Reaction of Cassava Leaves to *Microcyclus ulei*, Causal Agent of South American Leaf Blight of Rubber Tree

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ABSTRACT

Young cassava (*Manihot esculenta*) leaves inoculated with conidiospores or treated with an autoclaved conidiospore suspension of *Microcyclus ulei*, a pathogen to several species of the genus *Hevea*, react strongly with induction of blue fluorescing compounds in the contact areas, demonstrating local cell growth and irregular tissue formation, leaf distortion and, occasionally, leaf abscission. Restricted hyphal growth also occurs in areas inoculated with living spores, but without formation of new conidiospores. The plant reaction resembles a complex resistance reaction to a biotrophic pathogen rather than a typical non host defense reaction.

Additional keywords: host range, defense reactions, *Manihot esculenta*, *Hevea* spp.

RESUMO

Reação de folhas de mandioca ao *Microcyclus ulei*, agente causal do mal-das-folhas da seringueira

Folhas jovens de mandioca (*Manihot esculenta*) inoculadas com uma suspensão de conídios viáveis ou autoclavados de *Microcyclus ulei*, um patógeno de diversas espécies do gênero *Hevea*, reagiram fortemente com indução de compostos azuis fluorescentes nas áreas de contato, com a morte das células locais e a formação irregular dos tecidos, distorção das folhas e, ocasionalmente, com a abscisão da folha. Também ocorreu crescimento restrito das hifas, mas s em produção de novos conídios. A reação das plantas foi mais semelhante a uma reação de resistência complexa a um patógeno biotrófico do que uma reação típica de defesa de uma planta não hospedeira.

INTRODUCTION

The ascomycete *Microcyclus ulei* (P.Henn.) v. Arx reveals a very narrow host range. According to Chee (1976), who used data of field observations of large rubber (*Hevea* spp.) experimental fields in Trinidad, the specialized fungus attacks only plants of the genus *Hevea* and within the genus only the species or the respective hybrid genotypes of the species *H. brasiliensis* (Wild. ex. A. Juss) Muell. A rg., *H. guianensis* Aub., *H. benthamiana* Muell. Arg., *H. spruceana* (Bth.) Muell. Arg., Observations in gene pool collections of *Hevea* in Manaus, AM, Brazil, revealed, that the genotypes of *H. camargoana* Pires and *H. camporum* Ducke are also severely attacked by *M. ulei* (Junqueira *et al*., 1989). Unfortunately it is not possible to decide if the latter species were attacked by new races of the fungus occurring in Manaus or if the plant material had not been available for testing according Chee (1976). Gasparotto & Junqueira (1994) described a high physiological variability of a number of isolates of *M. ulei*, derived from different agroclimatic regions of Brazil, ranging from the hot and humid environment of central Amazonas to the temperate, semidry areas in the southern areas of Brazil. Changes of host ranges combined with the changes in adaptation to a wide range of abiotic environments cannot be excluded for this economically important fungus. In addition to this fact, an enormous amount and variability of potential host genotypes were provided to the pathogen during the colonization of Amazonas with rubber being one of the most important crop plants for small landholders. During this phase (1972 to 1989) under the auspices of the SUDHEVEA (1971), more than 75,000 ha were planted with rubber (Gasparotto *et al*., 1997), which turned out to be completely susceptible to *M. ulei*.

Lieberei *et al*. (1989) reported on field observations and preliminary tests, in which two isolates of the *M. ulei*-collection of the National Brazilian Rubber Research Institute (Junqueira *et al*., 1986) were applied in greenhouse studies to cassava (*Manihot esculenta* Crantz) leaves, which led to a strong morphological reaction against spores of *M. ulei* including leaf deformation and premature leaf fall. In order to evaluate the potential threat of this fungus to the tropical staple food plant cassava, experiments were carried out to describe in more detail the reactions of cassava leaves to spore samples of *M. ulei*.

