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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Alterações no Conteúdo e nas Propriedades Antioxidantes de

Compostos Fenólicos Isolados de Folhas Galhadas de

***Rollinia laurifolia* Schdtl. (ANNONACEA):**

Correlação com o Dimorfismo Sexual do Coccideo Indutor da Galha

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"Energy is eternal delight"

William Blake

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que com amor dedicou-se ao filho

de uma maneira marcante e inspiradora...

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ABREVIATURAS

EAO   Espécies ativas de oxigênio

C6   Composto de 6 carbonos

C6-C3   Composto de 6 carbonos + 3 carbonos

C6-C3-C6   Composto de 6 carbonos + 3 carbonos + 6 carbonos

D. C.   Depois de Cristo

HCAs   Ácidos hidroxicinâmicos

H_2O_2 Peróxido de Hidrogênio

m. a.   Milhões de anos atrás

NADH   Nicotinamida dinucleotídeo reduzida

NADPH   Nicotinamida dinucleotídeo fosfato reduzida

OH   Grupamento hidroxil

$O_2^{\bullet-}$ Radical Superóxido

PPO's   Polifenol oxidases

SOD   Superóxido dismutase

UV   Ultra-violeta

UV-A   Ultra-violeta A

UV-B   Ultra-violeta B

RESUMO

As galhas ou cecídeas, são caracterizadas por um crescimento anormal do tecido a estímulos específicos de um organismo invasor, que pode ser: bactéria, fungo, nematódeo ou artrópodo. A interação organismo invasor/planta hospedeira altera os estados químicos e fisiológicos dos tecidos da planta durante a cecidogênese, e provavelmente do organismo indutor. Vários tipos de estímulos bióticos e abióticos são conhecidos como potenciais alteradores da produção de compostos fenólicos nas plantas. Estes compostos estão envolvidos na defesa da planta contra pestes e patógenos. Uma espécie de coccídeo, ainda não identificada, induz galhas foliares em espécimes de *Rollinia laurifolia* Schdtl. (ANNONACEAE). Os coccídeos são caracterizados por um acentuado dimorfismo sexual, que também é expresso nas galhas que produzem. As galhas fêmeas são relativamente grandes, enquanto as galhas macho são menores e menos complexas. Os objetivos deste estudo foram verificar a relação ecológica/bioquímica entre inseto e planta averiguando possíveis diferenças entre folhas sadias e folhas galhadas, além também de verificá-las entre galhas fêmeas e galhas macho. Técnicas de determinação de fenóis totais, HPLC, TRAP e TBARS foram usadas neste estudo. Observou-se que a ocorrência de galhas nas folhas de *R. laurifolia* está relacionada à significativa redução no conteúdo fenólico total das mesmas. Qualitativamente também houve alteração no perfil fenólico das folhas galhadas em comparação com as folhas sadias. TRAP e TBARS também mostraram alterações no conteúdo fenólico das folhas galhadas. Alterações no conteúdo fenólico e na capacidade antioxidante relacionada ao sexo do galhador, também foram observadas. Nossos resultados sugerem que a

interação galhador/*R. laurifolia* altera o perfil fenólico das folhas, consequentemente é alterada também a capacidade antioxidante dos extratos de folhas com galhas macho e galhas fêmeas. Uma causa provável para estas mudanças seria a difusão de oxigênio para o tecido foliar e a oxidação de biomoléculas, disparando sinais para a produção de diferentes compostos fenólicos, em conseqüência do estresse biótico durante a cecidogênese, possivelmente em uma tentativa de a planta buscar o equilíbrio fisiológico com o galhador.

1. INTRODUÇÃO

1.1 Definição

Ao longo de toda história de vida das plantas e dos insetos, estes dois grupos de organismos têm evoluído muito proximamente. Entre ambos, existem relações múltiplas de todo tipo: trófica, reprodutiva, protetora...mas poucas relações como as galhas vegetais, representam uma interação tão elaborada e complexa do ponto de vista evolutivo na interação inseto-planta.

As galhas ou cecídias podem ser definidas como "estruturas anormais de partes dos tecidos ou órgãos da planta que se desenvolvem pela reação específica à presença ou atividade de um organismo indutor" (Meyer, 1987; Shorthouse e Rohfritsch, 1992). Sob a ação do agente indutor, a planta produz uma reação que inclui basicamente o desenvolvimento anormal ou patológico de suas células, tecidos ou órgãos. O organismo indutor utiliza a galha como um ambiente seguro fora do alcance de inimigos naturais, de variações ambientais e com nutrição especializada. Na natureza existem uma grande variedade de estruturas anormais de plantas que são produzidas por diversos animais e plantas, mas para serem consideradas galhas, estas estruturas devem ser ativamente produzidas pelas plantas como resultado de uma atividade anormal de crescimento. O caráter fundamental de uma galha, que o distingue de outras, é que a reação da planta perante o ataque do organismo estranho, inclui sem exceção fenômenos de hipertrofia (crescimento anormal das células) e hiperplasia (multiplicação anormal das células), associados ao processo de crescimento anormal (Meyer, 1987; Shorthouse e Rohfritsch, 1992).

1.2 Histórico

As galhas em vegetais têm chamado a atenção de observadores desde tempos antigos, intrigando-os pela sua presença e origem. Por outro lado, as galhas têm sido utilizadas pelo homem para diversos fins ao longo da história. O uso de certas galhas com fins médicos e curativos está documentado desde a antigüidade greco-romana por autores como Hipócrates, Plinio e Teofrasto, este último considerado o pai da Botânica (fig. 1), que elucidaram os seus usos farmacológicos no tratamento de diarréias, inflamações bucais e hemorróidas (Fernandes e Martins, 1985; Csóka, 1997). Foram encontrados restos de galhas atribuídos ao Cinipídio *Andricus kollari*, provavelmente a venda para uso médico, entre os produtos expostos em um mercado na cidade de Herculanum sepultada pela erupção do vulcão Vesúvio no ano 79 D.C. (Larew, 1987). Aproximadamente 2000 anos atrás, o célebre naturalista Caius Plinius Secundos (23-79 D.C) idealizou um teste qualitativo para verificar a adulteração de sulfeto de cobre, geralmente adulterado com sulfato de ferro. Neste teste, ele usava uma tira em papiro, umedecida em uma solução de extrato de noz-de-galha, que era mergulhada na solução a ser examinada. Se a solução contivesse sulfeto de ferro, o papiro tornava-se preto. O componente ativo desse extrato é o ácido tânico, uma mistura de ésteres de glicose do ácido gálico.

A noz-de-galha ou bugalho é a excrescência desenvolvida nos grelos dos ramos novos de uma espécie de carvalho, *Quercus infectoria*, que habita na Ásia Menor, além de outros carvalhos da Europa (Fig. 2). A água, o álcool e o éter extraem facilmente os princípios ativos da galha. Ela serve para preparar tanino. Adstringente e tônico, aconselhado internamente na diarréia, leucorréia, como antídoto emético e em gargarejo. Outras galhas de Cinipídios produzidas por *Andricus gallaetinctoriae*

foram utilizadas desde a antigüidade para curtir couro e na fabricação de tintas e tinturas para o cabelo e roupas, graças a seu alto conteúdo de ácido tânico. Na escrita, a noz-de-galha ou bugalho de carvalho, diluída em vinho branco e fixada com substâncias minero-metálicas, era o corante mais usado no fabrico de tintas para escrita em Portugal na Idade Média (Meirinhos, 1995). Aproveitamento comercial foi especialmente intenso ao longo do século XVII, em especial na indústria do couro. Outros usos das galhas foram no campo da etnobotânica, como sua utilização em adornos corporais, tatuagens e fabricação de colares por certas tribos da África Oriental e Amazônia. Por último pode-se mencionar também o uso alimentar de algumas galhas produzidas por um Cinipídio em *Salvia pomifera* que eram consumidas na ilha de Creta.

1.3 A Cecidologia

O primeiro cientista que descobriu a reação de causa e efeito entre um inseto e sua galha foi o italiano Malpighi no século XVII. Posteriormente Bijerink no final do século XIX foi o primeiro a associar o processo de formação de uma galha como resultado da atividade de uma larva dentro da mesma. Este descobrimento coincide no tempo com o posterior auge e rápido desenvolvimento da ciência da Cecidologia (Folliot, 1964). Inicia-se o inventário das zoocecídias do globo com trabalhos monumentais de Houard (1908, 1909, 1913, 1922-1923, 1933, 1940) muito mais tarde continuados por Buhr (1964). As obras mais citadas nas referências dos trabalhos com galhas são de Felt (1940), Mani (1964), Docters van Leeuwen (1982), Ananthakrishnan (1984), Price (1986, 1987), Meyer (1987), Redfern e Askew (1992) e Shorthouse e Rohfritsch (1992).

Fig 1 - Teofrasto na Grécia Antiga, considerado o pai da Botânica
Fonte: Phillipson, 2001

Fig. 2 - A galha do Carvalho (*Quercus sp*), talvez a galha melhor conhecida e estudada desde a antiguidade

Fonte: www.chrisraper.org.uk

A Cecidologia ou ciência que ocupa-se do estudo das galhas de plantas, é de âmbito interdisciplinar como poucas no campo das Ciências Naturais, já que a compreensão da íntima relação entre o organismo que induz a galha e a planta hospedeira, leva consigo estudos de tipo muito distintos, morfologia e estrutura, etiologia, citologia, fisiologia, bioquímica e patologia; estudos de ciclo de vida dos agentes indutores, comportamento, especificidade frente ao hospedeiro, fisiologia da nutrição, natureza, significado e evolução da interação planta-indutor ou o estudo das relações tróficas das complexas comunidades associadas a galhas (Mani, 1992).

Plenamente classificadas dentro do conceito de galhas ou cecídias, encontramos na natureza uma ampla variedade de formações vegetais diferenciadas enormemente enquanto sua estrutura e complexidade e produzidas também por organismos muito distintos. Conhece-se mais de 15000 espécies de organismos capazes de induzir a formação de galhas entre eles: vírus, bacterias, algas, fungos, protozooários, rotíferos, nematóides, ácaros e insetos (Felt, 1940). Referimo-nos neste trabalho ao caso mais notório, que são as galhas produzidas pelos artrópodos. A grande diversidade, sugere que esta associação particular surgiu independentemente neste grupo muitas vezes e de formas muito diversas (Roskam, 1992). Abundam as diferenças entre os diversos grupos de insetos galícolas. Estas diferenças incluem os mecanismos de indução das galhas, efeitos sobre os hospedeiros, métodos de alimentação e diferentes ciclos de vida. O que é de particular interesse neste processo é a capacidade desenvolvida independentemente em muitos táxons, de reproduzir o processo de crescimento da planta hospedeira, orientando-as a proporcionar abrigo e nutrientes adequados ao inseto galícola.

As estruturas botânicas atribuidas a insetos galícolas mais antigas que tem-se conhecimento datam do Cretácio (130 m.a.) encontradas na Lauraceae *Sassafras potomacensis* em Maryland, EUA (Larew, 1986). Um dos primeiros grupos de insetos a desenvolver a via cecidogênica no final do Cretácio, foram alguns himenópteros *Symphyta*, e ao que parece o resto dos insetos cecidogênicos adquiriram esta qualidade e desenvolveram-na ao longo do Terciário (65 m.a.), período que presume-se correu a estabilização e diversificação das Angiospermas (Diéguez et al, 1996). Porém existem evidências mais antigas da interação artrópode-planta em folhas fósseis do período Triássico (245 m.a.), que aparentemente forneceram alimento e abrigo a um artrópode em um tipo bem definido de galha (Ash, 1996).

1.4 Coccideos Galhadores

Os coccideos (Coccoidea: Eriococcidae) são caracterizados por um pronunciado dimorfismo sexual (Cook et al, 2000). As fêmeas são apteras e mantém um modo de vida completamente séssil, patas antenas e olhos podem ser reduzidos ou ter desaparecido por inteiro, podem não ter segmentação corporal, sendo dificilmente reconhecidas como insetos. Somente as ninfas no primeiro estádio de vida desenvolvem motilidade sobre a planta. Os machos são muito menores que as fêmeas, mais ativos e de vida mais curta. Eles podem ter asas reduzidas, vestigiais ou não possuí-las, os olhos são simples ou compostos (Ben-Dov e Hodgson, 1997). Este pronunciado dimorfismo sexual também é expresso nas galhas que produzem. As galhas fêmeas ou ginoceccideas são relativamente grandes, enquanto as galhas machos ou androceccideas são muito reduzidas em tamanho. A diferenciação morfológica destas galhas especialmente ginoceccideas são extremamente

elaboradas. Muitos gêneros são monófagos em um hospedeiro e em muitas espécies desconhece-se os indivíduos machos (fig. 3). Galhas de Eriococcidae foram muito estudadas na Nova Zelândia e Na Austrália, mas somente do ponto de vista taxonômico (Gullan 1978; 1984).

