

La fitobatteriologia verso il Duemila

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I batteri agenti di malattie delle piante sono stati oggetto di studio fin dalla seconda metà dell'Ottocento. La fitobatteriologia è la branca della patologia vegetale che raccoglie le conoscenze acquisite in poco più di un secolo sui batteri fitopatogeni, inclusi i cosiddetti batteri fastidiosi scoperti circa vent'anni or sono. A pochi anni dalla fine del secolo le batteriosi ad agente causale correttamente descritto ammontano a poco più di 270. Alcune batteriosi causano gravi perdite di raccolto in varie parti del mondo. Il progresso più significativo della fitobatteriologia è avvenuto negli anni sessanta dopo la scoperta della reazione di ipersensibilità nelle interazioni piante-batteri e la conseguente distinzione tra reazione ipersensitiva e normosensitiva, tra interazione omologa ed eterologa. La scoperta del DNA e il progresso della sua tecnologia sono stati determinanti per il progresso della fitobatteriologia negli ultimi 20 anni. La classificazione e l'identificazione dei batteri fitopatogeni hanno travagliato i fitobatteriologi da più di un secolo. Fino agli anni ottanta la classificazione basata su caratteri fenotipici (inclusa la capacità di causare malattia con tratti sintomatici ben definiti su una o più piante ospiti) è consistita in un sistema artificiale da cui il fitobatteriologo doveva trarre un insieme di caratteri distintivi ordinati in tabelle diagnostiche o chiavi dicotomiche utili all'identificazione della specie nominale. L'avvento della tassonomia numerica ha consentito dapprima la definizione delle tassospecie fitopatogene e poi ha costretto a rivalutare l'importanza della specie-specificità degli agenti causali. Di fatto è entrato in uso in fitobatteriologia il rango infraspecifico di pathovar contraddistinto da nomenclatura ternaria. Nelle pathovar sono state poi individuate le razze in relazione all'esistenza di interazioni differenziali con certe cultivar della pianta ospite, confermate poi nell'ultimo decennio dalla presenza di altrettanti geni di avirulenza caratterizzati con tecniche di biologia molecolare. Dalla metà degli anni ottanta in poi, l'uso dei caratteri genomici per la classificazione ha consentito la definizione delle specie genomiche o genospecie. La classificazione è diventata naturale o filogenetica. A fine secolo i criteri per la definizione della specie batterica fitopatogena sono quelli universalmente accettati in batteriologia: una riassociazione DNA/DNA uguale o superiore al 70% e una differenza in stabilità termica uguale o superiore a 5 °C tra coppie di catene omologhe ed eterologhe. Sebbene la soglia di definizione della specie sia divenuta meno rigida negli ultimi anni (50-70% di riassociazione DNA/DNA e 5-7 °C DTm), il valore di omologia DNA/DNA rimane il criterio essenziale. Alti valori di omologia DNA/DNA devono comunque essere correlati ad alti valori degli indici di somiglianza fenotipica. Conseguentemente, per la definizione della specie batterica in fitobatteriologia, a fine secolo è universalmente ritenuto corretto l'approccio polifasico. Questi criteri stanno rivoluzionando la classificazione dei batteri fitopatogeni Gram-negativi, di cui in tabella sono riportati generi, specie e sottospecie. La classificazione dei batteri Gram-positivi non è stata ancora rivista alla luce dei nuovi criteri.

Parole chiave: Fitobatteriologia, Procarioti fitopatogeni, Batteri fitopatogeni, Classificazione, Specie, Sottospecie, Genospecie, Genomovar, *Candidatus*, Tassonomia polifasica, Batteri fastidiosi.

Phytobacteriology towards two thousand.

Phytopathogenic bacteria have been studied since the second half of the nineteenth century. Phytobacteriology is a branch of plant pathology which deals with the knowledge acquired on phytopathogenic bacteria in little more than a century, including the so-called fastidious bacteria discovered around twenty years ago. At the eve of the millennium, little more than 270 bacterial diseases and causal agents have so far been correctly described. Some bacterial diseases cause severe harvest losses in various parts of the world. The most important progress in phytobacteriology occurred in the sixties with the discovery of the hypersensitive reaction in plant-bacteria interactions and the consequent distinction between the hypersensitive and normosensitive reactions, between homologous and heterologous interactions. The discovery of DNA and the advances in its technology have been crucial for the progress in phytobacteriology over the last 20 years. The classification and identification of phytopathogenic bacteria has challenged phytobacteriologists for more than a century. Up until the 1980's, classification based on phenotypic traits (including the ability to cause diseases with clearly defined symptoms on one or more host plants) consisted of an artificial system from which the phytobacteriologist had to extract a series of differential traits, arranged in diagnostic tables, or dichotomous keys used for the identification of the nomenclatures. In the first place, the advent of numeric taxonomy made it possible to define phytopathogenic taxospecies and which then made it necessary to reassess the importance of causal agent species-specificity. In fact the infrasubspecific rank of pathovar was adopted in phytobacteriology and indicated by the ternary nomenclature. The pathovar races were then identified on the basis of the existence of differential interactions with certain host plant cultivars, which have been confirmed over the last decade by the presence of correspondent avirulence genes characterized by molecular biology techniques. The use of genomic traits for classification from the mid-eighties onwards made possible the definition of genetic species or genospecies. Classification became natural or phylogenetic. At the end of the century, the criteria for defining phytopathogenic bacterial species are those universally accepted in bacteriology: 70% or greater DNA/DNA reassociation and 5 °C or less difference in thermal stability between the homologous and heterologous duplexes. Although the species definition threshold has become less rigid in recent years (50-70% DNA/DNA reassociation and 5-7 °C DT_m), the value of DNA/DNA homology remains the essential criterion. High DNA/DNA homology values must however be correlated to high phenotypic similarity indices. Consequently, at the eve of the millennium, in phytobacteriology the polyphasic approach is universally accepted for the definition of bacterial species. These criteria are revolutionising the classification of Gram-negative phytopathogenic bacteria; the genera, species and subspecies are shown in a table. The classification of Gram-positive bacteria has not yet been reviewed in the light of the new criteria.

