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ARTICLE

Exploiting intra-cultivar variation to select for *Barley yellow dwarf virus-PAV* (BYDV-PAV) resistance in barley

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Abstract: Selection within elite barley cultivars is assumed to be ineffective due to the belief that inbred cultivars are highly homogeneous. The objective of the present work was to evaluate a selection procedure (Honeycomb design) applied within five barley cultivars (Manel, Rihane, Kounouz, Lemsi, and Imen) and two Tunisian landraces (Ardhaoui and Djebali) under ultra-low plant density (1.2 plants m^{-2}) towards selecting high-performance lines with resistance to *Barley yellow dwarf virus-PAV* (BYDV-PAV). Lines selected through this process were further field-evaluated in hill plots under artificial BYDV-PAV inoculation and uninoculated control conditions during the 2016–2017 cropping season. Artificial inoculation in the field caused a severe reduction in agronomic performance traits, with yield loss reaching around 60%. However, two lines (IH16-H1 and IH4-L0) originating from cultivar Imen were significantly superior over the mother variety in the control field, showing at the same time minimum yield loss after BYDV-PAV inoculation not exceeding 10%, similar to the resistant check. Genotyping of the lines for the *Ryd2* and *Ryd3* resistance genes and assessment of visual symptoms in the field associated with reduction in yield revealed an additive effect of the genes conferring resistance to BYDV-PAV. However, there were lines with genotypic patterns that did not match the patterns of the source material, providing insights for exploitable intra-cultivar diversity within the barley cultivars and landraces assessed.

Key words: barley, BYDV-PAV, intra-cultivar variation, single plant selection, ultra-low plant density.

Résumé : Bien que, les cultivars d'orge sont supposés homogènes et stables, la sélection à très faible densité s'est avérée un outil efficace pour l'exploitation de la variabilité intra-cultivar et la sélection de lignées de hautes performances agronomiques et résistantes au virus de la jaunisse nanisante de l'orge (BYDV-PAV). La présente étude a été abordé en se référant à la méthode «Honeycomb design» basée sur le choix des individus performants au sein d'une population/cultivar existante, semée à très faible densité (1.2 plantes m^{-2}) et a porté sur 5 variétés inscrites (Manel, Rihane, Kounouz, Lemsi, et Imen) et 2 populations locales (Ardhaoui et Djebali). En sus des performances étudiées, les lignées plantes évaluées pour leurs comportements agronomiques ont été jugées pour leur résistance à l'égard du au virus de la jaunisse nanisante de l'orge (BYDV-PAV) dont l'importance en Tunisie a été prouvée. Cette évaluation a été faite en conditions d'inoculation artificielle durant la campagne agricole 2016–2017. Les résultats ont révélé que la perte de rendement n'a pas dépassé les 10% chez les deux lignées (IH16-H1 et IH4-L0) provenant de la variété Imen. La résistance relative au BYDV de ces deux lignées, a été également prouvée sur le plan moléculaire par la présence des deux gènes de résistance *Ryd2* et *Ryd3* moyennant l'utilisation de marqueurs SSR. [Traduit par la Rédaction]

Mots-clés : orge, BYDV-PAV, variabilité intra-cultivar, sélection de plante, très faible densité.

Introduction

Barley (*Hordeum vulgare* L.) is considered the fourth most important cereal crop in the world after wheat, corn, and rice, with nutritional benefits for both livestock and humans (Elke and Emanuele 2013). Barley is a member of the grass family and is one of the eight

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founder crops (einkorn wheat, emmer wheat, barley, lentil, pea, chickpea, bitter vetch, and flax) (Lakshmi et al. 2016). It is mainly used as animal feed (around two-thirds of global production) (Schulte et al. 2009) and is predominantly considered as a food crop in many parts of the globe like the semiarid regions of North Africa (Morocco, Algeria, Libya, and Tunisia), the Middle East (Saudi Arabia, Iran, Iraq, and Syria), the highlands of Nepal, Ethiopia, Tibet, the Andean countries of South America (Peru and Chile), and in some Asian countries (China and North Korea) and the Himalayas (Lakshmi et al. 2016).

Yellow dwarf viruses (YDVs) cause a serious disease affecting small grain crops around the world (Lister and Ranieri 1995; Miller and Rasochova 1997). The disease was first described in barley by Oswald and Houston (1951) and affects all major cereal crops (rice, maize, wheat, oat, and rye) as well as other grass species. The disease is caused by a group of phloem-limited luteoviruses known as yellow dwarf viruses that are strictly transmitted via aphid vectors (Hewings 1995). Based on molecular characterization, causal agents of YDVs are upgraded to the species level and 10 viruses are now included in the family Luteoviridae, viz. *Barley yellow dwarf virus* (BYDV)-PAV, BYDV-PAS, BYDV-MAV, BYDV-KerII, BYDV-SGV, BYDV-GAP, BYDV-KerIII, *Cereal yellow dwarf virus-RPV* (CYDV-RPV), CYDV-RPS, and *Maize yellow dwarf virus* (MYDV-RMV) (Adams et al. 2014).

The main symptoms produced by BYDVs are dwarfing of the roots and shoots associated with yellowing (Scheurer et al. 2001) or discoloration and the severity of symptoms is highly dependent on the host genotype. Moreover, infected plants are more prone to fungal infection and abiotic stresses, with a negative effect on yield and delay in heading date (D'Arcy 1995).

Šíp et al. (2004) previously showed on a selected set of Atlas 68 × Igri double haploid lines that the resistance to BYDV mediated by the *Ryd2* gene was strongly dependent on ambient environmental conditions. BYDVs induce yield losses ranging from 5% to 80%, with an average of 30% in affected fields (Perry et al. 2000). Tolerance to BYDV-PAV has been well-studied (Riedel et al. 2011). Several genes have been reported as contributing tolerance/resistance to BYDV: *ryd1*, a recessive gene identified in the spring barley cultivar 'Rojo' (Suneson 1955); *Ryd2* and *Ryd3*, identified in Ethiopian landraces (Schaller et al. 1964; Niks et al. 2004). *Ryd2* is the most widely used gene for BYDV tolerance in commercial barley breeding (Burnett et al. 1995; Ovesna et al. 2002; Šíp et al. 2004). This gene is located on chromosome 3HL (Collins et al. 1996) and is associated with a reduction in virus concentration for BYDV-PAV and BYDV-MAV but not for CYDV-RPV (Baltenberger et al. 1987; Banks et al. 1992; Ranieri et al. 1993; Capettini et al. 2002). *Ryd4 Hb* has been transferred to common barley from the wild relative *Hordeum bulbosum* L. (Johnston et al. 2009; Scholz et al. 2009; von Bothmer et al. 1995) but has not been used in barley breeding programs due to linkage drag. Several other quantitative trait loci have been identified in barley in regions near the *Ryd2* gene on chromosome 3HL (Scheurer et al. 2001). Marker-assisted selection has been used in barley for the identification of *Ryd2* (Ovesna et al. 2000).

Earlier surveys of the different BYDV species infecting barley in Tunisia have also reported the natural occurrence of BYDV-PAV as the predominant species in cereal crops (Makkouk et al. 2001). It is also assumed to be the most widespread (D'Arcy 1995) and usually causes the most severe symptoms in most cereal crops. Other studies conducted in Tunisia have reported the wide occurrence of BYDV in barley, reaching 30% incidence when infection starts at the early growth stage of the plant (Bouallegue et al. 2014; Najar et al. 2017*a*). Previously, only BYDV-PAV isolates were identified in Tunisia on the basis of serological detection, but the presence of BYDV-PAS and BYDV-MAV species was recently reported (Najar et al. 2017*a*). Methods of control for BYDVs include insecticides to control aphid populations; however, insecticides may only be feasible in highly intensive agricultural systems. The most effective and sustainable control method is the use of genetic resistance/tolerance to the virus complex (Burnett et al. 1995). Two types of resistance to BYDVs have been distinguished: virus resistance and field resistance. Virus resistance refers to low virus titer in infected plants, whereas field resistance (tolerance) refers to the reduction of symptoms of infection independent of the virus titer (Kosova et al. 2008). In this paper, resistance will be defined as a reduced viral replication in infected plants (Cooper and Jones 1983). Tolerance will, therefore, be defined as the development of mild or negligible symptoms in infected plants. It can also be stated as the ability of plants to maintain yield under BYDV infection.