MATERIAL AND METHODS

Cultivation of *Microcyclus ulei*

Isolate 40R7 was cultivated in 100 ml tubes on Potato-Sucrose-Agar (PSA) 0.5% Sucrose, pH 5.0. In a 2-month period the fungus (mycelium and spores) was transferred to fresh medium and was cultivated in the dark at 23 ºC. One week before transfer to the new tube the cultures were exposed two times to 45 min of light of a Osram DULUX 11 W / 21. This treatment enhanced induction of conidiophores and of spore production. Spore mass production was carried out according to Lieberei *et al*. (1983).

Plant cultivation

The experiments were carried out with non-defined material and with clone CG 165-7 of CIAT, Colombia. The plants were cultivated in pots of 90 cm diameter, at a day-night regime of 12 to 12 h, 70 to 90% relative humidity, 18 to 31 ºC, and 450 to 600 µE of light. Fertilization was carried out using 1.2 g Plantosan® per plant per six weeks (Aglucon Spezialduenger GMBH, Kleinkaribach, Germany).

Inoculation and incubation

Conidia were isolated as an aqueous suspension by adding 5 ml of sterile tap water to a sporulating culture of *M. ulei*, grown in 250 ml Erlenmeyer flasks on 70 ml PSA (0.5% sucrose). The conidia were collected, the washing procedure was repeated two times and the three washings of 15 ml were pooled and submitted to low speed centrifugation (300 g, 5 min). The spores were washed two times using sterile tap water, the spore number was adjusted after microscopically counting to 1 x 10^6 spores per ml and the suspension was applied to the leaves by a paintbrush type H, Paasch, USA. The distance of the paintbrush to the six days old leaves was 15 cm, at an initial pressure of 1.5 bar. Inoculated leaves were kept in humid polyethylene bags until harvest.

For control experiments, washed spore suspensions were autoclaved at 120 ºC, 15 min, 1 bar. Control solutions were tap water and diluted culture medium (PSA), which were applied in the same way.

Microscopy and staining

The microscopic studies were done with a BH-2 Research microscope of Olympus, which was equipped with a fluorescence device and interference contrast system.

Lignin was stained using phloroglucin-HCl according to Nultsch *et al*. (1979), phenolic cell wall-bound phenolics were detected with toluidin blue dye, according to Feder & O'Brien (1968) and fungal mycelium was detected applying the Pianese-stain (Gerlach, 1984).

RESULTS

Macroscopic and microscopic symptoms

Cassava leaves inoculated with living conidiospores or autoclaved conidiospore suspensions of *M. ulei* reacted in a similar way to the treatment, revealing leaf deformations and necrotic leaf areas (Figure 1a,b). Leaves treated with tap water or fresh culture medium did not show any visible change in leaf morphology and did not reveal any necrotic lesion.

The first day after inoculation there were no macroscopic changes visible. Using fluorescence microscopy with excitation 300 to 400 nm, and a 20 UB-W-2 filter, blue fluorescing spots were visible in the epidermal and subepidermal cell layers underneath the spores, regardless of whether living spores or autoclaved spores had been used for inoculation.

Four days after inoculation the blue fluorescence was less expressed, but still detectable.

On the lower leaf side, globose swollen structures developed directly in the contact zone of the leaf surface with the spores. The small swollen areas were water soaked and the epidermal cells were slightly enlarged. Within these areas of hypertrophic cell growth browning occasionally occurred (Figure 1c, d).

After ten days blue fluorescence was no longer detectable. The swollen areas revealed larger brown areas. Many cells collapsed and formed small necrotic lesions of less than 1 mm in size. Many leaves were abscised after about two weeks, but some remained attached to the plants. No spore formation was to be seen within the following two months of observation on the fallen leaves nor on the living leaves that remained attached to the treated plants.

