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# *Glasilo Future*

## Stručno-znanstveni časopis

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## Riječ gostujućeg urednika

Poštovani čitatelji Glasila Future,

pred Vama je specijalno izdanje časopisa posvećeno profesoru emeritusu Bogdanu Cvjetkoviću. Svojim znanstvenim i stručnim radom, koji traje više od pola stoljeća, prof. Cvjetković ostavio je značajan i neizbrisiv trag ne samo u Hrvatskoj nego i svjetskoj fitopatologiji i fitofarmaciji. Ostavljajući iza sebe brojne generacije diplomiranih inženjera agronomije svojim entuzijazmom i predanošću fitopatologiji uspio je „zaraziti“ te biti predani mentor 18 magistara znanosti te 8 doktora znanosti. Širokog znanja i znanstvenih interesa magistrirao je na Prirodoslovno-matematičkom fakultetu u Zagrebu iz području biljne virologije, a doktorirao na svojem *alma mater* Agronomskom fakultetu u Zagrebu kod profesora Josipa Kišpatića na području biljne mikologije. U želji da ovim brojem djelomično oslikamo široko područje interesa prof. Cvjetkovića, ovo specijalno izdanje obuhvaća tri izvorna znanstvena rada te dva prethodna priopćenja iz područja biljne mikologije, bakteriologije te virologije. Kraj ovog specijalnog izdanja posvećen je crticama iz života dr. Željka Jurjevića, jednog od doktora znanosti koji je doktorirao pod mentorstvom prof. Cvjetkovića, a trenutno s uspješnom karijerom u Sjedinjenim Američkim državama (EMSL Analytical, Inc.). Izrazito mi je drago da su se sudjelovanju u ovom broju odazvali znanstvenici koje se bave fitopatologijom na području Hrvatske, ali i kolege iz inozemstva, dajući svoj značajan doprinos kvaliteti ovog specijalnog izdanja, ali i izražavajući pijetet prof. Cvjetkoviću.

Prvi rad kolegica Dušice Kovačević, Katarine Zečević te Ivane Stanković s Poljoprivrednog fakulteta Univerziteta u Beogradu govori o djelomičnoj molekularnoj karakterizaciji izrazito polifagnog virusa mozaika krastavca izoliranoga iz dvije biljke božura sa simptomima mozaika i klorotičnih prstenova. Nakon potvrde virusa serološkim i molekularnim metodama sekvenciranjem dijela genoma proteinskog omotača utvrđeno je da izolati iz božura pripadaju u podgrupu IA. Autorice skreću pozornost da bi božur kao trajnica mogao imati značajnu epidemiološku ulogu u kontekstu značajnog izvora ovog virusa.

Rad kolega Kirila Bahcevandzieva te Antónia A. Monteira (Research Centre for Natural Resources, Environment and Society - CERNAS, Portugal) vodi nas u područje fenotipskih i genotipskih interakcija između različitih kupusnjača te ekonomski značajnog uzročnika plamenjača kupusnjača (*Hyaloperonospora brassicae*). Kroz istraživanje je utvrđeno da izolati navedenog patogena iz različitih područja Europe pokazuju različite stupnjeve patogenosti. Analizirani model gen-za-gen otvara nove mogućnosti istraživanja rezistentnosti kod različitih kupusnjača te gena za patogenost uzročnika plamenjače.

Da su na gljivične patogene osjetljive i invazivne biljne vrste govori rad autora Darija Ivića i Adrijane Novak (Hrvatska agencija za poljoprivredu i hranu). Analizom stabala pajasena sa simptomima

sušenja i propadanja na području Nacionalnog parka Krka utvrđena je prisutnost 15 različitih vrsta polifagnih gljiva iz rodova *Diaporthe*, *Diplodia*, *Dothiorella*, *Fomitiporia*, *Fusarium*, *Paraconiothyrium*, *Peroneutypa*, *Rosellinia*, *Schizophyllum* te *Verticillium*. Autori ističu da je ulogu utvrđenih gljiva u sušenju i propadanju ove invazivne vrste potrebno utvrditi testovima patogenosti.

Prethodno priopćenje doktorice znanosti Katarine Martinko i studentice Ivone Novaković sa Sveučilišta u Zagrebu Agronomskog fakulteta donosi preliminarne rezultate *in vitro* istraživanja protugljivičnog djelovanja esencijalnih ulja timijana, divljeg mažurana i lovora na uzročnika crne truleži plodova različitih poljoprivrednih kultura (*Aspergillus niger* Tiegh.). Autorice zaključuju da prvenstveno eterična ulja timijana i divljeg mažurana imaju veliki potencijal kao fumiganti u kontroli crne truleži uskladištenih poljoprivrednih proizvoda, te kao takvi predstavljaju svojevrsnu alternativu trenutno često korištenim fungicidima.

Prethodno priopćenje doktorice znanosti Jelene Plavec (Hrvatska agencija za poljoprivredu i hranu) opisuje uzročnika bakterioznog paleža lijeske (*Xanthomonas arboricola* pv. *corylina*) utvrđenog metodom lančane reakcije polimerazom iz rasadnika i komercijalnih nasada lijeske na području Hrvatske. Imajući u vidu sve veću popularnost ove kulture u našoj zemlji autorica skreće pozornost da će u budućnosti biti potrebno povesti više pažnje u praćenju ovog ekonomski značajnog patogena svrstanog na listu reguliranih nekarantenskih štetnika ne samo lijeske, već i drugih vrsta iz roda *Corylus*.

Crtice doktora znanosti Željka Jurjevića sažimlju različite dijelove profesionalnog razvoja prof. Cvjetkovića, ali ujedno predstavljaju i jednu toplu životnu priču protkanu zajedničkim trenucima provedenima s profesorom.