Regarding its reproductive system, barley is an inbred crop and as such, elite barley cultivars are considered to be genetically homogeneous. Nevertheless, even within fairly homogeneous gene pools, an intrinsic amount of latent genetic variation may still occur, whereas mechanisms that generate de novo variation may also be present. Residual heterozygosity due to segregation of parental loci during the breeding process is presumably one source of genetic variation (Haun et al. 2011; Tokatlidis 2015). On the other hand, additional heterogeneity might stem from de novo generated variation, resulting from spontaneous mutations (Shaw et al. 2000; Ossowski et al. 2010) or via genetic and epigenetic mechanisms, such as intragenic recombination, unequal crossing over, gene duplications or deletions, DNA methylation, excision or insertion of transposable elements, and chromatin alterations (Rasmusson and Phillips 1997; Sani et al. 2013, Cavrak et al. 2014; Kim and Zilberman 2014).

While genetic variation derived from crosses is the primary source of selection for improved cultivar performance, evidence from selection experiments within narrow gene pools of many crops substantiates a significant gain through the exploitation of intra-cultivar

Table 1. Pedigree of source material (El Felah et al. 2015) evaluated at the R-7 honeycomb trial.

No.	Name	Cross	Origin	Year of registration
1	Ardhaoui	Tunisian landrace	Tunisia	—
2	Djebali	Tunisian landrace	Tunisia	—
3	Kounouz	Alanda//Aths/4/Pro/Toll//Cer*2/Toll/3/5106/6/24569	Tunisia/ICARDA	2010
4	Rihane	As 46/Avt//Aths	ICARDA	1987
5	Lemsi	Selection from Rapidan	USA	2009
6	Manel	Lignee527/5/As54/Tra//2*Cer/TolI/3/Avt/TolI//Bz/4/Vt/Pro//Toll	Tunisia/ICARDA	1996
7	Imen	QB813-2/3/Lignee527/NK1272//JLB70-63	Tunisia/ICARDA	2011

variation (Fasoula 1990; Traka-Mavrona et al. 2000; Fasoula and Boerma 2005; Tokatlidis et al. 2006; Tokatlidis et al. 2008; Fasoula 2011). In terms of plant breeding, intra-cultivar variation refers to the genetic variation from plant to plant within a named cultivar (Tokatlidis 2015). To exploit this variation, a specific condition for selection under ultra-low density that excludes plant-to-plant interference for resources (i.e., nil competition) is a prerequisite. Nil competition maximizes the phenotypic expression of genetic differences among individuals within a narrow gene pool, thus facilitating the detection of desirable genotypes (Kyriakou and Fasoulas 1985; Fasoula and Fasoula 2002; Tokatlidis et al. 2010). Furthermore, selection under ultra-low densities erases the confounding effects of competition on the identification of high-yielding genotypes induced by the negative relationship between yielding and competitive ability (Kyriakou and Fasoulas 1985; Chatzoglou and Tokatlidis 2012; Ninou et al. 2014), while an additional merit is that nil competition attains greater heritability by minimizing the acquired variance arising from nongenetic sources (Fasoula and Fasoula 2002; Tokatlidis 2015).

The objective of this study was to investigate the presence of genetic variation for tolerance/resistance to BYDV-PAV within five released cultivars and two Tunisian landraces by screening under artificial field inoculation and use of simple sequence repeat marker first and second generation lines derived by single-plant selection at ultra-low density. The potential of this novel approach to exploit latent or de novo variation within barley cultivars for the development of high-yielding lines combining virus tolerance is also discussed.

Material and Methods

Plant material and selection history

Source material

The source material was comprised of five commercially released barley cultivars and two Tunisian landraces, characterized by different susceptibility levels to BYDV-PAV infection. These seven cultivars/landraces are either the ones most widely grown in the barley fields in Tunisia (Manel, Rihane Ardhaoui, and Djebali) or considered as the most promising newly released cultivars (Imen, Kounouz, and Lemsi) (El Felah et al. 2015) and, therefore, were selected for experimentation. Information on the pedigree, origin, and year of registration regarding this material is presented in Table 1.

Single plant selection to form first cycle selection lines

Seed lots were provided by the Field Crop Laboratory of the National Agricultural Research Institute of Tunisia (INRAT). A mass selection process had been applied for both of the landraces during the previous year to upgrade moderately their agronomic performance traits while improved cultivars had been maintained according to the ear-to-row model. On 5 Dec. 2013, the seven entries listed in Table 1 were planted in a field trial at the Kef experimental station of INRAT under the ultra-low density of 1.2 plants m^{-2} according to an R-7 honeycomb field layout (Fasoulas and Fasoula 1995). Three seeds were sown in each hill, thinned to a single plant per hill after emergence. A total of 22 rows 0.86 m apart were planted, each with 98 individual plants 1.00 m apart within each row so that at the full establishment of each of the seven entries in the trial was represented approximately by 308 individual plants.

Selection between entries was based on the estimation of three parameters for each of the entries as described by Fasoula and Fasoula (2000), which were (*i*) the entry's mean ($\bar{x}$), (*ii*) the entry's standardized mean ($\bar{x}/s$), and (*iii*) the entry's standardized selection differential ($\bar{x}_{sel} - \bar{x}/s$), for which the individual plant yields of the top 15 plants were used to calculate for each particular case the $\bar{x}_{sel}$. The top three entries that combined the highest scores for these parameters were the ones selected: Imen, Ardhaoui, and Djebali in this case.

To form the first cycle lines, divergent single plant selection for high and low yield within the top three entries was applied by the moving-circle procedure (Fasoulas and Fasoula 1995). In particular, the selected high-yielding plants were those that had the highest grain weight compared with the mean of 36 surrounding plants (i.e., 0.027 selection pressure). Similarly, the moving-circle approach identified the lowest-yielding individual plants, but in this case, selected low-yielding plants should weigh at least 10 g of grains, to get enough seeds for the following steps of the procedure.

Thus, by applying this approach, five plants from Imen, two from Ardhaoui, and three from Djebali, were selected as high yielders; at the same time, two plants

Table 2. Genealogy of the single-plant progeny lines selected through divergent selection in BYDV trial and source material.

Source material	First-cycle HY lines	First-cycle LY lines	Second-cycle HY lines	Second-cycle LY lines
Ardhaoui	AH9, AH10	AL0	AH9-H1, AH9-H2, AH9-H3, AH10-H1, AH10-H2, AH10-H3	AH9-L0, AH10-L0
Imen	IH4, IH16, IH17	IL0	IH4-H1, IH4-H2, IH4-H3, IH4-H4, IH16-H1, IH16-H2, IH16-H3, IH17-H1, IH17-H2, IH17-H3, IH5-VS	IH4-L0, IH16-L0, IH17-L0
Djebali	DH2, DH12	DL0	DH2-H1, DH2-H2, DH2-H3, DH2-H4, DH2-H5, DH12-H1, DH12-H2, DH12-H3, DH14-VS	DH2-L0, DH12-L0
Manel	MH18	ML0	MH18-H1, MH18-H2, MH18-H3	MH18-L0
Rihane	—	—	RH8-VS	—

Note: HY, high yield; LY, low yield. Coding of lines is based on two letters and the number of the selected plant. In the case of bulk sampling this is indicated with 0. The first letter indicates the source material from which the line has been selected (A, Ardhaoui; I, Imen; D, Djebali; M, Manel; R, Rihane). The second letter indicates whether the selection is based on high yield (H) or low yield (L). Cases indicated with VS stand for visual selection.

from each of the three entries were picked as low yielders. Another plant, from cultivar Manel, was selected visually due to its outstanding performance in the field. Seeds of each selected plant constituted a separate line and the 17 first cycle lines were selected for further evaluation and selection.

