuged (1400 × *g*, 4 min) and washed by successive stirring and centrifugation as follows: twice with phosphate buffer pH 7.0 (7 ml); 3 × with 1% (v/v) Triton® X-100 in pH 7.0 buffer (7 ml); 2 × with 1 M NaCl in pH 7.0 buffer (7 ml); 2 × with distilled water (7 ml); and 2 × with acetone (5 ml). The pellet was dried in an oven (60°C, 24 hr) and cooled down in a vacuum desiccator. The dry matter obtained was defined as a protein-free cell wall fraction. Further, all dry protein-free tissue was placed into a screw-cap centrifuge tube containing the reaction mixture (1.2 ml of thioglycolic acid plus 6 ml of 2 M HCl) and heated (95ºC, 4 hr). After cooling at room temperature, the sample was centrifuged

(1400 × *g*, 5 min) and the supernatant was discarded. The pellet contained the complex lignin-thioglycolic acid (LTGA). The pellet was washed 3 × with distilled water (7 ml) and the LTGA extracted by shaking (30ºC, 18 hr, 115 oscillations min^{-1}) in 0.5 M NaOH (6 ml). After centrifugation (1400 × *g*, 5 min), the supernatant was stored. The pellet was washed again with 0.5 M NaOH (3 ml) and mixed with the supernatant obtained earlier. The combined alkali extracts were acidified with concentrated HCl (1.8 ml). After precipitation (0°C, 4 hr), LTGA was recovered by centrifugation (1400 × *g*, 5 min) and washed 2 × with distilled water (7 ml). The pellet was dried at 60°C, dissolved in 0.5 M NaOH, and diluted to yield an appropriate absorbance for spectrophotometric determination at 280 nm. Lignin was expressed as mg LTGA g^{-1} dry weight.

Statistical Design. The experimental design was completely randomized and each plot was represented by one glass container with 25 seedlings. Data are expressed as the mean of four to five independent experiments ± S.E. The one-way variance analysis to test the significance of the observed differences was performed by Sisvar® package (Version 4.6, UFLA, Brazil). The difference among parameters was evaluated by the Scott–Knott test and *P* values <0.05 were considered as statistically significant.

RESULTS

Effects of ferulic acid on root growth. As may be judged visually, differences in the roots were apparent (Fig. 1). Primary root elongation of treated seedlings was inhibited by the compound. Roots became brown, thicker and less flexible. After 24 and 48 hr, the allelochemical prompted significant decreases in the root lengths when compared to the control conditions (Table 1).

Light and electron microscopy analyzes. Subsequent experiments were carried out to evaluate anatomical changes in roots by light microscopy (Table 1). The effects of ferulic acid on diameter (root and vascular cylinder) measures of medial and basal sections were examined after root treatments. After 24 hr, the vascular cylinder diameters increased in both medial (40.9%) and basal (14.3%) sections in comparison to controls. Significant effects of ferulic acid were evident at 48 hr; the root diameter decreased (11.3%) while the vascular cylinder diameter increased (19%) in the medial section. Cross sections of the medial region in untreated roots showed a normal pattern of growth (Fig. 2A). However, in ferulic acid-treated roots the metaxylem showed lignification (Fig. 2D). In addition, longitudinal sections of the transition

zone in treated roots indicated initiation of lateral roots and lignification of the primary xylem (Fig. 2B, E). Similar sections revealed a smaller length between the quiescent center and the differentiated cells in ferulic acid-treated roots when compared to the untreated roots (Fig. 2C, F).

Transmission electron microscopy observations of ferulic acid-treated root caps showed a reduced number of starch granules in comparison to control (Fig. 3A, D). Micrographs of the quiescent center in treated roots revealed that the cells were smaller and compressed, and contained irregular and enlarged nucleoli and many lipid globules in comparison to the untreated roots (Fig. 3B, E). Finally, scanning electron microscopy photomicrographs showed disintegration of the root cap in the ferulic-acid treated roots in comparison to control (Fig. 3C, F).

Effects of ferulic acid on CAD and POD activities. Ferulic acid-affected CAD activity was significantly different from control (Fig. 4). The allelochemical decreased the enzymatic activity by 42.8% at 1.0 mM treatment. In addition to this earlier finding, electrophoretic patterns for soluble and cell-wall bound POD isozymes were determined in soybean roots after allelochemical treatment (Fig. 5). Zimograms of starch gel electrophoresis of soluble POD in ferulic acid-treated roots revealed that only the anionic form PODa5 increased in comparison to the control condition (Fig. 5A). PAGE zimograms confirmed this increase (Fig. 5B). Starch gel electrophoresis did not reveal changes in cell wall-bound POD isozymes of ferulic acid-treated roots in comparison to control. Similar behavior has been noted in PAGE isozymes.