1.5 *Rollinia laurifolia*

Rollinia laurifolia Schldtl. pertence à familia ANNONACEAE (fig.4), que é uma família pantropical com interesses taxonômicos, ecológicos e farmacológicos (Leboeuf et al. 1982; Hufford e Oguntimein 1982; Santos e Salatino 2000). Popularmente conhecida como: Achecou, Araticum, Jangada-preta, Pindaiba ou Pinha-da-Mata. Muitos estudos fitoquímicos foram feitos sobre o gênero *Rollinia*, e foram identificadas diversas acetogeninas (Dabrah and Sneden 1984; Shi et al. 1997; Pimenta et al. 2001) que são agentes antitumorais encontrados somente na família ANNONACEAE (Alali et al. 1999). Particularmente, as acetogeninas têm um efeito inibidor seletivo da NADH-ubiquinona oxidoredutase (complexo I) da cadeia transportadora de elétrons, apesar de não haver qualquer similaridade estrutural entre essas moléculas e inibidores clássicos como piericidina A e rotenona (Takada, 2000).

1.6 Oxigênio e Espécies Ativas de Oxigênio (EAO)

O aparecimento de seres aeróbicos foi um marco na evolução biológica, pois o emprego do oxigênio aumenta consideravelmente a extração de energia dos substratos energéticos. As reações que utilizam o oxigênio no metabolismo energético são chamadas de óxido-redução, em que há trocas de elétrons entre os reagentes. O oxigênio é parte essencial da vida aeróbica, porém uma conseqüência indesejada é a
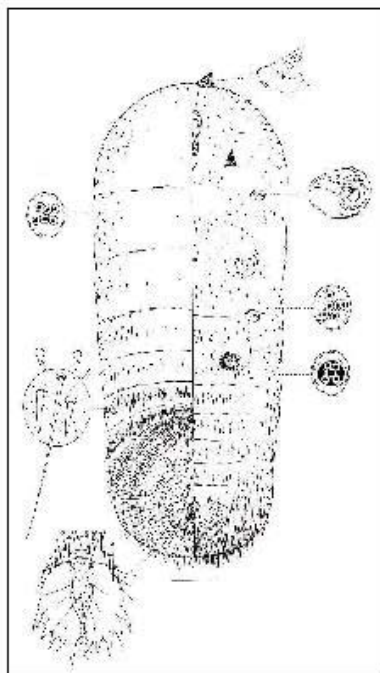
Fig. 3 - Fêmea adulta de *Cylindrococcus spiniferus* um galhador do mesmo grupo (Homoptera: Eriococcidae) do galhador de *Rollinia laurifolia*
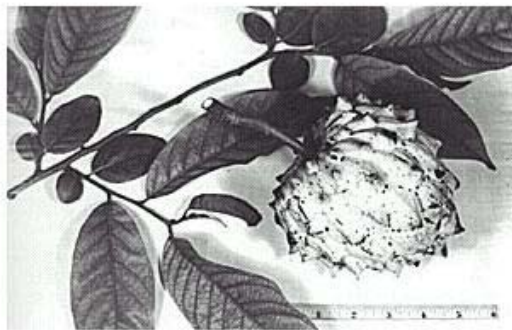
Fonte: Gullan, 1984

Fig. 4 - Um fruto típico do gênero *Rollinia*

Fonte: www.hort.purdue.edu

formação de radicais livres e outras espécies ativas de oxigênio (EAO) (Halliwell e Gutterdge, 1998). O oxigênio não é toxico *per se*, mas os produtos originados pelas reações onde o oxigênio é parte essencial, podem ser danosos a importantes componentes celulares como lipídios, proteínas e ácidos nucléicos. Entretanto tem sido conclusivamente demonstrado que as espécies ativas de oxigênio que sempre são formadas durante o metabolismo regular dos organismos, não são de todo danosas, servindo por exemplo, como sinalizadores de estado redox (Halliwell e Gutterdge, 1998). A produção aumentada de EAO é considerada como uma aspecto universal e comum na condição de estresse.

1.6.1 Espécies Ativas de Oxigênio (EAO) e as Plantas

As plantas em particular, desenvolveram uma variedade de mecanismos em resposta a este problema e tem tornado-se evidente que EAO que são formadas durante o ataque de um patógeno ou em situação de estresse abiótico, são reconhecidas pelas plantas como um sinal para disparar respostas de defesa (Vranová et al, 2002). O exemplo melhor conhecido é a ativação de resposta de resistência durante uma interação incompatível planta-patógeno. Sob infecção, uma NADPH oxidase de membrana plasmática é ativada, produzindo radicais superóxido ($O_2^{-\cdot}$) (Desikan et al, 1996) que são convertidos expontaneamente ou via superóxido dismutase (SOD) a peróxido de hidrogênio (H_2O_2). Altos níveis de H_2O_2 são tóxicos tanto para o patógeno, quanto para a célula vegetal, matando as células da planta entorno do ponto de infecção, inibindo a propagação do patógeno. O H_2O_2 também serve como substrato para reações peroxidativas cruzadas de precursores de lignina e induz reações cruzadas com proteínas da parede celular. Uma parede celular

reforçada diminui a propagação do patógeno, impedindo novos pontos de infecção (Levine et al, 1996). Além disto EAO podem atuar diretamente como transdutores de sinais através da rota de fenilpropanóides (Grace e Logan, 2000).

1.7 Os Compostos Fenólicos

Compostos fenólicos, constituem uma grande classe de compostos naturais onipresentes no reino vegetal. Os compostos fenólicos são substâncias químicas caracterizadas por possuir pelo menos um anel aromático (C6) carregando um ou mais grupamentos hidroxil (OH). Ácidos hidroxicinâmicos (HCAs) e flavonóides têm esqueleto básico de carbono C6-C3 e C6-C3-C6, respectivamente. HCAs e flavonóides são produzidos a partir da fenilalanina, via rota do chiquimato, rota geral de fenilpropanóides e rota específica de flavonóides (Herrmann, 1995) (Fig. 5).

1.7.1 Propriedade Antioxidante de Compostos Fenólicos

Esses metabólitos secundários são reconhecidos por demonstrar uma notável variedade de interações bioquímicas que acredita-se vir de suas propriedades antioxidantes (Rice-Evans et al, 1995). Essas moléculas podem modificar os efeitos de EAO em um grande número de processos biológicos. O fato de conter um ou mais grupos OH ligado à estrutura em anel, confere a estes compostos atividade antioxidante, porém às vezes podem ocorrer na forma glicosilada. Os polifenóis de plantas são multifuncionais e podem atuar como agentes redutores, antioxidantes doadores de hidrogênio ("scanvenger"), como eliminadores ("quenchers") de oxigênio singlet e, em alguns casos, podem ser quelantes de metais (Rice-Evans et al, 1996). Para um polifenol ser definido como um antioxidante ele deve atender duas condições

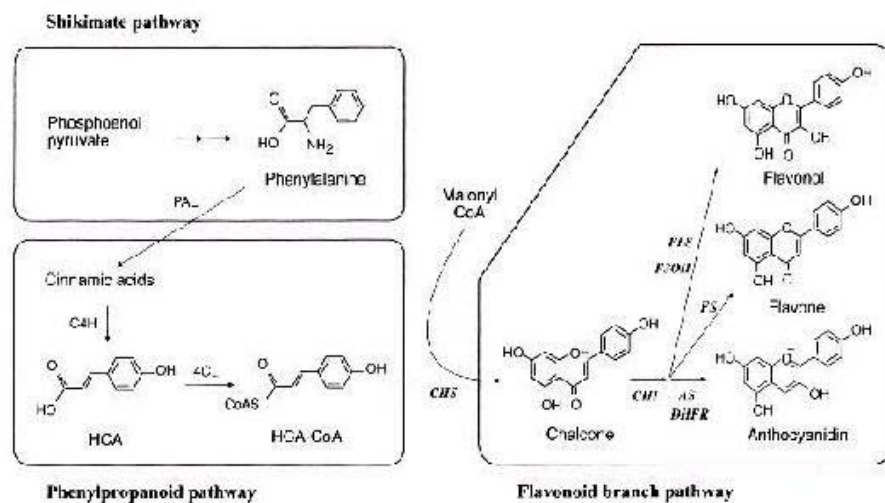Fig. 5 - Diagrama da rota biossintética de fitofenóis. Fenilalanina amonia liase (PAL); cinamato-4-hidroxilase (C4H); 4-coumarato:CoA ligase (4CL); ácido hidroxicinâmico (HCA); hidroxicinamoil CoA (HCA - CoA); chalcona sintetase (CHS); chalcona isomerase (CHI); flavanona 3-hidroxilase (F3OH); flavona sintase (FS); flavonol sintase (FLS); dihidroflavonol redutase (DHFR) e antocianina sintase (AS)

Fonte: Sakihama et al, 2002

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básicas: primeira, quando presente em baixas concentrações relativa ao substrato a ser oxidado, ele deve adiar, retardar ou prevenir a auto-oxidação ou a oxidação mediada por radicais livres (Halliwell, 1990); segunda, o radical resultante formado após a captura deve ser estável e menos reativo, diretamente pela ligação intramolecular de hidrogênio na oxidação adicional (Shahidi e Wanasundara, 1992). Por outro lado, há trabalhos que demonstram que o comportamento destes compostos como antioxidantes e até mesmo como próxidantes em sistemas oxidativos, são relacionados em função de sua estrutura/atividade (Cao et al, 1997; Rice-Evans et al, 1996).

1.7.2 Compostos Fenólicos e Estresse em Plantas

Muitas plantas sintetizam constitutivamente polifenóis, entretanto o acúmulo de compostos fenólicos nas mesmas, pode ser induzido por estresse biótico e abiótico. Por exemplo: radiação UV, iluminação excessiva, baixas temperaturas, ferimentos, baixo nível de nutrientes e ataque de patógenos (Lichtenthaler, 1998). Os polifenóis constituem portanto uma resposta importante ao estresse ambiental, sendo substrato para enzimas polifenol oxidase (PPO's), que catalisam reações dependentes de oxigênio, trasformando fenóis em quinonas que são tóxicas. Essas enzimas e esses fenóis estão presentes em delgadas estruturas chamadas tricomas, que ao serem tocadas por invasores rompem-se, colocando seu conteúdo em contato com o oxigênio atmosférico formando quinonas, intoxicando assim o organismo invasor (Constabel e Ryan, 1998). A formação de fenóis absorvedores de UV-A e UV-B como os flavonóides na camada epidérmica, protege o aparelho fotossintético do mesofilo contra os danos da radiação UV (Lichtenthaler, 1998). Os taninos que diminuem a

eficiência digestiva do fitófago, é outro exemplo considerado clássico para função de defesa dos compostos fenólicos (Meyer e Richardson, 1993).

1.7.3 Compostos Fenólicos e as Galhas

Diferenças no conteúdo e na complexidade de compostos fenólicos em galhas, tem sido bastante examinadas (Kraus e Spiteller 1997; Nyman e Julkunen-Tiitto 2000) e podem ser influenciadas pela espécie de galhador (Hartley 1998). Estudos anteriores encontraram uma correlação entre a resistência a insetos galhadores e o conteúdo fenólico aumentado da planta hospedeira; e correlação negativa com a sobrevivência do inseto, sugerindo em alguns casos, que altos níveis de compostos fenólicos na planta hospedeira pode ser uma defesa contra insetos galhadores (Westphal et al, 1981). Entretanto, o oposto também já foi observado (Nyman e Julkunen-Tiitto 2000). Em alguns casos, quando várias espécies de galhadores atacam a mesma espécie de planta hospedeira, cada um induz o tecido vegetal a reorganizar-se de formas distintas, produzindo estruturas espécie-específicas (Shorthouse e Rohfritsch 1992). Padrões gerais de composição química entre tecidos sadios e galhados são complexos de estabelecer-se (Hartley 1998), porém pouca informação está disponível sobre mudanças bioquímicas e antioxidantes relacionadas à cecidogênese.

Os coccideos, que incluem mais de 4000 espécies, são distribuídos por todo mundo e estão entre as pragas mais destrutivas para as plantas, compreendendo as chamadas cochonilhas (Linsenmaier, 1972). Estes insetos apresentam grande importância por parasitarem plantas de interesse econômico e ornamental. Uma espécie de coccideo ainda não identificada, forma galhas foliares em *Rollinia laurifolia*. Existem fortes indícios de que as galhas foliares de *R. laurifolia* sejam

induzidas por um inseto deste grupo (Soares et al. 2000). As galhas formadas apresentam um forte dimorfismo sexual (fig. 6). A identificação do animal, nas folhas galhadas, está atualmente sendo feita no País de Gales. Mudanças no perfil químico nas folhas de *R. laurifolia* devido à cecidogênese foram recentemente descritas (Soares et al. 2000).
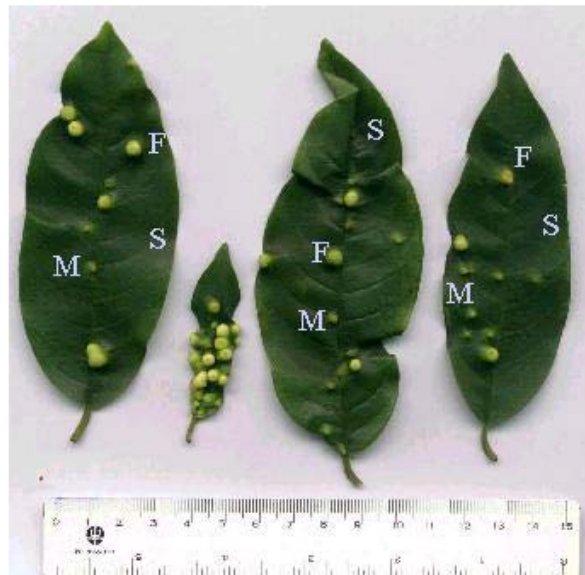
Fig. 6 - Folhas galhadas de *Rollinia laurifolia* com dimorfismo sexual das galhas. (F) galhas fêmeas que são maiores; (M) galhas macho que são menores e (S) tecido sadio das folhas com galha

Fonte: Samuel J.M.R. Gonçalves, Juiz de Fora, MG

2. OBJETIVOS

Levando em consideração a importância e a falta de conhecimento sobre a cecidogênese em plantas da família ANNONACEAE, os objetivos deste trabalho foram:

1 - Investigar as relações ecológicas/bioquímicas entre coccideo/folhas galhadas e folhas sadias de *R. laurifolia*, examinando se a presença de um inseto galhador altera o conteúdo fenólico e as propriedades antioxidantes dos extratos etanólicos de folhas de *R. laurifolia.*

2 - Averiguar se as possíveis alterações nos parâmetros anteriores, também são observadas em galhas masculinas e galhas femininas isoladas das folhas atacadas e também do tecido sadio (sem galhas) destas folhas, em comparações com as folhas totalmente sadias de *R. laurifolia*.

