UNIVERSIDADE FEDERAL DE PERNAMBUCO

CENTRO DE CIÊNCIAS BIOLÓGICAS

MESTRADO EM BIOQUÍMICA E FISIOLOGIA

PURIFICAÇÃO, CARACTERIZAÇÃO, PROPRIEDADES BIOLÓGICAS DA LECTINA DE RIZOMA DE *Microgramma vaccinifolia* E ESTUDO MOLECULAR DE *Fusarium oxysporum* f. sp. *lycopersici*

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Recife, 2009

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LIDIANE PEREIRA DE ALBUQUERQUE

"Purificação, caracterização, propriedades biológicas da lectina de rizoma de *Microgramma vaccinifolia* e estudo molecular de *Fusarium oxysporum* f. sp. *lycopersici*"

Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco

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LISTA DE ABREVIATURAS

Introdução

AH	**Atividade Hemaglutinante**
ACL-I	**Lectina de *Axinella corrugata***
AFLP	**Do inglês *Amplified Fragment Length Polymorphism***
ARDRA	**Do inglês *Amplified Ribosomal DNA Restriction Analysis***
BmoLL	**Lectina de folhas de *Bauhinia monandra***
CL_{50}	**Concentração letal média**
Con A	**Concanavalina A**
CM	**Carboximetil**
CT_{50}	**Concentração tóxica média**
cDNA	**DNA complementar**
DEAE	**Dietilaminoetil**
DGGE	**Do inglês *Denaturing Gradient Gel Electrophoresis***
DNA	**Ácido dexoxirribonucléico**
EDTA	**Ácido etilenodiaminotetraacético**
FPLC	**Do inglês *Fast Performance Liquid Chromatography***
HPLC	**Do inglês *High Performance Liquid Chromatography***
HSL	**Lectina de rizoma de *Holothuria scabra***
ISSR	**Do inglês *Inter Simple Sequence Repeat***
OJL	**Lectina de rizoma de *Ophiopogon japonicus***
PAGE	**Do inglês *Polyacrylamide gel electrophoresis***
PCL	**Lectina de sementes de *Phaseolus coccineus***
PCR	**Do inglês *Polymerase Chain Reaction***
RAPD	**Do inglês *Random Amplified Polymorphic* DNA**
RISA	**Do inglês *Ribosomal Intergenic Spacer Analysis***
RFLP	**Do inglês *Restriction Fragment Length Polymorphism***
SCL	**Lectina de *Schizophyllum commune***
SGM-2	**Lectina de rizoma de *Smilax glabra***
SSR	**Do inglês *Simple Sequence Repeat*s**

Artigo:

ADL	**Lectina de *Arundo donax***
CFU	**Do inglês *Colony Forming Units***
CTAB	**Do inglês Cetyltrimethylammonium Bromide**
HA	**Do inglês *Hemmaglutinating activity***
LC	**Do inglês *Lethal Concentration***
MvRL	**Lectina de rizoma de *Microgramma vaccinifolia***
NA	**Do inglês *Nutrient Agar***
PAS	**Do inglês *Schiff periodic acid***
PDA	**Do inglês *Potato Dextrose Agar***
PVP	**Do inglês *Polyvinylpyrrolidone***
RNA	**Ácido Ribonucléico**
SDS	**Do inglês *Sodium Dodecyl Sulphate***
SHA	**Do inglês *Specific hemagglutinating activity***
TE	**Tampão Tris-EDTA**
VCG	**Do inglês *Vegetative compatibility group***
UPGMA	**Do inglês *Unweighted Pair Group Method Using Arithmetic Average***

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RESUMO

Lectinas são proteínas que se ligam a carboidratos e glicoconjugados. Rizomas de *Microgramma vaccinifolia* têm ampla utilização na medicina popular no tratamento de hemoptises e hematúria. Os objetivos deste trabalho foram isolar, caracterizar, avaliar as atividades tóxica, antibacteriana e antifúngica da lectina de rizoma de *M. vaccinifolia* (MvRL) e identificar as raças 1, 2 e 3 de *Fusarium oxysporum* f. sp. *lycopersici* por biologia molecular. As proteínas do extrato de rizoma (ER) foram fracionadas com sulfato de amônio fornecendo a fração 0-60% (F0-60%). Atividade hemaglutinante (AH) e concentração de proteína foram determinadas em ER e F0-60%. MvRL foi isolada por cromatografia da F0-60% em Sephadex G-25. A AH de MvRL foi avaliada em presença de carboidratos, glicoproteínas, preparações contendo carboidrato das raças 1, 2 e 3 de *Fusarium oxysporum* f. sp. *lycopersici*, em diferentes temperaturas, valores de pH e na presença de íons. Eletroforese em gel de poliacrilamida de MvRL foi realizada em condições nativas (PAGE) e desnaturadas (SDS-PAGE). O efeito de MvRL sobre *Artemia salina*, bactérias e fungos foi também avaliado. O DNA das raças 1, 2 e 3 de *Fusarium oxysporum* f. sp. *lycopersici* foi isolado utilizando marcadores moleculares ISSR e RAPD. MvRL aglutinou eritrócitos humanos e sua AH foi inibida por manose, soro fetal bovino e preparações de *F. oxysporum* f.sp. *lycopersici*. A AH de MvRL foi termoestável, ativa em pH 5,0 e dependente de íons. PAGE revelou MvRL como uma proteína ácida e SDS-PAGE revelou banda polipeptídica glicosilada de massa molecular 17 kDa. Cromatografia de gel filtração definiu a massa molecular nativa de MvRL como 100 kDa indicando a lectina como um agregado molecular. MvRL foi tóxica sobre *A. salina* (CL_{50} de 154,16 µg/mL), não exibiu atividade antibacteriana e apresentou atividade antifúngica. MvRL foi mais ativa em inibir o crescimento da raça 3 de *F. oxysporum* f.sp. *lycopersici.* Os marcadores moleculares foram adequados para avaliar a variabilidade genética das raças. Em conclusão MvRL é uma lectina com atividade antifúngica e o efeito sobre *F. oxysporum* f.sp. *lycopersici* foi diferente para as três raças.

Palavras-chave: Atividade antifúngica, *Fusarium oxysporum* f.sp. *lycopersici*, ISSR, lectina, *Microgramma vaccinifolia,* RAPD.

ABSTRACT

Lectins are proteins that bind carbohydrates and glycoconjugates. Rhizomes of *Microgramma vaccinifolia* have widespread use in folk medicine in the treatment of hemoptysis and hematuria. The objectives of this study were to isolate, characterize and evaluate the toxic, antibacterial, antifungal activities of lectin from rhizome of *M. vaccinifolia* (MvRL) and identify the races 1, 2 and 3 of *Fusarium oxysporum* f. sp. *lycopersici* by molecular biology. The protein of rhizome extract (RE) was fractionated with ammonium sulfate providing the 0-60% fraction (F0-60%). Hemagglutinating activity (HA) and concentration of protein were determined in RE and F0-60%. MvRL was isolated by chromatography of F0-60% in Sephadex G-25. The HA MvRL was evaluated in the presence of carbohydrates, glycoproteins, preparations containing carbohydrate from races 1, 2 and 3 of *Fusarium oxysporum* f. sp. *lycopersici* and in different temperatures, pH values and in presence of ions. Polyacrylamide gel electrophoresis of MvRL was performed under native (PAGE) and denatured (SDS-PAGE) conditions. The effect of MvRL on *Artemia salina*, bacteria and fungi was also assessed. The DNA of races 1, 2 and 3 of *Fusarium oxysporum* f. sp. *lycopersici* was isolated using molecular markers ISSR e RAPD. MvRL agglutinated human erythrocytes and its HA was inhibited by mannose, bovine fetal serum and preparations of *F. oxysporum* f.sp. *lycopersici*. The HA MvRL was thermo-stable, active at pH 5.0 and dependent on ions. PAGE revealed MvRL as an acidic protein and SDS-PAGE revealed glycosylated polypeptide band of molecular mass 17 kDa. Chromatography of gel filtration defined the native molecular mass of MvRL as 100 kDa indicating the lectin as a molecular aggregate. MvRL was toxic on *A. saline* (LC_{50} of 154.16 g/mL), exhibited no antibacterial activity and showed antifungal activity. MvRL was more active in inhibiting the growth of race 3 of *F. oxysporum* f.sp. *lycopersici*. The molecular markers were adequate to assess the genetic variability of races. In conclusion MvRL is a lectin with antifungal activity and the effect on *F. oxysporum* f.sp. *lycopersici* was different for all three races.

Keywords: Antifungal activity, *Fusarium oxysporum* f.sp. *lycopersici*, ISSR, lectin, *Microgramma vaccinifolia,* RAPD.

1 INTRODUÇÃO

1.1 LECTINAS

1.1.1 Histórico e distribuição na natureza

Hermann Stillmark, em 1888, realizou a primeira descrição de uma lectina, quando a isolou de *Ricinus communis* (mamona) e observou que esta proteína, denominada ricina, apresentava efeitos de toxicidade e capacidade de aglutinar eritrócitos de diferentes espécies. A princípio, as proteínas presentes em plantas eram denominadas de fitohemaglutininas, hemaglutininas ou fitoaglutininas (Sharon & Lis, 1988). O termo "lectina" (do latim *lectus*, que significa selecionado, escolhido) foi proposto por Boyd & Shapleigh (1954) para designar a habilidade desse grupo de proteínas de interagir seletivamente com carboidratos.

A definição mais usada para as lectinas, atualmente, é aquela proposta por Peumans & Van Damme (1998) que as definem como proteínas, de origem não imunológica, que se ligam específica e reversivelmente a carboidratos ou grupo de carboidratos sem promover modificações químicas na estrutura covalente dos mesmos. Baseado na estrutura das lectinas, Peumans & Van Damme (1998; 2001) classificam as lectinas de plantas em:

- ✓ Merolectinas são proteínas de pequeno tamanho e que possuem um único domínio ligante ao carboidrato sendo, portanto, incapazes de precipitar glicoconjugados ou aglutinar células;
- ✓ Hololectinas possuem, no mínimo, dois domínios idênticos ou bastante homólogos que se ligam ao mesmo carboidrato ou a açúcares de estrutura similar.

Normalmente são denominadas como moléculas di ou multivalentes, capazes de aglutinar células e precipitar glicoconjugados;

- ✓ Quimerolectinas são moléculas contendo um ou mais domínios ligantes a carboidratos, associados a outro domínio distinto, bem definido e que possui atividade enzimática ou outra atividade biológica que independe do domínio ligante a carboidratos;
- ✓ Superlectinas compreendem proteínas que possuem, no mínimo, dois domínios ligantes a carboidratos que são estruturalmente diferentes, e que reconhecem açúcares distintos.

A origem não-imune das lectinas serve para diferenciá-las dos anticorpos anticarboidratos que aglutinam células. Enquanto os anticorpos são estruturalmente similares, as lectinas diferem entre si quanto à composição aminoacídica, requerimentos de metais, peso molecular e estrutura tridimensional (Moreira *et al.*, 1990).

As lectinas têm ampla distribuição na natureza, sendo encontradas em microrganismos (Bhowal *et al.*, 2005), animais (Adhya *et al.*, 2008; Battison & Summerfield, 2009) e plantas (Chen *et al.*, 2008; Kavitha *et al.*, 2009).

Normalmente, a avaliação da presença de lectinas em materiais biológicos se dá pelo ensaio de hemaglutinação com eritrócitos humanos e de animais. As lectinas ligam-se a carboidratos localizados na superfície dos eritrócitos formando uma malha de hemaglutinação entre as células (Figura 1). A capacidade de aglutinação por lectinas pode ser intensificada quando os eritrócitos são submetidos a tratamentos com enzimas (tripsina, papaína) ou com soluções químicas (glutaraldeído ou formaldeído) (Coelho & Silva, 2000). O ensaio da atividade hemaglutinante (AH) é comumente realizado pela técnica de diluições seriadas da lectina e posterior incubação com eritrócitos (Santos *et al.*, 2005).

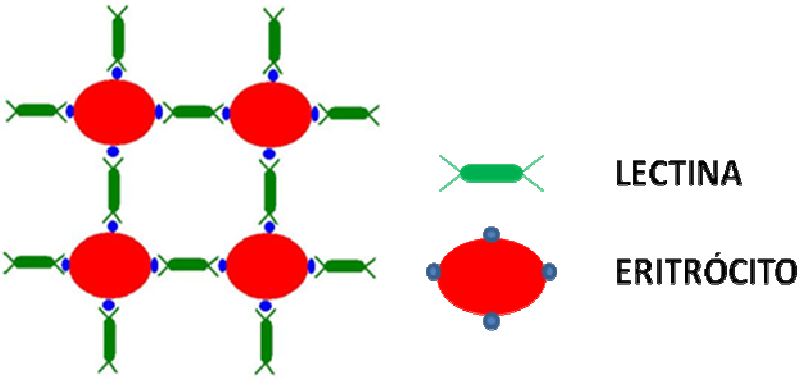


Figura 1. Representação esquemática da aglutinação de eritrócitos por lectina.

Ilustração: Lidiane Albuquerque.

Somente a aglutinação de eritrócitos não é capaz de comprovar a presença de uma lectina, pois alguns agentes como taninos, lipídeos ou cátions bivalentes, em altas concentrações na amostra, podem causar a dispersão de eritrócitos (Rüdiger, 1998). Logo, a inibição da AH por carboidratos e/ou glicoproteínas é um processo fundamental para comprovar que o agente aglutinante é uma lectina. As lectinas, ao ligarem carboidratos ou glicoproteínas da solução teste (Figura 2), não interagem com os carboidratos de superfície dos eritrócitos e a AH é inibida. Adicionalmente o ensaio define a especificidade da lectina. (Kawagishi *et al.*, 2001). A interação da lectina com carboidrato ocorre por meio de pontes de hidrogênio e interações hidrofóbicas (Kennedy *et al.*, 1995) estabelecidas entre os carboidratos e os resíduos de aminoácidos aromáticos favorecendo a especificidade dos complexos formados (Sharon, 1993).

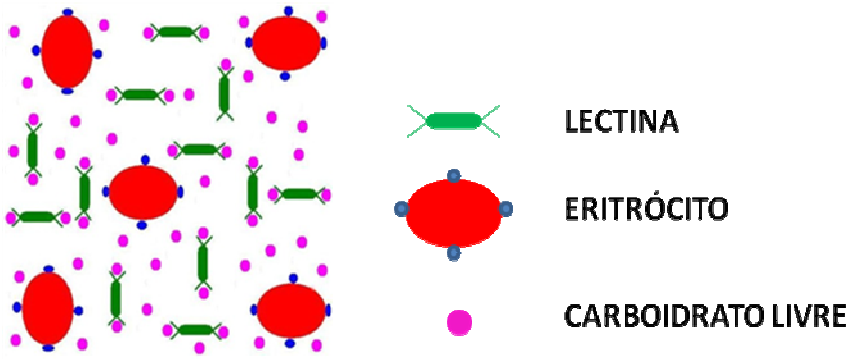


Figura 2. Inibição da aglutinação de eritrócitos por lectinas em presença de carboidratos. Ilustração: Lidiane Albuquerque.

1.1.2 Purificação e Caracterização de Lectinas

O isolamento de lectinas pode ser feito explorando-se algumas características da proteína, tais como: afinidade específica de ligação a carboidratos, carga, massa molecular e solubilidade.

Geralmente a etapa inicial para a purificação de lectinas consiste na extração de proteínas com solução salina ou tampão (Moure *et al.*, 2001; Mladenov *et al.*, 2002; Sitohy *et al.*, 2007). Uma vez apresentando AH, os extratos são submetidos à purificação parcial com sais (Paiva & Coelho, 1992) ou pelo tratamento do extrato com elevadas temperaturas (Bezerra *et al.*, 2001). A adição de sal tornou-se um dos procedimentos mais utilizados para purificar proteínas, pois estas possuem muitos grupos carregados e por isso, a sua solubilidade depende da concentração dos sais dissolvidos; esta solubilidade aumenta com o acréscimo de sais (*salting in*) e volta a decrescer à medida que mais sal é adicionado (*salting out*) (Lehninger, 2006). A precipitação com sulfato de amônio, por exemplo, além de promover purificação, pode estabilizar a AH da proteína, mesmo após longos períodos de armazenamento (Kennedy *et al.*, 1995; Coelho & Silva, 2000).

Outro processo utilizado para purificação parcial de lectinas é a diálise, técnica que separa as lectinas de moléculas pequenas através da utilização de uma membrana (celulose) semipermeável (Kabir *et al.*, 1998).

O uso de técnicas cromatográficas purifica as lectinas de acordo com os seguintes critérios: massa molecular, carga e afinidade específica de ligação a carboidratos. A cromatografia de filtração em gel ou exclusão molecular (Rego *et al.*, 2002; Jung *et al.*, 2007; Pohleven *et al.*, 2009) purifica as proteínas de acordo com a massa molecular; moléculas maiores migram em maior velocidade que as menores devido a sua exclusão dos poros do gel. Adicionalmente, a comparação com a velocidade de migração de proteínas com massas moleculares conhecidas define a massa molecular da lectina em estudo. Na cromatografia de troca iônica a proteína é separada em função de sua carga ao se ligar a um suporte com carga contrária a sua. DEAE-Celulose (Ng & Yu, 2001; Lam & Ng, 2008) e DEAE-Sepharose (Chen *et al.*, 2008; Parisi *et al.*, 2008) são exemplos de trocadores aniônicos, enquanto CM-Celulose (Pajic *et al.*, 2002; Feng *et al.*, 2006; Zhao *et al.*, 2008) é exemplo de trocador catiônico usado na purificação de lectinas. As lectinas também podem ser purificadas por cromatografia de afinidade, através da propriedade de reconhecimento a carboidratos. As lectinas adsorvem especificamente e com alta afinidade à glicoproteína imobilizada em suporte insolúvel ou a matrizes polissacarídicas (Coelho & Silva, 2000; Molchanova *et al.*, 2007; Santana *et al.*, 2008).

FPLC (cromatografia líquida de rápida resolução) e HPLC-RP (cromatografia líquida de alta resolução em fase reversa) têm sido amplamente utilizadas como um processo final de purificação mais refinada de lectinas após a utilização de outros métodos cromatográficos, além de ser um meio de caracterização da massa molecular destas proteínas quando ocorre em uma matriz de gel filtração (Ng *et al.*, 2003; Wong &

Ng, 2003; Jiang *et al.*, 2009). FPLC e HPLC podem estabelecer a homogeneidade de lectinas assim como podem separar subunidades protéicas determinando se a lectina é ou não monomérica (Wang *et al.*, 2003; Kawsar *et al.*, 2008; Silva *et al.*, 2008).

