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Chemometric analysis of the multidrug resistance in strains of *Penicillium digitatum*[1]

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Multidrug resistance activities pEC_{r50} of diverse strains of pathogenic fungus *Penicillium digitatum* against seven toxicants were studied by Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). Fungal growth data (radii, circumferences, surface areas of fungal colonies, radius differences and ratios) in absence and presence of toxicants were used to derive eight new descriptors for 35 fungal strains. This data set was studied by PCA and HCA, and was correlated with the genome descriptor PCR for expression of gene *CYP*51 by Partial Least Squares (PLS) regression. Both analyses of pEC_{r50} data and of fungal growth data have identified baseline resistance character, origin and target fruits of the fungal strains. In addition, the analysis of fungal growth data shows that fungal growth morphology is multivariate by nature, meaning that experimental data can be explored more intensely than in usual practice. Fungal growth is directly related to the production of enzyme $P450_{14DM}$ as the main resistance mechanism of *P. digitatum* against demethylation inhibitors. This is visible from a parsimonious PLS model (two principal components, $Q^2 = 0.985$, $R^2 = 0.991$, SEV = 0.028), validated with eight strains in the external validation set ($Q^2_{ev} = 0.982$, $R^2_{ev} = 0.990$, $SEC_{ev} = 0.025$). Chemometric methods in exploring bioassay data are promising approaches to obtain useful information on fungal resistance and to apply these findings in practice.

Keywords: chemometrics; demethylation inhibitors; multidrug resistance; exploratory analysis; partial least squares; green mould

1. Introduction

A group of Japanese researchers has made a systematic characterization and fungicide sensitivity bioassays of several strains of *Penicillium digitatum* [1–6]. This phytopathogenic fungus is known as the green mould or green rot that causes one of the most important postharvest diseases of citrus fruits [7, 8] and is also pathogen to apples [9] at broad temperature range (from 4°C to 30°C [10]). The resistance of this fungus to the first generation fungicides (aromatic hydrocarbons) has been known since 1960s [11, 12]. Several reports about *P. digitatum* resistance to newer azole-type fungicides, which

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belong to Class 1 inhibitors or demethylation inhibitors (DMIs) class of sterol biosynthesis inhibitors [13, 14], have been published in 1970s [15, 16], 1980s [11], and even recently [1–6, 17–20]. The Japanese researchers have reported two main mechanisms of DMI resistance (DMIR) in *P. digitatum* [1–6]: a) CYP51 mechanism which is based on the inability of DMIs to inhibit the ergosterol biosynthesis enzyme known as CYP51 or $P450_{14DM}$, one of the most frequent 14α-methyl sterol demethylases which is encoded by the gene *CYP*51 or *ERG*11 [21–23], and b) multidrug resistance (MDR) mechanism mediated by efflux pumps from the ATP-binding cassette (ABC) family of transporters [24–26]. In a recent work [27], we have shown that various biological activity data for *P. digitatum* strains can aid in the identification of pesticide characteristics and are quantitatively related to the fungal genome responsible for expression of the CYP51 and efflux pump defence mechanisms. In this work, multidrug resistance activities related to seven toxicants I–VII (Figure 1) and morphological characteristics of fungal cultures [1–5], were explored by means of chemometric methods. I–IV are DMIs from different azole subclasses (I: an imidazole, II: a pyrimidine, III: a triazole, and IV: a pyridine) to which *P. digitatum* strains have already developed resistance [17], and V–VII are non-DMIs. Hierarchical Cluster Analysis (HCA), Principal Component Analysis (PCA), and Partial Least Squares (PLS) regression [28–31] were used to analyse the studied data sets. This work proposes new chemometric approaches for better exploration of experimental data from DMI sensitivity tests. Reported findings based on fungal growth data can be useful for practical purposes, as for example, to classify fungal strains with respect to their baseline resistance and other characteristics including genome structure related to the CYP51 resistance mechanism.

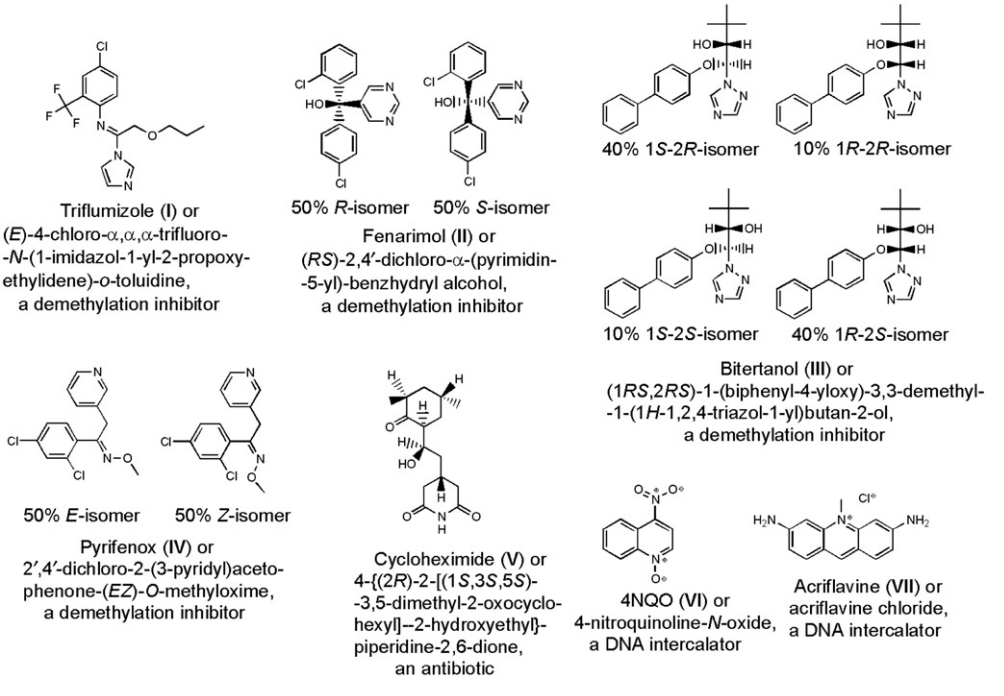

Figure 1. Structures, isomeric compositions, trivial and IUPAC names of toxicants I–VII.