Effects of ferulic acid on lignin content. Lignin content in soybean roots increased following 1.0 mM ferulic acid treatment by about 46% at 24 hr (Fig. 6, 7). Fig. 6 reveals that PIP, a potent quasi-irreversible inhibitor of cinnamate 4-hydroxylase (C4H), reduced the lignin content of soybean roots 37.6% in comparison to the control. Similar to ferulic acid, the treatment of roots with allelochemical plus PIP increased the lignin content 26.5% when compared to the control condition. Experiments with MDCA, a competitive inhibitor of 4-coumarate:CoA ligase (4CL), may be seen in Fig. 7. Lignin content was not affected by MDCA, alone or jointly with ferulic acid (FA plus MDCA), in comparison with the control experiment.

DISCUSSION

Root growth is characterized by high metabolic rates, and at this time roots are highly susceptible to environmental stresses, such as allelochemicals present in soils (Cruz-Ortega et al., 1998). The experimental condition used in this work was chosen because the net uptake of ferulic acid by the root system is high (Shann and Blum, 1987a), and lignification begins during the early stages of seedling growth (dos Santos et al., 2004; Passardi et al., 2005). At the final stages of xylem cell differentiation, lignin is deposited within the carbohydrate matrix of the cell wall by infilling of interlamellar voids and, at the same time, by the formation of chemical bonds with the non-cellulosic carbohydrates (Donaldson, 2001). In this sense, an important fact revealed here is that the inhibition of soybean root growth (Table 1) under 1.0 mM ferulic acid treatment had been associated with an increase in the lignin contents (Fig. 6, 7).

Structural changes of the root cells have been associated with root growth inhibition induced by stress. The increase in the root diameter, especially of vessel cylinder, may be attributed to the early differentiation of vessel tissues verified by the lignification of metaxylem. As reported earlier, cell walls may be lignified when stressed or when they differentiate to xylem (Christensen et al., 1998). Anatomical observations showed that the diameter of the vascular cylinder in the medial and basal regions was enhanced after 24 and 48 hr of treatments, compared with control (Table 1). Treated roots showed induction of lateral roots with lignification of the metaxylem (Fig. 2D) and an increase of the central cylinder (Fig. 2E), which was accompanied by an increase in the lignin production (Fig. 6, 7). Furthermore, a smaller distance between the quiescent center and the first differentiated cells together with clustered cells in the longitudinal section of treated roots (Fig. 2F) indicate premature cessation of the root growth. There is evidence corroborating these results. For example, Jankay and Muller (1976) demonstrated that umbelliferone, an allelochemical found in grasses, reduced cell elongation and increased radial cell expansion in cucumber (*Cucumis sativus*) roots. Coumarin-treated alfalfa (*Medicago sativa*) roots revealed a significant increase in its diameter due to an expanding of the vascular cylinder and cortex cell layers. Moreover, this allelochemical inhibited root elongation and cell division, indicating that the thickness of roots was enlarged due inhibition of the root longitudinal growth. The water-soluble extract of alfalfa leaves, which contain mainly ferulic acid among other allelochemicals, showed similar behavior (Chon et al., 2002).

At the ultrastructural level, treatment with ferulic acid adversely affected soybean root cells.

Electron micrographs showed a clear reduction in the number of starch granules (Fig. 3D), an increase in lipid globules, and compression of cells with enlarged nucleoli (Fig. 3E) in ferulic-acid-treated roots. Relevant disintegration of root cap in treated roots (Fig. 3F) suggests death of cells after direct contact with the allelochemical. In simplest terms, ferulic acid inhibited the root growth and led to cellular ultrastructural abnormalities. Some studies have shown that allelochemicals affect the cellular structure of growing roots. For example, white mustard (*Sinapis alba*) radicle exposed to gramine and hordenine showed vacuolation, disorganization of organelles and damage of the cells walls (Liu and Lovett, 1993). Burgos et al. (2004) reported that 2-benzoxazolinone (BOA), an allelopathic hydrocinnamic acid present in cereals, caused significant changes in the root cell ultrastructure of cucumber. BOA increased cytoplasmatic vacuolation and reduced the number of mitochondria, ribosomes and starch granules. For these authors, inhibition of the root growth was due to reduced lipid catabolism, protein synthesis and DNA modifications. Benzoic acid inhibited root growth of mustard (*Brassica juncea*) seedlings and caused damage at the cellular level; cells were disorganized, distorted and deformed due to adverse effects of this allelochemical on cell division, mineral uptake, water balance or respiration (Kaur et al., 2005). In this aspect, it is well known that ferulic acid inhibits protein synthesis, decreases stomatal conductance, affects membrane permeability and decreases uptake of nutrients, such as phosphate, potassium, nitrate and ammonium (Weir et al., 2004). The utilization of the energy necessary for cells to grow and multiply in response to ferulic acid has been affected in plants. Ferulic acid reduces lipid mobilization followed by accumulation of unsaturated fatty acids in canola (*Brassica napus*) seeds during germination (Baleroni et al., 2000). It also increases the contents of saturated and unsaturated fatty acids of the polar and non-polar lipid fractions and xylose, fructose and sucrose in soybean root (Ferrarese et al., 2001). Thus, cellular structure changes reported here appear to be, at least partially, associated with changes in the lipid and carbohydrate metabolism (*see also* Ho, 1988).