Métodos eletroforéticos são utilizados para caracterizar estruturalmente as lectinas, assim como para estabelecer o grau de pureza das mesmas. As separações eletroforéticas são quase sempre feitas em gel, onde os de poliacrilamida são os escolhidos por serem quimicamente inertes e porque o tamanho dos seus poros pode ser controlado (Stryer, 2004). Eletroforese em gel de poliacrilamida (PAGE) sob condições desnaturantes (na presença de dodecilsulfato de sódio) e redutoras (na presença de ß-mercaptoetanol) revelam o grau de pureza, a composição de subunidades e a massa molecular da proteína (Paiva *et al.*, 2006). Além disso, a eletroforese em gel de poliacrilamida pode revelar a natureza glicoprotéica da molécula através de coloração específica (Coelho & Silva, 2000; Chumkhunthod *et al*., 2006; Feng *et al.*, 2006). PAGE é uma ferramenta ímpar para avaliação da pureza de uma proteína, podendo ser realizada a cada etapa da purificação (Kennedy *et al.*, 1995).

As lectinas podem ser caracterizadas em relação a sua AH, através do ensaio com eritrócitos de diferentes animais, da inibição da AH por carboidratos e/ou glicoconjugados (Kawagishi *et al.*, 2001; Yang *et al.*, 2007), bem como do efeito da temperatura, pH e íons na AH. Para a utilização da lectina em biotecnologia, é importante o conhecimento sobre a faixa de pH e a temperatura ideal onde a proteína se mantém estável, desempenhando sua função. A verificação da faixa de estabilidade do pH pode ser feita submetendo-se a lectina a tampões em diferentes valores de pH (Machuka *et al.*, 1999; Mansour & Abdul-Salam, 2008). A avaliação da termoestabilidade revelou que algumas lectinas permanecem estáveis até 75º C e a partir de então, com a elevação da temperatura, a AH diminui até ser abolida, como no caso

da lectina de *Fenneropenaeus chinensis* (Sun *et al.*, 2008). Diversas lectinas precisam de cátions bivalentes (Ca^{2+} e Mn^{2+}, por exemplo) para exibir sua atividade. A lectina de *Erythrina speciosa* é uma metaloproteína que contém Ca^{2+} e Mn^{2+}, e quando tratada com EDTA perde sua AH sendo a mesma restaurada após a adição de Ca^{2+} e Mn^{2+} (Konozy *et al.*, 2003). Por outro lado, a lectina da esponja marinha *Axinella corrugata* (ACL-I) não perde sua AH quando tratada com EDTA e, portanto, a AH é independente de íons metálicos (Dresch *et al.*, 2008). A presença de cátions na estrutura da proteína confere estabilidade térmica e resistência relativa à ação de enzimas (Moreira *et al.*, 1990).

1.1.3 Lectinas de plantas

As sementes de leguminosas têm sido as principais fontes de lectinas (Sharon & Lis, 2004; Alencar *et al.*, 2005), entretanto, estas proteínas ou glicoproteínas também podem ser isoladas de outros tecidos tais como raízes (Naeem *et al.*, 2001), folhas (Moriyama *et al.*, 2003; Ghosh, 2008), flores (Liu *et al.*, 2002), frutos (Sampietro *et al.*, 2001; Thakur *et al.*, 2007), cerne (Sá *et al.*, 2008), entrecasca (Rojo *et al.*, 2003; Nascimento *et al.*, 2008), rizomas (Lin *et al.*, 2008; Ooi et al., 2008) e cladônias (Santana *et al.*, 2008).

Os papéis fisiológicos das lectinas nos vegetais ainda não foram completamente esclarecidos, embora algumas hipóteses vêm sendo apontadas. Algumas delas seriam: manutenção, armazenamento, transporte de carboidratos e extensão da parede celular (Santos, 2004); defesa contra ataque de insetos e outros predadores (Sengupta *et al.*, 1997). Diversos estudos mostram, ainda, que estas proteínas possuem função simbiótica

entre bactérias fixadoras de nitrogênio (*Rhizobium* spp.) e as raízes de leguminosas (Cavada *et al.*, 2001).

Rizomas, de vários grupos de vegetais, têm demonstrado ser excelentes fontes de lectinas. A lectina de rizoma de *Calystegia sepium*, denominada Calsepa, foi isolada e baseado na sua estrutura molecular e no seqüenciamento de aminoácidos foi demonstrada certa similaridade com lectinas previamente isoladas de espécies de Moraceae (Peumans *et al.*, 1996). Lectina específica para manose, designada SGM2, foi isolada do rizoma de uma erva medicinal chinesa, a *Smilax glabra*, através de extração salina, fracionamento por sulfato de amônio e cromatografia de afinidade. SGM2 mostrou ser uma proteína trimérica constituída por subunidades de massa molecular 12,5 kDa com atividade antiviral contra o vírus do herpes simples tipo 1 (Ooi *et al.*, 2004). Outra lectina com atividades antiproliferativa e mitogênica foi isolada de rizomas da gramínea *Arundo donax*. A mesma é uma proteína homodimérica e termoestável até 55 °C durante 15 min (Kaur *et al.*, 2005). Dos rizomas de *Ophiopogon japônicas* foi isolada uma lectina homodimérica, estável em pH 5,0-9,0 e com atividade antivirviral contra o vírus do herpes simples tipo II com uma concentração tóxica (CT_{50}) de 3,93 mg/ml (Tian *et al.*, 2008).

1.1.4 Propriedades biológicas de lectinas

Dentre as propriedades biológicas das lectinas estão as atividades mitogênica (Zheng *et al.*, 2007), antifúngica (Chen *et al.*, 2008), inseticida (Wong *et al.*, 2008) e antibacteriana (Gowda *et al.*, 2008)**,** aglutinação de células bacterianas (Ourth *et al.*, 2008; Sun *et al.*, 2008) e agregação plaquetária (Radis-Baptista *et al.*, 2006). As lectinas

são valiosas ferramentas em pesquisas nas áreas bioquímica, médica, biológica, farmacológica e afins.

A lectina de *Canavalia ensiformis*, concanavalina A (Con A) é um mitógeno e esta atividade pode ser inibida por baixas concentrações de monossacarídeos, como manose. A inibição da atividade levou à conclusão que a estimulação mitogênica resultou da ligação da lectina a carboidratos da superfície celular dos linfócitos (Sharon, 1993). Lectinas de *Belamyia bengalensis* (Banerjee *et al.*, 2004), de *Cratylia mollis* (Maciel *et al.*, 2004) e de *Chinese evergreen* (Wong *et al.*, 2008) também apresentam atividade mitogênica.

Lectinas, como a de sementes de *Phaseolus coccineus* (PCL) e a do cogumelo *Schizophyllum commune* (SCL) foram citotóxicas para células de mamíferos tanto *in vitro* quanto *in vivo*. A ação tóxica das lectinas sobre células é geralmente seletiva; elas são muito mais ativas sobre células transformadas, que são mais sensíveis aos seus efeitos quando comparadas a células normais (Chumkhunthod *et al.*, 2006; Chen *et al.*, 2008).

Algumas lectinas vêm sendo utilizadas em ensaios citoquímicos para localizar glicoconjugados em diferentes tecidos de invertebrados (Franceschini *et al.*, 2000), em ensaios histoquímicos e imunohistoquímicos para detecção de resíduos glicosilados em superfícies de tecidos animais, inclusive de humanos, e como ferramentas glicohistoquímicas para análises de tecidos normais e alterados de animais e de humanos (Barou *et al.*, 2002; Pedini *et al.*, 2002).

As lectinas também têm sido investigadas quanto à sua aplicação em sistemas de liberação de drogas, já que possuem a capacidade de mediar mucoadesão, citoadesão e citoinvasão de drogas (Gabor *et al.*, 2004). Por exemplo, a lectina de folhas de *Bauhinia monandra* (BmoLL) foi incorporada e adsorvida na superfície de nanopartículas,

mostrando ser uma potencial ferramenta para a utilização dessas nanopartículas em medicamentos de administração oral com liberação controlada (Rodrigues *et al.*, 2003).

Algumas lectinas vegetais atuam como bio-inseticidas contra larvas de insetos que causam perdas econômicas na agricultura ou que causam doenças em humanos. Estas proteínas provavelmente se ligam a receptores glicoprotéicos do trato digestivo destes insetos e provocam efeitos deletérios locais e sistêmicos que podem repelir ou retardar o crescimento ou, até mesmo matar o inseto (Peumans *et al.*, 2001). A lectina BmoLL de *B.monandra* foi ativa contra larvas de *Zabrotes subfasciatus* e *Anagasta kuehniella* (Macedo *et al.*, 2007) e atividade larvicida contra *Aedes aegypti* foi detectada em extratos, frações de sulfato de amônio e lectinas isoladas de cerne e entrecasca de *Myracrodruon urundeuva* (Sá *et al.*, 2008).

No mecanismo de defesa da planta, as lectinas agem inibindo o crescimento de algumas bactérias fitopatogênicas (Sá *et al.*, 2008), na interação parasito/hospedeiro (Piazza *et al.*, 1996) e na adesão de agentes infecciosos às células hospedeiras (Hirsch, 1999).

1.1.5 Atividade antimicrobiana

Muitas proteínas, inclusive lectinas, vêm sendo avaliadas quanto ao seu efeito antimicrobiano. As proteínas antimicrobianas, em animais, constituem parte do sistema imune nato. Peptídeos e pequenas proteínas, com atividade antimicrobiana, são usados contra inúmeros microrganismos patogênicos (Wang *et al.*, 2003). Em plantas e animais as lectinas antimicrobianas estão envolvidas com o mecanismo de defesa (Lee *et al.*, 2002). Lectina recombinante do anfioxo *Branchiostoma belcheri*, expressada em *Pichia pastoris*, foi capaz de inibir o crescimento da *Escherichia coli* e foi sugerido que a

proteína está envolvida na defesa imune do hospedeiro (Ju *et al.*, 2008). A lectina de *Anguilla japônica* apresentou atividade aglutinante frente à bactéria patogênica *Streptococcus difficile*, atuando como fator de defesa (Tasumi *et al.*, 2004). HSL, lectina de *Holothuria scabra* inibiu eficientemente o crescimento de bactérias gram-negativas (*Serratia* sp., *Proteus* sp., *Shigella* sp. e *E. coli*) e gram-positivas (*Streptococci* grupo D), indicando seu amplo efeito bactericida (Gowda *et al.*, 2008).

Lectinas isoladas também podem ser usadas como ferramentas na inibição do crescimento micelial de diversas espécies de fungos. Possivelmente, esta propriedade é devido à interação destas proteínas à porção glicídica da parede celular do fungo levando a inibição do seu crescimento ou morte do mesmo (Xu *et al.*, 1998). Lectinas de *Pseudostellaria heterophylla* (Wang & Ng, 2006), *Pisum sativum* (Sitohy *et al.*, 2007), *Capparis spinosa* (Lam & Ng, 2008), *Myracrodruon urundeuva* (Sá *et al.*, 2008), *Castanea mollisma* (Wang & Ng, 2003) e *Phaseolus coccineus* (Chen *et al.*, 2008) são exemplos de lectinas com atividade antifúngica. A lectina de rizoma de *Ophiopogon japonicus* (OJL) inibiu o crescimento dos fungos fitopatogênicos *Gibberella saubinetii*, *Rhizoctonia solani*, sendo a concentração mínima da lectina de 0,06 mg/ml e 0,05 mg/ml, respectivamente (Tian *et al.*, 2008).

1.2 *Artemia salina* como indicador da toxicidade

Artemia salina, pertecente à classe Brachiopoda, é um microcrustáceo de habitat marinho medindo de 8 a 10 mm de comprimento (Figura 3). Reproduz-se de forma bastante rápida e com facilidade. Seus ovos, quando secos, podem ser conservados durante 10 anos estando sempre aptos a eclodirem, contanto que sejam colocados em

água salgada. A regulação iônica é mantida pela absorção ou excreção dos sais através das brânquias (Ruppert & Barnes, 1996).

Figura 3. *Artemia salina*. Fonte: www.britannica.com/crustaceos/artemia

Este tipo de camarão é amplamente utilizado em aplicações toxicológicas para estabelecer a toxicidade de produtos químicos e naturais através da estimativa da concentração letal média, valor de CL_{50}, como parâmetro da avaliação da atividade biológica (Barahona & Sánchez-Fortun, 1996; Parra *et al.*, 2001).

Considera-se este teste um bioindicador de contaminação ambiental, devido à susceptibilidade desses microcrustáceos frente aos elementos químicos chumbo, mercúrio, selênio, arsênico, cobre, zinco e cádmio (Parra *et al.*, 2001). O método apresenta algumas vantagens, tais como a facilidade de execução, baixo custo, reprodutibilidade, rapidez, disponibilidade comercial dos ovos, a não exigência de equipamentos especiais e a necessidade de pequenas quantidades da amostra-teste para a realização dos experimentos. Larvas de *A. salina* vêm sendo utilizadas para avaliar a toxicidade de extratos de plantas pois, devido a sua sensibilidade, pode indicar compostos de menor toxicidade e, portanto, menos nocivo a organismos não-alvo (Favilla *et al.*, 2006). A técnica ainda permite a detecção da toxicidade e fototoxicidade

simultaneamente, sendo utilizada como prognóstico para atividades antitumoral e pesticida (Sánchez *et al.*, 1993; Ojala *et al.*, 1999).

1.3 Fungo fitopatogênico *Fusarium oxysporum* f. sp. *lycopersici*

O fungo *Fusarium* spp. é amplamente distribuído ao redor do mundo, encontrado em todos os tipos de solo ou associados a inúmeras espécies vegetais. Este fungo pode sobreviver por longos períodos de forma saprofítica sobre a matéria orgânica do solo e quando possível pode causar inúmeras doenças em diferentes espécies vegetais, sobretudo em culturas de importância econômica (tais como tomate, feijão, milho e banana), causando grandes prejuízos (Godoy & Colombo, 2004).

O gênero *Fusarium* é caracterizado pelo seu crescimento rápido, colônias com coloração pálida ou colorida (violeta à púrpura escuro ou do creme à laranja), com micélio aéreo e difuso (Domsch *et al.*, 1980). A maioria das espécies de *Fusarium* é composta por fungos de solo com distribuição cosmopolita e ativos na decomposição de substratos celulósicos, sendo que alguns isolados são parasitas de plantas (Leslie *et al.,* 2001; Zemankova & Lebeda, 2001; Godoy & Colombo, 2004).

As duas principais formas de esporos de *Fusarium* são os microconídios e os macroconídios. Os microconídios são unicelulares e uninucleados; os macroconídios mais comuns são multicelulares, mas cada célula tem somente um núcleo. Todos os núcleos de um macroconídio, contudo, são descendentes mitóticos de um mesmo núcleo progenitor e são, portanto geneticamente idênticos. Os estágios sexuais de *Fusarium* são ascomicetos; o esporo sexual é o ascósporo (Puhalla, 1981).

Segundo Oliveira & Costa (2002) existe um conjunto de variações nas características morfológicas e patogênicas no gênero *Fusarium*, resultando em uma

complexa classificação dividida em seções, *formae speciales* (f. sp.) e raças. O conceito *formae speciales* foi aplicado por Snyder & Hansen (1953) para reconhecer isolados patogênicos que foram morfologicamente semelhantes a isolados saprofíticos de mesma espécie, mas que diferem em sua habilidade para parasitar hospedeiros específicos.

Fusariose, causada pelo fungo *F.oxysporum* f. sp. *lycopersici* é uma doença que ocorre em todas as regiões onde o tomate é cultivado, podendo se manifestar em quaisquer estádios de desenvolvimento da planta, sendo mais comum no início de florescimento e frutificação sendo o tomate o único hospedeiro deste fungo de solo. Este patógeno penetra na planta pela raiz, através dos pêlos absorventes ou por ferimentos, colonizando o sistema vascular das plantas suscetíveis no sentido ascendente, podendo atingir frutos e sementes. A doença causa o amarelecimento geral da planta, iniciando-se pelas folhas inferiores seguido de uma murcha generalizada e secagem das folhas (Figura 4A). Cortando-se o caule longitudinalmente, nota-se uma coloração marrom típica acompanhando os vasos lenhosos (Figura 4B). O vento, a água e os implementos agrícolas são considerados os principais disseminadores deste fungo a curta distância. O desenvolvimento da doença é favorecido por temperaturas entre 21 a 33°C, sendo 28°C a melhor temperatura (Agrios, 2005). Como em todas as doenças vasculares de plantas, o controle químico não é eficiente. O uso de variedades resistentes ao fungo constitui o meio mais viável no controle da doença, já que o fungo *F. oxysporum* f. sp. *lycopersici* pode permanecer no solo por décadas (Brayford, 1992).

Três raças fisiológicas do fungo foram constatadas. Existe a predominância da raça 1, mas, em muitas regiões do mundo, a raça 2 tem aumentado de importância. A raça 3 apresenta uma distribuição geográfica mais limitada e foi registrada no Brasil no estado do Espírito Santo. Esta doença pode se tornar importante no Brasil, devido ao fato de cultivares adaptados e com resistência à raça 3 ainda não estarem amplamente

disponíveis, daí a preocupação dos melhoristas em incorporar genes de resistência a estas raças em novos híbridos e variedades (Agrios, 2005; Reis *et al.*, 2005).

A B

Figura 4: Sintomas da fusariose no tomateiro. Tomateiro infectado (A); corte longitudinal do caule (B). Fonte: www.avrdc.org/LC/tomato/fusarium.html.

Os métodos fenotípicos, algumas vezes não permitem à correta identificação das raças desta espécie, constituindo-se em métodos laboriosos e demorados, podendo gerar resultados conflitantes. Pelas limitações em desenvolver estudos morfológicos, os métodos moleculares podem auxiliar no esclarecimento da taxonomia evitando classificações artificiais sem consistência com a filogenética da espécie e das raças (Manicom *et al.*, 1987; Thomas *et al.*, 1994).