2. Methodology

2.1 *Exploratory analysis of the data set A*

Biological activities of seven *P. digitatum* strains against I–VII are defined in Table 1. The activities in standardized experiments were effective mass concentrations EC_{50} [1–5]. EC_{50} values for six strains and the standard strain PD5 were averaged whenever possible, and then transformed into pEC_{50} values. Fungal resistance activities relative to the

Table 1. Definition of all descriptors used in analyses related to multidrug resistance and germination of *Penicillium digitatum* strains.

Descriptor	*Definition*
Biological activities of fungal strains	
pEC_{50}	$pEC_{50} = -\log(EC_{50}/\mathrm{mol\,dm^{-3}})$, where EC_{50} is Effective Concentration for 50% inhibition[a]
$pECr_{50}$	$pECr_{50} = pEC_{50}(PD5) - pEC_{50}$, where PD5 is a *P. digitatum* strain used as a standard
Morphological descriptors of fungal cultures[b]	
1: $R_{max\text{-}ni}$	Maximum diameter of a culture in absence of toxicants
2: $R_{min\text{-}ni}$	Minimum diameter of a culture in absence of toxicants
3: $R_{mean\text{-}ni}$	Mean diameter of a culture in absence of toxicants, $R_{mean\text{-}ni} = (R_{max\text{-}ni} + R_{min\text{-}ni})/2$
4: C_{ni}	Circumference of a culture in absence of toxicants
5: S_{ni}	Projected surface area of a culture in absence of toxicants
6: $R_{est\text{-}ni}$	Diameter of an ideally round culture in absence of toxicants, $R_{est\text{-}ni} = (S_{ni}/\pi)^{1/2}$
7: D_{ni}	Diameters difference of a culture in absence of toxicants, $D_{ni} = R_{max\text{-}ni} - R_{min\text{-}ni}$
8: T_{ni}	Diameters ratio of a culture in absence of toxicants, $T_{ni} = (R_{max\text{-}ni} + 1)/(R_{min\text{-}ni} + 1)$
9: $R_{max\text{-}wi}$	Maximum diameter of a culture in presence of triflumizole
10: $R_{min\text{-}wi}$	Minimum diameter of a culture in presence of triflumizole
11: $R_{mean\text{-}wi}$	Mean diameter of a culture in presence of triflumizole, $R_{mean\text{-}wi} = (R_{max\text{-}wi} + R_{min\text{-}wi})/2$
12: C_{wi}	Circumference of a culture in presence of triflumizole
13: S_{wi}	Projected surface area of a culture in presence of triflumizole
14: $R_{est\text{-}wi}$	Diameter of an ideally round culture in presence of triflumizole, $R_{est\text{-}wi} = (S_{wi}/\pi)^{1/2}$
15: D_{wi}	Diameters difference of a culture in presence of triflumizole, $D_{wi} = R_{max\text{-}wi} - R_{min\text{-}wi}$
16: T_{wi}	Diameters ratio of a culture in presence of triflumizole, $T_{wi} = (R_{max\text{-}wi} + 1)/(R_{min\text{-}wi} + 1)$
17: R_{maxR}	Maximum diameters ratio $R_{maxR} = R_{max\text{-}wi}/R_{max\text{-}ni}$
18: R_{minR}	Minimum diameters ratio $R_{minR} = R_{min\text{-}wi}/R_{min\text{-}ni}$
19: R_{meanR}	Mean diameters ratio $R_{meanR} = R_{mean\text{-}wi}/R_{mean\text{-}ni}$
20: C_R	Circumferences ratio $C_R = C_{wi}/C_{ni}$
21: S_R	Surface areas ratio $S_R = S_{wi}/S_{ni}$
22: R_{estR}	Ideal diameters ratio $R_{estR} = R_{est\text{-}wi}/R_{est\text{-}ni}$
23: D_R	Diameter differences ratio $D_R = D_{wi}/D_{ni}$
24: T_R	Ratio of diameter ratios $T_R = T_{wi}/T_{ni}$
Genome descriptors of fungal strains	
PCR	Length of the promoter fragment in the *PdCYP51* gene (the gene that encodes the target enzyme $P450_{14DM}$ of the DMIs in the strain PD5), corresponding to one or more copies of the *CYP51* transcriptional enhancer [3]

[a]EC_{50} – Effective Concentration is the concentration inhibiting radial growth of a fungal culture by 50%.

[b]Descriptors were generated for 35 strains which germinated in absence of toxicants [3]. Numbering of these descriptors was introduced to distinguish three types of descriptors: ni-type descriptors (“no inhibitor” descriptors 1–8), wi-type descriptors (“with inhibitor” descriptors 9–16) and ratios of the wi-type descriptors with analogue ni-type descriptors (17–24).

DMI-sensitive (DMI-S) strain PD5 were defined as pEC_{r50}, making the data set A (matrix (6×7)), where each row corresponds to one strain and the columns are related to toxicants I–VII. PCA and HCA with complete linkage method were performed with this data set in order to explore the activity data for classification of fungal strains with respect to their baseline resistance level (DMI-S: DMI-sensitive, DMI-R: resistant, or DMI-M: moderately resistant), origin and target fruits. In all chemometric analyses in this work, data were autoscaled prior to the analyses. All these operations were carried out using programs Matlab 6.1 [32] and Pirouette 3.11 [33].