Key words: Phytobacteriology, Phytopathogenic prokaryotes, Phytopathogenic bacteria,

Classification, Species, Subspecies, Genospecies, Genomovar, *Candidatus*, Polyphasic taxonomy, Fastidious bacteria.

Petria 9(1-2), 27-41, (1999) Rassegna/Review

Fisiologia di specie coltivate infette da virus. *Solanum tuberosum*.

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La coltivazione della patata ha avuto ed ha un'importanza primaria nell'economia agraria mondiale. È quindi naturale che quelle virosi che influenzano qualità e resa del prodotto di questa solanacea abbiano attratto l'attenzione dei fitopatologi sin dai primissimi anni del secolo. Le alterazioni fisiologiche provocate dalle virosi non hanno per contro sollevato lo stesso interesse. Dall'esame della bibliografia emergono dati relativamente coerenti su di un generale incremento del metabolismo ossidativo e sulla diminuzione dell'attività fotosintetica. Per entrambe le funzioni, tuttavia, non si conoscono i meccanismi molecolari. Per quanto riguarda il metabolismo dei composti azotati, in particolare proteine ed acidi nucleici, sono reperibili solamente alcuni dati relativi all'accumulo in amminoacidi e nucleotidi, a testimonianza indiretta di un'azione idrolitica. Infine, poco o nulla è noto sulle alterazioni fitormonali, eccezion fatta per lecorrelazione tra alterazioni di attività citochinica ed espressione sintomatologica: risultati preliminari suggeriscono un meccanismo di inattivazione delle basi citochiniche mediante glucosilazione. La delucidazione delle alterazioni nella fisiologia della pianta dovrebbe costituire il fondamento per la comprensione dell'azione dei virus sul ciclo vitale — e produttivo — delle specie di interesse agrario. I risultati di questa rassegna testimoniano viceversa dello scarso interesse mostrato dalla maggioranza dei virologi, fisiologi e biochimici vegetali su questo tema.

Parole chiave: Fisiopatologia vegetale, Patata, Virus.

The physiology of crop species systemically infected with viruses. *Solanum tuberosum*.

The viruses infecting potatoes have been an object of intense and careful research from the beginning of plant virology. Alterations of physiology in virus-infected potato plants have instead raised poor interest. Increased respiration and decreased photosynthesis have been generally described, but the molecular mechanisms relative to both the functions are still unknown. Protein and nucleic acid metabolisms revealed an increase of amino acids and nucleotides, a finding suggesting some hydrolytic reaction which supplies metabolic precursors to virus replication. Alterations of plant hormone levels represent an hitherto neglected subject with the exception of cytokinins which were investigated in PVY-infected potatoes. Investigations have shown a relation between cytokinin activity and symptom severity, and preliminary results suggest that the active cytokinin bases may be inactivated by a glucosylation process. The elucidation of the alteration in plant physiology ought to be the basis for understanding the effect of

viruses on the life-cycle of cultivated plants, including crop production. On the contrary, the result of this review speaks against the interest of most plant virologists, physiologists and biochemists on this subject.

Key words: Physiological plant pathology, Plant Viruses, Potato.

Petria 9(1-2), 43-52, (1999) Articolo Scientifico/Scientific paper

Effetti morfologici e produttivi dell'infezione del *Potyvirus* latente del carciofo (ALV)

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Si espongono i risultati di un biennio di ricerche effettuate in agro di Melfi (PZ), per valutare gli eventuali effetti dell'infezione del *Potyvirus* latente del carciofo (ALV) sui caratteri morfoproductivi di 3 cultivar di carciofo ('H 137', 'H 044' e 'Talpiot'). Allo scopo sono stati allestiti tre campi, due dei quali, A e B, distanti 200 m l'uno dall'altro, con piante sane delle suddette cultivar ed il terzo, C, adiacente al campo B e da esso separato con una parete di rete antinsetti, costituito da piante artificialmente infettate con ALV prima del trapianto. Nel primo anno, l'infezione virale ha avuto effetti negativi sui caratteri morfoproductivi delle tre cultivar. Infatti, le piante del campo C hanno prodotto un numero di capolini minore del 38% rispetto a quelle degli altri due campi, hanno manifestato un marcato accorciamento degli steli fiorali ed accusato un calo produttivo del 53%. Nel secondo ciclo produttivo, le piante infette (campo C) delle tre cultivar hanno mantenuto inalterata, rispetto al primo anno, la produzione commerciabile, ma hanno fatto registrare un incremento del numero di capolini per singola pianta. Le stesse piante hanno prodotto, negli altri due campi, meno che nella prima annata. Alla fine dei due cicli colturali, ALV è risultato presente nel 29% delle piante del campo B e in nessuna pianta del campo A. Tra le cultivar in prova, 'Talpiot' è risultata più colpita dalle infezioni di ALV, che, nel campo B, hanno interessato il 45% delle sue piante contro il 21,4%, in media, delle piante delle altre due cultivar, risultate, conseguentemente, più produttive. In entrambi gli anni, le piante infette di 'Talpiot' e di 'H 044', sono risultate meno produttive rispetto a quelle sane. Al contrario, l'infezione di ALV della cv H 137 ha determinato, nel secondo ciclo colturale, soltanto una leggera riduzione dell'altezza del capolino.

Parole chiave: Carciofo, *Potyvirus*, ALV.

Morphoproductive effects of artichoke latent *Potyvirus* (ALV) infection.