This is evidence that the effects of ferulic acid on soybean are related to a premature lignification of roots since enzyme (PAL and POD) activities of the phenylpropanoid pathway increased jointly with lignin production (dos Santos et al., 2004). Independently of the phenylpropanoid metabolism, a direct incorporation of exogenous ferulic acid into lignin structure was initially hypothesized in this work. This possibility might not be discarded because there is evidence in this sense. The radiotracer [U-ring-^{14}C]ferulic acid] was found in residues of lignin isolated from cucumber seedlings treated with allelochemical (Shann and Blum, 1987b).

In robinia (*Robinia pseudoacacia*), labeled ferulic acid was incorporated into guaiacyl (G) and syringyl (S) lignin, and these incorporations increased as cell-wall lignification proceeded (Yamauchi and Fukushima, 2004). To strengthen the assumption that exogenous ferulic acid induces the lignification process, CAD activity was determined in treated soybean roots. An interesting verified fact is that CAD activity decreases in ferulic acid-treated roots (Fig. 4) in spite of the lignin production (Fig. 6, 7). Increase in the CAD activity would be anticipated, since it is considered to be a lignification marker (Boerjan et al., 2003). Increased lignification under reduced CAD activity might, at least in principle, strengthen the hypothesis of direct incorporation of exogenous ferulic acid into lignin polymer. However, plants are able to circumvent the block in CAD activity by shipping its substrates, the cinnamaldehydes, to the cell wall for polymerization (Boerjan et al., 2003). This indicates that the impact of CAD on lignin biosynthesis may not be critical. Cross-coupling of hydroxycinnamyl aldehydes into lignin compensates the reduced availability of monolignols in CAD-deficient plants (Kim et al., 2000; Li et al., 2001).

Another fact is that ferulic acid increased the activity of anionic isoform PODa5 in treated roots (Fig. 5). Although the existence of isoperoxidases specific to lignification has not been conclusively proved, there are arguments that POD participates in lignin polymerization. It is able to dehydrogenate monolignols, to induce lignification after addition of H_2O_2 in tissue sections, and to reveal specific co-localization of isoforms in lignifying tissues (Ros Barceló et al., 2004; Passardi et al., 2005). Anionic isoperoxidases are often held to be those most directly involved in lignification of xylem cells (Wallace and Fry, 1999; Passardi et al., 2005). In agreement, the anionic peroxidase gene *swpa4*, which is not expressed in any tissue of sweet potato (*Ipomoea batatas*) during normal growth, was up-regulated in leaves after incubation with H_2O_2 or NaCl (Park et al., 2003). This suggests that gene *swpa4* may be involved in several responses, including biotic or abiotic stresses. So, it is feasible that these facts may, in part, explain the increase in anionic isoform POD in ferulic acid-treated roots (Fig. 5).

Hamada et al. (2003) demonstrated that the exogenously supplied ferulic acid was converted to feruloyl and then to coniferyl and sinapyl alcohols, in poplar (*Populus alba*) callus. Since feruloyl CoA is an intermediate of phenylpropanoid metabolism (Fig. 8), a possible entry of free ferulic acid into the pathway, by the 4CL reaction, must be considered. To elucidate, subsequent experiments were made by growing roots with two inhibitors of the pathway enzymes: PIP, a quasi-irreversible inhibitor of cinnamate 4-hydroxylase (C4H) and MDCA, a competitive

inhibitor 4-coumarate:CoA ligase (4CL). Fig. 6 shows that roots grown under ferulic acid treatment produce more lignin while PIP-treated roots synthesize less lignin, in comparison to standard conditions. This is in agreement with the fact that PIP is an effective inhibitor of C4H, by acting before the entry point of ferulic acid in the pathway (Schoch et al., 2002). When applied jointly with PIP, ferulic acid prompted an increase in lignin content, suggesting its entrance into pathway by the 4CL reaction. To strengthen this assumption, roots were incubated with MDCA, an inhibitor of 4CL (Schoch et al., 2002). Lignin content did not change after MDCA or MDCA plus ferulic acid treatments in comparison to controls (Fig. 7), indicating that the access of exogenous allelochemical has been blocked at this metabolic point. Taken together, these results (Fig. 6, 7) suggest that ferulic acid has been channelled into the phenylpropanoid pathway and, later on, increased the lignin production.