Vežući se na životopisne crtice dr. Jurjevića, i osobno kao jedan od doktoranada, mogu reći da bi se o liku i djelu prof. Cvjetkovića mogla napisati ne jedna, nego više knjiga. Na kraju mogu reći da mi je bila iznimna čast i zadovoljstvo intenzivno surađivati s profesorom sve do njegovog odlaska u mirovinu. I danas, sa životopisnim pričama i neograničenim praktičnim iskustvom, dragi mi je sugovornik na Zavodu za fitopatologiju u čiji razvoj je utkao značajno razdoblje svojega života i kojem je dao svoj neprocjenjivi obol!

Prof. dr. sc. Darko Vončina

# *Glasilo Future*

## Stručno-znanstveni časopis

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**SADRŽAJ:**

# Identification of *Brassica oleracea* accessions with specific reaction to *Hyaloperonospora brassicae* isolates

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*izvorni znanstveni rad (original scientific paper)*

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## Abstract

The variability or polymorphism of the response between the host and the parasite is controlled by the gene-to-gene system, which is responsible for the "genetic wars" between the host and parasite populations, the mechanism by which the host recognizes a locus or some loci particulars in the parasite. A standard set of *B. oleracea* plants at the cotyledon stage, which could aloww a reproducible classification of *H. brassicae* pathotypes, is poorly characterised. The aim of this study was to identify differentiating plants selected from a collection of *B. oleracea* accessions characterised by possible specific reactions with *H. brassicae* isolates to verify the existence of races among the isolates and to serve as a basis for differentiation among brassica cultivars. The experiment was divided into two tests to evaluate the host-isolate interaction. In the first test, the interactions between thirteen brassica accessions and three *H. brassicae* isolates were evaluated. In the second test, sixteen accessions were inoculated with eight different isolates and analysed. Seven-day-old seedlings were inoculated by applying two 10 µl drops of the spore suspension of the different isolates to each cotyledon. The resistance found in some accessions showed different frequencies with respect to the isolates, ranging from 10% (HRI4302 with H501 and CGN18451 with Hb006) to 93% (ISA 207 with Hb006). In the second test in group D2, accession KB01 showed specific resistance to Hb005, in D3 accession KB091 was resistant to isolate Hb-FP06 and in group D4, accessions KB14/00, KB566 and KB092 showed resistance to isolate Hb-FP06. Two gene-to-gene models presented in this work were designed to explain the relationships between differentiated accessions and *H. brassicae* isolates. These resistant accessions were considered as potential sources of downy mildew resistance and for that purpose need to be genetically characterised and further exploited in breeding programmes.

**Key words**: host resistance, *Brassica oleracea*, downy mildew, cotyledon inoculation, gene-to-gene.

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[3] Bahcevandziev, K., Monteiro, A. A. (2024). Identification of *Brassica oleracea* accessions with specific reaction to *Hyaloperonospora brassicae* isolates. *Glasilo Future, 7*(4), 14–33.

## Introduction

The phenotypic expression of the interaction between the host and the pathogen depends on the interaction between the genotypes of the two in an environment that favours their development. Variations in this phenotypic expression may result from individual characteristics of the host and/or pathogen. This interaction is so close that it is sometimes difficult to recognize whether the parasite influences the host or the host influences the performance of the parasite (Lebeda and Schwinn, 1994).

*Hyaloperonospora brassicae* (syn. *Hyaloperonospora parasitica* subs. *brassica*) causing a downy mildew in brassica crops, is an economically destructive disease and can be found in many regions of the world where these crops are grown. Comparison of the pathogenic variability of *H. brassicae* isolates with the interaction phenotype of brassica plants is a likely indicator for finding accessions with specific reactions to the isolates, and provides a basis for defining whether potential parasite races exist.

*Brassica oleracea* plants from different regions of the world show different interaction phenotypes with *H. brassicae* isolates (Moss et al., 1991; Dias et al., 1993; Nashatt and Awasthi, 1995; Nashatt and Rawlinson, 1994; Branca et al., 2005; Coelho et al., 2012), which has been a challenge to better define the behaviour of this parasite.

Some variability in the pathogenicity of *H. brassicae* isolates to *Brassica oleracea* plants has been reported (Natti et al., 1967; Monteiro and Williams, 1989; Ohguchi et al., 1990; Dias et al., 1993a; Silué et al., 1995; Leckie et al., 1996; Masheva, 1996a; Masheva, 1996b; Silué et al., 1996; Sousa, 1996 and Jensen et al., 1999; Branca et al., 2005; Coelho et al., 2012), recognizing the existence of potential races of this parasite in Europe and the USA (Gaumann, 1918; Gaumann, 1926; Felton and Walker, 1946; Wang, 1949; Natti et al., 1967; McMeekin, 1969, Thomas and Jourdain, 1990; Thomas and Jourdain, 1992). This specific interaction between *H. brassicae*, and different Brassica hosts was also observed in *Arabidopsis* ecotypes (Koch and Slusarenko, 1990).

The evaluation of resistance of nuclear collection accessions (Bahcevandziev, 2003) showed the possibility of heterozygote interaction phenotypes with *H. brassicae* isolates. These accessions showed that among *B. oleracea* plants there is a specific reaction material capable of producing good sources of differentiating plants. The study of such variable material is only possible if there is a host-specific parasite reaction by comparing different isolates in relation to a defined host (Monteiro at al., 2005; Coelho et al., 2018).

In brassicas it is possible to apply two isolates to the same seedling, each isolate being inoculated on a different cotyledon. This technique allows an analysis of pathogenicity differences between the isolates and the potential hosts with differentiating characteristics (Bahcevandziev, 2003).

The evaluation methods used to define the presence of potential races in *H. brassicae* were based on numerical scales (Thomas and Jourdain, 1990; Thomas and Jourdain, 1992; Silué et al., 1995; Silué et al., 1996), observing the phenotypic host response (Wang, 1949; Natti et al., 1967) or simply in the form of *H. brassicae* spores (Gauman, 1918; Gauman, 1926; McMeekin, 1969). To date, no author has conducted a systematic study to detect potential *H. brassicae* races on different *B. oleracea* accessions.