Results of a two year research carried out in Melfi area (Potenza province, southern Italy) to evaluate the eventual effects of artichoke latent *Potyvirus* (ALV) infection on morpho-productive features of three artichoke cultivars ('H 137', 'H 044' and 'Talpiot') are reported. Three artichoke fields were constituted two of which, A and B, 200 m far

each from the other, with healthy seedlings of the above cultivars and the third, C, adjacent to field B and from it separated with a wall of anti-insect net, with seedlings artificially infected with ALV before transplanting. The viral infection had negative effects on morphoproductive features of the three tested cultivars. In fact, in the first cultural cycle, plants of field C produced 38% and 53% less artichoke heads and total yield, respectively, than those of the other two fields and showed a significant shortening of head stems. In the second cultural cycle, infected plants (field C) of the three cultivars maintained unaltered the value of marketable yield but increased the number of heads per plant. The same cultivars produced a minor average yield than the first year in the other two fields. ALV resulted naturally transmitted to only 29% of plants of field B and to none of those of field A at the end of the two cultural cycles. Among tested cultivars, 'Talpiot' resulted the most infected by ALV which reached, in field B, 45% of its plants and only 21.4% of plants belonging to the other two cultivars that consequently resulted more productive. In both cultural cycles, the artificially infected 'Talpiot' and 'H 044' plants resulted less productive than the healthy ones. At contrary, ALV infection caused only a light reduction of artichoke head length in 'H 137' plants in the second cultural cycle.

Key words: Artichoke, *Potyvirus*, ALV.

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L'avvizzimento del kenaf da *Fusarium oxysporum*

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Nel presente lavoro sono riportati i risultati di uno studio eseguito allo scopo di isolare ed identificare gli isolati di *F. oxysporum* f. sp. *vasinfectum* che causano avvizzimento delle piante di kenaf coltivate in Basilicata. Inoltre sono state condotte prove di patogenicità allo scopo di selezionare genotipi di kenaf resistenti a *F. oxysporum* f. sp. *vasinfectum*. L'identificazione degli isolati di *F. oxysporum* f. sp. *vasinfectum* ottenuti da kenaf è stata eseguita sulla base di caratteri morfologici, colturali e biologici. La patogenicità di due isolati di *F. oxysporum* ottenuti da kenaf è stata saggiata in serra su 13 specie ospiti e confrontata con quella di un isolato *F. oxysporum* f. sp. *vasinfectum* proveniente da cotone, di un isolato di *F. oxysporum* f. sp. *melonis* da melone e di un isolato di *F. oxysporum* f. sp. *lycopersici* da pomodoro. I risultati della ricerca hanno mostrato che la patogenicità su kenaf degli isolati di *F. oxysporum* da kenaf è simile a quella dell'isolato di *F. oxysporum* f. sp. *vasinfectum* da cotone. Inoltre, tra i genotipi di kenaf esaminati non è stato osservato materiale genetico resistente agli attacchi di *F. oxysporum* f. sp. *vasinfectum*.

Parole chiave: *Fusarium oxysporum*, Avvizzimento, Kenaf, Cotone.

The wilting of kenaf by *Fusarium oxysporum*

Symptoms of wilting and necrosis caused by *Fusarium oxysporum* f.sp. *vasinfectum* were observed on kenaf (*Hibiscus cannabinus* L.) plants cultivated in southern Italy (Basilicata). The results of the isolation and of the identification of isolates of *F. oxysporum* obtained from kenaf, and the results of the selection of kenaf genotypes resistant to *F. oxysporum* f. sp. *vasinfectum* are reported in this work. The identification of the isolates of *F. oxysporum* f. sp. *vasinfectum* obtained from kenaf was effected on the basis of morfological, cultural and biological characters. The pathogenicity of 2 isolates of *F. oxysporum* from kenaf was tested in greenhouse on 13 host plants in comparison with one isolate each of: *F. oxysporum* f. sp. *vasinfectum* from cotton (*Gossypium hirsutum* L.), *F. oxysporum* f. sp. *melonis* from muskmelon (*Cucumis melo* L.) and *F. oxysporum* f. sp. *lycopersici* from tomato (*Lycopersicon esculentum* Mill.). The results of this research showed that the fungus *F. oxysporum* f. sp. *vasinfectum* is the cause of the symptoms observed. *F. oxysporum* from kenaf is similar in pathogenicity to *F. oxysporum* f. sp. *vasinfectum* isolated from cotton, but it is different from *F. oxysporum* f. sp. *melonis* and *F. oxysporum* f. sp. *lycopersici*. Moreover, it was not observed any kenaf genotype resistant to *F. oxysporum* f. sp. *vasinfectum* between the genotypes tested.

Key words: *Fusarium oxysporum*, Wilting, Kenaf, Cotton.

Petria 9(1-2), 61-203, (1999) Atti del Convegno/Proceedings of the Meeting

Mass scale diagnosis of plant pathogens by nucleic- acid amplification methodologies

Faro (Portugal) July 9-10, 1998

Preface

These are the proceedings of the "Mass scale diagnosis of plant pathogens by nucleic-acid amplification methodologies" meeting, sponsored by the COST 823 Action "New Technologies" to improve phytodiagnosis and held in Portugal, at the University of Algarve, Faro, on July 9-10, 1998. Historically, the diagnosis of plant pathogens has been based on the direct microscopic detection of pathogens in plant tissues and, more recently, on a number of serological assays such as immunocytochemistry tests and solid-phase immunoassays (ELISA). Frequently, however, such diagnostic tests are insufficiently sensitive to detect pathogens or to distinguish infected from uninfected plant tissues. Currently, alternative diagnostic tools using nucleic-acid hybridisation and amplification methodologies are being developed and these appeared as a consequence of the emerging pressures to improve the sensitivity, the specificity and, no less important, the rapidity and reliability of the assays. A growing body of evidence exists now and shows that these methodologies will play, in the near future, a major role in plant pathogen detection.