The main interest of the present work was to investigate how the mode of action of ferulic acid is related to the lignification process. Light and electron microscopy studies, combined with biochemical assays, suggest a possible mechanism of soybean response to ferulic acid (Fig. 8). Exogenously applied ferulic acid induces premature cessation of the soybean root growth, with disintegration of the root cap, and cellular modifications, such as compression of cells in the quiescent center, early lignification of the metaxylem and increase of the vascular cylinder diameter. At metabolic level, ferulic acid is channelled into the phenylpropanoid pathway and converted to feruloyl CoA by the 4CL reaction. Catalyzed by subsequent enzymatic reactions, feruloyl CoA is then converted to coniferal- and sinapaldehydes. As an endwise enzyme of the pathway, CAD might be not a limiting step. So, these metabolites are converted in the respective alcohols by CAD or, eventually circumvent the inhibited CAD reaction (Kim et al., 2000; Li et al., 2001, Boerjan, 2003) by polymerizing toward lignin in the cell wall. Lignin polymerization requires a sufficient supply of H_2O_2, which is produced by the pH-dependent POD and NADPH oxidase complex, after changes in the membrane permeability (Baziramakenga et al., 1995; Wojtaszek, 1997). Whether this is the true way, it seems quite plausible to assume that ferulic acid-induced inhibition in root growth of the soybean may be due to excessive production of monolignol from exogenously applied ferulic acid. Monolignol polymerization forms a complex network that solidifies the plant cell wall and restricts plant growth.

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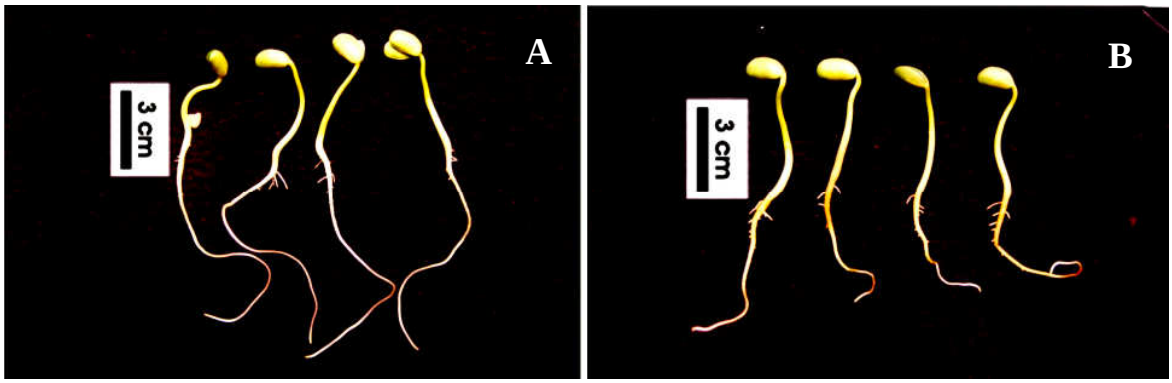

Fig. 1. Effects of ferulic acid on soybean root length. Control (A) and treated (B) roots with 1.0 mM ferulic acid after 48 hr.