2.2 *Exploratory analysis of the data set B and Quantitative Morphology-Genome Relationship (QMGR)*

Hamamoto et al. [3] have performed DMI sensitivity assays for 39 *P. digitatum* strains and published fungal radial growth results in absence of toxicants and in presence of triflumizole. Thirty five from 39 strains were successfully germinated in their experiments and thus could be studied in this work. An empirical graphics method with internal standards [34–37] was applied to published photographs of the fungal colonies [3], in order to generate fungal morphological (shape-size) descriptors. The 16 obtained descriptors from 35 fungal cultures are defined in Table 1 (numbered with 1–16). The values of these descriptors, after being obtained from graphical measurements, were normalized with respective values for the PD5 colony in absence of toxicants. This normalization has been performed because it was not possible to know the natural size of the colonies based on photographs without a scale. Data set B comprises eight descriptors (descriptors 17–24, forming a data matrix (35×8)), calculated as ratios of the descriptors for fungal growth in presence of triflumizole (wi-type descriptors or ''with inhibitor'' descriptors 9–16) and the analogous descriptors for fungal growth in absence of toxicants (ni-type descriptors or ''no inhibitor'' descriptors 1–8). A genome variable PCR (Table 1), originated from polymeraze chain reaction assays [3], was considered as the dependent variable with which we expected that descriptors 17–24 would be correlated. PCR is the length of the promoter region of the *PdCYP51* gene originated from the PD5 strain: 0.25 kb for DMI-S and 0.75 kb for DMI-R strains. The promoter region corresponds to one or five units of a 126 bp transcriptional enhancer in DMI-S and DMI-R strains, respectively. The length of the promoter region in the DMI-M strain LC2M is 0.37 kb, what corresponds to two units of the 126 bp transcriptional enhancer in this strain. The data set B was analysed by means of PCA and HCA with complete linkage method in order to recognize the strain differentiations with respect to strain baseline resistance character, origin and target fruits. The descriptor PCR was modelled from the autoscaled data set B by means of PLS regression. The PLS model was further validated by leave-*N*-out crossvalidation, where *N* varied from 1 to 10. The robustness of the model was tested by performing ten Y-randomizations according to Wold and Eriksson [38]. The original data was randomized prior to leave-*N*-out crossvalidation and Y-randomization. The final validation procedure consisted of an external validation, in which the original data set for 35 strains was divided into training and external validation sets with 27 and 8 strains, respectively. Selection of strains for the external validation set was carried out with the aid of HCA: a strain from each cluster was selected for external validation arbitrarily.

3. Results and discussion

3.1 *Exploratory analysis of the data set A*

Toxicants I–VII belong to three classes of compounds which interfere into different biochemical processes of *P. digitatum*, and thus undergo to distinct fungal resistance mechanisms [1–4]. I–IV are demethylation inhibitors (DMIs), frequently used as fungicides against the cytochrome enzyme P450 sterol 14α-demethylase ($P450_{14DM}$) [14, 17]. V is an antibiotic that interferes into microbial protein synthesis [39], and VI and VII are effective nucleic acid intercalators and modifiers [40, 41]. Seven strains have different baseline resistance characters with respect to I-VII. Field isolates PD5, DF1 and U1 are DMI-S strains. Another field isolate, LC2, is a DMI-R strain. PD5-21 is a mutant similar to PD5, and is also a DMI-S strain. PD5-7 and PD5-15 are mutants which act similarly to LC2 with respect to DMIs and behave similarly to PD5 with respect to non-DMIs. Therefore, these two mutants are considered demethylation inhibitor-moderately resistant (DMI-M) strains. All three mutants are PD5 transformants with the original gene *CYP*51 from the PD5 strain [2]. PD5-7, PD5-15 and PD5-21 were originally named PD5(*PdCYP*51-L)-7, PD5(*PdCYP*51-L)-15 and PD5(*PdCYP*51-P)-21, respectively, by Hamamoto *et al*. [2].

Bacterial [42] and fungal [27] MDR activities have shown to be able, when treated by PCA and HCA, to distinguish microbial strains in accordance with their respective higher and lower resistance. Similarly, cluster analysis methods and PCA are used today in taxonomy of living beings, taking into account functional, morphological, structural, phylogenetic, and physiological, biochemical and other characterizations of species and strains [43–46]. These facts justify the analysis of fungal resistance activities by chemometric methods in this work.

Exploratory analysis of biological activities $pECr_{50}$ for six strains relative to the DMI-S strain PD5 (data set A, Table 2) provide interesting information about strains classification (Figure 2). The first two principal components (PCs) contain 89.1% of the total variance. The strains are differentiated according to their baseline DMIR character along PC1: sensitive strains U1, DF1 and PD5-21 are placed at negative values of PC1, medium resistant strains PD5-17 and PD5-7 are at low positive PC1, whilst the resistant

Table 2. Data set A: multidrug resistance activities[a] $pECr_{50}$ of six *Penicillium digitatum* strains relative to the strain PD5.