1.4 Metodologias moleculares para o estudo da variabilidade genética de fungos

Nas últimas décadas, várias metodologias moleculares têm sido desenvolvidas contribuindo significativamente para um grande avanço do conhecimento sobre a variabilidade genética de microrganismos. Dentre as técnicas mais utilizadas destacam-se: RFLP *(Restriction Fragment Length Polymorphism*); RAPD *(Random Amplified Polymorphic DNA*); SSR (*Simple Sequence Repeats*); ISSR (*Inter Simple Sequence Repeat*); AFLP *(Amplified Fragment Length Polymorphism*); seqüências de

subunidades do rDNA; ARDRA *(Amplified Ribosomal DNA Restriction Analysis*); RISA *(Ribosomal Intergenic Spacer Analysis*); DGGE *(Denaturing Gradient Gel Electrophoresis)* (Vos *et al.*, 1995; McDonald, 1997; Van Elsas *et al.*, 1998; Amicucci *et al.*, 2001; Pennanen *et al.*, 2001; Taylor *et al.*, 2001; Oliveira & Cost*a,* 2002; Marshall *et al.*, 2003; Fagbola & Abang, 2004). Entretanto, apesar do grande número de técnicas que revelam polimorfismo de DNA, é importante considerar o tipo de organismo em estudo.

A técnica da reação em cadeia da polimerase (PCR) consiste na amplificação de uma seqüência específica de DNA, que visa à produção de milhões de cópias desta seqüência (Marshall *et al.*, 2003).

A PCR é considerada uma técnica simples, rápida e flexível. Desde sua criação um grande número de variáveis da técnica tem sido descritas tornando cada vez mais ampla a aplicabilidade da mesma. A PCR pode ser utilizada, por exemplo, na amplificação de regiões específicas do genoma ou cDNA, análise de polimorfismos, estudos de evolução molecular, medicina forense e no diagnóstico de doenças genéticas (Zaha, 1996).

A técnica de RAPD apresenta a vantagem de não necessitar de conhecimento prévio da seqüência de DNA em estudo, podendo-se utilizar oligonucleotídeos universais de 10 nucleotídeos aproximadamente. Esses oligonucleotídeos são simples e amplificam fragmentos arbitrários do DNA. Durante a amplificação, fragmentos de variados tamanhos são produzidos (Power, 1996).

Com esta técnica foi possível o desenvolvimento de marcadores moleculares, baseados em polimorfismos encontrados em proteínas ou no DNA, os quais contribuem para estudos em filogenia, ecologia, taxonomia e genética. A molécula de DNA é o principal alvo de estudo quando visa obter informações sobre polimorfismos, pois a

seqüência é única para cada indivíduo e a mesma pode ser explorada para estudos de diversidade genética e correlação entre os indivíduos (Weising *et al.*, 1994).

Várias são as vantagens dos marcadores RAPD, tais como o baixo custo, a facilidade e a rapidez no emprego da técnica. Estudos sugerem que os RAPDs fornecem marcas relativamente específicas de populações, espécies ou raças (Peakall *et al.*, 1998).

O RAPD vem sendo amplamente utilizado com grande sucesso por vários pesquisadores na tipagem e identificação de inúmeros fungos patogênicos, tais como *Histoplasma capsulatu*m, *Aspergillus fumigatu*s, *Cryptococcus neoformans* e espécies do gênero *Candida* (Lehmann *et al.*, 1992; Brandt *et al.*, 1995; Mondon *et al.*, 1995; Melo *et al.*, 1998; Poonwan *et al.*, 1998). O RAPD também tem sido utilizado para distinguir isolados de diferentes espécies de *Fusarium.* Amoah *et al.* (1995) determinaram as características genéticas dos isolados de *F. moniliforme* (=*F. verticillioide*s) coletados de milho, arroz e sorgo de diferentes regiões de Gana, usando as técnicas de RAPD e RFLP. Hering & Niremberg (1995) utilizaram a técnica de RAPD para o estudo de populações do gênero *Fusariu*m. Eles obtiveram padrões informativos típicos para populações de *F. sambuci*n, *F. torulosum* e *F.venenatum* utilizando 10 oligonucleotídeos arbitrários e seqüências repetitivas simples.

Marcadores moleculares baseados em ISSR têm sido empregados com sucesso nas estimativas de variabilidade genética em espécies de plantas silvestres e cultivadas, tanto intra quanto inter populacionais. A técnica de ISSR tem a capacidade de gerar um grande número de marcadores multiloci e podem ser aplicados para analisar praticamente qualquer organismo mesmo aqueles para os quais se dispõe de pouca ou nenhuma informação genética prévia (Fagbola & Abang, 2004). ISSR é uma dentre muitas técnicas de *fingerprinting* baseada em PCR e é definida como um variante da reação da polimerase em cadeia que utiliza oligonucleotídeo de seqüência simples e

repetitivas para amplificar regiões entre seqüências alvo. Este método foi inicialmente desenvolvido em 1994 e explora a abundância e a distribuição aleatória de SSRs em genomas de diferentes organismos, amplificando seqüências de DNA contidas entre estes SSRs (Amicucci *et al.*, 2001).

A técnica ISSR é altamente polimórfica e útil no estudo de diversidade genética, filogenia, caracterização molecular, mapeamento e biologia evolutiva. Nesse método, os microssatélites de 16 a 25 pb são utilizados como oligonucleotídeo iniciador único para amplificar as regiões internas dos SSR. É uma técnica baseada em PCR, a qual envolve a amplificação do segmento de DNA presente em uma distância amplificável entre dois microssatélites idênticos, orientados em direções opostas (Reddy *et al.*, 2002). Os marcadores ISSR apresentam alta reprodutibilidade devido ao uso das seqüências longas de 16-25 pb quando comparadas ao RAPD e permite o uso de altas temperaturas de anelamento mostrando alta estrigência (Reddy *et al.*, 2002).

Os marcadores ISSR têm várias aplicações em genética de populações, por exemplo, no estudo de fluxo gênico, análise de paternidade ou ainda na identificação de cultivares, onde tem sido empregado com mais freqüência. (Reddy *et al.*, 2002).

1.5 *Microgramma vaccinifolia* (Langsd. & Fisch)Copel.

Conhecida vulgarmente por “cipó cabeludo”, “cipó peludo”, “polipódio vacinifólio”, “erva-silvina”, “erva-teresa”, é uma planta epífita, preferencialmente de zona costeira, onde ocorre sobre as árvores e muros de residências. Possui rizoma longo, recoberto por escamas rúfulas, com diâmetro variando de 0,2 a 0,3 cm; apresenta dimorfismo laminar (frondes), sendo sempre inteiras e simples (Farias, 1994).

Rizomas de *M. vaccinifolia* (Figura 5) são caules modificados bastante utilizados na terapêutica como adstringentes no tratamento de diarréias e disenterias, e largamente usados como balsâmicos nas infecções das vias respiratórias, tosses, bronquites, coqueluche, laringites, entre outros. Também são empregados no tratamento de hemoptises e em casos de hematúria; inclusive, destaca-se a propriedade sudorífera, e quando cozidos, são usados em banhos quentes, no tratamento de hidropisia (Barros & Andrade, 1997).

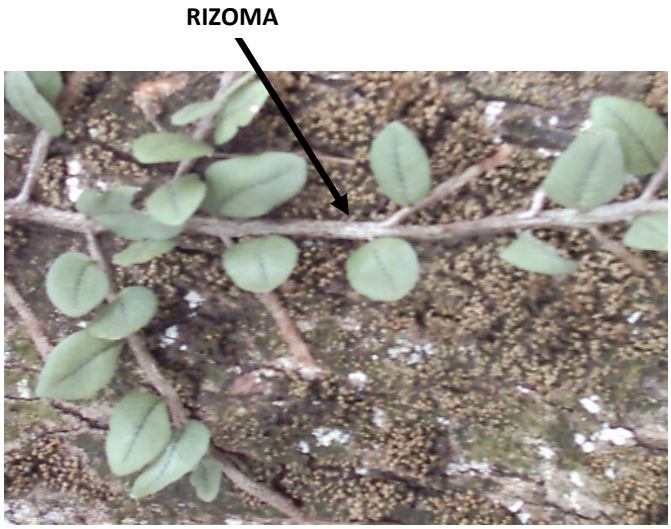


Figura 5: *Microgramma vaccinifolia*. Campus UFPE.

Foto: Lidiane Albuquerque.

2 OBJETIVOS

2.1 Objetivo geral

Purificar, caracterizar e determinar propriedades biológicas da lectina de rizoma de *M. vaccinifolia* (MvRL). Investigar a eficiência das técnicas de biologia molecular ISSR e RAPD na identificação da variabilidae genética das raças de *F. oxysporum* f. sp. *lycopersici*.

2.2 Objetivos específicos

- Definir o protocolo para o isolamento da lectina de rizoma (MvRL);

- Caracterizar a AH em presença de diferentes eritrócitos, carboidratos e íons e em valores distintos de pH e temperatura;

- Avaliar a lectina por eletroforese em gel de poliacrilamida em condições nativas (natureza da carga líquida) e desnaturantes (massa molecular de subunidades);

- Identificar a presença de carboidratos na estrutura da lectina por coloração para glicoproteínas em gel de poliacrilamida; e determinar o conteúdo de carboidrato pelo método fenol-sulfúrico;

- Determinar a massa molecular da lectina por cromatografia de gel filtração;

- Avaliar a atividade antimicrobiana de MvRL contra bactérias Gram-positivas e gram-negativas e contra fungos;

- Verificar a citotoxicidade *in vitro* de MvRL pela determinação da concentração letal (CL_{50}) da lectina frente ao microcrustáceo *Artemia salina*;

- Obter preparações das raças de *F. oxysporum* f. sp. *lycopersici* e determinar a presença de carboidratos nas mesmas;

- Determinar a AH da lectina em presença de preparações das raças 1, 2 e 3 de *F. oxysporum* f. sp. *lycopersici*;

- Comparar a diversidade genética das três raças de *F. oxysporum* f. sp. *lycopersici*, através de técnicas de PCR, utilizando marcadores moleculares dos tipos ISSR e RAPD;

- Analisar as seqüências obtidas por PCR utilizando ferramentas da bioinformática.

3. REFERÊNCIAS BIBLIOGRÁFICAS

ADHYA, M.; SINGHA, B.; CHATTERJEE, B. P. Purification and characterization of an N-acetylglucosamine specific lectin from marine bivalve *Macoma birmanica*. **Fish and Shellfish Immunology**, 2008.

AGRIOS, G.N. **Plant Pathology**. 5th ed. Amsterdam: Elseuier Academic. 922p. 2005.

ALENCAR, V. B. M.; ALENCAR, M. N. N.; ASSREUY, A. M. S.; MOTA, M. L.; BRITO, G. A. C.; ARAGÃO, K. S.; BITTENCOURT, F. S.; PINTO, V. P. T.; DEBRAY, M.; RIBEIRO, R. A.; CAVADA, B. S. Pro-inflammatory effect of *Arum maculatum* lectin and role of resident cells. **The International Journal of Biochemistry & Cell Biology**, Oxford, v. 37, n. 9, p. 1805-1814, 2005.

AMICUCCI, A.; ZAMBONELLI, A.; GUIDI, C.; STOCCHI, V. Morfological and molecular characterisation of *Pulvinula consellatio* ectomycorrhizae. **FEMS Microbiology Letters**, v.194, p.121-125, 2001.

AMOAH, B.K.; REZNOOR, H.N.; NICHOLSON, P.; MACDONALD, M.V. Variation in *Fusarium* section Liseola: pathogenicity and genetic studies of isolates of *Fusarium moniliforme* Sheldon from different hosts in Ghana. **Plant Pathology**, v.44, p.563-572, 1995.

BANERJEE, S.; CHAKI, S.; BHOWAL, J.; CHATTERJEE, B. P. Mucin binding mitogenic lectin from freshwater Indian gastropod *Belamyia bengalensis*: purification

and molecular characterization. **Archives of Biochemestry and Biophysics,** v. 421, p. 125-134, 2004.

BARAHONA, M. V.; SÁNCHEZ-FORTÚN, S. Comparative sensitivity of three age classes of *Artemia salina* larvae to several phenolic compuonds. **Bulletin of Environmental Contamination and Toxicology**, v. 56, p. 271-278, 1996.

BAROU, O.; MEKRALDI, S.; VICO, L.; BOIVIN, G.; ALEXANDRE, C.; LAFAGE-PROUST, M. H. Relationships between trabecular bone remodeling and bone vascularization: a quantitative study. **Bone**, v. 30, n. 4, p. 604-612, 2002.

BARROS, I. C. L.; ANDRADE, L. H. C. **Pteridófitas medicinais (samambaias, avencas e plantas afins)**. Recife: Editora Universitária da UFPE, 1997, 223 p.

BATTISON, A. L.; SUMMERFIELD, R. L. Isolation and partial characterisation of four novel plasma lectins from the American lobster *Homarus americanus*. **Developmental and Comparative Immunology**, v. 33, p. 198–204, 2009.

BEZERRA, S. F.; VIEIRA, V. L. A.; CARVALHO JR, L. B. Proteases no trato digestivo de peixes. **Biotecnologia Ciência & Desenvolvimento**, v. 22, p. 46-49, 2001.

BHOWAL, J.; GUHA, A. K.; CHATTERJEE, B. P. Purification and molecular characterization of a sialic acid specific lectin from the phytopathogenic fungus *Macrophomina phaseolina*. **Carbohydrate Research**, v. 340, p. 1973-1982, 2005.

BOYD, W. C.; SHAPLEIGH, E. Specific precipitating activity of plant agglutinins (lectins). **Science**, p. 119- 419, 1954.

BRANDT, M. E.; HUTWAGNER, L. C.; KUYKENDALL, R. J.; PINNER, R. W. The Criptococcal disease active surveillance group – Comparison of multilocus enzyme electrophoresis and random amplified polymorphic DNA analysis for molecular subtyping of *Cryptococcus neoformans*. **J Clin Microbiol**, v. 33, p. 1890-5, 1995.

BRAYFORD, D. Description of fungi *Fusarium oxysporum* f. sp. *lycopersici*. **Mycopathol**, v.118, p.51-53, 1992.

CAVADA, B. S., BARBOSA, T., ARRUDA, S., GRANGEIRO, T. B., BARRAL-NETTO, M. Revisiting proteus: Do minor changes in lectin structure matter in biological activity? Lessons from and potencial biotechnological uses of *Diocleinae* Subtribe lectins. **Current Protein and Peptide Science**, v. 2, n. 2, p. 123-135, 2001.

CHEN, J.; LIU, B.; JI, N.; ZHOU, J.; BIAN, H.; LI, C.; CHEN, F.; BAO, J. A novel sialic acid-specific lectin from *Phaseolus coccineus* seeds with potent antineoplastic and antifungal activities. **Phytomedicine**, 2008.

CHUMKHUNTHOD, P.; RODTONG, S.; LAMBERT, S. J.; FORDHAM-SKELTON, A. P.; RIZKALLAH, P. J.; WILKINSON, M. C.; REYNOLDS, C. D. Purification and characterization of an N-acetyl-D-galactosamine-specific lectin from the edible mushroom *Schizophyllum commune*. **Biochimica et Biophysica Acta**, v. 1760, p. 326–332, 2006.

COELHO, L. C. B. B.; SILVA, M. B. R. Simple method to purify milligram quantities of the galactose-specific lectin from the leaves of *Bauhinia monandra*. **Phytochemical Analysis**, v. 11, p. 295-300, 2000.

COLOMBO, C.; SECOND, G.; VALLE, T. L.; CHARRIER, A. Genetic diversity characterization of cassava cultivars (*Manihot esculenta* Crantz) with RAPD markers. **Genetics and Molecular Biology**, Ribeirão Preto, v. 21, n. 1, p. 105-113, 2001.

DRESCH, R. R.; ZANETTI, G. D.; LERNER, C. B.; MOTHES, B.; TRINDADE, V. M. T.; HENRIQUES, A. T.; VOZÁRI-HAMPE, M. M. ACL-I, a lectin from the marine sponge *Axinella corrugata*: Isolation, characterization and chemotactic activity. **Comparative Biochemistry and Physiology**, v. 148, p. 23–30, 2008.

DOMSCH, K.H.; GAMS, W.; ANDERSON, T.H. **Compendium of soil fungi.** New York: Academic Press, 1980, 859p.

FAGBOLA, O.; ABANG, M.M. *Colletotrichum circinans* and *Colletotrichum coccodes* can be distinguished by DGGE analysis of PCR-amplified 18S rDNA fragments. **African Journal of Biotechnology**, v.3, p.195-198, 2004.

FARIAS, M. C. A. **Teor de taninos em *Microgramma vaccinifolia* (Langsd. & Fisch.) Copel. (Pteridófita).** 1994. 119 f. Dissertação (Mestrado em Criptógamos - Departamento de Botânica, Departamento de Micologia) - Universidade Federal de Pernambuco, Recife, 1994.

FAVILLA, M.; MACCHIA, L.; GALLO, A.; ALTOMARE, C. Toxicity assessment of metabolites of fungal biocontrol agents using two different (*Artemia salina* and *Daphnia magna*) invertebrate bioassays. **Food and Chemical Toxicology**, v. 44, p. 1922-1931, 2006.

FENG, K.; LIU, Q. H.; NG, T. B.; LIU, H. Z.; LI, J. Q.; CHEN, G.; SHENG, H. Y.; XIE, Z. L.; WANG, H. X. Isolation and characterization of a novel lectin from the mushroom *Armillaria luteo-virens*, **Biochemical and Biophysical Research Communications**, v. 345, p. 1573–1578, 2006.

FRANCESCHINI, V.; LAZZARI, M.; CIANI, F. Lectin cytochemical localization of glycoconjugates in the olfactory system of the lizards *Lacerta viridis* and *Podarcis sicula*. **Anatomy and Embriology**, v. 202, n. 1, p. 49-54, 2000.

GABOR, F.; BOGNER, E.; WEISSENBOECK, A.; WIRTH, M. The lectin-cell interaction and its implications to intestinal lectin-mediated drug delivery. **Advanced Drug Delivery Reviews**, v. 56, p.459-480, 2004.

GODOY, P.; COLOMBO, A.L. Biologia e relevância clínica do gênero *Fusarium* spp. **Prática Hospitalar**, v.34, p.136-140, 2004.

GHOSH, M. Purification of a lectin-like antifungal protein from the medicinal herb, *Withania somnifera*. **Fitoterapia**, 2008.

GOWDA, N. M.; GOSWAMI, U.; KHAN, M. I. T-antigen binding lectin with antibacterial activity from marine invertebrate, sea cucumber (*Holothuria scabra*):

Possible involvement in differential recognition of bacteria. **Journal of Invertebrate Pathology**, v. 99, p. 141–145, 2008.

HERING, O.; NIRENBER, H.I. Differentiation of *fusarium sambuciunum* Fuckel sensu lato and related species by RAPD PCR. **Mycopathologia**, v. 129, p.159-164, 1995.

HIRSCH, A. M. Role of lectins (and rhizobial exopolysaccharides) in legume nodulation. **Current Opinion in Plant Biology**, v. 2, p. 320-326, 1999.

