

Part 5: Appendices

Workshop Programme

| Time | Topic | Resource person |
|--|---|---|
| Registration and Opening session | | |
| 08:30-09:00 | Registration | |
| 09:00-09:10 | Welcome address | Dr. A.B. Salifu, SARI |
| 09:10-09:20 | Chairman's address | Dr. Joseph Cobbina, CSIR |
| 09:20-09:30 | Project background and objectives | Dr. S. Sreenivasaprasad, HRI |
| Breeding for blast resistance and varietal diffusion | | |
| 09:30-10:00 | <i>Interspecific and intraspecific breeding for tolerance to blast in Burkina Faso working with WARDA/ breeding –task force</i> | Dr. M. Sié, INERA |
| 10:00-10:30 | <i>Screening strategy for durable resistance to blast fungus</i> | Dr. Y. Séré, WARDA |
| 10:30-11:00 | <i>Impact assessment of agricultural technology: concept methodology and application to rice pests and diseases</i> | Dr. A. Diagne, WARDA |
| 11:00-11:15 | COFFEE BREAK | |
| DFID-CPP Blast project activities and outputs | | |
| 11:15-11:45 | <i>Survey of rice blast and varietal screening in Ghana (joint presentation by SARI and CRI)</i> | Dr. S.K. Nutsugah, SARI SARI/CRI |
| 11:45-12:15 | <i>Analysis of Magnaporthe grisea population structure in Côte d'Ivoire as a prerequisite for the deployment of varieties with durable blast resistance</i> | Dr. Y. Séré, WARDA |
| 12:15-12:45 | <i>Diversity of blast pathogen populations in four West African countries and strategies for resistance management</i> | Dr. S. Sreenivasaprasad |
| 12:45-13:45 | LUNCH BREAK | |
| Blast management in some West African countries | | |
| 13:45-14:15 | <i>Integrated management approach for rice blast in Burkina Faso</i> | Dr. B. Kaboré, INERA |
| 14:15-14:45 | <i>Genotype by environmental reaction of some improved exotic rice varieties to the blast pathogen in The Gambia</i> | Dr. L. Jobe, NARI |
| 14:45-15:15 | <i>Pathogenic variability and effective management of blast disease in Nigeria</i> | Dr. D.D Kuta, NCRI |
| 15:15-15:30 | COFFEE BREAK | |
| Discussion session - Outputs, Linkages and Further work | | |
| 15:30-15:45 | DFID-CPP Rice projects cluster | Dr. T.C.B. Chancellor, DFID-CPP |
| 15:45-16:00 | Further work for sustainable blast resistance/ management | Dr. S. Sreenivasaprasad |
| 16:00-17:00 | Outputs, Linkages, Further work and Recommendations Open Discussion | led by Prof. K.A. Oduro, Univ. of Ghana |
| 17:00-17:15 | Closing remarks and Farewell | Dr. E. Otoo, CRI |

Rice blast pathogen *Magnaporthe grisea*: Methodologies for assessing genetic and pathogenic diversity

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Summary

Methodologies used for characterisation of genetic and pathogenic diversity of *Magnaporthe grisea* populations are described. These include isolation and storage of *M. grisea* isolates, various steps in MGR586 DNA fingerprinting and pathotyping under controlled conditions. Some of these methods can be further adapted to suit varying laboratory conditions in developing countries.

1. Isolation of *Magnaporthe grisea* from blast samples and storage

The infected plant part for e. g. leaf, stem or sheath is cut into small pieces around the area showing the blast lesion including the edge of the lesion. All pieces from a sample are placed in a small Petri dish. Surface sterilisation and washings are done in this container. Surface sterilisation with 1% sodium hypochlorite (bleach) for 1:30 min is followed by 3 washes with sterile distilled water. Then these plant pieces are placed in Petri dishes lined with moist filter papers and incubated at 25°C for 24h to encourage sporulation. After incubation, these plant pieces are examined under stereo-dissecting microscope. Abundant *M. grisea* growth and sporulation can be seen from and around the lesions with grey, dense and bushy appearance (Fig. 10).