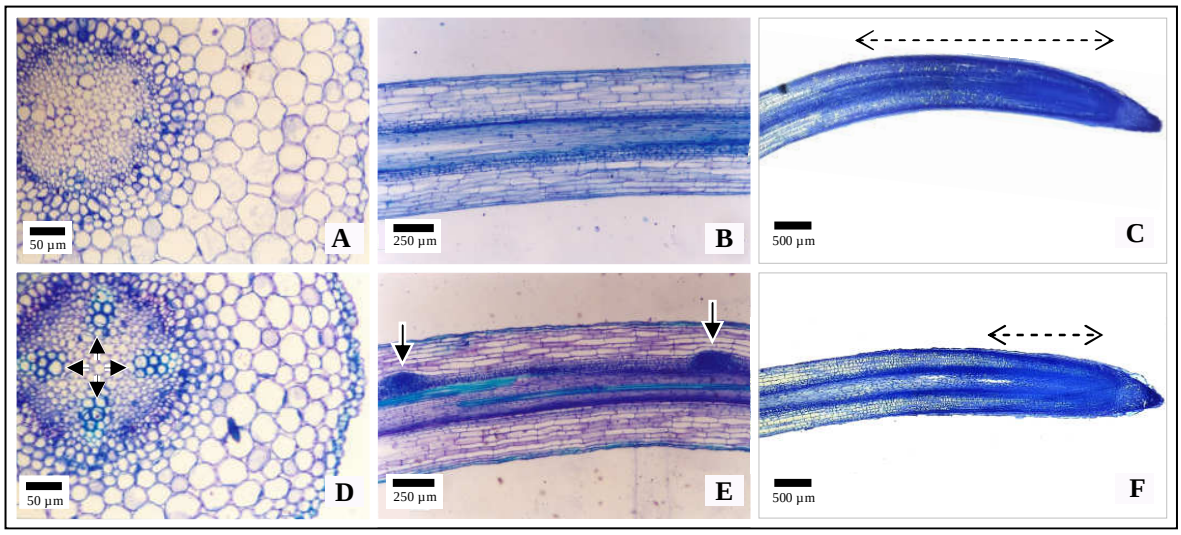

Fig. 2. Light microscopy photomicrographs of control (A, B, C) and treated (D, E, F) soybean roots with 1.0 mM ferulic acid for 48 hr. A and D: cross sections of the medial zone (arrows indicate lignification of metaxylem). B and E: longitudinal sections of the central cylinder in the basal region (arrows indicate induction of lateral roots and lignification). C and F: longitudinal sections (dotted lines indicate the distance between the quiescent center and first differentiated cells).

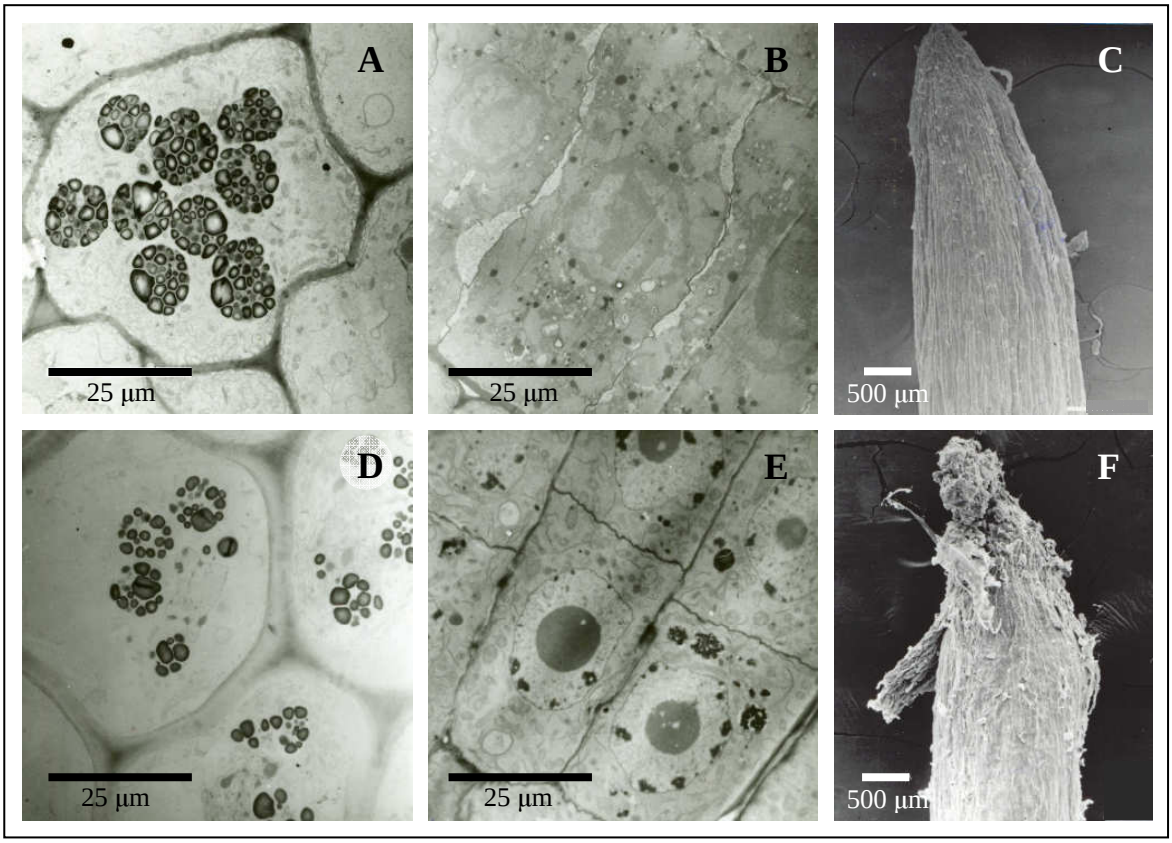

Fig. 3. Electron microscopy photomicrographs of control (A, B, C) and treated (D, E, F) soybean roots with 1.0 mM ferulic acid for 48 hr. A and D: transmission electron microscopy of root cap showing starch granules. B and E: transmission electron microscopy of quiescent center cells showing compression with enlarged nucleoli and lipid globules in treated roots. C and F: scanning electron microscopy of root cap showing epidermic tissue disintegrated in treated roots.