So often, themes of scientific conferences focus not only on particular groups of plant pathogens but also deal with a broad range of fields, from detection methods for the pathogen to its biology, pathogenicity and control. Less often, however, do Mycologists,

Bacteriologists and Virologists interested in the molecular aspects of plant pathogen detection get a chance to interact. This conference offered such a forum. The purpose of this meeting was to gather relevant researchers in the field of nucleic acid-based plant pathogen diagnostics, and have them interact in a short intense meeting. It was hoped that bringing together such diverse groups of Plant Pathologists, with sufficient time for discussion both at paper and poster presentations, would engender collaborative interactions among the researchers attending the meeting. Therefore, the presentations in this volume give brief insights into the areas of research which have been carried out in the plant molecular diagnostic field. The researchers who presented papers at this meeting represent a broad spectrum of European plant pathogen research and came from Universities, Research Institutes and Government Agencies from all over Europe. The organisers are sincerely indebted to the COST 823 European Programme, and to the Universidade do Algarve, whose contributions made the meeting possible.

Editor MARINA BARBA

Diagnosi massale di patogeni vegetali utilizzando tecniche di amplificazione degli acidi nucleici

Faro (Portogallo), 9-10 luglio 1998

Prefazione

Questi sono gli Atti del convegno "**Diagnosi massale di patogeni vegetali utilizzando tecniche di amplificazione degli acidi nucleici**", svoltosi in Portogallo, presso l'Università di Algarve, Faro, dal 9 al 10 luglio 1998 e organizzato, nell'ambito della Azione COST 823 "Nuove tecnologie", con lo scopo di migliorare la diagnosi. Nei tempi passati la diagnosi dei patogeni vegetali si è basata sul loro rilevamento diretto nei tessuti vegetali mediante osservazioni al microscopio e, più recentemente, sull'impiego di differenti metodologie sierologiche come i saggi immuno-citochimi o immunoenzimatici su fase solida (ELISA). Spesso, tuttavia, questi metodi di diagnosi non risultano sufficientemente sensibili per rilevare i patogeni o per discriminare tessuti infetti da quelli sani. Oggigiorno si stanno sempre più perfezionando tecniche molecolari basate sulla ibridazione o sulla amplificazione degli acidi nucleici. Questi studi sono stimolati dalla necessità sempre più impellente di disporre di metodologie di diagnosi altamente sensibili, specifiche e, aspetto di non secondaria importanza, rapide e attendibili. È sempre più evidente che queste tecniche molecolari rivestiranno, nel prossimo futuro, un ruolo prioritario nel rilevamento dei patogeni vegetali. Spesso, gli argomenti oggetto di conferenze scientifiche riguardano non solo un particolare gruppo di patogeni vegetali ma anche un ampio spettro di problematiche che spaziano dalla diagnostica allo studio della loro biologia, patogenicità o controllo. Meno frequentemente, invece, i Micologi, i Batteriologi e i Virologi, interessati agli aspetti della diagnosi molecolare, hanno la possibilità di interagire tra loro. Questo Convegno ha consentito ciò e ha permesso di fare il punto della situazione sulle attività di ricerca in essere nel campo della diagnosi molecolare dei patogeni vegetali, consentendo, nel corso di questo breve ma intenso incontro, un ampio e proficuo scambio di conoscenze. Finalità dell'Incontro, infatti, era consentire agli specialisti nella diagnosi di differenti gruppi di patogeni vegetali di

avere sufficiente tempo per discutere dei risultati presentati oralmente o sotto forma di poster e di gettare le basi per future collaborazioni. Pertanto, la pubblicazione di questi Atti fa il punto della situazione nel settore della ricerca che si occupa della diagnosi molecolare dei patogeni vegetali. Gli Autori dei lavori qui pubblicati rappresentano gran parte delle aree di ricerca europee e provengono da Università, Istituti di Ricerca e Agenzie pubbliche. Gli organizzatori sono profondamente riconoscenti al Programma Europeo COST 823 e all'Università di Algarve, i cui contributi hanno reso possibile questo Incontro.

A cura di MARINA BARBA

Petria 9(1-2), 67-72, (1999) Atti del Convegno/Proceedings of the Meeting

New developments in detection of plant pathogenic bacteria by combining methods based on microbial enrichment, serology and PCR-amplification

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The advantages of techniques for the detection of plant pathogenic bacteria by combining methods based on different principles is illustrated with two procedures to detect *Xanthomonas campestris* pv. *pelargonii* (Xcp) in pelargonium leaf extracts. (1) Immunofluorescence colony-staining and confirmation of fluorescent colonies by PCR-amplification allowed specific quantitative detection of Xcp at a level of 10² – 10³ cfu per ml of leaf extract within three days. The primers reacted with all Xcp strains tested, but not with related *Xanthomonas* species, or with bacteria cross-reacting in immunofluorescence procedures. (2) Bio-PCR based on growth of Xcp on 10% TSA plates for 3 days, harvesting of (all) bacteria from plates and PCR-amplification allowed specific detection of Xcp within three days with a similar sensitivity as IFC. The application of both methods for plant testing and ecological research is discussed.

Key words: *Xanthomonas campestris* pv. *pelargonii*, *Pelargonium*, Immunofluorescence colony-staining, IFC, bio-PCR, cross-reactions, primer selection.

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Diagnostics of pectinolytic *Erwinia* isolated from infected potato plants and tubers by PCR

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The genus *Erwinia* contains economically important pectinolytic species which are *E. chrysanthemi* and *E. carotovora* with two subspecies: *E. carotovora* spp. *atroseptica* and *E. carotovora* spp. *carotovora*. About 230 samples of potato plants and tubers with blackleg and soft rot symptoms were obtained from the State Inspection of Plant Protection in Poland. For estimation of *Erwinia* subspecies biochemical, serological and molecular methods were used. The use of PCR identified 1469 pectinolytic isolates as belonging to the species *E. carotovora*. It was estimated that about 51% of the strains isolated were *E. carotovora* spp. *atroseptica*. It has been estimated by indirect ELISA, with monoclonal antibody that only about 66% of *E. carotovora* spp. *atroseptica* strains belong to serotype I. However, serotype I constituted 86% of tuber isolates. None of the isolates from the area of Poland was identified as *E. chrysanthemi*.

Key words: black leg, Soft rot, molecular and serological diagnostics.