Figure 10. Growth and sporulation of *Magnaporthe grisea* from a blast lesion, following incubation in a moist chamber during the pathogen isolation process.

A sterile moistened needle is used to pick some conidia by brushing the needle across the sporulating lesion. The conidia are placed on oatmeal agar plate containing aureomycin. Plates were incubated at 25°C for about 7-10 days with 12 h darkness and 12 h light. The identity of *M. grisea* can be verified by checking the conidia under light microscope.

Mono-conidial cultures from the field isolate are derived by streaking a loopful of conidial suspension across a water agar (4% w/v) plate in a 'W' pattern, thus spreading the conidia. A guideline can be drawn on the undersurface the plate. Following 24h incubation at 25°C, a germinating conidium can be easily picked and subcultured on to a fresh OMA plate amended with aureomycin using a fine scalpel. *M. grisea* cultures are preserved for long term storage as dried cultures on filter papers. Several sterile filter paper squares (approx. 0.8 cm²) are placed around the actively growing edge of colonies on OMA plates. The culture is allowed to grow over the filter papers (7-10 days). The filter papers are then removed under aseptic condition and placed in a small Petri dish, in a desiccator and allowed to dry thoroughly. The dried filter papers are then stored at -20° C until required (Valent *et al.*, 1991).

2. DNA extraction, electrophoresis and quantification

2.1. Liquid culture preparation

Each *M. grisea* isolate was grown in 2 X Yeast Extract Glucose medium (YEG) (1 l contained glucose, 10.0 g; yeast extract, 2.0 g). Approximately ten plugs (5 mm in size) from an actively growing culture on antibiotic OMA medium were transferred to 100 ml of 2 X YEG medium contained in 250 ml Erlenmeyer flasks and grown at 25°C for 7 days in an orbital shaker (120 rpm). Mycelium was harvested by filtration through No. 3 Whatman filter paper and immediately frozen in liquid nitrogen. The frozen mycelium was pulverised, freeze-dried and ground to a fine powder using a sterile pestle and mortar. The mycelial powder was stored at -20°C until needed.

2.2. DNA extraction

CTAB method (Valent *et al.*, 1991; Hamer and Givan, 1990; Sreenivasaprasad, 2000) described below is widely used with *M. grisea* isolates.

1. Mix approx. 300 mg mycelial powder with 4 ml CTAB buffer prewarmed to 65° C in a suitable centrifuge tube. Sterile disposable needles can be used to aid in mixing. Add 40 µl β-mercaptoethanol in hood.
2. Incubate the tubes at 65°C for 30 min; mix once every 10 min.
3. Add 4 ml CHISAM, stand tubes uncapped for 2 min and mix by incubating on a platform shaker for 15 min (see notes).
4. Centrifuge for 30 min at 13,000 - 18,000 *g*.
5. Transfer the upper aqueous phase into a fresh centrifuge tube, add equal volume of isopropanol and mix.
6. Centrifuge for 10 min at 10,000 *g* to pellet DNA.
7. Discard the supernatant, vacuum dry the pellet briefly to remove traces of isopropanol.
8. Dissolve the DNA pellet in 600 µl sterile distilled water and add 100 µl RNase (20 mg ml⁻¹ stock), incubate at 37° C for 30 min.
9. Transfer DNA to a 2.0 ml microfuge tube and add an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) and mix well by inversion.

10. Centrifuge for 15 min at 12,000 - 14,000 *g*.
11. Collect aqueous phase, add equal volume of CHISAM and incubate on a shaker for 15 min.
12. Centrifuge as above and collect aqueous phase.
13. To precipitate DNA, add 1/10 volume of 3 M sodium acetate, pH 5.2 and 2.5 volumes of cold 100% ethanol (stored at -20° C), mix well and incubate at -20° C for 15 min.
14. Pellet DNA by centrifugation for 5 min as in step 10.
15. Discard supernatant, wash pellet with 1 ml cold 70% ethanol (stored at -20° C) and centrifuge for 2 min as in step 10.
16. Vacuum dry the pellet briefly to remove traces of ethanol.
17. Dissolve the pellet in 300 µl of TE 8 by incubating on a rotawheel/platform shaker; electrophorese a 5 µl aliquot.