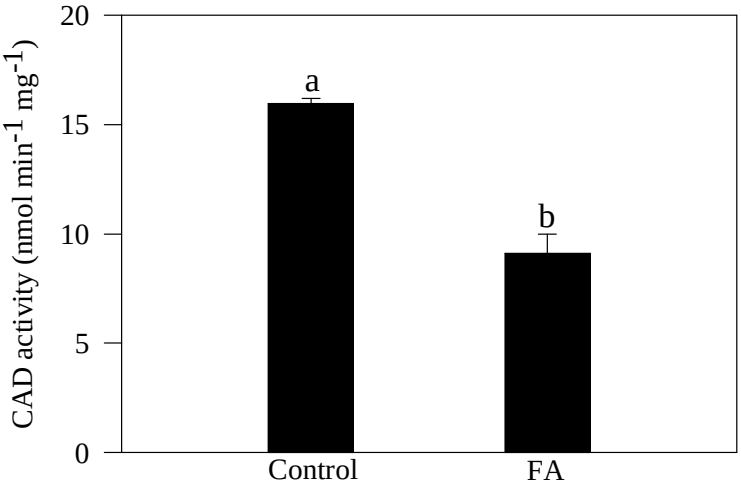

Fig. 4. Effects of ferulic acid (FA) on CAD activity in soybean roots. Mean ± SE values (N = 4) followed by the different letter are significantly different according to the Scott–Knott test ($P \leq 0.05$).

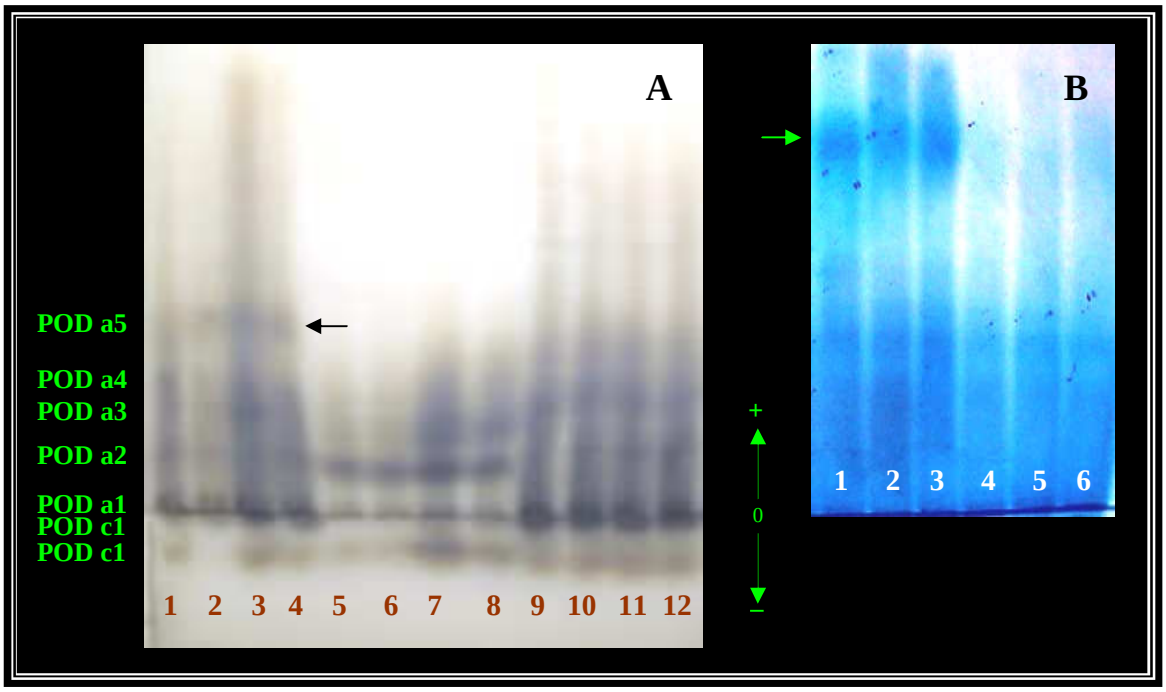

Fig. 5. Isozyme patterns of POD in soybean roots. A, starch gel: 1 to 4, treated roots showing increase of the anionic isoform of soluble enzyme (PODa5) in comparison to control (9 to 12); 5 and 6, cell wall-(ionically)-bound POD isozymes of treated roots and their corresponding controls (7 and 8). B, polyacrylamide gel: 1 to 3, treated roots confirming the increase of PODa5 in comparison to control (4 to 6). POD activity was stained using H_2O_2 as the substrate.