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Optimizing PCR technique for large scale diagnosis of angular leaf spot of strawberry in *Fragaria* plants

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Xanthomonas fragariae, the causal agent of angular leaf spot of strawberry, is a quarantine bacterium under the UE and EPPO regulations. In Portugal, sporadic infections occur, presumably when asymptomatic infected runners are used. In these cases, convenient phytosanitary measures are implemented to avoid the spread of the pathogen. Laboratory techniques used for detection and identification of the organism are slow and frequently lead to erroneous results. Recently, biomolecular techniques have been described to detect *X. fragariae* from infected strawberry plant tissues. These procedures are however quite laborious for routine diagnostic application. To overcome this problem a rapid and sensitive immunocapture-PCR (IC-PCR) was designed, allowing the detection of 10³ cells·ml⁻¹ of *X. fragariae* from infected plant tissues.

Key words: *Xanthomonas fragariae*, Diagnosis, Polymerase chain reaction.

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Identification of *Agrobacterium vitis* by amplification of molecular markers

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Twenty tumourigenic isolates of *Agrobacterium* spp. (nineteen of *A. vitis* and one of *A. tumefaciens*) obtained from galled grapevine plants were characterised by amplification of molecular markers by means of the polymerase chain reaction (PCR).

Five sets of primers were used, allowing the amplification of specific sequences of the genes *acs* (agrocinopine synthase), *6b/vis* (vitopine synthase), *ipt* (isopentenyl transferase), and the following IS elements, IS866, IS868 and IS869. Based on the amplification of the above mentioned molecular markers, two isolates of *Agrobacterium* spp. were identified as nopaline strains, fourteen as octopine strains and four as vitopine strains. Apparently, the *A. tumefaciens* isolate harbours a Ti plasmid of *A. vitis*. According to the distinct spectra of IS elements it was possible to detect different genomic groups within *A. vitis* strains isolated from grapevine tumours in Portugal.

Key words: Polymerase chain reaction, IS elements, Grapevine, Crown gall.

Petria 9(1-2), 85-88, (1999) Atti del Convegno/Proceedings of the Meeting

Introduction of nucleic acid based techniques for routine diagnosis of plant pathogenic bacteria in Slovenia

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In order to prevent the introduction and spread of certain plant pathogenic quarantine bacteria through imports and domestic production in Slovenia, routine laboratory testing procedures for potato brown rot, potato ring rot and fireblight have been introduced. PCR technology is currently used in pathogen testing protocols for potato brown rot (*Ralstonia solanacearum*) and for grapevine phytoplasma and viruses. Problems and advantages of introducing and using PCR technology as a part of routine testing protocols in our specific situation, especially regarding potato brown rot bacteria, are presented.

Key words: Polymerase chain reaction, Potato brown rot, *Ralstonia solanacearum*, Routine testing

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Ribosomal and non-ribosomal primers for sensitive detection and identification of phytoplasmas

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Mycoplasmalike organisms, now renamed phytoplasmas, are associated with severe plant diseases and because of the inability to isolate them in pure culture their identification has been difficult. Recently this has become possible due to the introduction of molecular tools such as DNA probes and PCR/RFLP on conserved ribosomal phytoplasma region (16SrDNA) and/or on unidentified chromosomal DNA. These approaches provides rapid and reliable means for classification and are also useful

in epidemiological studies of phytoplasma associated diseases.

Key words: Phytoplasma, Detection, Identification, PCR, RFLP.

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Possible improvement in phytoplasma detection

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The phytoplasmas are wall-less, non culturable prokaryotes that cause severe diseases in several cultivated plants. In several cases these pathogens are efficiently transmitted in the field by leafhopper insect vectors. The use of phytoplasma-free material is effective for planting of new orchards and vineyards. Up to now sensitive phytoplasma detection has been obtained using PCR-based techniques from herbaceous hosts while intense work is still in progress to set up suitable procedures for detection for these pathogens in woody plant material. The present work shows the specific amplification of ribosomal RNA (16SrRNA gene), by use of RT-PCR, of 4 different phytoplasmas infecting *Chataranthus roseus* and *Arabidopsis thaliana*. This result may suggest the use of RT-PCR-based procedure for phytoplasma detection of propagating material: its use, also, may allow to use of the same RNA extracts prepared for plant virus detection.

Key words: Phytoplasmas, Diagnosis, RT-PCR.

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AFLP DNA fingerprinting of plantpathogenic fungi at IPO-DLO

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AFLP (amplified fragment length polymorphism) is a powerful DNA fingerprinting technique to identify fungi and bacteria and to study their genetic variation. At IPO-DLO this method is used to study the population dynamics of different AGs of *Rhizoctonia solani*. In *Septoria tritici* AFLP is used to construct a genetic map. In *Phytophthora* spp. and *Fusarium oxysporum* markers linked to race specific avirulence genes will be identified using AFLPs.

Key words: AFLP, DNA fingerprint, Genetic variation.

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PCR based detection of latent infections of *Botrytis aclada* fres. in onion bulbs

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The ribosomal internal transcribed spacers (ITS1 and ITS2) were sequenced from isolates of *Botrytis aclada*, the causal agent of onion neck rot, and isolates of each of the closely related species *B. byssoidea* and *B. squamosa*. Alignment of the obtained ITS1 and ITS2 DNA sequences and published sequences from other closely related species showed very little variation between species of *Botrytis*. Differences were only found at four positions in the ITS1 DNA region and none were found in the ITS2 DNA region. A *B. aclada* specific primer pair (ITS1ba and ITS2rev) was designed based on one base pair difference, giving a PCR amplification product of 280 base pairs. Using pure fungal cultures it was possible to exclusively amplify DNA from *B. aclada*.

Key words: PCR, ITS, *Botrytis aclada*, *Botrytis allii*, *Botrytis squamosa*, *Botrytis cinerea*.