3. ARTIGO 1

"A Gall-Fomer Coccid Alters The Phenolic Content and Antioxidant Properties of

Rollinia laurifolia (ANNONACEAE) Leaves"

(Submetido à Oecologia)

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A GALL-FORMER COCCID ALTERS THE PHENOLIC CONTENT AND ANTIOXIDANT

PROPERTIES OF *Rollinia laurifolia* (ANNONACEAE) LEAVES

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24 ***Abstract***

25 Galls are characterised by an abnormal plant tissue growth in response to specific stimuli

26 from invading organisms: bacteria, fungi, nematodes and arthropods. The host plant-

27 inducer interaction changes the chemical and physiological status of plant tissues during

28 cecidogenesis. The ability to induce galls on plants had multiple independent origins.

29 Several types of biotic and abiotic stimuli are known to alter the production of plant

30 phenolic compounds involved in plant defence against pests and pathogens. A species of

31 coccid induces galls in leaves of *Rollinia laurifolia* specimens. With the aim of studying the

32 chemical relationships between galled (GL) and healthy (HL) leaves of *R. laurifolia* the

33 purpose of this study was to examine the influence of the gall-former in the phenolic

34 contents of the leaves, and the alterations in the phenol antioxidant properties due to gall

35 formation. Total phenolic content, HPLC analyses, total radical-trapping antioxidant

36 parameter (TRAP) and TBARS were performed. The presence of a live gall-former is

37 required to maintain the chemical differences between GL and HL tissues. The occurrence

38 of galls in *R. laurifolia* is related to significative reduction in leaves total phenol content.

39 Total antioxidant potential and TBARS index were altered in GL in comparison with HL.

40 Our results suggest that gall inducer/*R. laurifolia* interaction change the polarity of phenolics in

41 the plant in order to protect plant membrane lipids and possibly coccid from the oxidative stress

42 established during cecidogenesis. Oxygen diffusion in the *Rollinia laurifolia* leaf tissue during

43 cecidogenesis may be a signal for triggering responses for radical scavenging by the plant

44 phenylpropanoid pathway. The role of phenolics in adaptive significance and evolutionary

45 consequences of gall induction and triggering signals are discussed.

46

47 *Keywords: gall, Rollinia laurifolia, coccid, phenolic compounds, antioxidant*

Introduction

48

49   Galls result from a close association between an inducer (bacteria, fungi, nematodes

50 and arthropods) and a plant species characterised by an abnormal plant tissue growth in

51 response to specific stimuli from the invading organism (Meyer 1987). The host plant-

52 inducer interaction changes the growth and development of plant tissues during

53 cecidogenesis and is specific to each type of gall (Hartley 1998). Probably this specificity

54 also occurs in chemical and physiological levels both in plant and in gall-inducer during

55 cecidogenesis. The ability to induce galls on plants had multiple independent origins, and

56 there are evidences of arthropods-plant interactions in fossil leaves from the Upper Triassic,

57 which should have provided nourishment and shelter for several types of arthropods, and

58 included a well-defined type of gall (Ash 1996). One of the most plausible explanations for

59 this interaction is suggested by the nutrition hypothesis (Price et al. 1986), which assumes

60 that gall inducers are able to manipulate their hosts into producing tissues that are

61 nutritionally superior to other plant parts because they contain high amounts of nutrients

62 and/or low concentration of defensive chemicals.

63   As an unfortunate consequence of aerobic life, free radicals and another active oxygen

64 species (AOS) are formed by biological redox reactions (Halliwell and Gutteridge, 1998). The

65 increased production of toxic oxygen derivatives is considered to be a universal or common

66 feature of stress conditions. AOS inactivate enzymes and damage important cellular

67 components that include the induction of membrane lipid peroxidation and fatty acid de-

68 esterification. Plants developed a wide range of mechanisms in response to this problem and

69 it is becoming evident that AOS, which are generated during pathogen attack and abiotic

70 stress situations, are recognised by plants as a signal for triggering defence responses

71 (Vranová et al. 2002). Adequate responses to enhanced production of AOS within several

72   subcellular compartments of the plant cell are crucial for plant growth and survival. The level

73   and kind of AOS are determining factors for the type of response (Van Breusegem et al.

74   2001).

75   Several types of biotic and abiotic stimuli are known to alter the production of phenolic

76   compounds in plants (Rhodes 1985), which constitute an important response to environmental

77   stress. Phenols are substrates for polyphenol oxidase enzymes (PPO's), which catalyse the

78   oxygen-dependent reactions of these compounds and are assumed to be involved in plant defence

79   against pests and pathogens (Constabel and Ryan 1998). Formation of UV-A and UV-B-absorbing

80   phenols as flavonoids in epidermal layer protects the photosynthetic apparatus of mesophyll

81   against damaging UV radiation (Lichtenthaler 1998). As well, tannins decrease digestive efficiency,

82   and are assumed to be classical defences against herbivores (Meyer and Richardson 1993).

83   Differences in the content and complexity of phenolic compounds in galled and healthy

84   leaves have been examined (Kraus and Spiteller 1997; Nyman and Julkunen-Tiitto 2000) and

85   could be influenced by gall inducer (Hartley 1998). In cases where several gall-inducing species

86   attack the same host plant species, each one causes the plant tissues to be reorganised in a

87   diverse way, producing characteristic and species-specific structures (Shorthouse and Rohfritsch

88   1992). General patterns in chemical composition of galled in relation to ungalled tissues across a

89   range of species may prove hard to be established (Hartley 1998); however very little information is

90   available on the biochemical and antioxidant changes associated with cecidogenesis.

91   *Rollinia laurifolia* Schldtl. belongs to the Annonaceae, a large pantropical family, with

92   taxonomical, ecological, and pharmacological interest (Leboeuf et al. 1982; Hufford and Oguntimein

93   1982; Santos and Salatino 2000). Many phytochemical studies were made on the *Rollinia*

94   genus, identifying several acetogenins (Dabrah and Sneden 1984; Shi et al. 1997;

95   Pimenta et al. 2001) which are antitumor agents that are only found in the Annonaceae

(Alali et al. 1999). An unidentified species of coccid (Coccoidea: Eriococcidae) induces galls in leaves of *R. laurifolia* specimens. Eriococcid galls were largely studied in Australia and New Zealand, but just from the gall inducers taxonomic standpoint (Gullan 1978; 1984). Changes in chemical profile of *R. laurifolia* leaves, due to cecidogenesis were recently described (Soares et al. 2000). The aims of this study were to investigate the ecological/biochemical relationships between coccid/galled leaves (GL) and healthy leaves (HL) of *R. laurifolia,* by examining if the presence of gall-inducer changes the antioxidant properties and/or the phenolic contents of the leaves. This investigation should provide new insights into gall-inducer/host plant interactions in Annonaceae and evaluate their secondary compounds antioxidant potentials.

Materials and Methods

Chemicals

AAPH (2,2'-Azobis [2-methylpropionamidine] dihydrochloride), lipid pattern (cholesterol and triglycerides at 1200 mg/dl), luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione), glycine (amino acetic acid), DMSO (dimethyl sulfoxide), Folin-Ciocalteau (phenol reagent), and TBA (4,6-Dihydroxypyrimidine-2-thiol) were purchased from Sigma Chemicals, St. Louis, MO, USA. TMP (1,1,3,3-Tetramethoxypropane), Trolox (6-hydroxy-2, 5,7,8-tetramethyl-chroman-2-carboxilic acid) and tannic acid were purchased from Aldrich Chemicals, Milwaukee, WI, USA. Cellulose nitrate membrane was purchased from Merck chemicals, Darmstadt, Germany. Deionised water was used for all experiments and all other reagents used in this study were of analytical grade.

Plant material and extraction

The leaves of *R. laurifolia* tree were collected at the Ecological Station of Universidade Federal de Minas Gerais (UFMG), Pampulha *Campus*, Belo Horizonte,

120 Minas Gerais, Brazil. The reference plant material is BHCB 253331, deposited at the

121 herbarium of the Departamento de Botânica of UFMG.

122   The extraction method used was the classical phytochemical approach for polar

123 phenolic derivatives rich extraction (Harbone 1984). GL and HL were collected in February

124 2001, dried and lyophilised. Samples (20 g) of both materials were exhaustively extracted in

125 Sohxlet with hexane (24 h) to eliminate low polarity compounds, followed by extraction with

126 ethanol (24 h). The crude ethanolic extracts were dry at low pressure, lyophilised and stored

127 at -5°C. Stock solutions of both extracts were prepared immediately before use with 25 mg

128 of dry extract and 5 mL of solvent (distilled water or DMSO 0.07%), sonicated for 2 min. (4 X

129 30'') with potency 4 (Sonicator XL 2020 Heat Systems Inc.), and filtered with cellulose

130 nitrate membrane (pore size of 0.45 µm). These stock solutions with final concentration of 5

131 mg/mL were diluted as indicated in the experiments.

132 *Total phenolic content*

133   The total phenolic contents (Waterman and Mole 1994) were determined in both HL

134 and GL extracts by colorimetric assay, in which purified tannic acid was used as standard.

135 Extracts (25 mg) were dissolved in 5 mL of water or DMSO 0.07% and homogenised as

136 describe above. A sample of 0.5 mL from each extract had its volume completed up to 7

137 mL with water, 0.5 mL of Folin reagent (1N) was added, the mixture vortexed, 1mL of

138 Na_2CO_3 saturated solution was added and homogenised. The final volume was completed

139 up to 10 mL with water and used for determination of phenolic contents. The absorbance

140 was measure at 725 nm in a spectrophotometer (Beckman DU-640) and the results were

141 expressed as µg of phenol/mg of extract.

142 *HPLC profile*

The high performance liquid chromatography (HPLC) profiles was made in a Shimadzu® chromatograph (Column RP18 250x4mm, Ø 5μm, detector UV/Visible SPD 10A) using acetonitrile /H_2O (1:1, v/v), with injections of 20μL at a speed of 0,5μL/min. The detection of the substances were performed at 220nm using the guidelines suggested by Markhan (1982) and Harborne (1984), modified by Soares et al. (2000) for analyses of phenolic derivatives.

Total radical-trapping antioxidant parameter (TRAP)

An adapted method of TRAP assay was used to determine the capacity of ethanolic extract homogenised in distilled water or DMSO 0.07% to trap a flow of water-soluble peroxyl radical produced at constant rate, through thermal decomposition of AAPH (Ghiselli et al. 2000). Briefly, the reaction mixture (4 mL) containing the free radical source (AAPH 10 mM) in glycine buffer (0.1 M) pH 8.6, test samples (10 μL) at the concentrations of 0.5 - 5 mg/mL, and luminol (4 mM) as external probe to monitoring radical production, were incubated at 25°C. The chemiluminescence produced was directly proportional to the radical generation and measured in out of coincidence mode in a liquid scintillation counter (Wallac 1409) as counts per minute (CPM). The relation between antioxidant potential and reading (CPM) was inverse, i.e. high readings indicate poor antioxidant potential. One cycle was the time to read all samples (10 min.) and repeated eight times to monitor the decline of free radical source and the consume of samples antioxidant potential.

TBARS *in vitro* assay

TBARS (thiobarbituric acid reactive species) assay (Esterbauer and Cheeseman 1990) is a method for quantify lipid peroxidation and an adapted TBARS method was used to measure the pro/antioxidant capacity of extracts *in vitro* using egg yolk homogenate as

167 lipid rich substrate in which TMP was used as a standard. Briefly, 0.5 mL of 0.1% (w/v)

168 tissue homogenate (egg yolk) in phosphate buffer pH 7.4 was sonicated for 40 seg. (4 X

169 10'') potency 4 (Sonicator XL 2020 Heat Systems Inc.) and 0.1 mL of extracts in different

170 concentrations (0.1 - 5 mg/mL ressuspended in water or DMSO 0.07% prepared

171 immediately before use) was added to a test tube. 0.2 mL of AAPH solution (0.12 M) or

172 $FeSO_4$ (1,5 mM) was added to induce lipid peroxidation. The tubes were incubated at room

173 temperature for 30 min. 0.5 mL of tricloroacetic acid (10%) was added and mixed with

174 TBA (0.67% - 0.5 ml) vortexed, and heated at 95°C for 30 min. After cooling, samples

175 were centrifuged at 1200 x g for 10 min. The absorbance of supernatant was measure

176 using a spectrophotometer (Beckman DU-640) at 532 nm. The results were normalised by

177 lipid content (Postma 1994) using a purified lipid solution as standard for egg yolk

178 homogenate. The results were expressed as nmol malondialdehyde equivalents

179 (MDA)/mg of lipid.