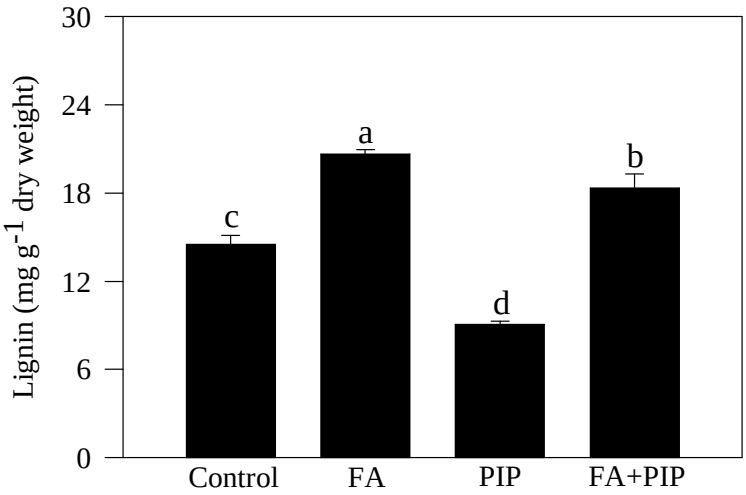

Fig. 6. Changes in the lignin contents of soybean roots untreated (Control) or treated with 1.0 mM ferulic acid (FA), 0.1 mM piperonylic acid (PIP) and 1.0 mM ferulic acid plus 0.1 mM piperonylic acid (FA+PIP) for 24 hr. Mean ± SE valus (N = 5) followed by different letters are significantly different according to the Scott–Knott test ($P < 0.05$).

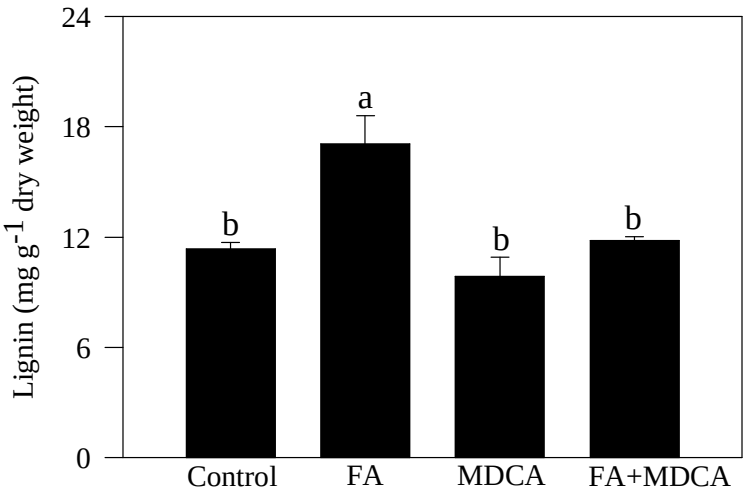

Fig. 7. Changes in the lignin contents of soybean roots untreated (Control) or treated with 1.0 mM ferulic acid (FA), 2.0 mM methylene dioxocinnamic acid (MDCA) and 1.0 mM ferulic acid plus 2.0 mM methylene dioxocinnamic acid (FA+MDCA) for 24 hr. Mean ± SE values (N = 5) followed by same letter are not significantly different according to the Scott–Knott test (P < 0.05).