Toxicant	*DF1*[b]	*U1*[b]	*LC2*[b]	*PD5-21*	*PD5-7*	*PD5-15*
I	−0.097	−0.699	1.681	0.477	1.505	1.505
II	0.329	0.286	1.125	0.222	0.630	0.802
III	0.243	0.287	1.243	0.340	0.875	1.266
IV	0.727	0.796	2.544	0.368	1.097	1.412
V	−0.398	−0.046	0.949	−1.208	−0.097	−0.155
VI	0.085	−0.188	0.485	−0.050	−0.024	−0.106
VII	0.222	−0.176	0.542	−0.041	−0.473	0.067

[a]DMI resistance activity data referring to five *P. digitatum* strains, based on experimental values of EC_{50} [1, 2, 4] and expressed relative to the values of EC_{50} for the SMI-S strain PD5: DMI-S strains DF1, U1, and PD5-21; DMI-R strain LC2; DMI-M strains PD5-7 and PD5-15.
[b]Averaged experimental values of EC_{50} for these strains and the strain PD5 were used, taking into account the number of repetitions [1, 2, 4].

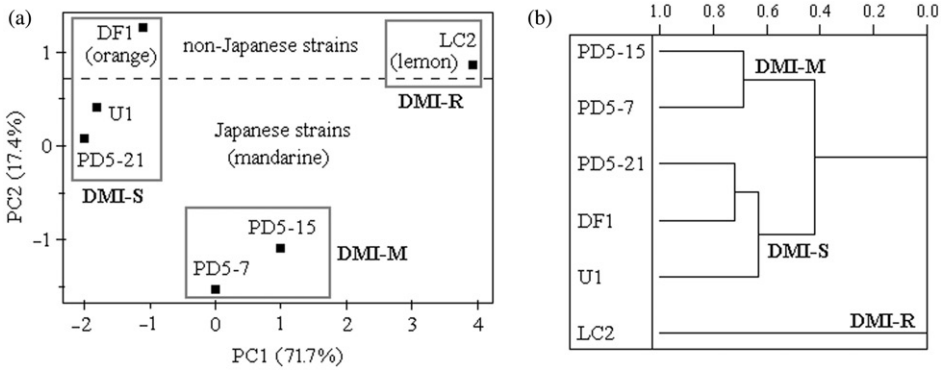

Figure 2. Exploratory analysis of the data set A. (a) PCA loadings plots showing the discrimination of *Penicillium digitatum* strains with respect to baseline resistance (DMI-S, DMI-M and DMI-R), origin (Japanese or non-Japanese) and target citric fruits (orange, mandarin and lemon mould). (b) HCA dendrogram with *Penicillium digitatum* strains differentiated according to their resistance.

strain LC2 is at high positive value of PC1. The HCA dendrogram confirms this strains differentiation even clearer. PC2 distinguishes four Japanese strains from two non-Japanese strains DF1 and LC2 (dashed line in Figure 2b).This principal component also distinguishes the strains with respect to their target citrus fruits: mandarin moulds from non-mandarin (lemon and orange) moulds. It is interesting to note that both PCA and HCA exhibit relative closeness of orange and mandarin moulds whilst the lemon mould is isolated. This fact agrees well with trends that have been observed in phylogenetic studies of citrus fruits (genus *Citrus*) [45, 46]. It can be concluded that exploratory analysis of biological activities of *P. digitatum* strains relative to the standard strain PD5 with known resistance can be a promising tool in identification and characterization of field isolate strains.

3.2 *Exploratory analysis of the data set B: fungal morphology and strains characterization*

Morphological ni-type descriptors (descriptors 1–8) and wi-type descriptors (descriptors 9–16) of 35 strain cultures are defined in Table 1. Measured and calculated values of these descriptors are presented in Tables 3 and 4. The PD9 culture was considered as not germinated by Hamamoto *et al.* [3], but our careful inspection of their published photographic results revealed the existence of a very small culture. Morphological characteristics are important in taxonomy [43, 47] and are good indicators of the morphology/physiology dependence on environmental conditions [48]. These observations suggest chemometric exploration of morphological descriptors in this work. In this sense, descriptors 17–24 (Table 1) were calculated as simple ratios of descriptors 8–16 with analogue descriptors 1–8, because they include fungal morphology both in absence of toxicants and in presence of triflumizole ($0.5\,\mu g\,ml^{-1}$). It is important to note that Hammamoto *et al.* [3] have carried out fungal growth experiments with fenarimol (II), bitertanol (III) and pyrifenox (IV) and have obtained the same results with fungal colonies as when using triflumizole (I). This fact can be rationalized in terms of chemical similarity among these DMIs (Figure 1).

Table 3. Measured[a] and estimated[b] morphological and class[c] descriptors used for characterization of the growth of 35 *P. digitatum* strains[d] in absence of demethylation inhibitors.