Histological reactions

Normally the cells that were in direct contact with the spore material reacted with cell enlargement, but in some cases the neighboring cell layers were also modified. The deformation never passed over the contact area between palisade to spongy parenchyma. The morphological aspects of other side of the leaf, which did not come into contact with the spores, remained unchanged. Within the malformed areas, some cells turned brown and revealed an accumulation of wall-bound phenolic compounds in the cell walls next to them. Fungal mycelium was detected in small tissue areas after inoculation with living spores. This tissue did not grow out of the lesion area and was stopped by necrotic response. Mycelium did not develop, and intercellular mycelium growth or formation of conidiophores or spores did not occur.

DISCUSSION

Inoculated cassava leaves react very strongly to *M. ulei*. The reaction is independent of whether the spores used for inoculation are infective, living spores, or if autoclaved spore material is used. The response is complex and bound to signal compounds which induce morphogenetic reactions. It comprises the induction of phytoalexin-like compounds such as the blue fluorescing substance(s), which occur(s) in areas which are in direct contact with the inoculation material as well as cell growth stimulation and, later on, necrotic responses.

This reaction type is different from typical non-host resistance reactions, in which generally fast responses are

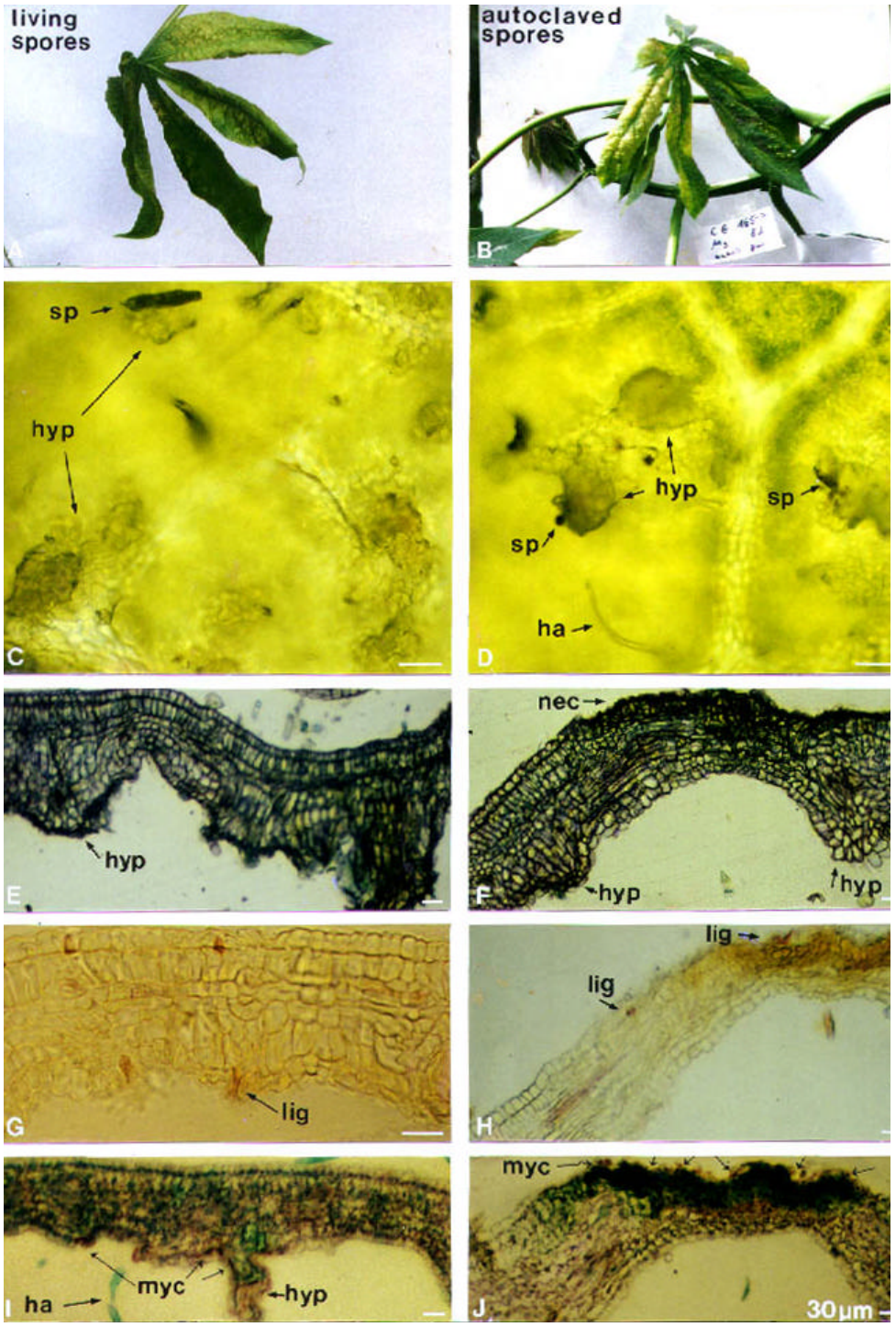

FIG. 1 - Morphological and microscopically visible reactions of cassava (*Manihot esculenta*) leaves to *Microcyclus ulei* after eight days of incubation. Inoculation with living (A, C, E, G, I) or autoclaved (B, D, F, H, J) spores; A,B macroscopic reaction pattern; C, D: lower leaf side revealing globose hypertrophic structures (hyp) in contact areas with spores (sp), (ha = hairs); E, F wall bound phenolics in hypertrophic (hyp) and necrotic (nec) areas are marked by darkened wall regions; G, H indication of lignin-like substances (lig) in necrotic and mesophyll areas by red or orange stain; restricted development of hyphae (myc) in subepidermal intercellular spaces (I, J).

induced by pathogen signals, leading to an oxidative burst (e.g. Lamb and Dixon, 1997) and subsequent necrosis of a limited number of cells which are in tight contact to the signal substances. The same symptom, the hypersensitive response with restricted tiny necroses, is known in host specific reactions of highly resistant hosts to avirulent or weakly virulent pathogen races (Scheel, 1998).