The interaction between brassica hosts and *H. brassicae* isolates is considered specific because it is qualitative in nature. To evaluate this type of interaction, a scale with discrete classes based on the description of the interaction phenotype (IP) should be used. This evaluation mode enables the detection of genes leading to specific IP. This type of evaluation facilitates more detailed distinction between different isolates and better determines hosts as potential differentiators.

The aim of this study was to identify differentiating plants, selected from a collection of *B. oleracea* accessions characterized by possible specific reactions with *H. brassicae* isolates, to verify the existence of putative strains among the isolates and to serve as a basis for differentiation between brassica cultivars.

## Material and methods

The work consisted of two evaluation tests of the host-isolate interaction. In the first test, interactions between thirteen accessions and three *H. brassicae* isolates (Tables 1 & 2) were evaluated. In the second test, sixteen accessions were inoculated with eight different isolates and analysed (Tables 4 & 5).

Two tests were performed to confirm the existence of plants with a differential reaction and to compare Portuguese isolates with those from Europe, which already showed different molecular characteristics (Casimiro, 2001).

### Seedling inoculation

The *H. brassicae* inoculum was prepared by placing sporulated cotyledons of the susceptible Portuguese variety ‘Coração de Boi’ (*Brassica oleracea* var. *capitata*), separated from the plantlets and used as a maintenance stock, in 40 ml of distilled water with agitation for 2 min with a vortex to dislodge the conidia. The spore suspension was filtered through cheesecloth and centrifuged at 2,000 rpm for 3 min. The supernatant was discarded, and the pellet re-suspended in distilled water and centrifuged again. This was repeated two times. Finally, the spore concentration was adjusted to $5\times10^4$ spores/ml with a haemocytometer (Neubauer Improved). Seeds from three brassica host plants were sown in multicell trays (3×5×5 cm cells) filled with peat-based substrate Levington M2 (Fysons, UK) and maintained in a growth room at 20±1°C, 70% RH, 20h photoperiod under cool white light (Osram) at 250 μmol $m^{-2}$ $s^{-1}$. The emerged seedlings were randomly thinned to one seedling per cell. Cotyledons of seven-day-old seedlings were inoculated with a micropipette by applying two 10 μl

drops of the spore suspension. One of the two cotyledons was marked with a pinch, to separate the isolates. Normally, the pinched cotyledon was inoculated with a Portuguese isolate when possible. After inoculation each tray was enclosed in a black polyethylene bag and placed in an incubation chamber at 16± 1°C and 100% RH to induce infection of the cotyledons with the isolate. In the case of Hb005, CrGC 3.4 (*Brassica oleracea* var. *acephala*) seedlings were used for inoculum preparation, as Portuguese variety 'Coração de Boi' showed resistance to this isolate.

**1st experiment**

The three isolates used in this experiment were Hb501, Hb005 and Hb006 (Table 1). For this test six styrofoam trays were used as repetition. In each tray seeds from thirteen accessions were sown with 10 seedlings each, randomly distributed, thus occupying 130 cells per tray, totalling 60 seedlings/accession. Inoculations were performed by applying two different isolates on the cotyledons of the same seedling, placing one drop of the conidia suspension from each isolate on a respective cotyledon. Thus, each plant was inoculated simultaneously with two isolates (Hb501 x Hb005; Hb501 x Hb006; Hb005 x Hb006). This facilitated the analysis of the differences that might occur between the isolates with respect to their pathogenicity in the same accession.

***Table 1.*** *Isolates of Hyaloperonospora brassicae (Hb) used in the 1st experiment.*

| Isolate | Origin |
|---|---|
| Hb501 | ISA (Portugal) |
| Hb005 | HRI Wellesbourne (United Kingdom) |
| Hb006 | HRI Wellesbourne (United Kingdom) |

The 'Coração de Boi' (*Brassica oleracea* var. *capitata*) is a commercial variety (Soares and Rebelo, Portugal) while CrGC 3.4 (*Brassica oleracea* var. *acephala*) was used as a control plant.

***Table 2.*** *Brassica oleracea accessions used in the 1st experiment.*

| Nr | Code | Variety | Comon name | Origin |
|---|---|---|---|---|
| **1** | ISA 207 | *Tronchuda* | Couve Algarvia | Portugal |
| **2** | HRI 4302 | *Acephala* | Covo | Zimbabwe |
| **3** | HRI 5389 | *Gongylodes* | Cavolo | Italy |
| **4** | HRI 5443 | *Gongylodes* | Cavolo forte | Italy |
| **5** | HRI 5555 | *Acephala* | Arsis Ra F1 | Netherland |
| **6** | HRI 5652 | *Capitata* | Shetland cabbage | United Kingdom |
| **7** | HRI 6226 | *Acephala* | Giant Gersey kale | United Kingdom |
| **8** | HRI 6254 | *Botrytis* | Tasman | Australia |
| **9** | CGN 18450 | *Capitata* | Raketa | Chech Republic |
| **10** | CGN 18451 | *Capitata* | Predzvest | Chech Republic |
| **11** | Commercial | *Capitata* | Coração de Boi | Portugal |
| **12** | ISA 62 | *Tronchuda* | Murciana | Portugal |
| **13** | CrGC 3.4 | *Acephala* | Rapid Cycling Brassica | USA |

Resistant phenotype was defined by the appearance of necroses (mild and/or dense) on the adaxial side of the cotyledons, with no sporulation on the abaxial side. On the other hand, conidiophores formation on the abaxial side was considered as a susceptible phenotype (Table 3). The same scale was used in both tests.

