Chemometric investigations of the multidrug resistance in strains of the phytopathogenic fungus *Penicillium digitatum*

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THE OBJECTIVES OF THIS WORK

1) *P. digitatum* (green mold), like other *Penicillium* species, contaminates fruits, nuts, vegetables, and even cereals causing serious losses in agriculture worldwide, and causes various respiratory problems, allergic diseases and other non-inflammatory symptoms that may be extremely dangerous to immunocompromised persons. To get more insight into the multidrug resistance (MDR) mechanisms of this microbe, particularly CYP51- (cytochrome 51 – ergosterol biosynthesis) and efflux pump PMR1-mediated resistance to demethylation inhibitors (DMIs), and to propose ways how to use new findings, are the main objectives of this work;

2) To present multivariate use of fungal morphology data and novel types of relationships: QGSAR (Quantitative Genome/Structure-Activity Relationship) and QMGR (Quantitative Morphology-Genome Relationship).

1 - GENERAL: MOLD AND ANTIFUNGALS

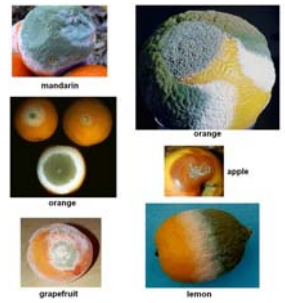

Figure 1. The most frequent targets of *P. digitatum* are fruits, especially citric fruits.

Figure 2. *P. digitatum* under microscope. The brush-like heads (Lat. *penicillus* = brush) have finger-like shape (Lat. *digitatum* = fingered) at their spore-producing ends.

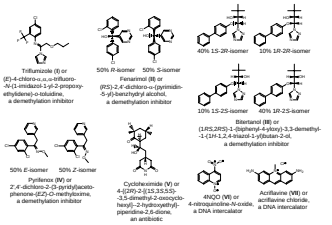

Figure 3. Molecular structure & isomeric composition of commercial agents used as azole-based fungicides (demethylation inhibitors, DMIs: I-IV), an antibiotic (V) and mutagens (VI, VII).

Resistance of *P. digitatum* strains against these compounds is studied in this work. The activity data are MIC (Minimal Inhibitory Concentration) and EC_{50} (Effective Concentration for 50% radial growth inhibition) from literature: R. Nakaune et al, Microbiol. 64 (1998) 3983; H. Hamamoto *et al*., *Appl. Env. Microbiol*. 66 (2000) 3421; H. Hamamoto *et al*., *Pestic. Biochem. Physiol*. 70 (2001) 19; H. Hamamoto *et al*., *Pest Manag. Sci*. 57 (2001) 839; R. Nakaune *et al*., *Mol. Genet. Genom.* 267 (2002) 179. Chemometric methods used in this work on autoscaled data are: Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA), and Partial Least Squares (PLS) regression.

3 - CHEMOMETRIC EXPLORATION OF FUNGAL GROWTH MORPHOLOGY

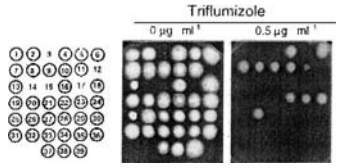

Figure 5. 35 of 39 *P. digitatum* colonies (1 strain=1 colony). Left: colonies arrangement. Middle: free growth. Right: inhibited growth. From: Hamamoto *et al*., *Pest. Manag. Sci.* 57 (2001) 839-843.

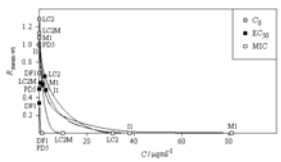

Figure 6. Examples of dose-response curves for various *P. digitatum* strains with different DMI resistance levels. Legend:
C_0 – no inhibition; EC_{50} – 50% radial growth inhibition; MIC – 100% inhibition.

Data set of 8 morphological parameters: matrix 35x8, where:
rows → *P. digitatum* strains,
columns → the parameters taking account free and inhibited growths (Figure 5) such as radii, circumferences and surface areas of the colonies.

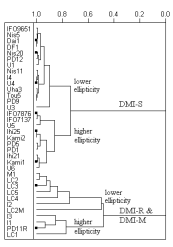

Figure 7. HCA analysis with complete linkage. Two clusters distinguish sensitive (DMI-S) from resistant (DMI-R&DMI-M) strains. Two sub-clusters in each cluster show more round and more elliptical colonies.

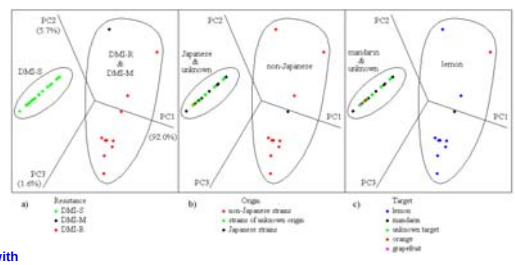

Figure 8. PCA scores plot showing two clusters from the HCA (Figure 7). Reasonably well are distinguished: a) resistance: sensitive (DMI-S) from resistant (DMI-R&DMI-M) strains; b) origin: non-Japanese from Japanese&unknown strains; c) target fruits: lemon molds from mandarin&unknown molds.