JIANG, L.; PENG, L.; ZHANG, Y.; CHEN, J.; ZHANG, D.; LIANG, S. Expression, purification and characterization of a group of lectin-like peptides from the spider *Ornithoctonus huwena*. **Peptides**, *article in press*, 2009.

JU, L.; ZHANG, S.; LIANG, Y.; SUN, X. Identification, expression and antibacterial activity of a tachylectin-related homolog in amphioxus *Branchiostoma belcheri* with implications for involvement of the digestive system in acute phase response. **Fish and Shellfish Immunology**, 2008.

JUNG, E. C.; KIM, K. D.; BAE, C. H.; KIM, J. C.; KIM, D. K.; KIM, H. H. A mushroom lectin from ascomycete *Cordyceps militaris*, **Biochimica et Biophysica Acta**, v. 1770, p. 833–838, 2007.

KABIR, S. Jacalin: a jackfruit (*Artocarpus heterophyllus*) seed-derived lectin of versatile applications in immunobiological research. **Journal of Immunological Methods**, v. 212, p. 193-211, 1998.

KAUR, A.; SINGH, J.; KAMBOJ, S. S.; SEXANA, A. K.; PANDITA, R. M.; SHAMNUGAVEL, M. Isolation of an N-acetyl-D-glucosamine specific lectin from the rhizomes of *Arundo donax* with antiproliferative activity. **Phytochemistry**, v. 66, p. 1933–1940, 2005.

KAWAGISHI, H.; TAKAGI, J.; TAIRA, T.; MURATA, T.; USUI, T. Purification and characterization of a lectin from the mushroom *Mycoleptodonoides aitchisonii*. **Phytochemestry**, v. 56, p. 53-58, 2001.

KAWSAR, S. M. A.; FUJII, Y.; MATSUMOTO, R.; ICHIKAWA, T.; TATENO, H.; HIRABAYASHI, J.; YASUMITSU, H.; DOGASAKI, C.; HOSONO, M.; NITTA, K.; HAMAKO, J.; MATSUI, T.; OZEKI, Y. Isolation, purification, characterization and glycan-binding profile of a D-galactoside specific lectin from the marine sponge, *Halichondria okadai*. **Comparative Biochemistry and Physiology Part B**, v.150, p. 349–357, 2008.

KAVITHA, M.; SULTAN, N. A. M.; SWAMY, M. J. Fluorescence studies on the interaction of hydrophobic ligands with *Momordica charantia* (bitter gourd) seed lectin. **Journal of Photochemistry and Photobiology B: Biology**, v. 94, p. 59–64, 2009.

KENNEDY, J. F.; PAIVA, P. M. G.; CORREIA, M. T. S.; CAVALCANTI, M. S. M.; COELHO, L. C. B. B. Lectins, versatile proteins of recognition: a review. **Carbohydrate Polymers**, v. 26, p. 219-230, 1995.

KONOZY, E. H. E.; BERNARDES, E. S.; ROSA, C.; FACA, V.; GREENE, L. J.; WARD, R. J. Isolation, purification, and physicochemical characterization of galactose-binding lectin from seeds of *Erythrina speciosa*. **Archives of Biochemistry and Biophysics**, v. 410, p. 222-229, 2003.

LAM, S. K.; NG, T. B. A protein with antiproliferative, antifungal and HIV-1 reverse transcriptase inhibitory activities from caper(*Capparis spinosa*) seeds. **Phytomedicine**, *article in press*, 2008.

LEE, D. G.; PARK, Y.; KIM, H. N.; KIM, P.; CHOI, B. H.; HAHM, K. S. Antifungal mechanism of an antimicrobial peptide, HP (2-20), derived from N-terminus of *Helicobacter pylori* ribosomal protein L1 against *Candida albicans*. **Biochemical and Biophysical Research Communications**, v. 291, p. 1006-1013, 2002.

LEHMANN, P. F.; LIN, D.; LASKER, B. A. Genotypic identification and characterization of species and strains within the genus *Candida* by using Random Amplified Polymorphic DNA. **J Clin Microbiol**, v. 30, p. 3249-3254, 1992.

LEHNINGER, A. L.; NELSON, D. L.; COX, M. M. **Princípios de Bioquímica**. Sarvier, 4ª ed., São Paulo, 2006.

LESLIE, J.F.; ZELLER, K.A.; SUMMERELL, B.A. Icebergs and species in populations of *Fusarium*. **Physiological and Molecular Plant Pathology**, v.59, p.107-117, 2001.

LIN, L.; LU, J.; ZENG, H.; LIANG, Z.; ZHOU, Y.; LIN, J.; SUN, X.; TANG, K. Molecular cloning and characterization of a mannose-binding lectin gene from *Pinellia cordata*. **Mol. Biol. Rep.**, 2007.

LIU, W.; HU, Y. L.; WANG, M.; GY, H. U. Z.; WANG, D. C. Purification, crystallization and preliminary X-ray diffraction analysis of a novel mannose-binding lectin from *Gastrodia elata* with antifungal properties. **Acta Crystallogr. D-Biological Crystallography**, v. 58, p. 1833-1835, 2002.

MACEDO, M. L. R.; FREIRE, M. G. M.; DA SILVA, M. B. R.; COELHO, L. C. B. B. Insecticidal action of *Bauhinia monandra* leaf lectin (BmoLL) against *Anagasta kuehniella* (Lepidoptera: Pyralidae), *Zabrotes subfasciatus* and *Callosobruchus maculatus* (Coleoptera: Bruchidae). **Comparative Biochemistry and Physiology**, v. 146, p. 486-498, 2007.

MACHUKA, J. S.; OKEOLA, O. G.; ELS, J. M. V. D.; CHRISPEELS, M. J.; LEUVEN, F. V.; PEUMANS, W. J. Isolation and partial characterization of galactose-specific lectins from African Yam beans, *Sphenostyles stenocarpa* Harms. **Phytochemistry**, v. 51, p. 721-739, 1999.

MACIEL, E. V. M.; ARAUJO-FILHO, V. S.; NAKASAWA, M.; GOMES, Y. M.; COELHO, L. C. B. B.; CORREIA, M. T. S. Mitogenic activity of *Cratylia mollis* lectin on human lymphocytes. **Biologicals**, v. 32, p. 57-60, 2004.

MANICOM, B. Q.; BAR-JOSEPH, M.; ROSNER, A.; VIGODSKY-HAAS, H.; KÓTZE, J. M. - Potential applications of random DNA probes and restriction fragment length polymorphisms in the taxonomy of the *Fusaria*. **Phytopathology**, 77: 669-672, 1987.

MANSOUR, M. H.; ABDUL-SALAM, F. Characterization of fucose-binding lectins in rock- and mud-dwelling snails inhabiting Kuwait Bay. **Immunobiology**, 2008.

MARSHALL, M.N.; COCOLIN, L.; MILLS, D.A.; VANDERGREYNST, J.S. Evaluation of PCR primers for denaturing gradient gel electroforesis analysis of fungal communities in compost. **Journal of Applied Microbiology**, v.95, p.934-948, 2003.

McDONALD, B.A. The population genetics of fungi: tools and techniques. **Phytopathology**, v.87, p.448-453, 1997.

MELO, A. S. A.; ALMEIDA, L. P.; COLOMBO, A. L.; BRIONES, M. R. S. Evolutionary distances and identification of *Candida* species clinical isolates by Randomly Amplified Polymorphic DNA (RAPD). **Mycopathologia**, v. 142, p. 57-66, 1998.

MLADENOV, I. V.; HARALAMBIEVA, I. H.; IANKOV, I. D.; MITOV, I. G. Characterization of 20-kDa lectin-spermagglutinin from *Arum macalatum* that prevents *Chlamydia pneumoniae* infection of L-929 fibroblast cells. **FEMS Immunology and Medical Microbiology**, v. 32, p. 249-254, 2002.

MOLCHANOVA, V.; CHIKALOVETS, I.; CHERNIKOV, O.; BELOGORTSEVA, N.; LI, W.; WANG, J. H.; YANG, D. Y. O.; ZHENG, Y. T.; LUKYANOV, P. A new lectin from the sea worm *Serpula vermicularis*: Isolation, characterization and anti-HIV activity. **Comparative Biochemistry and Physiology, Part C**, v. 145, p. 184–193, 2007.

MONDON, P.; THÉLU, J.; LEBEAU, B.; AMBROISE-THOMAS, P.; GRILLOT, R. Virulence of *Aspergillus fumigatus* strains investigated by Random Amplified Polymorphic DNA Analysis. **J Med Microbiol**, v. 42, p. 299-303, 1995.

MOREIRA, R. A., MONTEIRO, A. C. O,; HORTA, A. C. G.; OLIVEIRA, J. T. A.; CAVADA, B. S. **Plants lectins: Proceeding of the First Brazilian Congress Proteins**. (Oliveira, B. & Sgaburi, S., ed). Ed. Unicamp, Campinas, p. 71-76, 1990.

MORIYAMA, H.; IIZUKA, T.; NAGAI, M.; MIYATAKA, H.; SATOH, T. Antiinflamatory activity of heat-treated *Cassia alata* leaf extract and its flavonoid glycoside. **Yakugaku Zasshi**, v. 123, p. 607-611, 2003.

MOURE, A.; SINEIRO, J.; DOMÍNGUEZ, H. Extraction and functionality of membrane-concentrated protein from defatted *Rosa rubiginosa* seeds. **Food Chemistry**, v.74, p. 327-339, 2001.

NAEEM, A.; KHAN, R. H.; VIKRAM, H.; AKIF, M. Purification of Cajanus cajan root lectin and its interaction with rhizobial lipopolysaccaharide as studies by different

spectroscopic techniques. **Archives of Biochemistry and Biophysics**, v. 396, p. 99-105, 2001.

NASCIMENTO, C. O.; COSTA, R. M. P. B.; ARAÚJO, R. M. S.; CHAVES, M. E. C.; COELHO, L. C. B. B.; PAIVA, P. M .G.; TEIXEIRA, J. A.; CORREIA, M. T. S.; CARNEIRO-DA-CUNHA, M. G. Optimized extraction of a lectin from *Crataeva tapia* bark using AOT in isooctane reversed micelles. **Process Biochemistry**, v. 43, p. 779–782, 2008.

NG, T. B.; LAM, Y. W.; WOO, N. Y. S. The immunostimulatory activity and stability of grass carp (*Ctenopharyngodon idellus*) roe lectin. **Veterinary Immunology and Immunopathology**, v. 94, p. 105-112, 2003.

NG, T. B.; YU, Y. L. Isolation of novel heterodimeric agglutinin from rhizomes of *Smilax glabra*, the Chinese medicinal material tufuling. **The International Journal of Biochemistry & Cell Biology**, v. 33, p. 269-277, 2001.

OJALA, T.; VUOPRELA, P.; KIVIRANTA, J.; VUOPRELA, H.; HILTUNEM, R. A bioassay using *Artemia salina* for detecting phototoxicity of plant coumarins. **Planta medica**, v. 65, I. 8, 715-718, 1999.

OLIVEIRA, V.C.; COSTA, J.L.S. Análise de restrição de DNA ribossomal amplificado (ARDRA) pode diferenciar *Fusarium solani* f. sp. *phaseoli* de *F. solani* f. sp. *glycines*. **Fitopatologia Brasileira**, Brasília, DF, v.27, p.631-634, 2002.

OOI, L. S. M.; SUN, S. S. M.; WANG, H. AND OOI, V. E. C. New Mannose-Binding Lectin Isolated from the Rhizome of Sarsaparilla *Smilax glabra* Roxb. (Liliaceae). **Journal of Agricutural and Food Chemistry**, v. 52, p. 6091-6095, 2004.

OOI, L. S. M.; WONG, E. Y. L.; CHIU, L. C. M.; SUN, S. S. M. and OOI, V. E. C. Antiviral and Anti-proliferative Glycoproteins from the Rhizome of *Smilax glabra* Roxb (Liliaceae). **The American Journal of Chinese Medicine**, v. 36, n. 1, p. 185–195, 2008.

OURTH, D. D.; ROSE, W. M.; SIEFKES, M. J. Isolation of mannose-binding C-type lectin from sea lamprey (*Petromyzon marinus*) plasma and binding to *Aeromonas salmonicida*. **Veterinary Immunology and Immunopathology**, v. 126, p. 407–412, 2008.

PAIVA, P.M.; OLIVA, M.L.; FRITZ, H.; COELHO, L.C., SAMPAIO, C.A. Purification and primary structure determination of two Bowman-Birk type trypsin isoinhibitors from *Cratylia mollis* seeds. **Phytochemistry,** v. 67, n. 6, p. 545-552, 2006.

PAIVA, P. M. G.; COELHO, L. C. B. B. Purification and partial characterization of two lectin isoforms from *Cratylia mollis* Mart. (Camaratu bea). **Applied Biochemistry and Biotechnology**, v. 36, p. 113-118, 1992.

PAJIC, I.; KLJAJIC, Z.; DOGOVIC, N.; SLADIC, D.; JURANIC, Z.; GASIC, M.J. A novel lectin from the sponge *Haliclona cratera*: isolation, characterization and

biological activity. **Comparative Biochemistry and Physiology Part C**, v. 132, p. 213-221, 2002.

PARISI, M. G.; MORENO, S.; FERNÁNDEZ, G. Isolation and characterization of a dual function protein from *Allium sativum* bulbs which exhibits proteolytic and hemagglutinating activities. **Plant Physiology and Biochemistry**, v. 46, p. 403-413, 2008.

PARRA, A. L.; YHEBRA, R. S.; SARDIÑAS, I. G.; BUELA, L. I. Comparative study of the assay of *Artemia salina* L. and the estimate of the medium lethal dose (LD50 value) in mice, to determine oral toxicity of plant extracts. **Phytomedicine: International Journal of Phytotherapy and Phytopharmacology**, v. 8, p. 395-400, 2001.

PEAKALL, R.; GILMORE, S.; KEYS, W.; MORGANTE, M.; RAFALSKI, A. Cross species amplification of soybean (Glycine max) simple sequence repeats (SSRs) within the genus and other legume genera: Implications for the transferability of SSRs in plants. **Mol Biol Evol**, v. 15, p. 1275-1287, 1998.

PEDINI, V.; SCOCCO, P.; GARGIULO, A. M.; CECCARELLI, P.; LORVIK, S. Glycoconjugate characterization in the intestine of *Umbrina cirrosa* by means of lectin histochemistry. **Journal of Fish Biology**, v. 61, p. 1363-1372, 2002.

PENNANEN, T.; PAAVOLAINEN, L.; HANTULA, J. Rapid PCR-based method for the direct analysis of fungal communities in complex environmental samples. **Soil Biology & Biochemistry**, v.33, p.697-699, 2001.

PEUMANS, W. J.; WINTER, H. C.; BEMER, V.; VAN LEUVEN, F.; GOLDSTEIN, I. J.; TRUFFA-BACHI, P. and VAN DAMME, E. J. M. **Glycoconjugate J.**, 1996.

PEUMANS, W. J.; VAN DAMME, E. J. M. Plant lectins: Versatile Proteins with important perpectives in biotechnology. **Biotechnology and Genetic Engineering Reviews**, v. 15, p. 199-228, 1998.

PEUMANS, W. J., VAN DAMME, E. J. M., BARRE, A. & ROUGÉ, P. Classification of plant lectins in families of structural and evolutionary related proteins. In: The molecular immunology of complex carbohydrates-2, (Wu A. M. ed), p. 27-54. Kluwer Academic, 2001.

POHLEVEN, J.; OBERMAJER, N.; SABOTIČ, J.; ANŽLOVAR, S.; SEPČIĆ, K.; KOS, J.; KRALJ, B.; ŠTRUKELJ, B.; BRZIN, J. Purification, characterization and cloning of a ricin B-like lectin from mushroom *Clitocybe nebularis* with antiproliferative activity against human leukemic T cells, **Biochimica et Biophysica Acta**, *article in press*, 2009.

POONWAN, N.; IMAI, T.; MEKHA, N.; YASAWA, K.; MIKAMI, Y.; ANDO, A.; NAGATA, Y. Genetic analysis of *Histoplasma capsulatum* strains isolated from clinical specimens in Thailand by a PCR-based Random Amplified Polymorphic DNA method. **J Clin Microbiol**, v. 36, p. 3073-3076, 1998.

POWER, E. G. M. RAPD typing in microbiology - A technical review. **J Hosp Infect**, 34: 247-265, 1996.

PUHALLA, J.E. Genetic considerations of the genus *Fusarium.* In: Nelson, P.E.; Toussoun, T.A.; Cook, R.J. (Ed) **Fusarium: diseases, biology, and taxonomy.** Pennsylvania: Pennsylvania State University, v.27, p.291-305, 1981.

RADIS-BAPTISTA, G.; MORENO, F. B.; NOGUEIRA, L. L.; MARTINS, A. M.; TOYAMA, D. O.; TOYAMA, M. H.; CAVADA, B. S.; AZEVEDO JR., W. F. and YAMANE, T. Crotacetin, a novel snake venom C-type lectin homolog of convulxin, exhibits and unpredictable antimicrobial activity. **Cell Biochem. Biophys.**, v. 44, p. 412-423, 2006.

REDDY, M. P.; SARLA, N.; SIDDIQ, E. A. Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. **Euphytica**, v. 128, p. 9-17, 2002.

REGO, E. J. L.; CARVALHO, D. D.; MAGANRONI, S.; OLIVEIRA, B.; NOVELLO, J. C. Lectins from seeds of *Crotalaria pallida* (Smooth rattlebox). Phytochemistry, v. 60, p. 441-447, 2002.

REIS, A.; COSTA, H.; BOITEUX, L.S.; LOPES, C.A. First report of *Fusarium oxysporum* f. sp. *lycopersici* race 3 on tomato in Brazil. **Fitopatologia Brasileira,** Brasilia, DF, v. 30, p. 426-428, 2005.

RODRIGUES, J. S.; SANTOS-MAGALHÃES, N. S.; COELHO, L. C. B. B.; COUVREUR, P.; PONCHEL, G.; GREF, R. Novel core (polyester)-shell (polyposaccharide) nanoparticles: protein loading and surface modification with lectins. **Journal of Controlled Release**, v. 92, p. 103-112, 2003.

ROJO, M. A.; YATO, M.; ISHII-MINAMI, N.; MINAMI, E.; KAKU, H.; CITORES, L.; GIRBÉS, T.; SHIBUYA, N. Isolation, cDNA cloning, biological properties, and carbohydrate binding specificity of siebolding-b, a type II ribosome-inactivating protein from the bark of japanese elderberry (*Sambucus sieboldiana*). **Archives of Biochemestry and Biophysics,** v. 340, n. 2, p. 185-194, 2003.