***Table 3.*** *Scale used to evaluate host x parasite interaction.*

| Reaction to the parasite | DI | IP | Characteristics |
|---|---|---|---|
| **Resistant** | **0** | **NN** | Lack of host response and sporulation |
| | **1** | **HN** | Small necrosis; absence of sporulation |
| | **3** | **FN** | Necrosis confined to the inoculation site; absence of sporulation |
| **Susceptible** | **5** | **SS** | Necrosis confined to the inoculation site; weak sporulation (up to 5 conidiophores), confined to the inoculation site |
| | **6** | **CS-C** | Necrosis confined to the inoculation site; sporulation confined to the site of inoculation |
| | **7** | **CS-D** | Absence of localized necrosis; sporulation dispersed throughout the cotyledon |
| | **9** | **HS** | Absence of localized necrosis; abundant and dispersed sporulation throughout the cotyledon |

DI – disease index; IP – interaction phenotype.

**2nd experiment**

In this assay, eight *H. brassicae* isolates (Table 4) originated from various countries were used to inoculate sixteen brassica accessions (Table 5).

The accessions used in this second test were sown on six styrofoam trays (repetitions). In each tray sixteen accessions were randomly distributed with 10 seedlings per tray. Two different isolates were inoculated onto the same seedling, thus adding 60 seedlings/accession/isolate. The inoculations were performed as described in the 1st experiment.

***Table 4.*** *H. brassicae (Hb) isolates used in the 2nd experiment.*

| | Isolate | Origin |
|---|---|---|
| 1 | Hb501 | ISA (Portugal) |
| 2 | Hb005 | HRI Wellesbourne (UK) |
| 3 | Hb006 | HRI Wellesbourne (UK) |
| 4 | Hb-FP06 | University of Bretagne (France) |
| 5 | Hb-Italian | Universidade of Catánia (Italy) |
| 6 | Hb-Murcia | Nunhems (Germany) |
| 7 | Hb502 | ISA (Portugal) |
| 8 | Hb517 | ISA (Portugal) |

Some of the accessions used in this assay resulted from resistant plants selected from the nuclear collection assessments (Bahcevandziev, 2003), which were then self-pollinated (S2 and S3) or crossed (F2 and F3). Therefore, these accessions carry the same codes as their parents, (ISA 62, ISA 207, HRI 5443, and HRI 4302). The KB 91 and KB 92 accessions were provided by prof. Ales Lebeda from the University

of Prague (Czech Republic). The KB 4/00 accession was a traditional variety. The accessions 'Coração de Boi' (*Brassica oleracea* var. *capitata*), (Soares and Rebelo, Portugal),'Caramba' (*Brassica oleracea* var. *capitata*) (Germiplanta, Portugal), 'Beira' (*Brassica oleracea* var. *tronchuda*) (Germiplanta, Portugal) and 'Hunter Hybrid' (*Brassica oleracea* var. *botrytis*) (Yates, Australia) were the only commercial varieties used in the trial. The CrGC 3.4 accession was used as a control plant. (Table 5).

***Table 5.*** *Brassica oleracea accessions used to identify the differentiator sources.*

| Nº | Code | Variety | Common name | Origin/cross |
|---|---|---|---|---|
| 1 | **KB 207/00** | *tronchuda* | Couve Algarvia ($S_3$) | Portugal / $S_3$ ISA207 (FN) |
| 2 | **KB 62/00** | *tronchuda* | Murciana ($S_3$) | Portugal / $S_3$ ISA 62 (HS) |
| 3 | **KB 01** | *capitata* | Coração de Boi | Portugal / Soares e Rebelo |
| 4 | **KB 85** | *capitata* | Caramba | Portugal / Germiplanta |
| 5 | **KB 87** | *tronchuda* | Beira | Portugal / Germiplanta |
| 6 | **KB 4/00** | *tronchuda* | Tronchuda ($S_2$) | Portugal / $S_2$ Horto (FN) |
| 7 | **KB 5/00** | *gongylodes* | Cavolo forte ($F_2$) | $F_2$ (CrGC x HRI 5443) (FN) |
| 8 | **KB 14/00** | *tronchuda* | Couve Algarvia ($F_2$) | $F_2$ (CrGC x ISA 207) (FN) |
| 9 | **KB 91** | *capitata* | Predzvest | Univ. Praga / Chech Rep. |
| 10 | **KB 92** | *capitata* | Raketa | Univ. Praga / Chech Rep. |
| 11 | **KB 10/00** | *gongylodes* | Cavolo forte ($F_3$) | $F_3$ (CrGC x HRI 5443) (FN) |
| 12 | **KB 16/00** | *acephala* | Covo ($F_2$) | $F_2$ (CrGC x HRI 4302) (FN) |
| 13 | **KB 11/00** | *gongylodes* | Cavolo forte ($F_3$) | $F_3$ (CrGC x HRI 5443) (FN) |
| 14 | **KB 13/00** | *gongylodes* | Cavolo forte ($F_3$) | $F_3$ (CrGC x HRI 5443) (SS) |
| 15 | **KB 566** | *botrytis* | Hunter Hybrid | Austrália / Yates |
| 16 | **CrGC 3.4** | *acephala* | Rapid Cycling Brassica | USA |

Interaction phenotype assessments were performed by applying the same scale defined in the 1st experiment (Table 3).

**Trial design and statistical analysis of the obtained results**

Sampling and distribution of accessions in both trials was performed in randomized blocks with 6 replicates, testing a total of 60 plants per accession. To compare the pathogenicity of the isolates, the percentage of susceptible plants was calculated in relation to the total number of plants infected with the same isolate. The aggressiveness of the isolates was analyzed by calculating the disease index (DI) using the analysis of variance (ANOVA) from STATISTICA, version 12.0. The Scheffé test was applied to compare the means of accession DI.

## Results

### 1st experiment

The IP evaluation showed a clear interaction between host and isolate genotypes. 5 reaction groups were identified, which were designated as G1, G2, G3, G4 and G5 to facilitate analysis of the results (Tables 6 & 7).