180 *Statistical analysis*

181 All data are expressed as mean ± S.E. and were analysed by ANOVA. *p* values

182 lower than 0.05 were considered significant and Newman-keuls test was used to compare

183 groups when adequate.

184 **Results**

185 *Total phenolic content*

186 The determination of total phenolic content of *R. laurifolia* ethanolic extracts

187 showed that HL have higher amounts of phenolics when compared with GL (Fig. 1). HL

188 material has a phenolic content higher than GL when water is used as solvent. This

189 difference between the phenolic content of the extracts is reduce when DMSO 0,07% is

190 used as solvent, although HL keep the higher level of phenolics. Actually, the addition of

191 DMSO 0.07% causes another two distinct changes on this result. While HL show a

192 significant reduction of detectable phenolics; GL phenolic content is slightly increased. So

193 we may speculate that phenolics present in HL are more polar than phenolics present in

194 GL.

195 **Fig. 1**

196 *HPLC profile*

197 The HPLC analyses of *R. laurifolia* ethanolic extracts provide two chemical profiles

198 that allow us to distinguish between HL and GL. HL have a greater number of detectable

199 phenolic compounds than GL (Fig. 2), even when the signals with area <1% were omitted

200 (Table 1). It is very clear that there are few correspondences between the retention times

201 of the compounds detected in the both extracts. In general, HL and GL do not produce the

202 same phenolics. At 4,02/4,13 minutes the chromatograms show a peak that could indicate

203 the same compound (Table 1). Nevertheless the amount of this compound is increased in

204 GL (RT=4,13; A=34,55%), when compared with the correspondent peak in health leaves

205 profile (RT=4,02; A=7,02%). GL show a greater tendency to produce less polar

206 compounds. About 41% of detectable phenolics in this extract have retention times

207 greater than 19 minutes. In other hand, the majority of phenolics of HL were detected at

208 retention times shorter than 19 minutes.

209 **Fig. 2**

210 **Table 1**

211 *Total radical-trapping antioxidant parameter (TRAP)*

212 The data obtained by TRAP assay firstly indicate that extracts possess antioxidant

213 capacity (figs. 3 and 4). The antioxidant capacity of extracts solubilised either in water or

214 DMSO 0.07% was indicated by reduction in chemiluminescence intensity inversely

215 proportional to extracts concentration. These results also indicate that in concentration of

216 5 mg/mL the extracts are more effective than Trolox (7µM) which is the water-soluble

217 analogous of α-tocopherol and used as the reference scavenger molecule (insert box of

218 figs. 3A and 4A). As antioxidant, the extract from HL was more efficient than extract from

219 GL (figs. 3 and 4). Another important observation is the difference in efficiency of HL in

220 comparison to GL, which was increased when the solvent was water (fig.3), than DMSO

221 0.07% (fig.4). These data can be related to polar phenolic compounds that known to be

222 better antioxidants in TRAP assay.

223 **Fig. 3**

224 **Fig. 4**

225 *TBARS in vitro assay*

226 The results of lipoperoxidation assay show what when solubilised in water and

227 tested alone (without free radical inductor) HL extract was prooxidant at 5 mg/mL (fig.5A

228 and, 5C), GL extract alone has no pro or antioxidant effect in none of the concentrations

229 tested (fig.5A and, 5D). At of 0.1 and 1 mg/mL HL extract has a protective effect on egg

230 yolk lipid peroxidation induced by AAPH (peroxyl radical inducer) (fig. 5D). The GL extract

231 has the same protective effect in all concentrations tested (fig. 5D) at 1 mg/mL the

232 protective effect of GL was superior that of the HL extract (fig. 5D). The protective effect of

233 extracts solubilised in water against Fe was detected at 1 and 5 mg/mL (fig 5A) and at

234 these concentrations the HL is more effective than GL extract.

235 **Fig. 5**

236 A different protective profile was observed when extracts were solubilised in DMSO

237 0.07%. Firstly, both extracts protect the lipid rich substrate against AAPH and Fe (fig 5B

238 and 5C). When Fe was used the protective effect seen to follow a dose dependent patter

41

239 (fig 5B). When the inductor of free radicals was AAPH, there was no difference in

240 protective effect. There was only one difference between the protective effects of extracts

241 at 5mg/mL in assay with Fe in which GL was more effective than HL extract (fig 5B).

242 ***Discussion***

243   Gall-formers are a group of herbivores with a high specific mode of nutrition and

244 with a particularly close relationship with their hosts. The presence of a live gall-former is

245 required to maintain the chemical differences between GL and HL tissues. Furthermore,

246 the photosynthetic rates in galled leaves may be reduced (Larson 1998), what is likely to

247 cause changes in nutrient status. Synchronisation of herbivores with their host plant

248 phenology determines quality and quantity of food resources and affects the preference-

249 performance linkage and abundance of herbivores. In addition, synchronisation has a

250 more critical meaning for such gall-former than for other insects (Yukawa 2000).

251   Phenolic compounds can act as chemical signals of several environmental stresses

252 (Karban and Baldwin 1997; Waterman and Mole 1994; Rosenthal and Berenbaum 1992)

253 and the participation of these substances in plant-insect interactions is incontestable

254 (Harborne 1984). The quantitative and qualitative changes observed on the phenolic

255 derivatives of *R. laurifolia* GL likely to be a metabolic response related to gall formation

256 and reinforces the importance of these compounds as signals of plant-inductor interaction.

257   Increases on levels of phenolic compounds were reported in several plant galls

258 (Abrahamson et al. 1991). According to Hartley (1998), gall tissue generally contained

259 lower levels of nitrogen and higher levels of phenolic compounds than ungalled plant tissue.

260 In these cases phenols could potentially play a role in gall formation by influencing the

261 hormonal control of growth and/or in protecting the gall-former from its natural enemies

262 (Abrahanson et al. 1991). Amudhan et al. (1999) suggest that variation of total phenols at

263   the stem bases in genetically related rice varieties in relation to the gall midge infestation

264   can be a expression of plant resistance involving hypersensitive reaction against the

265   insect conferred by the gene Gm2.

266   Phenolic derivatives are found to be the principal constituent of ethanolic extracts of

267   GL and HL of *R. laurifolia* (Soares et al. 2000). We measure the phenolic content in

268   materials, showing that the occurrence of galls in *R. laurifolia* is related to significant

269   reduction of leaves total phenol content. Our results are similar to previous studies that

270   found negative correlation between phenolic content and presence of galls. Nyman and

271   Julkunen-Tiitto (2000) studied the changes in phenolic chemistry of willows (*Salix* genus)

272   by gall-inducing sawflies, and found that the concentrations of most defensive phenolics are

273   substantially lower in gall interiors than in leaves. On the other hand, Espirito-Santo and

274   Fernandes (1998) found no relationship between tannin concentration and gall abundance

275   in *Baccharis dracunculifolia* infected with *Neopelma baccharidis* (Homoptera: Psyllidae) and

276   associated this absence of chemical impact of *Neopelma* galls on *B. dracunculifolia* with the

277   feeding habit of galling insects.

278   The interaction between *Picea glauca* (Pinaceae) and *Adelgis abietes* (Aphidae) is

279   another gall model that shows a decrease in phenolic content in infected host tissues (Kraus

280   and Spiteller 1996). Spruce shoots contain two to ten folds more phenolic than galls and

281   qualitative changes were also detected in these plant materials. Shoots have higher

282   amounts of phenolic glycosides and the authors observed an accumulation of lignans, e.g.

283   cinnamic alcohol dimmers, and a decrease of cinnamic alcohol glucosides in galls

284   associated with the aphid hatching.

285   The model *P. glauca-A. abietes* shows qualitative changes in phenolic production,

286   which surpass in complexity the content changes. The reduction of glycosidic derivatives

and the increase of dimeric derivatives could affect the solubility of gall phenolics. The increase of detectable phenolics when DMSO 0.07% is used to solubilise the extract of galled leaves suggests that similar phenomena occur in *R. laurifolia* galls. The increase in retention times of the phenolics detected by HPLC analysis of this extract when compared to the retention times of the phenolics detected in healthy leaves indicates the production of less polar compounds in *R. laurifolia* infected tissues.

The most common methodology of analysis of phenolic content in plant extracts, by the use of Folin-Ciocalteu as reagent and tannic acid as standard (Waterman and Mole 1994), detects mainly water-soluble phenolic derivatives. Some frequent biosynthetic modifications on phenolic compounds (O- and C-methylation, glycoside hydrolysis, condensation) produce less polar derivatives that could be less water-soluble than its precursors. Therefore the combination of the analysis of phenol content with chromatographic techniques can show more clearly the changes in phenolic metabolism related with plant galls.

Plant phenolics constitute a large group of natural compounds, ubiquitous in the plant kingdom. These secondary metabolites are known to display a remarkable array of biochemical interactions, which are believed to come from its antioxidant properties (Rice-Evans et al. 1995). These substances may act as potent metal chelators and/or free radical scavengers (Hanasaki et al. 1994). However, it has been demonstrated that the behaviour of these compounds in oxidative systems is function of activity-structure relationships (Cao et al. 1997; Rice-Evans et al. 1996). To validate the hypothesis that cecidogenesis alters the biochemical properties of phenolic compounds of *R. laurifolia,* we evaluated the antioxidant potential of GL and HL ethanolic extracts by means of antioxidant approaches.

310   In order to determine the antioxidant potential of extracts a simple method based on

311 luminol-enhanced chemiluminescence was used, which is based on the measurement of

312 oxidation of AAPH, a free radical source. This method is based on the capacity of some

313 compounds to trap peroxyl radicals (ROO$^\bullet$), and is capable of detecting most of the

314 compounds with significant antioxidant activity present in complex mixtures such as plant

315 extracts. The addition of increasing concentrations of both extracts solubilised in water or

316 DMSO 0.07% to the reaction medium resulted in a reduction of luminol-enhanced

317 chemiluminescence, indicating the presence in both extracts of compounds with peroxyl

318 radicals scavenger capacity. The concentration of DMSO 0.07% or water did not interfere

319 in the flow of radical produced by the system. Extract from HL was more efficient than

320 extract from GL in the TRAP assay as observed by a significant difference in decrease of

321 CPM in all concentrations and some points of the time course experiment when water was

322 used as solvent. However when extracts were solubilised in DMSO 0.07% the differences

323 were less significant and only at 0.5 and 5mg/mL.

324   The result of phenolic content together with result of TRAP assays suggest that the

325 decrease in phenolic content of GL was related with the decrease in antioxidant potential

326 observed with TRAP in comparison to HL when water was used as solvent. However

327 when solubilised in DMSO 0.07% the phenolic content decreased in HL and increased

328 slightly in GL; according by the antioxidant potential of extracts which did not differ when

329 DMSO was used as solvent. It is plausible that the antioxidant potential was in part due to

330 phenolic content of extracts once what these metabolites are known to display antioxidant

331 properties (Hanasaki et al. 1994). Nevertheless increased phenolic content do not necessarily

332 imply in higher antioxidant potential since the behaviour of these compounds in oxidative

333 systems may alternate from prooxidant to antioxidant, as a function of activity-structure

334 relationships (Cao et al. 1997).

335   Oxidative stress is a situation of imbalance between production of AOS and

336 antioxidant defences, leading to potential damage. A wide range group of biotic and

337 abiotic agents are recognised as potentially harmful to plants. A common aspect of all

338 these adverse conditions is the enhanced production of AOS within subcellular

339 compartments of cell (Lamb and Dixon, 1997). AOS can be extremely reactive and oxidise

340 biological molecules, such as DNA, proteins and lipids (Halliwell and Gutteridge, 1998).

341 Aside from their harmful nature, AOS can have an important role in inducing protection

342 mechanisms during both biotic and abiotic stresses, acting as signal transduction

343 molecules (Vranová et al. 2002).

344   The membranes that surround cells and cell organelles contain large amounts of

345 lipids. The major constituents of biological membranes are lipid and protein and

346 lipoperoxidation can therefore cause damage to proteins as well as to lipids weakening

347 cell membranes (Halliwell and Gutteridge, 1998). Membrane lipids are amphipathic

348 molecules; i.e. they contain hydrocarbons regions that tend to cluster together away from

349 water, together with polar parts that like to associate with water. When a lipid rich substrate

350 are sonicated in aqueous solution they form micelles and lipossomes that are used in studies

351 of lipid peroxidation. A way of stimulating peroxidation of lipids *in vitro* is to add azo-initiators

352 as AAPH, since the decomposition of these compounds to peroxyl radicals induces the chain

353 reaction of lipid peroxidation (Halliwell and Gutteridge, 1998). Scavengers of free radicals are

354 able to inhibit the resulting lipoperoxidation. Another way to stimulate lipoperoxidation is to

355 add transition metals, such as iron, to a lipid system which, in presence of O_2, forms

356   superoxide radical ($O_2\bullet-$) (Halliwell and Gutteridge, 1998). Chelating agents that bind iron and

357   prevent its participation in free-radical reactions should inhibit the resulting lipoperoxidation.