Strain	*R*	*O*	*T*	$R_{max\text{-}ni}$	$R_{min\text{-}ni}$	$R_{mean\text{-}ni}$	$R_{est\text{-}ni}$	C_{ni}	S_{ni}	D_{ni}	T_{ni}
PD1	S	J	m	1.14	0.97	1.05	1.03	0.92	1.06	0.17	1.086
PD5	S	J	m	1.08	0.92	1.00	1.00	0.88	1.00	0.16	1.083
PD9	S	J	m	0.12	0.09	0.10	0.10	0.16	0.01	0.03	1.028
PD11R	R	J	m	1.25	1.17	1.21	1.16	1.10	1.34	0.09	1.037
PD12	S	J	m	0.99	0.91	0.95	0.93	0.88	0.87	0.09	1.042
LC1	R	N	l	1.76	1.56	1.66	1.26	1.15	1.59	0.20	1.078
LC2	R	N	l	1.37	1.20	1.28	1.28	1.15	1.64	0.16	1.077
LC3	R	N	l	1.08	0.92	1.00	1.00	0.94	1.00	0.16	1.083
LC4	R	N	l	1.23	0.92	1.07	1.04	0.94	1.09	0.31	1.162
LC5	R	N	l	1.12	1.05	1.09	1.03	1.00	1.06	0.08	1.034
LC2M	M	N	l	1.14	1.10	1.12	1.09	1.00	1.19	0.04	1.019
DF1	S	N	o	0.70	0.64	0.67	0.67	0.59	0.44	0.06	1.037
U1	S	J	m	1.02	0.93	1.30	0.95	0.80	0.89	0.09	1.047
U3	S	J	m	1.33	1.30	1.32	1.23	1.15	1.52	0.03	1.013
U4	S	J	m	1.03	0.97	1.00	0.97	0.86	0.94	0.06	1.031
U5	S	J	m	0.98	0.86	0.92	0.73	0.80	0.54	0.12	1.065
U6	S	J	m	1.20	0.99	1.10	1.03	0.98	1.06	0.21	1.106
I1	R	N	l	1.03	0.93	0.98	0.96	0.86	0.92	0.09	1.052
I2	R	N	l	1.08	0.90	0.99	0.98	0.86	0.97	0.18	1.095
I3	R	N	o	1.19	1.14	1.17	1.15	1.04	1.32	0.05	1.023
I4	S	N	g	0.71	0.66	0.68	0.65	0.61	0.43	0.05	1.030
M1	R	N	l	1.11	1.02	1.07	1.03	0.96	1.05	0.09	1.045
Kami1	S	U	u	0.90	0.73	0.81	0.80	0.74	0.64	0.16	1.098
Kami2	S	U	u	0.97	0.83	0.90	0.87	0.80	0.76	0.14	1.077
Tou5	S	U	u	0.88	0.83	0.86	0.85	0.80	0.72	0.05	1.027
Dai1	S	U	u	0.99	0.92	0.96	0.61	0.90	0.37	0.07	1.037
Uha3	S	U	u	1.17	1.11	1.14	1.10	1.04	1.22	0.06	1.028
Ihi21	S	U	u	1.07	0.88	0.97	0.92	0.84	0.84	0.19	1.101
Ihi25	S	U	u	0.96	0.82	0.89	0.90	0.80	0.81	0.14	1.077
Nis5	S	U	u	0.95	0.88	0.92	0.90	0.82	0.80	0.07	1.037
Nis11	S	U	u	0.88	0.82	0.85	0.83	0.74	0.69	0.07	1.033
Nis20	S	U	u	1.14	1.05	1.09	1.05	0.98	1.10	0.09	1.044
IFO7137	S	U	u	0.93	0.83	0.88	0.86	0.82	0.74	0.10	1.055
IFO7876	S	U	u	1.08	0.97	1.03	0.97	0.88	0.94	0.10	1.056
IFO9651	S	J	o	1.39	1.30	1.34	1.32	1.23	1.75	0.09	1.039

[a]Measured morphological descriptors for 35 *P. digitatum* strains in absence of demethylation inhibitors, as defined in Table 1: $R_{max\text{-}ni}$, $R_{min\text{-}ni}$, C_{ni} and S_{ni}.
[b]Morphological descriptors for 35 *P. digitatum* strains in absence of demethylation inhibitors, estimated from measured diameters and surfaces and expressed relatively to the strain PD5, as defined in Table 1: $R_{mean\text{-}ni}$, $R_{est\text{-}ni}$, D_{ni} and T_{ni}.
[c]Class descriptors according to the literature [3]: DMI resistance (R) – susceptible (S), resistant (R) and moderately resistant (M) to DMIs; origin of the strains – Japanese (J), non-Japanese (N) and unknown strains (U); target types (fruits, T) – mandarin (m), lemon (l), orange (o), grapefruit (g), and unknown (u) moulds.
[d]The measured descriptors are expressed relative to those normalized for the PD5 strain i.e., based on the normalized R_{mean} for this strain.

HCA results for the data set B (descriptors 17–24) are presented in Figure 3. Strains are grouped into two clusters: the larger cluster with 24 DMI-S strains and the smaller one with 10 DMI-R strains and the DMI-M strain LC2M (LC2M is clustered with the DMI-R strains at similarity index 0.50). The clusters are rather homogeneous with respect to

Table 4. Measured[a] and estimated[b] morphological descriptors, and the experimental[c] genome variable PCR used for characterization of the growth of 35 *P. digitatum* strains[d] in presence of triflumizole.

Strain	$R_{\text{max-wi}}$	$R_{\text{min-wi}}$	$R_{\text{mean-wi}}$	$R_{\text{est-wi}}$	C_{wi}	S_{wi}	D_{wi}	T_{wi}	*PCR*
PD1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
PD5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
PD9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
PD11R	0.86	0.74	0.80	0.76	0.74	0.58	0.12	1.069	0.75
PD12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
LC1	1.19	0.89	1.04	1.02	0.98	1.03	0.30	1.159	0.75
LC2	0.94	0.79	0.87	0.84	0.82	0.71	0.15	1.084	0.75
LC3	0.69	0.56	0.62	0.62	0.59	0.39	0.13	1.083	0.75
LC4	0.70	0.48	0.59	0.61	0.67	0.37	0.22	1.149	0.75
LC5	0.72	0.65	0.68	0.75	0.68	0.56	0.07	1.042	0.75
LC2M	0.39	0.33	0.36	0.36	0.39	0.13	0.06	1.045	0.37
DF1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
U1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
U3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
U4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
U5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
U6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
I1	0.71	0.56	0.63	0.63	0.63	0.40	0.14	1.096	0.75
I2	0.65	0.59	0.62	0.61	0.67	0.37	0.05	1.038	0.75
I3	0.83	0.73	0.78	0.76	0.78	0.58	0.10	1.058	0.75
I4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
M1	0.76	0.68	0.72	0.69	0.72	0.47	0.08	1.048	0.75
Kami1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
Kami2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
Tou5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
Dai1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
Uha3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
Ihi21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
Ihi25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
Nis5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
Nis11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
Nis20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
IFO7137	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
IFO7876	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25
IFO9651	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.000	0.25