RÜDIGER, H. Plant lectins – more thanjust tools for glycoscientlists: **Acta Anatômica**, v. 161, p. 130-152, 1998.

RUPPERT, E. E. & BARNES, D. R. **Zoologia dos invertebrados**. São Paulo, Rocca, 6 [a] ed., 1029 p., 1996.

SÁ, R. A.; GOMES, F. S.; NAPOLEÃO, T. H.; SANTOS, N. D. L.; MELO, C. M. L.; GUSMÃO, N. B.; COELHO, L. C. B. B.; PAIVA, P. M. G; BIEBER, L. W. Antibacterial and antifungal activities of *Myracrodruon urundeuva* heartwood. **Wood Science and Technology**, *article in press*, 2008.

SÁ, R. A.; SANTOS, N. D. L.; SILVA, C. S. B.; NAPOLEÃO, T. H.; GOMES, F. S.; CAVADA, B. S.; COELHO, L. C. B. B.; NAVARRO, D. M. A. F.; BIEBER, L. W.;

PAIVA, P. M. G. Larvicidal activity of lectins from *Myracrodruon urundeuva* on *Aedes aegypti*. **Comparative Biochemistry and Physiology**, Part C, *article in press*, 2008.

SAMPIETRO, A. R.; ISLA, M. A.; QUIROGA, E. N.; VATTUONE, M. A. An N-acetylglucosamine oligomer binding agglutinin lectin from ripe *Cyphomandra betacea* Sendt. Fruits. **Plant Science**, v. 160, p. 659-667, 2001.

SÁNCHEZ, C.; GUPTA, M.; VASQUEZ, M.; DE NORIEGA, Y. M.; MONTENEGRO, G. Bioassay with brine *Artemia* to predict antibacterial and pharmacologic activity. **Revista medica de Panama**, v. 18, p. 62-69, 1993.

SANTANA, G. M. S.; ALBUQUERQUE, L. P.; SIMÕES, D. A.; GUSMÃO, N. B.; COELHO, L. C. B. B.; PAIVA, P. M. G. Isolation of lectin from *Opuntia ficus indica* cladodes. **Acta Horticulturae**, 2008.

SANTANA, M. A.; SANTOS, A. M. C.; OLIVEIRA, M. E.; OLIVEIRA, J. S.; BABA, E .H.; SANTORO, M. M.; ANDRADE, M. H. G. A novel and efficient and low-cost methodology for purification of *Macrotyloma axillare* (Leguminosae) seed lectin. **International Journal of Biological Macromolecules**, v. 43, p. 352–358, 2008.

SANTOS, A. C. O.; PEIXOTO, C. A.; COELHO, L. C. B. B. Ultrastructural analysis and immunocytochemical localization of isolectins. *Cratylia mollis* seeds. **Micron**, Oxford, v. 35, n.7, p. 613-618, 2004.

SANTOS, A.F.S.; ARGOLO, A.C.C.; COELHO, L.C.B.B.; PAIVA, P.M.G. Detection of water soluble lectin and antioxidant component from *Moringa oleifera* seeds. **Water Research,** v. 39, p. 975-980, 2005.

SENGUPTA, S.; SINGH, S.; SENGUPTA, L. K.; BISEN, P. S. Phytolectins: Natural molecules with immense biotechnological potential. **Indian Journal of Experimental Biology**, v. 35, p. 103-110, 1997.

SHARON, N. Trends Biochemical. **Science**, v. 18, p. 221-226, 1993.

SHARON, N.; LIS, H. A century of lectins: impact on immunology. *In*: **Cellular Basis of Immune Modulation**, p. 609-619, 1988.

SHARON, N., LIS, H. History f lectins: from hemagglutinins to biological recognition molecules. **Glycobiology**, Cary, v. 14, n. 11, p. 53R-62R, 2004.

SILVA, M. D. C.; SÁ, R. A.; NAPOLEÃO, T. H.; GOMES, F. S.; SANTOS, N. D. L.; ALBUQUERQUE, A. C.; XAVIER, H. S.; PAIVA, P. M. G.; CORREIA, M. T. S.; COELHO, L. C. B. B. Purified *Cladonia verticillaris* lichen lectin: Insecticidal activity on *Nasutitermes corniger* (Isoptera: Termitidae). **International Biodeterioration & Biodegradation**, *article in press*, 2008.

SITOHY, M.; DOHEIM, M.; BADR, H. Isolation and characterization of a lectin with antifungal activity from Egyptian *Pisum sativum* seeds. **Food Chemistry**, v. 104, p. 971–979, 2007.

SNYDER, W.C.; HANSEN, H.N. Species concept, genetics and pathogenicity in hypomyces solani. **Phytopathology,** v. 44, p.338-342, 1953.

SOLL, D. R. The ins and outs of DNA fingerprinting the infectious diseases. **Clin Microbiol Rev**, 13 (2): 332-70, 2000.

STRYER, L. **Bioquímica**. 5ª Edição. Rio de Janeiro: Guanabara Kooga, 2004.

SUN, J.; WANG, L.; WANG, B.; GUO, Z.; LIU, M.; JIANG, K.; TAO, R.; ZHANG, G. Purification and characterization of a natural lectin from the plasma of the shrimp *Fenneropenaeus chinensis*. **Fish & Shellfish Immunology**, v. 25, p. 290-297, 2008.

TASUMI, S.; YANG, W-J; USAMI, T.; TSUTSUI, S.; OHIRA, T.; KAWAZOE, I.; WILDER, M. N.; AIDA, K.; SUZUKI, Y. Characteristics and primary structure of galactin in the skin mucus of the Japanese eel, *Anguilla japonica*. **Developmental Comparative Immunology**, v. 28, p. 325-335, 2004.

THAKUR, A.; RANA, M.; LAKHANPAL, T. N.; AHMAD, A.; KHAN, M. I. Purification and characterization of lectin from fruiting body of *Ganoderma lucidum*. **Biochimica et Biophysica Acta**, v. 1770, p. 1404–1412, 2007.

TAYLOR, E.; BATES, J.; KENYON, D.; MACCAFERRI, M.; THOMAS, J. Modern molecular methods for characterization and diagnosis of seed-borne fungal pathogens. **Journal of Plant Pathology**, v.83, p.75-81, 2001.

THOMAS, V.; RUTHERFORD, M. A.; BRIDGE, P. D. - Molecular diferentiation of two races of *Fusarium oxysporum* especial form *cubense*. **Letters in Applied Microbiology**, 18: 193-196, 1994.

TIAN, Q.; WANG, W.; MIAO, C.; PENG, H.; LIU, B.; LENG, F.; DAI, L.; CHEN, F.; BAO, J. Purification, characterization and molecular cloning of a novel mannose-binding lectin from rhizomes of *Ophiopogon japonicas* with antiviral and antifungal activities. **Plant Science**, v. 175, p. 877–884, 2008.

VAN ELSAS, J.D.; DUARTE. G.F.; ROSADO. A.S.; SMALLA, K. Microbiological an molecular biological methods for monitoring microbial inoculants and their effects in the soil environmental. **Journal of Microbiological Methods**, v.32, p.133-154, 1998.

VOS, P.; HOGERS, R.; BLEEKER, M.; REIJANS, M.; VAN, L. T.; HORNES, M.; FRITJERS, A.; PELEMAN, J.; KUIPER, M.; ZABEAU, M. AFLP: a new tool for DNA finger-printing. **Nucleic Acids Research,** v. 23, p. 4407-4414, 1995.

YANG, H.; LUO, T.; LI, F.; LI, S.; XU, X. Purification and characterization of a calcium-independent lectin (PjLec) from the haemolymph of the shrimp *Penaeus japonicus*. **Fish and Shellfish Immunology**, v. 22, p.88-97, 2007.

WANG, H. X.; NG, T. B.; LIU, Q. A novel lectin from the wild mushroom *Polyporus adusta*. **Biochemical and Biophysical Research Communications**, v. 307, p. 535-539, 2003.

WANG, H. X.; NG, T. B. Purification of Castamollin, a novel antifungal protein from *Chinese chestnuts*. **Protein Expression & Purification**, v. 32, p. 44-51, 2003.

WANG, H. X.; NG, T. B. Concurrent isolation of a Kunitz-type trypsin inhibitor with antifungal activity and a novel lectin from *Pseudostellaria heterophylla* roots. **Biochemical and Biophysical Research Communications**, v. 342, p. 349–353, 2006.

WEISING, K.; NYBOM, H.; WOLFF, K.; MEYER, W. PCR - based DNA fingerprinting. **DNA fingerprinting in plants and fungi**, CRC Press, Florida, 1994. p. 112-135.

WINDISH, P. G. **Pteridófitas da Região Norte - Ocidental do Estado de São Paulo - Guia para estudo e excursões.**2º Ed. Campus de São José do Rio Preto. Universidade Estadual Paulista: UNESP. 1992. 110p.

WONG, J. H.; NG, T. B. Purification of a trypsin-stable with antiproliferative and HIV-1 reverse transcriptase inhibitory activity. **Biochemical and Biophysical Research Communications**, v. 301, p. 545-550, 2003.

WONG, J. H.; CHAN, H. Y. E.; NG, T. B. A mannose/glucose-specific lectin from Chinese evergreen chinkapin (*Castanopsis chinensis*). **Biochimica et Biophysica Acta**, v. 1780, p. 1017–1022, 2008.

XU, Q.; LIU, Y.; WANG, X. C.; GU, H. Y.; CHEN, Z. L. Purification and characterization of a novel anti-fungal protein from *Gastrodia elata*. **Plant Physiol. Biochem.**, v. 36, p. 899–905, 1998.

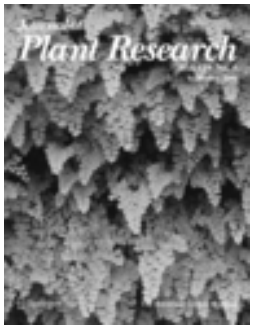
ZAHA, A. **Biologia Molecular Básica**. Ed Mercado Aberto, Porto Alegre, 1996.

ZEMANKOVA, M. LEBEDA, A. *Fusarium* species, their taxonomy, variability and significance in plant pathology. Review. **Plant Protection Science UZPI**, v.37, p.25-42, 2001.

ZHAO, J. K.; WANG, H. X.; NG, T. B. Purification and characterization of a novel lectin from the toxic wild mushroom *Inocybe umbrinella*, **Toxicon**, 2008.

ZHENG, P.; WANG, H.; ZHAO, J.; SONG, L.; QIU, L.; DONG, C.; WANG, B.; GAI, Y.; MU, C.; LI, C.; NI, D.; XING, K. A lectin (CfLec-2) aggregating *Staphylococcus haemolyticus* from *scallop Chlamys farreri*, **Fish & Shellfish Immunology**, v. 24, p. 286-293, 2007.

4. ARTIGO A SER SUBMETIDO AO PERIÓDICO JOURNAL OF PLANT RESEARCH



TOXIC AND ANTIFUNGAL LECTIN ISOLATED FROM RHIZOME OF *Microgramma vaccinifolia*

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Abstract

Rhizome of *Microgramma vaccinifolia* is used as a popular medicine for the treatment of diarrhea and human respiratory tract infections. This study reports the rhizome hemagglutinin (lectin) from *M. vaccinifolia* (MvRL), an acidic and glycosylated protein of 100 kDa constituted by 17 kDa subunits which hemmaglutinating activity is thermo-stable, active at pH 5.0, dependent on ions and inhibited by mannose and glycoproteins. Preparations of glycoproteins from three races of *Fusarium oxysporum* f. sp. *lycopersici* similarly inhibited MvRL hemagglutinating activity. MvRL was toxic on *Artemia salina* (LC_{50}154.62 µg ml^{-1}) and not showed antibacterial activity on Gram-positive and Gram-negative bacteria. Antifungal activity of MvRL was detected on *F. decemcellulare*, *F. lateritium*, *F. moniliforme*, *F. oxysporum* f.sp. *lycopersici* races 1, 2 and 3 and *F. solani.* This paper also described molecular variability of *Fusarium oxysporum* f. sp. *lycopersici* races using inter simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) methods. Toxicity and effect of MvRL on *Fusarium* species are indicative of its potential biotechnological use as antifungal and antitumor agent.

Keywords: Antifungal activity, *Artemia salina*, *Fusarium oxysporum* f.sp. *lycopersici*, ISSR, lectin, *Microgramma vaccinifolia,* RAPD, Toxicity.

Introduction

Lectins are proteins that have the ability to bind to carbohydrates expressed on different cell surfaces (Correia et al. 2008). Although first identified in plants (Chen et al. 2008), they are now known to exist throughout nature, including both prokaryotic (Bhowal et al. 2005) and eukaryotic (Adhya et al. 2008) organisms. The specificity of lectin-carbohydrate interaction makes them useful in medical, biological,

pharmacological and biochemistry researches (Beltrão et al. 2003, Bains et al. 2005, Santana et al. 2008, Zhao et al. 2008, Battison et al. 2009). Plant lectins have already being suggested as biological insecticide (Sá et al. 2008), antibacterial (Gowda et al. 2008) and antifungal (Chen et al. 2008) agents as well as for cancer therapy (Schumacher et al. 2003). Bioassay with *Artemia salina* has been used to establish the toxicity of synthetic and natural compounds via estimation of lethal concentration LC_{50} (Barahona & Sánchez-Fortune, 1996, Parra et al. 2001). The detection of toxicity in extracts of plants has been used as a prognosis of their antitumor and pesticide activities (Sánchez et al. 1993, Ojala et al. 1999).

Lectins isolated from rhizome have showed activity on microorganisms as well as on normal and tumor cells. SGM2 isolated from *Smilax glabra* exhibited antiviral activities against herpes simplex virus type 1 (Ooi et al. 2004), ADL from *Arundo donax* showed antiproliferative activity towards human cancer cell lines and was mitogen to human peripheral blood mononuclear cells (Kaur et al. 2005), PCL from *Polygonatum cyrtonema* induced apoptosis and autophagy in human melanoma A375 cells (Liu et al. 2008), OJL from *Ophiopogon japonicus* inhibited herpes simplex virus type II and showed antifungal activity against *Gibberella saubinetii* and *Rhizoctonia solani* (Tian et al. 2008).

Fusarium oxysporum f. sp. *lycopersici* occurs in all Brazilian regions and wind, water and agricultural products are considered to be the main disseminators of this fungus in short distance (Agrios, 2005). The genus *Fusarium* varies in morphology and pathogenic resulting in a complex classification divided into sections, formae speciales (f. sp.) and races (Oliveira & Costa 2002). There is a predominance of race 1, but the race 2 has increased in importance. Race 3 shows a more limited geographical distribution and adapted cultivars with resistance to race 3 are not yet widely available

(Agrios, 2005, Reis et al. 2005). Due to limitations in developing morphological studies, molecular methods have aided in the definition of species and races (Manicom et al. 1987, Thomas et al. 1994). Tomato is the unique host for this soil fungus and the beginning of flowering and fructification of plant are the most affected periods of life cycle (Reis et al. 2005). *F. oxysporum* f. sp. *lycopersici* causes a plant vascular disease and the chemical control is not efficient. The use of resistant strains to fungus constitutes is the more viable manner for disease control, since *F. oxysporum* f. sp. *lycopersici* can remain in soil for decades (Brayford, 1992).

Microgramma vaccinifolia is an epiphytic plant broadly distributed in world found preferentially at the tropics. In Brazil, there are registers of this species in all states mainly in Amazonas and across of the Brazilian littoral (Windisch, 1992). Rhizomes from *M. vaccinifolia* have widespread use in folk medicine in the treatment of diarrhea and as balsamic in infections in the respiratory tract (Barros and Andrade, 1997). This paper describes the isolation and characterization of lectin from *M. vaccinifolia* rhizome (MvRL) and its effect on *A. salina*, Gram-negative and Gram positive bacteria as well as on *Fusarium* species. Additionally was investigated the efficiency of molecular methods inter simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) as tools for genetic variability identification of *F. oxysporum* f. sp. *lycopersici* races.

Materials and Methods

Plant material

M. vaccinifolia (Langsd. & Fisch) Copel. belongs to the Division Pteridophyta, Class Filices, Subclass Leptosporangiatae, Order Filicales, Family Polypodiaceae (Joly, 1985) and in Brazil its common name is “erva-silvina”. Rhizomes were collected in

Recife City, State of Pernambuco, Northeastern Brazil, and stored at -20º C. A voucher specimen is archived under number 63,291/2003 at the herbarium Dárdano de Andrade Lima (Empresa Pernambucana de Pesquisa Agropecuária, Recife, Brazil).

Isolation of MvRL

Dry rhizome was milled to a fine powder. The powder (10 g) was then homogenised in a magnetic stirrer (16 h at 4 °C) with 0.15 M NaCl (100 ml), filtered through gauze and centrifuged at 3,000 *g* (15 min). The supernatant was then submitted to ammonium sulphate fractionation (saturation of 60%) and the precipitated protein (0-60 fraction) was collected by centrifugation (Green and Hughes, 1955). The 0-60 fraction was dissolved in 0.15 M NaCl and after dialysis (3.5 kDa cut-off membrane) against 0.15 M NaCl (8 h at 4 °C) was applied (6.0 mg of proteins) onto a Sephadex G-25 column (21.0 x 1.0 cm) equilibrated (20 ml min^{-1} flow rate) with 0.15 M NaCl and the fractions with hemagglutinating activity collected in the washing chromatographic step were pooled (MvRL).

Gel filtration chromatography

The molecular mass of native MvRL was determined by gel filtration chromatography on Hiprep 16/60 Sephacryl S-300 column (60 cm x 16 mm)/Äkta FPLC System (Amersham Pharmacia Biotech, Sweden) equilibrated (1 ml min^{-1} at 24 °C) with 0.5 M NaCl. Sample (2.0 ml; 1 mg of protein) was injected and eluted (3.0 ml fraction) with the same solution. The mass molecular markers (Sigma, USA) similarly chromatographed were bovine serum albumin (66,000 Da), fetuin (64,000 Da), ovoalbumin 45,000 Da), type III-O trypsin inhibitor from chicken (28,000 Da) and trypsin (25,000 Da).

Protein content

The protein concentration was estimated in all samples according to Lowry et al. (1951) using bovine serum albumin (31–500 μg ml^{-1}) as standard.