358   To evaluate the potential antioxidant property of HL and GL extracts in a lipid

359   system, we evaluated these extracts using a lipid rich substrate and AAPH or $FeSO_4$ as

360   free radicals generators. Differences in susceptibility of lipids to peroxidation depend on

361   the nature of the inductor (Halliwell and Gutteridge, 1998); in addition the ability of

362   scavenger AOS and/or metal chelating is related to the chemical structure of phenolic

363   compounds (Cao et al. 1997; Rice-Evans et al. 1996). The results obtained with this approach

364   showed us that DMSO 0.07% soluble phenolics from either HL or GL showed a more effective

365   lipid protection against AAPH and Fe than water-soluble phenolics. HL extract when solubilised

366   in water had a prooxidant effect different from GL that was only antioxidant (fig. 5).

367   We suggest that the coccid gallicolae and the plant in cecidogenous process have

368   the ability to manipulate the phenolic biosynthesis on infected leaves of *R. laurifolia.* This

369   ability is achieved by decreasing water-soluble defensive chemicals and by altering the

370   polarity of phenolics. Taken together, our results allow us to suggest that gall inducer/*R.*

371   *laurifolia* interaction change the polarity of phenolics in the plant in order to protect membrane

372   lipids of *R. laurifolia*, and possibly of coccid, due to oxidative stress during cecidogenesis.

373   Oxygen diffusion in the leaves tissue during cecidogenesis may be a signal for triggering

374   responses for radical scavenging by the plant phenylpropanoid pathway (Grace and Logan,

375   2000) given that phenolic actuating as chain breaking agents and prevent metal-induced

376   lipid peroxidation. However adaptive significance and evolutionary consequences of gall

377   induction and AOS triggering signals in galled tissues are still unknown.

378　　　　Differences in phenolic content may be a good marker in cecidogenesis (Nyman

379　and Julkunen-Tiitto 2000). TRAP assay is a good tool to assess antioxidant potential of

380　extracts since TRAP measurements indicate the quantity and the quality of antioxidants

381　(Ghiselli et al. 2000) present in the plant extracts. Antioxidant properties of phenolics may

382　be excellent as a comparison index and we suggest that an antioxidant approach may be a

383　good indicative for the alterations in phenolic contents between GL and HL.

384　　　　Insect-induced galls are observed on plants throughout the world, but a general

385　model of gall-inducing insects across a range of species may be complex to establish

386　(Hartley 1998). Many studies have reported galls related to phenolic compounds

387　nevertheless, there are no studies which analysis antioxidant properties of phenolics from

388　GL in comparison with HL. Regarding the great biodiversity of Brazilian forests, there are

389　numerous species of plants with galls. *Avicennia germinans* in mangrove and varzea

390　forest that are interesting for comparative studies among interstitial salinity and population

391　density of galling insect (Gonçalves-Alvim et al. 2001) or in cerrado vegetation (savanna)

392　for investigation effects of fire on cecidogenesis in *Palicourea rigida* (Vieira et al. 1996).

393　The Annonaceae represent a large family comprising approximately 120 genera and

394　2000-2200 species (Cronquist 1988) and there are some genera and several species of

395　Annonaceae native to Brazil. *R. laurifolia*/coccid interaction also may be a good model of

396　cecidogenous studies however additional approaches are needed to gain a more complex

397　landscape of gall-former impact on this host plant tissues and about the ecological

398　signification of this interaction.

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Legends

Fig. 1 Total phenolic content of ethanolic extracts from healthy leaves (HL) and galled leaves (GL) of *R. laurifolia*. solubilised in water and DMSO 0.07%. Experiments were performed in tetraplicate and tannic acid was used as standard. * Indicate significant difference between HL and GL $p<0.05$

Fig. 2 HPLC chromatograms (detection at 220 nm) of ethanolic extracts from healthy leaves (A) and galled leaves (B) of *R. laurifolia*

Table 1 HPLC peak areas (detection at 220 nm) of ethanolic extracts from healthy leaves (HL) and galled leaves (GL) of *R. laurifolia*

Fig. 3 Total radical-trapping antioxidant parameter (TRAP) of ethanolic extracts from healthy leaves (HL, open symbols) and galled leaves (GL, filled symbols) of *R. laurifolia* solubilised in water. Experiment was performed in triplicate. Trolox 7µM (----■----) was used as reference molecule, control group (——◆——) was water. Fig. 3A, 0.5 mg/mL (——◤——, ----◤----) and in insert box 5 mg/mL (——↓——, ----↓----). In fig. 3B, 1 mg/mL (——◤——, ----◤----) and 2 mg/mL (——↓——, ----↓----). *Indicate significant difference between HL (----◤----, ----↓----) and GL (——◤——, ——↓——) for each concentration in time indicated, $p<0.05$

Fig. 4 Total radical-trapping antioxidant parameter (TRAP) of ethanolic extracts from healthy leaves (HL, open symbols) and galled leaves (GL, filled symbols) of *R. laurifolia* solubilised in DMSO 0.07%. Experiment was performed in triplicate. Trolox 7µM (----■---) was used as reference molecule and control group (——◆——) was only DMSO 0.07%. Fig 4A, 0.5 mg/mL (——◤——, ----◤----) and in insert box 5 mg/mL (——↓——, ----↓---) In fig. 4B, 1 mg/mL (——◤——, ----◤----) and 2 mg/mL (——↓——, ----↓----). *Indicate

543 significant difference between HL (----↖----, ----↓----) and GL (——↖——, ——↓——) for

544 each concentration in time indicated $p < 0.05$

545 **Fig. 5** TBARS *in vitro* of ethanolic extracts from HL and GL of *R. laurifolia* in which egg

546 yolk homogenate was used as lipid rich substrate for peroxidation. (A) an (C) water was

547 solvent of extracts, (B) and (D) the solvent was DMSO 0.07%. $FeSO_4$ (1.5 mM) or AAPH

548 solution (0.12 M) were used as free radicals inductors in (A, B) and (C, D) respectively.

549 [a]Indicate significant difference between HL and GL for each concentration. *Indicate

550 antioxidant effect when comparate with control. [#]Indicate prooxidant effect comparate with
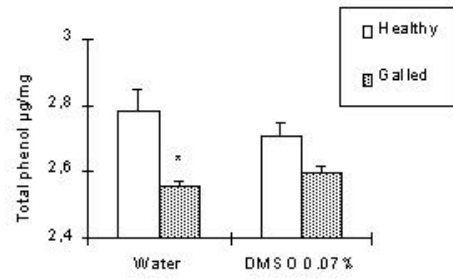
551 control. $p < 0.05$

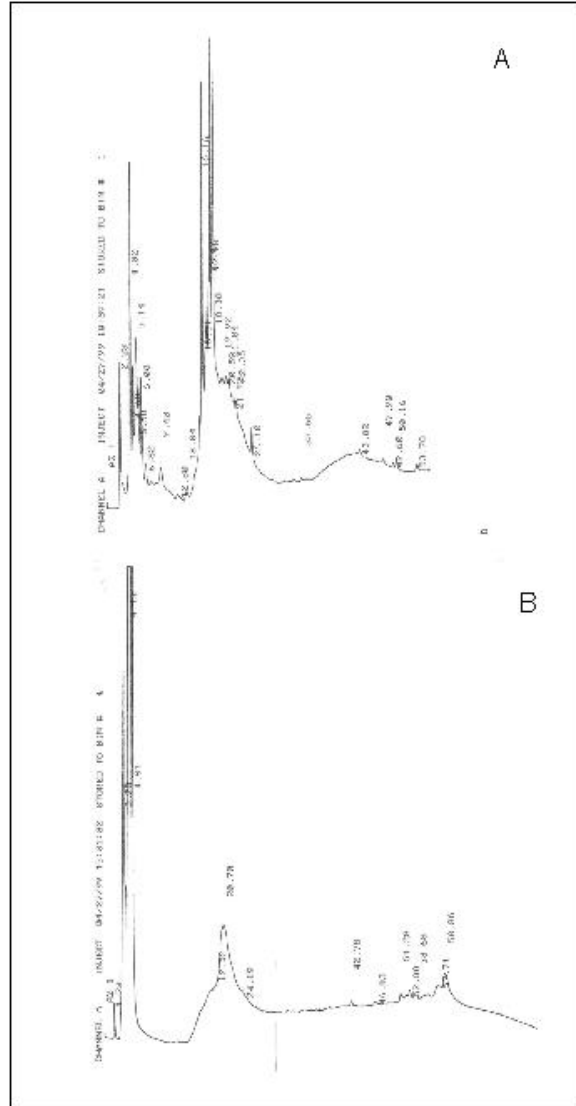Fig.1 Da Silva EG

Fig.2 Da Silva EG

Table1 Da Silva EG

SIGNAL	HEALTH LEAVES		GALLED LEAVES	
	TR (min)	A (%)	TR (min)	A (%)
1	2,28	2,26	-	-
2	-	-	3,20	12,55
3	4,02	7,02	4,13	34,55
4	4,60	2,58		
5	-	-	4,81	10,73
6	5,14	5,06	-	-
7	5,58	1,71	-	-
8	6,08	2,34	-	-
9	16,16	3,42	-	-
10	16,51	8,54	-	-
11	17,47	7,45	-	-
12	17,98	11,56	-	-
13	18,38	42,71	-	-
14	-	-	19,39	8,69
15	-	-	20,70	23,16
16	-	-	24,19	5,65
17	-	-	57,71	1,00
18	-	-	58,86	2,37

A= signal area in %, RT= retention time in minutes).
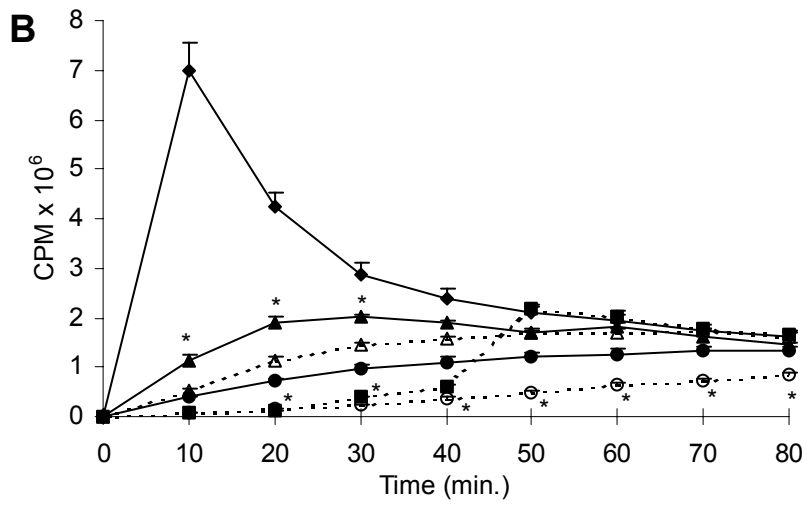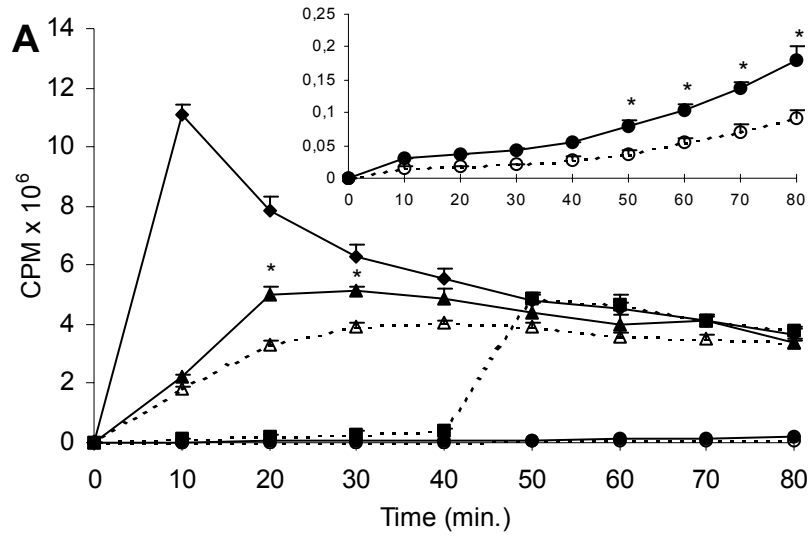Signals with area < 1% were omitted.