Progeny evaluation and selection to form second cycle selection lines

On 19 Nov. 2014, the 17 first cycle lines, along with the original seed lots of Imen, Ardhaoui, Djebali, and the cultivar Rihane (as the most widely cultivated cultivar in Tunisia), were sown in a field trial at Kef experimental station using an R-21 honeycomb layout (Fasoulas and Fasoula 1995). Each entry was represented approximately by 120 individual plants at the ultra-low density of 1.2 plants m^{-2}. Similar to the previous season, divergent selection was applied to select the plants with the highest and lowest grain weight, compared with the mean of the 36 surrounding plants (0.027 selection pressure). A total of 30 high-yielding and 40 low-yielding plants were selected through this approach, the latter ones representing eight different entries. These low-yielding plants were then bulked for each of the eight entries to have enough seeds for the following season trials. Thus, seeds of each of the selected 30 individual high-yielding plants and the bulk low-yielding selected samples from the eight entries formed the 38 second cycle lines.

These 38 lines along with the 12 first cycle lines were planted in the next cropping season (2015–2016) for seed multiplication.

BYDV nursery

On 27 Nov. 2016, the 38 second cycle lines along with the 12 (8 high-yielding and 4 low-yielding) first cycle lines and 6 checks (Imen, Ardhaoui, Djebali, Manel, Rihane, and ICB01) were planted in a field trial at the Mornag INRAT experimental station in Tunisia. The ICB01 check had been developed by the International Center for Agricultural Research in the Dry Areas (ICARDA) (Cross: Sutter*2/Numar/4/Baca'S/3/AC253//CI0887/CI05761) and was included in the trial as check resistant to BYDV (Najar et al. 2017*b*). Genealogy of the progeny lines and the involved checks is given in Table 2. Two nearby fields were planted, one to be used as a control and the other as the inoculated field. For both, a nonreplicated augmented design was used with five uncompleted blocks and 16 entries per block. Checks were randomly allocated in all of the five blocks (i.e., five replications per check). The trials were established as hill plots with a seeding rate of 25 seeds per hill.

For the control plots, seeds were treated before planting with Celest top [diféconazole (25 g L^{-1}) + fludioxonil (25 g L^{-1}) + thiamethoxam (262.5 g L^{-1})] at a rate of 200 mL hL^{-1} of seeds to protect uninoculated control plots from both aphids and viruses. Basic fertilizer in the form of diammonium phosphate (18–46–0) was applied before planting at a rate of 100 kg ha^{-1}. Complete weed control was attained by chemical applications [axial: pinoxaden (100 g L^{-1}) + cloquintocet-methyl (25 g L^{-1}) at a dose of 1 L ha^{-1} for the narrow leaf weeds and zoom: dicamba (66%) + triasulfuron (4%) at a dose of 180 g ha^{-1} for the broad leaf weeds] and hand-weeding. Two spring foliar spray applications of Ogam [kresoxim-methyl (125 g L^{-1}) + epoxiconazole (125 g L^{-1})] at a rate of 0.7 L ha^{-1} were applied to minimize yield reductions due to fungal diseases and thus measuring the pure effect of virus inoculation.

BYDV-PAV isolate used in this study was characterized (serologically and molecularly) earlier (Najar et al. 2017*b*). BYDV inoculation was carried out through the aphid vector *Rhopalosiphum padi* L. (Comeau 1984). A pure, virus-free colony of this species was established from a single apterous aphid collected from asymptomatic *Avena fatua* L. plants from northern Tunisia and reared in a screen cage in a glasshouse under controlled conditions

of 19 °C ± 1 °C and 16:8 h photoperiod for 2 wk to increase aphid numbers to a level satisfactory for the number of hills to be inoculated. Four weeks after planting at the tillering stage, Feekes growth stage 3, aphids were transferred and kept on infected barley plants (cultivar Rihane) for 48 h for virus acquisition. About 10–15 viruliferous *R. padi* were then placed on each test plant to be inoculated. To minimize the chances of disease escapes, all tested plants were inoculated twice, at 3 d intervals. Two days after the second virus inoculation, an insecticide was applied to kill the viruliferous aphids and minimize the spread of the virus to the control plots. After symptoms development (3–4 wk post inoculation), selected plants were then tested again. Five to six weeks post-inoculation, each plant was tested for virus presence by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) according to Clark and Adams (1977). Virus presence was also checked by tissue blot immunoassay 15 d after inoculation (Makkouk and Kumari 1996).

DNA extraction and markers detection

Total genomic DNA was extracted by the cetyl trimethylammonium bromide method of Saghai-Maroof et al. (1984) with some modifications as adapted by Udupa et al. (1999). Fresh young leaves were collected from greenhouse-grown plants of all entries. The isolated DNA was estimated both qualitatively and quantitatively using 1.0% (*w*/*v*) agarose gels by comparing bands to known concentrations of lambda DNA.

Polymerase chain reaction (PCR) was performed in a reaction volume of 10 μL containing 1× PCR buffer (1.5 mmol L^{-1} $MgCl_2$), 200 μmol L^{-1} of each dNTPs, 0.01 nmol L^{-1} of each primer, 0.5 U of *Taq* DNA polymerase (Promega), and approximately 50 ng of genomic DNA. The amplified DNA was run in 6% native polyacrylamide gels, prepared in a vertical electrophoresis unit (CBS Scientific) using 0.5× TBE buffer. Gels were stained with ethidium bromide and visualized under UV light.

For the detection of the *Ryd2* gene, the CAPS-Marker Ylp PCRM was used according to Ford et al. (1998). Screening for the presence of the *Ryd3* gene was conducted using the microsatellite marker HVM74 (Niks et al. 2004).

Disease readings and data collection of agronomic and physiological traits

Disease readings following BYDV inoculation were recorded twice, initially at 4 wk after inoculation and later at the full heading stage. Symptoms were evaluated on a scale from 0 to 9 (where 0 represents no symptoms and 9 represents yellowing over the whole plant and stunting) (Qualset 1984; Hewings et al. 1992).

Chlorophyll fluorescence, to determine the F_0, F_m, F_v, F_v/F_m, and F_v/F_0 parameters of the photosystem II (PSII) as described by Baker (2008), was measured at the fully expanded flag leaves of three representative plants of each hill plot at the heading stage using an OPTI-SCIENCE OS30+ handheld portable fluorometer.

The number of fertile tillers per plot was counted at maturity and the height of five randomly selected plants within each plot was recorded by measuring the distance in centimeters from the soil level to the tip of spikes excluding awns. Hill plots were individually hand-harvested and grain yield was recorded after threshing all the plants of the plot.

Data analysis

Raw data values for agronomic and physiological traits were first analyzed for differences between control and inoculated fields. Then the percentage of loss due to the inoculation with BYDV-PAV was calculated for all the agronomic performance traits. JMP version 13.0.0. was used to conduct the analysis of variance for an augmented design with entries as the fixed factor and blocks as random. Best linear unbiased estimates (BLUEs) were derived and appropriate standard errors of means (i.e., between checks, between entries of the same block, between entries of different blocks, and between entries and a check) were used to determine significant differences.

To identify both high-yielding and resistant entries, a biplot graph was generated using as reference axes the BLUEs values for the grain yield per hill plot at the control field and the percentage of yield loss due to BYDV inoculation. Those entries that surpassed the intersection of the lines traced at the axis point of 25% cut for higher-yielding entries (*x* axis) and for the upper threshold of 20% yield loss (*y* axis) were considered jointly as high-yielding and resistant to BYDV (Fig. 1).

JMP was also used to determine partial correlations among all agronomic and physiological traits along with the disease scoring. A box plot graph was also generated based on the presence or absence of *Ryd2* and *Ryd3* genes in the entries and their response to yield reduction and disease symptoms development (Fig. 2).

Independent sample *t* tests for the four entry categories (i.e., absence of both genes, presence of *Ryd2* with absence of *Ryd3*, absence of *Ryd2* with presence of *Ryd3*, and presence of both genes) to reveal differences for yield loss and visual symptoms due to BYDV infection were conducted using the SPSS version 24.0.0.0. statistical package.