Hemagglutinating activity

Rabbit and human (A, B, AB and O types) erythrocytes obtained as described by Bukantz et al. (1946) were treated with glutaraldehyde (Bing et al. 1967). Hemagglutinating assay of rhizome preparations (50 μl) was carried out in microtiter plates (Kartell S.P.A., Italy) according to Paiva and Coelho (1992) using 2.5% (v/v) erythrocyte suspension (50 μl). Hemagglutinating activity (titer), defined as the reciprocal of the highest dilution of the sample promoting full agglutination of erythrocytes, was expressed as one hemagglutination unit. Specific hemagglutinating activity was defined as the ratio between the titer and protein concentration (unit/mg).

Hemagglutinating activity inhibitory assay was performed by incubation (45 min) of MvRL (50 μl containing 40 μg) with inhibitor solution (50 μl) before addition of type O^+ erythrocyte suspension (50 μl). The solutions evaluated were: 200 mM carbohydrate ((+)-arabinose, D(-)-fructose, D(+)-galactose, D(+)-glucose, α-D-glucopyranoside, D(+)-lactose, D(+)-mannose, methyl-α-D-mannopyranoside, N-acetyl-D-glucosamine, D(+)-raffinose, L(+)-rhamnose, sucrose, threalose, D(+)-xylose), 0.5 mg commercial glycoproteins (bovine serum albumin, casein, bovine fetal serum, ovalbumin) or glycoprotein solutions from *Fusarium oxysporum* f. sp. *lycopersici* race 1, 2 and 3 prepared by maceration of fungal mycelium in liquid nitrogen followed by solubilization of race 1 (0.1639 g), race 2 (0.0898 g) and race 3 (0.1149 g) in 0.15 M NaCl (200 μl).

The effects of pH and temperature on hemagglutinating activity were evaluated by incubating (45 min at 28 °C) of MvRL in selected solutions (HCl pH 2.5 and 3.5; 10 mM citrate phosphate buffer pH 4.0, 5.0 and 6.0; sodium phosphate buffer, pH 7.0; Tris-HCl buffer pH 8.0 and 9.0; NaOH, pH 10 and 11) or at 30, 40, 50, 60, 70, 80, 90 or 100 °C for 30 min before type O^+ erythrocyte suspension addition.

MvRL was dialyzed against 0.005 M EDTA (16 h at 4 ºC) followed against 0.15 M NaCl (6 h at 4 ºC) to eliminate EDTA and the hemagglutinating assay was performed at presence of 0.01, 0.02 and 0.04 M Ca^{2+} solution.

Polyacrylamide gel electrophoresis (PAGE)

PAGE (10 % w/v) of MvRL was performed in the presence of sodium dodecyl sulphate (SDS), with and without β-mercaptoethanol according to Laemmli (1970). The molecular mass of polypeptide was determined by comparison of its electrophoretic mobility with those of molecular mass marker (phosphorilase b, 97,000 Da; bovine serum albumin, 66,000 Da; glyceraldehyde-3-phosphate dehydrogenase, 36,000 Da; trypsin inhibitor 20,000 Da and α-lactalbumin 14,200 Da from Sigma, USA). PAGE for native basic (7.5 %, w/v) and acidic (12 %, w/v) proteins were made according to Reisfeld et al. (1962) and Davis (1964), respectively. The Schiff periodic acid (PAS) method was also used for glycoprotein gel staining (Pharmacia Fine Chemicals, 1980). Glycoprotein preparations previously described of races 1, 2 and 3 of *F. oxysporum* f.sp. *lycopersici* were also submitted to SDS-PAGE (10% w/v) and after electrophoresis the gel was stained with Schiff's reagent.

Carbohydrate content

The concentration of total sugars was estimated according to phenol-sulphuric acid method described by Dubois et al. (1956) using mannose as standard.

Toxic activity

Toxicity was evaluated by determination of effect of MvRL on survival rate of brine shrimp larvae (*Artemia salina* Leach) according to the procedure described by Meyer et al. (1982). *A. salina* eggs (25 mg) were hatched in natural seawater at 20-30 ºC. The pH was adjusted to 8.5 using Na_2CO_3 to avoid risk of death to the larvae by decrease of pH during incubation (Lewan et al. 1992). The eggs were placed in container with two compartments. The compartment with the eggs was covered in order to keep the eggs in a dark ambient. The other compartment of the container was illuminated in order to attract *A. salina* through perforations at the boundary plate. After 24 h, the phototropic brine shrimps, which went to the illuminated compartment, were collected using Pasteur pipette. The tested MvRL concentrations were 25, 50, 75, 100, 125, 250, 500 e 750 μg ml^{-1} and the control solution (natural seawater) was set with vehicle used for dilutions. The test and control tubes with sample containing 12-15 larvae of *A. salina* were filled with 5 ml volume total with seawater. The time of exposition of the target organisms was 24 h. After this time, survival rate was determined and used to estimate MvRL concentration required to kill 10% (LC_{10}), 50% (LC_{50}) and 90% (LC_{90}) of larvae. The assay was carried out in triplicate and repeated in three experiments.

Antibacterial activity

Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Streptococcus pyogenes*) and

Gram-negative (*Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*) bacteria from Hospital Barão de Lucena, Pernambuco, Brazil were used. Stationary cultures were maintained in Nutrient Agar (NA) and stored at 4 °C. Bacteria were incubated under permanent shaking at 43 °C overnight. The culture concentrations were adjusted turbidimetrically at a wavelength of 600 nm to 10^5–10^6 colony forming units (CFU) per ml. Disk diffusion method (Bauer et al. 1966) was used. The solution was distributed in sterile Petri plates (90 x 15 mm) in portions of 10 ml and allowed to solidify. Aliquots (15 µl) of MvRL (12 µg) was impregnated on sterile paper disks (6 mm diameter) and placed on agar. Negative and positive controls were 0.15 M NaCl and Amoxicilin (1 mg ml^{-1}), respectively. Plates were incubated at 37 °C for 24 h. A transparent inhibition zone around the paper disk revealed antibacterial activity. The assay was performed in triplicate.

Antifungal activity

Fungi were from Culture Collections of University Recife Mycologia (URM) of Departamento de Micologia, Universidade Federal de Pernambuco, Brazil. Antifungal activity of MvRL was evaluated against *F. decemcellulare* (URM-3006), *F. lateritium* (URM-2491), *F. moniliforme* (URM-3226), *F. oxysporum* (URM-2489) and *F.solani* (URM-2490) identified by Dr. Débora Maria Massa Lima (Departamento de Micologia, Universidade Federal de Pernambuco, Brazil) as well as against races 1, 2 and 3 of *F. oxysporum* f.sp. *lycopersici* from Empresa Pernambucana de Pesquisa Agropecuária, Recife, Brazil.

Antifungal activity was performed according to Cunico et al. (2004). The method has been modified by application of samples in solid potato-dextrose agar (PDA) medium rather than liquid medium used by Cunico et al. (2004). MvRL was

filtered using a 0.45 µm sterile syringe filter (Minisart®). Next, aliquot (50 µl; 40 µg) was spread on solidified PDA medium in Petri plates (100 x 15 mm) and a fungal mycelium disk (0.625 cm in diameter) was disposed in the center of Petri plate. 0.15 M NaCl solution and 10 ppm Cercobin were used as negative and positive controls, respectively. The plates were incubated at 28 °C for 72 h. Antifungal activity was indicated by a reduction of the fungal growth zone (diameter) in the plates in comparison to negative control. The assays were carried out in triplicate.

Extraction of Genomic DNA from *F. oxysporum* f.sp. *lycopersici* races

Cultures (10^7 conidia ml^{-1}) of *F. oxysporum* f.sp. *lycopersici* races 1, 2 and 3 and *Metarhizium* isolates were grown for 72 h in PDA medium at 28 °C. The fungal mycelium was harvested by filtration and washed with distilled water.

The CTAB (cetyltrimethylammonium bromide) method was performed as described by Doyle and Doyle (1990) with slight modification. Samples (1.0 g of grounded and homogenised *F. oxysporum* f. sp. *lycopersici* races and *Metarhizium* isolates) were transferred to a 2 ml sterile reaction tube followed by the addition of 1.0 ml of CTAB extraction buffer [1% polyvinylpyrrolidone (PVP), 2% CTAB (p/v), 1.4 M NaCl, 2% (v/v), β-mercaptoethanol, 20 mM EDTA, and 100 mM Tris-HCl, pH 8.0] and mixed. After the incubation at 65 °C for 1 h, with occasional stirring, the suspension was centrifuged (15 min, 18,000 *g*) and 500 µl of the supernatant were extracted with 400 µl of chloroform, stirred and centrifuged (5 min, 12,000 g). The upper phase was transferred to a new tube (1.5 ml), mixed with 2/3 from volume of CTAB precipitation solution (5 g l^{-1}, 0.04 M NaCl) and incubated for 1 h at 28 ºC. After centrifugation (5 min, 12,000 g), the supernatant was discarded and the precipitate was dissolved in 350 µl of 1.2 M NaCl and extracted with 350 µl chloroform. The mixture was centrifuged

(10 min, 12,000 *g*), the supernatant was discarded and the pellet was washed with 1.0 ml of ethanol solution (70 %, v/v) and air-dried at 28 ºC. After centrifugation, the supernatant was discarded carefully by pipeting, the pellet was dried and the DNA was dissolved in 30 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8,0) containing 3 µl of RNAse and stored at 4 or -20 °C. DNA quality was tested using 1 % agarose gel electrophoresis.

ISSR and RAPD amplifications

Following the DNA extraction, amplifications by PCR were carried out in a 25 µl volume of a mixture containing sterilized milli–Q water, PCR amplification buffer (10 mM Tris-HCl pH 8.0, 100 µM dNTPs, 0.4 µM primer from Invitrogen, 2 mM $MgCl_2$, 1 U (unit) Taq DNA-polimerase and 30 ng of genomic DNA). DNA amplifications were carried out using the thermocycler model MJ Research, Inc. PTC100 Programmable Thermal Controller (Watetown, USA). ISSR reactions were carried out with initial denaturing step at 94 °C for 15 min, followed by 30 cycles with a denaturing step at 94 °C for 30 seconds. RAPD program involved initial denaturing step of 94 °C for 5 min followed by 30 steps of 1 min at 94 °C, 1 min at 42 °C and 1 min at 72 °C with a final extension step of 5 min at 72 °C. Amplified products were size-fractionated by electrophoresis in 2.0 % ISSR or 1.2 % RAPD (w/v) agarose gel containing TBE buffer (0.09 M Tris-borate and 0.002 M EDTA) and bands were stained with SyBr Gold (1X, Invitrogen) and then photographed under UV light (Vilber Lourmat).

Statistical analysis

Statistical analysis of antifungal activity was performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA) and data were expressed as a mean of three assays ± SD.

Statistical analysis of the experimental data from assay of brine shrimp lethality activity was performed using the computer software StatPlus® 2006 (AnalystSoft, Canada) to find the lethal protein concentrations required to kill 10% (LC_{10}), 50% (LC_{50}) and 90% (LC_{90}) of larvae in 24 h by probit analysis with a interval of confiance of 95%

The ISSR and RAPD bands were scored using the binary scoring system that recorded the presence and absence of bands as 1 and 0, respectively. Smeared and weak bands were excluded. Bands with the same migration distance were considered homologous. A pair-wise similarity matrix was computed and analysed with GENES software (Cruz, 1997) on the basis of Jaccard's similarity coefficients. The resulting matrix of genetic similarity was used to construct the dendrogram through the unweighted pair group method with arithmetic mean (UPGMA- *Unweighted Pair Group Method Using Arithmetic Average*).

Results and Discussion

The lectin from *M. vaccinifolia* rhizome, MvRL, could be isolated by protocol that employed only one chromatographic step of gel filtration on Sephadex G-25 matrix. 0-60% fraction was used to isolate the lectin since it showed high specific hemagglutinating activity (341) and MvRL was recovered in unadsorbed fractions from column. The fact of MvRL not bind to Sephadex G-25 avoids the use of an eluent solution of high ionic strength (Yang et al. 2007, Pohleven et al. 2009) or low pH value (Jung et al. 2007, Kavitha et al. 2009) to remove the lectin and thus dialysis step

generally used for elimination of eluent solution from chromatographic fractions was not need. Sephadex G-25 column was successfully employed for MvRL isolation (purification factor of 5 fold) and 4.2 mg of chromatographed protein yielded 1.6 mg of MvRL (Table 1). The established protocol for MvRL was similar to that used by Ghosh (2008) to purify *Withania somnifera* lectin, and constitute a cheap method since the consecutive use of Sephadex G-25 matrix (six times) showed same yielded.

MvRL by gel filtration on Sephacryl S-300 was a 100 kDa protein (Figure 1A) and PAGE for native acidic proteins showed a single band (Figure 1B1); no protein was detected on PAGE to native basic proteins. SDS-PAGE at presence or absence of reducing agent β-mercaptoethanol revealed polypeptide of 17 kDa (Figure 1B2). These results indicate MvRL as molecular aggregate similar to homodimer lectin isolated from *Ophiopogon japonicas* rhizome (Tian et al. 2008). Lectins exhibit different molecular arrangements and many of them are monomeric (Jung et al. 2007), dimeric (Matsuura et al. 2007; Pohleven et al. 2009) or tetrameric (Huang et al. 2008; Tian et al. 2008) proteins.

Glycoprotein staining on SDS-PAGE revealed polypeptide band indicating the presence of carbohydrate (Figure 1B3). The lectins of *Schizophyllum commune* (Chumkhunthod et al. 2006), *Penaeus japonicus* (Yang et al. 2007) and *Smilax glabra* (Ooi et al. 2008) were considered as glycoproteins because they were reactive to the Schiff's reagent. In fact 32.5% of carbohydrate was determined in MvRL by Dubois method and carbohydrate content of MvRL was higher than 2.1% searched for *Arundo donax* rhizome lectin (Kaur et al. 2005).

Rhizome extract, 0-60% fraction and MvRL were able to agglutinate A, B and O human erythrocytes at different degrees and this difference in hemagglutinating activity may be due to the nature of the glycoproteins present on erythrocyte surface, which are

weakly or not totally recognized by the lectin (Oliveira et al. 2002). Highest specific hemagglutinating activity of extract (16.5), 0-60% fraction (341.3) and MvRL (160) were detected with human type O erythrocytes. The erythrocyte recognizing by MvRL was similar to those of lectins from *Penaeus japonicas* (Yang et al. 2007), *Holothuria scabra* (Gowda et al. 2008), and *Fenneropenaeus chinensis* (Sun et al. 2008). Human AB and rabbit erythrocytes were not agglutinated by preparations of rhizome from *M. vaccinifolia*

The carbohydrate recognition of MvRL was evaluated by effect of monosaccharide and glycoprotein on hemagglutinating activity. The activity of MvRL was reduced (specific hemagglutinating activity of 20) by mannose, casein, ovalbumin and glycoproteins from races 1, 2 and 3 of *F. oxysporum* f.sp. *lycopersici*, whereas bovine fetal serum abolished the activity. Table 2 shows carbohydrate and protein contents of the glycoprotein solutions from races 1, 2 and 3 of *F. oxysporum* f.sp. *lycopersici* and Figure 2 reveals the glycoproteins by staining of SDS-PAGE with Schiff´s reagent. The linkage of monosaccharide or carbohydrate moiety of glycoprotein to lectin carbohydrate binding sites avoids binding of lectin to glycoconjugates of erythrocyte surface and hemagglutinating activity is reduced or abolished. The hemagglutinating activity inhibition assays is used to indicate the specificity of lectin carbohydrate binding site (Ng and Lam 2003; Thakur et al. 2007). The rhizome lectin purified from *Arundo donax* agglutinated rabbit erythrocytes and the agglutination was inhibited by N-acetyl-D-glucosamine (Kaur et al. 2005).

The activity of MvRL on human O erythrocytes was dependent of Ca^{+2}. Treatment with chelant agent EDTA abolished the hemagglutinating activity and addition of Ca^{+2} (0.02 M) to EDTA-treated MvRL restored the activity (specific hemagglutinating activity of 80). This result suggests that MvRL is similar to lectins

from *Cliona varians* (Moura et al. 2006) and *Fenneropenaeus chinensis* (Sun et al. 2008) that are Ca^{2+} dependent lectins.

The effect of pH on hemagglutinating activity showed that MvRL was active at pH range 5.0-6.0 (Figure 1C). The lectin was more sensible to pH value than others rhizome lectins that showed optimum activity in the range of 7.0–9.0 and 5.0-9.0 (Kaur et al. 2005, Tian et al. 2008). The hemagglutinating activity of MvRL was resistant to high temperature (Figure 1D) similar to *Holothuria scabra* lectin that remained active after incubation by 1 h at 80 °C (Gowda et al. 2008). MvRL was more thermo-stable than lectins from *Arundo donax* and *Ophiopogon japonicas* rhizomes that were active up to 55 °C and at temperatures below 60 ºC, respectively. The thermo-stability can be due to amino acid composition and Vieille et al. (2001) found that thermo-stable proteins have an increased content of charged amino acid residues. Temperature and pH may interfere on hydrogen bridges and ionic interactions between amino acids that maintain protein structure and a disruption of forces may result in protein denatured with lose of hemagglutinating activity (Santana et al. 2008). The definition of conditions where the hemagglutinating activity is maxima is essential to explore the potential biotechnological of lectin.

MvRL promoted mortality of *A. salina* with LC_{10}, LC_{50} and LC_{90} of 109.93, 154.62±1.22 and 199.30 μg ml^{-1}, respectively. The LC_{50} value reveals the high toxicity of MvRL since according to Meyer et al. (1982) crude extracts and pure substances with LC_{50} value lower than 1,000 μg ml^{-1} are toxic. It has been suggested correlation between toxicity and antitumor activity (McLaughlin, 1995) and thus this biological property of MvRL stimulates the evaluation of effect of MvRL on cancer cells. Induction of apoptosis and autophagy of human melanoma A375 cells were already described for *Polygonatum cyrtonema* lectin (Liu et al. 2008).

The effect of MvRL on bacteria growth revealed that the lectin had no antibacterial activity on both Gram-positive and Gram-negative bacteria tested. Antifungal activity of MvRL was detected on phytopathogenic fungi and the lectin was mainly active on *F. oxysporum* f.sp. *lycopersici* Race 3 (Table 3). Antifungal activity of rhizome lectin was already reported (Tian et al. 2008) and it is suggested that lectin with specificity for N-acetylneuraminic acid, N-acetylglucosamine, mannose or fucose might bind glycosylated components in the fungal cell wall affecting the fungal growth (Lis and Sharon 1998). The antifungal activity of MvRL may be due to mannose recognizing property and its effect on phytopathogenic *Fusarium* races may indicate the potentiality of lectin as antifungal agent. The greater efficiency of MvRL to inhibit the growth of race 3 stimulated the study molecular of races.