Fig.3 Da Silva EG
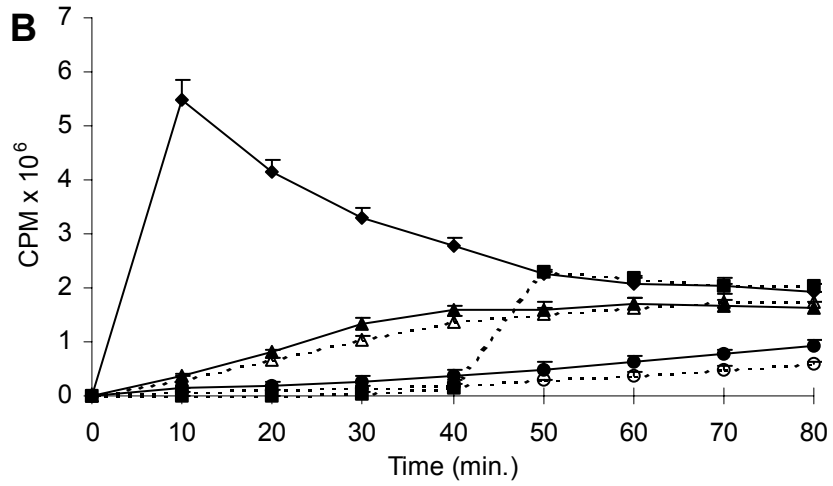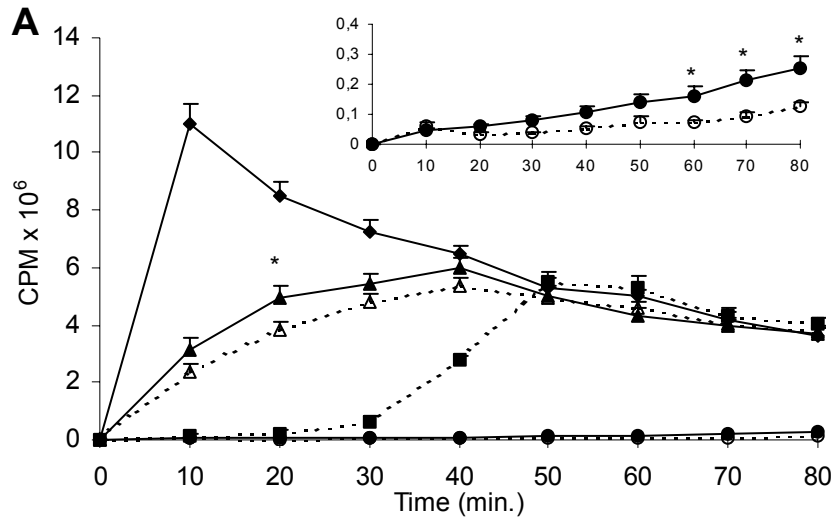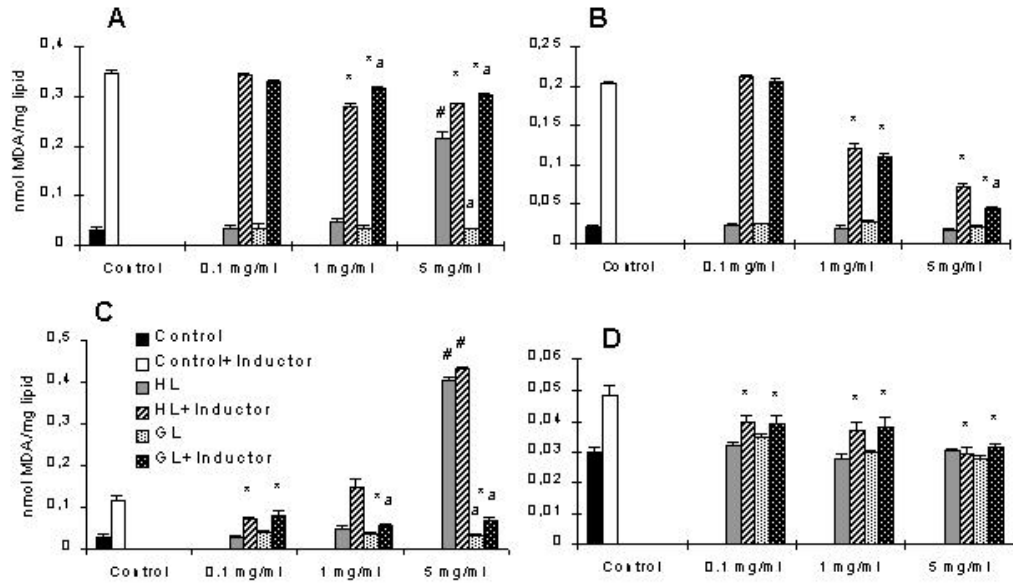
Fig.4 Da Silva EG

Fig.5 Da Silva EG

4. ARTIGO 2

"Pronouced Sexual Dimorphism of a Gall-Former Coccid Correlate with Alterations in Phenolic Content and Antioxidant Properties of *Rollinia laurifolia* (ANNONACEAE) Leaves"

(Submetido à Oikos)

Pronounced sexual dimorphism of a gall-former coccid correlate with alterations in phenolic content and antioxidant properties of *Rollinia laurifolia* Schldtl. (ANNONACEAE) leaves

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Abstract

Galls are characterised by an abnormal plant tissue growth in response to specific stimuli from the invading organism: bacteria, fungi, nematodes and arthropods. The host plant-inducer interaction changes the chemical and physiological status of plant tissues during cecidogenesis. Several types of biotic and abiotic stimuli are known to alter the production of phenolic compounds in plants. These compounds are involved in plant defence against pests and pathogens. A species of coccid induces galls in leaves of *Rollinia laurifolia* specimens. The coccids are characterised by pronounced sexual dimorphism, which is expressed even in the galls. Females galls are relatively large, whereas male galls are reduced in size. The aim of this study was to verify the chemical/ecological relationships between females galls (FG), males galls (MG), healthy tissue of galled leaves (HTGL) and healthy leaves (HL) of *R. laurifolia* by examining the influence of gall-former sex in the phenolic contents of the leaves, and the alterations in antioxidant properties related to gall sex formation. Total phenolic content and total radical-trapping antioxidant parameter (TRAP) were analysed. The presence of FG and MG induced chemical differences between galled and healthy tissues. The occurrence of galls in *R. laurifolia* is related to a significative reduction in total phenolic content of the leaves. Total antioxidant potential was altered in FG, MG and HTGL in comparison with HL. Our results suggest that gall inducer/*R. laurifolia* interaction change the phenolics content in plant related to sexual dimorphism of gall former in order to protect the plant and possibly the coccid due oxidative stress established during cecidogenesis. Oxygen diffusion in the *Rollinia laurifolia* leaf tissue during cecidogenesis may be a signal for triggering responses. The role of phenolics in adaptive significance and evolutionary consequences of sexes of galls are discussed.

Keywords: *Rollinia laurifolia,* Coccid, galls, sexual dimorphism, phenolic compounds, antioxidant

Introduction

Galls result of a close association between an inducer (bacteria, fungi, nematodes and arthropods) and a plant species characterised by an abnormal plant tissue growth in response to specific stimuli from the invading organism (Meyer 1987). The host plant-inducer interaction changes the growth and development of plant tissues during cecidogenesis and is specific to each type of gall (Hartley 1998). The ability to induce galls on plants had multiple independent origins, and there are evidences of arthropods-plant interactions in fossil leaves from the Upper Triassic, which could have provided nourishment and shelter for several types of arthropods, and included a well-defined type of gall (Ash 1996). One of the most plausible explanations for this interaction is suggested by the nutrition hypothesis (Price et al. 1986), which assumes that gall inducers are able to manipulate their hosts into producing tissues that are nutritionally superior to other plant parts, because they contain high amounts of nutrients and/or low concentration of defensive chemicals.

As an unfortunate consequence of aerobic life, free radicals and another active oxygen species (AOS) are formed by biological redox reactions (Halliwell and Gutteridge 1998). The increased production of toxic oxygen derivatives is considered to be an universal or common feature of stress conditions. Plants developed a wide range of mechanisms in response to this problem, and it is becoming evident that AOS (which are generated during pathogen attack and abiotic stress situations) are recognised by plants as a signal for triggering defence responses (Vranová et al. 2002). Adequate responses to enhanced production of AOS within several subcellular compartments of plant cell are crucial for plant growth and survival. The level and kind of AOS are determining factors for the type of response (Van Breusegem et al. 2001).

Several types of biotic and abiotic stimuli are known to alter the production of phenolic compounds in plants (Van Sumere and Lea 1985), which constitute an important response to environmental stress. Differences in the content and complexity of phenolic compounds in galled and healthy leaves have been

examined (Kraus and Spiteller 1997, Nyman and Julkunen-Tiitto 2000), and could be influenced by gall inducer (Hartley 1998). In cases where several gall-inducing species attack the same host plant species, each one causes the plant tissues to be reorganised in a diverse way, producing characteristic and species-specific structures (Shorthouse and Rohfritsch 1992). General patterns in chemical composition of galled in relation to ungalled tissues across a range of species may prove hard to be established (Hartley 1998), however very few information is available about biochemical and antioxidant changes related to cecidogenesis.

Rollinia laurifolia Schldtl. belongs to the Annonaceae, a large pantropical family, with taxonomical, ecological, and pharmacological interest (Leboeuf et al. 1982, Hufford and Oguntimein 1982, Santos and Salatino 2000). An unidentified species of coccid (Coccoidea: Eriococcidae) induces galls in leaves of *R. laurifolia* specimens. The coccid or scale insects are characterised by pronounced sexual dimorphism, which is expressed even in the galls (Cook et al. 2000). Females galls (gynocecidia) are relatively large, whereas male galls (androcecidia) are reduced in size. The morphological differentiation of these galls, especially gynocecidia is extremely elaborate (Ben-Dov and Hodgson 1997) and several genera are monophagous on the host. Eriococcid galls were largely studied in Australia and New Zealand, but just from the gall inducers taxonomic standpoint (Gullan 1978, 1984).

Changes in chemical profile of *R. laurifolia* leaves, due to cecidogenesis were recently described (Soares et al. 2000). The aims of this study were to investigate the ecological/biochemical relationships between female gall (FG), male gall (MG), healthy tissue of galled leaves (HTGL) and healthy leaves (HL) of *R. laurifolia,* by examining if the presence of sexual dimorphism of gall-inducer changes the antioxidant properties and/or the phenolic contents of the leaves. This investigation should provide new insights into gall-inducer/host plant interactions in Annonaceae, and evaluate its secondary compounds antioxidant potentials.

Materials and Methods

Chemicals

AAPH (2,2'-Azobis [2-methylpropionamidine] dihydrochloride); luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione); glycine (amino acetic acid); DMSO (dimethyl sulfoxide) and Folin-Ciocalteau (phenol reagent), were purchased from Sigma Chemicals, St. Louis, MO, USA. Trolox (6-hydroxy-2, 5,7,8-tetramethyl-chroman-2-carboxilic acid) and tannic acid were purchased from Aldrich Chemicals, Milwaukee, WI, USA. Cellulose nitrate membrane was purchased from Merck chemicals, Darmstadt, Germany. Deionised water was used for all experiments and all other reagents used in this study were of analytical grade.

Plant material and extraction

The leaves of *R. laurifolia* tree were collected in Ecological Station of Universidade Federal de Minas Gerais, Pampulha *Campus*, Belo Horizonte, Minas Gerais, Brazil. The reference plant material is BHCB 253331 deposited at the herbarium of Departamento de Botânica of UFMG.

The methodology used was the classical phytochemical approach for polar phenolic derivatives rich extraction (Harbone 1984). Galled and healthy leaves were collected in 2001 February. Samples of female gall (FG), male gall (MG) and healthy tissue of galled leaves (HTGL) were cut with a leaf cutter from galled leaves and lyophilised. Approximately 2 g of these plant materials and healthy leaves (HL) lyophilised were extracted in Sohxlet, Warburg equipment with hexane P.A. (6h) to eliminate low polarity compounds, followed by exhaustively extraction with ethanol P.A. (24 h). The crude ethanolic extracts were dry at low pressure, lyophilised and stored at -5°C. Stock solutions of extracts were prepared immediately before use with 25 mg of dry extract and 5 mL of solvent (deionised water or DMSO 0.07%). Solutions were sonicated for 2 min. (4 X 30'') with potency 4 (Sonicator XL 2020 Heat Systems Inc.) and filtered in cellulose nitrate membrane (pore size of 0.45 μm). These stock solutions with final concentration of 5 mg/mL were diluted up to the other concentrations used in this study.

Total phenolic content

The total phenolic contents (Waterman and Mole 1994) were determined in FG, MG, HTGL and HL extracts by colorimetric assay, in which purified tannic acid was used as standard. Extracts (25 mg) were dissolved in 5 mL of water or DMSO 0.07% and homogenised as describe above. A sample of 0.5 mL from each extract had its volume completed up to 7 mL with water, 0.5 mL of Folin reagent 1N was added, the mixture vortexed, and 1mL of Na_2CO_3 saturated solution was added and homogenised. The final volume was completed up to 10 mL with water and used for determination of phenolic contents. The absorbance was measure at 725 nm in spectrophotometer (Beckman DU-640), and results are expressed as µg of phenol/mg of extract.

Total radical-trapping antioxidant parameter (TRAP)

An adapted method of TRAP assay was used to determine the capacity of ethanolic extracts homogenised in water or DMSO 0.07% to trap a flow of water-soluble peroxyl radical produced at constant rate, through thermal decomposition of AAPH (Ghiselli et al. 2000). Briefly, the reaction mixture (4 mL) containing the free radical source (AAPH 10 mM) in glycine buffer (0.1 M) pH 8.6; test samples (10 µL) at concentrations of 0.5 - 5 mg/mL and luminol (4 mM) as external probe to monitoring radical production, were incubated at 25°C. Chemiluminescence produced was directly proportional to radical generation and measured in out of coincidence mode in a liquid scintillation counter (Wallac 1409) as counts per minute (CPM). The relation between antioxidant potential and reading (CPM) was inverse, i.e. high reading indicates poor antioxidant potential. One cycle was the time to read all samples (10 min.) and repeated eight times to monitor the decline of free radical source and the consume of samples antioxidant potential.

Statistical analysis

All data are expressed as the mean ± S.E. and analysed by ANOVA. *p* values less than 0.05 were considered significant and Newman-keuls test was used to compare the groups when adequate.

Results

Total phenolic content

The determination of total phenolic content of *R. laurifolia* ethanolic extracts showed that HL have higher amounts of phenolics when compared with FG, MG and HTGL solubilised in water or DMSO 0,07% (Fig. 1). FG material has a higher phenolic content than MG and HTGL when water or DMSO 0,07% were used as solvents (Fig 1). MG contain more phenolics than HTGL only when water (Fig. 1A), but not DMSO 0.07% (Fig. 1B) was used as solvent.