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Molecular identification methods for a *Gliocladium catenulatum* strain promising in biological control

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In a RAPD-PCR study concerning variation and molecular identification of Finnish *Gliocladium* isolates, *G. roseum* and *G. nigrovirens* isolates were nested within the clusters of *G. catenulatum* isolates. Differences between the strains were also found by UP-PCR. In sequence analysis identical ITS1 and ITS2 sequences were obtained. Only in *G. virens* ITS sequences were clearly different. A strain-specific amplification product was searched for one isolate (J1446), which has been promising in biological control of plant disease. Primer pair OPA1/OPA3 produced a band, which separated isolate J1446 from other *G. catenulatum* isolates. This band was isolated from the gel, purified with b-agarase and sequenced from both ends using OPA1 and OPA3 primers. SCAR-primers were also formed by cloning the strain-specific and selected clones were sequenced. Three specific primer pairs were designed and they amplified fragments of ca. 230 and 300 bp, which were specific to *G. catenulatum* J1446. Primers were also used in PCR assay with DNA isolated from cucumber leaves treated with a solution made from *Gliocladium* powder. Amplification of the expected product was obtained from treated plants, but not from control plants.

Key words: *Gliocladium catenulatum*, ITS, RAPD-PCR, rDNA, UP-PCR.

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Use of DNA heteroduplex analysis to distinguish the *Seiridium* isolates causing cypress canker in the mediterranean region

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A novel assay was employed to differentiate the cypress canker pathogens *Seiridium cardinale* and *S. cupressi*. The approach was based on PCR-amplification of the informative Internal Transcribed Spacer 2 region from the ribosomal DNA. Aliquots of the amplification products of each isolate were combined in pairs and subjected to electrophoretic migration as double strands in DNA Heteroduplex (HPA) Polymorphism analysis. *S. cardinale* was distinguished from *S. cupressi* through the conformation of the double helix structure and its perturbation by base mispairings in the heteroduplex molecules. The combination of PCR technology with the HPA technique allowed rapid screening of *Seiridium* isolates for polymorphism detection.

Key words: *Seiridium cardinale*, *S. cupressi*, PCR, HPA analysis.

Petria **9**(1-2), 119-122, (1999) Articolo Scientifico/*Scientific paper*

Polymerase chain reaction-single strand conformation polymorphism to differentiate the cypress canker pathogens *Seiridium cardinal* and *Seiridium cupressi*

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A rapid and sensitive method has been developed for the differentiation of the species of *Seiridium* causing cypress canker in the Mediterranean region. The assay was based on the PCR-amplification of the Internal Transcribed Spacer 2 (ITS2) region from the ribosomal RNA. Once the piece of DNA had been generated, the SSCP technique was applied to detect sequence polymorphism in the investigated fungal species. Minor sequence variations in the single-stranded amplified DNA fragments caused subtle changes in conformation and thus a different mobility on the polyacrylamide gels, allowing the species *S. cardinale* and *S. cupressi* to be differentiated. The SSCP assay showed inter-, but not intra-specific differences in the patterns. PCR-SSCP analysis provides a novel method by which to recognise and distinguish important plant pathogenic fungi and should have obvious applications in taxonomic classification.

Key words: *Seiridium cardinale*, *S. cupressi*, PCR, SSCP analysis.

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Development of ligase chain reaction assays for detection of potato viruses

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This paper describes the development of ligase chain reaction (LCR) assays for potato virus A and Y. Results are presented comparing the sensitivity of ELISA, LCR and PCR for detection of virus in tuber material. The obstacles to the introduction of such methods in routine diagnostic testing are discussed.

Key words: Ligase Chain Reaction, potato virus A, potato virus Y.

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Fluorescent PCR for the detection of potato viruses

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In order to facilitate the routine use of the polymerase chain reaction (PCR) for the detection of plant viruses, a number of fluorescent-based PCR detection systems have been investigated. Most work has focused on the PE Biosystems TaqMan® system, utilising 5'-exonuclease fluorogenic assay technology, which permits quantitative gel-free detection of PCR products. Using this system, assays have been developed for the detection of various potato viruses direct from tubers, including potato virus Y (PVY), tobacco rattle virus (TRV) and potato mop-top virus (PMTV). The performance and advantages of these tests are discussed.

Keywords: Polymerase chain reaction, Potato mop-top virus, Tobacco rattle virus, Potato virus Y, TaqMan®, 5'-exonuclease fluorogenic assay.

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PCR-based detection and strain typing of citrus tristeza virus

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Viruses exist as entities which have large number of strains that differ in the biological symptoms caused. To adopt an efficient protection strategy there is the need not only to detect a certain virus but also to identify the strains that are present. The introduction of PCR in plant diagnosis has enabled the development of sensitive assays that are evolving for user friendly systems. However, the strain typing aspects of diagnosis still require time consuming techniques. These aspects are illustrated with Citrus Tristeza Virus. In this work we describe a PCR-based diagnostic test that provides information about the

type of strains present in the isolates assayed. The assay is done in two steps: 1) Immunocapture/RT-PCR amplification of part of the coat protein (CP) gene coupled to a 5'-fluorogenic exonuclease assay for a sensitive broad spectrum detection of the virus, 2) re-amplification of an internal segment in the presence of strain specific fluorogenic probes; the extent of the reaction of each specific probe giving an insight of the strain composition of the isolate. This assay clearly discriminated among isolates containing severe stem-pitting strains from mild isolates.

Key words: Citrus tristeza virus, Diagnosis, PCR.

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Application of a simple IC/PC-PCR-ELISA technique for plum pox potyvirus detection

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The use of PCR methodology has greatly improved plum pox potyvirus (PPV) detection the last years. To further elaborate simplifications of the standard techniques, a PCR-ELISA assay was developed for amplicon revelation. PCR products were directly adsorbed on a microplate and hybridized with a DIG-labelled probe prepared with the same pair of primers as those used to amplify the target sequence. A gain in sensitivity of at least 100-fold was obtained over agarose electrophoresis, permitting high detection levels both with symptomless apricot leaves processed by immunocapture (IC)-PCR and single aphids processed by print capture (PC)-PCR.