Analyses of genetic variability of the three races of *F. oxysporum* f. sp. *lycopersici* were carried out with ISSR and RAPD molecular markers using oligonucleotides of arbitrary sequences. With 14 RAPD primers, 106 amplicons were generated and used for examining *F. oxysporum* f. sp. *lycopersici* races. Each oligonucleotide analyzed revealed a distinct amplification pattern. Example of electrophoresis profile obtained with one oligonucleotíde using RAPD technique is presented in Figure 3A. Amplicon numbers varied in size between 400 and 1500 bp. UPGMA-based cluster analysis grouped *Metarhizium* into one main group (extern group) and *F. oxyporum* races were grouped uniquely into other group which contained Subgroup 1 (Races 1 and 2) which was genetically distinct from the Race 3 (Subgroup 2) (Figure 4A). Jaccard's similarity coefficient between Subgroups 1 and 2 was 0.58 (58 %).

Seventeen ISSR primers were provided by University of British Columbia (UBC) of which 8 yielded good amplification products and reproducibility (UBC 812,

817, 848, 858, 864, 878, 888 and 890). A total of 76 amplicons were produced, with a mean of 8 amplicons per oligonucleotide. The amplicon sizes ranged between 150 bp and 2000 bp. Example of electrophoresis profile obtained with ISSR primers is showed in Figure 3B. Jaccard's similarity coefficient ranged to 0.38 (38 %). These groupings contained the same accessions as after RAPD-UPGMA-based analysis i.e., Group 1 (*Metarhizium*) and Group 2 that contains *F. oxysporum* races (Figure 4B). Group 2 could be further partitioned into two distinct subgroups (Subgroup 1: Races 1 and 2; Subgroup 2: Race 3). According to Colombo et al. (2001), 7 to 30 primers yielding 50 to 200 polymorphic amplicons are sufficient to estimate genetic relationships between fungal species.

The accessions demonstrated genotypic differences [i.e., RAPD (58%) and ISSR (38%) polymorphisms]. In our study, ISSR marker assessment was more effective (i.e., more banding morphotypes were identified) than RAPD analysis. Similar results were obtained when RAPD and ISSR marker analyses were applied to relationship analyses in *F. luffae* and *F. oxysporum* f. sp. *cucumerinun* (Wang et al. 2001). When compared to other arbitrary primers like RAPDs, ISSRs offers enormous potential for resolving intra- and inter-genomic relationships (Zemankova and Lebeda, 2001). The results obtained with ISSR and RAPD markers suggested that the races 1 and 2 were very genetically close and that both markers were not able to differentiate them. However, the race 3 was the more distinct from the three races.

ISSR and RAPD markers have been used to evaluate genetic variability of *Fusarium***.** ISSR discriminated pathogenic forms of *F. luffae* and *F. oxysporum* f. sp. *cucumerinun* and through of ISSR profile of 13 isolates of *F. oxysporum* selected DNA bands that can be used to differentiate these two pathogenic species (Wang et al. 2001)**.** Bentley et al. (1995) used RAPD technique in studies with *F. oxysporum* f. sp. *cubense*

aiming to evaluate genetic variation of a collection of isolates of races 1, 2, 4 and 11 with different vegetative compatibility groups (VCGs). Using this technique, it was possible to subdivide the isolates of *F. oxysporum* f. sp. *cubense* in two distinct genetically groups.

Conclusions

The present work indicates a simple protocol to isolate MvRL and reveals one toxic lectin with antifungal activity on *Fusarium* species. The highest efficiency of MvRL in inhibits the growth of *F. oxysporum* f. sp. *lycopersici* race 3 may be related to interaction with glycosylated components specific of race 3 since this race was genetically distinct of races 1 and 2 that were very genetically close and similarly inhibited.

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References

Adhya M, Singha B, Chatterjee BP (2008) Purification and characterization of an N-acetylglucosamine specific lectin from marine bivalve *Macoma birmanica*. Fish Shellfish Immunol (in press)

Agrios GN (2005) Plant Pathology. 5th ed. Amsterdam: Elseuier Academic

Bains JS, Dhuna V, Singha J, Kamboja SS, Nijjara KK, Agrewala JN (2005) Novel lectins from rhizomes of two *Acorus* species with mitogenic activity and

inhibitory potential towards murine cancer cell lines. Int Immunopharmacol 5:1470– 1478

Barahona MV, Sánchez-Fortún S (1996) Comparative sensitivity of three age classes of *Artemia salina* larvae to several phenolic compuonds. Bull Environ Contaminat Toxicol 56:271-278

Barros ICL, Andrade LHC (1997) Pteridófitas medicinais (samambaias, avencas e plantas afins). Recife: Editora Universitária da UFPE

Battison AL, Summerfield RL (2009) Isolation and partial characterisation of four novel plasma lectins from the American lobster *Homarus americanus*. Dev Comp Immunol 33:198–204

Bauer AW, Kirby WMM, Sherrie JC (1966) Turck, M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45:493–496

Beltrão EIC, Medeiros PL, Rodrigues OG, Figueredo-Silva J, Valença MM, Coelho LCBB, Carvalho Jr LB (2003) *Parkia pendula* lectin as histochemistry marker for meningothelial tumor. Eur J Histochem 47:139-142

Bentley S, Pegg KG, Dale JL (1995) Genetic variation among a world-wide collection of isolates of *Fusarium oxysporum* f. sp. *cubense* analysed by RAPD-PCR fingerprinting. Mycol Res 99:1378-1384

Bing DH, Weyand JGM, Stavistsky AB (1967) Hemegglutination with aldehyde-fixed erythrocytes for assay of antigens and antibodies. Proceedings Society Experim Biol Med 1166-1170

Bhowal J, Guha AK, Chatterjee BP (2005) Purification and molecular characterization of a sialic acid specific lectin from the phytopathogenic fungus *Macrophomina phaseolina*. Carbohydr Res 340:1973-1982

Brayford D (1992) Description of fungi *Fusarium oxysporum* f. sp. *lycopersici*. Mycopathol, 118:51-53

Bukantz CSC, Rein LCCR, Kent CJF (1946) Studies in complement fixation preservation of sheep's blood in dextroses mixtures (modified Alsever's solution) for use in the complement fixation reaction. J Laborat Clin Med 31:394-399

Chen J, Liu B, Ji N, Zhou J, Bian H, Li C, Chen F, Bao J (2008) A novel sialic acid-specific lectin from *Phaseolus coccineus* seeds with potent antineoplastic and antifungal activities. Phytomedicine (in press)

Chumkhunthod P, Rodtong S, Lambert SJ, Fordham-Skelton AP, Rizkallah PJ, Wilkinson MC, Reynolds CD (2006) Purification and characterization of an N-acetyl-D-galactosamine-specific lectin from the edible mushroom *Schizophyllum commune*. Biochim Biophys Acta 1760:326–332

Correia MTS, Coelho LCBB, Paiva PMG (2008) Lectins, carbohydrate recognition molecules: are they toxic? In: Siddique, Y.H. (Ed.), (Org.), Recent Trends in Toxicology, vol. 37. Transworld Research Network, Kerala, pp. 47–59

Cruz CD (1997) Programa GENES-Aplicativo Computacional em Genética e Estatística. Ed. UFV, Viçosa, MG, 442p

Cunico MM, Carvalho JLS, Silva VC, Montrucchio DP, Kerber VA, Grigoletti Júnior A, Auer CG, Miguel MD, Miguel OG (2004) Avaliação antifúngica de extratos obtidos de *Ottonia martiana* Miq. (Piperaceae) sobre três fitopatógenos. Arq Inst Biol 71:141–143

Davis BJ (1964) Annals of New York of Sciences. vol.121, pp. 404-427

Doyle IJ, Doyle JL (1990) Isolation of plant from fresh tissue. Foccus 12:13-15

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugar and related substances. Anal Chem 28:350-356

Ghosh M (2008) Purification of a lectin-like antifungal protein from the medicinal herb, *Withania somnifera*. Fitoterapia (in press)

Gowda NM, Goswami U, Khan MI (2008) T-antigen binding lectin with antibacterial activity from marine invertebrate, sea cucumber (*Holothuria scabra*): Possible involvement in differential recognition of bacteria. J Invertebr Pathol 99:141–145

Green AA, Hughes L (1995) Protein fractionation on the basis of solubility in aqueous solution of salts and organic solvents.In: Methods Enzymol (Colowick, S. & Kaplan, N., eds.) Academic Press, pp. 67-90

Huang L, Adachi T, Shimizu Y, Goto Y, Toyama J, Tanaka H, Akashi R, Sawaguchi A, Iwata H, Haga T (2008) Characterization of lectin isolated from *Momordica charantia* seed as a B cell activator. Immunol Lett 121:148–156

Joly AB (1985) Botânica: Introdução a Taxonomia Vegetal. São Paulo: Companhia Editora Nacional, pp.777

Jung EC, Kim KD, Bae CH, Kim JC, Kim DK, Kim HH (2007) A mushroom lectin from ascomycete *Cordyceps militaris*. Biochim Biophys Acta 1770:833–838

Kaur A, Singh J, Kamboj SS, Sexana AK, Pandita RM, Shamnugavel M (2005) Isolation of an N-acetyl-D-glucosamine specific lectin from the rhizomes of *Arundo donax* with antiproliferative activity. Phytochemistry 66:1933–1940

Kavitha M, Sultan NAM, Swamy MJ (2009) Fluorescence studies on the interaction of hydrophobic ligands with *Momordica charantia* (bitter gourd) seed lectin. J Photochem Photobiol B: Biol 94:59–64

Laemmli U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. pp. 680-685

Lewan L, Andersson M, Morales-Gomez P (1992) The use of *Artemia salina* in toxicity testing. ATLA, Altern Lab Anim 20:297-301

Lis H, Sharon N (1998) Lectins: carbohydrate-specific proteins that mediate cellular recognition. Chem Rev 98: 637–674

Liu B, Cheng Y, Zhang B, Bian H, Bao J (2008) *Polygonatum cyrtonema* lectin induces apoptosis and autophagy in human melanoma A375 cells through a mitochondria-mediated ROS–p38–p53 pathway. Cancer Lett (in press)

Lowry DH, Rosebrough NJ, Farr AL, Randal RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265

Manicom BQ, Bar-Joseph M, Rosner A, Vigodsky-Haas H, Kótze JM (1987) Potential applications of random DNA probes and restriction fragment length polymorphisms in the taxonomy of the *Fusaria*. Phytopathology 77: 669-672

Matsuura M, Yamada M, Saikawa Y, Miyairi K, Okuno T, Konno K, Uenishi J, Hashimoto K, Nakata M (2007) Bolevenine, a toxic protein from the Japanese toadstool *Boletus venenatus*. Phytochemistry 68:893–898

McLaughlin JL, Chang CJ, Smith DL (1995) Benchtop bioassays for the discovery of bioactivity natural products: an update. In: Rahman, A. V. (Ed.) Studies in natural products chemistry. Elsevier, Amsterdam

Meyer BN, Ferrigini NR, Putman JE, Jacobson LB, Nichols DE, McLaughlin JL (1982) Brine shrimp: a convenient general bioassay for active plant constituints. Planta Med 45:31-34

Moura RM, Queiroz AS, Fook JMSLL, Dias ASF, Monteiro NKV, Ribeiro JKC (2006) CvL, a lectin from the marine sponge *Cliona varians*: isolation, characterization

and its effects on pathogenic bacteria and *Leishmania promastigotes*. Comp Biochem Phys 145C:517-23

Ng TB, Lam YW (2003) The immunostimulatory activity and stability of grass carp (*Ctenopharyngodon idellus*) roe lectin. Vet Immunol Immunopathol 94:105-112

Ojala T, Vuoprela P, Kiviranta J, Vuoprela H, Hiltunem R (1999) A bioassay using *Artemia salina* for detecting phototoxicity of plant coumarins. Planta med 65:715-718

Oliveira VC, Costa JLS (2002) Análise de restrição de DNA ribossomal amplificado (ARDRA) pode diferenciar *Fusarium solani* f. sp. *phaseoli* de *F. solani* f. sp. *glycines*. Fitopatol Brasil 27:631-634

Oliveira JT, Melo VM, Camara MF, Vasconcelos IM, Beltramini LM, Machado OL (2002) Purification and physicochemical characterization of a cotyledonary lectin from *Luetzelburgia auriculata*. Phytochemistry 61:301–310

Ooi LSM, Sun SSM, Wang H, Ooi VEC (2004) New Mannose-Binding Lectin Isolated from the Rhizome of Sarsaparilla *Smilax glabra* Roxb. (Liliaceae). J Agric Food Chem 52:6091-6095

Ooi LSM, Wong EYL, Chiu LCM, Sun SSM, Ooi VEC (2008) Antiviral and Anti-proliferative Glycoproteins from the Rhizome of *Smilax glabra* Roxb (Liliaceae). Am J Chin Med 36:185–195

Paiva PMG, Coelho LCBB (1992) Purification and partial characterization of two lectin isoforms from *Cratylia mollis* Mart. (Camaratu bea). Appl Biochem Biotechnol 36:113-118

Parra AL, Yhebra RS, Sardiñas IG, Buela LI (2001) Comparative study of the assay of *Artemia salina* L. and the estimate of the medium lethal dose (LD50 value) in

mice, to determine oral toxicity of plant extracts. Phytomed: Int J Phytother Phytopharmacol 8:395-400

Pharmacia Fine Chemicals (1980) Polyacrylamide Electrophoresis. Laboratory Techniques. Uppsala: Pharmacia pp.54

Pohleven J, Obermajer N, Sabotič J, Anžlovar S, Sepčić K, Kos J, Kralj B, Štrukelj B, Brzin J (2009) Purification, characterization and cloning of a ricin B-like lectin from mushroom *Clitocybe nebularis* with antiproliferative activity against human leukemic T cells. Biochim Biophys Acta (in press)

Reis A, Costa H, Boiteux LS, Lopes CA (2005) First report of *Fusarium oxysporum* f. sp. *lycopersici* race 3 on tomato in Brazil. Fitopatol Brasil 30:426-428

Resfield RA, Lewis VJ, Williams DE (1962) Disk electrophoresis of basic protein and peptides on polyacrylamide gels. Nature 195: 281-283

Sá RA, Napoleão TH, Santos NDL, Gomes FS, Albuquerque AC, Xavier HS, Coelho LCBB, Bieber LW, Paiva PMG (2008) Induction of mortality on *Nasutitermes corniger* (Isoptera, Termitidae) by *Myracrodruon urundeuva* heartwood lectin. Int Biodeterior Biodegrad (in press)

Sánchez C, Gupta M, Vasquez M, De Noriega YM, Montenegro G (1993) Bioassay with brine *Artemia* to predict antibacterial and pharmacologic activity. Rev med Panama 18:62-69

Santana MA, Santos AMC, Oliveira ME, Oliveira JS, Baba EH, Santoro MM, Andrade MHG (2008) A novel and efficient and low-cost methodology for purification of *Macrotyloma axillare* (Leguminosae) seed lectin. Int J Biol Macromol 43:352–358

Schumacher K, Schneider B, Reich G, Stiefel T, Stoll G, Bock PR (2003) Influence of postoperative complementary treatment with lectin-standardized mistletoe

extract on breast cancer patients: a controlled epidemiological multicentric retrospective cohort study. Anticancer Res 23:5081–5087

Sun J, Wang L, Wang B, Guo Z, Liu M, Jiang K, Tao R, Zhang G (2008) Purification and characterization of a natural lectin from the plasma of the shrimp *Fenneropenaeus chinensis*. Fish Shellfish Immunol 25:290-297

Thakur A, Rana M, Lakhanpal TN, Ahmad A, Khan MI (2007) Purification and characterization of lectin from fruiting body of *Ganoderma lucidum*. Biochim Biophys Acta 1770:1404–1412

Thomas V, Rutherford MA, Bridge PD (1994) Molecular diferentiation of two races of *Fusarium oxysporum* especial form *cubense*. Lett Appl Microbiol 18:193-196

Tian Q, Wang W, Miao C, Peng H, Liu B, Leng F, Dai L, Chen F, Bao J (2008) Purification, characterization and molecular cloning of a novel mannose-binding lectin from rhizomes of *Ophiopogon japonicas* with antiviral and antifungal activities. Plant Sci 175:877–884

Vieille C, Epting KL, Kelly RM, Zeikus JG (2001) Bivalent cations and amino-acid composition contribute to the thermostability of *Bacillus licheniformis* xylose isomerase. Eur J Biochem 268:6291-301

Yang H, Luo T, Li F, Li S, Xu X (2007) Purification and characterization of a calcium-independent lectin (PjLec) from the haemolymph of the shrimp *Penaeus japonicus*. Fish Shellfish Immunol 22:88-97

Wang PH, Lo HS, Yeh Y (2001) Identification of *F. o. cucumerinum* and *F. o. luffae* by RAPD-generated DNA probes. Lett Appl Microbiol 33:397-401

Windisch PG (1992) Flora da Serra do Cipó, Minas Gerais: Hymenophyllaceae. Boletim do Instituto de Botânica, vol.13. São Paulo, pp. 133-139

Zemankova M, Lebeda A (2001) *Fusarium* species, their taxonomy, variability and significance in plant pathology. Review. Plant Protect Sci UZPI 37:25-42

Zhao JK, Wang HX, Ng TB (2008) Purification and characterization of a novel lectin from the toxic wild mushroom *Inocybe umbrinella*, Toxicon (in press)

Figure captions

Fig. 1. Characterization of MvRL. (A) Gel filtration chromatography on Sephacryl S-300. (B) Electrophoresis profiles of MvRL on (1) PAGE (12%) for native and acidic protein, (2) SDS-PAGE (10%) stained with Coomassie blue and (3) SDS-PAGE (10%) stained with Schiff´s reagent. Effect of pH (C) and temperature (D) on hemagglutinating activity of MvRL. Log specific hemagglutinating activity with type O^+ human erythrocytes before heating: 2.20412

Fig. 2. SDS-PAGE (10%) of *F. oxysporum* f.sp. *lycopersici* race 1 (R1), 2 (R2) and 3 (R3) stained with Schiff's reagent.

Fig. 3. RAPD profiles of DNA from *F. oxysporum* f. sp. *lycopersici* races using primer OPC-06 (A). ISSR profiles of DNA from *F. oxysporum* f. sp. *lycopersici* races using primer UBC-808 (B). The numbers 1, 2 and 3 represent *F. oxysporum* f. sp. *lycopersici* races. Letter M represents marker of 1kb molecular weight ladder.