2.3. Materials and notes for DNA extraction

1. CTAB buffer: 2% CTAB (hexadecyltrimethylammonium bromide), 100 mM Tris, 10 mM EDTA and 700 mM NaCl. Add β-mercaptoethanol (1% by volume) just before use (mercaptoethanol is toxic and smells horribly; work in fume-hood, wear gloves).
2. CHISAM: 24:1 (v/v) chloroform/ isoamyl alcohol mixture (volatile; work in fume-hood, wear gloves).
3. Phenol equilibrated to neutral pH can be purchased from Sigma. Store phenol in amber-coloured bottles under a layer of extraction buffer. Phenol is highly toxic and appropriate health and safety regulations such as wearing gloves, goggles, lab coat and working in fume-hood should be followed while handling.
4. RNase solution - Dissolve Ribonuclease I-A (Sigma) 20 mg ml⁻¹ in TN buffer (10 mM Tris and 10 mM NaCl, pH 7.5), boil for 15 min to make it DNase free and allow to cool at room temperature for 1 h to permit renaturation of RNase. Store RNase solution at -20° C.
5. TE 8: 10 mM Tris and 1 mM EDTA, pH 8.0.
6. 20 x SSC: 3 M NaCl and 0.3 M sodium citrate, pH 7.0
7. Ethidium bromide: Use a 10 mg ml⁻¹ stock (ethidium bromide is a carcinogen; purchase of stock solutions is recommended; adequate care should be taken in handling and disposal).
8. Avoid excess agar with the inoculum while setting up liquid culture for DNA extraction.
9. Break the frozen mycelium into as small pieces as possible to increase the efficiency of freeze drying. Do not allow the frozen mycelium to thaw.
10. While taking powered mycelium for DNA extraction, leave out agar pieces, if any.
11. Some freeze dried mycelial samples do not grind well; using liquid nitrogen (take adequate precautions while handling) or sterile sand while grinding helps the process.
12. Complete mixing of the mycelial powder and extraction buffer into a homogenate increases the efficiency of DNA extraction.
13. Using pipettoman for aliquoting/adding chloroform damages the rubber O-rings; preferable to use a pasteur pipette.
14. After adding chloroform or chloroform and isoamyl alcohol (CHISAM) to the homogenate, wait a couple of minutes before closing the cap and mixing; else the caps pop-off. With hot chloroform the tubes can even explode; loosen the cap to release the pressure, in between mixing.
15. While taking the aqueous layer, leave a clear layer of the inter/solvent phase; purity of the extracted DNA depends on avoiding these contaminants.

16. Use cut pipette tips or sterile transfer pipettes, while removing the aqueous phase, to avoid shearing of DNA.
17. Do not over dry DNA pellets; leads to problems in dissolving the DNA. Leaving the tubes on a rotawheel for several hours at room temperature or warming at 37-55° C for 15 - 30 min is useful; but do not pipette the solution up and down as it shears the DNA. Undissolved residues, if any, can be removed by brief centrifugation and collect the supernatant into a clean tube.
18. If the extracted DNA is not sufficiently pure (i.e. not amenable to restriction enzyme digestion and/or PCR amplification), repeat phenol : chloroform extraction and ethanol precipitation. Alternatively, Qiagen columns (QIAquick) can be used to purify 5-10 µg DNA, following the manufacturer's instructions.

2.4. Agarose gel electrophoresis and DNA quantification

DNA samples were electrophoresed on 0.8% agarose gels prepared with 1 X Tris Borate-EDTA (TBE) pH 8.3 (Tris, 108 g; Boric acid, 55 g; EDTA, 7.44 g) and using 1 X TBE as the running buffer. All agarose gels were processed in a constant electric field, in a horizontal configuration. The gel contained ethidium bromide, the DNA intercalating fluorescent dye at a concentration of 0.5 µg ml⁻¹. The stock solution for ethidium bromide was purchased ready prepared (e.g. Life Tech, UK). For loading agarose gels, 4 X loading buffer (100 ml contained 40 ml 10 X TBE, 60 ml of 50% glycerol and 250 mg bromophenol blue) was added at an appropriate volume to the samples.