Results

Performance of source material and selected lines in honeycomb trials

The average yield of all cultivars and landraces tested in the joint R-7 trial reached 58 g $plant^{-1}$. Regarding the first parameter assessed in honeycomb methodology, cultivar Imen was the one outperforming all the others with its average mean being 70.3 g $plant^{-1}$ followed by the two landraces Ardhaoui and Djebali (63.2 and 62.6 g $plant^{-1}$, respectively). Among all the entries tested, cultivar Lemsi showed the lowest yield, not

Fig. 1. Percentage of yield reduction due to virus inoculation against grain yield at the control field [the latter expressed as the best linear unbiased estimates (BLUE)]. The reference lines were traced at the position of the 25% best entry for the *x* axis and for a reduction threshold value of 20% for the *y* axis. The entries in the bottom right corner of each graph can be considered as both having high yield performance and sustain low losses due to virus inoculation. A dark black circle is used to identify these "top-performing" genotypes.

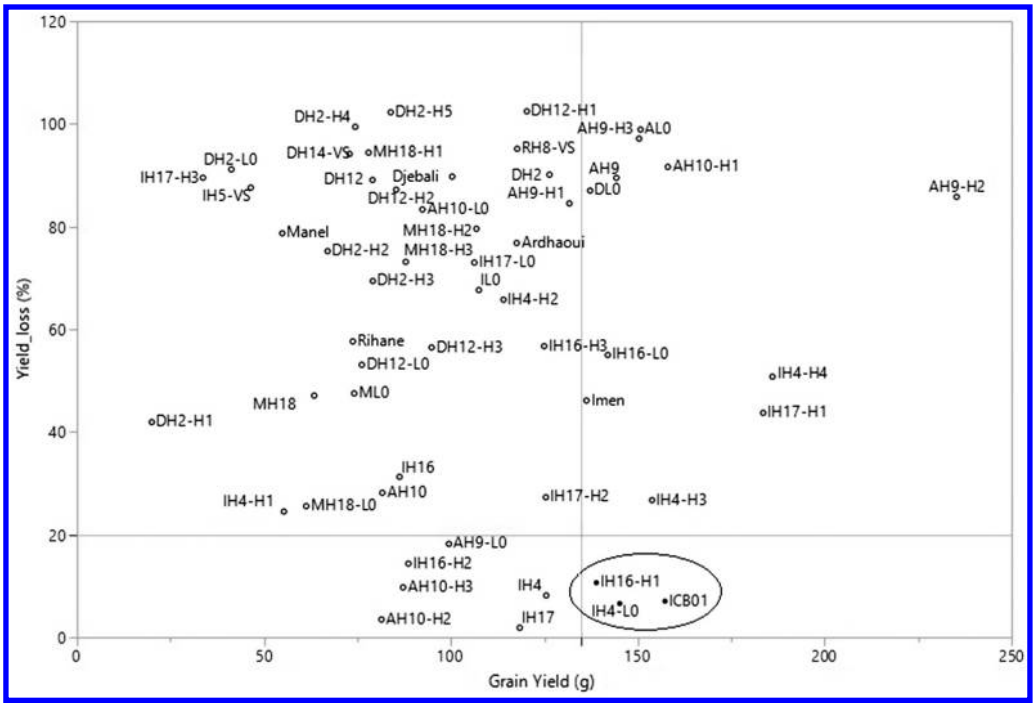
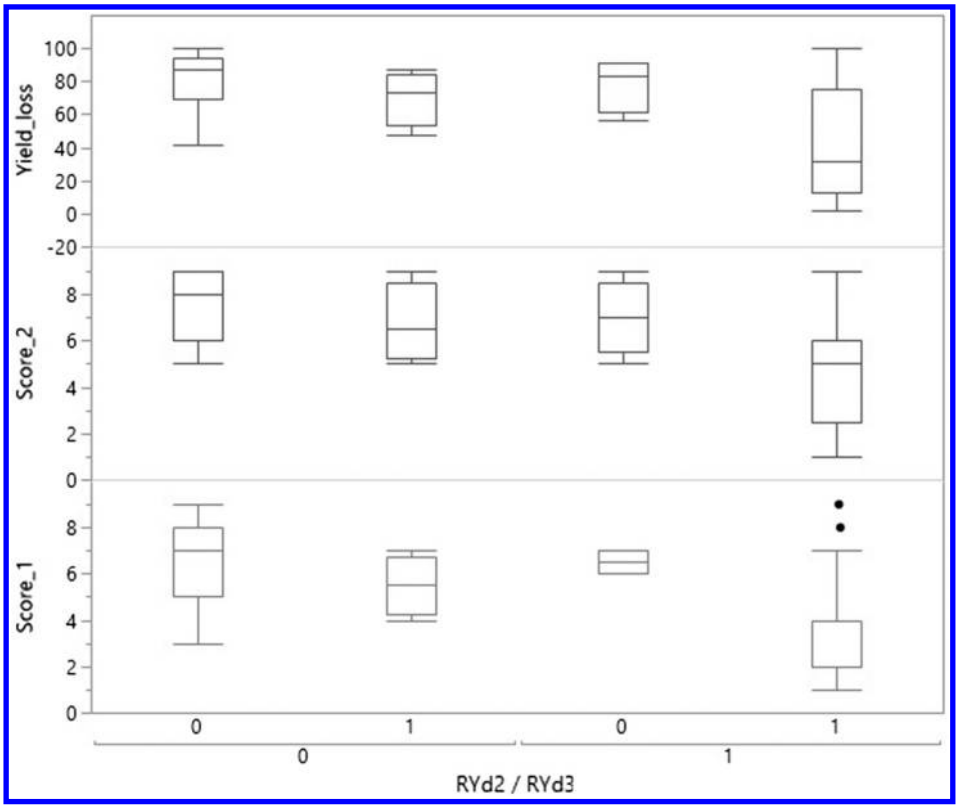


Fig. 2. Yield losses and visual symptom scoring box plots graph for the different combinations of *RYd2* and *RYd3* genes as expressed in the 56 barley entries.



exceeding 43 g plant^{-1}. The same three entries were also top-scoring for the second parameter of honeycomb methodology with $\bar{x}/s$ values being 1.85, 1.87, and 1.83 for Imen, Ardhaoui, and Djebali, respectively (data not shown). The latter implies a relatively narrow genetic variation for the specific seed lots of the two landraces to almost similar levels with the improved cultivars, possibly due to the mass selection that these two landraces had previously been subjected to. As far as for the third criterion, the ranking was to some extent reversed, with cultivars Lemsi and Kounouz being the top-scoring followed by Imen (data not showed).

For the next season's R-21 trial, the average yield of all first-cycle selected lines was 46.4 g plant^{-1}. As a general pattern, most of the first generation lines that had been selected as high-yielding proved consistency in terms of yield, with 13 of them outperforming the best check, in this case cultivar Rihane (data not shown).

Performance of selected lines in BYDV-PAV nursery

Growth and yield parameters

The average yield for the control trial was estimated at 105 g per hill plot (Table 3). Among the top 14 (best 20%) of the entries six lines selected from Imen, five from Ardhaoui, and one from Djebali were recorded, while the checks ICB01 and Imen were also included (Table 3). Line AH9-H2, a second-cycle high-yielding line selected from the landrace Ardhaoui, was the one that surpassed significantly the yield of the best check, ICB01 (235.28 vs 157.28 g per plot) (Table 3). Among the bottom 14 entries, 6 lines were from Djebali, 3 from Manel, and 3 from Imen, including the checks Manel and Rihane (Table 3).

The BLUEs values for the number of fertile tillers in the control field ranged from 11.07 to 99.4, with an average of 46.4 tillers per plot (Table 3). Again, line AH9-H2 outperformed the best check ICB01 with 99.4 tillers vs 63.8 tillers per plot (Table 3). All checks other than Rihane had at least one of their lines included among the top 14 entries. The 14 inferior lines, in terms of number of fertile tillers, included lines selected from all the checks during the selection history, with the original population of Manel being the only one of the checks included in this group (Table 3).

Plant height ranged from 62.83 to 99.83 cm, with an average of 81.56 cm (Table 3). Among the group of 20% tallest entries, six lines were from landrace Djebali, whereas the original population of Djebali along with the checks Rihane and ICB01 were also included in this group. The rest of the entries came from Imen (three lines), Manel (one line), and Ardhaoui (one line) (Table 3). Among the group of 20% shortest, six lines were selected from Imen, three from Djebali, three from Ardhaoui, and one from Manel. In addition, the original population of Manel was the only one of the checks among the group of the shortest entries (Table 3).