[a]Measured morphological descriptors for 35 *P. digitatum* strains in presence of triflumizole, as defined in Table 1: $R_{\text{max-wi}}$, $R_{\text{min-wi}}$, C_{wi} and S_{wi}.
[b]Morphological descriptors for 35 *P. digitatum* strains in presence of triflumizole, estimated from measured diameters and surfaces and expressed relatively to the strain PD5, as defined in Table 1: $R_{\text{mean-wi}}$, $R_{\text{est-wi}}$, D_{wi} and T_{wi}.
[c]Experimental values of the dependent genome variable PCR from Hamamoto *et al.* [3].
[d]Measured descriptors are expressed relative to those normalized for the PD5 strain i.e., based on the normalized R_{mean} for this strain.

strains origin: the smaller cluster consists mostly of non-Japanese strains (only one strain is Japanese), whilst the larger cluster contains mainly Japanese and unknown strains (only one strain is non-Japanese). It is also interesting to note strains classification with respect to target fruits. All lemon moulds are placed in the smaller cluster, whilst the larger cluster consists mainly of mandarin and unknown moulds. Besides these strain characterizations,

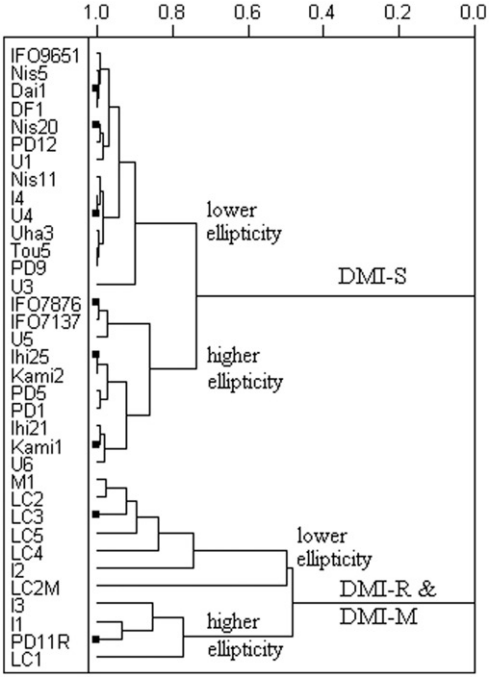

Figure 3. HCA dendrogram with 35 fungal strains (data set B). The strains are clustered with respect to the baseline resistance and morphology of the colonies. Strains from the external validation set are marked with solid squares.

the shape of fungal colonies is also visible in the dendrogram. Each cluster contains a sub-cluster of almost round colonies (lower ellipticity) and another one of elliptical shape colonies (higher ellipticity). Ellipticity of fungal colonies under specified growth conditions seems to be an intrinsic characteristic of a *P. digitatum* strain. Descriptor T_R can be considered as a measure of fungal colony's ellipticity in absence of toxicants, with values ranging from 0.96 to 0.99 for more round DMI-S colonies (ideal value for T_R is 1.00), and with values ranging from 0.90 to 0.95 for more elliptical DMI-S colonies. T_R values for two sub-clusters in the DMI-R & DMI-M cluster are 0.95–1.03 for more round colonies and 1.03–1.07 for more elliptical colonies. Detailed inspection of descriptors T_{ni} (Table 3) and T_{wi} (Table 4) which define T_R shows that these two sub-clusters exhibit the opposite ellipticity trends when the colonies are treated with triflumizole.

PCA scores plot for the data set B is presented in Figure 4, with two clusters as in HCA. The first three principal components contain 99.4% of the original data. All radius-based, circumference- and surface area-based descriptors are tightly grouped in the loadings plot (not shown) whilst D_R and T_R are two isolated descriptors. The scores plot clearly distinguishes the DMI-S cluster from DMI-R and DMI-M cluster (Figure 4a). Besides, origin (Figure 4b) and target (Figure 4c) of *P. digitatum* strains also show clustering tendencies, as has been noticed in the HCA dendrogram. Non-Japanese strains are reasonably well distinguished from Japanese and unknown strains (which could be Japanese also), with exception of three strains (DF1, I3 and PD11R). Regarding the targets, the smaller cluster consists of mostly lemon moulds, whilst most moulds in the larger cluster are mandarin and unknown moulds (which in fact could be

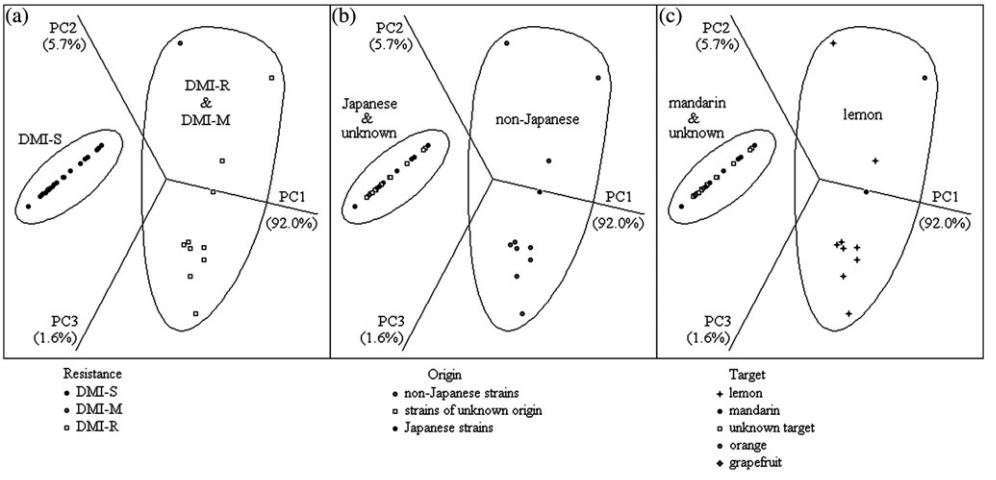

Figure 4. PCA scores plot for the data set B. 35 strains are discriminated according to: (a) baseline DMI resistance character, (b) geographic origin, and (c) target fruits.

mandarin moulds). It is clear that lemon moulds are rather well separated from mandarin and unknown moulds both in PCA and HCA, what has been already observed in the exploratory analysis of data set A, and what is consistent with phylogenetic analyses of *Citrus* species [45,46].