The DNA samples were viewed using an UV transilluminator (254 nm) and photographic records made when necessary. The concentration of DNA in a sample was estimated by ethidium bromide fluorescence comparing the intensity of known quantities of marker DNA and/or by spectrophotometry. Absorbance was recorded at 260 nm and 1 OD corresponds to 50 µg ml⁻¹ of double-stranded DNA (Sambrook *et al.*, 1989). DNA extracted as above is usually high quality and is suitable for a range of molecular analysis such as fingerprinting and RFLPs by hybridisation, RAPDs, AFLPs and SSRs by PCR as well as nucleotide sequencing. We have also adapted two other DNA extraction methods (incorporating DNA clean-up columns from Qiagen) which are simple and quick and DNA preparations from these can also be used for various applications mentioned above.

3. Molecular typing

3.1. DNA restriction and Southern blotting

Genomic DNA (2-3 µg) was digested with 20-25 U of *Eco* RI (Boehringer Mannheim) at 37°C overnight. Digested DNA (usually 20 µl reactions) was fractionated by electrophoresis in 0.8% agarose gels for 24 h at 60 V with *Hind* III-digested DNA (Boehringer Mannheim) as a molecular size marker. On completion of electrophoresis, mobility (cm) of the molecular size marker was recorded using an UV transilluminator. The bottom left-hand corner of the gel was cut to record its orientation.

DNA was denatured by soaking the gel for 45 min in denaturing buffer containing 1.5 M NaCl and 0.5 N NaOH with constant gentle agitation (up to 1 l denaturing solution/gel) at room temperature. The gel was rinsed twice with de-ionised water and then soaked for 30 min in a neutralization solution containing 1 M Tris and 1.5 M NaCl (pH 7.4) at room temperature with constant gentle agitation. The neutralization step was repeated for 15 min with fresh neutralization solution. When removed from this solution, the gel was inverted and placed on a platform covered with 3 layers of Whatman No. 1 filter paper which acted as a wick by dipping into transfer buffer (10 X SSC) held in a tray. Nylon membrane

(Hybond N, Amersham) was cut to the size of the gel, rinsed with sterile distilled water, soaked in 10 X SSC for 5 min and placed over the gel. Air bubbles between the gel and the membrane were expelled and the edges of the gel were covered with strips of parafilm to ensure buffer flows through the gel only. Four layers of Whatman No. 3 filter paper, cut to the size of the nylon membrane, were soaked in 2 X SSC and placed on top of the membrane. To draw the transfer buffer through the gel 2-ply tissue paper folded to the size of the gel was stacked to approximately 6 cm on top of the filter paper and parafilm at the edge of the gel. A weight of approximately 500 g was placed on the tissue pad (Sambrook *et al.*, 1989). Capillary transfer proceeded for 16-20 h, at the end of which the membrane was removed and the orientation of the gel and positions of the slots were marked. The membrane was then air-dried on benchkote (Sambrook *et al.*, 1989) for about 20 min. DNA fragments, which transferred from the gel, were immobilized on the membrane by baking for 90 min in an oven at 80°C (Gallenkamp) and also by UV cross-linking at 35 mw cm⁻² on an Appligene transilluminator. The membrane was then wrapped loosely in aluminium foil and stored at room temperature until use. Use of N⁺ membranes, alkali blotting (Sambrook *et al.*, 1989) and maintaining the membrane wet throughout the process further improves the resolution level of the fingerprints.

3.2. Preparation of MGR586 probe and hybridization

MGR586 probe (Levy *et al.*, 1991) was labelled by the random priming method with 50 μ Ci Redivue (³²P) dCTP (Amersham) using the rediprime DNA labelling system (Amersham) according to the manufacturer's instructions. Removal of the unincorporated ³²P improved the fingerprint resolution and background.