Chlorophyll fluorescence responses

For the PSII-related parameters, no significant differences were detected in most of the cases among entries for the control trial (Table 3). The ratio F_v/F_m, which according to Baker (2008) determines the maximum efficiency at which light is absorbed by light-harvesting antennae of PSII and is converted to chemical energy, ranged from 0.56 to 0.75, with an average of 0.70. The checks Imen and Djebali outperformed all the other checks, whereas another six lines from Djebali, three from Imen, two from Manel, and one from Ardhaoui were among the ones forming the top 14 group (Table 3). On the other hand, the lower BLUEs values for the F_v/F_m ratio were derived from five lines originating from Ardhaoui, the original population of this landrace used as a check, three lines selected from Imen, three from Djebali, one from Manel, and one from Rihane (Table 3).

Similar patterns with the F_v/F_m ratio were recorded when the other PSII-related parameters were assessed (i.e., F_v, F_m, and F_v/F_0) with almost the same entries and checks being among the top and low scoring ranking positions (Table 3). The pattern was different for the F_0 parameter, for which the highest scores were equally shared among 12 lines selected from Ardhaoui, Imen, and Djebali, along with two lines from Manel, none of them being significantly higher than the higher-scoring check Imen (Table 3). The lowest F_0 scores were prevalent among lines selected from Imen (seven lines), followed by two lines from Djebali, two from Ardhaoui, and two from Manel, with the check ICB01 also included within the entries of this group (Table 3).

Response of selected lines to BYDV inoculation

Combined analysis of variance for both the control and inoculated fields revealed significant reduction in all agronomic performance traits due to BYDV inoculation treatment (Table 4). On average, grain yield per plot was reduced by 60.43%, the number of fertile tillers dropped to half (50.27% reduction), and plant height in the inoculated plots was reduced by 23.59% compared with the control plots (Table 5). Significant interactions between entries × BYDV treatment were also revealed (Table 4), with the line AH9-H2 being the top yielder in the control field and the least performing in the inoculated field, showing >85% reduction in grain yield per plot (Table 5). Three lines (two selected from Imen and one from Ardhaoui) outperformed the resistant check ICB01 by showing lower yield loss, although these differences were not significant (Table 5). Among the rest of checks, only cultivar Imen showed <50% yield reduction, whereas there were at least five lines selected from Imen (IH17, IH4-L0, IH4, IH16-H1, and IH16-H2) that showed significantly lower yield reduction after BYDV inoculation than their source material (Table 5). On the other hand, Djebali was the check with the higher grain yield reduction, reaching almost 90%, while 6 out of the

Table 3. Entries' BLUEs values for agronomic performance traits and photosynthesis-related parameters for the control field (blocks are indicated to facilitate comparisons for appropriate standard error).

Entries	Block	Grain yield (g plot^{-1})	Fertile tillers	Plant height (cm)	F_0	F_v	F_m	F_v/F_m	F_v/F_0
AH9-H2	3	235.28	99.4	89.67	188.33	494.37	682.70	0.73	2.61
IH4-H4	1	186.03	56.07	77.67	167.50	376.53	544.03	0.69	2.26
IH17-H1	5	183.54	39.9	76.17	164.33	423.37	587.70	0.72	2.58
AH10-H1	5	158.04	51.9	80.17	182.33	358.37	540.70	0.67	2.00
ICB01	—	157.28	63.8	87.40	156.80	350.80	507.60	0.69	2.31
IH4-H3	1	153.83	49.07	81.67	167.50	375.53	543.03	0.69	2.25
AL0	5	150.74	57.9	78.17	174.33	439.37	613.70	0.72	2.53
AH9-H3	2	150.36	18.4	62.83	189.83	379.03	568.87	0.66	1.93
IH4-L0	2	145.16	52.4	83.83	184.83	467.03	651.87	0.71	2.49
AH9	3	144.28	57.4	80.67	166.33	406.37	572.70	0.71	2.44
IH16-L0	3	141.98	27.4	70.67	168.33	430.37	598.70	0.72	2.55
IH16-H1	5	138.92	49.9	77.17	175.33	493.37	668.70	0.74	2.81
DL0	2	137.26	40.4	99.83	190.83	565.03	755.87	0.75	2.96
Imen	—	136.28	41.8	80.00	176.20	504.00	680.20	0.74	2.86
AH9-H1	4	131.69	69.23	74.67	189.00	426.70	615.70	0.69	2.28
DH14-VS	3	126.38	28.4	96.67	177.33	367.37	544.70	0.68	2.07
IH4	1	125.51	41.07	80.67	172.50	279.53	452.03	0.62	1.64
IH17-H2	1	125.41	39.07	75.67	154.50	325.53	480.03	0.67	2.12
IH16-H3	2	124.96	40.4	77.83	195.83	363.03	558.87	0.64	1.78
DH12-H1	1	120.33	62.07	88.67	172.50	395.53	568.03	0.69	2.30
IH17	2	118.44	44.4	79.83	182.83	398.03	580.87	0.68	2.11
RH8-VS	2	117.76	11.4	78.83	173.83	334.03	507.87	0.64	1.83
Ardhaoui	—	117.62	55.4	83.40	171.20	355.60	526.80	0.67	2.06
IH4-H2	3	114.08	42.4	85.67	190.33	456.37	646.70	0.71	2.39
IL0	4	107.49	65.23	74.67	203.00	620.70	823.70	0.75	3.09
MH18-H2	5	106.84	48.9	78.17	171.33	471.37	642.70	0.73	2.75
IH17-L0	4	106.29	70.23	88.67	186.00	475.70	661.70	0.72	2.59
Djebali	—	100.36	49.6	87.40	177.20	494.20	671.40	0.74	2.79
AH9-L0	4	99.47	48.23	81.67	175.00	268.70	443.70	0.61	1.55
DH2	2	94.86	46.4	89.83	202.83	480.03	682.87	0.70	2.33
AH10-L0	4	92.39	52.23	81.67	192.00	280.70	472.70	0.59	1.48
IH16-H2	5	88.64	57.9	88.17	177.33	377.37	554.70	0.68	2.15
MH18-H3	1	87.93	58.07	81.67	166.50	211.53	378.03	0.56	1.30
AH10-H3	1	87.23	40.07	76.67	171.50	486.53	658.03	0.73	2.83
IH16	3	86.28	33.4	73.67	170.33	497.37	667.70	0.75	2.91
DH12-H2	3	85.38	34.4	95.67	182.33	502.37	684.70	0.74	2.74
DH2-H4	5	83.94	15.9	83.17	176.33	405.37	581.70	0.70	2.32
AH10	3	81.66	46.4	78.67	179.33	525.37	704.70	0.75	2.92
AH10-H2	3	81.46	32.4	84.67	171.33	329.37	500.70	0.67	1.93
DH2-H2	5	79.14	68.9	84.17	169.33	392.37	561.70	0.70	2.33
DH12	2	79.06	35.4	79.83	194.83	539.03	733.87	0.73	2.75
MH18-H1	4	77.99	75.23	86.67	179.00	526.70	705.70	0.75	2.98
DH12-H3	3	76.18	40.4	99.67	175.33	279.37	454.70	0.63	1.60
DH2-L0	5	74.44	55.9	75.17	179.33	499.37	678.70	0.74	2.78
ML0	2	74.14	53.4	81.83	195.83	532.03	727.87	0.73	2.70
Rihane	—	73.78	50	88.80	180.60	448.60	629.20	0.71	2.49
DH2-H5	4	72.89	26.23	77.67	191.00	524.70	715.70	0.73	2.78
DH2-H1	1	67.03	48.07	77.67	167.50	499.53	667.03	0.74	2.97
MH18	2	63.46	52.4	88.83	189.83	448.03	637.87	0.70	2.32
MH18-L0	1	61.31	27.07	74.67	167.50	459.53	627.03	0.73	2.74
IH4-H1	5	55.34	67.9	95.17	148.33	432.37	580.70	0.74	2.90
Manel	—	54.92	33.8	71.00	184.00	412.00	596.00	0.69	2.23
IH5-VS	4	46.49	43.23	74.67	189.00	459.70	648.70	0.71	2.46
DH2-H3	4	41.29	18.23	73.67	161.00	204.70	365.70	0.56	1.29

Table 3. (*concluded*).