A resistant accession was considered when more than 10% of the seedling/accession had a resistant phenotype. With more than 10% of resistant plants in an accession, the systematic behaviour of the accession was considered, thus showing the presence of genes responsible for the determination of resistance in the host. Susceptible accessions were classified in group G1 and accessions that were resistant to the three isolates were classified in group G5 (Table 6).

***Table 6.*** *B. Oleracea accession groups with differentiator reaction to the isolates.*

| Groups | **Characteristics** |
|---|---|
| G1 | Accessions susceptible to all isolates |
| G2 | Accessions susceptible to isolates H501 and H006 |
| G3 | Accessions susceptible to isolates H501 and H005 |
| G4 | Accessions susceptible to isolate H501 |
| G5 | Accessions resistant to all isolates |

Four genotypes resistant to one of the isolates (groups G2 and G3) were observed. The commercial 'Coração de Boi' variety showed resistance to Hb005 (Group G2), while accessions CGN 18451, HRI5652 and CGN18450 were only resistant to Hb006 (Table 7).

***Table 7.*** *Accessions distributed in groups with differentiating reactions, the percentages of seedlings resistant (-) to the three H. brassicae isolates and the DI.*

| **Group** | **Accessions** | **Percentage of seedlings (%) resistant to respective isolate *** | | |
|---|---|---|---|---|
| | | **Hb501** | **Hb005** | **Hb006** |
| | | (+) | (+) | (+) |
| **G1** | CrGC 3.4 | 0 | 0 | 0 |
| | ISA 62 | 0 | 0 | 0 |
| | | (+) | (-) | (+) |
| **G2** | Coração de Boi | 3 | 70 | 0 |
| | | (+) | (+) | (-) |
| **G3** | CGN 18451 | 3 | 0 | 10 |
| | HRI 5652 | 0 | 0 | 12 |
| | CGN 18450 | 0 | 4 | 58 |
| | | (+) | (-) | (-) |
| **G4** | HRI 5555 | 0 | 20 | 13 |
| | HRI 6254 | 8 | 27 | 27 |
| | | (-) | (-) | (-) |
| | HRI 4302 | 10 | 47 | 59 |
| | HRI 5389 | 15 | 34 | 37 |
| **G5** | HRI 6226 | 20 | 12 | 34 |
| | HRI 5443 | 39 | 67 | 43 |
| | ISA 207 | 66 | 82 | 93 |
| | **DI**** | **6,9 a** | **6,1 b** | **6,3 b** |

* (-) resistant; (+) susceptible.

** In each sub-column the mean values followed by the same letter did not differ significantly (P≤0.05).

Among the 21 accessions analysed in this test, no plants resistant only to the Portuguese isolate and susceptible to the two UK isolates were found. Host-isolate interactions in resistant plants showed the existence of resistance genes (Table 8). These genes were expressed through phenotypes with necrosis and without any sporulation.

***Table 8.*** *Presence of possible resistance genes to the isolates of H. brassicae in B. oleracea accessions analysed in the 1st experiment.*

| **Group** | **Accessions** | **H. brassicae isolates *** | | |
|---|---|---|---|---|
| | | **Hb501** | **Hb005** | **Hb006** |
| **G1** | CrGC 3.4<br>ISA 62 | 0 | 0 | 0 |
| **G2** | Coração de Boi | 0 | 1 | 0 |
| **G3** | CGN 18451<br>HRI 5652<br>CGN 18450 | 0 | 0 | 1 |
| **G4** | HRI 5555<br>HRI 6254 | 0 | 1 | 1 |
| **G5** | HRI 4302<br>HRI 5389<br>HRI 6226<br>HRI 5443<br>ISA 207 | 1 | 1 | 1 |

***** (1) Presence of resistance gene (s); (0) lack of resistance gene (s)

To elucidate the relationship between differentiating accessions and H. brassicae isolates, Table 9 was constructed. This model posits the involvement of three resistance (R) genes and three corresponding avirulence (Avr) genes.

***Table 9.*** *Gene-to-gene relationship between the accessions and the three H. brassicae isolates.*

| | | | | | **Isolates with avirulence genes (A)** | | |
|---|---|---|---|---|---|---|---|
| | | | | | **Hb501** | **Hb005** | **Hb006** |
| **Group** | **Accessions** | **Resistance Genes (R)** | | | **A1**<br>**.**<br>**.** | **.**<br>**A2**<br>**.** | **.**<br>**.**<br>**A3** |
| **G1** | CrGC 3.4<br>ISA 62 | **.** | **.** | **.** | + | + | + |
| **G2** | Coração de Boi | **.** | **R2** | **.** | + | **-** | + |
| **G3** | CGN 18451<br>HRI 5652<br>CGN 18450 | **.** | **.** | **R3** | + | + | **-** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **G4** | HRI 5555<br>HRI 6254 | **.** | **R2** | **R3** | + | **-** | **-** |
| **G5** | HRI 4302<br>HRI 5389<br>HRI 6226<br>HRI 5443<br>ISA 207 | **R1** | **R2?** | **R3?** | **-** | **-** | **-** |

+ = compatible reaction (susceptibility); - = incompatible reaction (resistance).

The r1 gene present in hri 4302, hri 5389, hri 6226, hri 5443 and isa 207 confers resistance to isolate hb501 which has the avirulence gene a1.The 'Coração de boi' gene r2 confers resistance to isolate hb005, the latter with avirulence gene a2 and the same gene confers resistance in accessions hri5555 and hri6254 to isolate hb005, which has the same avirulence gene a2. The r2 gene is probably found in the g5 group accessions, where it expresses incompatible interactions with the same isolate hb005, this with the a2 avirulence gene. The r3 gene characterizes the hri5652, cgn18450 and cgn18451 accessions and confers resistance to isolate hb006, which has the avirulence gene a3. The r3 gene is present in the g4 group and probably in the g5 group accessions, where it expresses interactions incompatible with the hb006 isolate (Table 9).