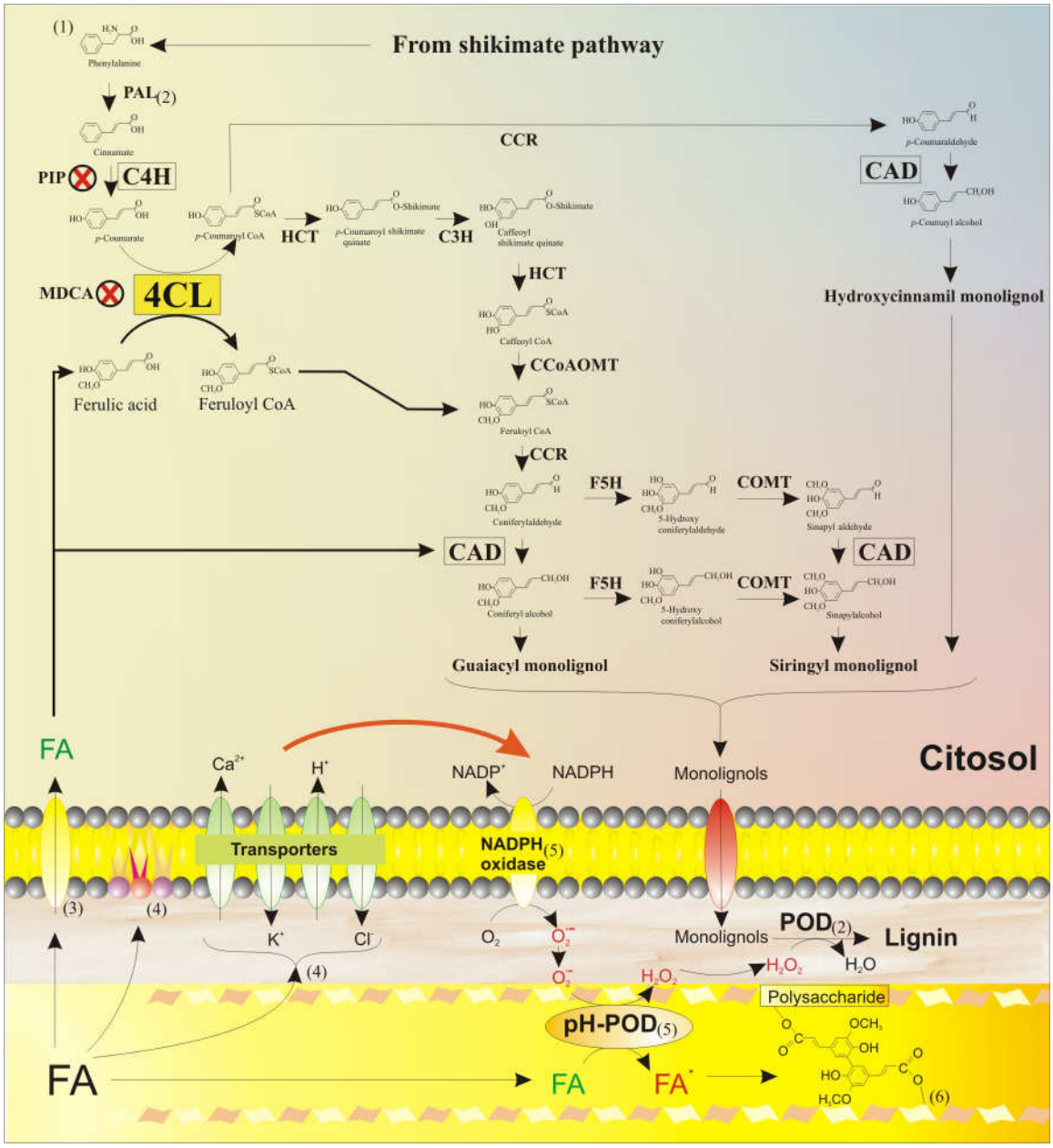

Fig. 8. Proposed mode of action for ferulic acid on lignification of soybean roots. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxilase; 4CL, 4-coumarate:CoA ligase; HCT, hydroxycinnamoyl-CoA: quinate/shikimate hydroxycinnamoyltransferase; C3H, *p*-coumarate 3-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid/5-hydroxy ferulic acid *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; peroxidase (POD); ferulic acid (FA); piperonylic acid (PIP); 3,4-(methylenedioxy) cinnamic acid (MDCA). (1), Chen et al. (2006); (2), dos Santos et al. (2004); (3), Shann and Blum (1987a); (4); Baziramakenga et al. (1995); (5), Wojtaszek (1997); (6), Boerjan et al. (2003).

TABLE 1. ANATOMICAL CHARACTERISTICS OF FERULIC ACID-TREATED SOYBEAN ROOTS AFTER 24 AND 48 HR.

			Medial Region		Basal Region	
		Root length (cm)	Root diameter (µm)	Cylinder diameter (µm)	Root diameter (µm)	Cylinder diameter (µm)
24 hr	C	3.3 ± 0.21	5.8 µm ± 0,37	2.2 µm ± 0.07	7.7 µm ± 0.50	2.8 µm ± 0.10
	T	2.2 ± 0.09*	7.5 µm ± 0.59^{ns}	3.1 µm ± 0.06*	8.2 µm ± 0.22^{ns}	3.2 µm ± 0.09*
	%	-33.3	ns	+40.9	ns	+14.3
48 hr	C	7.0 ± 0.14	7.1 µm ± 0.05	2.1 µm ± 0.02	8.0 µm ± 0.32	3.0 µm ± 0.11
	T	2.7 ± 0.16*	6.3 µm ± 0.44*	2.5 µm ± 0.07*	7.2 µm ± 0.26^{ns}	2.9 µm ± 0.11^{ns}
	%	-61.4	-11.3	+19.0	ns	ns

Note. Means (N = 4 ± SE) significantly ($P \leq 0.05$) smaller than the control experiment (Scott-Knott´s test) are marked *. *Abbreviations:* C: control; T: treatment; ns: not significant. The symbol % represents inhibition (–) or activation (+) of statistically significant means after treatment in comparison to control.

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