In contrast to hypersensitive reactions, the responses

of cassava leaves to autoclaved inoculum include synthesis dependent resistance responses like phenolic compound formation in cell wall regions and the formation of fluorescing phytoalexins. In addition, cell growth and tissue reactions are visible. It cannot be excluded that the typical hypersensitive response was initially induced but subsequently inhibited by liberation of HCN from dead cells, as shown by Lieberei *et al*. (1989) for *Hevea* leaves, which like cassave leaves are also cyanogenic. It is known that HCN inhibits peroxidases and phenolase (Bolwell *et al*., 2002) and gives rise to additional reactions, leading to the lesion development reported here. Within about one day after cell death, at the latest the HCN content of the tissue is below any inhibitory concentration, synthesis dependent reactions to take place (Lieberei *et al.*, 1996), and phytoalexins and phenolics can be produced. It is of great interest to study the factors released by *M. ulei*, which give rise to the expressed reaction of cassava leaves.

Using living spores as inoculum, a restricted growth of hyphae takes place in the intercellular spaces between the epidermal and the subepidermal layer. This is a first indication of the development of a potential colonization phase in this tissue by the biotrophic non host fungus. It may be a response that is again enabled by the occurrence of HCN, liberated from dying cells. It has been shown that the fungus is highly tolerant to HCN (Lieberei *et al*., 1983) whereas the host plant is inhibited in its defense reactions.

In the case studied here, the reaction pattern gives rise to the assumption, that in the course of this intensive interaction, a biochemical selection process to a new fungus-plant relationship may be on its way, which may result in the development of cassava as a new host of the biotrophic pathogen *M. ulei*.

This assumption may be premature, but as already pointed out, the pathogen *M. ulei* is physiologically highly variable (Junqueira *et al*., 1986; Gasparotto & Junqueira, 1994), and the enlargement of the host range to other members in the family of *Euphorbiaceae* which reveal the same biochemical properties of cyanogenesis seems to be possible.