Key words: Plum pox potyvirus (PPV), Immunocapture, Print capture, PCR, PCR-ELISA.

Petria 9(1-2), 141-143, (1999) *Atti del Convegno/Proceedings of the Meeting*

An immunocapture/PCR method for the detection of petunia flower mottle potyvirus in petunias

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In the last years, a new viral disease infecting petunias has occurred in Germany. Electron microscopic investigations revealed flexuous, filamentous particles typical of a potyvirus infection. Serological tests showed no cross-reactivity with other known petunia-infecting potyviruses. We have used an immunocapture/PCR method to amplify the 3' end of the petunia-infecting viral genome, corresponding to the 3' end of the CP gene and the 3' non-coding region, with a specific antiserum and degenerate primers. The resulting PCR products were cloned and sequenced. Maximum homology levels of 51% in the 3' non-coding region, and 71% in the amino acid sequence of the partial coat

protein were found between the petunia-infecting virus and other potyviruses. From these results, specific primers were designed for the detection of the PetFMV in petunias by IC/PCR.

Key words: Potyvirus-degenerate primer, Specific antiserum.

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Optimized IC-RT-PCR method for detection of the black currant reversion associated virus

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Reversion is the most wide-spread and serious virus-like disease infecting black currant. Recently, we have isolated a new nepovirus from reverted black currant and shown by Immunocapture-RT-PCR (IC-RT-PCR) detection method that the virus is consistently associated to the reversion disease suggesting that this virus is the causal agent of the reversion disease. Optimized method for the detection of the disease agent includes sampling of the tissue by the dot-printing method, immunocapture of the print-extract, RT-PCR reaction preferably in a one-tube reaction format, combined with Southern blot detection of the PCR products.

Key words: Nepovirus, Print capture PCR, Southern blot.

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Developing of an immunocapture RT-PCR for the detection of potato mop-top virus in potato tubers

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A Finnish PMTV (potato mop-top virus) isolate, Fin2b, was maintained in the propagation host *Nicotiana clevelandii*, purified and used to produce a polyclonal antiserum. Purified IgG was used to develop an immunocapture RT-PCR. The test primers were designed according to the sequences of RNA 2 from a Scottish isolate PMTV-T. All the potato tuber samples with disease symptoms tested were confirmed to be infected using this method. Healthy potato tubers were used as negative controls. Comparison with ELISA results indicate that IC-RT-PCR is at least 100-fold more sensitive. The results indicate that IC-RT-PCR is a promising method for testing PMTV from seed-tubers when ELISA is not sufficiently reliable.

Key words: Potato mop-top virus, IC-RT-PCR, ELISA.

Establishing a protocol for IC-RT-PCR detection of prune dwarf virus in almond

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Most of the Portuguese almond trees, are strongly infected with viruses. Prune dwarf virus (PDV) is widespread in Portuguese almond orchards and is thought to be one of the main factors leading to their low productivity. To monitor the sanitary status of the material obtained from the sanitation programmes, a protocol using Immunocapture/Reverse Transcriptional-Polymerase Chain Reaction has been established for PDV detection in almond. Conditions like composition of the extraction buffer, age of the extract, incubation period and magnesium concentration were tested to optimise the IC-RT-PCR reactions. Using the optimised conditions, a cDNA fragment with 722 bp, containing the coat protein gene of PDV, was amplified from the infected samples.

Key words: Almond, Ilarvirus, Prune dwarf virus, Diagnosis, PCR, IC-RT-PCR.

Sample preparations and RT-PCR assays for large scale detection of fruit tree viruses covered by certification standards

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The European Union has recently issued directives indicating that propagative material must meet qualitative and phytosanitary standards in order to be accepted commercially by Member States. The most endangering pathogens; (essentially the systemic ones such as viruses, viroids, and phytoplasmas); must be excluded from certified material, therefore, different studies have been initiated for improving and optimizing detection methods for these pathogens. Polymerase chain reaction (PCR) is a very sensitive and relatively reliable molecular technique that has been applied with increasing success to the large scale sanitary evaluation of fruit tree propagative material. We evaluated different RNA extraction methods followed by single — or multiplex — reverse transcription (RT)-PCR in order to find the simplest way to detect different viruses present in single or mixed infections in fruit tree species. Samples of pome (apple) and stone fruit (peach, plum, apricot, almond) trees, naturally infected with plum pox virus, prunus necrotic ringspot virus, apple mosaic virus, prune dwarf virus, and apple chlorotic leafspot virus were analyzed by single or multiplex one-step RT-PCR using a mixture of primers specific for each virus. RNA was extracted from viruses by trapping virions with a mixture of antisera specific for each tested virus (IC-RT-PCR) or by a

simple rapid extraction method (REM-RT-PCR). Both procedures needed no more than 0.3 g of green tissue and eliminated laborious and hazardous extraction procedures. We have determined that it is possible to determine the presence of a virus or viruses in a mixed infection in a large number of samples (50-80) in one or two days.

Key words: Diagnosis, Virus, IC-RT-PCR, REM-RT-PCR, Multiplex-IC-RT-PCR.

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Nested PCR for detection of plum pox potyvirus in cherries

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Different PCR systems using various sets of primers were tried for detection of plum pox potyvirus (PPV) in Hungarian cherries. Combining 3 different primers specific for the 3' non-coding region of PPV a nested PCR assay was set up. Total RNA and captured virions extracted from cherry leaves were amplified with primers NCR1/NCR3 in the first RT-PCR, followed by a second nested PCR amplification using primer pair NCR1/NCR2. Bands of 220 bp were detected in case of M, D, El-Amar and cherry strains of PPV as well as symptomatic cherry samples, when total RNA and captured virions were used in nested PCR.

Key words: PPV, Detection, Cherry, Nested PCR, RT-PCR, IC-PCR.