6 - CONCLUSIONS

1) Chemometric approaches to fungal radial growth data (EC_{50} and morphological data) are novel and promising procedures to identify and characterize *P. digitatum* strains in terms of their resistance to demethylation inhibitors, origin and target fruits.
2) PLS regression models show direct quantitative relationships between genome structure related to the fungal resistance and fungal growth data. This means that *P. digitatum* strains can be well characterized knowing only one of the two types of data.
3) Molecular structures of toxicants also affect resistance of the fungal strains, as is visible from the QGSAR model.

ACKNOWLEDGEMENT FAPESP

2 – CHEMOMETRIC EXPLORATON OF EC_{50} ACTIVITIES

Data set of pEC_{50} values: matrix 7x7, where:
rows → toxicants I-VII,
columns → *P. digitatum* strains.
Definitions:
$pEC_{50} = -\log(EC_{50}/\text{mol dm}^{-3})$

Data set of $pECr_{50}$ values: matrix 7x6, where:
rows → toxicants I-VII,
columns → *P. digitatum* strains.
Definitions:
$pECr_{50} = pEC_{50}/pEC_{50}(PD5)$
PD5 – the standard DMI-S strain

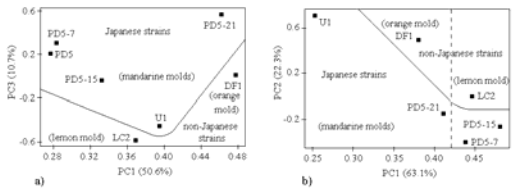

Figure 4. Loadings plots for the pEC_{50} data (a) and $pECr_{50}$ data (b), compared in terms of discriminatory power for fungal strains (resistance level, origin and target fruits). $pECr_{50}$ data are better than pEC_{50} data for discrimination purposes.

Demethylation inhibitor resistance:
-sensitive strains: DMI-S
-resistant strains : DMI-R
-moderately resistance strains: DMI-M

4 - QUANTITATIVE MORPHOLOGY-GENOME RELATIONSHIP (QMGR)

Data set of 8 morphological descriptors: matrix 35x8, from the above section (Figures 7 and 8). 8 samples excluded in external validation (solid squares in Figure 7). The genome variable PCR is modeled from morphology descriptors by means of PLS regression.

PLS statistics:
2 PCs (97.6%)
Leave-1-out CV: SEV=0.028, Q^2=0.991
Prediction: SEP=0.023, R^2=0.985
Relative errors: mean 4.1%, max. 21.6%
No. samples with relative error >10%: 1

External validation:
2PCs
SEV=0.030, SEP=0.025,
Q^2=0.982, R^2=0.990.

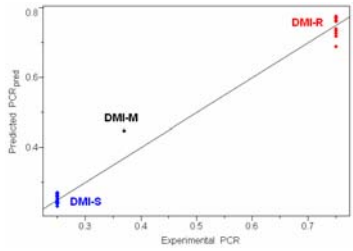

Figure 9. Experimental against predicted PCR values. DMI resistance levels are colored differently.

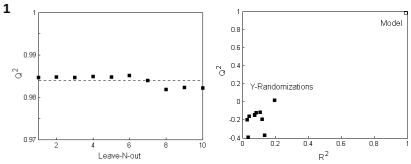

Figure 10. Validations of the PLS model. Left – Leave-*N*-out: *N* varies from 1 to 10, whilst the mean value of Q^2 stays high. Right – Y-Randomization: no chance correlation is observed.

5 - QUANTITATIVE GENOME/STRUCTURE-ACTIVITY RELATIONSHIP (QSGAR)

Data set of 3 genome and 5 mixed genome/molecular descriptors: matrix 86x8, where:
-rows → strain-toxicant-experiment combinations
-columns → the descriptors

Initial set of descriptors consisted of 8 genome descriptors related to fungal resistance *via* production of CYP51 (a cytochrome) and PMR1 (an efflux pump) proteins. Products of these descriptors with 2 molecular descriptors (No. π systems and No. of single bonds between these systems) gave 12 new descriptors. Variable selection resulted in 8 from 20 descriptors for the final PLS model to predict pEC_{50} values.

PLS statistics:
5 PCs (96.8%)
Leave-1-out CV: SEV=0.286,
Q^2=0.851
Prediction: SEP=0.271, R^2=0.874
Relative errors:
mean 3.3%, max. 13.3%
No. samples with >10%: 2

Eternal validation: 5PCs,
SEV=0.305, SEP=0.279, Q^2=0.841,
R^2=0.881.

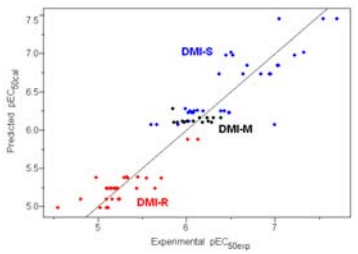

Figure 11. Experimental against predicted pEC_{50} values. DMI resistance levels are colored differently.

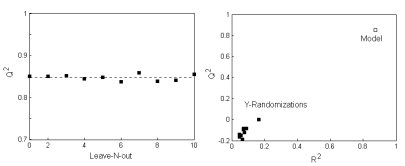

Figure 12. Validations of the PLS model. Left – Leave-*N*-out: *N* varies from 1 to 10, whilst the mean value of Q^2 stays high. Right – Y-Randomization: no chance correlation is observed.