**2nd experiment**

The accessions that had more than 10% of the seedling/accession with the hn and fn interaction phenotype were considered resistant and therefore carrying possible resistance genes.

Based on the host-isolate interaction, the accessions were divided into 8 groups (Table 10). The accessions belonging to each of these groups were characterized by differential and specific reactions with the isolates.

***Table 10.*** *Groups of B. oleracea accessions with differential reaction to the H. brassicae isolates.*

| Groups | Characteristics |
|---|---|
| **D1** | Accessions susceptible to all isolates |
| **D2** | Accessions resistant to isolate 2 |
| **D3** | Accessions resistant to isolate 4 |
| **D4** | Accessions resistant to isolate 5 |
| **D5** | Accessions resistant to isolates 2, 4 and 5 |
| **D6** | Accessions susceptible to isolates 1, 3 and 6 |
| **D7** | Accessions susceptible to isolates 7 and 8 |
| **D8** | Accessions resistant to all isolates |

In D1 group were placed susceptible accessions on the eight isolates. Five accessions from D2, D3 and D4 groups showed resistance to a different isolate. The segregation of resistance found in KB 4/00 (Group D5) showed that its genotype recognized three of the eight isolates used. The accessions from group D6 and those from D7 showed greater differentiation from the eight isolates (Table 11).

The resistance to the eight H. brassicae isolates characterized the accessions from group D8, where 'Cavolo forte' (HRI 5443) offspring predominated (Table 11).

***Table 11.*** *Sixteen accessions distributed in groups with differential reactions and the percentages of seedlings resistant (-) to the eight H. brassicae isolates, characterized by their respective ID.*

| | | **Percentage (%) of seedlings resistance to each isolate*** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Gr.** | **Accessions** | **Hb501** | **Hb005** | **Hb006** | **Hb-FP06** | **Hb-Italian** | **Hb-Murcia** | **Hb502** | **Hb517** |
| **D1** | | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) |
| | **CrGC 3.4** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | **KB 62/00** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **D2** | | (+) | (-) | (+) | (+) | (+) | (+) | (+) | (+) |
| | **KB 01** | 0 | 45 | 0 | 0 | 0 | 0 | 0 | 0 |
| **D3** | | (+) | (+) | (+) | (-) | (+) | (+) | (+) | (+) |
| | **KB 091** | 0 | 0 | 0 | 15 | 0 | 0 | 0 | 0 |
| **D4** | | (+) | (+) | (+) | (+) | (-) | (+) | (+) | (+) |
| | **KB 092** | 0 | 0 | 0 | 0 | 16 | 0 | 0 | 0 |
| | **KB 566** | 0 | 0 | 0 | 0 | 21 | 0 | 0 | 0 |
| | **KB 14/00** | 0 | 0 | 0 | 0 | 42 | 0 | 0 | 0 |
| **D5** | | (+) | (-) | (+) | (-) | (-) | (+) | (+) | (+) |
| | **KB 4/00** | 0 | 30 | 0 | 20 | 33 | 0 | 0 | 0 |
| **D6** | | (+) | (-) | (+) | (-) | (-) | (+) | (-) | (-) |
| | **KB 11/00** | 0 | 31 | 0 | 35 | 67 | 0 | 21 | 19 |
| **D7** | | (-) | (-) | (-) | (-) | (-) | (-) | (+) | (+) |
| | **KB 16/00** | 60 | 26 | 15 | 25 | 50 | 43 | 0 | 0 |
| | **KB207/00** | 53 | 44 | 41 | 38 | 63 | 27 | 0 | 0 |
| **D8** | | (-) | (-) | (-) | (-) | (-) | (-) | (-) | (-) |
| | **KB 5/00** | 15 | 60 | 35 | 35 | 85 | 28 | 37 | 38 |
| | **KB 10/00** | 28 | 74 | 85 | 74 | 95 | 47 | 67 | 67 |
| | **KB 13/00** | 19 | 29 | 22 | 55 | 58 | 29 | 29 | 19 |
| | **KB 85** | 78 | 80 | 95 | 22 | 70 | 53 | 52 | 53 |
| | **KB 87** | 78 | 18 | 90 | 18 | 84 | 59 | 82 | 85 |
| **DI**** | | **6,4ab** | **5,5ab** | **5,9ab** | **5,8ab** | **5,2a** | **6,1ab** | **6,7b** | **6,6b** |

* (-) resistant; (+) susceptible; ** In each sub-column the mean values followed by the same letter did not differ significantly (P≤0.05).

The pathogenicity, recognized by DI, matched with the degree of virulence of the isolates. Isolates Hb502 and Hb517 showed the highest pathogenicity and were significantly different from Hb-Italian. The pathogenicity of the remaining five isolates was similar, showing no significant differences with the three isolates reported (Table 11).

For resistance, accessions with differentiating reaction were observed. In group D2 there was specific resistance in accession KB01 to Hb005, in group D3 accession KB091 was resistant to isolate Hb-FP06 and in group D4 accessions KB14/00, KB566 and KB092 expressed resistance to isolate Hb-Italian (Table 11). The accessions of these three groups showed different degrees of resistance (% resistant plants) in relation to their isolates.

Group D5 was characterized by specific resistance expressed in KB4/00 to isolates Hb005, Hb-FP06 and Hb-Italian, while in group D6 specific resistance was observed in the accession KB11/00 in relation to Hb502 and Hb517 (Table 11).On the other hand, accessions from group D7 showed specific resistance to isolates. Therefore, accessions KB207/00 and KB16/00 showed resistance type with Hb501, Hb005, Hb006, Hb-FP06, Hb-Italian and Hb-Murcia. In group D8 accessions, non-specific resistance was observed. The Hb-Italian isolate showed a high incompatibility with all accessions in this group (Table 11).