The present exploratory analysis, like that of the data set A, indicates various benefits of applying chemometric methods to fungal growth data for *P. digitatum*. Such approach, taking into account data for one or more standard strains, could be useful in the identification of baseline resistance and other characteristics of unknown field isolates. It seems that correct diagnostics would be able to prescript adequate fungicide and reduce time of the whole experimental methodology, once bioassays to determine genome characteristics relative to baseline DMIR would not be mandatory. However, larger data sets are necessary to be treated by chemometric methods to confirm that chemometric approaches would improve the exploration of experimental data.

3.3 *Quantitative Morphology-Genome Relationship (QMGR)*

Eight descriptors 17–24, already employed in exploratory analysis of the data set B, were used to model the genome variable PCR by means of PLS regression. Statistics of the PLS model, including leave-*N*-out, Y-randomization and external validation, is presented in Table 5. External validation set is highlighted in Figure 3, and predicted PCR values from the external validation are presented in Figure 5.

Hamamoto *et al*. [3] have developed a fast method for identification of baseline fungal resistance by carrying out bioassays for PCR values. This method has been introduced to replace DMI sensitivity assays in which fungal radial growth is monitored in absence and presence of demethylation inhibitors. However, quantitative relationships between these two methods and possible benefits from such findings have never been explored. The PLS model (Table 5) shows linear relationships between PCR and descriptors 17–24, with high correlation coefficients for PCR-descriptor correlations (0.71–0.99). Positive values of

Table 5. PLS statistics, regression vector[a] and PCR-descriptor correlation coefficients.[b]

Parameters	*Values*	*Regression vector*	
Training/Ext. val. set[c]	35/0	R_{maxR}	0.177
PCs (%Var)[d]	2 (97.6%)	R_{minR}	0.197
SEV[e]	0.028	R_{meanR}	0.187
SEC[e]	0.023	C_{R}	0.180
Q^{2f}	0.985	S_{R}	0.171
R^{2f}	0.991	R_{estR}	0.173
R.e.>10.00%[g]	1	D_{R}	−0.027
Max. R.e.[g]	21.6%	T_{R}	−0.085
Mean R.e.[g]	4.1%		
Leave-N-out crossvalidation[h]			
$<Q^2_{\text{LNO}}>$	0.984	Correlation coefficients	
Y-randomization		R_{maxR}	0.992
Q^2_{Yrand}	0.016	R_{minR}	0.993
R^2_{Yrand}	0.195	R_{meanR}	0.993
		C_{R}	0.989
External validation		S_{R}	0.977
Training/Ext. val. Set[c]	25/8	R_{estR}	0.990
PCs (%Var)[d]	2 (97.5%)	D_{R}	0.829
$\text{SEV}^{\text{j}}_{\text{ev}}$	0.030	T_{R}	0.712
$\text{SEC}^{\text{j}}_{\text{ev}}$	0.025		
$Q^{2\text{j}}_{\text{ev}}$	0.982		
$R^{2\text{j}}_{\text{ev}}$	0.990		
R.e.>10.00%[g]	1		
Max. R.e.[g]	19.9%		
Mean R.e.[g]	4.4%		

[a]Regression vector components (in autoscaled form) for the PLS model with 35 strains in the training set.
[b]PCR-descriptor correlation coefficients for 35 strains.
[c]Number strains in the training and external validation sets.
[d]Number of used principal components and the corresponding % variance of the **X** data matrix.
[e]Standard deviations of the PLS model: SEV – standard error of leave-one-out crossvalidation, SEC – standard error of calibration.
[f]Squares of correlation coefficients of the PLS model: Q^2 – correlation coefficient of leave-one-out crossvalidation, R^2 – correlation coefficient of calibration.
[g]Relative errors: R.e.≥10.00% – number of samples with relative error ≥10.00%, Max. R.e. – maximum relative error, Mean R.e. – mean relative error.
[h]Average Q^2 correlation coefficient of leave-N-out crossvalidations where $N = 1, 2, \ldots, 10$.
[i]Maximum Q^2 and R^2 correlation coefficients of ten Y-randomisations.
[j]Parameters for external validation: SEV_{ev} – standard error of leave-one-out crossvalidation, SEC_{ev} – standard error of calibration, Q^2_{ev} – correlation coefficient Q^2, R^2_{ev} – correlation coefficient R^2.

these correlation coefficients mean that more resistant colonies (i.e., those with high PCR) grow more (i.e., have high values of morphology descriptors) in presence of toxicants than sensitive colonies. It can be seen from the correlation coefficients and regression vector components (in autoscaled form) that there are two groups of descriptors: six descriptors obtained from fungal morphology (descriptors 17–22: originated from radii,