Fig. 4. Dendrograms of *F. oxysporum* f. sp. *lycopersici* races generated by applying the using UPGMA to the data gathered using RAPD (A) and ISSR (B) primers. Genetic similarity relationships analyzed on the basis of Jaccard's similarity coefficients.

Figure 1

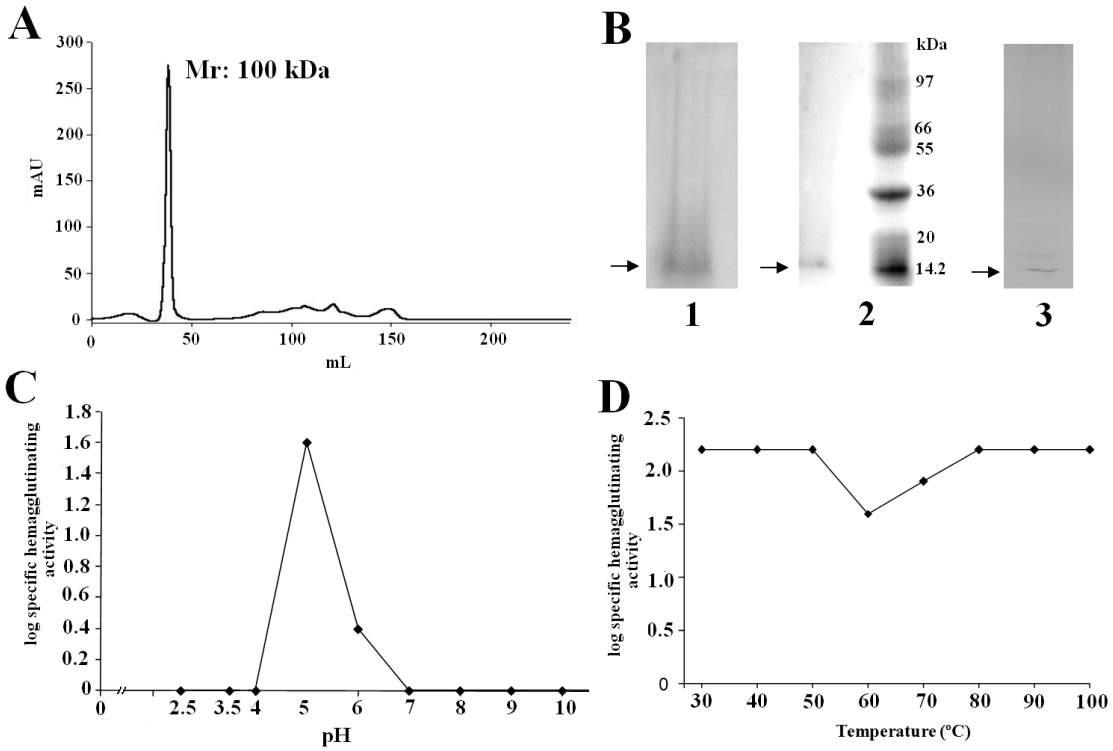
A
Mr: 100 kDa
mAU
mL
B
kDa
97
66
55
36
20
14.2
1
2
3
C
log specific hemagglutinating activity
pH
D
log specific hemagglutinating activity
Temperature (°C)

Figure 2

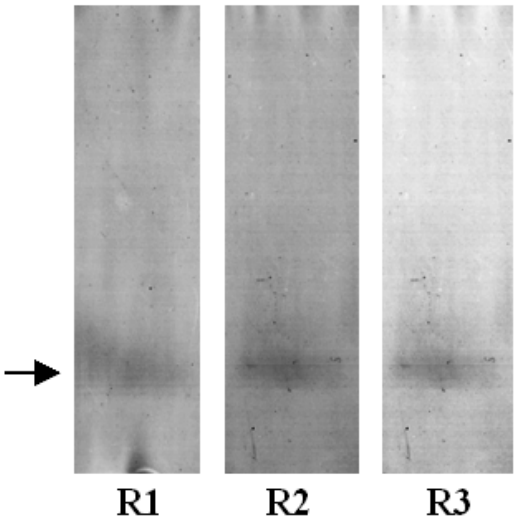

Figure 3

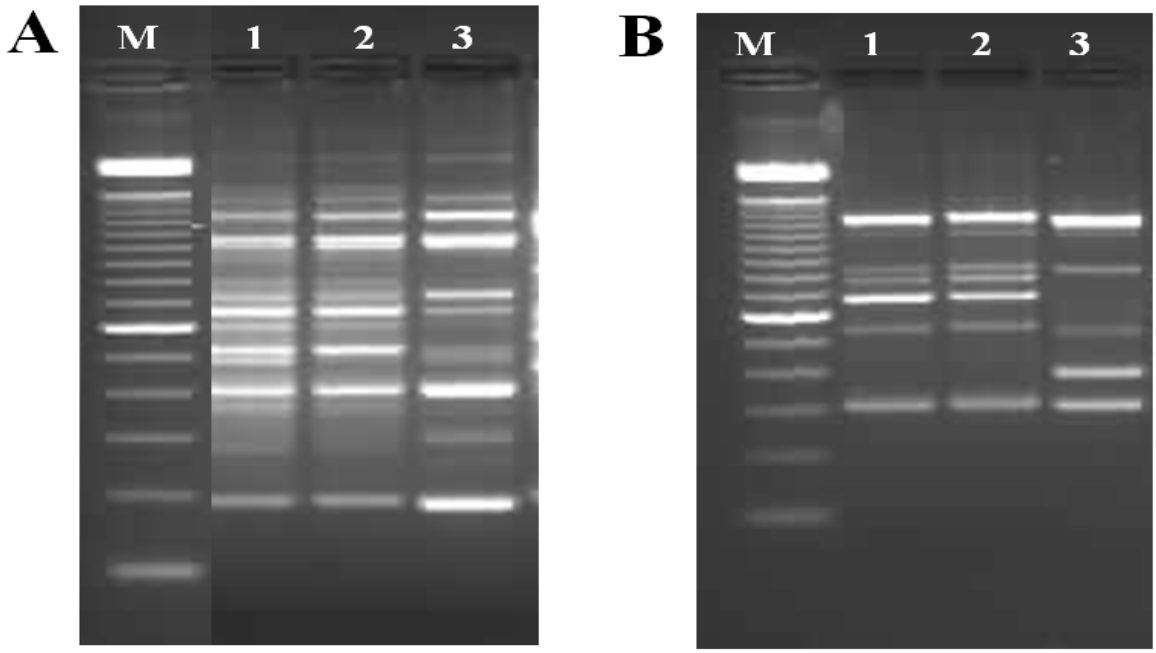

Figure 4

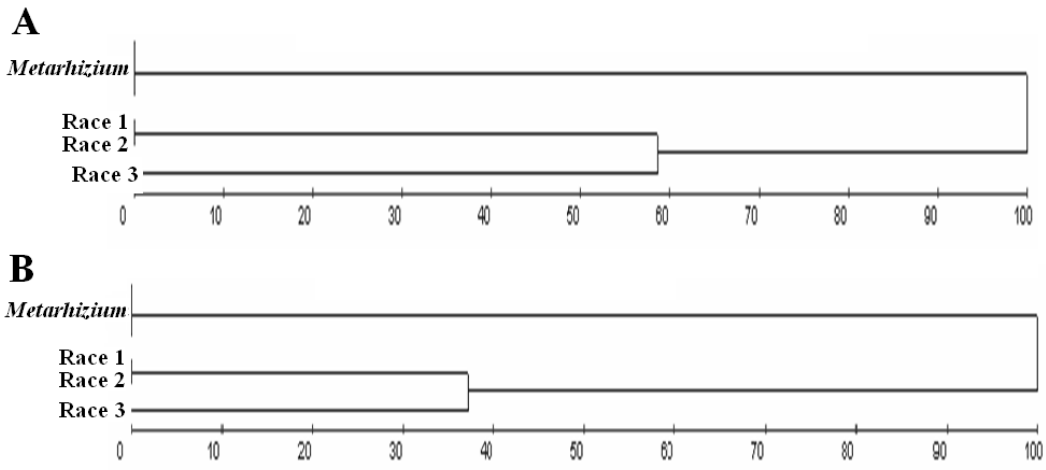
A
Metarhizium
Race 1
Race 2
Race 3
0
10
20
30
40
50
60
70
80
90
100
B
Metarhizium
Race 1
Race 2
Race 3
0
10
20
30
40
50
60
70
80
90
100

Table 1. Summary of MvRL purification

Sample	Total protein (mg)	Total hemagglutinating activity	Specific hemagglutinating activity	Purification (fold)
Extract	256.1	4,224	16.5	1.0
0-60 Fraction	4.2	1,443	341.3	20.7
MvRL	1.6	128	80	5.0

Hemagglutinating activity with type O^+ human erythrocytes. Specific hemagglutinating activity corresponds to the ratio between titer and protein concentration (mg ml^{-1}). Purification was measured as the ratio between the specific hemagglutinating activity in the stage and specific hemagglutinating activity of extract.

Table 2. Carbohydrate and protein contents of solutions from races 1, 2 and 3 of *F. oxysporum* f.sp. *lycopersici* and specific hemagglutinating activity of MvRL at presence of these solutions.

Race	Carbohydrate content (mg ml^{-1})	Protein content (mg ml^{-1})	Specific hemagglutinating activity
1	1.66 ± 0.54	10.0 ± 1.1	20
2	1.02 ± 0.13	7.0 ± 0.4	20
3	2.36 ± 0.35	10.7 ± 0.2	20

Hemagglutinating activity assay performed with type O^+ human erythrocytes. Specific hemagglutinating activity in 0.15 M NaCl = 160.

Table 3. Antifungal activity of MvRL on *Fusarium* species

Fungus	Growth Inhibition (%)
Fusarium decemcellulare	56 ± 0.0
Fusarium lateritium	60 ± 1.9
Fusarium moniliforme	37 ± 2.5
Fusarium oxysporum	45 ± 3.5
Fusarium solani	52 ± 3.7
Race 1 *F. oxysporum f.sp. lycopersici*	55 ± 1.6
Race 2 *F. oxysporum f.sp. lycopersici*	45 ± 1.4
Race 3 *F. oxysporum f.sp. lycopersici*	61 ± 3.8

Inhibition percentual (antifungal activity) calculated in relation to fungi growth zone (diameter) at MvRL presence in comparison to growth in 0.15 M NaCl (negative control).

5. CONCLUSÕES

- A fração 0-60% de maior atividade hemaglutinante foi o material de escolha para purificação da lectina de rizoma (MvRL).

- A cromatografia da fração 0-60% em coluna de Sephadex G-25 resultou no isolamento de MvRL.

- A atividade de MvRL foi maior para eritrócitos humanos tipo O, inibida por monossacarídeos e glicoproteínas, sensível a variação de pH, termoestável e dependente de íon cálcio.

- Eletroforese em gel de poliacrilamida para proteínas nativas revelou a natureza acídica de MvRL.

-Comparação da massa molecular nativa da lectina determinada por cromatografia em gel filtração com a massa molecular da subunidade determinada por eletroforese em condições desnaturantes indica MvRL como agregado molecular.

- A toxicidade de MvRL foi detectada pelo seu efeito sobre a sobrevivência de *A. salina*.

- MvRL não apresentou afetou o crescimento de bactérias Gram negativas e Gram positivas, nas condições utilizadas.

- MvRL apresentou atividade antifúngica contra espécies de *Fusarium* e inibiu diferentemente o crescimento das raças 1, 2 e 3 de *F. oxysporum* f. sp. *lycopersici*.

- Estudo molecular das raças de *Fusarium* usando marcadores moleculares ISSR e RAPD, revelaram similaridade genética entre as raças 1 e 2 de *Fusarium oxysporum* f. sp. *lycopersici* sendo a raça 3 mais distinta geneticamente.

- O marcador ISSR foi considerado o mais informativo no estudo da diversidade genética, pois os produtos amplificados atingiram 2000 pb (pares de bases).

6. ANEXO

Instructions to Authors Journal of Plant Research

(Revised: 16 Jan 2009)

Editorial Policy

Scope of the journal
The Journal of Plant Research is an international journal dedicated to the dissemination of fundamental knowledge in all areas of basic plant sciences including taxonomy, phylogenetics, evolutionary biology, ecology, ecophysiology, environmental biology, morphology, anatomy, structural biology, genetics, developmental biology, physiology, biochemistry, molecular and cellular biology, biophysics, and theoretical and systems biology. In addition to these disciplines, the journal welcomes papers in interdisciplinary areas and in newly developing areas of basic plant biology. The journal especially encourages publication of work carried out through unique approaches and those reporting unprecedented findings.

Reviewing policies

- While the scope of this journal is diverse, topics should be significant with regard to basic plant sciences. Thus, papers whose topics are confined to the field of applied sciences such as agricultural and pharmaceutical sciences are not within the scope of the journal. These papers will not be considered for reviewing.
- The journal does not encourage the submission of papers that merely confirm well-investigated phenomena in different species or under different experimental conditions.
- Manuscripts not conforming to the style and format specified by these Instructions to Authors will not be considered for reviewing (See Manuscript preparation). As well, manuscripts that are not written in clear and grammatical English may not be considered for reviewing.

Manuscript types

The following types of papers are accepted by the editors: Regular papers, short communications, technical notes. Review articles are generally invited and published in the sections Current Topics in Plant Research and JPR Symposium.

- Regular papers report full-length research articles that describe original and fundamental findings of significance that contribute to understanding of plants. Regular papers should not exceed 10 printed pages.
- Short communications report significant new findings in concise form and should not exceed 6 printed pages.

Technical notes report new methodology that contributes to exploring new aspects in plant sciences and should not exceed 10 printed pages.

Submission of review articles is also welcome and encouraged. Please send the title and a short summary of the proposed topic to the Editor-in-Chief for approval by the Editorial Committee before submitting a manuscript.
Manuscripts submitted are subject to review and editing by two reviewers and a handling editor. Acceptance of manuscripts, revision of manuscripts, and the order of publication are decided by the Editorial Committee. All accepted manuscripts are subject to copyediting. The publisher and the editorial office reserve the right to edit the language of accepted papers.

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However, manuscripts for review articles such as JPR Symposium and Current Topics in Plant Research may include figures or text passages that already have been published elsewhere, provided that the original sources are cited appropriately and that authors have obtained permission from the copyright owner(s) to use the material.

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JPR Ethics Investigation Committee Bylaws (pdf)

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Editorial Office, Journal of Plant Research
Department of Developmental Biology and Neurosciences,
Graduate School of Life Sciences, Tohoku University, Sendai 980-8578, Japan
E-mail: jpr@mail.tains.tohoku.ac.jp

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New nucleotide data must be deposited in the DDBJ/EMBL/GenBank databases and an accession number obtained before a paper can be accepted for publication.

For the use of Latin names, consult the International Code of Botanical Nomenclature. For abbreviations of herbaria, consult Index Herbariorum and indicate them with small capitals (e.g., TI, KYO, TNS, K). For the authority of a species, consult the Authors of Plant Names published by the Royal Botanic Gardens, Kew. Units of measurement should be in the metric system.

Manuscripts should be typed double-spaced with 3-cm wide margins on A4 sheets. Each manuscript page usually consists of 250 words. Three typewritten pages are approximately equivalent to one printed page.

Cover letter:
Manuscript submission should be followed by submission of a cover letter that contains a brief explanation of the essence of the work to be reported in the manuscript. In the case of a resubmission of a revision, the cover letter should contain detailed explanations for individual revisions, including

responses to the reviewers, in the revised manuscript.

The manuscript should be arranged in the order listed below, with all pages numbered consecutively.

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  - Whether the corresponding author is a member or non-member of the Botanical Society of Japan.

  - Subject area that is most relevant to the work, selected from among
  (1)Taxonomy/Phylogenetics/Evolutionary,Biology
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- Title page (the second page of the manuscript):
  Title of the paper, names of all authors, and institutions with their mailing addresses. The title should include binomial names for organisms without abbreviation of genus names. The author(s) who described the species should not be included in the title. (See "Scientific names")

- Abstract:
  Should be a clearly stated digest of the paper, not exceeding 200 words for a full regular paper, technical note, and review article, and less than 100 words for a short communication.

- Key words:
  Not more than six words or phrases should be selected to identify the most important subjects covered by the article. Key words should be arranged in alphabetical order.

- Text:
  Should be prepared with the headings Introduction, Materials and methods, Results, and Discussion. Short communications should follow the style of full papers, but the headings may be omitted.

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For scientific names of plants, authors are advised to refer to The International Plant Names Index, which is the standard database for plant names presented collaboratively by The Royal Botanic Gardens, Kew, The Harvard University Herbaria, and the Australian National Herbarium, at the website http://www.ipni.org/index.html.

In the title of an article, the species name should be binomial, in which the genus names should not be abbreviated and the author(s) who described the species should be omitted (e.g., Ginkgo biloba and Arabidopsis thaliana).

In the text, the binomial name for a species should include the author(s) who described the species. The binomial name should not be abbreviated at first use. The genus name may be abbreviated to its first letter (uppercase, with a period) when subsequently used.

If two genera with the same initial letter are used in a section, the genus name should normally be written out on each occasion. If, however, the organisms are common and there is no danger of confusion, the genus names may be abbreviated as usual (e.g., Avena sativa and Arabidopsis thaliana on first use and A. sativa and A. thaliana on subsequent occurrences).

In some model plants, such as Arabidopsis thaliana, the genus name in roman (Arabidopsis or

arabidopsis) may be used as a common noun for the plant species. Note that a common noun should not be italicized.
The International Plant Names Index

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- Book chapter:
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  Kamiya N (1964) The motive force of endoplasmic streaming in the amoeba. In: Allen RD, Kamiya N (eds) Primitive motile systems in cell biology. Academic, New York, pp 257–277

- Journal article:
  Tsukaya H, Iokawa Y, Kondo M, Ohba H (2005) Large-scale general collection of wild-plant DNA in Mustang, Nepal. J Plant Res 118:57–60
  If available, the Digital Object Identifier (DOI) of the cited literature should be added at the end of the reference in question, e.g.,
  Ebinuma H, Sugita K, Matsunaga E, Endo S, Yamada K, Komamine A (2001) Systems for the removal of a selection marker and their combination with a positive marker. Plant Cell Rep 20:383–392. DOI 10.1007/s002990100344
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- Taxonomic treatment:
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  Adema F (1999) Notes on Malesian Fabaceae (Leguminosae-Papilionoideae). 4. Millettia chrysamaryssa, a new species from Peninsular Malaysia. Novon 9: 289–291
  Adema F (2000) Notes on Malesian Fabaceae (Leguminosae-Papilionoideae). 7. The genus Millettia. Blumea 45: 403–422

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