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RT-PCR of cucumber mosaic virus: A quick and informative way to characterise isolates in a COST 823 ringtest on zucchini viruses

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During a COST Ringtest on zucchini viruses held in Torino, Italy, 44 isolates of cucumber mosaic virus (CMV) together with one isolate of peanut stunt virus (PSV) and one of tomato aspermy virus (TAV), were tested by RT-PCR using four sets of primers. Isolates came from Europe, Asia, America and Australia mainly from *Cucurbitaceae* and some of them, already characterised, served as reference isolates. All isolates were tested with primers specific for RNA3, followed by *MspI* digestion and most of them could be assigned to subgroup I or subgroup II but in some cases different patterns came out. Some isolates were also tested with primer sets specific for RNA2. Only one set of primers detected PSV and TAV. In conclusion, the method appears to be a reliable and informative alternative to monoclonal antibody-based ELISA.

Key words: CMV, RT-PCR, zucchini.

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Use of RT-PCR methods for the detection of garlic potyviruses

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Two PCR primer pairs were designed for detection of onion yellow dwarf potyvirus (OYDV) and leek yellow stripe potyvirus (LYSV). Garlic samples were tested by RT-PCR and IC-RT-PCR. We evaluated optimal conditions for RT-PCR, IC-RT-PCR, the use of different sample treatments and compared their sensitivity to DAS-ELISA. Furthermore, a single tube IC-RT-PCR was developed using garlic leaf extract, found to be 100 times more sensitive than ELISA, and more convenient method for testing leaf samples.

Key words: Garlic, *Potyvirus*, Leek yellow stripe virus, Onion yellow dwarf virus, Polymerase chain reaction, Immunocapture-RT-PCR, Single tube RT-PCR.

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Detection of geminivirus genomes by print PCR

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A rapid and simple procedure is described to amplify efficiently geminivirus DNA genomes. This method, named print-PCR (P-PCR), allows direct amplification of DNA from infected plant or whitefly tissues printed directly on Whatman 3MM paper, without the need of any grinding, incubation, or washing steps previous to the amplification reaction, thereby diminishing the possibilities of cross-contamination between samples. P-PCR has been successfully applied to whiteflies and various plant species infected by two different tomato yellow leaf curl virus (TYLCV) species, TYLCV-Sr and TYLCV-Is.

Key words: Geminivirus, TYLCV, Detection, Print-PCR, Tomato, *Lycopersicon esculentum*, Bean, *Phaseolus vulgaris*, Whiteflies, *Bemisia tabaci*.

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Use of Spot-PCR for the detection of grapevine phloem-limited viruses

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PCR amplification is a most sensitive method for grapevine virus detection but it can hardly be used for large scale diagnosis. Spot-PCR represents a simple, reliable and cost effective technique that eliminates the laborious procedures for template purification before enzymatic reaction, allowing sampling of hundreds vines per day. A drop of crude sap, squeezed from leaf petioles is deposited on an alkali-treated nylon membrane and let to dry. A small fragment is excised from the spotted membrane and boiled in a buffered solution to release nucleic to be amplified with standard Rt-PCR protocols. With this technique, successful amplification of grapevine virus B (GVB) and grapevine fleck virus (GFkV) was obtained from samples collected in early June. Detection of GLRav-3 in the same vines was possible only in September, likely because of a different kinetics of virus accumulation in host tissues.

Key words: diagnosis, RT-PCR, grapevine viruses.

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Detection of grapevine viruses by RT-PCR of double stranded RNA templates

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The non-availability of adequate immunological reagents has prevented the use modern diagnostic techniques for all the viruses that are considered in grapevine certification programs. In this work we study the feasibility of using double stranded RNA (ds-RNA) as a template for PCR amplification and detection of eight grapevine viruses, aiming at the development of an integrated system for viral diagnosis. All the viruses could be detected in variable amounts in nursery material. Rupestris stem pitting associated virus (RSPaV 1) and Grapevine virus B were very frequent. RSPaV 1 seems to be a very variable virus. A significant number of samples previously assayed by ELISA were found positive for GLRV 3.

Key words: PCR, Rupestris stem-pitting, Grapevine leafroll, Vitiviruses, Double stranded RNA.

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RT-PCR procedure to amplify S RNA sequences of iris yellow spot virus, a distinct tospovirus

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The full length S RNA of a new tospovirus species, named iris yellow spot virus (IYSV), obtained by RT-PCR reaction, was sequenced. The first clones were obtained using a

very short primer and a non-specific primer, that together with the use of a mixture of enzymes on the PCR assay, instead of the traditional enzyme, enabled the amplification of specific viral fragments. The alignments of the available sequences of the tospoviral N proteins showed that IYSV is a distinct species, based on the amino acid identity values, being more related with WSMV and PBNV (44%) and genetically more distant from PYSV (21%).

Key words: Tospovirus, Iris yellow spot virus, PCR assay.

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A ligation-anchored/PCR method for the cloning of the 3' end of the impatiens isolate of oilseed rape mosaic tobamovirus

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The development of DNA-based diagnostic methods has greatly improved the sensitivity and specificity of the detection of pathogens. However, these techniques require at least partial knowledge of the sequence of the pathogen, for the design of primers for example. In the case of previously undescribed viruses, or new strains of viruses, this sequence information is not always available. We have developed a ligation-anchored/PCR technique (LA/PCR) to clone a new tobamovirus infecting *Impatiens neuguinea*. Purified viral RNA was ligated to an EcoRI-linearized plasmid using T4 RNA ligase, and the ligation product subjected to an RT/PCR, with a degenerate tobamovirus primer located in the tobamovirus 3' non-coding region and the universal reverse primer corresponding to plasmidic sequence. The resulting PCR product, which contained some plasmidic sequence and the 3' end of the viral genome, was cloned and sequenced. Sequence comparisons with the corresponding sequences of other tobamoviruses revealed very high homologies with the oilseed rape mosaic virus.

Key words: ORMV-Impatiens, Tobamovirus degenerate primers, LA/PCR.