Entries	Block	Grain yield (g plot^{-1})	Fertile tillers	Plant height (cm)	F_0	F_v	F_m	F_v/F_m	F_v/F_0
IH17-H3	4	33.69	52.23	77.67	173.00	460.70	633.70	0.73	2.69
DH12-L0	1	20.02	11.07	67.67	178.50	430.53	609.03	0.70	2.41
Average	—	105.05	46.40	81.56	177.84	423.95	601.79	0.69	2.38
Standard error of difference									
Between checks	—	20.714	9.526	4.238	15.593	56.599	66.417	0.031	0.282
Between augmented entries (same block)	—	46.318	21.301	9.477	34.866	126.559	148.514	0.069	0.631
Between augmented entries (different block)	—	50.029	23.008	10.236	37.661	136.700	160.413	0.074	0.681
Between an augmented entry and a check	—	37.818	17.392	7.738	28.469	103.305	121.261	0.056	0.514

Table 4. Combined analysis of variance for agronomic performance traits and PSII-related parameters for control and inoculated fields, indicating values for degrees of freedom (df) and mean squares (MS).

		MS							
Source of variation	df	Grain yield	Fertile tillers	Plant height	F_0	F_v	F_m	F_v/F_m	F_v/F_0
Entries (adj.)	55	2121.797**	231.332	111.805*	122.836	2805.226	3876.601	0.00045	0.0418
Treatment (unadj.)	1	125108.425**	15117.521**	11786.349**	14.106	28017.118*	26774.020*	0.01492**	1.1850**
Entries × Treatment	55	1241.531*	330.156**	94.818*	454.048	10295.830**	13541.949*	0.00235**	0.2039**
Block (unadj.)	4	563.326	45.105	3.357	158.714	4069.058	6200.401	0.00024	0.0205
Residuals	44	563.948	124.292	45.492	267.149	3948.242	5685.833	0.00070	0.0608

Note: *, significant differences for $\alpha = 0.05$; **, significant differences for $\alpha = 0.01$.

10 lines that showed >90% grain yield loss were derived also from the Djebali landrace (Table 5). Reduction patterns for the number of fertile tillers and plant height were almost similar to the ones derived for the grain yield loss, with most of the lines that have recorded minimum yield loss and coming from Imen to be ranked again among the ones less affected by BYDV inoculation (Table 5).

To select simultaneously for high yield performance and resistance to BYDV inoculation, the biplot graph of Fig. 1 was generated using as reference axes the grain yield per plot at the control field (*x* axis) and yield reduction (%) due to BYDV inoculation (*y* axis). High-yielding entries were considered those being within the top 20% for grain yield (i.e., the ones within grain yield per plot >135 g), plotted all at the right side of the reference line traced vertically on the *x* axis at the point of 135 g. At the same time, a 20% yield reduction was considered as the upper acceptable threshold, therefore, entries plotted below the horizontal reference line traced from the *y* axis at the point of 20% were the ones considered as resistant to BYDV (Fig. 1). Thus, by applying the joint process, three entries (marked with dark black circle) were identified that combined both high yield per plot and resistance to the virus, with one being the resistant check ICB01 and the other two lines being IH4-L0 and IH16-H1, both selected from variety Imen (Fig. 1).

Except for F_0, PSII-related parameters were significantly affected by BYDV inoculation, revealing lower values for the inoculated field in comparison with the control field (Table 4). Similarly, significant interactions between entries × BYDV treatment were also observed for all the PSII-related parameters except F_0 (Table 4).

Molecular markers detection and disease symptoms

Considerable intra-cultivar/intra-landrace variation was revealed from molecular analyses for the presence of the *Ryd2* and *Ryd3* genes (Table 6). In all cases, lines were identified that revealed either novel diversity in reference to the presence of *Ryd2* and *Ryd3* genes or absence of the previously existing genes in relation to the source material from which they have been selected (Table 6). Thus, while both the *Ryd2* and *Ryd3* genes were present in the original cultivar Imen, in 2 (IH17-L0 and IH5-VS) out of the 18 total selected lines from Imen, the *Ryd2* gene was not traced (Table 6). The opposite situation occurred for cultivar Manel, for which none of the *Ryd2* and *Ryd3* genes have been detected in the original population, whereas two (MH18-H3 and MH18) out of the six lines selected from Manel revealed the

Table 5. Entries' BLUEs values for percentage reduction in agronomic performance traits due to inoculation with BYDV (blocks are indicated to facilitate comparisons for appropriate standard error).

Entries	Block	Grain yield	Fertile tillers	Plant height
IH17	2	1.88	0.00	4.12
AH10-H2	3	3.48	0.00	19.24
IH4-L0	2	6.59	0.00	5.28
ICB01	—	7.05	12.06	8.72
IH4	1	8.17	19.39	6.91
AH10-H3	1	9.74	19.39	12.31
IH16-H1	5	10.65	8.74	8.97
IH16-H2	5	14.37	39.65	21.63
AH9-L0	4	18.20	2.80	21.76
IH4-H1	5	24.50	47.20	24.34
MH18-L0	1	25.55	19.39	16.60
IH4-H3	1	26.72	19.39	16.89
IH17-H2	1	27.29	19.39	12.40
AH10	3	28.15	40.17	15.90
IH16	3	31.27	42.66	9.15
DH12-L0	1	41.92	19.39	38.98
IH17-H1	5	43.72	8.74	14.34
Imen	—	46.13	25.95	13.16
MH18	2	47.10	49.08	22.38
ML0	2	47.51	40.59	22.84
IH4-H4	1	50.78	36.41	12.22
DH12-H3	3	53.08	81.38	24.87
IH16-L0	3	55.00	0.00	4.10
DH2	2	56.46	41.65	22.15
IH16-H3	2	56.73	34.49	9.42
Rihane	—	57.65	44.38	26.81
IH4-H2	3	65.77	45.88	41.98
IL0	4	67.64	65.68	16.57
DH2-H2	5	69.40	67.83	30.94
IH17-L0	4	72.99	45.52	14.07
MH18-H3	1	73.15	60.20	9.39
DH2-H1	1	75.22	96.31	34.59
Ardhaoui	—	76.81	56.15	19.31
Manel	—	78.70	57.55	23.58
MH18-H2	5	79.58	41.34	8.87
AH10-L0	4	83.33	62.41	32.48
AH9-H1	4	84.52	65.50	20.46
AH9-H2	3	85.79	78.92	42.21
DL0	2	87.03	51.56	26.31
DH12-H2	3	87.19	79.59	24.99
IH5-VS	4	87.59	70.39	25.66
DH12	2	89.08	65.59	31.04
AH9	3	89.50	84.64	49.59
IH17-H3	4	89.53	82.06	36.94
Djebali	—	89.74	80.49	29.83
DH14-VS	3	90.12	71.65	29.80
DH2-H3	4	91.14	78.32	27.40
AH10-H1	5	91.64	63.84	14.93
DH2-H5	4	94.16	80.75	44.44
MH18-H1	4	94.41	84.88	52.74
RH8-VS	2	95.16	65.59	48.29
AH9-H3	2	97.13	43.23	11.03
AL0	5	98.91	99.65	39.64
DH2-L0	5	99.44	97.42	13.18
DH2-H4	5	99.99	99.99	62.62
DH12-H1	1	99.99	99.99	42.42

Table 5. (*concluded*).

Entries	Block	Grain yield	Fertile tillers	Plant height
Average	—	60.43	50.27	23.59
Standard error of difference				
Between checks	—	12.057	13.402	6.487
Between augmented entries (same block)	—	26.961	29.969	14.505
Between augmented entries (different block)	—	29.121	32.370	15.667
Between an augmented entry and a check	—	22.014	24.469	11.843

Table 6. Distribution of lines selected by each check cultivar/landrace according to the presence (+) or absence (–) of the *Ryd2* and *Ryd3* genes.