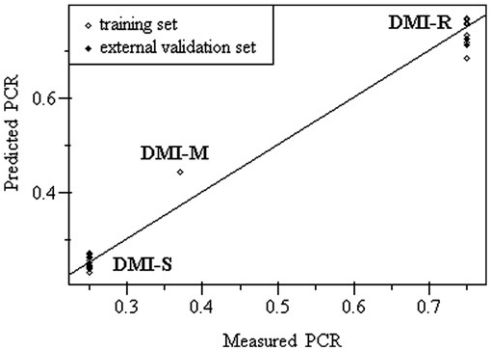

Figure 5. PLS plot for external validation of the data set B. DMI-S, DMI-M and DMI-R stand for demethylation inhibitor sensitive, medium resistant and resistant strains, respectively. Strains from the training and external validation sets are marked with different symbols.

circumferences and surface areas), and two more complex descriptors derived from radii descriptors (descriptors 23 and 24). This explains why two principal components with 97.6% of the total variance were used for the PLS model, what is consistent with the fact that PCA was also based on two principal components. It is important to note that the regression vector components show that higher fungal resistance i.e., greater PCR is followed by pronounced fungal growth in presence of a toxicant, as has been observed from PCR-descriptor correlation coefficients. One can observe that fungal size ni-type descriptors (descriptors 1–6 in Table 3) and wi-type descriptors (descriptors 9–14 in Table 4) tend to have closer values for DMI-R colonies than for DMI-S colonies. Consequently, the descriptors used for PLS modelling have either positive (descriptors 17–22, Table 5) or negative (D_R and T_R) contributions to PCR. The former descriptors, as has been already observed, mean more intense growth in presence of toxicants, what is a characteristic of DMI-R strains i.e., strains with high resistance. The latter descriptors (differences and ratios of fungal radii in free and inhibited growth, Table 1) have different behaviour than the former descriptors in terms of PCR-descriptor correlations (obviously lower correlation coefficients), what reflects in negative regression vector components. There is only one strain (LC2M) with a medium resistance (DMI-M), but the model can predict PCR for any putative resistance level. This strain has been reported as "a spontaneous mutant isolated from LC2 with a tandem repeat of two units of the transcriptional enhancer" [3]. The values 0.25, 0.37 and 0.75 of PCR for DMI-S, DMI-M and DMI-R strains, respectively, are in direct linear relationship with the number of copies of the *PdCYP*51 transcriptional enhancer (1, 2, and 5, respectively): one enhancer copy contributes to PCR increase by 0.12. Besides LC2M, other natural or artificial mutations of *P. digitatum* strains may result in strains with other values of PCR besides those in Table 4. This is an additional reason to establish a fungal morphology-genome relationship at quantitative level.

High-level prediction power of the PLS model can be seen from acceptable errors and high correlation coefficients of validations and calibration (Table 5). Relative errors range from 0% to 8%, with exception of the strain LC2M whose relative error is above 10%. The PCR value for this strain is estimated as expected for a DMI-M strain with two copies of the *PdCYP*51 transcriptional enhancer, what confirms the validity of the PLS

model for practical purposes (Figure 5). Y-randomization shows no chance correlation. Stability of the model is visible from the high average correlation coefficient from leave-*N*-out crossvalidation.

The data set B was divided into groups of 27 and 8 strains for training and external validation sets (see Figure 3), respectively, to perform external validation of the proposed PLS model. The new regression model has been obtained with a good statistics, very similar to that one of the proposed PLS model (Table 5). The corresponding predicted values PCR have relative errors ranging from 0% to 9% for the training set, with exception of the strain LC2M which has relative error of 19% and is still reasonably well predicted (Figure 5). PCR values for the eight samples from the external validation set have relative errors from 0% to 8%. These external validation results confirm the practical validity of the proposed PLS model.

The usual practice in bioassays is still, unfortunately, univariate. Dose-response plots are calibration curves from which EC_{50} or other biological activity parameters are derived. In contrary, this work and the previous one [27] have demonstrated the multivariate character of biological activity data sets for *P. digitatum* strains. It is visible from exploratory analysis of the data set B that interesting strain characterizations can be obtained and used in practice. Newly established QMGR relationship via a PLS model is a quantitative confirmation that fungal growth data are multivariate by their nature. Another interesting characteristic of the PLS model is that the fungal growth is directly related to the expression level of the gene *CYP*51, i.e., elevated synthesis of the enzyme $P450_{14DM}$. This resistance mechanism results in more intense consumption of demethylation inhibitors, after which fungal ergosterol biosynthesis stays untouched. It has been shown previously at qualitative and quantitative level [27] that the CYP51 resistance mechanism is the major contributor to *P. digitatum* resistance to DMIs, but efflux pumps-mediated mechanisms as well as toxicant molecular properties also play an important role. Chemometric methods present powerful tool in detecting these biological items, and therefore, development of their applications for practical purposes is an open field with promising results.

4. Conclusions

Biological activity data EC_{50} in logarithmic form and relative to a standard fungal strain have pointed out that exploratory analysis can show baseline resistance character, origin and target fruits of *P. digitatum* strains. This approach can be further developed by using larger data sets of diverse pathogenic microbes. The other data set, derived from fungal growth morphology in absence and presence of demethylation inhibitors, leads to the following conclusions. Fungal growth morphology is not univariate by nature, meaning that usual bioassay practices explore only a fraction of obtained data (one variable instead of many). Furthermore, descriptors from fungal morphology can distinguish the same strain characteristics as the $pECr_{50}$ data set. Finally, the fungal growth is directly related to the production of the enzyme $P450_{14DM}$ as the main resistance mechanism of *P. digitatum* against demethylation inhibitors. This work has shown that chemometric methods, when exploring bioassay data from a different perspective than in common practice, are promising approaches to obtain useful information on fungal resistance and to apply these findings in practice.

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