The virulence of the isolates was defined by the distinguishing characteristics of the accessions in relation to the isolates. When evaluating the degree of virulence (number of accessions resistant to the respective isolate), the three Portuguese isolates showed a higher virulence compared to the other isolates. To this group we can add Hb-Murcia and Hb006, which had almost the same degree of virulence with the accessions. The least virulent of all was the Hb-Italian.Within the groups, accessions KB5/00 and KB10/00 were identified that presented more stable resistance in relation to the eight isolates (Table 11).

The differentiating reactions in the accessions related to 8 isolates were expressed by the incompatible interaction phenotype which was characterized by necrosis on the adaxial side and no sporulation on the abaxial side of the cotyledons. These interactions in resistant accessions showed the existence of resistance genes (Table 12).

***Table 12.*** *Possible presence of resistance genes to eight H. brassicae isolates in the sixteen B. oleracea accessions analysed in the 2nd experiment, characterized with their degree of virulence.*

| | | **H. brassicae isolates*** | | | | |
|---|---|---|---|---|---|---|
| **Group** | **Accessions** | **Hb502 Hb517** | **Hb501 Hb006 Hb-Murcia** | **Hb005** | **Hb-FP06** | **Hb-Italian** |
| **D1** | **CrGC 3.4 KB 62/00** | 0 | 0 | 0 | 0 | 0 |
| **D2** | **KB 01** | 0 | 0 | 1 | 0 | 0 |
| **D3** | **KB 091** | 0 | 0 | 0 | 1 | 0 |
| **D4** | **KB 092 KB 566 KB 14/00** | 0 | 0 | 0 | 0 | 1 |
| **D5** | **KB 4/00** | 0 | 0 | 1 | 1 | 1 |
| **D6** | **KB 11/00** | 1 | 0 | 1 | 1 | 1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **D7** | **KB 16/00 KB 207/00** | 0 | 1 | 1 | 1 | 1 |
| **D8** | **KB 5/00 KB 10/00 KB 13/00 KB 85 KB 87** | 1 | 1 | 1 | 1 | 1 |
| | **Degree of virulence** | **6** | **7** | **10** | **10** | **12** |

***** (1) presence of resistance gene(s); (0) lack of resistance gene(s).

To try to explain the differentiating relationships between the 16 accessions and the eight H. brassicae isolates, a model presented in Table 13 was created. This model shows the expression of 5 resistance genes in the accessions and five avirulence genes in the isolates.

***Table 13.*** *Gene-to-gene relationships between sixteen B. oleracea accessions and eight H. brassicae isolates.*

| | | | | | | | **Isolates with avirulence gene (A)** | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | **Hb502 Hb517** | **Hb501 Hb006 Hb-Murcia** | **Hb005** | **Hb-FP06** | **Hb-Italian** |
| | | | | | | | **A1 . . . .** | **. A2 . . .** | **A1 A2 A3 . .** | **A1 A2 . A4 .** | **A1 A2 . . A5** |
| **Gr.** | **Accession** | **Resistance genes (R)** | | | | | | | | | |
| **D1** | **CrGC 3.4 KB 62/00** | **.** | **.** | **.** | **.** | **.** | + | + | + | + | + |
| **D2** | **KB 01** | **.** | **.** | **R3** | **.** | **.** | + | + | - | + | + |
| **D3** | **KB 091** | **.** | **.** | **.** | **R4** | **.** | + | + | + | - | + |
| **D4** | **KB 092 KB 566 KB 14/00** | **.** | **.** | **.** | **.** | **R5** | + | + | + | + | - |
| **D5** | **KB 4/00** | **.** | **.** | **R3** | **R4** | **R5** | + | + | - | - | - |
| **D6** | **KB 11/00** | **R1** | **R2 ?** | **.** | **.** | **.** | - | + | - | - | - |
| **D7** | **KB 16/00 KB 207/00** | **R1 ?** | **R2** | **.** | **.** | **.** | + | - | - | - | - |
| **D8** | **KB 5/00 KB 10/00 KB 13/00 KB 85 KB 87** | **R1** | **R2** | **.** | **.** | **.** | - | - | - | - | - |

+ = compatible reaction (susceptibility); - = incompatible reaction (resistance).

## Discussion

The aim of combining of *H. brassicae* isolates from different European countries and analyzing them in the same laboratory under uniform conditions was to compare the virulence of the isolates and to verify whether these parasitic *H. brassicae* races are putative.

The *B. oleracea* accessions showed great variability in the interaction between the host and *H. brassicae* isolates. According to Milgroom and Fry (1997), host-parasitic interaction variability characterizes hosts as genetically heterogeneous populations. Regarding the variability in the reaction with the respective isolates, the accessions in the two tests were divided into 5 (first test) and 8 groups (second test) after evaluation.

The accessions belonging to groups 2, 3 and 4 (first test), and those from groups 2, 3, 4, 5, 6 and 7 (second test) showed a different reaction with the isolates due to host-parasite interactions. These accessions recognized different isolates. This recognition is in agreement with Beynon (1997), who considers that certain plants have the particular resistance gene that is able to recognize some isolates (or races), while others do not. According to this author, the parasite has genes with which it interacts with host genes and this interaction can lead to resistance or susceptibility.

The commercial variety 'Coração de Boi' was resistant to isolate H005 in both tests. The gene that confers resistance in 'Coração de Boi' to Hb005 is probably the only resistance gene that this variety has. According to Kuhn (1987) differentiating accessions usually have a single resistance gene. Thus, there is a possibility that 'Coração de Boi' could be used as a differentiating plant for the Hb005 isolate. In this sense, group G3 accessions from the first test can be used as differentiating plants for Hb006. On the other hand, in the second test, accessions KB092, KB566 and KB14 / 00 can probably be considered differentiating plants for Hb-Italian, such as KB 091 for Hb-FP06.