	No. of lines			
	RYd2, RYd3			
Source material	– –	+ –	– +	+ +
Imen (+ +)	No line	No line	IH17-L0, IH5-VS	IH4-H1, IH4-H2, IH4-H3, IH4-H4, IH16-H1, IH16-H2, IH16-H3, IH17-H1, IH17-H2, IH17-H3, IH4-L0, IH16-L0, IH4, IH16, IH17, IL0
Ardhaoui (+ –)	AH10-H1, AL0	No line	No line	AH9-H1, AH9-H2, AH9-H3, AH10-H2, AH10-H3, AH9-L0, AH10-L0, AH9, AH10
Djebali (+ –)	DH12-H2, DH2-H1, DH2-H2, DH2-H3, DH2-H4, DH2-H5, DH14-VS, DH12-L0, DH2, DH12, DL0	DH12-H3, DH2-L0	No line	DH12-H1
Manel (– –)	MH18-H1, MH18-H2, ML0	No line	MH18-H3, MH18	MH18-L0
Rihane (– –)	RH8-VS	No line	No line	No line

presence of the *Ryd3* gene and another line (MH18-L0) indicated the presence of both *Ryd2* and *Ryd3* (Table 6). With respect to the landrace Ardhaoui, the original population revealed the presence of only the *Ryd2* gene. However, in 9 out of the 11 selected lines from Ardhaoui (AH9-H1, AH9-H2, AH9-H3, AH10-H2, AH10-H3, AH9-L0, AH10-L0, AH9, and AH10) both the *Ryd2* and *Ryd3* genes were identified, whereas in the other two lines (AH10-H1 and AL0), none of the genes were present (Table 6). For the original population of the landrace Djebali, molecular analysis revealed the presence of only the *Ryd2* gene. A similar pattern was also confirmed for another two lines selected from Djebali. However, 11 of the lines selected from this landrace appeared to miss the *Ryd2* gene being present in the original population, while there was one line (DH12-H1) for which both the *Ryd2* and *Ryd3* genes were detected (Table 6). For the cultivar Rihane, no resistance genes were detected in the original population and also in the unique line selected from this cultivar (Table 6).

An additive effect can be inferred by the interaction of the *Ryd2* and *Ryd3* genes (Fig. 2), as the presence of only one of the two genes did not improve significantly a line's performance, and low disease scoring and yield reduction were achieved only in the presence of both genes (Fig. 2; Table 7). Thus, lines carrying both genes indicated significantly less yield reduction and showed less symptoms than lines with the absence of a resistance gene (Table 7). However, differences were narrowed to nonsignificant when comparisons were done between lines carrying one of the genes and lines with the absence of both genes (Table 7).

Correlation analysis

Correlation among all traits revealed that disease scoring (score 1 and 2) was highly correlated with the yield loss ($r = 0.72$–0.81, $p < 0.01$), fertile tillers loss ($r = 0.66$–0.75, $p < 0.01$), and plant height reduction ($r = 0.60$–0.69, $p < 0.01$) (Table 8). The correlations between disease scoring and PSII fluorescence parameters were significant for F_v ($r = 0.50$, $p < 0.05$) and F_m ($r = 0.49$-0.52, $p < 0.05$), while those for the rest of photosynthetic parameters were not significant (Table 8). F_m and F_v parameters also showed a significant correlation with the yield, fertile tillers, and plant height reduction ($r = 0.51$–0.52, $p < 0.05$; $r = 0.53$–0.54, $p < 0.05$ and

Table 7. Yield losses and visual symptoms scoring *t* values for the different combinations of *RYd2* and *RYd3* genes as expressed in the 56 barley entries (degrees of freedom in parentheses for each comparison).

			RYd2, RYd3			
RYd2	*RYd3*	Trait	– –	+ –	– +	+ +
–	–	Yield loss (%)	—	0.201 (21)	1.035 (21)	5.055** (45)
		Score 1	—	0.249 (21)	1.262 (21)	6.130** (46)
		Score 2	—	1.076 (21)	1.383 (21)	5.056** (46)
+	–	Yield loss (%)	—	—	0.715 (6)	3.498** (7)
		Score 1	—	—	1.414 (6)	3.149** (31)
		Score 2	—	—	0.212 (6)	1.777 (31)
–	+	Yield loss (%)	—	—	—	2.587* (7)
		Score 1	—	—	—	2.207* (31)
		Score 2	—	—	—	1.562 (31)
+	+	Yield loss (%)	—	—	—	—
		Score 1	—	—	—	—
		Score 2	—	—	—	—

Note: *, significant differences for $\alpha = 0.05$; **, significant differences for $\alpha = 0.01$.

$r = 0.58$, $p < 0.05$, respectively) but no correlation was revealed between the other PSII-related parameters and the reduction of agronomic performance traits (Table 8).

Discussion

In this study, a set of 50 lines selected by applying single-plant selection at ultra-low density within three commercial cultivars and two Tunisian landraces was exposed in the field to highly contrasting conditions created by BYDV inoculation. Results from the control field indicated that the selection process applied within each commercial cultivar and landrace succeeded in isolating single-plant progeny lines of high performance. In particular, two second-cycle lines selected as high-yielding from cultivar Imen (IH4-H4 and IH17-H1) outperformed significantly the source material, whereas the same was valid for one second-cycle line selected for high yield from cultivar Manel (MH18-H2) and one second-cycle line for high yield from cultivar Rihane (RH8-VS), both outyielding significantly the corresponding checks. The results obtained from the two landraces were comparable, as two second-cycle lines selected for high yield within the Ardhaoui landrace outperformed the original population. However, this was not fully confirmed for the case of the Djebali landrace, for which only one first-cycle selection identified through divergent selection as low-yielding material out-yielded marginally the original landrace.

These results give some insights for exploitable intracultivar variation for grain yield not only within landraces but also within improved commercial inbred cultivars. Landraces, in contrast to improved cultivars, are considered to be genetically more diverse and have been defined as dynamic populations of cultivated plants that have historical origin, distinct identity, and lack of formal crop improvement, as well as often being genetically diverse, locally adapted, and associated with traditional farming systems (Camacho Villa et al. 2006). Therefore, a certain extent of exploitable genetic diversity is expected within a landrace. It used to be thought highly contradictory in the past, the existence of exploitable variation within an inbred commercial cultivar.

Nonetheless, new debates come into light in this regard. In bread wheat, divergent single-plant selection at a density of 1.2 plants m^{-2} within the cultivar Siete Cerros produced lines with 8% higher and 9% lower yield than the original cultivar (Fasoula 1990). Selection within the bread wheat cultivar Nestos for two generations at 1.2 plants m^{-2} confirmed improvement of grain yield up to 22% per unit area under typical farmers' dense stands, as well as of stability across variable plant densities (Tokatlidis et al. 2006). In another predominantly inbred crop, such as cotton, Tokatlidis et al. (2008) selected for 2 yr within three commercial cultivars at the density of 1.2 plants m^{-2} and succeeded in identifying lines with higher cotton seed yield than the original cultivars. In another study, Fasoula and Boerma (2005) performed divergent selection for protein and oil content within three soybean cultivars using a density of 1.4 plants m^{-2}. After 3 yr evaluation of the selected lines, they reported significant variation for seed protein and oil content within each of the three soybean cultivars, while they also traced variation among selected progeny lines for specific fatty acid composition, even though selection had not been applied for the latter trait. Further assessment of these cultivars showed that they were also heterogeneous for seed weight and maturity time (Fasoula and Boerma 2007). By applying 1% selection pressure (Fasoula 2011) under an ultra-low density of 1.2 plants m^{-2} within the barley cultivar Athenaida, 6 out of the 600 totally established plants were used. The lines formed from this process were further

Table 8. Correlation analysis for percentage losses in agronomic and physiological-related traits and disease symptoms due to BYDV inoculation.