Pre-hybridization solution (6X SSC, 5 X Denhardt's solution, 0.5% SDS and 100 μ gml⁻¹ denatured herring sperm DNA) was prepared by dissolving one hybridization buffer tablet (Amersham) in 10 ml sterile distilled water. Pre-hybridization of membranes bearing restriction fragments was carried out in 10 ml pre-hybridization solution per membrane in a cylinder in an hybridization oven (Hybaid) at 65°C for at least 3 h. The labelled probe was denatured (boiled for 5 min and flash cooled on ice) and added to the pre-hybridization solution. Hybridization was performed overnight at 65°C. Post-hybridization washes consisted of three washes of 30 min each in 2 X SSC and 0.1% SDS and three washes of 30 min each in 0.1 X SSC and 0.1% SDS.

3.3. Autoradiography and analysis of fingerprints

After the final post-hybridization wash, the membrane was placed on backing paper (non-absorbent side of Whatman benchkote) and wrapped with cling film. It was then exposed to X-ray film (Genetic Research Instrumentation) using intensifying screens at -70°C. Autoradiographs were developed after 12–48 h. Similarity between MGR fingerprints among the *M. grisea* isolates was calculated using the formula: $S_{xy} = 2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of shared fragments, and n_x and n_y are the number of fragments in isolates x and y. Further, presence and absence of all hybridization bands in the 0.8-20 kb size range was scored and a binary matrix computed. Cluster analysis by the group average method, UPGMA was performed to generate dendrograms. Based on the fingerprint patterns, similarity values (approx. above 75%) and grouping on the dendrograms, genetic groups (lineages) were identified (Levy *et al.*, 1991).

3.4. RAPD- PCR and data analysis

A range of primers e.g. A1 (5' CAGGCCCTTC 3'), A13 (5' CAGCACCCAC 3') and B10 (5' CTGCTGGGAC 3') were used for RAPD PCR, as it is essential to build a composite picture of RAPD profiles for each isolate. These primers can be ordered from commercial companies such as Sigma-Genosys or Operon. Reaction mixtures contained 5 μ l of genomic DNA (10 - 20 ng), 5 μ l of 10 X Dynazyme buffer (Flowgen), 0.5 μ l of 10 mM dNTP mix, 2.5 μ l of primer (15 ng μ l⁻¹ stock), 0.5 μ l (1 U) of Dynazyme DNA polymerase and 36.5 μ l SDW to give a total volume of 50 μ l. Alternatively red-Taq ready mix (e.g. Sigma) that incorporates all of the PCR components can be used to achieve better consistency and to avoid cross-contamination etc. Amplification cycles were as follows: initial denaturation of 2 min at 95°C; 35 cycles of 1 min at 94°C, 30 s at 40°C and 2 min at 72°C followed by a final extension of 5 min at 72°C. After amplification the products (20 μ l) were separated in 20 x 20 cm 1.4% agarose gels for about 2 h at 100 V. Molecular size marker Type VI (Boeringher Mannheim) was loaded appropriately with every set of samples.

Each amplified fragment was treated as a separate character. DNA fragments of the same size were assumed to represent the same genetic locus and scored as a common fragment. For cluster analysis, a binary matrix with presence (1) and absence (0) of each fragment was constructed for all the isolates for each primer was generated and data from all ten primers was combined. Pairwise similarity coefficients were computed and the dendrogram was generated by cluster analysis following the group average method (UPGMA) using the Genstat programme (Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK).

3.5. Other markers and adaptations

PCR based markers such as AFLPs, SSR-PCR and REP-PCR as well as sequence data from ribosomal RNA gene block spacer regions are also useful for characterisation of *M. grisea* isolates and would offer varying levels of resolution. We have screened a wide range of AFLP and SSR-PCR primers against *M. grisea* isolates and some primer combinations are more informative to assess genetic diversity. Further, methodologies used for preparation of mycelial material, DNA extraction, electrophoresis and MGR586 hybridisation/fingerprinting could be adapted (Muthumeenakshi *et al.*, unpublished) to suit the requirements of laboratories in developing countries.