Total radical-trapping antioxidant parameter (TRAP)

The data obtained by TRAP assay firstly indicate that every extract possess antioxidant capacity in comparison with its solvent (Figs. 2 and 3). Antioxidant capacity of extracts solubilised either in water or DMSO 0.07% was observed by reduction in chemiluminescence intensity inversely proportional to extracts concentration (Figs. 2 and 3). These results also indicate that in concentration of 2 and 5 mg/mL the extracts (solubilised in water or DMSO 0.07%) are more effective than Trolox (7μM) which is a water-soluble analogous of α-tocopherol and used as reference scavenger molecule (Figs. 2C, 2D, 3C and 3D). The concentration of 1 mg/mL solubilised in water show us what all extracts are more effective than Trolox (Fig. 2B), whereas when the solvent was DMSO 0.07% only HL and FG were more efficient than Trolox in this concentration (Fig. 3B). MG and HTGL extracts demonstrate a comparable behaviour in all concentrations in TRAP assay when DMSO 0.07% was used as solvent (Fig. 3); nevertheless when the solvent used was water the course of action in TRAP assay was very distinct among every extracts in all concentrations tested (Fig. 2).

Discussion

The scale insects, which include more than 4,000 species in a number of families, are distributed throughout most of the world and are among the most destructive pests of plants (Linsenmaier 1972). Like aphids, but to a lesser extent, certain species of scale insects form galls, and others shift from one kind of plant to another. However scale insects do not have the diversity of generation-types that the aphids do, though the individual development stages of scale insects differ much more strongly, as do the two sexes (Cook et al. 2000). Females are wingless, and the highly specialised kinds are so greatly modified, in keeping with their completely sessile way of life, that they scarcely can be recognised as insect. Legs, antennae, and eyes may disappear more or less completely, and the broadly oval body, often with characteristic shield- or bowl-shape, hardly reveals any segmentation. Only the nymphs in the first stage of development are motile and spread over the plant; after these nymphs have settle down, all further developments, such as molting and reproduction, take place more or less in a single spot (Ben-Dov and Hodgson 1997, Cook et al. 2000). Male scale insects are much smaller than females, more active, and shorted-lived. They may have wings, wings reduced to vestiges, or no wings at all; when present, the hind wings are tiny. The eyes are present as simple eyes or compound eyes (Ben-Dov and Hodgson 1997, Cook et al. 2000). The Eriococcidae are a group of insects with a high specific cycle of life and with a particularly close relationship with the cycle of their hosts. Within this group there are species most of which produce galls that are species-specific in shape and sexually dimorphic (Cook et al. 2000). We observed that the presence of a gall-former is required to maintain the chemical differences between galled and healthy tissues and, this difference is related to sex of gall-former. Synchronisation of herbivores with their host plant phenology determines quality and quantity of food resources, and affects the preference-performance linkage and abundance of herbivores. In addition, synchronisation has a more critical meaning for such gall-former than for other insects (Yukawa 2000). The differences clearly

are present in gall formation and we also observed differences at the biochemical level. We suggest that sexual dimorphism of erioccoccids are present in extended phenotype (Dawkins 1982) of galls, in morphology and in biochemical levels.

Phenolic compounds can act as chemical signals of several environmental stresses (Karban and Baldwin 1997, Waterman and Mole 1994, Rosenthal and Berenbaum 1992) and is incontestable the participation of these substances in plant-insect interactions (Harborne 1984). The quantitative changes observed on phenolic derivatives of *R. laurifolia* FG, MG and HTGL are likely to be metabolic responses related to gall formation, and reinforces the importance of these compounds as signals of plant-inductor interaction.

Increased levels of phenolic compounds have been reported in several plant galls (Abrahamson et al. 1991). According to Hartley (1998), gall tissue generally contained lower levels of nitrogen and higher levels of phenolic compounds than ungalled plant tissue. In these cases phenols could potentially play a role in gall formation by influencing the hormonal control of growth and/or in protecting the gall-former from its natural enemies (Abrahanson et al. 1991). Amudhan et al. (1999) suggest that a variation of total phenols at the stem bases in genetically related rice varieties in relation to the gall midge infestation can be an expression of plant resistance involving hypersensitive reaction against the insect conferred by the gene Gm2. Our results are similar to few works that found negative correlation between phenolic content and presence of galls. Nyman and Julkunen-Tiitto (2000) studied the changes in phenolic chemistry of willows (*Salix* genus) by gall-inducing sawflies, and they found that the concentrations of most defensive phenolics are substantially lower in gall interiors than in leaves. In other hand, Espirito-Santo and Fernandes (1998) found no relationship between tannin concentration and gall abundance in *Baccharis dracunculifolia* infected with *Neopelma baccharidis* (Homoptera: Psyllidae) and associated this absence of chemical impact of *Neopelma* galls on *B. dracunculifolia* with the feeding habit of galling insects. The interaction between *Picea glauca*

(Pinaceae) and *Adelgis abietes* (Aphidae) is another gall model that shows a decrease of phenolic content in infected host tissues (Kraus and Spiteller 1996). Spruce shoots contains two to ten fold more phenolics than galls and qualitative changes were also detect in these plant materials. Our results suggests that similar phenomena occurs in *R. laurifolia* galls.

The most common methodology of phenolic content analysis in plant extracts, using Folin-Ciocalteu as reagent and tannic acid as standard (Waterman and Mole 1994), detects mainly water-soluble phenolic derivatives. Some frequent biosynthetic modifications on phenolic compounds (O- and C-methylation, glycoside hydrolysis, condensation) produce less polar derivatives that could be less water-soluble than its precursors. The analysis of phenol content can clearly show the changes in phenolic metabolism related to sexual dimorphism of plant galls.

Plant phenolics constitute a large group of natural compounds, ubiquitous in the plant kingdom. These secondary metabolites are known to display a remarkable array of biochemical interactions, which believed to come from its antioxidant properties (Rice-Evans et al. 1995). These substances may act as potent metal chelators and/or free radical scavengers (Hanasaki et al. 1994). However, it has been demonstrated that the behaviour of these compounds in oxidative systems is a function of activity-structure relationships (Cao et al. 1997, Rice-Evans et al. 1996). To validate the hypothesis that cecidogenesis alters the biochemical properties of phenolic compounds of *R. laurifolia,* we evaluated the antioxidant potential of FG, MG, HTGL and HL ethanolic extracts.

In order to determine the antioxidant potential of extracts a simple method based on luminol-enhanced chemiluminescence was used, which is based on the measurement of oxidation of AAPH, a free radical source. This method is based on capacity of some compounds to trap peroxyl radicals ($ROO^{\bullet}$), and is capable of detecting most of the compounds with significant antioxidant activity present in complex mixtures such as plant extracts. The addition of increasing concentrations of

extracts solubilised in water or DMSO 0.07% to the reaction medium resulted in a reduction of luminol-enhanced chemiluminescence, indicating the presence in all extracts of compounds with peroxyl radicals scavenger capacity. The concentration of DMSO 0.07% or water did not interfere in the flow of radical produced by the system. The increasing order of antioxidant potential of extract solubilised in water observed in TRAP assay was HTGL<MG<FG<HL (Fig. 2). However when solubilised in DMSO 0.07% no differences was observed among MG and HTGL. The increasing order of antioxidant potential of extract solubilised in DMSO 0.07% in TRAP assay was HTGL=MG<FG<HL (Fig.3).

Taking into account results of phenolic content and TRAP assays, we suggest that the decrease in phenolic content of FG, MG and HTGL was related with the decrease in antioxidant potential in the TRAP assay of the extracts in comparison with HL when water was used as solvent. However, when solubilised in DMSO 0.07%, the phenolic content in MG and HTGL was similar, as was antioxidant potential of the extracts. It is plausible that the antioxidant potential was in part due to phenolic content of extracts since these metabolites are known to display antioxidant properties (Hanasaki et al. 1994). Nevertheless, increased phenolic content do not necessarily imply in higher antioxidant potential, since the behaviour of these compounds in oxidative systems may alternate from prooxidant to antioxidant, as a function of activity-structure relationships (Cao et al. 1997).

Oxidative stress is a situation of imbalance between production of AOS and antioxidant defences, leading to potential damage. A wide range group of biotic and abiotic agents are recognised as potentially harmful to plants. A common aspect of all these adverse conditions is the enhanced production of AOS within subcellular compartments of cells (Lamb and Dixon 1997). AOS can be extremely reactive and oxidise biological molecules, such DNA, proteins and lipids (Halliwell and Gutteridge 1998). Aside from their harmful nature, AOS can have an important role in inducing

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protection mechanisms during both biotic and abiotic stresses, acting as signal transduction molecules (Vranová et al. 2002).

Based on our findings, we suggest that the coccid gallicolae and plant in cecidogenous process have the ability to manipulate the phenolic biosynthesis on infected leaves of *R. laurifolia*. This manipulation is achieved by decreasing water-soluble defensive chemicals and altering the polarity of phenolics. The results allow us to suggest that gall inducer/*R. laurifolia* interaction induced changes in the polarity of phenolics is related to sexes of insect in the plant to protect *R. laurifolia* and possibly the coccid from oxidative stress during cecidogenesis. Oxygen diffusion in the leaves tissue during cecidogenesis may be a signal for triggering responses for radical scavenging by the plant phenylpropanoid pathway (Grace and Logan 2000). Close and McArthur (2002) suggest an alternative role for most phenolics: antioxidant function for protection against oxidative pressure that produce EAO during photosynthesis generating photodamage. Larson (1998) observed that the photosynthetic rates in galled leaves may be reduced, what may likely cause changes in nutrient status. Understanding the primary role of phenolics, and hence their selective agents at the evolutionary level or elicitors at the ecological level, is also important (Close and McArthur 2002). However the adaptive significance and evolutionary consequences of gall induction and AOS triggering signals in galled tissues require further studies.

Differences in phenolic content may be a good marker in cecidogenesis (Nyman and Julkunen-Tiitto 2000). TRAP assay is a good tool to assess antioxidant potential of extracts since TRAP measurements indicate the quantity and the quality of antioxidants (Ghiselli et al. 2000) present in the plant extracts. Phenolics as main components of ethanolic extracts may be an excellent comparison index due to its antioxidant properties, and we suggest that an antioxidant approach may be a good indicative for the alterations in phenolic contents between healthy and galled leaves.

Insect-induced galls are observed on plants throughout the world, but a general model of gall-inducing insects across a range of species may be complex to establish (Hartley 1998). Many studies have reported phenolic compounds of galled leaves related to healthy leaves; nevertheless, there are no studies which analysis phenolics from HL in comparison with FG, MG and HTGL. To the best of our knowledge, no studies about antioxidant properties of extracts were performed in this context. The Annonaceae represent a large family comprising approximately 120 genera and 2000-2200 species (Cronquist 1988) and there are some genera and several species of Annonaceae native to Brazil. Eriococcidae comprise a particularly interesting group from the cecidogenetic standpoint. Unfortunately, little is known about their galls, apart from the gall morphology; no work has yet appeared concerning either their biochemical or ecology interaction with their host. Presently identification of the Eriococcidae is current in Gales. The Eriococcidae/*R. laurifolia* interaction certainly offers a potentially fruitful field of research for cecidology, however additional approaches are needed to gain a more complex picture of gall-former impact on this host plant tissues as well is the ecological signification of this interaction.

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Legends

Fig. 1 Total phenolic content of ethanolic extracts from healthy leaves (HL) female galls (FG), male galls (MG) and healthy tissue of galled tissue (HTGL) of *R. laurifolia*. solubilised in water (A) and DMSO 0.07% (B). Experiments were performed in tetraplicate and tannic acid was used as standard. a, b, c and d indicate significant difference between phenolic content of extracts $p < 0.05$.

Fig. 2 Total radical-trapping antioxidant parameter (TRAP) of ethanolic extracts solubilised in water of healthy leaves (HL), female galls (FG), male galls (MG) and healthy tissue of galled tissue (HTGL) of *R. laurifolia*. Experiments were performed in triplicate. Trolox 7μM (---- ----) was used as reference molecule and control group (----◆----) was solvent only. Fig. 2A, 0.5 mg/mL, Fig. 2B, 1 mg/mL, Fig. 2C 2 mg/mL (control and trolox not show), Fig. 2D 5 mg/mL (control and trolox not show). HL (----▲----), FG (----●----), MG (----↓----) and HTGL (----⇨----).