The accessions HRI 4302, HRI 5389, HRI 6226, HRI 5443, and ISA 207 used in the first test showed different levels of incompatibility with the three isolates, which was also confirmed in the second test with the accessions KB 05/00, KB 10/00, KB 13/00, KB 85 and KB 87 in relation to the eight isolates. According to Nielsen and Tikhomirov (1993), incompatibility does not define races; these accessions were recognized as resistant. The mentioned accessions probably do not have specific genes that can specify the isolates, but according to Moseman et al. (1984) and Nielsen and Tikhomirov (1993) must have other genes or the combination of two or more genes that condition their resistance response. On the other hand, the incompatibility between the hosts and the isolates (groups G5 and D8) was highly variable in their expression, showing low (10 or 15%) and high (93 or 95%) levels. This shows that the resistance observed in these accessions can be controlled by different genes. According to Roelfs (1984), variable incompatibility characterizes the heterozygous genotype that defines resistance.

Following this author, we can say that the *H. brassicae* isolates used in the tests do not have the same genotype for pathogenicity with the different accessions.

Isolates from different geographical regions showed a high degree of host specificity. According to Sherriff and Lucas (1987), *H. brassicae* shows specificity for its hosts. According to Nielsen and Tikhomirov (1993), the specificity of the host-parasite interaction, represented by different IFs, characterizes genetically different races. According to Cardwell and Wehrly (1997), parasite populations are composed of several races with different frequencies of host affinity. According to Slusarenko and Mauch-Mani (1991), *H. brassicae* is a complex race with at least two avirulent loci.

If we analyze the specificity of the host-isolate interaction in the two tests, we can say that the isolates Hb005, Hb-Italian, Hb502, Hb517 and Hb-FP06 can be considered as races due to their specific reaction with the accessions that are not fully recognized by the hosts.

The resistance observed in the accessions was characterized by necrosis at the inoculation site and without sporulation of the parasite. Necrosis, according to Rethage et al. (2000), depends directly on the host-parasite interaction. According to Bittner-Edy et al. (1999), this phenotype determines the presence of race-specific resistance in the host and is controlled by a gene-to-gene interaction.

According to Person et al. (1976) and Crute et al. (1985) parasite variability awakens the function of gene-to-gene interaction. Lebeda and Schwin (1994) consider that pathogenic variability in *H. brassicae* is expressed through gene-to-gene interaction, which is essential for the recognition of physiological races (Parlevliet, 1985; Kema et al., 1996). According to De Wit (1992), the two types of resistance, specific and non-race specific, are closely related to gene-to-gene interaction.

The two gene-to-gene models presented in Tables 9 and 13 should explain the relationships between the differentiated accessions and the *H. brassicae* isolates.

In the first model, three pairs of resistance and avirulence genes were determined. The R1 gene was found in the G5 group accessions. This gene is the only one that conferring resistance to Hb501 in the accessions and could originate from *B. oleracea* var. gongylodes, *B. oleracea* var. *acephala* and *B. oleracea* var. *tronchuda*. The R2 gene was found in 'Coração de Boi' (group G2) and in G4 group accessions where tohether with R3 it confers resistance to the two UK isolates. The R3 gene confers resistance to the isolate Hb006 in accessions from group G3. The three genes R1, R2 and R3 probably confer partial quantitative resistance to the three *H. brassicae* isolates in the G5 group accessions, which is also defended by Bahcevandziev et al. (2015), and may represent an interesting type of durable resistance.

The model presented in Table 13 is based on five pairs of resistance and avirulence genes. Several hypotheses were made to explain some of the accession-isolate interactions. The most important genes

were defined as R1 and R2. The R1 gene confers resistance to the isolates Hb502, Hb517, Hb005, Hb-FP06 and Hb-Italian in the accessions of groups D6 and D8 and probably to the isolates Hb501, Hb006 and Hb-Murcia in the accessions of group D7. This gene could originate from the genomes of *B. oleracea* var. *gongylodes* and *B. oleracea* var. *tronchuda*. The R2 gene confers resistance to the isolates Hb501, Hb006, Hb-Murcia, Hb005, Hb-FP06 and Hb-Italian in the accessions of groups D7 and D8 and probably to Hb502 and Hb517 in accession KB11 / 00. Its origin coincides with that of the R1 gene. In the D8 group accessions these two genes together conferred partial quantitative resistance to the eight *H. brassicae* isolates, which could be part of a durable resistance (Bahcevandziev et al., 2015).

The R3 gene is the only one that confers resistance to the isolate Hb005 in 'Coração de Boi'. The R4 gene confers resistance to the Hb-FP06 isolate in ‘Predzvest’, originating from this accession. The R5 gene confers resistance to the Hb-Italian isolate in the D3 group accessions. These three genes together (R3, R4 and R5) are also present in KB4 / 00 where they confer resistance to the isolates Hb005, Hb-FP06 and Hb-Italian.

Previously published studies show the existence of two *H. brassicae* races in relation to *Brassica oleracea* plants (Felton and Walker 1946; Wang 1949; McMeekin 1969; Natti et al. 1967; Thomas and Jourdain 1990; Thomas and Jourdain, 1992), but neither has been found in Europe. The only paper referring to the presence of three *H. brassicae* races in Europe, particularly in Switzerland, was published by Gaümann in 1926. Since then, nothing more has been published on *H. brassicae* races in Europe. This work should be considered as a preliminary basis for future studies on the potential of *H. brassicae* races in Europe.

## Conclusions

The results obtained in this study regarding the phenotypic/genotypic interactions between the different *Brassica oleracea* accessions and *H. brassicae* isolates from different regions in Europe, showed the existence of the downy mildew putative races. These isolates were distinguished by different levels of virulence and by the expression of genomes in the accessions, which demonstrated their specificity.

The gene-to-gene model analysed in this work can lead to a deeper investigation of the resistance genes present in brassicas and their avirulence genes that can be found in *H. brassicae* isolates.

This model, which identifies a potential source of downy mildew resistance in *Brassica oleracea* plants, needs to be genetically characterised and further exploited in breeding programmes.

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