4. Pathotyping and resistance screening

4.1. Inoculum preparation

For spray inoculation of rice seedlings in the pathotyping experiments, aqueous conidial suspensions (10^5 conidia ml⁻¹) were prepared from 2-3 week old cultures of *M. grisea*. The conidial suspensions contained 0.1% (w/v) gelatin to facilitate adhesion of the conidia to the leaves. Conidia from each mono-conidial *M. grisea* isolate grown on Oat Meal Agar plates were collected by washing with sterile distilled water containing gelatin and the suspensions were filtered through Miracloth to remove mycelial fragments. Conidia were counted using a haemocytometer and, where necessary, the suspensions were adjusted to 10^5 conidia ml⁻¹.

4.2. Inoculation, disease scoring and pathotype designation

Pathotyping of *M. grisea* isolates was undertaken using the international rice differential set : A, Raminad Str. 3; B, Zenith; C, NP-125; D, Usen; E, Dular; F, Kanto 51; G, Sha-tiao-tiao and H, Caloro including B40 and CO39 which were used as standard checks (seeds obtained from WARDA). The eight international rice differentials selected from different rice growing regions by a co-ordinated effort provides a common set of cultivars for comparing the virulence spectrum (uniform pathotype designations) of *M. grisea* populations in different parts of the world.

Ten seedlings of each international rice differential variety and checks were grown to three-to-four leaf stage (18-21 days after planting) under greenhouse conditions in a plastic tray using John Innes No. 2 compost and were replicated three times. The seedlings were spray inoculated (using a badger airbrush) with 30 ml aqueous conidial suspension (i.e. 10 ml per tray) held inside polythene bags. Controls included seedlings sprayed only with the gelatin solution. Following spray inoculation, the polythene bags were sealed-up for two days to maintain high humidity. The polythene bags were removed after 48 h and the trays were maintained in growth chambers with a 12h photoperiod and set at 25°C.

Host responses were scored visually for lesion type, 7 days after inoculation. Disease reaction was based on a 0-5 scale (Valent *et al.*, 1991). A score of 0 and 1 being recorded as an incompatible (R) reaction. Lesion type 2 or greater or if the majority of seedlings exhibited fully sporulating lesions was recorded as a compatible (S) reaction. Pathotype designation was based on the nomenclature of Ling and Ou (1969), who developed the system for differentiating *M. grisea* by their reactions (virulence spectrum) on the international set of rice differentials.

The international pathotypes/races are designated as 'I' (for international), followed by A, B, C, D, E, F, G, or H - each representing one of the differentials, according to the first susceptible variety when the eight varieties are examined in the same sequence and a specific number to indicate a particular virulence pattern. For example an *M. grisea* isolate that shows S, R, R, R, R, R, R, R reactions on A, B, C, D, E, F, G, H, respectively is designated as IA-128. Based on the eight differential varieties, with each variety showing either susceptible (S) or resistant (R) reaction, a maximum of 256 races (2^8) of pathotypes/races can be differentiated. The 256 pathotypes/races consist of 128, 64, 32, 16, 8, 4, 2 and 1 races in the IA, IB, IC, ID, IE, IF, IG and IH groups, respectively. Pathotype II-1 was added to accommodate *M. grisea* isolates incompatible to all eight differentials (Ling and Ou, 1969).

4.3. Resistance screening

Various rice cultivars provided by WARDA and NARS in Ghana were screened against some of the characterised blast isolates from different lineages following the seedling model used for pathotyping, with five replicates and a randomised block design. Mean disease reactions, subjected to REML variance components analysis, are expressed on the basis of the 0-5 scale of Valent *et al.* (1991).

5. References

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Dr. J. Chipili has recently re-joined the Zambian Ministry of Agriculture.

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Appendix II
Poster

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Reference List

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