Fig. 3 Total radical-trapping antioxidant parameter (TRAP) of ethanolic extracts solubilised in DMSO 0.07% of healthy leaves (HL), female galls (FG), male galls (MG) and healthy tissue of galled tissue (HTGL) of *R. laurifolia*. Experiments were performed in triplicate. Trolox 7μM (---- ----) was used as reference molecule and control group (----◆----) was solvent only. Fig. 3A, 0.5 mg/mL, Fig. 3B, 1 mg/mL, Fig. 3C 2 mg/mL, Fig. 3D 5 mg/mL (control and trolox not show). HL (----▲----), FG (----●----), MG (----↓----) and HTGL (----⇨----).
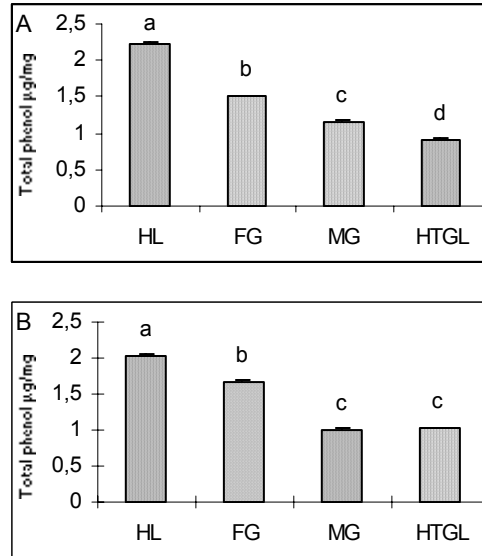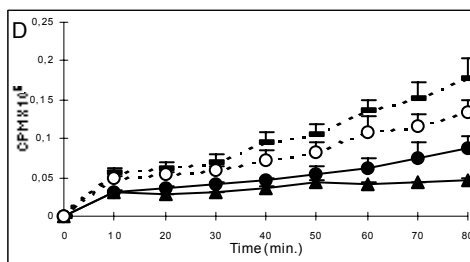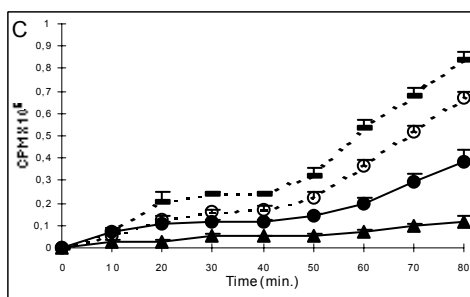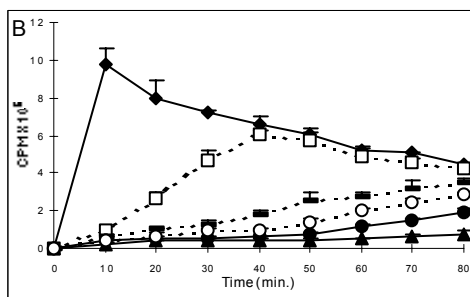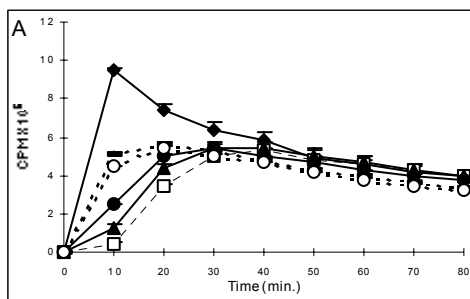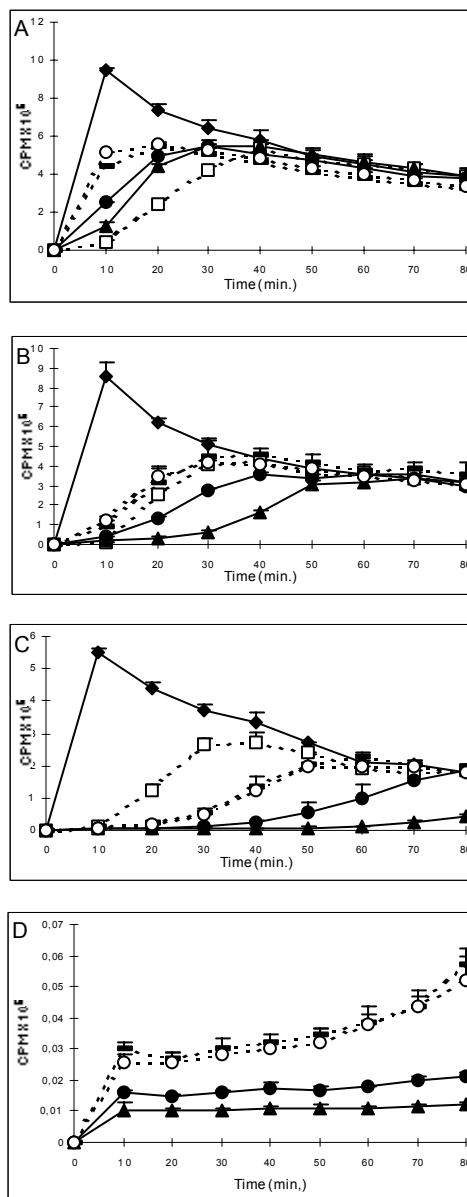
Fig. 1

Fig. 2

Fig. 3

5. DISCUSSÃO

Os insetos formadores de galhas são organismos com alto grau de adaptação ao ciclo de vida de seu hospedeiro. Diversos trabalhos demonstram que a presença de um agente cecidogênico é essencial para estabelecer diferenças no perfil químico entre folhas sadias e galhadas (Westphal et al, 1981; Kraus e Spiteller 1997; Soares et al, 2000; Nyman e Julkunen-Tiitto 2000). Muitos aspectos devem ser levado em conta ao analisar-se as diferenças estabelecidas entre os extratos de folhas sadias e galhadas. A taxa fotossintética pode ser diminuída em folhas galhadas (Larson, 1998), o que pode levar à diminuição na produção de compostos energéticos. Tal fato, nos faz pensar sobre o primeiro e um dos maiores dilemas encontrado pelas plantas: crescer ou defender-se (Herms e Mattson, 1992). A sincronização do ciclo de vida dos herbívoros com a fenologia da planta, determina a quantidade e qualidade dos recursos nutricionais, afetando a preferência e abundância de herbívoros. Além disto a sincronização parece ser mais crítica para os insetos galhadores do que para outros insetos (Yukawa, 2000). O fato de o coccideo galhador apresentar um dimorfismo sexual acentuado, e que este dimorfismo reflete-se não somente nas galhas formadas, mas também no conteúdo fenólico e nas propriedades antioxidantes destas galhas, mostra-nos que a planta hospedeira demonstra uma plasticidade genética que é explorada pelo inseto indutor, apresentando-se também no fenótipo estendido (Dawkins, 1982) de galhas machos e galhas fêmeas.

Os compostos fenólicos podem atuar como sinalizadores químicos de vários estressores ambientais (Karban e Baldwin 1997; Waterman e Mole 1994; Rosenthal e Berenbaum 1992) e é incontestável a participação destas substâncias na interação

inseto/planta (Harbone, 1984). As mudanças qualitativas e quantitativas nos derivados fenólicos de folhas galhadas demonstrada por nós primeiramente, reforçam a importância destes compostos como sinalizadores na interação inseto/planta. Secundariamente, observamos que o conteúdo fenólico também está alterado em galhas masculinas e femininas, sugerindo que estas alterações estão relacionadas ao sexo do organismo indutor de galhas. Nossos resultados sugerem que um possível mecanismo de alteração qualitativa e quantitativa no conteúdo fenólico no material coletado, pode estar associado a uma diminuição na polaridade do mesmo (fig. 7), tornando-o mais lipossolúvel possivelmente para proteger biomembranas, não somente da folha galhada, mas até mesmo do organismo indutor.

Os insetos conhecidos como ericoccideos estudados neste trabalho, têm um ciclo de vida intimamente relacionado ao ciclo da planta hospedeira, sincronizando seu desenvolvimento ao desenvolvimento sasonal das folhas de *R. laurifolia*. Diversos trabalhos confirmaram que a interação inseto/planta expressa em galhas, vem ocorrendo há milhões de anos (Larew, 1986; Diéguez et al, 1996; Ash, 1996), sendo possível sugerir que tanto galhador e *R. laurifolia* caminham para encontrar uma estratégia de sobrevivência evolutivamente estável para ambos. Uma das mais plausíveis explicações para isto é sugerida pela hipótese nutritiva (Price et al, 1986), que assume que o indutor de galhas é apto a manipular seu hospedeiro a produzir tecido que é nutricionalmente superior a outras partes da planta, porque contem altas concentrações de nutrientes e/ou baixa concentrações de defesas químicas. Neste caso observado por nós, a manipulação seria uma diminuição no conteúdo fenólico nas folhas atacadas, e tornando este conteúdo menos polar. Podemos também sugerir que esta interação pode ter algum valor adaptativo não somente para o inseto,

mas para a planta, podendo a mesma neutralizar os efeitos nocivos do galhador sobre suas células.

Os derivados fenólicos, são um grande grupo de moléculas, largamente distribuídas no reino vegetal. Estes metabólitos secundários são conhecidos pelas suas propriedades antioxidantes em diversos sistemas biológicos (Rice_evans et al, 1995). Diferenças no conteúdo fenólico podem ser um bom marcador na cecidogênese (Nyman e Julkunen-Titto). Os ensaios antioxidantes podem ser boas ferramentas para acessar a quantidade e qualidade de compostos antioxidantes presentes em misturas complexas como extrato vegetais (Ghiselli et al, 2000). No nosso conhecimento, não há trabalhos que examinem alterações na capacidade antioxidante de extratos de folhas galhadas em comparação com folhas sadias. Os compostos fenólicos são os principais componentes de extratos deste tipo estudado por nós; e sugerimos que ensaios antioxidantes podem ser um bom parâmetro de comparação para indicar alterações não somente no conteúdo, mas também na qualidade destes extratos.

Podemos especular que alterações quantitativas e qualitativas no conteúdo fenólico de plantas galhadas podem estar associados ao processo de estresse oxidativo. Considera-se comum e universal este processo desencadeado durante situações de estresse (Halliwell e Gutterdge, 1998). Particularmente no reino vegetal, a produção aumentada de EAO vivida pelas plantas durante situações de estresse biótico e abiótico, pode servir como uma sinalização para disparar defesas antioxidantes (Vranová et al, 2002). Sugerimos que durante o processo de cecidogênese a difusão de oxigênio atmosférico através do tecido foliar, pode estar interferindo diretamente na sinalização química da planta, disparando defesas e
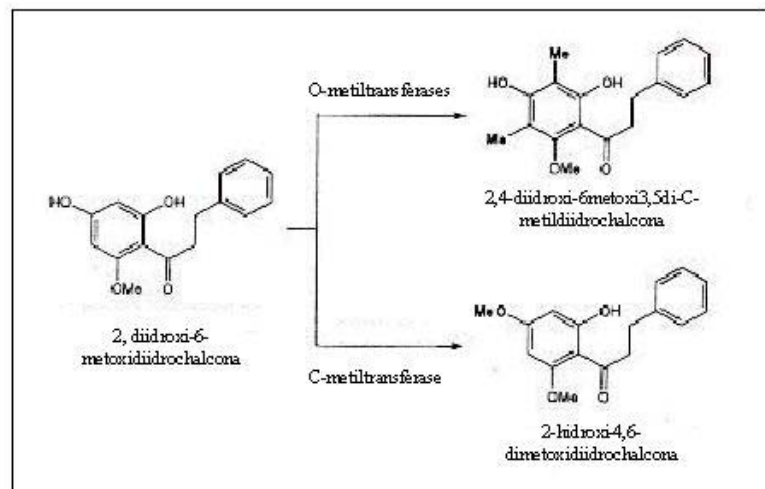
Fig. 7 - Formação de derivados fenólicos menos polares a partir de um mesmo precursor biossintético. Os flavonóides exemplificados foram isolados de *Uvaria angolensis* (ANNONACEAE)

Fonte: Soares et al, 2000

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preparando a planta para lidar melhor com esta situação, gerando compostos com menor potencial antioxidante e menos polares para proteger suas membranas e ser menos danosos ao galhador, mas que permanecem mais tempo ativos em sistemas lipofílicos, onde EAO iniciam seu ataque vindo através da atmosfera.

Insetos indutores de galhas são observados no mundo inteiro, porém padrões gerais de alterações na fenologia da planta são complexos de estabelecer (Hartley, 1998). Este é um trabalho inicial, e a interpretação do significado da formação das galhas vegetais está sujeita a controvérsias. A interação *R. laurifolia*/galhador pode ser um bom modelo de estudos na cecidogênese, entretanto estudos adicionais são necessários para obtermos uma melhor interpretação sobre o impacto do galhador sobre *R. laurifolia* e sobre o significado ecológico/bioquímico desta interação.

6. CONCLUSÕES

▶ Há alterações quantitativas e qualitativas no conteúdo fenólico de folhas galhadas de *R. laurifolia*;

▶ Houve uma diminuição no conteúdo fenólico das folhas galhadas, esta diminuição está relacionada à uma diminuição na polaridade dos mesmos;

▶ Os ensaios antioxidantes mostraram-se capazes de revelar estas diferenças;

▶ O sexo do galhador influencia o conteúdo fenólico das galhas;

▶ O dimorfismo do galhador influencia também na polaridade dos compostos extraídos;

▶ O ensaio antioxidante TRAP também mostrou a diferença qualitativa que existe na produção de compostos fenólicos em galhas macho e galhas fêmeas;

▶ O modelo *R. laurifolia*/galhador mostrou-se útil neste estudo inicial de cecidogênese;

▶ O dimorfismo sexual apresentado pelo galhador também foi útil neste estudo inicial de cecidogênese em *R. laurifolia*;

7. PERSPECTIVAS

Como perpectivas pretendemos:

▸ isolar os componentes que marcam as diferenças qualitativas dos extratos através de HPLC;

▸ confirmar a espécie do galhador;

▸ quantificar enzimas marcadoras de estresse nas folhas;

▸ traçar outros paralelos como: biometria, morfologia, ecologia e fisiologia tanto do galhador como de *R. laurifolia*;

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