	Trait								
Trait	Yield_loss (%)	Fertile tillers_loss (%)	Plant height_loss (%)	F_0_loss (%)	F_v_loss (%)	F_m_loss (%)	F_v/F_m_loss (%)	F_v/F_0_loss (%)	Score 2
Fertile tillers _loss (%)	0.82**	—	—	—	—	—	—	—	—
Plant height_loss (%)	0.59**	0.69**	—	—	—	—	—	—	—
F_0_loss (%)	0.38	0.39	0.50*	—	—	—	—	—	—
F_v_loss (%)	0.52*	0.54*	0.58*	0.73**	—	—	—	—	—
F_m_loss (%)	0.51*	0.53*	0.58*	0.83**	0.98**	—	—	—	—
F_v/F_m_loss (%)	0.37	0.40	0.34	0.22	0.77**	0.65**	—	—	—
F_v/F_0_loss (%)	0.37	0.41	0.36	0.21	0.79**	0.67**	0.97**	—	—
Score 2	0.81**	0.75**	0.69**	0.36	0.50*	0.49*	0.35	0.36	—
Score 1	0.72**	0.66**	0.60**	0.47*	0.50*	0.52*	0.29	0.28	0.83**

Note: *, significant probability for $\alpha = 0.05$; **, significant probability for $\alpha = 0.01$.

advanced next season by selection under ultra-low density and then tested under typical farm densities in two locations. An average yield increase up to 2.5-fold was recorded for the best performing line over the original cultivar across both locations (Fasoula 2011). Therefore, results of the present study can further enhance the perception for exploitable intra-cultivar variation not only within landraces but also within commercial cultivars.

BYDV inoculation caused severe reduction in all agronomic performance traits, with an average yield loss of 60.43%. Similar results were reported by Beoni et al. (2016) in a 4-yr trial under field conditions in Czech Republic, where after artificial BYDV inoculation of 22 barley cultivars, the average reduction for grain weight per spike was 55.33%. Disease symptoms recorded through visual scoring were highly correlated with the reduction of all agronomic performance traits ($r = 0.60$–0.81, $p < 0.01$). However, no or marginal correlations were traced between PSII-related parameters and reduction in yield and other agronomic traits ($r = 0.34$–0.58), implying that chlorophyll fluorescence cannot be considered as a strong indicator of the stress effect imposed on the plants due to BYDV inoculation. The effect of BYDV infection on PSII in plants have been controversial, with some studies showing that BYDV stress inhibited the PSII activity (Jensen 1968), whereas others demonstrated that BYDV had no effect on PSII (Livingston et al. 1998).

The combination of both of the *Ryd2* and *Ryd3* BYDV resistance/tolerance genes resulted in significant lower values for yield reduction and disease symptoms. The presence of only one of the two genes indicated a slight reduction in yield losses and milder disease symptoms but these differences were not significant compared with the response of the lines that were not carrying any of these genes. Riedel et al. (2011) attempted to get information whether the level of tolerance against a German isolate of BYDV in barley could be improved by a combination of different loci. The results of their study showed that in the lines carrying the combination of *Ryd2* and *Ryd3* genes, a significant reduction in virus titer was detected compared with lines carrying only one of these genes. In addition, lines carrying the two genes showed a significantly higher relative grain yield compared with lines carrying only *Ryd2* or *Ryd3*, thus the combination of *Ryd2* and *Ryd3* leads to quantitative resistance against BYDV-PAV instead of tolerance (Riedel et al. 2011). The results of our study confirm similar findings and an additive effect can be inferred by the interaction of the *Ryd2* and *Ryd3* genes on barley germplasm for conferring resistance to BYDV.

Unexpectedly, a high intra-cultivar/intra-landrace variation was revealed in our study for the presence of *Ryd2* and *Ryd3* genes. In almost all of the cases, lines were identified that revealed either novel diversity for the presence of the *Ryd2* and *Ryd3* genes or absence of previously existing genes in relation to the source material (original cultivar or landrace) from which they have been

selected. The extent of variation revealed cannot be easily interpreted, even for the cases of landraces, since in the case of the Djebali landrace only 2 of the total 14 selected lines have generated the same profile as the original landrace (i.e., presence of *Ryd2*), with the majority of them showing the absence of both genes. Furthermore, for the Ardhaoui landrace, none of the lines selected have shown the same profile sd the original population (i.e., presence of *Ryd2*) and the majority of them, contrary to the one from the Djebali landrace, revealed the presence of both genes. Similar incidences regarding novel variation or absence of resistance genes, even though to a lesser extent, were also identified for the lines selected within the commercial cultivars Imen and Manel. These data generated in the laboratory, in terms of genes presence, were confirmed in most of the cases in the field through the response of the lines to BYDV inoculation. Thus, for the case of Manel for example, the original cultivar as well as two out of the three lines with the absence of the resistance genes, showed more than 78% reduction in grain yield. The two lines MH18-H3 and MH18, which were testified as having the *Ryd3* gene, showed a yield reduction of 73.15% and 47.10%, respectively, whereas the line MH18-L0 with confirmed presence of both *Ryd2* and *Ryd3* showed a yield reduction of only 25.55%. Recently these lines were subjected to genotyping by sequencing analysis. Preliminary data showed no intra-polymorphism for the cultivars, whereas the landraces had approximately 20%–30% intra-polymorphism (unpublished data).

Molecular evidence of cultivar heterogeneity has well been documented in other narrow gene pools. Olufowote et al. (1997) detected significant variation using restriction fragment length polymorphism and microsatellite markers not only within landraces but also within cultivars, assumed to be pure lines. Gethi et al. (2002) estimated the level of genetic diversity among and within six inbred maize lines derived from different sources and found that 7.6% of the total variation observed was attributed among sources within inbred lines and 4.6% within sources. The researchers conclude that a small but significant amount of variation exists within inbreds, raising concerns in germplasm conservation (Gethi et al. 2002). In another study, Yates et al. (2012) applied SSR marker analysis to the single-plant selected lines under ultra-low density, which have been derived from three soybean cultivar and previously quoted by Fasoula and Boerma (2005) for heterogeneity in terms of protein and oil content and fatty acid composition. Most of the variant alleles detected among the lines were back-traced to the original cultivars. However, there was a percentage from 7% to 18% of the variant bands that could not be detected in the source material (Yates et al. 2012). The researchers concluded that this portion of genetic variation could be attributed to mutation or some other mechanism generating de novo variation (Yates et al. 2012).

Exploitable genetic variation within a cultivar for disease resistance has been reported by Fasoulas (2000), who identified two cotton lines with tolerance to *Verticilium* wilt after selection at an ultra-low density of 0.74 plants m^{-2} within the susceptible cotton cultivar, Sindos 80. In lentil, another inbred crop, Kargiotidou et al. (2014) selected at ultra-low density of 1.2 plants m^{-2} for three consecutive cycles within a Greek lentil landrace and succeeded in obtaining lines yielding up to 23% higher than the original population, showing simultaneously an improved response to virus presence [such as the aphid transmitted, *Bean leafroll virus* (BLRV), or the seed-borne *Pea seed-borne mosaic virus* (PSbMV)]. The source material in their case was a lentil landrace. However, this landrace was commercially cultivated in Greece and the seed stock of their study came from a private pulse company, implying possibly a lesser extent of genetic diversity rather than a typical landrace maintained and regenerated by local farmers. Our study provides additional evidence on upgrading fairly homogeneous genepools in terms of disease resistance, in this case tolerance to BYDV-PAV.

Conclusion

The results of the current study provide evidence of considerable variation within locally adapted landraces as well as within improved commercial cultivars. Single-plant selection at ultra-low density within the barley landraces and commercial cultivars by applying the principles of honeycomb methodology resulted in lines combining both high yield performance and virus tolerance. Ultra-low density is a prerequisite for efficient selection within narrow gene pools by maximizing phenotypic expression and differentiation among individual plants, erasing at the same time the confounding effect of competition. Phenotypic expression among lines for tolerance to BYDV-PAV at the field was for most of the cases consistent with data generated from the laboratory, providing molecular evidence for exploitable variation within homogeneous gene pools. In addition, an additive effect of the *Ryd2* and *Ryd3* genes conferring resistance to BYDV-PAV was revealed. The results can give further insights that selection within cultivars could be a beneficial approach to avoid gradual degeneration of seed stock and to exploit the latent or de novo generated genetic variation.

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