Cassava is a very important staple food. In order to be prepared and to avoid losses of cassava production, research on this plant – fungus interaction should be continued using a broad base of cassava genotypes and a representative range of *M. ulei* isolates.

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LITERATURE CITED

BOLWELL, G.P., BINDSCHEDLER, L.V., BLEE, K.A., BUTT. V.S., DAVIES, D.R., GARDNER, S.L., GERRISH, C. & MINIBAYEVA, F. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. Journal of Experimental Botany 53:1367-1376. 2002.

CHEE, K.H. Assessing susceptibility of *Hevea* clones to *Microcyclus ulei*. Annals Applied Biology 84:135-145. 1976.

FEDER, N. & O'BRIEN, T.P. Plant microtechnique: some principles and new methods. American Journal of Botany 55:123-142. 1968.

GASPAROTTO, L.& JUNQUEIRA, N.T.V. Ecophysiological variability of *Microcyclus ulei*, causal agent of rubber tree leaf blight. Fitopatologia Brasileira 18:22-24. 1994.

GASPAROTTO, L., SANTOS, A F., PEREIRA, J.C.R.& FERREIRA, F.A. Doenças da seringueira no Brasil. Brasília. Embrapa-SPI. 1997.

GERLACH, D. Botanische Mikrotechnik. Stuttgart. Thieme Verlag. 1984.

JUNQUEIRA, N.T.V., CHAVES, G.M., ZAMBOLIM, L., ROMEIRO, R.S. & GASPAROTTO, L. Variabilidade fisiológica do *Microcyclus ulei*. Fitopatologia Brasileira 11:823-833. 1986.

JUNQUEIRA, N.T.V., GASPAROTTO, L., KALIL Fº, A.H., LIEBEREI, R. & LIMA, M.I.P.M. Identificação de fontes de resistência ao *Microcyclus ulei*, agente causal do mal-das-folhas da seringueira. Fitopatologia Brasileira 14:147. 1989. (Resumo).

JUNQUEIRA, N.T.V., LIEBEREI, R., FILHO, A.H.K., & LIMA, M.I.P.M. Components of partial resistance in *Hevea* clones to rubber tree leaf blight, caused by *Microcyclus ulei.* Fitopatologia Brasileira 15:211-214. 1990.

LAMB, C. & DIXON, R.A. The oxidative burst in plant disease resistance. Annual Review of Plant Physiology and Plant Molecular Biology 48:251-275. 1997.

LIEBEREI, R., BIEHL,B., GIESEMANN, A. & JUNQUEIRA, N.T.V. Cyanogenesis inhibits active defense reactions in plants. Plant Physiology 90:33-36. 1989.

LIEBEREI, R., FOCK, H. & BIEHL, B. Cyanogenesis inhibits active defence in plants: Inhibition by gaseous HCN of photosynthetic CO_2 fixation and respiration in intact leaves. Journal of Applied Botany 70:232-238. 1996.

LIEBEREI, R., JUNQUEIRA, N.T.V. & FELDMANN, F. Integrated disease control in rubber plantations in South America. Proc, 3rd Intern, CTA/DLG Symposium integrated pest management in tropical and subtropical cropping systems. Bad Duerkheim, Germany 2:445-456. 1989.

LIEBEREI, R., SCHRADER, A., BIEHL,B. & CHEE, K.H. Effect of cyanide on *Microcyclus ulei* cultures. Journal of Rubber Research Institute of Malaysia 31:227-235. 1983.

NULTSCH, W. & GRAHLE, A. Mikroskopisch-botanisches Praktikum. Stuttgart. Thieme-Verlag. 1979.

SCHEEL, D. Resistance response physiology and signal transduction. Current Opinion in Plant Biology 1:305-310. 1998.

SMITH, C.J. Accumulation of phytoalexins: defense mechanism and stimulation response system. New Phytologist 132:1-45. 1996.

SUDHEVEA. Plano Nacional da Borracha. Anexo XI- Pesquisas e experimentação com seringueira. Rio de Janeiro, 1971.

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